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THE ROLE OF LEPTIN RECEPTORS IN THE DEVELOPMENT OF OBESITY

by

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JULY 1999

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SUBMITTED AS REQUIRED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
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ABSTRACT

The focus of this dissertation was leptin and the leptin receptor, and the role of these genes (OB and OB-R) in the development of obesity and type 2 diabetes in humans and Psammomys obesus, a polygenic rodent model of obesity and type 2 diabetes.

Studies in humans showed that circulating leptin concentrations were positively associated with adiposity, and independently associated with circulating insulin and triglyceride concentrations. Analysis of two leptin receptor sequence polymorphisms in a Caucasian Australian population and a population of Nauruan males, with very high prevalence rates of obesity, showed no associations between sequence variation within the OB-R gene and obesity- or diabetes-related phenotypic measures. In addition, these two OB-R polymorphisms were not associated with longitudinal changes in body mass or composition in either of the populations examined. A unique analysis of the effects of multiple gene defects in the Nauruan population, demonstrated that the presence of sequence alterations in both the OB and OB-R genes were associated with insulin resistance.

Psammomys obesus is regarded as an excellent rodent model in which to study the development of obesity and type 2 diabetes in humans. Examination of circulating leptin concentrations in Psammomys revealed that, as in humans, leptin concentrations were associated with adiposity, and independently associated with circulating insulin concentrations. This animal model was utilised to examine expression of OB-R, and the regulation of expression of this gene after dietary manipulation.

OB-R is known to have several isoforms, and in particular, OB-RA and OB-RB gene expression were examined. OB-RB is the main signalling isoform of the leptin receptors. It has a long intracellular domain and has previously been shown to play an important role in energy balance and body weight regulation in rodents and humans. OB-RA is a much shorter isoform of OB-R, and although it lacks the long intracellular domain necessary to activate the JAK/STAT pathway, OB-RA is also capable of signalling, although to a lesser degree than OB-RB. OB-RA is found to be expressed almost ubiquitously throughout the body, and this isoform may be involved in transport of leptin into the cell, although its role remains unclear.
OB-RA and OB-RB were both found to be expressed in a large number of tissues in *Psammomys obesus*. Interestingly, obese *Psammomys* were found to have lower levels of expression of OB-RA and OB-RB in the hypothalamus, compared to lean animals. This finding raises the possibility that decreased leptin signalling in the brain of obese, hyperlepticemic *Psammomys obesus* may contribute to the leptin resistance previously described in this animal model. However, the primary defect is unclear, as alternatively, increased circulating leptin concentrations may lead to down-regulation of leptin receptors.

The effect of fasting on leptin concentrations and gene expression of OB-RA and OB-RB was also examined. A 24-hour fast resulted in no change in body weight, but a reduction in circulating leptin concentrations, and an increase in hypothalamic OB-RB gene expression in lean *Psammomys*. In obese animals, fasting again did not alter body weight, but resulted in an increase in both circulating leptin concentrations and hypothalamic OB-RB gene expression. In the liver, fasting resulted in a large increase in OB-RA gene expression in both lean and obese animals. These results highlighted the fact that regulation of leptin receptor gene expression in polygenic models of obesity and type 2 diabetes is complex, and not solely under the control of circulating leptin concentrations. Sucrose-feeding is an established method of inducing obesity and type 2 diabetes in rodents, and this experimental paradigm was utilised to examine the effects of longer term perturbations of energy balance on the leptin signalling pathway in *Psammomys obesus*. Addition of a 5% sucrose solution to the diet of lean and obese *Psammomys* resulted in increased body weight in both groups of animals, however only obese *Psammomys* showed increased fat mass and the development of type 2 diabetes. The changes in body mass and composition with sucrose-feeding were accompanied by decreased circulating leptin concentrations in both groups of animals, as well as a range of changes in leptin receptor gene expression.

Sucrose-feeding increased hypothalamic OB-RB gene expression in obese *Psammomys* only, while in the liver there was evidence of a reduction in OB-RA and OB-RB gene expression in both lean and obese animals. The direct effects of sucrose on the leptin signalling pathway are unclear, however it is possible to speculate that
the effect of sucrose to decrease leptin concentrations may have been involved in the exacerbation of obesity and the development of type 2 diabetes in obese \textit{Psammomys}.

From these studies, it appears that sequence variation in the OB and OB-R genes is unlikely to be a major factor in the etiology of obesity in human populations. The ability to examine regulation of expression of these genes in \textit{Psammomys obesus}, however, has demonstrated that the effects of nutritional modifications on leptin receptor gene expression need closer attention. The role of the OB and OB-R genes in metabolism and the development of type 2 diabetes also warrants further examination, with particular attention on the differential effects of dietary modifications on leptin receptor gene expression across a range of tissues.
PUBLICIATION STATUS

A total of 7 manuscripts have either been prepared or are in preparation and each has constituted a separate chapter of this thesis. The publication status of each chapter is described below:

Chapter 3  Circulating leptin concentrations, obesity and lifestyle factors in Australian women. 

Chapter 4  Associations between leptin receptor polymorphisms and obesity in Australian women: A prospective study.
Manuscript in preparation.

Chapter 5  Combination of polymorphisms in OB-R and the OB gene associated with insulin resistance in Naurian males. 

Chapter 6  Cross-sectional characterisation of plasma leptin in Psammomys obesus. 
Manuscript in preparation

Chapter 7  Characterisation of leptin receptor gene expression in a polygenic animal model of obesity and diabetes, Psammomys obesus. 
Manuscript in preparation

Chapter 8  Regulation of leptin receptor gene expression with fasting in Psammomys obesus, a polygenic model of obesity and type 2 diabetes. 
Manuscript in preparation

Chapter 9  The effects of sucrose feeding on energy balance in Psammomys obesus: The role of leptin and leptin receptors. 
Manuscript in preparation
CHAPTER 1: INTRODUCTION

1.1 OBESITY

1.1.1 OBESITY: DEFINITION AND MEASUREMENT
Obesity may be defined as the presence of an abnormally large adipose tissue mass within the body (Gray, 1989). Measurement of the size of this depot is complex and both direct and indirect methods for the estimation of body composition have been used historically. As the only truly direct measurement of body composition involves the analysis of individual cadavers (Bray, 1985), methods for the indirect measurement of body fat have been used more widely.

Anthropometric measurements represent the cheapest and quickest method of gaining an estimation of body fat mass. These include measures of height, weight, various body circumferences and skinfold thicknesses. The most widely used index of overweight is that of body weight in relation to height, the body mass or Quetelet index (Bray, 1985). The body mass index (BMI) is body weight (in kg) divided by the square of the height (in metres) (BMI=kg/m²). This index correlates closely with body fat and is used widely in large-scale epidemiological studies (Gray, 1989).

Due to their ease of measurement, a large amount of epidemiological data is available about weight and height. The life insurance industry has historically been interested in the effect of increasing body weight on longevity and has conducted periodic surveys of mortality and body build (Bray, 1985, Gray, 1989). Life insurance statistics published from 1959 onwards established that excess weight was associated with a higher mortality risk. Numerous studies have confirmed this relationship and the relationship of BMI to health risks can be used as a guide for the treatment of obesity (see Section 1.1.5).

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<td>Obese</td>
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In Australia the WHO classifications are used to define lean, overweight and obese individuals. These classifications are based on large-scale, population-based studies which have shown an increasing morbidity and mortality as BMI increases above
25kg/m² (Lew and Garfinkel, 1979; Bray, 1985; WHO Study Group, 1990). Different classifications are used in some countries, for example, in Canada, individuals with BMI>27 kg/m² is regarded as obese, while in the USA individuals are considered obese if they have a BMI>25 kg/m² and are younger than 34 years of age, or a BMI >27 kg/m² and are older than 34 years (NIDDK, 1993). In other countries, gender-specific differences in BMI are also taken into account. These differences in classification make comparisons between studies from different countries difficult.

The major criticism of the use of BMI as a measure of obesity, is that it fails to distinguish between weight that is due to muscle, and weight that is due to fat. Therefore, the relationship between BMI and obesity is likely to vary in individuals and populations who differ in body build and body proportions (WHO Expert Committee, 1995). In addition, BMI fails to account for body fat distribution, an important determinant of health risks associated with obesity (see Section 1.1.6). Despite these criticisms, BMI remains the most widely used measure of adiposity, due to its low cost, ease of use and generally satisfactory accuracy.

It is now apparent that total fat mass may be more relevant to the health risks associated with adiposity than body weight. The simplest way to estimate the size of the fat mass is to measure skin fold thickness at four sites (biceps, triceps, subscapular and suprailiac) using a caliper, and then to apply these values to published equations (Durnin and Womersly, 1974). Several other techniques also exist for the estimation of adipose tissue mass, however often the practicality and/or cost of these methods may reduce their application. Measurement of body density provides a quantitative technique for measuring body fat. The density is determined from the specific gravity of the body, that is the weight of the body both in and out of water, and the residual volume of the lungs is determined. It is possible to divide the body into its fat and fat-free components as fat is lighter than water and other tissues are heavier than water (Bray, 1985). Isotope or chemical dilution methods may also be used, in which the distribution of a fat-soluble isotope such as cyclopropane can be used to estimate total fat mass. Body fat may also be calculated from measurements of body water by the distribution of tritiated water or antipyrine, which equilibrates with body water (Bray, 1985). Total body fat can also be estimated using impedance analysis, which relies on the difference in electrical resistance between lean tissue and fat (respectively good and poor conductors). A weak current is passed between electrodes attached to the limbs, and fat mass can be determined from the measured resistance and the subject’s height, weight, and sex, using standard equations (Segal
et al., 1988). Normal total body fat content is 10-20% of body weight in males and 20-30% in females (Lohman, 1981).

It is also imperative to establish the distribution of the body fat, as some diseases are associated not only with a high fat mass, but also with a particular pattern of body fat distribution. Waist-to-hip ratio (WHR) provides a reasonable indication of central (‘android’) obesity. Recently the use of waist circumference alone has been proposed as a better measure of central obesity (Lean et al., 1995). Other techniques for the measurement of specific fat depots include computerised topographic scans, nuclear magnetic resonance scans, neutron activation and dual energy x-ray absorptiometry (Bray 1985, Bray 1987). These methods can be quite costly while some involve exposing the subject to radiation.

### 1.1.2 EPIDEMIOLOGY OF OBESITY

The bodyweight of the world’s population appears to be increasing. In 1998 in the United States, approximately 22.5% of the population is considered obese while in 1980 this level was 14.5% (Taubes, 1998). This increase in bodyweight has become evident mainly from data originating from the National Health and Nutrition Examination Surveys (NHANES) carried out by the National Centre for Health Statistics (NCHS). There have been four surveys conducted between 1960 and 1994 and the increase in the degree of overweight and obesity in the American population can be tracked in these surveys. The NHANES completed in 1962 (known as the National Health Examination Survey; NHES) estimated that 12.8% of the population was obese. During the next two decades there was no major increase in this level with the NHANES of 1971-1974 and 1976-1980 reporting obesity prevalence rates of 14.1% and 14.5%, respectively (Abraham et al., 1983). NHANES III, conducted in the early 1990s, however, showed that the level of obesity had increased by more than half to 22.5% of the population, while more than 55% of the total population was considered overweight by the end of the survey (Pi-Sunyer, 1993). The increase in the degree of obesity appeared to be across all sectors of the community and in all age groups, including children and adolescents.

This phenomenon appears to be world-wide and is paralleled in a number of developed nations. In the United Kingdom obesity rates jumped from 6% to 15% in men, and from 8% to 16.5% in women between 1980 and 1994 (Seidell, 1995). In Australia the prevalence of obesity has increased from 9.3% to 11.5% in men and 8% to 13.2% in women between 1980 and 1989 (Bennet and Magnus, 1994). In Europe where there is great variability in the prevalence of obesity, studies from Finland, The
Netherlands and Sweden indicate only a slight increase in obesity levels in men, and no increase in women (Lee et al., 1993; Seidell, 1997). In contrast, the rates of obesity in the former East Germany have increased from 13.7% to 20.5% in men and 22.2% to 26.8% in women between the years of 1985 and 1992 (Lee et al., 1993).

Obesity levels in some lower-income and transitional countries are as high, or higher than those reported for the United States and other developed countries (Popkin, 1998). In Latin America, more than 10% of females are obese in Brazil (Monteiro et al., 1995) and Colombia (Dufour et al., 1994) and more than 50% of the population in Mexico is overweight (Sanchez-Castillo, 1998). In Asia, obesity prevalence is low and can often be related to a relatively higher level of economic development, especially in countries such as urban Thailand and Malaysia (Popkin, 1998).

The Western Pacific region boasts some of the world’s highest prevalence rates of obesity and related chronic diseases. On the island nations of Nauru, Samoa, Fiji and Melanesia approximately 50% of the population is obese and in most cases female obesity is more prevalent (Hodge et al., 1994; Taylor et al., 1994; Hodge et al., 1995). Modernisation has been associated with this high rate of obesity seen in this region and these rates mirror the rates found among Native American groups in the United States (Brown and Konner, 1987). In Nauru and Western Samoa the prevalence rates of obesity and overweight are among the highest in the world and appear to be increasing steadily (Hodge et al., 1994; Hodge et al., 1995).

The increase in the prevalence rates of obesity in affluent countries cannot be easily explained. Over the past three decades blood pressure and blood cholesterol levels have been declining and rates of coronary heart disease mortality have decreased by more than half (Vartiainen et al., 1994a; Vartiainen et al., 1994b). During this same time the fat content of the diet has decreased (Pietinen, 1996a) however the levels of overweight and obesity has been increasing steadily around the world (Pietinen 1996b, Siedell 1996; Popkin 1998).

If it is assumed that height has remained constant, an average weight increase of slightly less than 1 kilogram (kg) over ten years would be sufficient to increase the rates of obesity to that observed (Seidell et al., 1997). This increase in bodyweight would represent only a minute change in energy balance on a daily basis; a constant positive energy balance of about 2 kcal/day. Existing methods for measuring energy expenditure and energy intake in populations would not be expected to detect such a small change (Seidell et al., 1997).
In addition to changes in energy balance across the population, increases in the degree of overweight and obesity within a country may reflect lifestyle changes across the population. It has been estimated that perhaps 20% of the increase in overweight adults may be due to the cessation of smoking as men typically gain 3.5-4kg and women 5-6kg when they quit. Smoking behaviour particularly in men has changed since the 1970s, however this decrease seems to have plateaued in the 1980s (Ruward et al., 1996).

The level of physical activity across the population also appears to have changed, with an apparent increase in the amount of ‘sedentary behaviour’ (Hill and Saris, 1998). The increase in the number of hours spent in front of the television or computer is thought to be an important element in the increase of obesity in children and adolescents (Gortmaker et al., 1996; Seidell 1997). In addition, the increase in the number of labour-saving devices available in the workforce and in homes allows adults to become more sedentary in all aspects of their life (Popkin 1998).

In lower- and middle-income countries the effects of rapid changes in urbanisation are reflected in many aspects of the lifestyle of the population. A shift in the structure of employment has been occurring in lower-income countries during the past few decades with a movement towards more capital-intensive and knowledge-based employment that relies far less on physical activity (Popkin 1998). The shift towards much less physically demanding occupations appears to be a world-wide phenomenon (Popkin 1998). The shift in activity is also found in the increased use of transportation to get to school or work, more technology in the home and much more passive leisure time. These changes resulting from high rates of urbanisation facilitate inactivity among all age groups of the population (Popkin, 1998).

1.1.3 ECONOMIC COSTS OF OBESITY
The escalating rates of obesity globally have resulted in the World Health Organisation (WHO) and the International Obesity Task Force (IOTF) declaring an obesity epidemic worldwide. They suggest that the numbers of people affected are so large that sufferers from obesity problems already threaten to overwhelm countries' medical services. They add that the problem of obesity has been largely ignored as a public health issue and nation-wide prevention policies aimed at the entire population are imperative (WHO, 1998).
The 1994 National Health Interview Survey (NHIS) estimated the economic cost of obesity in the United States and found that the total cost attributable to obesity in 1995 amounted to $99.2 billion dollars. Approximately $51.64 billion was direct medical costs, while the remaining cost was associated with the cost of lost productivity with 39.2 million days of lost work. In addition it was calculated there were 239 million restricted activity days, 89.5 million bed-days, and 62.6 million physician visits attributable to obesity in 1994 (Wolf and Colditz, 1998). These figures have increased substantially from those recorded in the 1988 NHIS and now represent 5.7% of the National Health Expenditure in the United States (Wolf and Colditz, 1998).

In Australia in the early 1990s, the annual cost of obesity and obesity related diseases was estimated at $810 million (1992-1993 dollars; NHMRC, 1996). It was calculated that $510 million was a direct cost within the health system, while the remaining $300 million was the indirect cost of days of lost work and loss of earnings due to premature mortality (NHMRC, 1996). In addition there were 3,512 obesity-related surgical procedures performed in Australian hospitals, while an estimated $500 million is spent annually by consumers on weight loss programs (NHMRC, 1996). The Australian National Medical Health and Research Council has estimated the total economic burden of obesity to the health system is in excess of $1.5 billion annually (NHMRC, 1996).

1.1.4 OBESITY AND MORTALITY
A number of studies found an association between obesity and mortality and most data relating total mortality in men and women to bodyweight or the level of body fatness show a U- or J-shaped curve (Figure 1.1). Total mortality decreases with decreasing bodyweight until a threshold weight, below which mortality rate increases as bodyweight decreases further (Lew and Garfinkel, 1979; Bray, 1985; Stern, 1995). Based on the 1979 Life Insurance statistics, a bodyweight 10% above average is accompanied by an 11% increase in excess mortality for men, and a 7% increase in women. If bodyweight is 20% above average the excess mortality increases to 20% for men and 10% for women (Lew and Garfinkel, 1979; Bray, 1985). The increased mortality observed in lean subjects may be explained by cancer and various digestive diseases and, at least in men, the U- or J-shaped relationship only appears when smokers are included in analyses (Bray, 1985).
Figure 1.1 Relationship between BMI and Mortality rates (Bray, 1987)

Several prospective studies have examined the relationship between obesity and various diseases. The Framingham study is one such study conducted through the National Heart, Lung and Blood Institute (NHLBI) in Bethesda, Maryland on over 5000 men and women between the ages of 30 and 60 from the Framingham area (Hubert et al., 1983). The participants were initially weighed and examined in 1949 and after 26 years of study it was determined that the relative weight at entry into the study was an independent predictor of cardiovascular disease (CVD), particularly in women. The predictive effect of the initial degree of overweight was independent of age, smoking, glucose intolerance, blood pressure or cholesterol level (Hubert et al., 1983; Bray, 1985). The conclusion of this study was that obesity is an important long-term predictor of cardiovascular disease, particularly in younger individuals (Hubert et al., 1983). This conclusion was also supported by a prospective study of 750,000 men and women by the American Cancer Society which found a steady increase in heart disease with weight (Lew and Garfinkel, 1979).

This finding has more recently been supported by results from the prospective Nurses Health Study (Mason, 1995; Willett et al., 1995) in which more than 115,000 young and middle-aged female nurses were followed for 14 to 16 years and the rates of mortality and CVD morbidity were associated with BMI (Willett et al., 1995). The lowest rate of mortality was seen in women with a BMI less than 19 and for those women above this BMI the risk of death increased by 20% for women with BMIs
between 19 and 24.9, 60% for BMIs between 27 and 28.9 and more than 100% for BMIs of 29 and above (Willett et al., 1995).

The most recent study to support this hypothesis is the American Cancer Society’s Cancer Prevention Study I. This study followed more than 62,000 men and 262,000 women for 12 years and determined that the lowest risk of mortality from all causes was found in men and women with BMIs between 19 and 21.9 (Stevens et al., 1998). There is a degree of conjecture however, regarding the degree of risk associated with being slightly overweight in younger persons. Several studies have indicated that the risk of mortality is very low over a wide distribution of weights and that an optimal BMI is closer to 24 than 19. The 12 year follow up of 13,242 men and women participating in the original National Health and Nutrition Examination Survey (NHANES I), indicated that mortality was lowest at a BMI of 27.1 for black men, 28.8 for black women, 24.8 for white men and 24.3 for white women (Pamuk et al., 1993). These results highlight the problems associated with the use of standards of optimum weight-for-height values across different ethnic groups (Stern, 1995).

1.1.5 OBESITY AND MORBIDITY

The presence of increased body weight and obesity have been associated with higher rates of morbidity as well as mortality (see figure 1.1). Obesity has consistently been demonstrated to specifically increase the risk of developing cardiovascular disease, various cancers, hypertension, gall bladder disease and type 2 diabetes. These relationships will be discussed in detail below.

1.1.5.1 Cardiovascular Disease and Hypertension

Obesity appears to increase cardiovascular risk via both direct effects on the heart and indirect effects (mainly insulin resistance) via its effects on hypertension and lipids. Individuals with hypertension have two to three times the risk of CHD, and seven times the risk of stroke. The association between obesity and hypertension has been widely recognised, with obesity seen as a modifiable risk factor contributing to hypertension. The largest study of the association between obesity and hypertension is the Community Hypertension Evaluation Clinic study, which screened over one million people (Stamler et al., 1978). ‘Overweight’ middle aged people (40-64 years) were 50% more likely to be hypertensive than those of ‘normal’ weight, and had double the risk of ‘underweight’ individuals of the same age. In this study the association between obesity and hypertension was even more marked in younger people.
Subsequent large scale studies using more precise quantitative measures have confirmed this relationship. In NHANES II, overweight (BMI>27) Americans aged 20-75 years were three times more likely to be hypertensive than their non-overweight compatriots (Van Itallie, 1985). In the Framingham study, both systolic and diastolic blood pressure increased significantly and progressively with increasing BMI. This study also demonstrated a relationship between hypertension and waist circumference and skinfold thickness (Higgins et al., 1988). In the Chicago Heart Study of 33,600 subjects, there was a progressive increase in both blood pressure and established hypertension throughout the entire range of relative weight (Pan et al., 1986).

The association between obesity and hypertension has been found to vary with age as well as ethnicity. Within Australia, the Risk Factor Prevalence Study in a predominately Caucasian population determined that obesity may account for almost one-third of hypertension in the population as a whole, and for nearly two-thirds in young men (MacMahon et al., 1984).

Obesity can directly affect the mechanics of the heart. Alterations in the structure and function of the heart have been associated with the presence of obesity, even in the absence of hypertension. These structural abnormalities may actually represent an independent effect of obesity on CVD risk. The increase in cardiac output required to meet the additional metabolic needs of the obese individual is achieved by a rise in stroke volume rather than increased heart rate. This state of high cardiac output results in eccentric hypertrophy of the left ventricle and diastolic dysfunction (Alpert and Hasimi, 1993). Systolic dysfunction may eventuate if wall thickening fails to keep pace with dilation, which in turn leads to ‘obesity cardiomyopathy’ and congestive heart failure (Alpert and Hasimi, 1993). Left ventricular hypertrophy is recognised as a major risk factor for sudden death and other types of cardiac morbidity and mortality and the coexistence of obesity and hypertension has an even greater effect on left ventricular structure and function than either disorder alone (Alpert and Hasimi, 1993). In the Framingham Study, one in two men identified with left ventricular hypertrophy died within the next eight years (Kannel et al., 1969).

Obesity is also associated with a range of dyslipidemias that predispose to CHD, including hypercholesterolemia, high fasting and postprandial triglyceride levels, low high-density cholesterol, high apolipoprotein B and small dense low-density lipoprotein particles (Van Gaal et al., 1995). In the Framingham study, every 10% increase in relative weight was associated with an increase in plasma cholesterol of
0.3 mmol/l (Kannel and Gordon, 1979). In addition, NHANES II demonstrated that the relative risk of hypercholesterolemia for overweight Americans aged 20-75 years was 1.5 times that of those not overweight (Van Itallie, 1985). Among younger people (20-45 years), the relative risk doubled compared with that of non-overweight people.

1.1.5.2 Gallbladder Disease
Several studies have also highlighted an association between obesity and gallbladder disease (Nestle et al., 1973; Lew and Garfinkel, 1979; Bray, 1985; Ko and Lee, 1998). The life insurance statistics show that the risk of dying from gallbladder disease increases with obesity (Bray, 1985). In both these statistics and the American Cancer Society study, there was a relationship between increasing body weight and increased mortality from digestive diseases (Lew and Garfinkel, 1979). The underlying mechanism for this association appears to lie with the increased levels of cholesterol production and secretion observed in obese individuals. For each extra kilogram of body weight there is an increased cholesterol production of 20-22mg/day (Nestle et al., 1973). In addition, the bile of obese subjects is more saturated with cholesterol than non-obese subjects (Bennion and Grundy, 1978). Thus the increased biliary excretion of cholesterol in obesity is the likely cause of the increased risk of gallstones (Bray, 1987).

1.1.5.3 Cancer
Cancer also shows a significant association with bodyweight. The American Cancer Society study of 750,000 individuals with a 12-year follow-up period found as BMI increases there is an increase in death from cancer (Garfinkel, 1985). There are significantly higher rates of prostatic and colorectal cancer in obese males, while obese women experience higher rates of cancer of the gallbladder, breast, cervix, endometrium, uterus and ovary (Garfinkel, 1985). The Danish Record Linkage Study of almost 44,000 individuals and an 11-year follow-up found a higher risk of cancer of the oesophagus, liver and pancreas in obese men and women (Moller et al., 1994; Deslypere, 1995). While this epidemiological data supports the association between obesity and cancer there are many factors thought to confound this relationship, including fat distribution, insulin levels, sex hormone levels, nutrition and use of diuretics (Deslypere, 1995) which may all affect cancer risk.

1.1.5.4 Respiratory Problems
Obese subjects also show mechanical problems. Respiratory problems such as breathlessness, snoring, sleep apnea and decreased vital capacity as respiratory
muscles may function abnormally with disturbances in ventilation and perfusion (Ray et al., 1983). A Finnish study reported that one-quarter of all disability pensions for cardiovascular and musculo-skeletal causes in women, and one-eighth of those in men were attributed to the recipient being overweight (Rissanen et al., 1991). Similarly, epidemiological follow-up of the NHANES I found a two-fold increase in the risk of mobility disability in older women (>60 years) with a BMI over 27. This increased risk persisted even into very old age (>80 years; Kuczmarski et al., 1994).

1.1.5.5 Type 2 Diabetes

The development of type 2 diabetes appears to be exacerbated by the presence of obesity while the distribution of the fat may also be an important factor. The physiological relationship between obesity and type 2 diabetes will be discussed in detail later (Section 1.2) however obesity is considered the most important modifiable risk factor for type 2 diabetes. The US National Commission on Diabetes (1975) reported that the risk of developing type 2 diabetes was about two-fold in mildly obese, five-fold in moderately obese and ten-fold in severely obese people. Similarly, NHANES II found that for American adults self-categorised as overweight, the relative risk of diabetes was 2.9 times that for non-overweight people of similar age (Van Itallie, 1985). In younger people within this group (aged 20-45 years), the risk was 3.8 that for non-overweight individuals (Van Itallie, 1983).

Recent prospective studies further confirm the link between obesity and type 2 diabetes. The British Regional Heart Study followed 7,735 British middle-aged men for a mean of 12.8 years and demonstrated that BMI was the dominant risk factor for the development of diabetes (Perry et al., 1995). Men with a BMI >27.9 were found to have seven times the risk of diabetes compared to those with the lowest BMI. Interestingly, over 75% of the diabetes reported in this study was found in men with a BMI <30, highlighting the role played by other risk factors for diabetes, namely low levels of physical activity, high serum triglycerides, low HDL cholesterol, high heart rate, high uric acid concentration and existing CHD (Perry et al., 1995).

The US Health Professionals’ Follow-up Study conducted on 51,529 men (aged between 45 and 75) followed for 5 years also demonstrated a strong positive association between BMI and risk of developing diabetes (Chan et al., 1994). Overweight men (BMI between 25 and 27) had a risk 2.2 times greater than men with a BMI <23. This association was found after adjustment for age, family history and smoking habits. The risk rose markedly with increasing BMI and men with a BMI>35 had a relative risk 42.1 times greater than men with a BMI<23 (Chan et al.,
1994). Similar findings have been reported in women, with the Nurses' Health Study again confirming that BMI is a dominant predictor of risk for type 2 diabetes (Colditz et al., 1990; Colditz et al., 1995). This study followed 110,000 women between the ages of 30 and 55 for 14 years and found that even women towards the upper end of the 'normal' weight range showed excess risk of developing diabetes. Women with a BMI between 22-22.9 had 2.9 relative risk of developing diabetes, compared to women with a BMI<22 (relative risk of 1.0). This risk rose to an incredible 93.2 at a BMI >35 (Colditz et al., 1995).

1.1.6 BODY FAT DISTRIBUTION

Several studies have demonstrated that the risk of developing type 2 diabetes is increased not only with increasing BMI but additionally with a particular pattern of body fat distribution. This is true for other diseases also, including CHD. Excess adipose tissue may primarily be stored either in the abdominal area (upper body or 'android' obesity) or in the gluteal-femoral region (lower body or 'gynoid' obesity). In addition, abdominal fat comprises both abdominal subcutaneous fat and intra-abdominal visceral fat and these two fat depots exhibit metabolic differences (Vague, 1956).

Vague first reported that android obesity was associated with type 2 diabetes, atherosclerosis and gout more frequently than gynoid obesity (Vague 1947; Vague 1956) and subsequent cross-sectional and longitudinal studies have confirmed and further described this association. In a cross-sectional study of second-generation Japanese-American men, the amount of intra-abdominal fat was found to be greater in men with CHD than in men without, even after adjustment for BMI and glucose tolerance (Bergstrom et al., 1990).

The 13.5 year, longitudinal 'Study of Men Born 1913' included 792 Swedish men followed between at the ages of 54 and 67 (Larsson et al., 1984). The distribution of fat deposits was found to be a better predictor of stroke, ischemic heart disease and all-cause mortality than BMI or skinfold thickness, although in this study it was not an independent risk factor. The Study of Women in Gothenburg included 1462 Swedish women aged between 30 and 60 (Lapidus et al., 1984). A significant association was reported between WHR and the 12-year incidence of myocardial infarction, angina pectoris, stroke and death. In this study, the association was found to be independent of age, BMI, serum cholesterol and triglyceride concentrations and systolic blood pressure. Other measures of obesity including BMI and sum of
skinfold thickness were found not to be as good predictors of subsequent morbidity and mortality (Lapidus et al., 1984).

1.1.6.1 Central Obesity and Type 2 Diabetes
Several studies have now provided evidence of a relationship between central obesity and development of type 2 diabetes. A cross-sectional study of Japanese-American men demonstrated that intra-abdominal fat deposition was more closely correlated with type 2 diabetes than subcutaneous fat in the abdomen, while subcutaneous fat deposits in the abdomen, thorax or thigh were not significantly associated with diabetes development (Larsson et al., 1990). In the Study of Men Born 1913, WHR was positively associated with type 2 diabetes independent of BMI (Ohlson et al., 1985). A 16-year US study (the Normative Aging Study) demonstrated a 2.4 times greater risk of developing diabetes for men in the top tertile of the ratio of abdominal circumference to hip breadth, compared to men in the lowest tertile, independent of BMI, smoking habits and age (Cassano et al., 1992). Recently the US Health Professionals’ Follow-up Study reported a strong correlation between waist circumference and type 2 diabetes. In this study, WHR was found to be a good predictor of diabetes in the top 5% and waist circumference was positively associated with the risk of diabetes among the top 20% of the cohort (Chan et al., 1994).

1.1.6.2 Central Obesity and Endocrine Abnormalities
Endocrine abnormalities accompany the deposition of adipose tissue deep within the abdomen and Kissebah et al. (1982) were the first to provide evidence for a clear relationship between the distribution of body fat and plasma glucose and insulin homeostasis (Despres and Lamarche, 1993). They demonstrated that hyperinsulinemia and hypertriglyceridemia were significantly associated with abdominal visceral obesity in women (Kissebah et al., 1982). A similar association in a large cohort studied by Krook et al (1983) confirmed this relationship in both men and women and demonstrated that abdominal obesity is significantly correlated with disturbances in plasma glucose, insulin and lipid levels.

In women, the metabolic abnormalities associated with abdominal fat deposition appear to be closely related to the role of sex steroids, and in particular sex hormone status (Desypere, 1995). In addition, there appears to be a complex interaction between corticosteroid, sex steroid hormones and growth hormone, resulting in specific localisation of triglycerides in central or peripheral adipose tissue depots (Haffner, 1994; Bjorntorp, 1997). An excess of cortisol and a relative deficit of sex steroid hormones and/or growth hormone deficiency result in visceral fat
accumulation (Björntorp, 1995a; Björntorp, 1997). These findings imply that the increased cortisol secretion accompanied by decreased secretion of sex steroids and growth hormone is responsible for the accumulation of visceral fat. Intervention studies, where testosterone is administered to obese subjects support this interpretation and have demonstrated a specific decrease in the mass of visceral adipose tissue depots after testosterone treatment of abnormally obese men (Björntorp, 1995a).

An important relationship has also been demonstrated between the level of visceral abdominal fat and fasting as well as post-prandial insulin and glucose levels. As discussed earlier, prospective studies have also confirmed the relationship between abdominal obesity and increased CVD risk. Insulin resistance appears to be the key factor that links obesity to glucose intolerance, hypertension and dyslipidemia (Weidmann et al., 1993; Rocchini; 1995) and it is widely believed that insulin resistance and hyperinsulinemia are the initial triggers of the cascade of events leading to obesity-related hypertension and dyslipidemia (Krieger and Landsberg, 1988; Rocchini; 1995; Zemel, 1995).

1.1.7 OBESITY AND INSULIN RESISTANCE
The insulin resistance observed in obesity is characterised by several defects including decreased non-oxidative glucose disposal in muscle, impaired lipid metabolism in adipose tissue and impaired lipoprotein lipase activity (Turner, 1992; Walker 1995). The mechanisms underlying these defects involve increased levels of circulating free fatty acids (FFAs), although it is difficult to ascertain whether these defects are secondary to the excess availability of FFAs, to down-regulation of insulin receptors or to chronic hyperglycemia (Turner, 1992). The following sequence of events has been proposed for the development of these metabolic disturbances in obesity: excess caloric intake expands the adipose tissue mass and lipolysis starts to increase to a level which eludes control by insulin. Consequently, fat oxidation and FFA re-esterification both increase as a consequence and the increase in fat oxidation impedes glucose oxidation. In addition, FFA re-esterification conserves energy. The reduced efficacy of insulin action on the adipocyte mass is thought to alter the kinetics of insulin within the poorly perfused fat tissue, with insulin surges blunted and delayed. In addition, the hyperinsulinemia induced by increasing fat mass appears to down-regulate insulin receptor numbers, further reducing the insulin sensitivity of this tissue (Koltermann et al., 1980).
Severe hyperinsulinemia and insulin resistance have been demonstrated in abdominal obesity (Ohlson et al., 1985; Lundgren et al., 1989). This appears to be largely dependent on the amount of intra-abdominal visceral fat. Visceral abdominal adipocytes largely mobilise their FFA into the portal vein, exposing the liver to high concentrations of FFAs. High levels of FFAs in vitro have been associated with reduced binding and internalisation of insulin by rat hepatocytes suggesting that high concentrations of portal FFAs inhibit the uptake of insulin by the liver (Vague et al., 1985). This may in turn lead to peripheral hyperinsulinemia, followed by a relative insulin insensitivity (Vague, 1956; Vague et al., 1985; Bjorntorp, 1988). High portal FFA concentrations are also associated with hypertriglyceridemia. In addition, increased FFA availability from enhanced lipolytic activity and hyperinsulinemia enhances the formation of very low-density lipoprotein (VLDL) cholesterol in the liver (Eckel, 1989; Albu and Pi-Sunyer, 1998).

In 1963, Randle proposed a glucose-fatty acid cycle whereby circulating FFAs, by a substrate competition mechanism, can potentially increase liver glucose output and impair glucose removal by peripheral tissues, which in turn may lead to insulin resistance. The pattern of this phenomena is thought to begin with increased FFA disposal/oxidation, paralleled by an increased rate of lipid oxidation. The rise in FFA oxidation is paralleled by a concomitant decrease in glucose oxidation (and possibly in glucose storage) and by an impairment in insulin-mediated inhibition of hepatic glucose production (HGP). As glucose tolerance deteriorates, increasing amounts of lipids appear to be oxidised and decreasing amounts of glucose are oxidised or stored, with a parallel deterioration of hepatic insulin sensitivity also observed (Groop, 1991). Lipoprotein lipase activity may also be decreased which possibly results in decreased clearance of triglycerides from blood (Eckel, 1989). A pattern of dyslipidemia also emerges, with increased levels of VLDL, triglyceride, and low-density lipoprotein particles, and decreased concentrations of high-density lipoprotein cholesterol (Howard, 1987; Eckel, 1989).

Control of the cycle is thought to be modified by insulin as it enhances glucose uptake by muscle and adipose tissue, inhibits the release of FFAs in adipose tissue and increases esterification of fatty acids in adipose tissue and muscle (Groop, 1991).

In addition to alterations in glucose and insulin homeostasis, lipid metabolism is also affected by the accumulation of visceral abdominal adipose tissue. High levels of abdominal, visceral fat have been associated with dyslipidemia, namely elevated triglyceride concentrations and reduced concentrations of high-density lipoprotein
cholesterol (HDL-C) in the plasma (Lemieux and Despres, 1994). These associations have been demonstrated to be largely independent of obesity and provide one explanation of the link between the regional distribution of adipose tissue and CVD risk as decreased levels of HDL-C and increased levels of plasma triglyceride are well established risk factors for the development of CVD (Lemieux and Despres, 1994).

1.2 TYPE 2 DIABETES

Diabetes mellitus is a significant chronic disease in many parts of the world. It is characterised by hyperglycemia and disturbances of carbohydrate, fat and protein metabolism arising from absolute (type 1) or relative (type 2) deficiencies in insulin action and/or insulin secretion (WHO, 1994). Overall, type 2 diabetes accounts for between 85 and 90% of all cases of diabetes. This form of diabetes occurs primarily in adults and diagnosis is usually made after the age of 40, although this may occur earlier in high risk groups. Obesity is considered the most modifiable risk factor for type 2 diabetes, and the risk of developing type 2 diabetes is about 5 times higher in severely obese individuals compared to those who are only mildly obese, and about 10 times higher compared to non-obese subjects (United States Commission on Diabetes, 1975).

1.2.1 TYPE 2 DIABETES: DIAGNOSIS AND EPIDEMIOLOGY

The World Health Organisation (WHO) defined diabetes mellitus as “characterised by hyperglycemia and disturbances of carbohydrate, fat and protein metabolism that are associated with absolute or relative deficiencies in insulin action and/or insulin secretion” (WHO Study Group, 1994). The WHO classification of diabetes mellitus is based on fasting and/or post-glucose load hyperglycemia, assessed by an oral glucose tolerance test (OGTT) according to table 1.2.

<table>
<thead>
<tr>
<th>Normal glucose tolerance</th>
<th>Venous Plasma Glucose (mmol/l)</th>
<th>2-hour post load*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting</td>
<td>&lt;7.8</td>
<td>AND &lt;7.8</td>
</tr>
<tr>
<td>Impaired glucose tolerance</td>
<td>&lt;7.8</td>
<td>AND 7.8-11.0</td>
</tr>
<tr>
<td>Type 2 diabetes</td>
<td>&gt;7.8</td>
<td>OR &gt;11.0</td>
</tr>
</tbody>
</table>

*2 hours after an oral glucose load of 75g.

The category of impaired glucose tolerance (IGT) was defined by WHO as a risk category for type 2 diabetes, although there was difficulty associated with establishing where “normality” ended and diabetes began (WHO Study Group, 1994). Recently
the WHO classification of type 2 diabetes has come under scrutiny, with the American Diabetes Association (ADA) defining new diagnostic criteria for the classification of type 2 diabetes. This new criteria uses fasting plasma glucose concentrations only, and defines normal fasting glucose as <6.0 mmol/l, and diabetes as a fasting plasma glucose (FPG) of ≥7.0 mmol/l. In addition the ADA defines a new category of intermediate glucose metabolism, impaired fasting glucose (IFG). IFG is diagnosed by fasting glucose concentrations in the range of 6.1 to 7.0 mmol/l. This ADA criteria may make the OGTT redundant, and the merits of these two classification systems are still being debated in many countries. The majority of the epidemiological data which is discussed below however, utilises the WHO criteria.

Type 2 (or non-insulin-dependent) diabetes mellitus is a global public health problem which is expected to reach epidemic proportions during the next 10-20 years. In 1994, WHO estimated that there were approximately 100 million people world-wide affected by type 2 diabetes and further projected that in 15 years this number will increase to over 215 million.

As with other non-communicable diseases, the prevalence of diabetes varies widely among populations and ethnic groups. The lowest reported rates have been found among certain Chilean Indians and urban Chinese (less than 1%) and, in 1996, the WHO estimated that diabetes affected 4-15% of individuals from populations in the Seychelles, South Africa and Mauritius. The highest prevalence rates for diabetes have been reported in American Pima Indians. Several populations found within the Pacific have also recorded high prevalence rates of type 2 diabetes. The incidence of diabetes for adults over 20 years of age in the Pacific region are: Nauru, 33% (men) and 35% (women); urban Samoans, 12% and 16%; urban Wangelians (PNG), 31% and 40% and Creole Mauritians, 13% and 16%. The prevalence rate of diabetes in European Australians is 4% and 3% for men and women respectively. In Pima Indian populations, 61.3% of men and 68.3% of women exhibit diabetes.
Figure 1.2 Prevalence of type 2 diabetes amongst different ethnic groups (King, 1997).

1.2.2 ECONOMIC COST OF TYPE 2 DIABETES

As the population ages and urbanisation and industrialisation continues in developing countries, the number of people affected by diabetes will inevitably rise (as projected by the WHO), as will the cost of treatment. As type 2 diabetes accounts for such a high proportion of all diabetes this disease therefore represents the major financial burden of diabetes on health care systems around the world. In the United States, with roughly 16 million diabetics (90-95% type 2 diabetics), one out of every seven
health care dollars is spent on people with diabetes. The major cost of diabetes relates to long-term complications, including retinopathy, neuropathy and premature macrovascular disease. In the United States the estimated annual cost of diabetes has increased from US $20.4 million in 1987 to approximately US $100 million in 1994. In England and Wales, it has been calculated that the treatment of diabetes and its complications accounted for 5-8% of the total direct health care expenditure in 1996, while in Australia, the total costs of diabetes (both direct and indirect) was estimated to be approximately $A1 billion annually in 1996 (McCarthy et al., 1996).

1.2.3 AETIOLOGY OF TYPE 2 DIABETES
Cross-sectional data has indicated that as individuals progress from normal glucose tolerance through impaired glucose tolerance to type 2 diabetes there is an accompanying decrease in insulin sensitivity (Lillioja et al., 1988; Groop et al., 1993). Therefore it appears that insulin resistance becomes increasingly prominent with the progression of the disease. It has been further demonstrated that subjects with impaired glucose tolerance (IGT) have decreased insulin action which is associated with increased insulin secretion while subjects with type 2 diabetes had both impaired insulin action and secretory failure. These results have been taken to indicate that insulin resistance precedes impaired insulin secretion, a hypothesis which is supported by longitudinal studies (Groop et al., 1993; Ferrannini, 1997), although not agreed to by all investigators (Kahn and Porte, 1998).

A longitudinal study conducted in Pima Indians demonstrated that subjects who were later to develop diabetes, exhibited higher fasting and post-load insulin concentrations (when their glucose tolerance was normal) than in controls who did not become diabetic (Saad et al., 1989). The onset of IGT or diabetes was associated with a further increase in fasting insulin concentration, although the progression from IGT to diabetes was associated with little change in the insulin response to oral glucose. Once diabetes had developed, increases in glucose concentrations were associated with progressive declines in fasting insulin concentration and insulin response (Saad et al., 1989).

Early studies by DeFronzo and others characterised the natural alterations in glucose metabolism and insulin secretion during the progression from impaired glucose tolerance to type 2 diabetes. It was found that in individuals with IGT and mild diabetes, fasting plasma insulin concentration is invariably increased and basal insulin secretion is enhanced (Faber et al., 1978; Faber and Damsgaard, 1984; DeFronzo et al., 1992). Fasting plasma insulin continues to rise progressively as fasting glucose
increases from 4.5 to 7.8 mmol/l. When the fasting concentration of glucose exceeds 7.8 mmol/l, insulin secretion drops off markedly, as the pancreas is unable to maintain the high rate of insulin secretion (DeFronzo et al., 1989). At this glucose concentration, hepatic glucose production increases in absolute terms and begins to contribute to the elevation in fasting glucose concentrations (DeFronzo et al., 1989). In subjects with type 2 diabetes, the graph of glucose-stimulated insulin secretion plotted against fasting glucose displays an inverted 'U' shaped curve (Faber and Damsgaard, 1984).

![Graph showing the Starling Curve of the pancreas.](image)

**Figure 1.3** Starling Curve of the pancreas (DeFronzo 1992).

The early stages of type 2 diabetes are characterised by increased insulin secretion, which represents a compensatory response to insulin resistance (DeFronzo et al., 1989). The cause of the late-stage β-cell failure remains unclear, as beta-cell mass is not markedly reduced (Westmark and Wilander, 1978; Stefan et al., 1982), and genetic mutations in the insulin gene are rare (Steiner et al., 1990). Several subsequent prospective studies have examined the relationship between insulin resistance, insulin secretion and the development of type 2 diabetes (Haffner et al., 1990a, Haffner et al., 1990b; Lillioja et al., 1993). From these studies it was determined that insulin resistance predicted twice as many cases of diabetes as insulin secretion, and that these two factors were additive.

A prospective study of approximately 4500 normal, non-diabetic, normoglycemic individuals was undertaken in Malmö, Sweden over a 6 year period (Erickson and
This study demonstrated that a fasting glucose concentration \( \geq 5.5 \text{mmol/l} \) increased the relative risk of developing diabetes two-fold, while a 2-hour glucose \( \geq 8.3 \text{mmol/l} \) increased the risk four-fold (Ericksson and Lindgarde, 1996). In addition, if both of these factors were present and the subjects had a BMI \( \geq 27 \), and a family history of diabetes, the relative risk increased further. Additional risk factors in this study included hypertension, elevated 2-hour glucose and leg pain, and the presence of these risk factors at baseline increased the relative risk of developing diabetes within 6 years to 700 times the risk seen in subjects with a plasma glucose \(< 5.5 \text{mmol/l} \) (Ericksson and Lindgarde, 1996).

A longitudinal, epidemiological study carried out in San Antonio, Texas examined the 8-year incidence of diabetes in initially non-diabetic and normotensive individuals (Haffner et al., 1992). In this study, plasma insulin levels were associated with increased risk of developing diabetes within 8 years, further supporting the hypothesis that insulin resistance precedes, and is predictive of, IGT and subsequently diabetes (Haffner et al., 1992; Ferrannini, 1997).

1.2.4 **THE HERITABILITY OF TYPE 2 DIABETES**

Type 2 diabetes mellitus is widely acknowledged as a genetic disease, although the mode of inheritance, degree of heritability and number of loci involved are yet to be determined. Studies in both humans and rodent models of diabetes have revealed some of the genes that may be involved in the development of type 2 diabetes in humans.

A positive family history of type 2 diabetes has been described as possibly the single most important factor in determining susceptibility to this form of diabetes (Yki-Jarvinen, 1997). This appears to be particularly true if first-order relatives are affected, and the risk increases further if both parents have diabetes (Barnett et al., 1981; Helmiich et al., 1991; Kobberling et al., 1985; Knowler et al., 1981; Lillioja et al., 1987a; Newman et al., 1987; Vaag et al., 1993). The lifetime risk of a sibling or other first-degree relative of a person with type 2 diabetes developing the disease has been estimated to be approximately 40% (Kobberling et al., 1985; Rewers and Hamman, 1995). In high risk populations such as the Pima Indians, a positive family history of type 2 diabetes has been found to be a better predictor of the incidence of diabetes than the combined effects of obesity, gender and physical fitness (Knowler et al., 1981; Lillioja et al., 1987a), although the risks conferred by parental diabetes appear to act synergistically with the diabetogenic effect of obesity (Knowler et al., 1981).
In less highly selected populations, the same effect is also apparent. In American male graduates, a parental history of type 2 diabetes was associated with a three-fold higher risk of type 2 diabetes, an effect which was greater than that conferred by decreased physical activity, increased BMI or a history of hypertension (Helmrich et al., 1991). In identical twins, the reported concordance rates for type 2 diabetes range from 50 to 100% (Barnett et al., 1981; Harris et al., 1987; Newman et al., 1987).

The prevalence of type 2 diabetes varies between different ethnic groups by a factor of about 30 (figure 1.2), and this variability is generally not explained by environmental factors (Bennett et al., 1992). Given this, it appears likely that several genes predispose to type 2 diabetes, however, attempts to identify the susceptibility genes for common type 2 diabetes have been largely unsuccessful, even within defined ethnic groups (Bennett et al., 1992; Groop et al., 1993; Prochazka et al., 1993; Bjorbaek et al., 1994; Elbein et al., 1994).

The genetic defects that contribute to insulin resistance in type 2 diabetes are not yet known and although mutations in the insulin receptor have been found to cause marked insulin resistance, they are extremely rare. Functional abnormalities have also been described in various post-binding mechanisms including reduced tyrosine kinase activity of the insulin receptor, the insulin-sensitive GLUT-4 glucose transporter and of glycogen synthase, although these are likely to be secondary to hyperglycemia or some other aspect of diabetes. Certain polymorphisms of the genes encoding insulin receptor substrate (IRS)-1 (Almind et al., 1993; Clausen et al., 1995), glycogen synthase (Groop et al., 1993; Zouali et al., 1993) and the regulatory subunit of protein phosphotase 1 (Hansen et al., 1995) have been associated with type 2 diabetes and the inheritance of these genetic defects could theoretically contribute to the inherited susceptibility of this disease.

Much more progress has been made in defining the molecular genetics of certain subtypes of type 2 diabetes, namely maturity-onset diabetes of the young (MODY) and the syndromes associated with mutations in the mitochondrial DNA. Unlike most type 2 diabetic patients, individuals with MODY are generally not obese and they have predominant defects in insulin secretion with normal insulin sensitivity. Three MODY loci have been defined to date, MODY1, MODY2 and MODY3. MODY1 was localised to chromosome 20 by a genome-wide scan and has now been characterised as a nonsense mutation in the hepatocyte nuclear transcription factor 4α (HNF4α) (Yamagata et al., 1996a), although the precise mechanism by which this
mutation results in severe diabetes is unknown. MODY2 was identified by linkage studies of a candidate gene, glucokinase (Froguel et al., 1993). Glucokinase mutations account for 50% of MODY in France and have also been detected in other populations (Stoffel et al., 1992; Chiu et al., 1993; Froguel et al., 1993; McCarthy et al., 1993; Shimada et al., 1993; Wajngot et al., 1994). These mutations are relatively rare elsewhere, and do not contribute to late onset type 2 diabetes (Elbein et al., 1993; Elbein et al., 1994). MODY3 has been suggested to be the most important single-gene cause of type 2 diabetes (Elbein et al., 1997). The locus was initially mapped to chromosome 12q by linkage analysis of nonglucokinase-linked French MODY families (Vaxillaire et al., 1995) and accounts for 25% of French MODY diabetes (Elbein et al., 1997). This locus was identified as HNF1α (Yamagata et al., 1996b) and a large number of mutations were subsequently identified in English, French, German, Finnish and American families (Froguel 1996; Elbein et al., 1997).

The MODY loci described do not account for typical type 2 diabetes in the general population. Studies of typical type 2 diabetes have focused heavily on candidate genes implicated from both the known physiology of diabetes, and studies in rodents. In general, no single locus has been identified that can explain the genetic propensity to either type 2 diabetes or insulin resistance among the known candidate genes. Genome-wide screening of families with multiple diabetic siblings has been conducted in several populations, however only suggestive linkage at best has been described (Hanis et al., 1996; Mahtani et al., 1996; Stern et al., 1996; Elbein et al., 1997). Initial studies from multiple ethnic groups suggest that type 2 diabetes will result from a complex interaction of several susceptibility genes and the environment (Elbein et al., 1997). Obesity has been predicted to play an important role in the expression of these loci, although it appears that obesity loci per se are unlikely to define a unique subgroup of type 2 diabetes or that unique obesity loci will be found among type 2 diabetic families (Elbein et al., 1997).

1.2.5 THE THRIFTY GENOTYPE
As discussed previously, the highest rates of type 2 diabetes are seen in populations that have undergone rapid lifestyle changes. The increased susceptibility of traditional-living or hunter-gatherer populations such as Pima Indians, Nauruans and Australian aborigines has been suggested to have an evolutionary basis. In 1962, the American geneticist James Neel questioned why populations such as the American Indians displayed such high prevalence rates of diabetes, and he went further to hypothesise the presence of a ‘thrifty gene’ in this population (Neel, 1962). Neel suggested that under conditions of feast and famine which brought about fluctuating
and often sparse food supplies, people who possessed a thrifty gene were better able to store food as fat during the feast period, and thus demonstrated a survival advantage during times of famine. The phenotypic manifestation of the thrifty gene was proposed to be hyperinsulinemia or insulin resistance. The thrifty gene proposed involved a 'quick insulin trigger' which reduced caloric loss and allowed increased fat storage during periods of relative plenty (Neel, 1962).

The metabolic expression of the thrifty genotype is thought to be through selective tissue insulin resistance and high basal and insulin-stimulated insulin concentrations (Zimmet et al., 1990; Wendorf and Goldfine, 1991; Zimmet, 1992). Insulin resistance in muscle would blunt the hypoglycemia associated with fasting and allow energy storage in fat and liver during feeding, conferring the ability to efficiently convert excess energy to fat in times of plenty. Under modern conditions however, when food is plentiful and high in energy and physical activity levels are low, the presence of the thrifty genotype is disadvantageous as hyperinsulinemia may then result in the development of central obesity, increasing insulin resistance and hyperinsulinemia which eventually leads to pancreatic β-cell failure and type 2 diabetes. In addition significant postprandial hyperglycemia would occur, further exacerbating the cycle of events.

The thrifty gene hypothesis states that our nomadic ancestors of 10,000 years ago possessed a genome that enabled them to respond to seasonal food availability with extreme efficiency and fatten for times of famine (Zimmet et al., 1990; Zimmet, 1992; Dowse and Zimmet, 1993; Zimmet and O'Dea, 1993). The ability to demonstrate fluctuating food supplies in the evolutionary history of the populations who today have high prevalence rates of diabetes is therefore central to this hypothesis. The Pacific Islanders were subjected to periodic and regular food deprivation associated with long migratory canoe voyages, as well as natural phenomena such as droughts and hurricanes. The migration history from South-East Asia into the Pacific indicates three major migrations, with archeological evidence suggesting that New Guinea was first settled by Australoid populations 50,000 years ago. Between 3500 and 5000 years ago, Austronesian speakers arrived in New Guinea and intermarried with the Australoids who were living in the coastal areas. The Austronesian speakers moved on and intermarried with the coastal people of New
Britain, New Hebrides (Vanuatu) and New Caledonia, and by 3000 years ago had moved on to Fiji. They later continued their migration in canoes, moving further east into Polynesia, colonising Samoa, Tahiti and then other parts of Polynesia. The cycle of feast and famine through long canoe journeys and the effects of droughts and hurricanes on food productivity on atoll soil that was often barren would clearly mean that the acquisition of a thrifty gene would represent a survival advantage (Zimmet et al., 1990; Zimmet, 1992; Dowse and Zimmet, 1993; Zimmet and O'Dea, 1993).

In terms of the American Indians, it has been suggested that the thrifty genotype arose through natural selection during the peopling of North America south of the continental glaciers. At least 20,000 years ago early paleo-Indian hunter-gatherers may have been subject to these natural selection pressures as they moved from an unusual midlatitude tundra environment (the ‘ice-free’ corridor) and adapted to more typical mid-latitude environments during which there were periods of alternating feasts and famines (Wendorf, 1989). Populations of American Indians who did not make this transition and remained in high latitudes or migrated south after the glaciers had retreated were not subject to the sudden environmental changes encountered by the Paleo-Indians and therefore the ‘thrifty gene’ metabolism would not have conveyed any advantage. Today those populations (including the Athapaskans, Aleuts and Eskimos) do not exhibit the high rates of diabetes seen in the Pima Indians.

To add further weight to the thrifty gene hypothesis, several polygenic animals models of diabetes and obesity have been described which mirror the pattern of development of diabetes seen in susceptible human populations. A significant proportion of both Psammomys obesus and the spiny mouse (Acomys cahirinus) develop obesity and diabetes when taken out of their natural desert environment and are maintained in captivity on a relatively high-energy diet with a sedentary lifestyle (Shafrir and Gutman, 1993).

The thrifty genotype hypothesis remains controversial, and recently James Hill questioned the presence of the thrifty gene in Arizonan Pima Indians and suggested that behavioural responses to a changing environment may be the key to the development of obesity (Hill 1998). He refers to an ‘environmental creep’ which has
reached the Arizonan Pima Indians, making their food abundant, energy-dense, cheap and good-tasting, and is yet to reach the non-obese Mexican Pima Indians. Hill suggests that most people in the United States live in an environment more similar to that of the Arizonan Pima Indians than of the Mexican Pima Indians. In this environment, populations not necessarily thought to have a thrifty gene are experiencing obesity in epidemic proportions. Without substantial environmental changes, Hill suggests that we will all suffer the same fate as the Arizonan Pima Indians (Hill, 1998).

1.2.6 THE THRIFTY PHENOTYPE

In 1992 Hales and Barker proposed the concept of the ‘thrifty phenotype’. This hypothesis suggests an environmental origin for diabetes that is related to nutritional hardship in utero, with poor fetal and early post-natal nutrition imposing mechanisms of nutritional thrift upon the growing individual (Hales and Barker, 1992). Impaired development of the endocrine pancreas and an increased susceptibility to the development of type 2 diabetes are both then seen as major long term consequences of inadequate early nutrition. Support for this hypothesis comes from several places including data from British, Pima Indian and other populations which shows that low birth weight is associated with increased risk of diabetes and insulin resistance in adult life.

It has been hypothesised that insulin resistance may result from the possible diabetogenic effects of malnutrition in utero and during the first year of life. It is suggested that specific nutritional defects in fetal and early infant life predispose to diabetes, perhaps by compromising the development of the β-cells and possibly by inducing peripheral insulin resistance. Support for this hypothesis has come mainly from a study of the weights at birth and one year of age in a cohort of British men born between 1920-1930 (Hales et al., 1992). IGT or type 2 diabetes was found in 40% of men with a birthweight of 2.5kg or less, compared to 14% in those weighing 4.3kg or more. In addition, IGT was observed in 43% of men weighing 8.2kg or less at 12 months compared with 13% of those weighing 12.3kg or more.

Several subsequent studies have reported findings in support of the hypothesis that the in utero environment plays an important role in the development of diabetes.
(Lithell et al., 1995; Godfrey et al., 1996) although strong arguments have been raised against this hypothesis. Various factors are thought to undermine these studies (Paneth and Susser, 1995) including selection bias of the population, the indirect measurements of nutritional intakes of mothers and babies, with nutritional status inferred from fetal and infant growth, the compounding nature of limited geographical range (it may be that within particular geographical locations other factors such as poor socioeconomic conditions may also be present; Ben-Shlomo and Smith, 1991) and often no account is made of the possible influences of maternal smoking, age, obesity or diabetes on placental weight and birth weight (Clarson et al., 1989; Yki-Jarvinen and Williams, 1997).

The thrifty phenotype hypothesis suggests that although early developmental changes determine susceptibility to diabetes, additional factors such as obesity, aging and physical inactivity which lead to insulin resistance must also play a role in the time of onset and severity of the disease. In addition, the authors suggest that the clustering of metabolic abnormalities demonstrated by syndrome X (hypertension, dyslipidemia and diabetes) can also be explained by the thrifty phenotype hypothesis as these abnormalities are associated because of a common origin; suboptimal development at a particular stage of intrauterine life (Phillips and Barker, 1993).

Another alternative proposed by Meier and Cincotta is that the expression of the thrifty genotype/phenotype is actually regulated by innate circadian rhythms. This hypothesis suggests that rather than genetics, it is seasonality that plays a central role in the evolution of obesity and insulin resistance as a survival advantage (Meier and Cincotta, 1996). Briefly, support for this hypothesis comes from studying vertebrates in the wild with evidence that seasonal obesity is common among vertebrates (from teleosts to mammals). In addition the development and reversal of seasonal obesity can be demonstrated under laboratory conditions of constant photoperiod and food consumption. This annual cycle of obesity is hypothesised to be the net result of changing interactions of multiple circadian neural oscillations within the central nervous system. Temporal synergism of circadian neuroendocrine activities plays a central role in this model as the greatest net daily effect on body weight (maximal fattening) would occur when the stimulus peak (of insulin for example) coincides with the daily interval of greatest target tissue responsiveness (e.g. hepatic lipogenesis). All other phase relations of these two rhythms would thus result in a
gradation of lesser effects. Such a mechanism allows for the precisely timed seasonal
development of obesity in vertebrates in the wild, which is thought to represent the
ancestral predecessor of the 'thrifty gene' hypothesis for human obesity. Again, in a
Westernised society where food availability is not seasonal in either amount or
content this thrifty genome has become detrimental and is essentially turned on
continuously, leading to the pathological consequences of the obese, insulin-resistant
state.

1.2.7 PATHOGENESIS OF TYPE 2 DIABETES
Insulin resistance has been defined as 'the inability of insulin to produce its usual
biological effects at circulating concentrations that are effective in normal subjects
(Yki-Jarvinen and Williams, 1997). In the late 1930s, Himsworth was the first to
make the clinical distinction between insulin-sensitive and insulin-resistant
individuals (Himsworth 1936; Himsworth and Korr, 1939). Some of the actions of
insulin throughout the body include suppression of hepatic glucose production by
inhibition of gluconeogenesis and glycogen breakdown, and the promotion of glucose
transport from the bloodstream into the peripheral tissues.

Glucose circulating in the blood stream is transported to the brain, and several
peripheral tissues, including liver, adipose tissue, skeletal muscle and the
gastrointestinal tract. Most of the glucose from the blood is transported into muscle,
with adipose tissue accounting for only 1% of total insulin-stimulated glucose uptake.
Once the glucose is taken into the tissue it can be stored as glycogen (non-oxidative
glucose uptake) or it can be oxidised to produce energy. With the development of
hyperinsulinemia and insulin resistance, glucose homeostasis is altered and a number
of defects are evident in skeletal muscle, the major site of peripheral insulin resistance
in type 2 diabetes, and in adipose tissue and liver. The nature of these defects will be
discussed below.

Skeletal Muscle:
Within skeletal muscle resistance to insulin-mediated glucose disposal has been
demonstrated in both diabetic and non-diabetic subjects, and it is clear that a normal
fasting glucose concentration can be maintained by individuals with both normal and
impaired glucose tolerance (Hollenbeck and Reaven, 1987). In addition, the finding
that relatively large increases in fasting plasma glucose are associated with only
minor decreases in muscle glucose disposal rates indicates that insulin resistance at
the level of skeletal muscle does not account for the development of significant
hyperglycemia in patients with diabetes, and similarly this defect cannot explain the severity of fasting hyperglycemia in these subjects (Yki-Jarvinen and Williams, 1997).

The nature of the primary biochemical defects in insulin action in muscle remain unknown although there do not appear to be any major disturbances in insulin-receptor binding. Minor reductions in the tyrosine kinase activity of the receptor's β-subunit have been detected although they appear to be secondary to the metabolic consequences of diabetes (Rincon et al., 1996; Yki-Jarvinen and Williams, 1997). Skeletal muscle does show various morphological changes in insulin resistant subjects however, with a decrease in capillary density and an increase in the proportion of 'white', insulin-resistant muscle fibres compared to 'red', insulin-sensitive fibres (Krotkiewski and Bjorntorp, 1986; Lillioja et al., 1987b; Yki-Jarvinen and Williams, 1997). Overall, the effects of insulin resistance on skeletal muscle are not sufficient to account for the increase in glucose concentrations seen with the development of diabetes.

*Adipose Tissue:*

Lipolysis in adipose tissue is extremely insulin sensitive, with free fatty acid (FFA) concentrations found to be half-maximally suppressed at a plasma insulin concentration of 20mU/l (Howard et al., 1979). The sensitivity of FFA levels to rises in plasma insulin concentrations has been demonstrated in both normal and hyperglycemic subjects (Reaven, 1988). Several metabolic changes indicate that adipose tissue in diabetes is also insulin resistant. There is evidence that triglyceride breakdown is enhanced and plasma FFA concentrations are higher in diabetic patients compared to normal subjects with comparable insulin concentrations (Frazer et al., 1985; Groop et al., 1989) and further it appears that plasma FFA levels rise in parallel with plasma glucose (Lillioja et al., 1985). Overall, insulin resistance is associated with an increase in circulating FFAs.

*Pancreas:*

With insulin resistance in the peripheral tissues, circulating glucose concentrations increase and the pancreas responds by increasing insulin secretion, and thus increasing circulating insulin concentration. This results in down regulation of insulin receptors, and may exacerbate insulin resistance in tissues. A vicious cycle ensues, with a progressive rise in plasma glucose up to a point, as seen in 'Starling curve of the pancreas' (figure 1.3), after which point, the β-cell decompensates, insulin secretion falls, and this further exacerbates hyperglycemia (DeFronzo, 1992;
Caterson and Zimmet, 1998). The reason for β-cell failure with increasing glucose concentrations remains unclear, however it has been proposed that hyperglycemia per se plays a role in causing reduced insulin secretion, a phenomenon termed ‘glucose toxicity’ (DeFronzo and Ferrannini, 1991). More recently, however, there is increasing evidence that chronically elevated FFA levels may impair β-cell secretory function, in a phenomenon termed ‘lipotoxicity’ (Sako and Grill, 1990; Zhou and Grill, 1994).

Liver:
The fasting hyperglycemia seen in type 2 diabetes is thought to result from increased HGP. An increase in HGP may be mediated by decreased insulin action in the liver, or increased FFA delivery to the liver (Sindelar et al., 1997). Although several previous studies have reported 2-3 times higher levels of HGP in diabetic patients (Kolteman et al., 1981; DeFronzo et al., 1982; Nankervis et al., 1982; Best et al., 1982; Bogardus et al., 1984; Firth et al., 1986; Campbell et al., 1988) it appears that the measurement of HGP by isotope dilution methods under non-steady conditions in these studies may have overestimated the increase in glucose production by the liver (Yki-Jarvanin et al., 1989; Yki-Jarvanin and Williams, 1997). Under truly steady state conditions (Jeng et al., 1994), it appears that even in patients with the highest plasma glucose concentrations, HGP does not reach the high levels previously reported (DeFronzo et al., 1982; Nankervis et al., 1982; Best et al., 1982; Bogardus et al., 1984; Firth et al., 1986; Campbell et al., 1988; Jeng et al., 1994). It is now clear that subjects with mild hyperglycemia do not have higher levels of HGP than control subjects and those with progressively higher fasting glucose levels show mean increases of at most only 20-30% (Hother-Nielsen and Beck-Nielsen, 1991; Yki-Jarvanin and Williams, 1997).

It seems therefore that HGP is not grossly elevated and cannot solely explain fasting hyperglycemia, especially in mildly hyperglycemic individuals. Rather it appears that modest increases in HGP are found in subjects with more severe hyperglycemia and this contributes to the rise in blood glucose as the glucose generated is not cleared normally by the peripheral tissues. In addition, even ‘normal’ levels of HGP in diabetic subjects do not mean that the liver is acting normally as hyperglycemia should act to suppress HGP. Therefore glucose output from the liver remains inappropriately elevated in the face of hyperglycemia in these subjects (Yki-Jarvanin and Williams, 1997). Insulin resistance at the level of the liver clearly involves post-receptor defects, which may partially be attributable to the increased delivery of FFAs, in part generated by visceral fat.
Increased levels of FFA may contribute to worsening hyperglycemia in several ways. A large increase in plasma FFA concentration has been demonstrated to decrease insulin-stimulated glucose uptake (Bonadonna et al., 1990), and possibly more importantly, increased FFA delivery to the liver stimulates both hepatic FFA oxidation and HGP. The biochemical mechanisms by which hepatic FFA oxidation modulates HGP are encompassed in the Randle cycle (discussed above) and include the stimulation of gluconeogenesis and a decrease in the extraction of insulin from portal blood by the liver.

The changes that occur in muscle (glucose disposal), β-cell (plasma insulin concentration), adipose tissue (plasma FFA concentrations) and liver (glucose production) in various stages of glucose tolerance are summarised in figure 1.4.

**Figure 1.4** Effects of insulin resistance on major tissues in type 2 diabetes (Jung, 1997).
1.2.8 THE METABOLIC SYNDROME

Several of the epidemiological and clinical studies already discussed have also highlighted the association between insulin concentration and various metabolic and physiological abnormalities including hypertension, dyslipidemia (increased VLDL triglyceride levels and decreased HDL cholesterol levels) and glucose intolerance (Modan et al., 1985; Zavaroni et al., 1989; Haffner et al., 1988). This association of symptoms has been termed the 'Metabolic Syndrome' or 'Syndrome X' with insulin resistance proposed as the common underlying pathophysiology of all of these conditions (Reaven, 1988).

Hypertension is accompanied by high triglyceride, low HDL-C, and in some studies, high LDL-C, more frequently than expected by chance. The further association of insulin resistance or hyperinsulinemia, glucose intolerance or diabetes and haematopoietic abnormalities with dyslipidemia has been reported in a number of epidemiological surveys (Logan et al., 1978; Pyorala et al., 1985; Zimmet et al., 1986; Ferrannini et al., 1991; Walker and Alberti, 1993). The favoured explanation for the clustering of these metabolic features appears to involve a central role for hyperinsulinemia as there is evidence for a direct causal role for hyperinsulinemia in initiating and maintaining the principal features of the Metabolic Syndrome. In addition, hyperinsulinemia also plays a direct role in the increased risk of coronary artery disease (Jarrett 1988; Reaven 1988).

This hypothesis has been increasingly disputed however, and another postulated mechanism involves the presence of an inherited or acquired underlying predisposition from which various components of the Metabolic Syndrome can emerge (Hales and Barker, 1992; Barker et al., 1993). This hypothesis proposes that the existence of this predilection, most likely visceral, abdominal adipose tissue, shared in common by many individuals expressing the various components of this Syndrome explains both the clustering of the metabolic factors as well as the dissociation of some elements of the Syndrome from others. The absence, and thus dissociation, of hypertension from the Metabolic Syndrome in a number of populations adds support to this hypothesis and has been widely documented (Alberti et al., 1989; Saad et al., 1990; Asch et al., 1991).

Insulin resistance, therefore plays a central role in the metabolic syndrome as well as in obesity, type 2 diabetes and cardiovascular disease. Insulin resistance is considered
to be a fundamental link between these diseases, and the presence of hyperinsulinemia is associated with increased body mass, as well as with the presence of central obesity.

1.3 AETIOLOGY OF HUMAN OBESITY

1.3.1 HERITABILITY OF BODY FAT

The familial nature of obesity is well established, although the precise importance of genetic factors is still debated. The heritability of body mass index (BMI) has variably been reported to be between zero and 90%, although these differences may result from different study designs and the ability to separate the effects of genes from those of the environment shared by relatives living in the same household. Twin studies provide a unique opportunity to separate genetic effects from environmental effects, with monozygotic (MZ) twins being genetically identical and dizygotic (DZ) twins sharing on average, 50% of their genes.

The study of MZ twins reared apart and MZ twins reared together provides an opportunity to assess the effects of genes independent of confounding environmental influences. Several studies have provided evidence that shared familial environment has little effect as heritability estimates for BMI were very similar between MZ twins reared apart and MZ twins reared together (MacDonald and Stunkard, 1990; Stunkard et al., 1990; Price and Gottesman, 1991). According to these studies the heritability of BMI is in the range of 40 to 70%. Twin studies consistently yield higher estimates of heritability than family studies, while adoption studies generate the lowest heritability values (Bouchard et al., 1993b). In addition, the many studies conducted to date do not provide consistent evidence for a maternal, or early environmental effect on the inheritance of BMI (Bouchard, 1994).

Several adoption studies have demonstrated the effect of shared family environment on BMI is negligible, with the BMI of adoptees more closely resembling that of their biological, rather than adoptive parents for both adults and children (Price et al., 1987; Stunkard et al., 1986; Sorensen et al., 1989; Sorensen et al., 1992; Sorensen et al., 1992b; Sorensen et al., 1994; Sorensen et al., 1998). A large Norwegian study of approximately 75,000 individuals estimated BMI heritability in a large number of first- and second-degree relatives (Tambs et al., 1991). Correlations obtained were
about 0.12 for spouses, 0.20 for parent-offspring, opposite-sexed siblings and DZ twins, 0.26 for same-sexed siblings and 0.58 for MZ twins. Correlations among second degree relatives were found to be close to zero (Tambs et al., 1991). By using statistical modeling, the heritability level for BMI was estimated to be 40% with a moderate, but significant effect of cultural transmission. A similar estimate was obtained in a study of over 9000 individuals in Tecumeh, Michigan with the total familiarity of BMI estimated to be between 30 and 40% (Longini et al., 1984). Family studies have provided relatively low estimates of heritability (Vogler et al., 1995) which appears to be concordant with the low parent-offspring correlations observed for BMI in large population-based studies (Lissner et al., 1994).

Harris et al. (1995) demonstrated that there is a genotype by sex interaction on BMI heritability. In a population-based Norwegian twin panel, the quantitative genetic effects on BMI were not the same in men and women, with some genetic effects found to be shared by both sexes, but others were unique to each sex (Harris et al., 1995). In addition, Comuzzie et al. (1995) reported that the effect of the major gene component of fat mass depended on sex and accounted for slightly more variance in females than in males. In contrast, a longitudinal National Heart, Lung and Blood Institute Twin Study indicated no environmental effect of age on BMI heritability (Fabrisz et al., 1994) although recent studies have suggested that the age trends seen in the familial effects of BMI may be primarily genetic, but that the trends diverge in different directions between childhood and adulthood (Bouchard et al., 1998).

Although the heritability of BMI is well characterised, much less is known about measures such as fat mass, percent body fat or fat-free mass. Ramirez et al. (1993) reported that the familiarity for percent body fat (estimated by bioelectrical impedance) was about 25%, while in the Quebec Family Study, the familiarity of percent body fat (underwater weighing) was estimated to be about 36% (Savard et al., 1983; Bouchard, 1985). In Quebec twins and adopted and biological families, the genetic heritability for each of percent body fat and fat free mass was estimated to be about 25%, with an additional 30% due to familial environmental effects (Bouchard et al., 1988).
The regional distribution of fat is also thought to contain a genetic component, with data from the Quebec Family Study demonstrating that the distribution of subcutaneous fat between the trunk and the limbs or between the upper and lower parts of the body is characterised by a heritability ranging from 30% to 50% of the variance, after adjusting for total fat mass (Bouchard et al., 1993a). Similar results were found in the Iowa Women's Health Study, with the mother-daughter and sister-sister correlations for the WHR determined to be approximately 40% after adjustment for BMI (Sellers et al., 1994). Other WHR familiarity estimates vary from 28% in adoptive and biological families (Perusse et al., 1988) to 40-50% in traditional nuclear families (Donahue et al., 1992), and over 60% in longitudinal family data (Towne et al., 1993).

Genetic and environmental correlations among eight skinfolds were determined by pedigree analysis of Mexican Americans from San Antonio, Texas (Comuzzie et al., 1994). In this study, the genetic correlations were found to be highest between skinfolds from the same body region, while environmental correlations remained relatively constant across all skinfolds (Comuzzie et al., 1994). This study indicated that the pattern of central versus peripheral fat distribution is primarily a function of genetic correlations (Comuzzie et al., 1994). In French-Canadian biological and adoptive families trunk-to-extremities contrast was found to account for about 40% of the familial variance in fat distribution, of which 18% was determined to be genetic in origin (Bouchard, 1988; Bouchard et al., 1998). This estimate of genetic effect increased to 30% when adjusted for BMI and as high as 50% when adjusted for total body fat content (Bouchard, 1991).

The heritability of abdominal visceral fat is not well characterised although familial correlations in the Quebec Family Study for abdominal total, abdominal visceral and abdominal subcutaneous fat tissue were reported to be 0.35 (Perusse et al., 1996). The results of many studies examining regional body fat distribution suggest that there is a significant familial influence on the pattern of subcutaneous fat distribution and that adjusting for total body fat usually increases the familiarity estimate (Bouchard et al., 1998).
Studies conducted in MZ twins suggest that in addition to overall adiposity and regional adiposity having strong genetic components, the individual response to alterations in energy balance also appears to be influenced by genes. When MZ twins were overfed by 1000 kcal/day for a period of 22 consecutive days the differences in response were found not to be randomly distributed, with significant intra-pair resemblance observed despite the short length of the study (Poehlman et al., 1986a; Bouchard et al., 1988). The intrapair resemblance in response to overfeeding was estimated to be 0.88 for total fat mass and 0.76 for fat-free mass. A short term negative energy balance experiment was also performed, with exercise used to induce a 1000 kcal/day deficit over RMR and baseline energy intake maintained (Poehlman et al., 1986b). Again, in this study the changes were small, and only fat-free mass was associated with the twin lines, with changes found to be more similar within pairs than between pairs.

Two long term studies were also performed in which the protocols used in the first series of experiments were maintained but lengthened to 100 days (Bouchard et al., 1990). In the long term over feeding study there was significant within pair resemblance in changes in body weight and fat mass, with three times more variance in response seen between pairs than within the pairs of MZ twins. This study suggested that some individuals are more at risk of gaining fat than others when energy surplus is clamped at the same level and all subjects are confined to a sedentary lifestyle (Bouchard et al., 1990). In addition, the within twin pair response to the standardised energy surplus suggests that the amount of fat stored is likely to be influenced by the genotype. Similarly, in the long term energy deficit study, although there were large individual differences in response, significant intrapair resemblance was observed for the changes in body weight, fat mass, body energy content and sum of 10 skinfolds (Bouchard, 1994). Again these results suggest that subjects with the same genotype are more alike in responses to a given treatment than subjects with different genotypes (Bouchard, 1994).

With these studies establishing unequivocally that there is a genetic component to the inheritance of body mass and body fat, emphasis has now shifted from the question of whether or not obesity is influenced by genes to which specific genes are involved. Several sampling strategies have been used in an attempt to identify the genes

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involved in human obesity, these include: random sampling, sampling of large sibships or pedigrees, sampling of phenotypically extreme individuals and sampling of special populations (Comuzzie et al., 1998). Each one of these methods has its advantages although the statistical power of any association will be influenced by the sampling strategy employed. Once the population to be studied is determined, the best genetic approach must also be considered in order to conduct a powerful examination of the genes which may be involved in the development of human obesity. There are several types of approaches that may be considered.

1.3.1.1 Candidate Gene Studies
Candidate genes are genes identified \textit{a priori} on the basis of their effects in animal models or suspected physiological involvement in a particular disorder (Comuzzie et al., 1998). The study of candidate genes has been the primary strategy used in the search for potential obesity genes, and there are now many candidate genes described which can be linked with the obesity phenotype. Most of the candidate genes examined come from analysis of the pathophysiology and genetics of the development of obesity in a growing number of rodent models of obesity.
Table 1.3 Candidate Genes of Obesity

<table>
<thead>
<tr>
<th>Gene</th>
<th>Phenotype</th>
<th>Chromosomal Location</th>
<th>Mouse</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASIP</td>
<td>obesity</td>
<td></td>
<td>2-88.8</td>
<td>20q11.2-q12</td>
</tr>
<tr>
<td>CPE</td>
<td>obesity</td>
<td></td>
<td>8-32</td>
<td>4q28</td>
</tr>
<tr>
<td>LEP</td>
<td>obesity</td>
<td></td>
<td>6-10.5</td>
<td>7-q32</td>
</tr>
<tr>
<td>LEPR</td>
<td>obesity</td>
<td></td>
<td>4-46.7</td>
<td>1-p31</td>
</tr>
<tr>
<td>TUB</td>
<td>obesity</td>
<td></td>
<td>7-51.45</td>
<td>11p15.4-p15.5</td>
</tr>
<tr>
<td>UCP1</td>
<td>energy balance</td>
<td></td>
<td>8-37</td>
<td>4q31</td>
</tr>
<tr>
<td>UCP2</td>
<td>energy balance</td>
<td></td>
<td>7-50</td>
<td>11q13</td>
</tr>
<tr>
<td>UCP3</td>
<td>energy balance</td>
<td></td>
<td>7-50</td>
<td>11q13</td>
</tr>
<tr>
<td>MC3R</td>
<td>feeding behaviour</td>
<td></td>
<td>2-100</td>
<td>20q13</td>
</tr>
<tr>
<td>MC4R</td>
<td>feeding behaviour</td>
<td></td>
<td>1 or 18 predicted</td>
<td>18q21.3-q22</td>
</tr>
<tr>
<td>POMC</td>
<td>obesity (leptin levels)</td>
<td></td>
<td>12-4</td>
<td>2p23.2</td>
</tr>
<tr>
<td>NPY5</td>
<td>appetitc regulation</td>
<td></td>
<td>8-33</td>
<td>4q31-15.1</td>
</tr>
<tr>
<td>MSTN</td>
<td>skeletal muscle growth</td>
<td></td>
<td>1 or 2 predicted</td>
<td>2q32.1</td>
</tr>
<tr>
<td>CCKAR</td>
<td>satiety</td>
<td></td>
<td>5-34.0</td>
<td>4p15.1</td>
</tr>
<tr>
<td>TNFA</td>
<td>obesity</td>
<td></td>
<td>17-19.1</td>
<td>6p21.3</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>adipocyte differentiation</td>
<td></td>
<td>6-53.0</td>
<td>3p25</td>
</tr>
<tr>
<td>ADRB3</td>
<td>adipocyte differentiation</td>
<td></td>
<td>8-10</td>
<td>8p11.1-p12</td>
</tr>
</tbody>
</table>

Abbreviations-ASIP: agouti signalling protein; CPE: carboxypeptidase E; LEP: leptin; LEPR: leptin receptor; TUB: tubby; UCP: uncoupling protein; MCR: melancortin receptor; POMC: pro-opiomelancortin; NPYR: neuropeptide Y receptor; MSTN: myostatin; CCKAR: cholecystokinin A receptor; TNFα: tumor necrosis factor α; PPAR-γ: peroxisome proliferator activated receptor-γ; ADR3: beta-3-adrenergic receptor (Comuzzie and Allison, 1998).

Although candidate genes studies have not been overly successful in identifying the major genes involved in human obesity, several individuals have been identified in which the development of obesity can be associated with a specific mutation in a particular candidate gene.
Two independent groups have recently described frameshift mutations in the human melanocortin-4 receptor (MC4R) which are associated with a dominant form of early onset obesity (Vaisse et al., 1998; Yeo et al., 1998). The MC4R is a seven-transmembrane G-protein coupled receptor which has been implicated in the central regulation of body weight (Fan et al., 1997). It is a high-affinity receptor for α-melanocyte stimulating hormone (MSH), a product of the proopiomelanocortin (POMC) gene, which inhibits feeding when administered to rodents (Fan et al., 1997). Both of the mutations described alter the sequence of the receptor in such a way as to result in the expression of a non-functional receptor. Both of the subjects described (a 35-year old woman and a four-year old male) were of normal birth weight, but developed obesity progressively from infancy (Vaisse et al., 1998; Yeo et al., 1998), with a history of hyperphagia. The subjects did not display clinical endocrine abnormalities and had appropriate circulating leptin concentrations for their body weight. The frameshift mutation described in human MC4R represents the first locus at which mutations are associated with dominantly-inherited morbid obesity in humans (Yeo et al., 1998).

A genetic defect has also been detected in the human POMC gene itself (Krude et al., 1998). Two mutations were identified which resulted in structural changes in the POMC gene and a complete loss of POMC-derived adrenocorticotropic hormone (ACTH), α-MSH and β-endorphin. Alpha-MSH has been shown to have a dual role in regulating food intake and influencing hair pigmentation, and children found to have the POMC mutations were initially identified on the basis of alteration in pigmentation and ACTH deficiency (Krude et al., 1998). The individuals with the mutations had severe early-onset obesity, adrenal insufficiency and red hair pigmentation. These children represent the first reported cases of a POMC-deficiency syndrome and these observations underline the importance of the POMC gene in the aetiology of human obesity.

Within the OB gene, Montague et al. (1997a) have described two separate mutations which are linked to morbid obesity in subjects homozygous for the mutation. The subjects were identified initially as having abnormally low leptin concentration for their level of adiposity. Subsequently these subjects were determined as having altered OB gene sequences which resulted in the production of abnormal leptin, the
protein product of the OB gene, that was not secreted from the adipocyte (Montague et al., 1997a). At 8 and 2 years old, both subjects had body weights greater than the 99.9th centile of body weight for children that age. Similarly, Strobel et al. (1998) identified a recessive mutation in the OB gene which resulted in severe obesity and very low concentrations of circulating leptin when present in the homozygous form. Two adult subjects identified with this mutation were also found to have endocrine abnormalities and reproductive defects (Strobel et al., 1998). A mutation in human OB-R, the receptor for leptin, has also been identified (Clement et al., 1998). This mutation was found within a family with a strong prevalence of early-onset obesity and shown to result in morbid obesity and pituitary dysfunction in subjects homozygous for the mutation. The mutations in OB and OB-R are described in further detail in Section 1.6.2.4.

These studies represent an increasing list of subjects identified with monogenic defects leading to the development of severe human obesity. Although these mutations are rare, they nonetheless emphasise the importance of these candidate genes in the regulation of body weight in humans.

1.3.1.2 Association Studies
Table 1.3 summarises the positive associations between candidate genes and BMI or body fat phenotypes (Chagnon et al., 1998). The concept of association refers to a situation in which the correlation of a genetic polymorphism with a phenotype is investigated (Bouchard et al., 1998). These studies are usually performed on samples of unrelated individuals and if a significant association is observed with a polymorphism at a candidate gene locus, there are several likely explanations: 1) the locus is causally linked to the phenotype, 2) the locus is in linkage disequilibrium with the trait locus as a result of natural selection or chance, or 3) this is an artifact due to population admixture (Bouchard et al., 1998). This genetic approach appears to be particularly useful for the identification of genes that make only a minor contribution.

Several of the candidate genes listed in Table 1.3 have been examined in many different populations, including OB-R, tumour necrosis factor (TNF)-α and β3 adrenergic receptor (β3AR). Studies examining the leptin receptor are discussed in
Section 1.6.2 The β3AR was examined as a candidate gene for obesity in several human populations. A missense mutation at codon 64 of the gene that results in a tryptophan substitution by arginine was detected with allelic frequency of 0.31 in Pima Indians, three times higher than observed in Caucasians or African Americans (Walston et al., 1995). When Pima Indians were subsequently genotyped for the polymorphism, no linkage was observed between the mutation and obesity or type 2 diabetes, although there was linkage with age of onset of type 2 diabetes and subjects homozygous for the mutation also tended to have lower adjusted metabolic rates (Walston et al., 1995). The same polymorphism was examined in a Finnish population however in this study there was no difference in allelic frequency between diabetic and non-diabetic subjects, although there was an association with higher waist-to-hip ratio in women and with early onset of diabetes (Widen et al., 1995). In a French cohort of morbidly obese individuals the β3AR mutation was associated with increased capacity to gain weight (Clement et al., 1995). These studies indicate that the β3 AR may be involved in susceptibility to gain weight and age of onset of diabetes, although a host of other studies examining this gene have failed to find an association between this mutation and obesity (Elbein et al., 1996; Gagnon et al., 1996; Li et al., 1996; Hinney et al., 1997; Moriarty et al., 1997).

Overexpression of the TNF-α gene in adipose tissue is observed in obese humans, with weight loss found to correct this defect. In addition, adipose tissue TNF-α is strongly correlated with fasting insulin levels, an indirect measure of insulin resistance. For these reasons, the TNF-α gene was examined as a candidate gene for obesity in Pima Indians. Pima Indian families were scored for three simple sequence length polymorphism (SSLP) markers near the TNF-α gene and sib-pair analysis revealed a marker 10 kb from TNF-α was linked to percent body fat and BMI. Subsequent analysis of the entire coding region of the TNF-α gene identified only a single polymorphism in the proximal promoter although this was not associated with percent body fat in Pima Indians (Norman et al., 1995). A subsequent study involving Spanish individuals examined the polymorphism in the TNF-α promoter and found an association with body fat and leptin concentration (Fernandez-Real et al., 1997).
Overall, candidate gene studies have provided importance evidence suggesting that the genes identified as potential regulators of energy balance in humans play only a minor role in the development of obesity in the general population. It is likely that some of the genes which determine body weight in humans are not yet identified and so other genetic approaches must also be employed.

1.3.1.3 Linkage Studies

Linkage refers to the co-segregation of a marker or a trait locus together in families and linkage analysis can be performed with candidate gene markers or with a variety of other anonymous markers such as microsatellites (Bouchard et al., 1998). Although there are many candidate genes now identified, to date, most linkage studies of candidate genes have failed to reach the critical threshold of a LOD score (logarithm of the likelihood ratio for linkage) greater than 3, although suggestive linkage (LOD>2) has been reported in several studies (see table 1.4). The reasons for the low linkage seen between candidate genes and the obesity phenotype is unclear, however it may highlight the relatively small sample sizes and therefore low statistical power of many human studies (Comuzzie and Allison, 1998).
Table 1.4 Chromosomal regions linked to obesity related phenotypes through linkage studies.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosomal location</th>
<th>N pairs</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1S202</td>
<td>1q31-32</td>
<td>large pedigree</td>
<td>BMI</td>
</tr>
<tr>
<td>ACP1</td>
<td>2p25</td>
<td>&gt;300</td>
<td>BMI</td>
</tr>
<tr>
<td>GRL</td>
<td>5q31-q32</td>
<td>88</td>
<td>BMI&gt;27</td>
</tr>
<tr>
<td>BF</td>
<td>6p21.3</td>
<td>&gt;168</td>
<td>skinfolds</td>
</tr>
<tr>
<td>TNFA,</td>
<td>6p21.2</td>
<td>&gt;255</td>
<td>% body fat</td>
</tr>
<tr>
<td>Tnfr24, D6S273, 291</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLO1</td>
<td>6p21.2</td>
<td>&gt;168</td>
<td>skinfolds, relative weight</td>
</tr>
<tr>
<td>SUR (D11S419)</td>
<td>11p15.1</td>
<td>67</td>
<td>BMI&gt;21</td>
</tr>
<tr>
<td>D1S200</td>
<td>1p32-p22</td>
<td>137 sibships</td>
<td>BMI</td>
</tr>
<tr>
<td>ADA to MCR3</td>
<td>20p12 to 20q13.3</td>
<td>258</td>
<td>% body fat; BMI, fasting leptin</td>
</tr>
<tr>
<td>D2S1788</td>
<td>2p21</td>
<td>pedigrees</td>
<td>serum leptin</td>
</tr>
<tr>
<td>LEP region</td>
<td>chromosomal 7</td>
<td>&gt;1000</td>
<td>BMI</td>
</tr>
<tr>
<td>KEL</td>
<td>7q33</td>
<td>402</td>
<td>BMI, skinfolds</td>
</tr>
<tr>
<td>ESD</td>
<td>13q14.1-q14.2 194</td>
<td></td>
<td>% body fat, skinfolds</td>
</tr>
<tr>
<td>ADA</td>
<td>20q12-q13.11</td>
<td>428</td>
<td>BMI, skinfolds</td>
</tr>
<tr>
<td>PI</td>
<td>22q11.2-qter</td>
<td>&gt;168</td>
<td>relative weight</td>
</tr>
<tr>
<td>D3S2432</td>
<td>3p24.2-p22</td>
<td>874</td>
<td>% body fat</td>
</tr>
<tr>
<td>D11S2000, 2366</td>
<td>11q21-q22</td>
<td>874</td>
<td>% body fat</td>
</tr>
<tr>
<td>MCSR</td>
<td>18p11.2</td>
<td>242-289</td>
<td>BMI, %6 skinfolds, fat mass, % body fat</td>
</tr>
<tr>
<td>ADA, MC3R,</td>
<td>20q12-q13</td>
<td>258</td>
<td>BMI, %6 skinfolds</td>
</tr>
<tr>
<td>DS0S17, 120</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ACP1 acid phosphatase; SUR sulfonylurea receptor; MCR melanocortin receptor; GRL glucocorticoid receptor; BF properdin factor B; TNFir24 dinucleotide repeat marker locus near the tumor necrosis factor α gene; GLO1 glyoxylase 1; LEP leptin; KEL Kell blood group; ESD esterase D; ADA adenosine deaminase; P1 P blood group; BMI body mass index (Cornuzzie and Allison, 1998).
Table 1.4 provides a summary of the studies showing significant evidence of linkage with obesity-related phenotypes (Chagnon et al., 1998) and several interesting findings using this approach are listed. A study of Mexican Americans revealed a significant multipoint linkage for sum of extremity skinfolds and D7S514, an anonymous marker near the OB gene on chromosome 7q31.3. This marker was reported to account for over 50% of the variation in this trait (Duggirala et al., 1996). In a study of French Canadian families, four phenotypes were examined using three markers spanning a 5cM region around the gene for uncoupling protein (UCP)-2 on chromosome 11 (Bouchard et al., 1997). Based on a two-point linkage analysis, significant linkage was detected between the marker D11S911 and resting energy expenditure (Bouchard et al., 1997). Several groups have also investigated linkage between the chromosomal region encompassing the OB gene and obesity-related phenotypes. A recent meta-analysis of these studies suggests there may be linkage between this region and BMI (Allison and Heo, 1998).

1.3.1.4 Genome-Wide Scans
To date the results of two genome wide scans have been reported, one in the Mexican Americans (Comuzzie et al., 1997) and the other in Pima Indians (Norman et al., 1997). In a genome wide scan, linkage analysis is conducted using a series of anonymous polymorphisms, spaced at relatively constant intervals over the entire genome to identify quantitative trait loci (QTLs) affecting the phenotype of interest. Using this approach no prior assumption is made regarding the potential importance of specific genes or chromosomal regions and the results of the scan are used to identify candidate chromosomal regions, or in some cases positional candidate genes which then become the focus of subsequent analyses (Comuzzie and Allison, 1998).

In the Pima Indian population, a genome scan for percent body fat was conducted using an ~10cM map for 283 sibling pairs from 88 nuclear families. In two-point linkage analysis, two genomic regions were detected that showed evidence of suggestive linkage to percent body fat, one at chromosome 3p24.2-p22 and the other at chromosome 11q21-22 (Norman et al., 1997). As yet, no obvious candidate genes have been mapped to either chromosomal region (Comuzzie and Allison, 1998). The second genome scan in 10 families of Mexican Americans (representing 459
individuals and comprising 5667 relative pairs) were evaluated for several obesity-related phenotypes in a 20cM genomic scan (Comuzzie et al., 1997). Significant linkage was reported for QTLs on chromosome 2 (~74cM from the tip of the short arm) and chromosome 8 (~65cM from the tip of the short arm) and leptin levels, and the QTL on chromosome 2 and fat mass. This QTL was estimated to account for 47% of the variance in serum leptin levels and 32% of the variance in fat mass (Comuzzie et al., 1997). Both the regions identified contain known positional candidate genes for obesity including the POMC gene (chromosome 2) and the β3 AR gene (chromosome 8).

1.3.2 ENERGY BALANCE

The concept of energy balance is inherent to any discussion about body weight regulation and the development of obesity. Energy balance refers to the difference between metabolisable energy intake and total energy expenditure. The maintenance of a stable body weight and composition over many years requires a precise balance between energy intake and energy expenditure, as well as macronutrient intake and macronutrient oxidation. The precision required is exemplified by the finding that a daily excess of 5% of energy intake over total energy expenditure can lead to approximately 6-7 kg weight gain in a period of 5 years (Weinsier et al., 1993).

1.3.2.1 Energy Intake

_Sociological Influences_

Longitudinal and cross-sectional studies of thousands of adult Finns have illustrated the importance of socioeconomic and behavioural factors as determinants of body weight (Rissanen et al., 1991). In these studies, the prevalence of obesity was found to be inversely related to the level of education and physical activity, but positively associated with alcohol consumption in men and parity in women. Similarly, the risk of weight gain (>5kg/5yr) was greatest for individuals with a low level of education, little physical activity, or heavy alcohol consumption, and for those who got married or stopped smoking. The impact of these factors on body weight gain were found to be of similar strength to the impact of the metabolic factors that will be discussed here.
Nutritional Influences

The majority of dietary studies conducted between 1969 and 1981 were unable to demonstrate a clear difference in eating behaviours between normal- and over-weight individuals (Blundell and Stubbs, 1998). In addition obese individuals have been found to have higher total daily energy expenditure than the rest of the population (Prentice et al., 1989; Prentice et al., 1996). The increased energy expenditure has been proposed to be a result of both the increased energy cost of physical activity in the obese and the highly sedentary nature of the general population to whom they are compared (Blundell and Stubbs, 1998). Studies of dietary compliance suggested that people tended to under-report their intakes of energy during study periods (Bingham and Cummings, 1985; Bingham et al., 1989; Bandini et al., 1990; Black et al., 1991; Goldberg et al., 1991). This appears to be more of a systematic problem in obese compared to lean individuals (Bingham et al., 1989; Black et al., 1991). The findings of significant under-reporting of food intake in the obese, together with their absolutely higher energy requirements tends to support the contention that during study conditions, obese subjects are actually consuming less than their usual energy intakes (Spitzer and Rodin, 1981; Durrant et al., 1982; Bandini et al., 1990). The problems associated with food intake data must always be considered when interpreting data of this nature.

When examining the role of energy intake and, in particular, overconsumption of energy it is important to consider the composition of foods that form the basis of overconsumption. A large body of literature exists regarding the ability of particular macronutrients to influence energy intake, however the post-ingestive satiety effect of carbohydrate, protein and fat will only be examined briefly. Under both free-living and laboratory conditions, where fat contributes disproportionately to energy density, the available data suggests that there is a hierarchy in the satiating efficiency (the ability of each MJ of each macronutrient ingested to suppress subsequent energy intake) of the dietary macronutrients, such that protein suppresses subsequent energy intakes to a greater extent than carbohydrates, which in turn suppresses subsequent energy intake to a greater extent than dietary fat (Stubbs et al., 1995; Blundell and Stubbs, 1998).
Several studies have established that higher energy intakes are associated with higher BMI and body weight (WHO Study Group, 1990; Miller et al., 1990). In addition, it appears that the proportion of energy coming from fat is an important determinant of overall energy intake, and levels of fat intake appear to correlate with BMI and particularly with percent body fat (Dreon et al., 1988; Romieu et al., 1988; Tremblay et al., 1989; George et al., 1990; Miller et al., 1990; Tucker et al., 1992). Similarly, several studies have found a relationship between reduced dietary fat intake and reduced body fatness (Prewitt et al., 1991; Sheppard et al., 1991).

There are several reasons why the consumption of dietary fat may be a greater risk factor for subsequent increased body weight when compared to the other macronutrients. One of these reasons is the energy-dense nature of fat (36 kJ/g), compared to both carbohydrate (16 kJ/g) and protein (17 kJ/g) (Flatt, 1978; Flatt et al., 1985; Donato et al., 1985). The metabolisable energy derived from fat is therefore, more than twice that derived from the other macronutrients per gram of consumption.

In addition to the energy-density of dietary fat, this macronutrient is also converted to, and stored as, adipose tissue for a much smaller energy cost (2-7%) than the energy required to convert and store carbohydrate as adipose tissue (24-28%) (Flatt, 1978; Flatt et al., 1985). This finding suggests that dietary fat will be preferentially stored within the body, whereas little dietary carbohydrate will be used for synthesis of adipose tissue under normal feeding conditions because of the high energy costs (Bjorntorp and Sojostrom, 1978; Acheson et al., 1984; Acheson et al., 1988).

Another reason for the association between increased intakes of dietary fat and increased body weight is that fat oxidation is not directly regulated by fat intake (Flatt et al., 1985; Schutz et al., 1989). Overall substrate oxidation rates are dictated by the body's need to regenerate the energy used in performing its metabolic functions, maintaining temperature, and in moving, which all depend primarily on an individual's size and physical activity. It appears that evolution has led to the development of metabolic and endocrine regulatory processes that give higher priority to the adjustment of amino acids and glucose oxidation to protein and carbohydrate intakes than to the goals of maintaining fat and energy balance (Flatt, 1995). This is
not surprising given the importance of proteins and the need to ensure a sufficient supply of glucose to the brain, despite the fact that glycogen reserves in humans are limited to 0.4-0.8 kg (Bjorntorp and Sojostrom, 1978). In contrast, the body’s fat stores hold an energy reserve that is two orders of magnitude greater than its glycogen stores, so the daily differences in the overall energy balance can be readily accommodated by gains or losses of fat.

The composition of the fuel mix oxidised is controlled primarily by changes in circulating substrate and hormone concentrations (Flatt et al., 1974). These are most markedly influenced by nutrient intake because the impact of changes in substrate concentrations during the postprandial phase of their rates of utilisation is enhanced by the release of insulin. Insulin promotes storage of carbohydrates, amino acids and fats and it induces an increase in carbohydrate oxidation, revealed by a marked postprandial rise in the respiratory quotient (RQ); Acheson et al., 1982). The release of catecholamines and glucagon between meals activates the mobilisation of the body’s glycogen and fat stores. This, as well as a concomitant decline in insulin secretion, serves to provide glucose and fatty acids to sustain energy production and to ensure an adequate supply of glucose in the circulation (Cahill, 1971; Flatt, 1995).

The RQ is defined as the ratio between the amount of carbon dioxide produced for a given amount of oxygen consumed by the body, and provides an index of the relative proportion of carbohydrate and fat oxidised. The RQ of carbohydrate oxidation is 1.0 while fatty acid oxidation gives a value of approximately 0.7, depending on the composition of the fatty acids oxidised. A resting post-absorptive RQ in a non-obese individual ranges between 0.8 and 0.85 (Schutz, 1993), indicating that approximately 50% of the body’s energy needs are met by fat oxidation and 40% by carbohydrate utilisation, with the remaining 10-15% supplied by protein oxidation. Therefore, a person with a low RQ will naturally oxidise more fat and thereby store less as adipose tissue, than someone with an RQ closer to 1.0, who will naturally oxidise lower amounts of fat and greater amounts of carbohydrate, thereby driving all dietary fat into storage. The RQ therefore represents an important determinant of energy partitioning and substrate utilisation.
The oxidation of both protein and carbohydrate is spontaneously adjusted to protein and carbohydrate intakes. In contrast, fat oxidation is not regulated by fat intake. As a result, over a period of time longer than a few days, the gap between energy intake and total energy expenditure is accommodated by changes in the body's fat content. Thomas et al. (1992) have demonstrated that within one week of either a high carbohydrate or a high fat diet, protein and carbohydrate equilibrium is usually achieved, although this is not the case for fat balance. It appears that there is no short-term metabolic response stimulating fat oxidation to correct the deviation from fat equilibrium (Schutz and Jequeir, 1998).

In Pima Indians, a high RQ (suggestive of higher carbohydrate oxidation and lower fat oxidation) has been shown to predict future weight gain (Zurlo et al., 1990), and a similar finding has been reported in Caucasians (Eckel, 1992). In a study of weight gain over three years, individuals with a high RQ were more likely to gain weight than those with a low RQ. Studies have also shown that 24-hour RQ may vary among individuals within a population but is relatively constant within any one individual. In addition, RQ is a familial trait, and similar to metabolic rate, it is thought to be genetically determined and predictive of subsequent weight change (Ravussin et al., 1995).

1.3.2.2 Energy Expenditure

Energy expenditure is obviously a fundamental component of the energy balance equation. Energy expenditure can be estimated easily and accurately using indirect calorimetry by measuring respiratory gas exchange using either a ventilated hood or doubly-labeled water.

Energy expenditure can be divided conceptually into several components, with the resting metabolic rate (RMR) representing the largest of these. The RMR refers to the amount of energy expended while resting, several hours after meals or physical activity, and thereby represents the metabolic costs of maintaining the integrated systems of the body at rest (Danforth, 1985). The RMR accounts for approximately 65-75% of daily energy expenditure and is slightly higher than the basal metabolic rate (BMR), which is measured early in the morning, at the longest interval from meals or physical activity (Danforth, 1985).
Physical activity is the most variable component of daily energy expenditure and can account for the use of many calories in very active people. Physical activity can be divided into two main components: spontaneous physical activity (including fidgeting; SPA) and the energy cost of unrestricted activity. There are large differences between individuals with respect to SPA and therefore energy expenditure under the artificial conditions of a respiratory chamber. The finding by Ferraro et al. (1991) showing a negative correlation between the energy expended for activity on a metabolic ward and the degree of obesity accords with data in Pima Indians which suggests that physical activity is inversely related to both age and adiposity (Ravussin et al., 1991). Sedentary daily energy expenditure seems to be affected to some extent (10-15%) by many factors including physical fitness, meal composition, cigarette smoking, anxiety and temperature (Ravussin and Swinburn, 1993). However it is suggested that it is unlikely these factors are sufficient to explain large weight gains (Ravussin and Swinburn, 1992). Only differences in physical activity may be important in the pathogenesis of obesity (Ravussin and Swinburn, 1992).

The third component of energy expenditure is the energy expended above RMR over the several hours (usually 6 hours) following a meal. This is referred to as the thermic effect of food (TEF) and is usually expressed as a percentage of the ingested
calories measured above the RMR over the several hours following its ingestion (recently reviewed by Junge and Bray, 1997). The largest part of the TEF following a meal is due to the metabolic costs of processing the meal, which includes the cost of digestion, absorption, transport and storage. This has been referred to as ‘obligatory’ thermogenesis and can be predicted theoretically from the metabolic costs of these processes (Danforth, 1985; Junge and Bray, 1997). The TEF however, is usually greater than the obligatory or predicted energy expenditure and this difference is referred to as ‘facultative’ thermogenesis. Facultative thermogenesis is thought to represent increased SNS-induced thermogenesis and the operation of futile pathways of metabolism which are wasteful of energy, such as cycling of glucose through 3-carbon intermediates. The TEF accounts for approximately 10% of daily energy expenditure, but can vary depending on the amount and composition of the diet (Ravussin, 1993). Adaptive thermogenesis (AT) is a component of energy expenditure which expends energy and produces heat but does not yield energy. This component includes adaptation to cold and non-shivering thermogenesis and in fact overlaps the energy expenditure within RMR and TEF (Danforth, 1985).

Up to 85% of the variation in the overall rate of energy expenditure across a population can be explained by differences in fat mass and fat-free mass, with the remaining variation accounted for by activity of the sympathetic nervous system (SNS), androgens, thyroid hormones, age, gender, body temperature and genetics. Fat-free mass has been demonstrated to be the major single determinant of resting energy expenditure, 24-hour energy expenditure and free-living energy expenditure. Studies conducted in Pima Indians have demonstrated that variations in energy expenditure per volume of forearm tissue explained 40-50% of the variation in resting energy expenditure, after adjustment for differences in fat mass, fat-free mass, age and sex (Ravussin and Swinburn, 1993). As the absolute resting energy expenditure is positively correlated with fat-free mass, obesity is clearly associated with a high absolute resting energy expenditure. In addition, a low relative resting energy expenditure (expressed in relation to fat-free mass) is a risk factor for subsequent weight gain (Ravussin et al., 1988).

Ravussin et al. (1988) demonstrated a correlation between energy expenditure and rate of weight change in Pima Indians, with the estimated risk of gaining more than
7.5 kg in body weight over a two year period increased four-fold in subjects with a low adjusted 24-hour energy expenditure (200 kcal/day below predicted values). In subjects followed for four years, the risk of gaining more than 10 kg of body weight was approximately 7 times greater in subjects with the lowest adjusted RMR (Ravussin et al., 1988). In this study the rate of adjusted 24-hour energy expenditure was estimated to be responsible for up to 40% of the weight change. Familial analysis of RMR and energy expenditure revealed that values for adjusted 24-hour energy expenditure aggregated in families, indicating that a low rate of energy expenditure may contribute to the aggregation of obesity in families (Ravussin et al., 1988). Similar findings were reported by Griffith et al. (1990) in a study of 4-5 year old children. In this study, the RMR and estimated total energy expenditure of those children with an obese parent were 16% and 22% lower, respectively than those children whose parents had never been obese (Griffith et al., 1990).

1.3.3 CONTROL OF ENERGY BALANCE
The control of energy balance involves alterations in energy partitioning and substrate utilisation, as well as the regulation of feeding behaviour and energy expenditure. A number of factors have been found to play a role in the regulation of energy balance and some of these are discussed in detail below.

1.3.3.1 Central Regulation of Feeding Behaviour
Historically, feeding was believed to be controlled by reciprocally interacting centres in the ventromedial hypothalamus (VMH) and the lateral hypothalamic areas (LHA). This view was based on studies that showed that stimulation of the VMH suppressed food intake, whereas stimulation of the LHA increased food intake (Schwartz et al., 1992a). Destruction of the VMH was shown to induce hyperphagia and obesity, whereas LHA lesions caused anorexia and weight loss (Schwartz et al., 1992a). This 'dual-centre' hypothesis of the regulation of food intake has subsequently become more detailed as increasing amounts of information are generated regarding the roles of various brain regions, neurotransmitter systems, and hormonal and neural signals originating within the gut. Several of the host of neurotransmitters and peptides implicated as hypothalamic regulators of feeding are discussed below.
1.3.4 NEUROPEPTIDES:

1.3.4.1 Neuropeptide Y

Neuropeptide Y (NPY) is a 36 amino acid peptide expressed widely in the mammalian brain. Its role in energy homeostasis focuses on the NPYergic neurons in the hypothalamic arcuate nucleus (ARC) that project to the paraventricular nucleus (PVN), an area rich in NPY terminals and a site of NPY release (Frankish et al., 1995). These projections function in close association with both corticosterone and insulin, two circulating hormones involved in glucose homeostasis. NPY is distinguished from other appetite-modifying peptides by its potency, behavioural specificity, and ability to induce obesity.

NPY injected into the PVN of mice, dramatically alters energy balance in a co-ordinated fashion, causing hyperphagia and reduced energy expenditure, by inhibiting the sympathetic stimulation of brown adipose tissue. Overall obesity results, and it can be induced within a few days by continued NPY administration (Vettor et al., 1994). Insulin secretion is also stimulated by NPY administration, favouring the net energy gained to be stored as triglyceride. Several groups have demonstrated that NPY mRNA levels are inversely correlated with insulin concentrations (Davies and Marks, 1994; Strack et al., 1995). Sipols et al. (1995) demonstrated that insulin injected into the third ventricle of insulinopenic diabetic rats reduces the increased NPY mRNA levels in the ARC and decreases feeding. Dryden and Williams (1996) however, suggest that this does not necessarily point to a direct relationship between the two peptides as the very high local insulin levels in the hypothalamus may activate other receptors.

Other endocrine effects of NPY include stimulation of corticosterone secretion, which favours the availability and utilisation of glucose, and also allows NPY to integrate feeding with the hypothalamic-pituitary-adrenal (HPA) axis, which is important in nutrient balance. While central administration of insulin is known to inhibit feeding, possibly by inhibiting the ARC-NPY neurons, glucocorticoids are thought to stimulate NPY synthesis. The NPY gene carries glucocorticoid-response elements upstream in its coding region, and the ARC-NPY neurons carry abundant low affinity glucocorticoid receptors (Tempel and Leibowitz, 1993). NPY mRNA is positively correlated with corticosterone, and together insulin and corticosterone have been
reported to explain over 50% of the total variance in body weight (Strack et al., 1995).

The receptors involved in NPY-induced feeding have been investigated for many years. Initially it was thought that the response was mediated by the Y1 receptor subtype, as the Y1 agonist [Leu31, Pro28]-NPY stimulated food intake to about the same extent as the natural ligand NPY1-36. However, as the NPY2-36 was more potent than either NPY1-36 or [Leu31, Pro28]-NPY, this conclusion was questioned (Kalra et al., 1991; Stanley et al., 1992). A novel receptor, Y5, has since been isolated by expression cloning (Weinberg et al., 1996; Gerald et al., 1996; Hu et al., 1996). This receptor shows varying degrees of sequence homology to Y1 and displays a higher affinity for NPY2-36 than for the Y1 agonist [Leu31, Pro28]-NPY. In addition to the PVN, this receptor is localised in a number of hypothalamic sites, however whether the Y5 receptor alone is responsible for mediating the actions of NPY remains to be determined (Gerald et al., 1996).

Hypothalamic NPY mRNA or protein content has been shown to be increased in several models of genetic obesities (Beck et al., 1990; Sanacora et al., 1990; Schwartz et al., 1991; Bchini-Hooft et al., 1993; Dryden et al., 1994). It has been reported that in normal rats the NPY levels increase with fasting, and this increase is corrected by administration of insulin (Sanacora et al., 1990; Schwartz et al., 1991; Schwartz et al., 1992a; Schwartz et al., 1992b). In obese rodents, this regulation appears to be abnormal, with hypothalamic NPY levels found to be high even in the fed state, and not further increased by fasting. In addition, the high levels of NPY in the hypothalamus are not normalised by insulin (Schwartz et al., 1991; Schwartz et al., 1992b).

In an effort to further characterise the role of NPY, studies were conducted in which NPY was infused intracerebroventricularly (ICV) into lean rats, and the effects compared to lean rats infused with saline. These studies recorded behavioural and hormonal alterations, with NPY-infused rats found to rapidly increase food intake, and subsequently increase body weight and body fat mass, as well as increases in insulinemia and basal and stress-induced corticosteronemia (Zarjevski et al., 1993; Vettor et al., 1994; Zarjevski et al., 1994; Sainsbury et al., 1996a). Changes in fat
metabolism were also found, with increased adipose tissue lipoprotein lipase activity (Zarjevski et al., 1993; Zarjevski et al., 1994).

Further studies examined the effects of NPY administration when hyperphagia was prevented. These studies showed that even in the absence of hyperphagia, high hypothalamic NPY levels altered the metabolism of the animal in such a way as to prepare it store energy, either by producing actual changes favouring fat cell storage, or by resulting in a state of insulin resistance which favoured glucose channeling away from utilisation and into fat synthesis and storage pathways (Vettor et al., 1994; Zarjevski et al., 1993; Zarjevski et al., 1994; Cusin et al., 1990a,b).

Adrenalectomy is known to reverse obesity syndromes in rodents (Rothwell et al., 1984b), and the effects of NPY were examined in adrenalectomised rats and compared to non-adrenalectomised, control rats. The infusion of NPY into the control rats produced all of the abnormalities summarised above, however, NPY infused into adrenalectomised rats produced none of the hormonal or metabolic changes expected (Sainsbury et al., 1997). This latter study indicates that while NPY is an important peptide in the aetiology of obesity syndromes, central glucocorticoids are required for the effects of NPY to be seen (Rohner-Jeanrenaud and Jeanrenaud, 1997).

Genetically obese, ob/ob mice, have elevated levels of NPY mRNA in the hypothalamus, and recently, Erickson et al. (1996a) developed an ob/ob NPY ‘knock-out’ mouse, in which ob/ob mice were crossed with mice with a mutant NPY allele. Interestingly, the NPY"−−" ob/ob mice demonstrated reduced food intake and increased energy expenditure and were therefore less obese than normal ob/ob mice (Erickson et al., 1996a,b). These results suggest that NPY is required for full manifestation of the ob/ob phenotype and thereby implicate NPY-containing neural pathways as mediators of the hyperphagia, hypometabolism and endocrine alterations resulting from chronic deficiency of the OB protein (Erickson et al., 1996b).
1.3.4.2 Glucagon-like peptide-1

Glucagon-like peptide (GLP)-1 is a cleavage product of preproglucagon that is normally secreted from the gut by nutrient stimulation and potentiates the secretion of insulin (Loren et al., 1979). GLP-1 is also produced in the CNS and the sequence conservation of this peptide in all mammals suggests that GLP-1 may have an important physiological role (Orskov, 1992). Peripheral administration of GLP-1 has been shown to powerfully stimulate insulin secretion, suppress glucagon secretion and delay gastric emptying (Kreymann et al., 1988; Ritzel et al., 1995).

Turton et al. (1996) examined the central appetite-modulatory effects of GLP-1 and demonstrated that ICV administration of GLP-1 suppresses feeding (by 63% over the first two hours of the dark phase), while administration of a potent GLP-1 antagonist (exendin (9-39)) prior to GLP-1 completely abolishes the inhibitory effect of GLP-1 on food intake. In addition it was demonstrated that when exendin (9-36) alone was administered to animals satiated after a night’s feeding, there was an increase in 2-hour food intake compared with control animals (Turton et al., 1996). When exendin (9-39) was administered to fasted animals there was no effect on food intake. Interestingly at the neuronal levels, exendin (9-39) co-administered with GLP-1 completely inhibited the GLP-1-induced activation of the PVN and the central nucleus of the amygdala, however, when exendin (9-36) was administered on its own it had no effect on neuronal activation (Turton et al., 1996).

These results suggest that GLP-1 is a satiety factor as endogenous GLP-1 appears to be present in satiated animals but absent from fasted animals. In fact GLP-1 levels in the periphery have been demonstrated to increase in response to ingestion (Holst, 1994). In support of this, when NPY and GLP-1 are both injected into an animal, GLP-1 causes a decrease in the NPY-stimulated food intake. In contrast when NPY, GLP-1 and exendin (9-39) are all injected to satiated animals, the animals increase food intake over and above that seen following NPY alone (Gunn et al., 1996). The pathway through which GLP-1 exerts its inhibition on feeding remains unknown, however identification of this pathway and the interaction of GLP-1 with other regulatory peptides will provide useful information on the regulation of food intake.
1.3.4.3 Melanocortins

The melanocortins are peptides cleaved from the proopiomelanocortin (POMC) precursor polypeptide. In mammals, POMC gene expression is limited to ARC neurons that project to areas that participate in energy homeostasis, such as the PVN (Kiss et al., 1984). These brain areas also express melanocortin receptors, specifically MC3 and MC4 receptors, and agonists of these receptors elicit anorexia, whereas antagonists have the opposite effect (Fan et al., 1997). The endogenous melanocortin implicated most strongly in the control of food intake is α-melanocyte-stimulating hormone (α-MSH).

Melanocyte stimulating hormone (MSH) is produced during the processing of POMC with desacetyl-MSH (d-MSH) normally acetylated on the N-terminus to produce α-MSH, which binds with high affinity to MC3 and MC4 receptors (Schioth et al., 1997). In relation to obesity, the yellow agouti mouse shows an increased ratio of d-MSH to α-MSH in the pituitary (Shimizu et al., 1989b) and pharmacological studies in agouti mice demonstrate inhibition of feeding after central administration of α-MSH, in contrast to the stimulatory effect with peripheral injection of MSH (Shimizu et al., 1989b). The hyperphagia, reduced sympathetic tone and enhanced metabolic efficiency of the agouti mouse appears to be the result of over-expression of the agouti gene product in the brain, where it modulated MSH and antagonises the melanocortin receptor, MC4, in the hypothalamus (Lu et al., 1994).

ICV administration of MCII, a non-selective MC4R agonist, acutely suppresses food intake in ob/ob and agouti mice as well as fasted mice and mice injected ICV with NPY (Fan et al., 1997). Central administration of a MCR antagonist with partial selectivity for the MC-4 receptor was found to increase nocturnal food intake in normal mice (Fan et al., 1997). The agouti-related protein (AGRP), another product of ARC neurons, shares sequence homology with agouti and is an antagonist of MC3 and MC4 receptors (Graham et al., 1997). Transgenic overexpression of AGRP also produces an obesity syndrome (Graham et al., 1997).
1.3.5 OTHER REGULATORS OF FEEDING

1.3.5.1 Galanin

Galanin (GAL) is a 29-residue brain-gut peptide which, like NPY, is a potent stimulator of feeding when injected centrally. Galanin stimulates feeding and alters metabolism through local hypothalamic circuits although NPY and GAL are anatomically and functionally distinct (Leibowitz, 1995). The GAL system that is related to feeding behaviour and metabolism involves a population of neurons in the anterior portion of the PVN which project locally within the PVN, and contain a high concentration of GAL receptors. These neurons also project ventrally to the median eminence to control pituitary hormone secretion (Merchenthaler et al., 1993).

The behavioural and endocrine actions of GAL in the PVN are very different to those of NPY (Tempel et al., 1988; Kyrkouli et al., 1990; Tempel et al., 1990; Chae et al., 1995; Leibowitz, 1995). They include an increase in fat ingestion in addition to carbohydrate intake, a response that occurs in the same animals that respond with a specific increase in carbohydrate intake after NPY administration. Injection of the substituted galanin (1-12) fragment M40 into the PVN has been reported to decrease spontaneous fat intake, and to block the orexigenic effects of exogenously applied GAL (Leibowitz, 1992). ICV administration of M40, as well as the more potent analogue C7, also inhibited galanin-induced feeding, but had no effect on food intake after an overnight fast (Corwin et al., 1993; Crawley et al., 1993).

In fact, the effect of GAL on overall food intake or bodyweight warrants further investigation as Smith et al. (1994) demonstrated that although GAL induces acute hyperphagia, this appears to be followed by compensatory hypophagia. In addition, this group reported that repeated ICV administration of GAL for 14 days did not increase either food intake or weight gain during that period. One explanation proposed for the apparent lack of effect of GAL on body weight is that although GAL reduces energy expenditure and inhibits SNS activity (Mendendez et al., 1992; Corwin et al., 1993), unlike NPY, GAL has little effect on carbohydrate or fat utilisation (Stanley et al., 1986).
1.3.5.2 Cholecystokinin

Cholecystokinin (CCK) was the first well characterised endogenous peptide that has an anorectic action. Functioning as a paracrine hormone, CCK is released from secretory cells and nerve fibres in the upper intestine where it works locally to stimulate pancreatic secretion and gall bladder contraction. The peptide also inhibits gastric emptying by constricting the pyloric sphincter (Moran, 1996). CCK is one of several peptides secreted from the gut during meals that when administered exogenously reduces meal size. This was established in the early 1970s when it was found that administration of CCK to rats before the time of food availability caused a dose-dependent decrease in meal size (Gibbs, 1973).

The mechanism of action of CCK appears to be via CCK_A receptors on sensory endings of the vagus nerve, as evidenced by the finding that vagotomy completely blocks both the anorexic and brain activation induced by peripheral administration of CCK (Li et al., 1995). Several stable CCK agonist analogues, including those specific for the CCK_A receptor subtype, have proven to be effective anorectic agents in animals (Asin et al., 1992; Hirosee et al., 1993). In addition Mamoun et al. (1995) demonstrated that the ability of endogenously released CCK-8 (the octapeptide form of CCK) to induce satiety depends on the composition of the diet.

Although satiety peptides can alter the size of an individual meal, their repeated administration does not alter body weight. When CCK-8 is automatically administered to rats at the start of each spontaneous meal, the size of each meal is reduced, however the animals appear to compensate by initiating more meals, and thereby maintaining bodyweight (West et al., 1984). Therefore the effect of CCK to potently alter food intake over the course of an individual meal has only limited influence on adiposity, although investigations into agonists and antagonists of CCK have suggested that CCK may have a central satiety effect which could be used to develop anti-obesity drugs.

Rose et al. (1996) demonstrated that the membrane-bound isoform of tripeptidyl peptidase II is a CCK-inactivating enzyme found in neurons responding to CCK. Specific inhibitor of this enzyme was produced which had a significant satiety effect when administered to fasted rats, and significantly reduced spontaneous food intake at
the start of the dark period, the time of most feeding in control rats (Rose et al., 1996). This study highlighted the possibilities associated with manipulation of the CCK-pathways on the regulation of food intake.

1.3.5.3 Insulin

Insulin was the first hormonal signal to be identified as a contributor to the regulation of energy balance (Schwartz et al., 1992a). Insulin has been known for a long time to be secreted in proportion to adipose mass with plasma levels changing in response to excess or deficits of caloric intake (Bagdade et al., 1967). High concentrations of insulin receptors are found on CNS neurons, in areas known to be important in the regulation of food intake in the hypothalamus of animals (Baskin et al., 1987) and humans (Hopkins et al., 1997). The finding that insulin is present in the cerebrospinal fluid (CSF) at concentrations proportional to circulating plasma levels (Woods et al., 1978; Wallum et al., 1987), also indicates that insulin enters the brain to regulate neuronal function independent of its ability to stimulate glucose uptake in peripheral tissues. Entry of insulin into the CSF has been examined in many species (Schwartz et al., 1992a; Schwartz et al., 1991; Steffens et al., 1988) and it appears that a specialised transport system facilitates the passage of insulin across the blood-brain barrier epithelium, with subsequent diffusion throughout the neurophil interstitial fluid (ISF) and entry into the CSF (Schwartz et al., 1991; Pardridge, 1986; Schwartz et al., 1990).

Insulin injections into the brain reduce food intake, meal size and weight gain and stimulate the sympathetic nervous system (Woods et al., 1979; Iked et al., 1986; Plata-Salaman and Oomura, 1986; Plata-Salaman et al., 1986; Florant et al., 1991; Foster et al., 1991). In each case there is a dose-dependent reduction in food intake, which is slowly progressive over time, so while there is no immediate suppression of a single meal, 24-hour food intake is suppressed. This effect has been found to be evident within several hours and is maximal within 24-hours to 7 days, depending on the dose and species studied (Porte et al., 1998). Insulin, therefore is a central catabolic hormone that promotes a state of negative energy balance, which directly contrast its peripheral actions.
In the periphery, insulin has well established anabolic effects. Therefore during a period of positive energy balance induced by over-feeding, plasma insulin levels rise which promotes fat storage peripherally but simultaneously influences CNS regulatory sites to suppress food intake and limit weight gain (Porte et al., 1998). In the regulatory feed-back loop hypothesised by Porte et al. positive energy balance should result in expansion of adipose stores, an increase in CNS insulin delivery and subsequently inhibition of food intake. Conversely, during a period of negative energy balance, the peripheral anabolic action of insulin is reduced due to decreased food intake and alternative non-carbohydrate sources of fuel are metabolised to meet energy needs. The insulin deficiency will immediately stimulate increased food intake through pathways within the CNS, thereby facilitating the restoration of body adiposity (Porte et al., 1998).

1.3.5.4 Hypothalamic-pituitary-adrenal (HPA) axis

Corticotropin-releasing hormone (CRH) is known to have a regulatory role in the control of the hypothalamic-pituitary adrenal axis, however included in the CRH-induced responses are changes in feeding behaviour and metabolism, favouring a state of negative energy balance (Arase et al., 1988; Egawa et al., 1990; Rothwell, 1990; Glowa et al., 1992; Menzaghi et al., 1993). CRH is synthesised in the PVN and other forebrain areas, and there is extensive overlap between CRH neurons and NPY-containing terminals in the PVN. Evidence that these two systems overlap includes the findings that CRH blocks (Heinrichs et al., 1993), and immunotoxic lesion of CRH neurons enhances (Menzaghi et al., 1993), the orexigenic action of NPY in rats.

When CRH is injected centrally there is a marked suppression of food intake coupled with stimulation of sympathetic outflow that increases lipolysis and energy expenditure and raises blood glucose while inhibiting insulin secretion. Chronic central administration of CRH results in a sustained reduction in food intake and body weight in both lean and obese animals (Arase et al., 1988; Arase et al., 1989a; Arase et al., 1989b; Glowa et al., 1991; Hotta et al., 1991). Urocortin, a neuropeptide related to CRH, is 30-times more potent than CRH in suppressing feeding and more selective in binding to the CRH$_2$ receptor, which may mediate the anorectic response (Spina et al., 1996).
A role of endogenous CRH or urocortin in energy balance is suggested by evidence that antagonists of CRH receptors or manipulations that reduce CRH biosynthesis potentiate feeding (Heinrichs et al., 1993). In states of glucocorticoid insufficiency induced by adrenalectomy, CRH expression in the PVN is enhanced. This is associated with decreased bodyweight, impaired recovery of weight loss due to food deprivation, and experimental induced obesity (Glowa et al., 1992). Interestingly, inhibition of CRH may be mediated in part by inhibition of endogenous NPY, which normally potentiates eating and weight gain. Administration of CRH reduces the NPY-elicited feeding and decreases NPY gene expression in lean and obese rats. In addition, adrenalectomy, which enhances CRH expression in the PVN reduces NPY mRNA in the ARC (Beyer et al., 1988; Bray et al., 1990; Ponsaile et al., 1993). Thus conditions associated with changes in eating behaviour and body weight have opposite effects on CRH and NPY.

1.3.5.5 Glucocorticoids
Corticosteroid hormones (cortisol in humans; corticosterone in rodents) are produced in the adrenal gland, particularly in periods of stress (Munck et al., 1984; Dallman, 1993). These hormones reach many peripheral organs via the circulation and, in general, they act to enhance the availability of glucose in the blood by inhibiting cellular glucose uptake and affecting gluconeogenesis in the liver (Joels and de Kloet, 1995). Due to their lipophilic nature, glucocorticoids easily cross the blood-brain barrier and act in the hypothalamus, and the pituitary, as part of a neuroendocrine feedback loop, through which these steroids control their own secretion (Dallman, 1993).

Hypersensitivity and/or hyper-responsiveness of the hypothalamo-pituitary-adrenal (HPA) axis is viewed as a central lesion of endocrine malfunction in human visceral obesity (Bjorntorp et al., 1997). Other endocrine abnormalities in visceral obesity, including diminished secretions of sex steroids and growth hormones, may be derived from the perturbation of the HPA axis as both corticotropin releasing hormone (CRH) and cortisol are known inhibitors of gonadal and growth hormones (Chrousos and Gold, 1992). Maximal stimulation of the adrenals (Marin et al., 1992) and the pituitary (Pasquali et al., 1996) results in elevated activity of distal parts of the HPA in women.
The HPA is controlled by central glucocorticoid receptors via a negative feedback mechanism. These receptors (type I and type II) act in part via their permissive interaction with neurochemical systems that modulate nutrient balance (Tempel and Leibowitz, 1994). Under conditions of obesity and repeated stress, the adrenal steroids are elevated, resulting in chronic disturbances in the steroid-neurochemical interactions. In the absence of these steroids, such as after adrenalectomy, all forms of obesity are attenuated (Gray et al., 1996). The type I receptor is activated under conditions of low basal levels of glucocorticoids and functions tonically throughout the daily cycle to sustain feeding, particularly fat intake, and enhance fat deposition. Fat intake occurs at a fairly constant level across the feeding cycle in almost every meal, although in rodents, it rises towards the second half of the active phase (Leibowitz and Hoebel, 1998). The type II receptor, in contrast, is activated under conditions of moderate to high levels of glucocorticoids, which are reached during early hours of the active phase in rodents, and during periods of stress (Tempel and Leibowitz, 1994). A primary function of this receptor is to replenish and defend the body’s carbohydrate stores through both ingestion and storage, ensuring adequate glucose supplies to the brain (Sapolsky et al., 1986; Virgin et al., 1991).

Cortisol (and corticosterone) and insulin are well known to have antagonistic actions, particularly in their effects on glucose uptake and metabolism (Cigolini and Smith, 1979; Bjorntorp 1992a; Bjorntorp, 1993). This antagonism is also evident in their behavioural actions, where insulin is secreted in proportion to the body’s fat mass, acts to reduce food intake and attenuate the stimulatory action of steroids on feeding. The interaction between cortisol and insulin may occur within the brain as in peripheral tissues, possibly involving differential effects on brain neurochemicals such as NPY (Joels and de Kloet, 1995). In adipose tissue, cortisol in the presence of insulin increases the pathways for lipid accumulation, and inhibits pathways of lipid mobilisation, with a net effect on triglyceride retention. Both sex steroids and growth hormone exert opposite and balancing effects. Elevated cortisol and insulin as well as low sex steroid and growth hormone will thus result in an efficient accumulation of triglycerides in adipose tissue. As visceral adipose tissue contains a higher density of specific steroid hormone receptors, these effects will be more pronounced here.
compared to other adipose tissue depots (Bjorntorp, 1992b; York, 1993; Bjorntorp, 1995b; Bjorntorp, 1996).

The mechanism of visceral fat accumulation through abnormal activity of the HPA axis is supported by clinical observations and intervention studies. Dysbalance between the lipid accumulating hormones, cortisol and insulin, and the lipid mobilising hormones, growth and sex steroid hormones, are seen in several conditions with visceral fat accumulation. These include Cushing's Syndrome (high cortisol and insulin, low sex steroids and growth hormone), menopause or aging (low sex steroids and growth hormone), excess alcohol intake (periodically elevated cortisol, low sex steroids), smoking (periodically elevated cortisol), total growth hormone deficiency (low growth hormone) as well as depression and anxiety (elevated cortisol).

The paraventricular nucleus (PVN) has been recognised as a key hypothalamic area for the integration of both inhibitory and stimulatory inputs to feeding behaviour. The integrative function of the PVN arises from its innervation by neurons which project from a variety of areas involved in food intake, including brainstem, VMH and lateral hypothalamus (Schwartz et al., 1992a). The activity of these neurons, which release neurotransmitters and peptides (discussed above) is influenced by both intrinsic (e.g. nutritional, metabolic, gastrointestinal, emotional and cognitive stimuli) and extrinsic (e.g. temperature, light/dark cycles) factors. Defense of a regulated level of body weight, therefore, may arise via the interaction of metabolic and nutritional factors (possibly humoral) to modify the 'gain' on neural pathways which impinge on hypothalamic integration centers such as the PVN (Schwartz et al., 1992a).

1.3.5.6 Sex Hormones
The clearance of testosterone increases as sex hormone binding globulin (SHBG) decreases. In men, SHBG and both total and free testosterone levels are negatively associated with BMI and WHR (Seidell et al., 1990; Pasquali et al., 1991; Simon et al., 1992; Phillips, 1993; Haffner et al., 1993a; Haffner et al., 1993b; Haffner et al., 1994). In addition both total and free testosterone were negatively associated with the concentrations of insulin and c-peptide, independent of adiposity, or body fat distribution (Phillips, 1977; Lichtenstein et al., 1987; Seidell et al., 1990; Pasquali et
In general, increased testosterone levels are associated with decreased insulin resistance and a more favourable body fat distribution in men. Castrated male rats show decreased insulin sensitivity that is improved by low dose testosterone administration (Holmang and Bjorntorp, 1992). Although few prospective studies have been performed, Marin et al. (1992) demonstrated that administration of testosterone to centrally obese, hypogonadal, middle-aged men reduces central adiposity and decreases insulin resistance.

Changes in metabolic fuels are known to influence reproduction through actions at multiple sites. These include key sites in the brain and involve alterations in the activity of gonadotropin releasing hormone neurons and estradiol-binding effector neurons (Leibowitz and Hoebel, 1998). The ovarian steroids estradiol and progesterone play a major role in this process, producing changes in nutrient ingestion, partitioning and utilisation of fuels (Laferriere and Wurtman, 1989; Wade and Schneider, 1992).

Estradiol is associated with decreases in eating behaviour, body weight and adiposity in adults (Wade and Schneider, 1992). Ovariectomy produces the opposite effect, with animals found to gain weight in a manner that is reversible with estradiol. These effects may be mediated through the influence of estradiol on hypothalamic peptide systems, such as NPY, CRH and CCK (Swanson and Simmons, 1989; Bonavera et al., 1994; Baskin et al., 1995; Butera et al., 1996). An example of this is seen after ovariectomy. With estradiol replacement in ovariectomised animals, there is a reduction in NPY gene expression in the ARC and NPY release in the PVN, which are subsequently associated with decreased food intake and loss of body weight. The reverse pattern is seen with CRH and CCK. Estradiol stimulates the production or function of these peptides in the PVN, which acts normally to inhibit feeding and decrease weight gain. During high chronic levels of estradiol therefore, this combination of hypothalamic events (enhanced catabolic signaling combined with impaired compensatory activation of anabolic pathways) leads to decreased body weight.
Progestrone is another important gonadal steroid and it has been found to have diverse actions in relation to estradiol which may involve an early enhancing effect followed by an inhibition. When estradiol inhibits NPY therefore, progesterone actually stimulates the production of NPY in estradiol-primed animals (Laferriere and Wurtman, 1989), suggesting an antagonism between the two steroids. In contrast, GAL production is activated by estradiol alone, but to a greater extent in the presence of progesterone (Gabriel et al., 1992; Merchenthaler et al., 1993). This resulting pattern of increased NPY and GAL production has been suggested to explain the increased caloric intake, particularly fat intake, and body weight seen in females during puberty, a time when the gonadal steroids and peptides rise to peak levels (Leibowitz et al., 1991; Alexander et al., 1994; Leibowitz, 1995) The role of progesterone therefore contrasts with that of estradiol. Progesterone builds energy stores through behavioural as well as metabolic actions while estradiol has anorexic and lipolytic actions (Wade and Schneider, 1992; Puerta et al., 1996; Wade et al., 1996).

Many cross-sectional (Lanska et al., 1985; den Tonkelaar et al., 1989; den Tonkelaar et al., 1990; Ley et al., 1992; Razay et al., 1992; Zamboni et al., 1992; Kirchengast, 1993; Kotani et al., 1994; Poehlman et al., 1995a; Svendsen et al., 1995; Troisi et al., 1995; Hunter et al., 1996; Panotopoulos et al., 1996; Tremoilierces et al., 1996; Pasquali et al., 1997) and longitudinal (Lindquist, 1982; Poehlman et al., 1995b; Akahoshi et al., 1996; Bjorkelund et al., 1996) studies have examined the changes in adiposity associated with transition through menopause in women (Tchernof and Poehlman, 1998). Despite conflicting reports, in general, natural menopause transition appears to be related to modest increases in total fatness. However, central body fatness has been found to increase significantly at menopause, an effect found to be independent of age (Tchernof and Poehlman, 1998).

The focus of this dissertation is on the regulation of energy balance. A number of factors involved in this regulation were discussed above, however there are still many gaps in our knowledge of the primary determinants of energy balance in humans. As studies examining energy balance in humans can be difficult, often studies are
conducted in suitable animal models. A number of models are available for research into obesity and body weight regulation, and these will be discussed below.

1.4 ANIMAL MODELS OF OBESITY AND TYPE 2 DIABETES

As outlined earlier, both obesity and diabetes in humans exhibit high degrees of heritability however these diseases most likely represent complex, polygenic traits, which are also significantly influenced by environmental interactions. The identification of the genetic factors which predispose humans to obesity and diabetes is problematic and is complicated by factors such as the heterogeneity of the diseases, age-dependent penetrance, gene-environment interactions (diet, drug-use and physical activity level) and gene-gene interactions (i.e. susceptibility genes interacting with disease genes) (Naggert and Nishina, 1995).

The use of animal models for the study of human diseases such as obesity and diabetes has allowed the discovery of anatomical, neurochemical and endocrine systems involved in the regulation of food intake and energy expenditure. Historically, animal models of obesity have been focused on rodent species, however study of a limited number of non-rodent models such as non-human primates have also yielded important knowledge in this field.

The use of animal models for the study of human disease is advantageous for several reasons, including that animals can be inbred and their environments strictly controlled. The phenotype of rodent species can therefore be controlled and also more easily defined (York and Hansen, 1998). The ability to directly and precisely measure specific end points and outcomes in animals models such as food intake, body composition or neuropeptide activity also allows for a greater understanding of obesity and diabetes. These measures may be indirect, subject to large error or impossible in humans (York and Hansen, 1998).

The major concern regarding the use of animal models to study human diseases is the relevance of the animal model studied. A mutation in the homologous gene in mice does not always lead to the same phenotype seen in humans (Erickson et al., 1996). While the genes identified to date through animal experimentation may or may not be
involved in the development of obesity or diabetes in humans they may help to
further define the obese/diabetic phenotype and provide new avenues for the

For ease of classification the animal models used widely for the study of obesity and
type 2 diabetes may be sub-divided broadly into three groups: monogenic, polygenic
and induced, however there may be some overlap between the groups.

1.4.1 MONOGENIC MODELS:
Monogenic, or single-gene rodent models are those in which obesity develops either
as a dominant (yellow, KK) or recessive trait (ob/ob, db/db, fa/fa, fat or tub) and all
of these species and strains are characterised by an excess of body fat.

1.4.1.1 The Yellow (A/y) Obese Mouse
The first of the rodent models to be characterised at a molecular level was the yellow
obese mouse (Bultman et al., 1992). In this strain, a dominant mutation arose
spontaneously from an allele at the agouti locus on mouse chromosome 2. The
yellow obese mouse is one of only two models of dominant inheritance of obesity, the
other being the KK mouse (discussed below) and it exhibits a moderate obesity as
well as a high incidence of tumour growth. Although the homozygous (A/y/A/y) mouse
is lethal in utero, a number of different alleles (A/y/A/Y, A/y/A/Y) exist at the agouti
locus in which the defects are less severe and the level of obesity can be linked
directly to the level of yellow pigmentation in the coat (Wolff, 1965). The obesity of
the yellow mouse is less severe than that seen in obese (ob/ob) or diabetic (db/db)
mice and is of later onset (8-12 weeks of age; Gill et al., 1994). In addition, yellow
obese mice exhibit many of the characteristics common to all rodent obesities,
however the associated hyperglycemia is dimorphic between the sexes in this model
(Gill et al., 1994).

The mouse agouti gene encodes a protein containing 131 amino acids which is
expressed in hair follicles in normal mice (Bultman et al., 1992; Michaud et al.,
1997). Within the follicles it induces melanocytes to synthesise the yellow pigment
which characterises these mouse strains by inhibiting eumelanin synthesis in response
to stimulation by α-melanocyte-stimulating hormone (α-MSH; Michaud et al., 1997;
Lu et al., 1994; Bultman et al., 1992). Obese yellow mice exhibit a mutation within the agouti gene which results in ubiquitous expression of the agouti protein in a wide range of tissues including adipose tissue and brain (Bultman et al., 1992). Subsequent studies have confirmed that obesity in the lethal yellow mouse is caused by a dominantly inherited trait whereby a promoter rearrangement at the agouti locus results in constitutive ectopic expression of the agouti peptide (Michaud et al., 1993; Michaud et al., 1994). Agouti is a potent antagonist of the hypothalamic melanocortin-4 receptor (MC4-R). It is a high-affinity receptor for α-MSH, derived from expression of the pro-opiomelanocortin (POMC) gene in neurons within the arcuate nucleus (Schioth et al., 1997; Shimizu et al., 1989b). α-MSH inhibits feeding when administered to rodents and appears to play a tonic inhibitory role in feeding and energy storage (Schioth et al., 1997; Shimizu et al., 1989b). Interruption of MC4R signaling therefore increases feeding behaviour in mice (Boston, 1997, Vaisse, 1998).

1.4.1.2 The Obese (ob/ob) Mouse

Obese (ob) is an autosomal recessive mutation on chromosome 6 that occurred spontaneously in the V stock at the Jackson Laboratory in 1949. The mutation was propagated in both the C57BL/6J inbred strain and in C57BL/Ks mice (Coleman, 1978). Similar to the diabetes mouse and fatty Zucker rat, obesity in ob/ob mice begins to develop soon after birth and is associated with decreased thermogenesis in brown adipose tissue (BAT) and enhanced insulin secretion.

After weaning obesity progresses rapidly as the animals become hyperphagic and hyperinsulinemic. Prevention of the hyperphagia does not prevent the development of obesity in ob/ob mice, or in any other single-gene models (Bray et al., 1973; Dubuc, 1976; Cox and Powley, 1977). In addition, the severity of the hyperglycemia and diabetes that develops in parallel with insulin resistance is dependent on the background strain of the mouse upon which the OB gene is expressed. Adult ob/ob mice exhibit severe obesity with large excess deposits of adipose tissue in the intra-abdominal, subcutaneous and intra-thoracic compartments (Bray et al., 1990; Bultman et al., 1992). There are also many neuroendocrine abnormalities apparent, including impaired sympathetic stimulation of BAT thermogenesis, overactivity of the hypothalamic-pituitary-adrenal axis and infertility.
Undoubtedly one of the most exciting recent discoveries in the obesity field was the cloning of the obese gene by Friedman and colleagues and the subsequent demonstration of the biological potency of its gene product leptin (Zhang et al., 1994) (see Section 1.5 for a discussion of regulation and action of leptin). The OB gene encodes a 4.5-kb mRNA containing a 167-amino acid open reading frame, with the main site of OB gene expression being white adipose tissue.

The C57BL/6J ob/ob mouse has a C-to-T mutation in codon 105 which changes an arginine codon (CGA) to a stop codon (TGA), and therefore leads to the production of a truncated, inactive protein. A second mutation, ob$^b$/ob$^b$, has been mapped 7-kb upstream of the 4.5-kb OB RNA start site and results in the failure to produce mature OB mRNA. The ob$^b$ mutation has been found to result from the insertion of a retroviral-like transposon in the first intron of the OB gene which leads to the production of chimeric RNAs in which the OB first exon is spliced to sequences in the transposon insertion. As a consequence mature OB mRNA is not synthesised and leptin is not produced (Moon and Friedman, 1997). Administration of recombinant leptin, has subsequently been shown to decrease food intake and increase thermogenesis in ob/ob mice, thereby decreasing body weight and body fat and correcting the obesity seen in these animals (Campfield et al., 1995; Halaas et al., 1995; Pellymouther et al., 1995). Leptin (leptos in Greek meaning thin) was so named by Halaas et al. because of its weight-reducing effects when initially administered to ob/ob mice. In addition, exogenous leptin administration normalises the endocrine abnormalities characteristic of ob/ob mice, namely hyperinsulinemia and hyperglycemia as well as correcting the infertility. It is clear that the obese phenotype of the ob/ob mouse results from the failure of these animals to produce active leptin.

1.4.1.3 The Diabetes (db/db) Mouse

Diabetes (db) is an autosomal recessive mutation on mouse chromosome 4 that arose in a colony of the inbred strain C57BL/Ks. This mutation is characterised by an obese and diabetic phenotype that is indistinguishable from the obese (ob/ob) mouse when both genes are expressed on the same background strain. On the C57BL/6J background the obesity is severe and maintained throughout life, although the
hyperphagia is moderate. The diabetes is also moderate on this background, with transient hyperglycemia and hyperinsulinemia that is marked and maintained throughout life (Coleman and Hummel, 1973). On the C57BL/Ks background the hyperphagia and obesity of ob/ob mice is more severe and is associated with pancreatic β-cell failure and early death. A number of different alleles exist at the db locus such as \( db^{j+} \), \( db^{j} \) and \( db^{ad} \) (Herberg and Coleman, 1977; Leiter et al., 1981). On the B/LKs background, the \( db^{j} \) and \( db^{ad} \) phenotype expressed is the same as the original db mutation on the same background, \( db^{ad} \) however occurred spontaneously on the 129/J strain and is characterised by severe obesity with hypoglycemia, rather than hyperglycemia coupled with marked hyperinsulinemia (Coleman, 1978).

Classical parabiosis studies conducted on ob/ob and db/db mice and fa/fa rats during the late 1970s by Coleman and Hervey predicted the presence of a circulating factor that was secreted in response to increased body fat stores and would act to regulate feeding behaviour. Coleman hypothesised that ob/ob mice did not produce this factor while db/db mice and fa/fa rats were unresponsive to it. This circulating factor has since been shown to be leptin, and it is now apparent that db/db mice possess a mutation exists within the gene for the leptin receptor (Coleman and Hummel, 1969).

The leptin receptor gene (OB-R) is known to have several alternatively spliced isoforms, with the longest of these (OB-RB) demonstrated to be the major signaling isoform. OB-RB is not translated in db/db mice as a result of a G-to-T mutation which introduces a novel consensus splice donor site at lysine 889 (AGGGAAA to AGGTAAA sequence change) (Tartaglia et al., 1995; Lee et al., 1996). As a result of this mutation, a 106-base sequence of the terminal exon is spliced into the OB-RB mRNA. This abnormal splicing introduces a stop codon and prevents translation of the long intracellular domain of OB-RB. The inability of db/db mice to respond to circulating leptin because of a mutated receptor results in the obese and diabetic phenotype described and further supports the original hypothesis proposed by Coleman (Coleman, 1978).

While the \( db \) mutation is a result of abnormal splicing, specifically altering expression of OB-RB, the \( db^{l} \) and \( db^{pas} \) mutations truncate the receptor in the extracellular
region. In \( db^{3J} \)/\( db^{3J} \) mice a frameshift mutation has been identified in the extracellular region of OB-R, which ablates expression of all of the wild type receptor proteins by truncating the protein at amino acid 625 (Lee et al., 1997a). In \( db^{Pas} \)/\( db^{Pas} \) mice a similar mutation has been identified which results from a duplication of exons 4 and 5 of OB-R and introduces a premature stop codon at amino acid 281, thereby preventing the expression of OB-R in these mice (Li et al., 1998a).

1.4.1.4 The Zucker Fatty (fa/fa) Rat

The fatty rat was first described in 1961 by Zucker and Zucker as a model for studying metabolic, endocrine, neurochemical and behavioural aspects of obesity (Zucker and Zucker, 1961). The temporal sequence for the development of obesity and the phenotype of the \( fa \) rat are similar to that of the \( ob/ob \) and \( db/db \) mice, although hyperglycemia is rare. Excess fat deposition is evident by 3 days of age and decreased thermogenesis and increased insulin secretion apparent between days 5 and 10 (Truett et al., 1995; Bazin et al., 1984; Kortner et al., 1994). The \( fa \) gene is regarded as a recessive gene, however the effects of \( fa \) on body fat and serum insulin may be dominant in the suckling pup during the first 20 days of life (Truett et al., 1995).

There are a number of allelic variants of the \( fa/fa \) rat generated by altering the background strain upon which it is expressed. By introducing the corpulent (\( fa^{cp} \)) or Kolletskey gene onto an inbred strain of rats derived from the hypertensive Okamoto strain (SHR/N) and further crossing onto other strains, e.g. LA/N, a variety of strains have been developed which exhibit various aspects of the metabolic syndrome (Russell, 1992). The corpulent (\( fa^{cp} \)) SHR/N-cp and LA/N-cp rats display both pronounced hyperglycemia and hypertension and have been used for models of the metabolic syndrome in humans (Russell, 1992). When the \( fa \) gene is transferred from its Brown-Norway background strain, on which diabetes is normally absent, to other backgrounds, long-lasting, severe diabetes may become characteristic, e.g. diabetic fatty (ZDF/Drt-\( fa \)), Wistar Kyoto fatty (WKY/NDrt-fa) or Wistar Kyoto diabetic (WDF/TA-fa).
The \( fa \) gene has been mapped to chromosome 5 of the rat and is thought to be syntenic to the diabetes gene on mouse chromosome 4, therefore within the rat OB-receptor gene. A single A to C alteration at nucleotide 880 of this gene has been detected which results in a glutamine to proline substitution at position 269, a highly conserved region of the OB-receptor gene (Chua et al., 1996b; Phillips et al., 1996; Takaya et al., 1996; Lee et al., 1997a). The A to C change is only found in fatty strains and is homozygous in obese animals only. The subsequent amino acid alteration results in altered signaling by the leptin receptor in the brain thus conferring leptin resistance on animals homozygous for the mutation.

1.4.1.5 The Fat (\( fat/fat \) or \( CPE^{fa} /CPE^{fa} \)) Mouse

Unlike the obese and diabetes mice, the fat mouse is an example of a late-onset form of obesity. The obesity may be severe in these mice, however it is usually not apparent until 8-12 weeks of age, and is not pronounced until 16-20 weeks (Coleman and Eicher, 1990). The fat mouse is characterised by massive hyperinsulinemia which is found to precede the development of obesity by several weeks (Coleman and Eicher, 1990). This model is also interesting because the hyperinsulinemia is not accompanied by significant hyperglycemia and the mouse remains extremely sensitive to exogenous insulin. This paradox is explained by the finding that the fat gene, located on chromosome 8 has been shown to code for carboxypeptidase \( E \) (CPE) (Naggert et al., 1995). CPE is required for cleavage of two arginine residues from the B chain of insulin during its processing from proinsulin. The phenotype of the fat mouse has been linked to a single base mutation that results in a serine to proline substitution at amino acid 202 which dramatically reduces the activity of CPE in both the pancreatic islet and pituitary gland (Naggert et al., 1995). The impairment in processing of proinsulin in mice homozygous for the \( CPE^{fa} \) allele is reflected in the 10-fold increase in proinsulin:insulin ratios in the pancreas. This defect appears to precede the development of obesity, and explains the very high level of proinsulin, rather than insulin, in the circulation and pancreas of the \( fat/fat \) mouse in addition to their responsiveness to exogenous insulin (Naggert et al., 1995).

The link between the development of obesity and the defect in proinsulin processing is unclear, particularly as proinsulin has little biological activity. It is known that a number of prohormones and propeptides require similar processing to that of
proinsulin to become biologically active. It has been suggested that the obesity of the fat/fat mouse may result from a series of complex alterations in neuropeptide activity and secretion within the hypothalamic-pituitary system rather than the hyperproinsulinemia (Naggert and Nashina, 1995).

1.4.1.6 The Tubby (tub/tub) Mouse
This is an autosomal recessive mutation on chromosome 7 that was first identified by Coleman and Eicher in 1990. On the C57BL/6J background, the obese phenotype exhibited by tubby mice is very similar to that seen in fat mice although the obesity develops more slowly and is only apparent after 9-12 weeks of age (Coleman and Eicher, 1990). The tub mutation is associated with distinct sexual dimorphism, with blood glucosce and serum insulin concentrations being higher in males. Morphological changes in pancreatic islets, hypoglycemia and infertility are also features of this mouse when severe obesity develops (Coleman and Eicher, 1990).

In 1996, Kleyn et al. identified and characterised the tubby gene using positional cloning. The mutation responsible for the tubby obesity phenotype was found to involve a G->T transversion within a splice donor site that results in the incorrect retention of a single intron in the mature tub mRNA transcript. The consequence of this mutation is the substitution of the carboxy-terminal 44 amino acids with 24 intron-encoded amino acids and thus the partial deletion of an evolutionary conserved region of the protein (Kleyn et al., 1996).

1.4.1.7 The KK Mouse
The KK mouse was first described in 1967 and subsequently several different strains have been identified (Butler and Gerritsen, 1970). These mice develop age- and diet-dependent obesity, insulin resistance and hyperglycemia (Ikeda, 1994), with diabetes developing after obesity under appropriate dietary conditions. The obesity and diabetes seen in these animals were originally thought to be inherited as a polygenic trait, however it is now clear that the KK gene is a dominant gene with low (25%) penetrance (Butler and Gerritsen, 1970). The obesity which develops in the KK mouse is mild, however the hyperglycemia may be severe. The severity of the obesity is exacerbated when KK mice are crossed with A' mice, generating KKA' mice which develop more severe obesity and diabetes which is not as diet-dependent.
(Ikeda, 1994). KK mice are very sensitive to diet and obesity may be prevented in this model with dietary restriction.

The use of monogenic models of obesity have provided important discoveries in the field of obesity research, although their overall relevance to human obesity is limited. To date, only a very small number of humans have been identified with homologous mutations to those seen in the monogenic rodent models, and it is clear that the epidemic proportions of human obesity seen world-wide will not be explained by a single gene mutation. As human obesity is thought to be polygenic in nature, the use of polygenic animal models of obesity may provide an opportunity to investigate the interactions between various components of the overall phenotype in a situation more closely resembling that found in humans. Unfortunately the inherent complexity of these genetic interactions make it more difficult to isolate specific determinants of aspects of a phenotype as heterogeneous as body weight. Several polygenic models of obesity have provided important insights into the development of obesity, and these will be discussed below.

1.4.2 POLYGENIC MODELS

A number of polygenic inbred rodent models have arisen from selection for obesity and associated diabetes. The polygenic forms of obesity are normally of later-onset and sometimes less severe than the obesity observed in single gene models. In addition, the development of obesity is often associated with dietary manipulations.

1.4.2.1 The New Zealand Obese (NZO) Mouse

The New Zealand Obese mouse originated from a mixed colony of agouti mice selected for spontaneous obesity (based on their agouti coat colour) from F12 to F17, and the genotype was fixed by continuous inbreeding (Bielschowsky and Goodall, 1970). NZO mice exhibit a polygenic syndrome of hyperphagia, obesity, insulin resistance and hyperglycemia similar to that of young C57BL/KsJ-db/db mice (Herberg and Coleman). The obesity that develops is gradual with maturity-onset weight gain and a concomitant increase in plasma insulin levels, presumably as a result of insulin resistance, and mild-to-moderate hyperglycemia in some male, but not female, mice (Herberg, 1988). The increased deposition of adipose tissue in NZO mice appears to occur primarily in the abdomen (visceral), with relative sparing of the
limbs and neck (Proietto and Larkins, 1992). In addition, while hyperphagia is evident in young NZO mice, hypophagia has been reported in adult mice compared to random bred albino mice of similar length (Proietto and Larkins, 1992).

1.4.2.2 The Otsuka Long-Evans Tokushima Fatty (OLETF) Rat
This polygenic rat model of obesity is a relatively new inbred obese strain which displays a late onset of hyperglycemia in male rats only (Kawano et al., 1992). These animals are naturally hyperphagic and show progressive accumulation of abdominal visceral fat compared to lean control rats (Ishida et al., 1995). This accumulation of adipose tissue is followed by hyperinsulinemia, hypertriglyceridemia and hyperglycemia in male OLETF rats (Sato et al., 1995).

1.4.2.3 The NON Mouse
NON, initially defined as non-obese non-diabetic, mice were originally bred as a control strain for the NOD (non-obese diabetic) mouse (Igel et al., 1996a). However, contrary to their name, NON mice develop mild obesity, and males exhibit severely impaired clearance of glucose injected intraperitoneally (Ikeda, 1994; Igel et al., 1996a). The obesity develops similarly to that seen in KK mice however in contrast to ob/ob and db/db mice, basal glucose and insulin levels are normal in NON mice (Ikeda, 1994). This indicates that insulin resistance is not a feature of the polygenic obesity syndrome seen in NON mice (Igel et al., 1996a).

1.4.3 INDUCED MODELS
1.4.3.1 Dietary Induced Obese Models
Studies involving genetic crosses between AKR/J, a strain that is susceptible to diet-induced obesity, and SWR/J, an obesity-resistant strain, have resulted in the identification of 3 loci involved in the control of adiposity (West et al., 1994a; West et al., 1994b). These studies involved the development of mild obesity in the mice by feeding a high fat/condensed milk diet for 12 weeks. West et al. utilised QTL mapping to identify complex gene interactions linking three loci (Dob1, Dob2 and Dob3) to phenotypic differences in body fat. Similar work has been done by Warden et al. utilising the BSB mouse. These mice are derived from a backcross of Mus spretus and C57BL/6J strains and exhibit individual variation in body fat content (from 1% to 50%; Warden et al., 1995; Warden et al., 1993). Studies involving these
mice have resulted in the identification of the Mob1, Mob2, Mob3 and Mob4 loci
which have variously been linked to adiposity and lie in the vicinity of known obesity
loci such as the OB and tub genes (West et al., 1992; Warden et al., 1993; West et al.,
1994b; Warden et al., 1995; West et al., 1995).

1.4.3.2 Hypothalamic Obesity

It has been recognised for a long time that destruction of, or damage to, the
ventromedial hypothalamus (VMH) is associated with the development of obesity in
several animal species, including humans (Bray et al., 1990a; Bray et al., 1990b).
VMH lesions can be caused by electrical, chemical (gold thioglucose, bipiperidyl
mustard, monosodium glutamate, ibotenic acid) or viral (scrapie, and Coackie virus)
routes (Bernardis, 1985; Scaliet and Olney, 1986). The obesity that results from
lesions in the VMH is very similar to that observed in the genetic obesities of the fa/fa
rat and ob/ob and db/db mouse, although hyperphagia may or may not be present
(Bray et al., 1990a; Bray et al., 1990b; Rohner-Jeanrenaud, 1995). Damage restricted
to the VMH normally produces obesity without hyperphagia as a result of changes in
autonomic control of thermogenesis and insulin. In contrast, damage to neural
pathways innervating the PVN or the PVN itself predominately cause a hyperphagic
syndrome, that may be related to changes in NPY and CRH activity.

The changes that result from VMH lesions are primarily responsible for the
alterations in peripheral metabolism (Bray et al., 1990; Bray et al., 1990b). After
VMH lesions, there is an increase in the activity of the afferent vagus innervation
from the gastrointestinal tract and the efferent vagal drive to the endocrine pancreas
(Bertothoud, 1985). As a result, there is an increase in insulin secretion within
minutes of a VMH lesion. The hyperinsulinemia associated with VMH lesions have
also been linked to alterations in both hepatic and adipose tissue lipogenesis and
substantially increased fat deposition (Penicaud et al., 1986; Rohner-Jeanrenaud,
1995). VMH lesions are also known to cause reduced sympathetic drive to BAT and
subsequently a reduced level of thermogenesis (Assimiacopoulos-Jeannet and
Together these alterations lead to a rapid increase in fat deposition and body weight as
well as the development of insulin resistance (York and Hansen, 1998).
1.4.3.3 Induced Mutant Models
The recent application of transgenics and induced mutagenesis to the study of complex genetic diseases such as obesity and diabetes is providing valuable insights into the specific pathways involved in the development of these diseases. By increasing the ability to manipulate the mouse genome and change the level of expression of specific genes (e.g. transgenic mice overexpressing the PEPCK gene) (Valera, 1994), or selectively delete mouse genes (e.g. NPY-knockout mice) (Erickson, 1996a) researchers are gaining new information on the individual genes involved in the development of specific phenotypes. Transgenic insertions and embryonic stem cell ‘knockouts’ are also providing new information on the role of gene/gene interactions in the mouse, as often ‘knockout’ models show great variation in phenotype depending on the different genetic background of the strain (Darling and Abbott, 1992; Smithies, 1993 Melton, 1994; Searle et al., 1994).

1.4.4 NON-RODENT MODELS: Rhesus monkeys
Rhesus monkeys (Macaca mulatta) frequently spontaneously develop obesity after sexual maturation, when they are provided with a balanced ad libitum diet (Hansen and Bodkin, 1986). These monkeys have been well characterised and the adult-onset obesity that develops (usually between the ages of 10 and 25 years) is very similar to human middle-age onset obesity, making them a very attractive model in which to study the regulation of body weight and development of obesity.

If allowed ad libitum access to food, obesity will eventually develop in approximately 50% of the animals, however as there are no apparent differences in the food intake of the most obese and least obese groups of monkeys, differences in energy expenditure are thought to be fundamental to the development of obesity in these animals (Hansen and Bodkin, 1986; Hansen, 1995). Similar to several other models of obesity and diabetes, obese Rhesus monkeys exhibit hyperinsulinemia and insulin resistance (Hansen and Bodkin, 1986). In addition, there is a progressive decline in the function of pancreatic β-cells until overt diabetes results, with hypertriglyceridemia and altered insulin receptor splicing also noted in some diabetic animals.

Rhesus monkeys have also been suggested as a useful model for human central obesity as abdominal circumference in obese monkeys is the best predictor of body
fat and is strongly positively associated with plasma insulin and negatively associated with glucose tolerance and peripheral insulin-stimulated glucose uptake in these animals (Bodkin et al., 1989). In Rhesus monkeys, as in humans, the link between central obesity and abnormalities in lipid and glucose metabolism appears to be insulin resistance (Bodkin et al., 1993). The spectrum of metabolic abnormalities seen across a population of Rhesus monkeys allows for the characterisation of monkeys as insulin-sensitive or insulin-resistant and also a more detailed examination of the relationships between insulin-resistance and central obesity.

As monkeys are taxonomically very similar to humans, findings in monkeys can be applied to human physiology more easily than those from rodents. However, as monkeys are large animals, which are free-roaming in the wild there are inherent difficulties associated with confining these animals. In addition, monkeys, like humans, exhibit age-dependent penetrance of the disease phenotype and the presence of obesity and/or type 2 diabetes may not be evident in monkeys for many years (at least 10 in most cases). This will also increase the cost and difficulty of the research (Bodkin and Hansen, 1986; Bodkin et al., 1989).

All of the animal models of obesity and type 2 diabetes discussed above have contributed extensively to the study of these diseases, and will probably continue to expand our knowledge into the specific physiological pathways involved in the development of obesity. One rodent model of obesity and type 2 diabetes which exhibits a complete spectrum of phenotypic responses when held in captivity, *Psammomys obesus*, was the focus of several studies in this dissertation, and is discussed in more detail below.
1.4.5  *Psammomys obesus*

There are very few truly polygenic rodent models of obesity existing in outbred colonies, one of which is *Psammomys obesus*. *Psammomys obesus* is a unique animal model of obesity and type 2 diabetes, and several aspects of metabolism in this animal model closely resemble those found in human obesity and type 2 diabetes. In particular, *Psammomys obesus* are representative of those human populations which develop high prevalence rates of obesity and type 2 diabetes in response to the change from a traditional to a “Western” lifestyle, such as Pima Indians, Australian Aborigines and Pacific Islanders (Knowler et al., 1981; Zimmet, 1992).

*Psammomys obesus* (Israeli Sand Rat) are desert-dwelling, burrowing rodents who are native to the Saharo-Arabian deserts from Algeria to the Sudan in North Africa, and extending eastwards to Arabia. Several populations of *Psammomys obesus* are found in Israel around the Dead Sea where they exist on a meagre herbivorous diet eating in particular the salty-tasting saltbush *Atriplex halimus* (Harrison and Bates, 1991).

*Atriplex halimus* has a high moisture content but is low in energy and organic matter content (Frenkel and Kraicer, 1972; Degen, 1988; Kam and Degen, 1988). In addition it has a very high ash and electrolyte composition, with the electrolytes found to be highly concentrated in the leaves of the plant (Kenagy, 1973). Atriplex halimus is highly tolerant of the surrounding arid conditions and is usually freely available throughout the year (Degen, 1993). *Psammomys obesus* show a low metabolic utilisation of the energy in Atriplex halimus (Degen, 1993). When this is coupled with the low energy yield and high water content of the plant, *Psammomys obesus* are forced to eat large quantities of this food to maintain neutral energy balance. In fact, it has been estimated that in the field *Psammomys obesus* consume 50-65% of their body mass in plant material daily, and up to 68% under laboratory conditions (Kam and Degen, 1988; Pinshow, 1993).

In the 1960s specimens of *Psammomys obesus* were collected by a US Naval Medical Research Unit in Egypt. These first animals were trapped on the sandy beaches of the Nile delta and given the trivial nickname ‘sand rats’ (Hogstraal, 1961). However, this is a misnomer as these animals are not murines but gerbils belonging to the family Gerbillinae, therefore they are more correctly referred to as Psammomys (Shafrir and
Psammomys was described in the Nile delta as early as 1902 and 1908 (Shafirir and Gutman, 1993).

*Psammomys obesus* are relatively large rodents (mean adult bodyweight approximately 180g) (Harrison and Bates, 1991) however they have a relatively low basal metabolic rate (BMR). The BMR of *Psammomys obesus* is approximately 57-60% of the BMR expected for a rodent of similar size, and 88% of that expected for eutherian mammal of its body size (Pinshow, 1993; Degen, 1993). This decreased metabolic rate is thought to be an adaptation to the desert by decreasing the internal heat load in the hot environment (McNab and Morrison, 1963; Shkolnik and Schmidt-Nielsen, 1976). *Psammomys* are unique among other desert gerbils as they are active above ground during the day, while all other species are strictly nocturnal (Ilan and Yom-Tov, 1990).

In their native environment *Psammomys obesus* remain healthy, with no evidence of diabetes or obesity ever recorded in animals freshly trapped from the wild (Shafirir and Gutman, 1993). However it is now well established that when *Psammomys obesus* are allowed access to a relatively high energy diet of standard laboratory chow a substantial proportion of the animals develop mild to moderate obesity and severe type 2 diabetes. For this reason *Psammomys obesus* are thought to be an excellent model of animals with a putative ‘thrifty gene’.

### 1.4.5.1 Dietary Studies

The investigators at Duke University were the first to demonstrate that when *Psammomys obesus* are maintained *ad libitum* on a diet of regular laboratory chow, a large proportion of the animals develop diabetcs (Schmidt-Nielsen, 1964; Hackel *et al.*, 1966). The diabetes covers the wide spectrum of the disease, ranging from mild hyperglycemia with hyperinsulinemia to hypoinsulinemia with ketoacidosis, a terminal stage with short survival (Hackel *et al.*, 1965; Hackel *et al.*, 1966). In all studies however, a certain percentage of *Psammomys obesus* remain healthy and non-diabetic even on the high calorie diet.

Early dietary studies by Hackel *et al.* (1967) demonstrated a striking correlation between caloric intake and plasma insulin concentration in *Psammomys obesus*. They
studied three groups of animals to determine the effects of altered energy intake on glucose and insulin concentrations. Initially all animals still had relatively high insulin levels and decreased glucose tolerance from the predominantly beet diet they had been maintained on until two weeks prior to the experiments. One group of animals was fed an all vegetable diet with an energy content of about 30 cal/day. This group of animals lost on average 23% body weight and developed low plasma insulin concentrations and normal glucose tolerance and remained lean and healthy. A second group of animals was fed a chow diet in restricted amounts so they had an energy intake of approximately 30 cal/day. These animals lost an average of 14% body weight, had normal insulin and glucose levels. When these same animals were allowed *ad libitum* access to the chow diet they consumed about 49 cal/day, gained weight (on average by 28%) and developed markedly elevated plasma insulin concentrations and impaired glucose tolerance. The third group of animals was given the chow diet *ad libitum*, and then in a restricted manner. This group showed similar responses to the chow diet as those animals in group 2 (Hackel *et al.*, 1967).

Subsequent dietary studies have demonstrated that *Psammomys obesus* are extremely metabolically responsive to dietary manipulations. Adler *et al.* (1985) reported that animals lose body weight, body fat and exhibit normal insulin and glucose concentrations when maintained on a predominantly wheat straw diet. In addition, significant associations were found to exist between the level of energy intake and body fat, blood glucose and plasma insulin concentrations when *Psammomys obesus* were fed 3 diets based on pellets composed of different ratios of saltbush and standard laboratory chow (Adler *et al.*, 1986).

Studies in our laboratory have also demonstrated that when the energy intake of *Psammomys obesus* is restricted to 67% of their normal intake body weight and blood glucose decrease in all animals, however plasma insulin concentrations decreased in all but the most hyperinsulemic animals. This is despite no significant decrease in body weight with the energy restriction (Barnett *et al.*, 1994b). In addition, hyperinsulinemic animals showed a normalisation of both plasma triglyceride and cholesterol concentrations.
In the early 1970s Brodoff et al reported the effect of hypothalamic lesions in *Psammomys obesus* when the animals were maintained on a high fat diet. Although only 5 animals survived post-lesion, it was apparent that lesions in the posterior median eminence extending into the arcuate nucleus in the region of the ventromedial nuclei improves glucose tolerance and insulin sensitivity independent of weight change. Some of the metabolic changes observed were in the presence of marked obesity which was taken to imply the influence of a hypothalamic influence on insulin resistance separate to the effect of adiposity (Brodoff et al., 1971).

1.4.5.2 Characterisation

It became apparent that *Psammomys obesus* exhibited a spectrum of metabolic characteristics when held in captivity and fed a diet of laboratory chow, and the animals can be classified into several groups. Three main groups of animals have been identified, normoglycemic-normoinsulinemic, normoglycemic-hyperinsulinemic and hyperglycemic-hyperinsulinemic (Kalderon et al., 1983). The proportion of animals that fall into each group has remained relatively constant over the years, with about 32% of animals normoglycemic-normoinsulinemic (group A), about 26% normoglycemic-hyperinsulinemic (group B) and about 36% hyperglycemic-hyperinsulinemic (group C). In addition about 6% of the colony displayed frank diabetes with pronounced hyperglycemia and hypoinsulinemia, indicating pancreatic exhaustion (group D).

These early studies also demonstrated abnormalities in lipid and glucose metabolism in *Psammomys obesus*, with reduced glucose uptake seen in the muscle and adipose tissue of group B animals (Schafer et al., 1977; Kohler and Knospe, 1980). In addition, an increased uptake of VLDL-triglyceride and increased activity of lipoprotein lipase was demonstrated in group C and D animals (Kalderon et al., 1986; Gutman et al., 1990; Shafrir and Gutman, 1993). Although informative, these studies were conducted in animals who were not age-matched, and blood was collected only in the fed state, which may have increased the variability of the metabolic measures examined across the colony as a result of differing postprandial times. In addition, the alterations in lipid and glucose metabolism investigated in several of the early studies were judged in comparison to the albino rat and although a rodent, the validity of this comparison is unclear.
The colony of *Psammomys obesus* housed at Deakin University has also been studied in a longitudinal and cross-sectional manner in an effort to understand the development of obesity and diabetes in these animals. Cross-sectional examination of blood glucose and plasma insulin concentrations in 19-week-old *Psammomys obesus* in the fed state revealed an inverted U-shaped relationship between glucose and insulin (Barnett *et al*., 1994a). This relationship has previously been described in humans and termed 'Starlings curve of the pancreas' by DeFronzo (DeFronzo *et al*., 1992). This curve describes the relationship between glucose and insulin across a population, and as circulating glucose concentrations rise, insulin concentrations also increase. However, the increase in insulin concentrations continues with rising glucose concentrations only until a threshold level, the development of hyperglycemia. After this point, insulin concentrations fall off rapidly as insulin secretion is diminished as a result of β-cell failure, and the diabetes gets progressively worse, with increasing glucose concentrations.

The animals of the Deakin colony were also able to be divided into 4 groups based on age-matched, fed blood glucose and plasma insulin concentrations (Barnett *et al*., 1994a) and the characteristics of these groups have been further investigated. When compared to normoglycemic, normoinsulinemic group A animals, animals in group C displayed a number of metabolic abnormalities including hyperglycemia, hyperinsulinemia, high triglyceride and cholesterol levels, increased fat stores and increased body weight (Habito *et al*., 1995; Collier *et al*., 1997a). Subsequent studies in our laboratory have demonstrated a direct correlation between fasting plasma glucose concentrations and the rates of hepatic glucose production (Habito *et al*., 1995) as well as an inverse relationship between fasting plasma glucose and the metabolic clearance rates of glucose. Results of studies in which free fatty acid oxidation is inhibited, have indicated that elevated fatty acid oxidation is at least partly responsible for the increased basal glucose turnover (both at the whole body and tissue level) thought to be primarily responsible for the fasting hyperglycemia seen in group C animals, and that this occurs through increased hepatic glucose production (Habito *et al*., 1995; Barnett *et al*., 1996).
The obesity that develops in *Psammomys obesus* has also been characterised. It is evident at an early stage (between 4-8 weeks) that some animals (about 50%) will overeat and develop the subsequent metabolic abnormalities described (Collier *et al.*, 1997b). All animals that become obese develop hyperphagia early on, however at weaning (4 weeks of age) these animals are indistinguishable from animals that remain lean and healthy, in terms of their body weight, glucose, insulin and triglyceride levels at this age (Collier *et al.*, 1997b). In addition to hyperphagia, group C animals also display decreased spontaneous exercise levels when mature.

### 1.4.5.3 Additional Metabolic Abnormalities

*Insulin resistance:* When *Psammomys obesus* are maintained on salt bush they continue to have normal insulin levels. When transferred to a high-energy diet however, plasma insulin levels have been shown to rise early in the course of the development of diabetes with a subsequent drop in severely diabetic animals. Early examinations of muscle and adipose tissue revealed pronounced insulin resistance and only low-level binding of insulin to its receptors in the liver, with subsequently low levels of tyrosine kinase activation by the receptor (Kohler *et al.*, 1976; Fielder *et al.*, 1977; Shafirir and Gutman, 1993).

Early histochemical and ultramicroscopic investigations have demonstrated a wide spectrum of stages of β-cell degranulation and necrosis associated with glycogen deposition (Schmidt-Nielsen *et al.*, 1964; Hackel *et al.*, 1965). In severely diabetic *Psammomys obesus*, the presence of increased protein synthesis, glycogen accumulation and extensive degranulation result in cytoplasmic disorganisation and β-cell breakup, leaving pancreatic islets virtually devoid of β-cells (Molleson *et al.*, 1973). More recently the presence of marked increases in proinsulin and proinsulin conversion intermediates in the plasma and pancreas of diabetic *Psammomys obesus* have been detected in conjunction with 90% reduction in pancreatic insulin stores (Gadot *et al.*, 1994). Subsequently, it has been demonstrated that fully processed insulin in *Psammomys obesus* contains a phenylalanine residue, unique to this species however the unusual structure of *Psammomys obesus* insulin does not contribute to the proinsulinemia observed in diabetic animals (Kaiser *et al.*, 1997).
Fat Metabolism: Group A (normoglycemic, normoinsulinemic) *Psammomys obesus* have a relatively high VLDL-TG uptake and lipoprotein lipase (LPL) activity in adipose tissue, compared to albino rats (Kalderon et al., 1986; Gutman et al., 1990). The VLDL-TG uptake by adipose tissue of *Psammomys obesus* was 5-fold increased, and LPL activity 10-fold increased when compared to albino rats (Gutman et al., 1990). In addition, the activity of LPL in *Psammomys obesus* heart and skeletal muscle was relatively low compared with albino rats (Chajek-Shaul et al., 1988), while the high activity of LPL in adipose tissue was not reduced by a 20-hour fast or treatment with cholera toxin as it was in albino rats (Chajek-Shaul et al., 1988). These findings suggest that the metabolism of *Psammomys obesus*, as a species, is geared toward the channeling of lipoprotein triacylglycerols towards adipose tissue for storage, a key step for fat accumulation when fed an energy dense diet (Kalman, 1993).

In contrast to other rodents, fatty acid synthesis occurs almost entirely in the liver in *Psammomys obesus*. However, as with higher mammals, the adipose tissue in *Psammomys obesus* is rich in LPL and in the capacity to store the preformed lipids, but poor in the enzymes involved in lipogenesis compared with albino rats (Kalderon et al., 1983). Injection of labeled triglycerides in *Psammomys obesus* has been shown to result in a slower rate of disappearance from plasma and an increased recovery in adipose tissue than that observed in albino rats (Gutman et al., 1990). These factors indicate a state of readiness in *Psammomys obesus* to store lipids whenever available.

Liver metabolism:

Group A *Psammomys obesus* have been reported to have increased liver gluconeogenesis and phosphoenolpyruvate carboxykinase (PEPCK) activity, increased lipid synthesis and activity of NADP-malate dehydrogenase, and decreased pyruvate kinase activity compared to albino rats (Kalderon et al., 1986). These findings suggest that, relative to albino rats, *Psammomys obesus* have a high rate of hepatic lipogenesis and a concomitant increase in the uptake of VLDL-TG by adipose tissue. In addition, gluconeogenesis is higher in *Psammomys obesus*, although this appears to be at least partially compensated by a relative preference for oxidation of glucose rather than fatty acids in skeletal muscle (Kalderon et al., 1986).
In subsequent studies by Barnett et al. (1995), analysis of liver and muscle (soleus) revealed that active glycogen synthase activity is reduced in hyperglycemic group C and D animals. In addition, total glycogen synthase activity (in liver and muscle) and PEPCK activity (in liver) were not different between the four groups of *Psammomys obesus* (A-D) (Barnett et al., 1995). The enzymatic analysis in this study by Barnett et al. (1995) and earlier studies by Kalderon et al. (1986) suggest that a defect in active glycogen synthase and not PEPCK is in part responsible for the hyperglycemia seen in *Psammomys obesus* and that reduced glycogen synthesis may be an earlier predictor for insulin resistance than elevated gluconeogenesis.

Further studies found reduced activity of hepatic pyruvate dehydrogenase, an important enzyme in glucose oxidation, in *Psammomys obesus*, compared to albino rats (Nakai et al., 1997). In addition, the activity of hepatic 3-hydroxy-CoA dehydrogenase was found to be four-fold higher in *Psammomys obesus* compared to albino rats. These results suggest a low capacity for glucose oxidation, but a high capacity for fatty acid oxidation in the liver (Nakai et al., 1997).

The data summarised above indicate that *Psammomys obesus* offered a diet of laboratory chow develop a range of metabolic defects in carbohydrate and lipid metabolism. The extent of these defects appears to rely on an underlying genetic predisposition to the development of obesity and type 2 diabetes in susceptible animals. This spectrum of response indicates that *Psammomys obesus* is an excellent polygenic model in which to study the development of obesity and type 2 diabetes, as this species display a range of body weight and food intake in response to a diet of laboratory chow.

*Psammomys obesus* forms the basis of the animal component of this dissertation for the reasons outlined above. The main focus of this dissertation is the aetiology of obesity and the genes which regulate energy balance. Specifically, the importance of the OB gene and the OB receptor gene (OB-R) in the regulation of energy balance will be explored, and these genes are discussed in detail below.
1.5 THE OB GENE

1.5.1 THE LIPOSTAT THEORY

The lipostat theory of weight control was first proposed by Kennedy et al. as early as 1953. According to this theory, the adipose tissue was thought to secrete a hormone which was able to regulate body size. In 1958, Hervey et al. conducted classic parabiosis studies, the results of which supported the existence of such a circulating factor. In these studies, Hervey showed that a normal rat would become anorexic when parabiosed to a rat made hyperphagic by lesioning the ventromedial hypothalamus (VMH). It was proposed that as the VMH-lesioned rat became obese it secreted a factor that crossed the parabiotic union and inhibited food intake in the normal rats, the lesion rendering the satiety factor ineffective in the lesioned rat (Hervey, 1958). Further work by Hausberger (1959) and later by Coleman and Hummel (1969) extended Hervey’s observations through parabiotic studies between the genetically obese mice, ob/ob and db/db.

Coleman and Hummel showed that similar to a VMH-lesioned rat, when a db/db mouse was parabiosed to a normal mouse, the normal mouse would lose weight and die of apparent starvation (Coleman and Hummel, 1969). This finding suggested that db/db mice were producing a satiety/anti-obesity factor, but were unable to respond to it. In contrast, when an ob/ob mouse was parabiosed to a normal mouse the rate of weight gain in the normal mouse did not decrease, but rather body weight was decreased in the ob/ob mouse (Coleman, 1978). Hausberger first suggested that the ob/ob mouse was missing an anti-obesity factor that could be supplied by a normal mouse. Coleman furthered this hypothesis by demonstrating that when an ob/ob mouse was parabiosed to a db/db mouse, the ob/ob mouse lost weight and died of apparent starvation (Coleman, 1978). Together these data are consistent with the idea that ob/ob mice are unable to produce a satiety/anti-obesity factor, while db/db mice produce this anti-obesity factor but cannot respond to it; thus both obesity syndromes are caused by a common factor. This hypothesis has since been supported with the isolation and characterisation of the OB gene.
1.5.2 CLONING OF THE OB GENE

In late 1994, Zhang et al reported the positional cloning of the mouse OB gene and its human homologue. This group used exon trapping to isolate genes within a 650-kb interval of mouse chromosome 6 to which the OB gene had been mapped earlier. One of the exons trapped, 2G7, was hybridised to a northern blot of mouse tissues and a ~4.5-kb RNA was detected in white adipose tissue, with no single RNA detected in other tissues. The level of expression of the 2G7 exon was then assayed in the two available ob strains of mice by hybridisation to northern blots as well as by RT-PCR. The 2G7 RNA was found to be absent in ob^{+/ob} adipose tissue, however northern blots of adipose tissue from ob/ob mice revealed a ~20 fold increase in the level of OB RNA (Zhang et al., 1994). This suggested that the original OB allele was associated with a non-functional gene product and that the mRNA was increased as part of a possible feed-back loop.

A total of 22 cDNA clones were isolated from the 2G7 exon using a mouse adipose tissue cDNA library (Zhang et al., 1994). Sequencing of these clones revealed a methionine initiation codon in the 2G7 exon with a 167-amino acid open reading frame followed by a long 3'-untranslated sequence. RT-PCR products from the entire open reading frame prepared from adipose tissue RNA from C57B6/J ob/ob mice were used to identify the OB mutation in this strain. The coding sequences were found to be identical apart from a single C->T mutation, which results in an amino acid change of an arginine at position 105 to a stop codon (Zhang et al., 1994).

The polypeptide predicted from the OB gene was largely hydrophilic and had a putative N-terminal signal sequence. Sequence analysis suggested that both human and mouse RNA encoded a secreted protein and subsequent cloning revealed a 16kDa functional protein (Zhang et al., 1994). To determine if OB was a highly conserved gene, cross-species hybridisation was performed using Southern blots of vertebrate genomic DNA. OB was found to be highly conserved, with signals detected in all species tested, including rat, rabbit, cat, cow, sheep, pig, chicken and human. Alignment of the predicted human and mouse amino acid sequences showed ~84% overall homology (Zhang et al., 1994).
Subsequent studies have confirmed that in the original C57BL/6J ob/ob mouse the missense mutation at codon 105 results in the synthesis of a truncated protein that is apparently degraded in the adipocyte, as OB protein cannot be detected in mouse plasma. In addition, the mutation in the congenic ob^dd strain has been determined to be the result of an insertion of a retroviral-like transposon into the first exon of the OB gene. This insertion provides an alternative splice acceptor and polyadenylation site and leads to the expression of fusion RNAs between the OB first exon and the retrovirus. As a consequence, RNA from the coding sequence of the OB gene is not expressed (Moon and Friedman, 1997).

The mouse OB gene is found on mouse chromosome 6 within a region of conserved synteny with human chromosome 7q. Using physical mapping technology Green et al. (1995) localised the human OB gene using YAC contig mapping to 7q31.3. This study also identified eight established microsatellite markers in close proximity to the human OB gene.

Northern blot analysis revealed that the human OB gene is expressed predominately in adipose tissue with low levels detected in placenta (250-fold lower than in adipose tissue) and heart (5-fold lower than placenta). In this and subsequent experiments OB RNA was not detected in tissues other than adipose tissue including: brain, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood leukocytes or fetal brain, liver or kidneys (Green et al., 1995). It is also established that the OB gene is not expressed by preadipocytes (Hardie et al., 1996; Murakami et al., 1996; Slieker et al., 1996), with OB gene expression and secretion of leptin only seen in differentiated cells (Mitchell et al., 1997). More recently, in rats OB mRNA has been detected in the stomach, within the gastric epithelium (Bado et al., 1998), while Wang et al. (1998) also reported activation of OB gene expression in skeletal muscle by glucosamine.

1.5.3 STRUCTURE OF THE OB GENE

Subsequent to the initial cloning of the OB gene by Zhang et al., several investigators have further characterised the gene. He et al. (1995) reported that the coding sequence for the mouse OB gene was in exons 2 and 3 with the promoter of the gene found upstream of exon 1. A 762-base pair OB gene promoter was further studied
using mutational analysis and it was determined that a minimal 161-bp promoter was active in transfected adipose cells. This sequence of the promoter contained the Sp1 consensus sequence (GGGCGG) which is thought to bind a number of transcription factors from the Sp1-like family and also contained CCAAT/enhancer-binding protein (C/EBP) motifs. C/EBPα is a transcription factor which plays an important role in adipose cell differentiation (Lin and Lane, 1994) and co-transfection of the OB promoter with C/EBPα resulted in a 23-fold activation of transcription; suggesting that the OB promoter is a natural target of this transcription factor (He et al., 1995).

In addition He et al. reported evidence of alternative splicing of exon 1 of the mouse OB gene, with the existence of an alternative exon identified by sequencing analysis and further confirmed using RT-PCR (He et al., 1995). Evidence for alternative splicing of this gene has previously been demonstrated by Zhang et al. (1995) who reported the variable inclusion of the first codon in exon 3. The sequence of the predicted protein product would not be altered by the alternative splicing of exon 1, described as the initiation site, which is located downstream in exon 2 (He et al., 1995).

The structural organisation of the human OB gene has also been examined. Isse et al. reported that the human OB gene spanned roughly 20 kilobases and contained three exons separated by two introns (Isse et al., 1995). Splicing donor and acceptor consensus sequences were located at the putative exon/intron boundaries and the first intron (~10.5kb in size) occurred in the 5’-untranslated region, 29 bp upstream of the ATG start codon. The second intron (~2.3 kb in size) was located at glutamine +49.

The 172 bp 5’-flanking region of the human OB gene contained a TATA-like box sequence 27-30 bp upstream of the transcription initiation sites and the consensus sequences of the regulatory elements Sp1, C/EBP, E-box (CANNTG) and an AP-2 binding site (CCCAGGGGC) were also identified in this region (Isse et al., 1995). Although the 5’-untranslated region of the OB gene shares 93% nucleotide homology, much less homology was seen for this region between human and mouse (51%) or human and rat (47%) (Isse et al., 1995). Similar to the mouse OB gene, evidence for alternative splicing of the first exon of the human OB gene was also reported (Isse et al., 1995).
Gong et al. (1996) also isolated the promoter of the human OB gene in an effort to further characterise its structure, as earlier studies indicated that OB gene expression may be transcriptionally regulated. In this study different lengths of the 5'-flanking region of the first exon were sub-cloned into luciferase reporter vectors which were then transfected into F442A adipocytes (Gong et al., 1996). These analyses indicated that the 5'-flanking region of the OB gene contained all of the necessary elements to support basal transcription and serve as a promoter. In addition, a shorter 300-bp fragment of the 5'-flanking region appeared to be more active than the full length 3 kb fragment, indicating that transcription in F442A adipocytes may be inhibited by the remote 5'-flanking region (Gong et al., 1996).

C/EBPα is potentially a regulator of OB gene expression because of the presence of the consensus sequence of this regulatory element in the 5'-flanking region of the OB gene. However, Sloop et al. (1998) recently reported that acute changes which modulate OB mRNA expression are not accompanied by changes in C/EBPα mRNA expression. These studies were performed using comparative RT-PCR of rat epididymal fat and demonstrated that while both fasting and leptin treatment decreased OB mRNA expression in Sprague-Dawley rats, C/EBPα expression remained unchanged (Sloop et al., 1998). Previously Rolland et al. (1995) demonstrated that non-obese (fa/+ ) Zucker rats exhibit the same level of C/EBPα mRNA expression as obese (fa/fa) Zucker rats that have more than twice as much inguinal subcutaneous fat. These results suggest that reductions in OB mRNA expression are not a direct result of reduced activity of C/EBP-α (Sloop et al., 1998).

Transcriptional regulation of OB gene expression also appears to be controlled by peroxisome proliferator activating receptor γ (PPARγ), one of a family of transcription factors known to promote adipocyte differentiation (De Vos et al., 1996; Nolan et al., 1996). Thiazolidinediones (TZD), the pharmacological ligands for PPARγ have been demonstrated to down-regulate the level of OB mRNA in adipocytes (Kallen and Lazar, 1996; Nolan et al., 1996; Zhang et al., 1996). Consistent with this role for PPARγ in regulation of the OB gene promoter a canonical DR+1 PPARγ binding site between -3951 and -3939 of the mouse 5'-flanking sequence of the OB gene has also been identified (Hollenberg et al., 1997).
Hollenberg et al. (1997) further report that PPARγ2 mediates down-regulation of the OB promoter by inhibiting C/EBPα-mediated transactivation. It should be noted however that while TZD suppress leptin secretion from human cultured adipocytes (Kallen and Lazar, 1996), it has not been shown to affect leptin production in vivo (Nolan et al., 1996; Mantzoros et al., 1997).

Another recent report suggests that the transcription factor ADD1/SREBP1 plays a key role in linking changes in nutritional status and insulin levels to the expression of various genes regulating systemic energy metabolism, including the OB gene (Kim et al., 1998). The adipocyte differentiation dependent factor (ADD)-1 and its human homologue, sterol regulatory element binding protein (SREBP)-1 is a basic-helix-loop-helix protein that has unusual dual DNA-binding specificity (Kim et al., 1995). This protein is able to bind to E-box sequences in addition to the sterol regulatory element (SRE)-1 sequence (ATCACCCCA). This DNA binding specificity is of note as several identified insulin response elements appear to be E-boxes (Moustaid et al., 1994).

The expression of ADD1/SREBP1 is shown to alter dramatically during fasting and refeeding of mice, closely paralleling the regulation of the OB gene under the same conditions. Insulin appears to regulate ADD1/SREBP1 and Kim et al. (1995) describe this regulation to be on several levels. Insulin acts to greatly enhance expression of ADD1/SREBP1 at the mRNA level and in addition, this regulation occurs at insulin concentrations consistent with a function through the insulin receptor. Insulin has also been found to phosphorylate serine residue(s) on ADD1/SREBP1 in response to insulin.

Kim et al. (1998) also demonstrated that the promoter of the OB gene is transactivated by ADD1/SREBP1. This appears to occur in a manner reliant on both ADD1/SREBP1 DNA binding domains, as a mutation in the basic domain of ADD1/SREBP1 that allows E-box binding but destroys sterol regulatory element-1 binding prevents OB gene transactivation (Kim et al., 1998). Taken together, this study therefore provides a transcriptional mechanism by which nutritional status and insulin levels may be linked to regulation of OB gene expression.
1.5.4 OB GENE EXPRESSION AND LEPTIN, THE OB PROTEIN

Studies examining the expression of the OB gene in rodent models of obesity other than the ob/ob mouse demonstrate that obese animals have higher levels of OB mRNA expression in adipose tissue as well as higher levels of the OB protein, leptin circulating in their blood compared to lean controls. The rat OB gene was cloned separately by different groups and within the coding region of the OB cDNA the sequence was found to be conserved across rats, mice and humans, with 96% homology at the nucleic acid and amino acid levels between rat and mouse, and 84% and 82% homology with human at the nucleic acid and amino acid level respectively (Murakami and Shima, 1995; Ogawa et al., 1995). In addition, sequence alterations in the coding region of the OB gene were not detected in either Zucker (fa/fa) or Otsuka Long Evans Tokushima Fatty (OLETF) rats.

Murakami and Shima (1995) reported that expression of OB mRNA in retroperitoneal adipose tissue was 4-fold higher in Zucker fa/fa rats compared to lean controls. Ogawa et al also reported an augmentation of OB mRNA expression in Zucker fa/fa rats, however they further described regional differences in OB mRNA expression with a rank order of the level of OB mRNA in the adipose tissue reported to be: epididymal, retroperitoneal and pericardial white adipose tissue (WAT) > mesenteric and subcutaneous WAT > intrascapular brown adipose tissue (BAT) (Ogawa et al., 1995). In addition, the differences in OB mRNA expression between obese Zucker and lean rats were not uniform across all adipose tissue depots, with OB gene expression in obese Zucker rats -12, 6-, 6-, 2-, 86- and 22-fold higher compared to lean littermates in the epididymal, mesenteric, subcutaneous, retroperitoneal and pericardial WAT, and in the interscapular BAT, respectively (Ogawa et al., 1995). Similarly, in obese spontaneously hypertensive rats (SHR), a strain related to Zucker fa/fa rats, OB gene expression was markedly elevated compared to lean SHR (Hiraoka et al., 1997). In addition OB mRNA levels in the epididymal, mesenteric, subcutaneous and retroperitoneal WAT from obese SHR was reported to be 4.4-, 4.8-, 56- and 5.4-fold higher than the level of OB mRNA in these same fat depots from lean SHR (Hiraoka et al., 1997).

In db/db mice, with an obese phenotype identical to that of ob/ob mice, the level of OB mRNA expression was reported to be between 10- and 20-fold higher compared
with lean control mice (Igel et al., 1996b and Maffei et al., 1995b, respectively). Mice heterozygous for the db mutation (who have a normal phenotype) were found to have levels of OB mRNA comparable to lean control animals. Longitudinal analysis of OB mRNA expression revealed that both db/db mice and Zucker fa/fa rats have increased OB mRNA levels by three weeks of age and continued to rise thereafter. This effect was taken to represent a very early defect in the development of the metabolic disturbances seen in these animals as bodyweight and blood glucose levels are still normal at this time. However the rise in OB gene mRNA in both strains is paralleled by increases in serum insulin concentrations and the development of obesity (Igel et al., 1996b).

OB gene expression has also been examined in polygenic and non-genetic rodent models of obesity. Expression of OB mRNA was found to be ~11- and 9-fold higher in KK and NZO mice respectively, compared to lean control mice (Igel et al., 1996b). OB mRNA expression in NON mice was only 3-fold higher than controls but roughly corresponded to adiposity. In non-obese Sprague-Dawley rats of differing weights, an almost proportional increase in OB mRNA expression with increasing body weight was demonstrated (Igel et al., 1996a).

Several studies have recently been conducted in our laboratories examining the OB gene in Psammomys obesus. Examination of the DNA sequence of the OB gene in Psammomys obesus revealed a high degree of homology with mouse (90%), rat (88%) and human (79%) while the OB gene sequence in Psammomys obesus was found to be identical between lean and obese animals (Walder et al., 1997b). In addition, the mutation at codon 105 found in ob/ob mice is not present in the OB gene of Psammomys obesus. Examination of the tissue distribution of the gene revealed that the OB gene is expressed almost exclusively in adipose tissue in these animals while the level of OB gene expression was also found not to differ between the suprascapular, perirenal, intramuscular and mesenteric fat depots (Walder et al., 1997b). Overall OB gene expression in adipose tissue correlated positively with body weight, percent body fat and circulating plasma insulin concentrations in Psammomys obesus, with significantly elevated levels of OB gene expression found in obese compared to lean animals (Walder et al., 1997b).
Lesions to the ventromedial hypothalamus are known to result in obesity as part of a syndrome resembling that seen in ob/ob and db/db mice (Bray and Campfield, 1975). Animals with such lesions were used in early parabiosis studies and results suggested that these mice over-express but are unresponsive to a circulating factor known to suppress food intake and bodyweight (Hervey, 1959), this factor is now known to be leptin. Consistent with these early findings, mice with hypothalamic lesions induced by treatment with gold-thioglucose or monosodium glutamate exhibit higher OB mRNA expression (~20-fold) than lean controls (Maffei et al., 1995b; Frederich et al., 1995). Sprague-Dawley rats with electrolytic VMH lesions also show marked increases in OB mRNA expression when compared to unlesioned control rats (Funahashi et al., 1995). The increase in expression is seen in both the subcutaneous and mesenteric fat depots and appears to occur during marked fat accumulation after VMH-lesions (Funahashi et al., 1995).

In non-human primates such as the Rhesus monkey the sequence of the OB gene is reported to be 91% homologous with the human protein with expression of the gene correlating with body weight and fasting insulin (Hotta et al., 1996). Circulating plasma leptin in monkeys has also been shown to correlate with body weight and body fat (Hotta et al., 1996; Bodkin et al., 1996).

In humans the OB gene is expressed almost exclusively in adipose tissue and similar to rodents, the level of OB mRNA expression has been found by several groups to be higher in obese compared to lean subjects (Considine et al., 1995; Hamilton et al., 1995; Lonngqvist et al., 1995; Considine et al., 1996). In addition OB gene expression is highly correlated with both percent body fat and BMI (Considine et al., 1995; Maffei et al., 1995b; Considine et al., 1996).

Structural characteristics linking the OB protein to the cytokine family are well established. Several studies have provided evidence that leptin is related to a family of helical cytokines which includes interleukin (IL)-2 and growth hormone. Although there is no sequence homology among the members of this family, all contain a distinctive three-dimensional fold and a four-α-helix bundle structure that is recognised by the cognate family of hematopoietic cellular receptors. In addition, there is evidence that the 2 cysteine residues brought into close proximity as a result
of the helical fold form an intra-chain disulphide bridge that is critical for the structural integrity and stability of leptin (Rock et al., 1996). The structural characteristics of leptin have been elucidated by use of threading analysis (Madej et al., 1995), structure prediction algorithms (Rock et al., 1996), nuclear magnetic resonance techniques (Kline et al., 1997) and x-ray crystallography (Zhang et al., 1997).

![Figure 1.6 Three dimensional structure of Leptin (Rock et al., 1996)](image)

1.5.5 CIRCULATING LEPTIN CONCENTRATIONS

1.5.5.1 Animal

As discussed above, OB mRNA expression is markedly elevated in obese rodents and humans. Apart from in the NON mouse, the high levels of OB mRNA expression seen in these obese groups are associated with increased circulating levels of leptin (Frederich et al., 1995; Maffei et al., 1995b). Maffei et al. reported the development of an immunoassay for measurement of leptin in humans and rodents and further described a strong correlation between leptin and BMI in mice and rats (Maffei et al., 1995b).

Circulating leptin levels in plasma from several different rodent strains were measured and found to be increased 10-fold in db/db and A\textsuperscript{v} mice, 5-fold in fat mice
and 2-fold in tubby mice relative to lean littermate controls. Fatty mice exhibited 50-fold higher leptin levels compared to control rats. Non-genetic models of obesity were also described with leptin levels increased 15-fold and 25-fold in GTG-treated and diet-induced obese mice, respectively (Maffei et al., 1995). Similar results have been reported by other groups including Frederich et al. (1995) using a Western blot method for measurement of plasma leptin levels. The NON mouse is unique in that although obese animals have higher expression of ob mRNA, plasma leptin levels in these animals is comparable to those seen in lean control animals and markedly lower than those seen in db/db mice (Igel et al., 1996a).

In Psammomys obesus, the concentration of circulating plasma leptin is also positively associated with body weight and percent body fat, with obese animals found to have significantly higher leptin levels than lean animals (Walder et al., 1997b). In a longitudinal study examining changes in circulating leptin levels during the development of obesity and diabetes in Psammomys obesus it was demonstrated that animals who remained lean (increased their body weight by 154%) over the 8 weeks of the study (between 4 and 12 weeks of age) showed no change in circulating leptin during that time (Collier et al., 1997b). In contrast, animals who developed obesity (increased their bodyweight by 223%), hyperglycemia and hyperinsulinemia over the same time period also developed hyperleptinemia between 4 and 8 weeks of age. It is unclear from this study whether the increase in leptin levels preceded the increase in body weight, or if the higher leptin levels by 8 weeks of age were a consequence of the increased fat mass in these animals. As Psammomys obesus respond to only very high doses of exogenous leptin, the development of leptin resistance in Psammomys obesus may be a manifestation of the ‘thrifty gene’, which would represent a survival advantage for these animals in their natural desert environment, allowing the consumption of large amounts of food without leptin turning off appetite signals, thus enabling storage of excess energy as fat (Collier et al., 1997b; Collier et al., 1997c).

1.5.5.2 Human
In humans, the relationship between BMI or percent body fat and plasma leptin concentration has been found to be very strong in both men and women and this relationship has been described by many groups in several different populations
Women have consistently been reported to have higher leptin levels than men and this difference persists in most groups after adjustment for differences in adiposity between the sexes (Havel et al., 1996a; Rosenbaum et al., 1996; Bennett et al., 1997).

Regional differences in OB gene expression have also been reported in humans with OB mRNA levels found to be significantly lower in omental compared to subcutaneous adipose tissue (Hube et al., 1996; Montague et al., 1997b; Lefebvre et al., 1998, Van Harmelen et al., 1998), although findings of regional differences in human OB gene expression have been inconsistent, with negative findings reported by several other groups (Lonnqvist et al., 1995; Masuzaki et al., 1995). In addition, Montague et al. also reported sex-specific, regional differences in OB gene expression, with females having a markedly higher ratio of subcutaneous-to-omentai OB mRNA expression (~6-fold) compared to men (~2-fold) (Montague et al., 1997b).

Associations between regional body fat distribution and circulating leptin levels have also been investigated with conflicting results reported. Haffner et al. (1996a) reported that plasma leptin concentration was associated with total adiposity but not
central adiposity in a group of Mexican Americans. In this study measures of body fat were not direct, with anthropometry used to estimate individual adipose tissue depots. In contrast a study in African American women found that leptin levels were associated with total adiposity and not selectively with visceral adiposity when fat mass was measured using DEXA and computerised axial topography (Dua et al., 1996). A similar study comparing leptin levels in African American and Caucasian women reported that while serum leptin concentrations were correlated with BMI and fat mass (measured by DEXA) in both groups, leptin was associated with trunk-to-lower extremity fat ratio in Caucasian women and this relationship was not observed in African American women (Perry et al., 1997). Takahashi et al. reported that plasma leptin levels were associated with BMI and subcutaneous fat area at the umbilicus, however no relationship was found with visceral fat area. This study included both men and women with fat measured by computerised topography (Takahashi et al., 1996a). Bennett et al. (1997) demonstrated an association between peripheral fat accumulation and higher plasma leptin levels in women, after adjusting for fat mass.

Although there is conjecture regarding associations between regional fat distribution and circulating leptin levels the primary relationship between leptin concentration and body fat is found to remain under various conditions, with leptin levels in patients with depression (Deuschle et al., 1996), anorexia nervosa (Hebebrand et al., 1995; Grinspoon et al., 1996; Baranowska et al., 1997; Kopp et al., 1998), bulimia nervosa (Kopp et al., 1998), Prada-Willi syndrome (Pietrobelli et al., 1998), spinal cord injury (Bauman et al., 1996), ischemic heart disease (Couillard et al., 1998) and hypothalamic surgery (for pituitary adenoma) (Brabant et al., 1996) found to be directly related to the degree of adiposity, rather than the existing medical conditions. In addition plasma leptin levels in identical twins discordant for obesity were found to be directly related to the degree of adiposity (Ronnea et al., 1997), although subjects with Cushing’s syndrome were found to have a 2-fold increased level of plasma leptin over a 24-hour period when compared to BMI-matched controls (Leal-Cerro et al., 1996). As indicated by studies in subjects with anorexia nervosa and endurance athletes, the relationship between leptin levels and body fat/weight remains at the lower limits of adiposity. Studies conducted in populations such as Pima
Indians, Nauruans and W. Samoans indicate that at the higher limits of adiposity this relationship is also still apparent (Zimmet et al., 1996).

Many studies have been conducted in an effort to characterise the actions of leptin and determine both the central and peripheral effects of this hormone within the body. Peripheral and intracerebroventrical (ICV) administration of exogenous leptin to a number of rodent models have provided importance evidence regarding the central and direct effects of leptin on body weight regulation, as well as the presence of varying degrees of leptin sensitivity across different models and species.

1.5.6 LEPTIN ADMINISTRATION
1.5.6.1 Peripheral Administration-ob/ob mice
The obese phenotype of ob/ob is a result of a longenital lack of circulating leptin which can arise as a result of two different mutations in the OB gene. One mutation abolishes OB gene transcription and thereby prevents leptin synthesis, the other leads to the production of a truncated, inactive protein, which is not secreted from the adipocyte.

Single, daily IP injections of recombinant mouse leptin have been shown in several studies to decrease the body weight of ob/ob mice by 30% at 2 weeks and by 40% after 4 weeks (Campfield et al., 1995; Halaas et al., 1995; Pollymounter et al., 1995; Weigle et al., 1995; Levin et al., 1996). The reduction in body weight after leptin administration has been shown to be a result of both reduced food intake and increased energy expenditure, with metabolic rate, body temperature and activity levels increased after treatment (Campfield et al., 1995; Halaas et al., 1995; Pollymounter et al., 1995; Stephens et al., 1995; Weigle et al., 1995; Levin et al., 1996). The weight-reducing effect of the OB protein were dose- and time-dependent with even the lowest dose of leptin (0.1mg/kg/day) producing an effect. Hyperinsulinemia and hyperglycemia were also reversed by leptin treatment in a dose-dependent manner in ob/ob mice, while other endocrine abnormalities seen in this model were normalised and reproductive capability also restored (Chehab et al., 1996). Surprisingly, these effects were seen with both murine and human recombinant leptin (Pollymounter et al., 1995; Halaas et al., 1995).
In addition, Campfield et al. (1995) demonstrated that the effects of leptin were reversible, with ob/ob mice rapidly gaining weight over two days when leptin treatment was withdrawn. Subcutaneous infusion of leptin into ob/ob mice at doses substantially lower than those required IP provides a constant increase in circulating leptin levels and results in a dose-dependent decrease in body weight at incremental increases in plasma leptin levels within the physiologic range (Levin et al., 1996; Halaas et al., 1996).

A human subject has been identified with congenital leptin deficiency due to a mutation in the OB gene homologous to that seen in the ob/ob mouse (Montague et al., 1997a). Clinical trials are currently underway to assess the effectiveness of leptin treatment to this subject, with daily administration of recombinant-MetHionyl human leptin as a subcutaneous injection (Farooqi et al., 1998). Preliminary results suggest that this treatment induces significant and consistent weight loss, which appears to be mediated through changes in food intake, rather than energy expenditure. The effects of the exogenous leptin were apparent as early as 12 days after initiation of treatment, and after nine months of treatment the subject has lost 14.7 kg of weight, over 90% of which has been determined to be fat mass (Farooqi et al., 1998). The administration of exogenous leptin to this subject with congenital leptin deficiency appears to be very effective, and mimics the changes in body weight and food intake seen in the ob/ob mouse with leptin treatment.

The effects of leptin are also seen in wild-type (WT) mice, however a higher dose is required. Twice daily injections of high doses of leptin into WT mice resulted in a sustained 12% weight loss, decreased food intake and reduced body fat from 12.2 to 0.7% (Campfield et al., 1995; Halaas et al., 1995; Pellymounter et al., 1995; Stephens et al., 1995; Weigle et al., 1995 Levin et al., 1996). In WT mice, leptin appears to have minimal effects on food intake, but causes loss in body fat, presumably due to a direct action of leptin to increase energy expenditure (Levin et al., 1996). In addition, leptin has been shown to selectively increase norepinephrine turnover in brown adipose tissue, which suggests that leptin’s actions may be mediated via the activation of the sympathetic nervous system (Collins et al., 1996a). Recently Harris et al. (1998) conducted a study in which varying concentrations of leptin (0, 1, 2, 5, 10 or 42 µg/day) were infused into the peritoneal cavity from miniosmotic pumps for 7
days. In ob/ob mice, a dose of 2 μg/day decreased food intake, body weight and glucose and insulin concentrations. As only small amounts of leptin were required to illicit these responses, they were suggested to represent primary physiological responses to leptin. As a much higher dose of 45 μg/day was required to correct hypothermia and increase expression of BAT UCP in ob/ob the authors suggested that these effects are indirect responses to high concentrations of protein and are representative of responses to a pharmacological dose of leptin (Harris et al., 1998).

1.5.6.2 Central Administration
Intracerebroventricular (ICV) administration of leptin results in a more potent response compared to systemic administration of leptin, as a much lower concentration of leptin can be used to cause a reduction in body weight. This method of administration also overcomes peripheral leptin resistance seen in several rodent models. A single ICV injection of leptin at concentrations between 0.01 and 3.0 μg has been shown to reduce food intake and body weight in ob/ob mice (Campfield et al., 1995; Stephens et al., 1995; Hwa et al., 1996; Halaas et al., 1997). Hwa et al. (1996) demonstrated a lowering of food intake and body weight within 24 hours of administration, with leptin also increasing 22 hour energy expenditure and reducing the respiratory quotient in a dose-dependent manner. These findings have been taken to suggest a central effect of leptin on energy utilisation and indicate that leptin is also able to regulate energy partitioning to increase fat metabolism through a mechanism mediated through the CNS.

1.5.6.3 Leptin Administration—Other Models Of Obesity
Single-gene models
Leptin administration to db/db mice does not reduce food intake or body weight under any of the experimental protocols discussed, including central administration, which indicates that leptin resistance in this animal model cannot be overcome by exogenous leptin treatment (Halaas et al., 1995; Pellymounter et al., 1995; Stephens et al., 1995; Halaas et al., 1997). These mice exhibit hyperleptinemia and do not express functional OB-RB, the leptin receptor primarily responsible for transduction of the leptin signal within the brain. Interestingly, a study by Van Heck et al (1996) which examined the distribution and turnover of 125I-leptin injected IP into ob/ob and db/db mice found no differences in localisation, accumulation or clearance of 125I-
leptin between the two groups in any of the tissues examined, indicating that a lack of OB-RB does not affect clearance of leptin from the plasma. The mutation in the leptin receptor in db/db mice therefore, appears to confer complete resistance to the anorexigenic effects of leptin.

Agouti (A^v) mice also have high circulating leptin concentrations and do not respond to peripheral leptin administration, with high doses of leptin given subcutaneously or intraperitoneally failing to reduce food intake or body weight (Halaas et al., 1997). Similar to the NZO mouse, A^v mice have been shown to respond to leptin administered centrally, although agouti mice are 100-fold less sensitive to ICV leptin than control mice, with a dose of 500 ng/hr resulting in modest, but significant weight loss (Halaas et al., 1997). These studies show that agouti mice are resistant to peripheral leptin administration, and are responsive to only high doses of leptin administered centrally, suggesting an overall degree of leptin resistance.

Obesity in the fa/fa Zucker rat results from a missense mutation within the OB-R known to affect all isoforms of the leptin receptor. Obese Zucker rats have been shown to have high circulating leptin levels and do not respond to peripheral leptin administration. Several groups have demonstrated that obese Zucker rats do not respond to central administration of leptin at doses shown to elicit a response in non-obese Zucker rats (Cusin et al., 1996; Seeley et al., 1996; Al-Barazanji et al., 1997). Central leptin resistance can be overcome in obese Zucker rats, however a dose of leptin between 2- and 10-fold higher than required by lean controls is necessary to decrease food intake and bodyweight (Cusin et al., 1996). This reduced sensitivity to leptin, leptin resistance, appears to be a direct result of reduced signaling by leptin receptors containing the fa/fa mutation (Yamashita et al., 1997).

Polygenic models

AKR/J mice remain lean when fed a diet of standard laboratory chow, but develop obesity if fed a high-fat diet (45% energy from fat) (West et al., 1994b). These diet-induced obese (DIO) mice do respond to leptin administered IP, however a higher dose of leptin was required to elicit a response than necessary for chow-fed, non-obese AKR/J mice (Halaas et al., 1997). A dose of 12.5 mg/kg twice daily resulted in a reduction in body fat of about 30% in DIO mice, compared to a reduction of about
83% in lean AKR/J mice (Halaas et al., 1997). The response to leptin in DIO mice appears to alter with time however and Van Heek et al. (1997) have demonstrated that DIO mice exhibit a time-dependent development of peripheral leptin resistance. IP leptin administration was shown to be effective at decreasing food intake in AKR mice fed either 10%- or 45%-energy from fat, however this effect was not sustained after 56 days of treatment (Van Heek et al., 1997). After this time leptin resistance develops and the mice no longer respond to IP leptin administration. The mechanism for the development of this apparent peripheral leptin resistance is unknown, however throughout this period DIO mice retain full sensitivity to leptin administered ICV (Van Heek et al., 1997), indicating a reduction in peripheral leptin action, without an accompanying change in central leptin activity.

DIO rats are a similar model to DIO mice, with obesity developing in Wistar rats after they are transferred from a chow to a high-calorie diet (Widdowson et al., 1997). Lean Wistar rats are known to respond normally to ICV leptin administration (Wang et al., 1997) with a dose-dependent decrease in food intake observed at 1, 4 and 24 hours after injection of leptin into the third ventricle (Widdowson et al., 1997). In DIO rats although food intake decreased after ICV leptin administration, this effect did not persist after the first hour (Widdowson et al., 1997). When these same DIO rats are returned to a diet of standard laboratory chow their food intake decreases and they appear to regain at least partial responsiveness to their endogenous leptin satiety signal indicating that there is not a complete attenuation of peripheral leptin activity as seen in DIO mice, and that while the leptin satiety signal remains at least partially intact, it may be overridden by the attractions of a palatable diet (Widdowson et al., 1997).

NZO mice exhibit a polygenic syndrome of hyperphagia, obesity, hyperglycemia and hyperinsulinemia, in addition to elevated leptin levels. These mice have been found to exhibit peripheral leptin resistance, with doses of leptin up to 9-times that required to elicit a response in ob/ob mice failing to reduce body weight or food intake (Halaas et al., 1997; Igcl et al., 1997). Interestingly however, NZO mice have been found to be normally responsive to leptin administered ICV, with infusion of a low dose of leptin (5 ng/hr) into the third ventricle resulting in significant weight loss (Halaas et
al., 1997). These results indicate that leptin resistance in this model may be the result of reduced transport of leptin into the CNS or a defect located distal from OB-R.

In our laboratory several studies have been conducted examining the effects of leptin treatment in *Psammomys obesus*. Both intraperitoneal administration of 5mg leptin/kg body weight/day for 14 days and 0.8 mg/kg/day for 14 days by continuous infusion had no effects on food intake, body weight, blood glucose or plasma insulin concentrations in either lean or obese *Psammomys obesus* (Walder et al., 1999). Both of these protocols are successful at reducing food intake and body weight in both wild-type and *ob/ob* mice. The leptin resistance of *Psammomys obesus* was overcome by a very large dose of leptin, however it was only effective in lean, but not obese animals. A dose of 45 mg leptin/kg/day lowered body weight, body fat and food intake in lean animals (Walder et al., 1999). This dose increased circulating leptin levels on average to 125-times baseline levels in lean animals. In obese animals this dose increased circulating leptin levels to 80-times baseline although it was ineffective at decreasing body weight and food intake in this group (Walder et al., 1999).

In normal Sprague-Dawley rats, central administration of human leptin into the ventro-medial hypothalamus (VMH) has been found to decrease food intake to 56% of baseline levels and decrease bodyweight by approximately 5% during three days of leptin treatment (Jacob et al., 1997). Satoh et al. (1997) have also demonstrated however, that Sprague-Dawley rats with bilateral lesions of the VMH do not respond to central leptin administration (Satoh et al., 1997).

The varying response to exogenous leptin treatment in rodents demonstrates a range of leptin resistance, with *ob/ob* mice being the most sensitive to leptin and *db/db* mice being the most leptin resistant. These responses are summarised in figure 1.8 and demonstrate that obesity and high leptin concentration are associated with leptin resistance in rodents.
1.5.6.4 Gene Therapy

Leptin gene therapy has been demonstrated to be an effective method of increasing circulating leptin levels in both rats and mice (Muzzin et al., 1996; Chen et al., 1996b; Murphy et al., 1997; Koyama et al., 1998). Ob/ob mice treated intravenously with a recombinant adenovirus expressing murine OB cDNA showed normal levels of circulating leptin and decreased food intake and body weight in addition to normalisation of serum insulin levels and glucose tolerance (Muzzin et al., 1996). The effect a single injection of adenovirus lasted for 3-weeks. Murphy et al. (1997) demonstrated that a single intra-muscular injection of a similar recombinant adenovirus increased circulating leptin levels and prevented the development of obesity and diabetes in ob/ob mice. The single intra-muscular injection was found to last through the six-month time course of the study (Murphy et al., 1997).

Leptin gene therapy in normal Wistar rats resulted in the disappearance of body fat in treated animals. Hyperleptinemia was induced in these rats by treatment with a recombinant adenovirus containing the rat OB gene and this hyperleptinemia was accompanied by complete ablation of identifiable fat tissue, an effect which was thought to be a specific lipoatrophic action of leptin, as a group of rats pair-fed to the food intake of the treated rats did not show such an effect (Chen et al., 1996b). Koyama et al have recently demonstrated that although adenovirally induced hyperleptinemia is effective in reducing food intake and bodyweight in ob/ob mice
and normal rats, this therapy does not overcome the leptin resistance observed in VMH-lesioned rats (Koyama et al., 1998).

1.5.6.5 Primate Leptin Treatment

Rhesus monkeys frequently spontaneously develop obesity after sexual maturation when provided with a balanced ad lib diet and also exhibit hyperleptinemia (Bodkin et al., 1996). Recently it has been shown that peripheral administration of human leptin (1 mg/kg) to Rhesus monkeys is not effective at decreasing food intake or body weight (Tang-Christensen et al., 1999), however central administration of leptin (1 μg/kg) acutely increases plasma norepinephrine and also decreases food intake by 40-50% the day after leptin injection. These results suggest that in addition to decreasing food intake, central leptin administration of leptin to the Rhesus monkey also activates the sympathetic nervous system (Tang-Christensen et al., 1999).

Preliminary results from trials of subcutaneous recombinant methionyl human leptin treatment in humans have revealed a dose response for weight loss from baseline in both lean and obese subjects (Hemysfield et al., 1998). All subjects had decreased bodyweight by on average between 0.4 and 1.9 kg after one month of leptin treatment while obese subjects who completed 6-months of treatment decreased bodyweight by on average between 0.7 and 7.1 kg (Hemysfield et al., 1998). Throughout the study obese subjects consumed a 500-kcal deficient diet, while lean subjects maintained a eucaloric diet. In addition, the authors indicate that no notable adverse effects of the leptin treatment were observed on major organ systems. This preliminary data demonstrates that recombinant methionyl human leptin can reduce weight in humans and has an acceptable safety profile (Hemysfield et al., 1998) although obviously much work must be done in this area.

The results of leptin treatment to lean and obese humans are quite different to those seen with leptin treatment to the individual with congenital leptin deficiency, in which leptin treatment resulted in significant and consistent weight loss within two weeks of treatment. Similar to the results in rodents, the data in humans therefore also demonstrates that individuals which lack circulating leptin are most sensitive to leptin treatment, while humans with obesity and high concentrations of circulating leptin show degrees of leptin resistance.
1.5.7 LEPTIN KINETICS

1.5.7.1 Secretion

White adipose tissue (WAT) is the major site of OB mRNA expression in both rodents and humans. In addition adipose tissue has consistently been demonstrated to be the major source of leptin in these species. Leptin secretion from WAT has been measured directly in humans by several groups using standard arteriovenous balance principles (Klein et al., 1996; Esler et al., 1998). In addition Lonnqvist et al. (1996) have demonstrated that in humans, OB mRNA and leptin secretion reflect the lipid content of the fat cell in adipose tissue, with fat cell volume accounting for as much as 70-80% of the variations in leptin secretion rate, either expressed per lipid weight or per cell number. The rate of leptin secretion from adipose tissue has been found to correlate closely with adiposity, including percent body fat and BMI (Klein et al., 1996; Van Harmelen et al., 1998), while leptin is cleared from the plasma at a rate independent of body fat mass. In addition, Van Harmelen et al. (1998) reported that fat taken from the abdominal subcutaneous and omental depots of women during gastric banding demonstrated different rates of leptin secretion. Leptin secretion rates were found to be two to three times higher in subcutaneous than omental adipose tissue from both obese and non-obese women, while leptin secretion from both depots correlated closely with BMI and circulating plasma leptin levels (Van Harmelen et al., 1998).

Another interesting finding was reported by Esler et al. (1998) who measured arteriovenous plasma concentration gradients of leptin across the brain, kidneys, gut, liver, heart and forearm in middle aged, fasting men. There was no detectable flux of leptin to or from plasma in passage through the heart, the gut and liver or the forearm, however obese subjects cerebral leptin overflow was ~450 ng/min compared to ~90 ng/min in lean men, this difference was seen despite no difference in leptin clearance rates by the kidney in the two groups. These results suggest that the brain is a source of leptin and also that the rate of leptin secreted from the brain is higher in obese compared to lean men (Esler et al., 1998).
1.5.7.2 Circadian Rhythm

Sinha et al. (1996a) were the first to describe a nocturnal rise in plasma leptin levels in humans. 24-hour profiles of circulating leptin were measured in lean and obese subjects with blood collected at various times throughout the 24-hour period. This study confirmed that there is no postprandial decrease in circulating plasma levels and changes in leptin throughout the day could not be correlated with glucose or insulin concentrations (Sinha et al., 1996a,b). Despite the initial differences in leptin levels between lean and obese individuals, circulating leptin concentration was shown to peak between mid-night and early morning hours and drop to their lowest around noon to mid-afternoon (Sinha et al., 1996a). The peak night time leptin levels were reported to be between 50 and 80% higher compared to nadir afternoon leptin levels. A further study by Sinha et al. (1996b) described a pattern of ultraradian oscillations in leptin secretion in humans. When blood was sampled at 15-min intervals over a 12-hour period an average of 4.2 leptin pulses were detected, with approximately 3.5 hour periodicity (Sinha et al., 1996b). These collections were made during an overnight fast, however oscillating blood glucose was infused during this period. Both BMI and fasting plasma leptin levels were associated with the number of leptin pulses, while the number of pulses was positively correlated with mean absolute amplitude (Sinha et al., 1996b).

In rats a rhythmicity of OB mRNA gene expression in adipose tissue has also been described (Saladin et al., 1995). In this study OB mRNA levels were found to be lowest during the light cycle and increasing soon after the onset of feeding, reaching a maximum at approximately 04:00 hours. Subsequently, OB mRNA expression decreased steadily and reached a minimum in the afternoon. The rhythmicity of OB mRNA expression in rats was linked entirely to feeding in this study, although the circulating leptin levels during this time were not reported. If changes in OB mRNA are taken to reflect changes in plasma leptin concentrations then rodents and humans appear to demonstrate different diurnal rhythms for leptin. Ahima et al (1998) determined that the circadian rhythm of leptin is also linked to feeding in mice, with circadian rhythms of corticosterone, thyroxinc and leptin shown to be regulated by food intake in adult mice (Ahima et al., 1998). Leptin and corticosterone were found to have reciprocal rhythms in mice, as in humans, with leptin peaking late in the dark cycle and decreasing during the light cycle. This reciprocal relationship was
maintained even when animals were restricted to feeding in the light cycle for two weeks. During this time corticosterone decreased to nadir after feeding, increased during the rest of the light and dark cycles and peaked before the next meal and there was a parallel shift of the timing of peak levels of leptin, with leptin rising after feeding (Ahima et al., 1998). Interestingly leptin deficiency in ob/ob mice did not alter the circadian rhythm of corticosterone, indicating that leptin does not primarily regulate the corticosterone rhythm.

Approximately one year after the initial reports of diurnal variation in leptin levels in humans, Schoeller et al. reported the rhythmicity of plasma leptin concentrations is in fact entrained to meal timing. This study demonstrated that alterations in meal timing were accompanied by an acute phase shift in leptin that directly corresponded with the altered meal timing, while the diurnal cycle for plasma cortisol, a marker of circadian time, did not change. Despite this finding leptin levels were not acutely altered post-prandially. In addition sleep deprivation did not alter the leptin rhythm, indicating that the leptin rhythm was not entrained to the sleep cycle (Schoeller et al., 1997).

1.5.7.3 Clearance from Circulation
It has been established that while leptin is secreted predominately from adipose tissue, it is cleared from the circulation by the kidneys in both rodents and humans. The half-life of leptin in humans has been reported to be approximately 25 mins across a range of plasma leptin concentrations (Klein et al., 1996). No difference in leptin clearance rates have been detected between lean and obese subjects (Klein et al., 1996, Esler et al., 1998). In rodents (ob/ob, db/db mice and Sprague-Dawley rats) the half life of leptin was found to be 1.5 hours (Van Heek et al., 1996; Cumin et al., 1996), while in New Zealand white rabbits, the elimination half-life of leptin was 45 mins (Karonen et al., 1998). Meyer et al. (1997) reported that in humans the kidneys account for ~80% of overall systemic removal of leptin from plasma, while the removal of leptin by the kidneys was found to be a saturable process which followed Michalis-Menten kinetics. In addition little or no leptin was detected in the urine which indicated that leptin was degraded within the kidney, and not simply excreted from the body (Meyer et al., 1997).
A similar pattern of leptin clearance has been reported in rodents, with studies in mice and rats demonstrating that between 80-95% of endogenous leptin is cleared by the kidneys (Cumin et al., 1996; Cumin et al., 1997a,b). Extensive studies in both Sprague-Dawley and obese Zucker (fa/fa) rats have determined that the process of renal leptin extraction is unaltered over a wide range of plasma leptin concentrations and similar to humans, leptin is not detected in the urine (Cumin et al., 1996; Cumin et al., 1997a,b). In addition in rats, leptin clearance from the circulation is rapid and represents a high-capacity, non-saturable process most likely involving glomerular filtration with subsequent metabolic degradation in the renal tubules (Cumin et al., 1996; Cumin et al., 1997a,b). Interestingly, Cumin et al reported that after bilateral nephrectomy plasma leptin levels in Sprague-Dawley rats rise sharply, however after 3 hours leptin levels start to plateau and by 48-hours they have returned to control levels. It was demonstrated that the plateauing of leptin levels in these animals was not due to an inhibition of leptin secretion from adipocytes and the authors hypothesized that other elimination mechanisms for leptin must be present, with the liver thought to be the most likely candidate (Cumin et al., 1997a,b).

Several studies have examined plasma leptin levels in humans with chronic renal failure. Iida et al (1996) reported a significant increase in Japanese patients with chronic renal failure compared to normal controls, however serum leptin concentrations were not found to be correlated with several indices of impairment of renal function including serum creatinine or BUN (Iida et al., 1996b). In addition Merabet et al. (1997) reported a 2-fold increase in plasma leptin concentration in patients with end-stage renal disease. Together these studies further support the role of the kidneys in the clearance of leptin from the circulation in humans. Diabetic Pima Indians with and without nephropathy were reported to have similar plasma leptin levels, indicating that although diabetic nephropathy may alter renal metabolism, alterations in leptin metabolism by the kidneys which are reflected by increased plasma leptin levels may only occur with severe renal disease (Wilson et al., 1998).

1.5.7.4 Leptin Transport Into The Brain
Leptin has been detected in the CSF in both humans and rodents and there are two possible mechanisms for transport of leptin into the brain. Leptin receptors have been
identified on the choroid plexus which may be responsible for transport of leptin
across the blood-brain barrier and into the CSF (Tartaglia et al., 1995; Lynn et al.,
1996). In addition leptin receptors have recently been found at the brain capillary
endothelium which would allow leptin to be transported into brain ISF (Golden et al.,
1998). The Wu-Peng et al. that there are equivalent CSF leptin concentrations
between lean (ff) and obese (fa/fa) Koletsky rats, indicates that leptin may also enter
the CSF via a non-leptin receptor mediated mechanism as fa/fa rats have mutated
leptin receptors (Wu-Peng et al., 1997).

The relationship between circulating plasma levels of leptin and the amount of leptin
in the CSF was investigated as a possible mechanism for leptin resistance in human
obesity. Caro et al. (1996) reported that while plasma leptin levels in obese
individuals were 318% higher than those in lean individuals, the concentration of
leptin in the CSF was only 30% higher, thus the ratio of CSF/serum leptin was only
~4-fold higher in obese, compared to lean subjects (Caro et al., 1996). Schwartz et al.
(1996b) reported a similar finding around the same time with reduced efficiency of
brain leptin delivery seen in obese subjects and thought to mediate leptin resistance
(Schwartz et al., 1996b). In addition, both groups reported that leptin levels in the
plasma and the CSF correlated strongly with adiposity in a non-linear manner (Caro
et al., 1996; Schwartz et al., 1996b). There were a few differences between these
studies however, with CSF leptin reported not to plateau even at very high plasma
levels by Schwartz et al., while Caro et al., described a relationship between plasma
leptin and CSF leptin that appeared to plateau at high plasma leptin concentrations.
In addition, Schwartz et al. reported that CSF leptin levels were higher in women
(postmenopausal) than in men, even after adjustment for adiposity. Both studies
however, describe a saturable transport system for leptin into the brain, with reduced
efficiency of transport observed in obese individuals.

Banks et al. also described a saturable transport system for leptin across the blood-
brain barrier (BBB) in mice. Capillary depletion studies demonstrated that leptin
enters the brain parenchyma behind the BBB rather than being trapped by the
endothelial cells of the capillaries that constitute the BBB (Banks et al., 1996). Leptin
was found to be transported intact and at a rate 20-times faster than albumin,
although the rate was comparable to that of a limited number of regulatory substances
of similar size (Banks et al., 1996). In addition, leptin transport was unaffected by insulin, indicating that the transport mechanisms for the two peptides differs.

1.5.8 REGULATION OF OB GENE EXPRESSION AND LEPTIN

1.5.8.1 Human OB Gene Sequence Variation

Two different mutations in the mouse OB gene give rise to the obese ob/ob phenotype when present in homozygous form (Section 1.4.1). This raised the question of whether obesity in humans could be due to a similar mutation in the OB gene and subsequently leptin deficiency. In 1995 and early 1996 several DNA screening studies were conducted in human populations in an attempt to identify mutations responsible for human obesity. Considine et al. (1995) reported that the mouse mutation at amino acid 105 was not present in the OB gene of obese humans although a group of 8 obese subjects was found to have 72% more OB gene expression than lean controls. Maffei et al. (1995b) screened 105 obese/diabetic patients and also failed to find sequence polymorphisms within the coding region of the OB gene, although a simple polymorphic dinucleotide repeat DNA polymorphism was identified in the non-coding region. From these studies both groups reached the conclusion that mutations within the coding sequence of the OB gene are not a common cause of human obesity (Considine et al., 1995; Maffei et al., 1995b).

Subsequent screening of the human OB gene was conducted in several populations. Studies in both American subjects (Considine et al., 1996) and Japanese and Asian Indian subjects (Niki et al., 1996) again reported negative findings after screening the coding region of the OB gene for possible mutations and/or polymorphisms associated with human obesity. Shintani et al., (1996) described a highly polymorphic tetranucleotide repeat polymorphism in the 3' flanking region of the human OB gene, however there was only a tentative association between this polymorphism and obesity in a population of Japanese subjects (Shintani et al., 1996). Clement et al. (1996) used radiation hybrid mapping to examine the human OB gene and eight microsatellite markers spanning the OB gene region in 101 French obese families. This study reported that the OB gene was a candidate for genetic predisposition to extreme obesity in some families, as suggestive evidence for linkage was detected for three markers located within 2cM of the OB gene (D7S514, D7S680 and D7S530).
More recently Okansen et al. reported a novel polymorphism in the promoter of the human OB gene (Okansen et al. (1997) and Mammes et al. (1998) demonstrated that polymorphic areas within the 5' region of the OB gene were associated with changes in circulating leptin in response to a low calorie diet although none of the eight polymorphisms described were associated with BMI in subjects on their normal diets (Mammes et al., 1998).

The screening of hundreds of obese individuals for OB gene mutations over several years generally returned negative results although recently two separate mutations within the OB gene have been identified which are linked to the morbid obese phenotype of the individuals affected. Montague et al. described 2 morbidly obese cousins from an inbred Pakistani kindred found to have an OB gene mutation. The cousins, aged 8 yrs and 2 yrs, both had body weights greater than the 99.6th centile of body weight for children that age, however serum leptin levels were very low despite their markedly elevated fat mass (Montague et al., 1997a). Sequencing of the OB gene in these subjects revealed the deletion of a guanine nucleotide in codon 133, which results in five, rather than 6 guanine residues present between nucleotides 393 and 398. This alteration disrupts the open reading frame of the OB gene which in turn leads to the introduction of 14 aberrant amino acids after gly132 in the OB protein, followed by a premature stop codon. In vitro studies using CHO cells transfected with expression vectors encoding this OB gene mutation revealed that while OB mRNA is expressed within the cells and leptin is produced, the leptin is not secreted into the surrounding media (Montague et al., 1997a). This frame-shift mutation therefore results in the production of leptin that is not normally targeted for secretion, similar to the OB mutation seen in ob/ob mice (Zhang et al., 1995). In addition to developing obesity very early in life, the two children had a history of marked hyperphagia, and while both were normoglycemic, the older child was hyperinsulinemic. While it is too early to detect any reproductive abnormalities in these leptin-deficient children, mild dysfunction of the pituitary-thyroid axis was detected (Montague et al., 1997a). Taken together the syndrome of congenital leptin deficiency described in these subjects parallels the abnormalities described in the ob/ob mouse, which has a similar mutation in the OB gene.
Strobel et al. (1998) screened 203 Turkish subjects and identified one individual with a BMI of ~56 with very low levels of circulating leptin detected in serum. DNA sequencing revealed a C->T base substitution in codon 105 of the OB gene which results in an Arg->Trp replacement in the mature protein. Two other individuals homozygous for the mutation were subsequently identified in the same family, both of whom were found to have marked obesity and low levels of circulating leptin. The other members of the family were either lacking the mutation or were heterozygous for it and exhibited normal bodyweight, and normal levels of leptin, fasting blood glucose and plasma insulin, which indicated that a recessive mutation was responsible for the monogenic obesity seen in this family (Strobel et al., 1998). In addition to obesity, the two adults affected (1 male, 1 female) displayed amenorrhoea (female) and hypogonadism (male) with sex hormone levels found to be low or at the lower end of the normal range. These findings again describe abnormalities similar to the reproductive defects previously reported in ob/ob mice and also suggest a role for leptin in human reproductive function.

1.5.8.2 Endocrine Regulation of Leptin
Leptin concentrations have been shown to be regulated by factors other than just the size of the adipose tissue mass, and OB gene expression is altered with changing metabolic status. Several important regulators of metabolic status are circulating metabolites and hormones, nutritional status and energy balance. The importance of these factors on OB gene expression is discussed below.

Insulin
One important regulator of OB gene expression and leptin secretion is insulin. In rodents, OB mRNA and plasma leptin are decreased in insulin-deficient states including fasting and streptozotocin diabetes and increased with insulin treatment and refeeding (Cusin et al., 1995; MacDougald et al., 1995; Saladin et al., 1995; Trayhurn et al., 1995b; Igel et al., 1996b; Sivitz et al., 1996).

A direct effect of insulin on OB gene expression and leptin secretion in rat and human adipose cells has been demonstrated by several groups, although varied results in humans have been reported. In rodents the evidence that insulin acutely regulates OB gene expression is quite clear. Hyperinsulinaemia for 3-5 hours has been shown to
increase leptin levels in rodents (Cusin et al., 1995; Saladin et al., 1995; Utriainen et al., 1996; Vidal et al., 1996) and insulin administration to rodents stimulates OB gene expression in adipose tissue (Saladin et al., 1995; Cusin et al., 1995; Zheng et al., 1996) and increases plasma leptin levels (Hardie et al., 1996; Koopmans et al., 1998). The action of insulin on OB gene expression and leptin secretion is thought to be direct, as studies in cultured adipocytes or differentiated adipocyte cell lines produce similar results (Saladin et al., 1995; Gettys et al., 1996; Leroy et al., 1996; Rentsch et al., 1996; Slicker et al., 1996).

In humans the role of insulin is less clear. A short-term increase in plasma insulin (2-5 hours) does not increase leptin production (Kolaczynski et al., 1996; Muscelli et al., 1996; Prately et al., 1996; Vidal et al., 1996), whereas prolonged exposure to insulin (8-72 hours) has been shown to increase leptin production both in vitro and in vivo (Kolaczynski et al., 1996; Malmstrom et al., 1996; Utriainen et al., 1996). During hyperinsulinemic clamp studies, the reported minimum time required to obtain a significant rise in plasma leptin concentration ranged from 6 (Malmstrom et al., 1996) to 72 hours (Kolaczynski et al., 1996). When the clamp studies were of shorter duration they failed to demonstrate stimulation of leptin production (Dagogo-Jack et al., 1996; Prately et al., 1996; Ryan et al., 1996; Vidal et al., 1996). A significant increase in leptin levels has been observed after 4 hours however, but only with supraphysiological (~2,800 pmol/l) hyperinsulinemia (Utriainen et al., 1996). Interestingly, Saad et al. (1998) provided evidence suggesting that the effects of insulin infusion on leptin production may only become apparent when a saline infused control group is included in the study.

In the human studies discussed above, plasma leptin levels are correlated with basal insulin independently of adiposity, however it is difficult to determine a causal relationship from these studies as their relationship in regulating each other's production is unknown. Interestingly healthy subjects who have chronic primary hyperinsulinemia as a result of insulinoma show elevated levels of both OB gene expression and circulating leptin, both of which correlate positively with plasma insulin in these patients. After surgery, when the insulin-secreting tumour was removed, plasma leptin levels decreased and returned to normal (Popovic et al., 1998; D'Adamo et al., 1998).
The long-term regulation of OB gene expression and leptin levels by insulin may occur at a transcriptional level (Leroy et al., 1996; Kim et al., 1998). One reason for this is that to date no intracellular storage pool for leptin has been identified which could be rapidly mobilized in response to secretagogues such as insulin (Houseknecht et al., 1998), although leptin secretion is known to be pulsatile in nature (Licino et al., 1997; Sinha et al., 1996b). In addition, a candidate transcription factor (ADD1/SREBP1) has recently been reported which links changes in insulin levels to OB gene expression (Kim et al., 1998).

In human studies leptin and fasting insulin concentrations are often correlated independently of body weight or fat mass. In addition circulating leptin levels have been found to be correlated with insulin area and overall, non-oxidative and oxidative whole body glucose disposal during a hyperinsulinemic, euglycemic clamp (Haffner et al., 1997). In addition, Saad et al. have linked insulin-sensitivity with regulation of leptin by insulin, indicating that inadequate insulin-induced leptin production may contribute to the development or worsening of the obese state (Saad et al., 1998). In addition, Mueller et al. (1998) demonstrated that the insulin-stimulated increase in leptin may be mediated by glucose transport and metabolism. In cultured adipocytes the addition of 2-deoxy-D-glucose (a competitive inhibitor of glucose transport and phosphorylation; 2-DG) resulted in a concentration-dependent inhibition of leptin release in the presence of insulin (Mueller et al., 1998). The addition of two other inhibitors of glucose transport also inhibited leptin secretion, while inhibitors of glycolysis also inhibited leptin secretion in a concentration-dependent manner. These in vitro results indicate that the effect of insulin to increase adipocyte glucose utilisation is likely to contribute to insulin-stimulated leptin secretion. In vivo, decreased adipose glucose metabolism may represent one mechanism by which fasting decreases circulating leptin levels, and re-feeding, via increased glucose metabolism, increases circulating leptin (Mueller et al., 1998).

Glucocorticoids

Glucocorticoids are another known potent stimulator of OB gene expression and leptin secretion in both rodents and humans. In vivo administration of various glucocorticoids to rats demonstrated that OB gene expression is rapidly induced by
corticosteroids and this induction was followed by a concordant decrease in body weight gain and food consumption (De Vos et al., 1995). The effect of glucocorticoids is thought to be direct, as OB mRNA is increased by glucocorticoids in vitro in cultured adipocytes (Murakami et al., 1995; Slieker et al., 1996; Wabitsch et al., 1996). Murakami et al. demonstrated that the synthetic glucocorticoid dexamethasone rapidly increases OB gene expression in cultured rat adipocytes, with this effect observed within 1 hour, and reaching a maximum approximately 7 hours after stimulation (Murakami et al., 1995a). In cultured human fat cells, administration of cortisol at near-physiological concentrations potentiates the insulin-induced increase in circulating leptin (Wabitsch et al., 1996). It is possible that the effects of high glucocorticoid levels to increase leptin concentrations may be at least partly mediated by concurrent hyperinsulinemia (Wadjaja et al., 1998).

In vivo studies in humans have also demonstrated an increase in plasma leptin levels after glucocorticoid treatment (Berneis et al., 1996; Kiess et al., 1996; Miell et al., 1996) although this effect is not seen in all studies (Tataranni et al., 1997a). Glucocorticoids and leptin are seen to have reciprocal diurnal rhythms in both rodents and humans (Saladin et al., 1995; Ahima et al., 1996; Sinha et al., 1996a; Licinio et al., 1997), and this relationship is shown to be maintained even if the glucocorticoid rhythm is disturbed (Ahima et al., 1998), implying a regulatory relationship between these two factors.

In contrast, OB gene expression appears to be decreased by the activity of the sympathetic nervous system. The reducing effect of adrenergic stimulation on OB mRNA expression and leptin secretion is indicated in studies using either a range of β3-adrenergic agonists, cold exposure or dbcAMP (Gettys et al., 1996; Mantzoros et al., 1996; Slieker et al., 1996; Trayhurn et al., 1996). Studies examining the effect of cold exposure have demonstrated a large decrease in OB gene expression in brown (Moinat et al., 1995) and white (Trayhurn et al., 1995a) adipose tissue and a fall in circulating leptin levels (Hardie et al., 1996). The response to cold exposure also involves an increase in energy expenditure and fatty acid mobilisation (Girard et al., 1997).
Administration of norepinephrine, isoprenaline (a β-adrenoceptor agonist) or a selective β3-adrenoceptor agonist markedly decreases OB gene expression in adipose tissue (Moinat et al., 1995, Collins et al., 1996a; Trayhurn et al., 1995a; Mantzoros et al., 1996). This effect of β3-adrenoceptor agonist is thought to be direct as in vitro studies in cultured adipocytes show similar results (Gettys et al., 1996). These studies suggest that sympathetically-mediated suppression of OB gene expression occurs, with the sympathetic system acting as a key feedback loop to adipocytes (Girard et al., 1997). The increase in FFAs observed with some of these treatments may also contribute to inhibition of OB gene expression as in differentiated adipocytes FFAs inhibit OB gene expression (Rentsch et al., 1996).

**Cytokines**

Administration of endotoxin (LPS), a model for gram negative infections, induces profound anorexia and weight loss. A study in fasted hamsters by Grunfeld et al. (1996), demonstrated that administration of LPS to fasted animals increased expression of OB mRNA to levels similar of fed controls, and in addition there was a strong inverse relationship between OB mRNA levels and subsequent food intake (Grunfeld et al., 1996). TNF-α is known to be a mediator of the host response to LPS and TNF-α administration to fasted hamsters also increased levels of OB mRNA (Grunfeld et al., 1996). Circulating leptin was found to be regulated by LPS and TNF-α in parallel to regulation of OB mRNA in adipose tissue.

Similar results were demonstrated by Finck et al. (1998) in mice, where intraperitoneal injection of TNF-α increased plasma leptin levels in both endotoxin-sensitive and endotoxin-resistant mice. Studies in cultured fat cells from the mice demonstrated that LPS did not increase leptin secretion, however TNF-α induced a marked increase in the cell supernatant leptin concentration (Finck et al., 1998). This study indicates that TNF-α may mediate the effects of LPS on OB gene expression and also that TNF-α may directly stimulate leptin secretion by adipocytes.

In humans, TNF-α has also been reported to increase leptin secretion (Zumbach et al., 1997) although there are varying reports in this area. Yamaguchi et al. (1998) have described an apparent autocrine inhibition of leptin secretion from cultured murine
adipocytes by TNF-α. In this study TNF-α inhibited leptin secretion in a dose-dependent manner, with a TNF-α antibody completely blocking this inhibitory effect (Yamaguchi et al., 1998). Similar to leptin secretion, OB mRNA expression was also significantly inhibited by TNF-α treatment, and these results were duplicated in cultured human adipocytes taken from subcutaneous adipose tissue (Yamaguchi et al., 1998). By using an agonistic polyclonal antibody raised against the TNF-type-I receptor (TNF-R1), TNF-α was found to be significantly inhibited, although use of an anti-TNF-RII did not alter leptin secretion, implying that the TNF-α inhibition of leptin production occurs via TNF-R1 (Yamaguchi et al., 1998). Interestingly, Ranganathan et al. (1998) demonstrated that in a group of 3 subjects covering a range of adiposity, OB mRNA expression was found not to related to circulating plasma leptin, but to TNF-mRNA levels instead.

1.5.8.3 Dietary regulation of leptin
When body weight is maintained at a stable state, circulating plasma leptin concentrations correlate strongly with the degree of adiposity in both rodents and humans. When subject to dietary alterations either acutely or more long-term, there appears to be an uncoupling of this strong association between leptin and adiposity, with other regulators of leptin secretion becoming apparent.

Specific dietary components: Carbohydrate
In rodents a high-carbohydrate meal has been reported to induce OB mRNA expression in adipose tissue to levels close to those seen in rats fed ad libitum (Thompson, 1996). The increase in OB gene expression after the meal was inhibited by administration of actinomycin-D, a potent inhibitor of transcription, indicating that changes in OB gene expression and plasma leptin are probably under direct transcriptional control (Thompson, 1996). The role of glucose in regulation of OB gene expression was further examined by Mizuno et al. (1996a) who reported that mice sacrificed 15 minutes after an IP injection of glucose exhibited elevated OB mRNA expression compared to mice not stimulated in this way. This study is interesting as it indicates that OB mRNA expression is sensitive to acute changes in plasma glucose. A study by Jenkins et al. (1997) found a relationship between change in leptin levels and changes in carbohydrate (but not fat or protein) intake during 28 days energy restriction in humans. In this study, decreases in plasma leptin
were associated with reduced carbohydrate intake before changes in body weight or fat (Jenkins et al., 1997).

*Fat*

A high fat diet has been shown to increase OB gene expression and plasma leptin levels in normal and diabetic rats (Masuzaki et al., 1995; Chavez et al., 1998). Similarly in mice, a high fat diet results in increased OB gene expression and plasma leptin concentrations (Frederich et al., 1995; Ahren et al., 1997). Interestingly, Ahren et al. reported that feeding a high-fat diet to mice impairs the decrease in plasma leptin normally seen in fasted mice (Ahren et al., 1997).

In humans, consumption of a high fat, low carbohydrate diet has been reported to result in lowered 24-hour leptin concentrations (Havel et al., 1999). The high fat/low carbohydrate meals used in this study produced smaller excursions of plasma insulin and glucose than low fat/high carbohydrate meals; indicating that reduction in leptin secretion after a high fat meal may be a consequence of decreased insulin release with subsequent lower glucose metabolism in adipose tissue (Havel et al., 1999). However previous studies have reported no effect of dietary fat content on fasting plasma leptin in the absence of changes in adiposity (Havel et al., 1996b; Weigle et al., 1997; Schrauwen et al., 1997a). These apparently conflicting results may in fact reflect the necessity for measurement of 24-hour leptin profiles when trying to detect effects of dietary macronutrients on circulating leptin (Havel et al., 1999). Niksanen et al. (1997) examined a group of obese, middle-aged subjects and demonstrated that serum leptin concentrations were inversely associated with resting energy expenditure, respiratory quotient and carbohydrate oxidation rate and dietary fat intake, after adjustment for fat mass, age and sex.

*Energy Restriction: Fasting*

In both rodents and humans, fasting has extensively been shown to markedly decrease, and re-feeding markedly increase both OB gene expression and circulating leptin levels (Frederich et al., 1995; Saladin et al., 1995; Ahima et al., 1996; Boden et al., 1996; Igcl et al., 1996b; Mizuno et al., 1996a; Ahren et al., 1997; Grinspoon et al., 1997; Pratley et al., 1997; Weigle et al., 1997; Li et al., 1998b). These effects are seen only in lean mice however and not diet-induced obese or yellow agouti mice in
which elevated levels of OB gene expression appear to be insensitive to nutritional state (Mizuno et al., 1996a).

A recent study examining the effect of a 24-hour fast on plasma leptin levels in Psammomys obesus reported an apparent dysregulation of leptin in response to fasting in obese, insulin-resistant Psammomys obesus (Walder et al., 1998). This study demonstrated that in lean, insulin-sensitive animals leptin concentrations decreased by 44% after a 24-hour fast, despite no change in bodyweight. In obese, insulin-resistant animals plasma leptin levels increased by 18% after fasting, again despite no change in bodyweight. The decreases in both blood glucose and plasma insulin concentrations were of a similar magnitude in lean and obese animals (Walder et al., 1998). This study indicated that in Psammomys obesus, circulating leptin levels are not simply a marker of adipose tissue mass.

The acute changes in leptin and OB gene expression are seen with various fasting periods (between 12-48 hours in rodents; between 24-96 hours in humans) and are independent of changes in body weight or fat mass. Several studies have demonstrated that the reduction in OB gene expression and leptin levels as a result of fasting may be mediated by changes in circulating insulin and glucose concentrations during this time. Mizuno et al. found that the decrease in OB gene expression after a 72-hour fast could be somewhat reversed with intraperitoneal administration of either glucose or insulin. Thirty minutes after the injection, OB mRNA expression had doubled in these animals (Mizuno et al., 1996a). A similar effect has been reported in normal-weight humans. Boden et al., demonstrated that serum leptin is not decreased during a 72-hour fast when basal plasma glucose and insulin concentrations are maintained by IV infusion of small amounts of glucose (Boden et al., 1996). In addition Grinspoon et al. (1997) found that a continuous infusion of 5% glucose to women who had been fasted for 4-days resulted in an 80% increase in leptin levels within 24 hours.

Long-term dietary restriction
Similar to the effects seen with fasting, dietary restriction also results in decreases in both OB gene expression and leptin levels which are disproportionate to changes in body weight. Considine et al. (1996) reported that as little as a 10% decrease in body
weight (during 8-12 weeks of energy restriction) results in a 53% reduction in serum leptin and a 38% decrease in OB gene expression in obese humans (Considine et al., 1996). In addition this study demonstrated that during 4-weeks of weight maintenance both serum leptin and OB gene expression increased towards baseline levels despite no change in body weight.

Figure 1.9 Changes in OB gene expression and circulating leptin concentrations with energy restriction (Campfield et al., 1996)

Similar results have been reported by other groups (Geldszus et al., 1996; Wing et al., 1996), although other studies have also indicated that serum leptin levels decrease further in women than men as a result of energy restriction (Dubuc et al., 1998). This finding is in agreement with an early finding by Maffei et al. (1995b) that the decrease in leptin concentration during energy restriction is greater in subjects with higher baseline levels.
Changes in leptin during energy restriction have been reported to correlate independently with changes in other endocrine and metabolic parameters, including changes in glucose, FFA and β-hydroxybutyrate (Dubuc et al., 1998), although correlations between changes in plasma leptin and changes in body weight have not consistently been reported, with several studies finding an association (Havel et al., 1996b; Wing et al., 1996) and others not (Considine et al., 1996; Geldszus et al., 1996; Scholz et al., 1996; Shimizu et al., 1997). In addition leptin levels were negatively associated with self-reported hunger, desire to eat and prospective consumption during modest energy deficit in women (Keim et al., 1998). In this study the greatest hunger increase was associated with the largest decrease in leptin, although the relationship between leptin and hunger was not associated with the amount of weight or fat loss (Keim et al., 1998).

1.5.8.4 Other Regulators of Leptin Levels

Cigarette Smoking and Alcohol Intake

Alcohol intake and cigarette smoking have been found to be independently associated with plasma leptin concentrations in several populations (Hodge et al., 1997; Wei et al., 1997; Mantzoros et al., 1998). Hodge et al. (1997) demonstrated that in three populations (Nauruan, Western Samoan and Mauritian) plasma leptin was negatively associated with cigarette smoking in men, independent of adiposity. A similar finding was reported by Mantzoros et al. (1998) in young, healthy men. The association between leptin and smoking habits is thought to involve increased sympathetic nervous activity, which may itself be responsible for decreased leptin levels (Mantzoros et al., 1998). The study of Mantzoros et al. also reported a positive, independent association between plasma leptin and alcohol intake (drinks/week), which was suggested to be a function of the inhibitory effects of alcohol on lipolysis and FFA concentrations in humans. This comprehensive study did not find any independent associations between plasma leptin levels and exercise, education or coffee intake, while plasma DHEAS, cortisol GH and testosterone were also not associated with plasma leptin (Mantzoros et al., 1998).

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Exercise

In humans the effect of exercise training on OB gene expression is usually accounted for by changes in body composition during the training period (Hickey et al., 1996; Kohrt et al., 1996; Perusse et al., 1997) although a study by Landt et al. (1997) demonstrated that male marathon runners had significantly lower plasma leptin levels immediately (32% reduction) and 18- and 24-hours (16% reduction) after running an ultramarathon compared to prerace levels. The different results found in this study may reflect the intensity and duration of the exercise session. In rats, OB gene expression has been found to be transiently affected by exercise (Zheng et al., 1996). A single bout of exercise resulted in a reduction in OB gene expression in adipose tissue by approximately 30% in rats both immediately and 3 hours after exercise, in addition four weeks of exercise training was also associated with about a 50% reduction of OB gene expression, 2 hours after the last training session (Zheng et al., 1996).

1.5.9 LEPTIN AND ENERGY BALANCE

Wing et al. (1996) reported that weight change six months after completion of a four-month weight loss program was associated with changes in serum leptin levels during the six month follow-up period (Wing et al., 1996). The subjects who maintained their weight loss were those who had maintained low serum leptin levels, while subjects who regained their weight also had higher leptin levels. Whether the change in leptin levels in this latter group preceded the increase in body weight is not clear from this study. Havel et al. (1996b) reported that after 8 months on an ad libitum low fat diet, changes in plasma leptin levels were strongly correlated with changes in BMI, percent body fat and resting energy expenditure, while changes in plasma leptin were also associated with changes in plasma insulin independent of changes in adiposity.

Nikanen et al. (1997) examined a group of obese, middle-aged subjects and demonstrated that serum leptin concentrations are inversely associated with resting energy expenditure, respiratory quotient and carbohydrate oxidation rate and dietary fat intake, after adjustment for fat mass, age and sex. Serum leptin in particular was found to be a predictor of REE, accounting for 5% of the variation within the group studied. In a study of older African-Americans, daily energy expenditure was also
found to be associated with plasma leptin concentrations, however in this study a positive association was reported (Nicklas et al., 1997). After controlling for fat mass, plasma leptin concentrations were found to be associated with resting energy expenditure and tended to be related to total daily energy expenditure. These associations were found only in women (n=25), not in men (n=21) and were thought to suggest that leptin may partially contribute to the regulation of total energy expenditure in this population via effects on resting energy metabolism (Nicklas et al., 1997).

Other cross-sectional studies in humans have demonstrated an association between low circulating plasma leptin and low daily energy expenditure (Salbe et al., 1997) and low SNS activity (Snitker et al., 1997), both of which are known to be associated with weight gain (Ravussin et al., 1988; Tataranni et al., 1997b). In an effort to determine if low leptin levels were associated with the metabolic characteristics associated with the 'thrifty genotype', Fox et al. (1998) examined energy expenditure in non-diabetic Pima Indians living a traditional lifestyle in Mexico and compared them to non-diabetic non-Pima Mexicans living in the same environment. Ravussin et al. (1997) followed a group of Pima Indians (n=36) for approximately three years and demonstrated that those subjects who gained weight during this time had lower baseline mean plasma leptin concentration (adjusted for initial percent body fat) compared to subjects whose weight remained stable. This finding suggested that relatively low plasma leptin concentrations may play a role in the pathophysiology of obesity in Pima Indians, a population prone to this disease (Ravussin et al., 1997). In the subsequent study of non-diabetic non-Pima Mexicans living a traditional lifestyle in Mexico, although leptin levels were strongly correlated with percentage body fat as they were for the Pima Indians, plasma leptin concentrations and resting metabolic rates were not different between the two groups of traditional-living Mexicans (Fox et al., 1998). The findings therefore do not support the hypothesis that hypoleptinemia, a relatively low RMR or both are expressions of the thrifty genotype.

In contrast to this study, Chessler et al. (1998) recently reported that in Japanese Americans (n=492) followed for five years, changes in bodyweight and total fat were positively correlated with baseline leptin, after adjustment for baseline adiposity, fasting insulin and age (Chessler et al., 1998). Therefore this study, in direct contrast
to the earlier study, demonstrated that fat accumulation was associated with increased baseline leptin and possibly leptin resistance (Chessler et al., 1998).

Three further studies have been published recently in which baseline leptin does not predict weight change at all (Hodge et al., 1998; Haffner et al., 1998; Nagy et al., 1998). In a study of non-diabetic Mauritians (n=2888), changes in leptin levels during a five-year period were not predictive of changes in body weight or body fat distribution after adjustment for age and baseline BMI (Hodge et al., 1998). Similarly baseline leptin levels in non-diabetic participants in the Mexico City Diabetes Study (n=180) did not predict weight gain in either men or women followed for 3.25 years (Haffner et al., 1998). Nagy et al. (1998) examined the effect of baseline leptin on weight gain in a group of women (n=14) who had previously been obese, but whose body weight was reduced to normal by a mean weight loss of 12.0 kg. Four years after weight loss the women were reassessed for changes in body weight, however baseline leptin concentration (adjusted for fat mass) did not predict weight regain during the four-year period (Nagy et al., 1998).

The role of leptin in body weight regulation therefore remains unclear. The studies summarised above provide conflicting results from a number of different populations, and suggest that more studies are needed before the predictive nature of leptin concentrations on changes in body weight can be determined with certainty.

The OB gene was discovered in late 1994, and the receptor for leptin was identified in 1995. This discovery provided alternative avenues of investigation through which to examine the actions of leptin throughout the body, and the role of leptin in body weight regulation. Characterisation of the leptin receptor also identified novel metabolic pathways through which leptin may act to elicit a response, and highlighted potential direct effects of leptin in a range of tissues. The leptin receptor is the primary focus of this dissertation and is discussed in detail below.
1.6 LEPTIN RECEPTOR

1.6.1 DISCOVERY AND CHARACTERISATION

The receptor for the OB protein was identified by Tartaglia et al. (1995) utilising an expression cloning strategy. Tagged versions of leptin were generated and used to identify a tissue source expressing a cell surface leptin binding activity. The mouse choroid plexus demonstrated significant and specific leptin binding and subsequently a mouse choroid plexus cDNA library was constructed in an effort to clone this leptin binding activity. Cells transfected with this library were screened with a leptin-alkaline phosphatase fusion protein and cDNAs were identified that encoded a cell surface leptin receptor (OB-R) with an affinity for leptin of about 0.7 nM (Tartaglia et al., 1995).

Sequence analysis of the original cDNA identified revealed an encoded protein of 894 amino acids with a consensus amino terminal hydrophobic signal sequence (Tartaglia et al., 1995). In addition, the sequence predicted a single membrane-spanning receptor of the class I cytokine receptor family. The closest relatives of OB-R were the gp130 signal-transducing chain of the interleukin-6 (IL-6) receptor, the granulocyte colony-stimulating factor (G-CSF) receptor, and the leukemia inhibitory factor receptor α-chain. The homologous regions of these receptors and OB-R included a fibronectin type III domain and two cytokine receptor-like domains, each containing the Trp-Ser-X-Trp-Ser (WSXWS) motif which has been implicated in ligand binding and signal transduction by members of this cytokine family. In addition, gp130, granulocyte-colony stimulating factor receptor (G-CSFR) and leukemia inhibitory factor (LIF)-α also contain a conserved intracellular region of 55-60 amino acids that includes the sequence motifs necessary for signal transduction, termed box 1 and box 2 JAK kinase interaction sequences.

The predicted extracellular domain of OB-R was about 816 amino acids long while only a short (34 amino acids) intracellular domain was predicted which contained sequences corresponding only to the box 1 motif (Tartaglia et al., 1995). This structure suggested that the protein may not have signaling capabilities however subsequent screening of cDNA libraries was performed and it was soon revealed that there were multiple forms of OB-R in both mice and humans, including an isoform with an intracellular domain of about 303 amino acids. The intracellular domain of
this long form of OB-R was found to contain sequence motifs suggestive of intracellular signal-transducing capabilities.

1.6.1.1 OB-R Gene and Different Isoforms

Genetic mapping studies were subsequently conducted which localised the OB-R gene to mouse chromosome 4, within the 5.1 cM interval to which the diabetes (db) locus had previously been mapped, suggesting it as an excellent candidate for the db gene (Tartaglia et al., 1995). It was previously established that the rat fatty (fa) gene is a homologue of db with the fa gene found on rat chromosome 5 in a region syntenic to the mouse db gene (Truett et al., 1991). It was soon confirmed that the mouse OB-R gene was the db gene (Chen et al., 1996a; Lee et al., 1996) while the fa gene was subsequently demonstrated to encode rat OB-R (Chua et al., 1996a; Chua et al., 1996b; Phillips et al., 1996). It was also established that OB-R encoded several alternatively spliced forms in both mouse and rat (initially designated: OB-RA, OB-RB, OB-RC, OB-RD, OB-RE and OB-RF [rat only]) (Lee et al., 1996; Wang et al., 1996).

OB-RB was identified as the receptor isoform with the long intracellular domain, containing the motifs required for signal transduction (Lee et al., 1996). The other isoforms have a shorter intracellular domain and lack either some or all of these motifs.
Figure 1.10  Predicted organisation of OB-RA and OB-RB. The positions and lengths of the extracellular, transmembrane and intracellular domains are indicated (White and Tartaglia, 1996).

The extracellular domains of the OB-R isoforms are identical throughout their entire length until lysine 889, however, as the differences in the receptor forms result from alternative RNA splicing at the most C-terminal coding exon, the resulting OB-R isoforms have intracellular domains of differing length and sequence composition (Lee et al., 1996; Chua et al., 1997). One OB-R transcript was identified which lacked a transmembrane domain and therefore potentially encoded a soluble form of the receptor, OB-RE. Similar isoforms of the OB-R gene have been detected in both rodents and human, however OB-RD has only been demonstrated in the mouse, while an additional isoform, OB-RF, was identified exclusively in rat. The murine and human receptors are highly similar in amino acid sequences of both the intracellular (71% homology) and extracellular (78% homology) domains (Tartaglia et al., 1995; Chen et al., 1996a).

Chua et al. (1997) examined the fine structure of the murine OB-R gene to examine how the various forms of OB-R may be made. Isoforms OB-RE and OB-RC are formed by recognising 14'-14' and 17'-17' respectively, as 3' terminal exons and ignoring the consensus splice donor sites at the start of exons 14' and 17' (Chua et al.,
Isoforms OB-RA and OB-RB are formed by using splice donor sites 14' and 17' with competition between the 3' terminal exons 18a and 18b to complete their respective mRNAs. OB-RC competes with the combined strengths for the formation of OB-RA and OB-RB, since OB-RC can only be made if the splice donor site at the start of 17' is ignored, whereas use of the 17' splice donor leads to the formation of either OB-RA or OB-RB. For the mouse OB-R gene therefore, the mechanism of 3' terminal exon selection requires either the suppression of a splice donor site (for OB-RC and OB-RE) or the selection of competing 3' splice acceptor sites (for OB-RA and OB-RB; Chua et al., 1997). Splice site suppression is seen to occur even with the mutant OB-RBdb exon 18a, which contains a mutation-induced splice site donor site in the 3' untranslated region of coding exon 18a (Chua et al., 1997).

![Gene map of different isoforms of OB-R alternative splicing (Chua et al., 1997)](image)

Figure 1.11 Gene map of different isoforms of OB-R alternative splicing (Chua et al., 1997)

In humans, OB-R was thought to map to between 1q31 or 1p22 between the genetic markers PGM1 and D1S22. Chung et al. (1996) developed a genetic map of 1p in the region of the OB-R gene by physically mapping the OB-R gene using radiation hybrid mapping and placing it in a contig composed of 10 adjacent YACs and 5 PI artificial chromosomes (PACs). On the basis of rodent genetic maps for the regions surrounding OB-R<sup>ob</sup> and OB-R<sup>h</sup>, the human homologue of OB-R would have been predicted to lie between D1S85 and PGM1 based on conserved linkage of most of the telomeric half of mouse chromosome 4 with human 1p. However Chung et al. reported that the gene order on this segment of 1p has not been conserved between
rodent (Cen-/-Jun-Pgm1-OB-R^{sh}/OB-R^{a}-D4H1S85-C8B) and human (Cen-/-OB-R-PGM1-D1S85-C8B) (Chung et al., 1996).

**Figure 1.12** Comparative genetic maps in the region of the OB-R gene (Chung et al., 1996)

There appears to be an inversion of JUN and between OB-R and D4H1S85 to produce this order in humans, with OB-R located on the centromeric side of PGM1 which is located at 1p31. JUN and C8B, which map to 1p32-p31 and 1p32 respectively, were found to be located further telomeric of PGM1. It has previously been assumed that mouse chromosome 4 represented a conserved linkage group of >20 cM with human 1p, however it appears that this segment is only syntenic as the gene order has not been conserved (Tartaglia et al., 1995, Chung et al., 1996). In 1997 Chung et al further defined the OB-R gene by reporting the boundaries of 18 coding exons for OB-RB, although subsequent studies have found that the previously identified exon 18 actually contains 2 introns, changing the structure of the coding region of OB-R to 20 exons (Thompson et al., 1997; Matsuoka et al., 1997).
Figure 1.13 Coding region of OB-R and sequence variation within exons (Thompson et al., 1997)

1.6.2 Sequence Variation in the OB-R Gene:
The morbid obesity that develops in ob/ob mice has been shown to be the result of a mutation in the OB gene. This mutation does not explain the obesity seen in other rodent models, while only a very small number of humans have been identified with a homologous mutation in the OB gene. For this reason, the sequence of the OB-R gene has been examined in rodents and primates, including humans, and several mutations have been identified within this gene which can be directly associated with the development of morbid obesity.

1.6.2.1 Rodents: db/db Mice
In C57BL/6J db/db mice, a mutation was identified in OB-R which results in the abnormal splicing of the OB-R gene (Chen et al., 1996a; Lee et al., 1996). The resulting mutant protein was found to be truncated and defective in signal transduction (Ghilardi et al., 1997). In db/db mice, the level of expression of OB-RA and other splice variants is not altered, but instead this mutation converts the OB-RB isoform to OB-RA. There are a number of allelic variations of the db mutation, and several other OB-R mutations have been identified. While the original db mutation is the result of abnormal splicing, specifically altering expression of OB-RB, the $\text{db}^{31}$ and $\text{db}^{\text{Pas}}$ mutations truncate the receptor in the extracellular region. $\text{db}^{31}/\text{db}^{31}$ mice exhibit a frameshift mutation in the extracellular region of OB-R which ablates
expression of all of the wild type receptor proteins by truncating the protein at amino acid 625 (Lee et al., 1997a; Li et al., 1998a). \( \text{db}^{\text{Pas/DB}} \) mice have a mutation which results from a duplication of exons 4 and 5 of OB-R which introduces a premature stop codon at amino acid 281, thereby preventing the expression of OB-R in these mice (Li et al., 1998a).

As OB-RB is the main signaling isoform of the leptin receptor, this mutation renders \( \text{db/db} \) mice unresponsive to leptin, and thereby leptin resistant. \( \text{db/db} \) mice have been reported to have high circulating leptin concentrations, however the anorectic effects of leptin are not detected, resulting in a failure to regulate body weight, and the development of obesity in these animals.

1.6.2.2 Obese Zucker (fa/fa) Rat

In Zucker rats, a base mutation has been identified within the OB-R gene which is associated with the development of obesity in affected animals. An adenine to cytosine transversion was detected which results in a glutamine to proline substitution at residue 269 of OB-R (Gln269Pro) (Iida et al., 1996a). The \( \text{fa} \) mutation is in the amino terminus of OB-R and is homologous to the \( \text{db} \) mutation. This base change alters post-translational processing of the leptin receptor. This mutation is present in all isoforms of OB-R and early studies indicated that although the amino acid change does not alter the leptin binding site, the change may interfere with receptor dimer formation, and thus cause reduced signaling (White et al., 1997a). In vitro studies have demonstrated that cells expressing the \( \text{fa} \) mutation show constitutive and impaired signaling in OB-R. Yamashita et al (1997) used CHO cells that stably express OB-RB of either the wild-type or \( \text{fa} \) type and demonstrated that even if the levels of cell surface expression of OB-RB were similar, signal transduction was reduced in cells expressing OB-RB of the \( \text{fa} \) type compared to the wild-type OB-RB (Yamashita et al., 1997).

Recently Yamashita et al. (1998) further characterised the \( \text{fa} \) mutation by demonstrating that the \( \text{fa} \) mutation decreases the signal transduction of OB-RB via the activation of both STAT and MAPK. The decreased abilities in signal transduction in both pathways collectively resulted in a reduction in immediate early gene expression after leptin stimulation in CHO-OB-RB(\( \text{fa} \)) cells compared to cells expressing the
wild-type OB-RB (Yamashita et al., 1998). This reduction in signal transduction as a result of the fa mutation was not accompanied by constitutive activation of these molecules in this study however (Yamashita et al., 1998). These changes in signal transduction are thought to result in diminished leptin responsiveness in hypothalamic neurons which in turn causes the fa/fa rats to become obese, as they do not respond sufficiently to circulating leptin. In support of this hypothesis, fa/fa rats do not respond to peripheral leptin administration, and response to ICV leptin administration is only seen at greatly increased doses compared to lean controls.

1.6.2.3 Obese Koletsky Rats
Another OB-R mutation was identified in the OB-R sequence of obese Koletsky rats. A thymidine to adenine transversion at base 2349 results in a coding change from a tyrosine to a stop codon (Takaya et al., 1996). This nonsense mutation occurs just before the transmembrane domain and results in a truncated receptor with no transmembrane domain and no cytoplasmic signaling domain. There is no difference in hypothalamic OB-RB levels between lean and obese rats, however the truncated receptor means that the leptin signal is not transduced and the animals are resistant to the effects of leptin. Although studies of leptin treatment to obese Koletsky rats are not available, this mutation is homologous to the mouse db mutation and the rat fa mutation. Therefore, an inability for the truncated leptin receptors of the Koletsky rats to transduce a signal would be consistent with these other rodent models, and result in leptin resistance and obesity.

In these monogenic rodent models of obesity, a single base change within OB-R has been identified which explains the presence of obesity in these animal models. In NZO mice, a polygenic model of obesity, although several polymorphic regions have been identified in the OB-R sequence, these sequence changes do not explain the obesity seen in these mice (Igel, 1997). This important study indicates that in polygenic forms of obesity, the explanation for increased body weight and fat mass may not be as simple as that identified in the monogenic rodent models discussed above.
1.6.2.4 Humans

The sequence of the human OB-R gene has also been examined in an effort to identify mutations which would possibly explain the presence of obesity in humans. Initial examination of the human OB-R gene indicated that the db/db mouse and fa/fa rat single base mutations within the OB-R gene were not present in the human sequence (Considine et al., 1996). Many studies have however, identified a number of polymorphic regions within both the introns and exons of the OB-R gene (summarised in tables 1.5 and 1.6) although it is clear from this table that the sequence alterations in OB-R detected to date do not explain the common obese phenotype seen in humans.
<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Populations</th>
<th>Investigators</th>
<th>Linked to phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys109Arg (exon 4)</td>
<td>Pima Indian</td>
<td>Thompson et al., 1997</td>
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</tr>
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<td></td>
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<td>no</td>
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<td></td>
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<tr>
<td></td>
<td>American</td>
<td>Chung et al., 1997</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>Danish</td>
<td>Echwald et al., 1997</td>
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</tr>
<tr>
<td>Gln223Arg (exon 6)</td>
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<td>Thompson et al., 1997</td>
<td>polymorphism found almost exclusively in obese subjects</td>
</tr>
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</tr>
<tr>
<td></td>
<td>Japanese</td>
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Table 1.6 Common sequence polymorphism within introns of OB-R

<table>
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<th>Investigators:</th>
<th>Linked to phenotype:</th>
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<tr>
<td>-36 (A-&gt;T)</td>
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<td>% body fat</td>
</tr>
<tr>
<td>+37 (A-&gt;C)</td>
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<td>Thompson <em>et al.</em>, 1997</td>
<td>% body fat</td>
</tr>
<tr>
<td>+52 (C-&gt;T)</td>
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<td>Thompson <em>et al.</em>, 1997</td>
<td>no</td>
</tr>
<tr>
<td>hOB-R/MaelII</td>
<td>French</td>
<td>Rolland <em>et al.</em>, 1998</td>
<td>no</td>
</tr>
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</table>

Recently, a mutation in human OB-R has been detected that results in morbid obesity and pituitary dysfunction in subjects homozygous for the mutation (Clement *et al.*, 1998). This mutation was found in a consanguineous family of Kabilian origin with a strong prevalence of early-onset morbid obesity. The G-to-A base substitution within the splice donor site of exon 16 seen in this family resulted in amplification of abnormal OB-R mRNA in the affected (homozygous) subject, both normal and abnormal OB-R mRNA in heterozygous subjects, and only normal OB-R mRNA in the normal individuals (Clement *et al.*, 1998). The abnormal OB-R mRNA was found to lack exon 16 as a result of exon skipping. Therefore the OB-R protein would lack both the transmembrane and intracellular domains. Both lean and obese French Caucasians (n=402) were screened for this mutation, however it could not be detected. Therefore it appears this is a very rare mutation and not a common cause of obesity in humans (Clement *et al.*, 1998).

1.6.3 TISSUE DISTRIBUTION OF OB-R

The tissue distribution of the different isoforms of OB-R have been characterised in rodents, humans and monkeys, although the receptors OB-RA and OB-RB have been the main focus of investigations. Several different methods have been used to determine mRNA gene expression for these receptors in a variety of tissues, with different results often reflecting the different level of sensitivity for each method. Studies using the techniques of *in situ* hybridisation, Northern blotting and comparative RT-PCR have revealed that OB-RA is expressed almost ubiquitously in both the mouse and rat with varying levels of expression detected in the hypothalamus, brain (-hypo), thymus, lymph nodes, heart, lung, spleen, testes,
seminal vesicles, kidney, lung, liver, stomach, small intestine, colon, bladder, adipose tissue, skeletal muscle skin, bone, adrenal, pancreas, uterus and ovary (Lee et al., 1996; Chua et al., 1997; Fei et al., 1997; Hoggard et al., 1997; Lollman et al., 1997; Zamorano et al., 1997).

The longer OB-RB has been found to be expressed predominantly in the brain, particularly the hypothalamus, however expression of OB-RB has also been detected in a number of peripheral tissues including adrenal (medulla) lymph nodes, lung, spleen, colon, testis, seminal vesicles, adipose tissue (white and brown), T-cells, vascular endothelial cells and kidney (inner zone of medulla). A similar pattern of tissue distribution has been reported for the leptin receptor isoforms in humans (Luoh et al., 1997).

*In situ* hybridisation has been used to identify the arcuate nucleus, dorsomedial hypothalamic nucleus (DMH), paraventricular nucleus (PVN), ventromedial hypothalamic nucleus (VMH) and lateral hypothalamic nucleus (LH) as the principal sites of OB-RB expression in the central nervous system (Malik et al., 1996; Mercer et al., 1996b; Fei et al., 1997; Guan et al., 1997), although low levels of expression were recently reported in brain microvessels (Björbaek, 1997). In addition, high levels OB-RA expression have been detected in the choroid plexus and brain microvessels, while low levels of expression have been reported in cerebellum, hypothalamus and meninges (Björbaek et al., 1997; Golden et al., 1997; Savioz et al., 1997; Elmquist et al., 1998b; Hakansson et al., 1998a; Hakansson et al., 1998b).

The almost ubiquitous distribution of OB-RA and the widespread occurrence of OB-RB mRNA in several species suggests that leptin may play a role in a number of tissues throughout the body. In addition, the variety of tissues found to express leptin receptors indicates that leptin may also play a number of different roles within the body. The potential role of OB-RA and OB-RB in a number of tissues is explored in detail later.
1.6.4 LEPTIN BINDING

The fine structure of the murine leptin receptor gene was first described by Chua et al. in 1997. This group determined duplicated ligand binding domains in the receptor, which is a conserved feature among cytokine receptors. Several class 1 cytokine receptors including GH-R, IL-6R, EPO-R and GCSF-R are characterised by the presence of multiple domains, including CK, C2 and F3, with each domain characterised by unique consensus residues (Livnah et al., 1996; Wells and Devos, 1996; Fukunaga et al., 1991). High resolution examination of the GH-R and EPO-R determined that the combined CK-F3 domain forms the ligand binding site for these receptors. In contrast to other similar receptors, OB-R has been found to contain two repeating CK-F3 domains within its extracellular region (Livnah et al., 1996; Wells and Devos, 1996). Using analysis of an extensive array of deletion and substitution mutants of OB-RB Fong et al. (1998) recently demonstrated that the first CK-F3 domain is not required for leptin binding and activation. The binding site for leptin was localised to residues 323-640 of OB-R, a region shown to contain the second CK-F3 domain (residues 428-635). In addition, this study confirmed that only one molecule of leptin binds to each leptin receptor (Fong et al., 1998).

The functional significance of the first leptin binding site is unclear, however OB-RB was found to have a unique structure among the cytokine receptors as it was demonstrated that there is a long segment (residues 179-234) between the CK domain and the F3 domain within the first CK-F3 domain of OB-RB. The long connecting loop may confer a very high degree of flexibility, which in turn prevents the formation of a stable leptin binding site (Fong et al., 1998). These studies further supported the existence of OB-R as a preformed complex, with receptor activation occurring after a ligand-induced conformational change (Fong et al., 1998).

Haniu et al. (1998) recently demonstrated that the human leptin receptor has some unique features. When expressed in and purified from CHO cells, the extracellular domain of human OB-R was found to contain extensive N-glycosylation, seen in about 36% of the total protein. In addition, the purified protein had a molecular weight of 145,000 (the unglycosylated protein has a molecular weight of 90,000) and exhibited ligand binding ability, evidenced by the formation of ligand-receptor complexes followed by chemical cross-linking. When comparing human and murine
OB-R, the extracellular domain of the murine receptor lacked several cysteine residues present in the human receptor, and surprisingly, OB-R was found to contain two sites with an unusual N-glycosylation motif, indicated as an NCS sequence, where the cysteine residue is involved in the disulphide formation. Studies of other receptors have demonstrated that these disulfide and N-glycosylation motifs are not homologous with any known cytokine receptors. Furthermore, the potential importance of cysteine residues in maintaining peptide structure indicates that N-glycosylation within the C-terminal may alter the folded structure of OB-R in humans (Haniu et al., 1998).

1.6.5 CENTRAL LEPTIN ACTION

Regulation of body weight and energy balance is thought to be mediated by pathways within the hypothalamus, involving a number of neuropeptides and hormones (see Section 1.3.3). The high levels of OB-RB mRNA within the hypothalamus suggests that leptin’s effects on energy balance occur via central pathways. This hypothesis is supported by the findings that leptin administered directly into the brain produces effects at much lower doses than those required peripherally. Furthermore, leptin delivered centrally can overcome apparent peripheral leptin resistance in some animal models.

Leptin receptors have been found to be located in several regions of the brain (arcuate nucleus, ventromedial nucleus, dorsomedial nucleus), some of which are known to be involved in the regulation of feeding behaviour and body weight (Schwartz et al., 1996c; Tartaglia et al., 1995; Couce et al., 1997; Savioz et al., 1997). When $^{125}$I-leptin is injected intravenously, the protein is transported across the BBB at a rate 20 times faster than albumin, and 75% of extravascular $^{125}$I-leptin in brain crosses the BBB completely to reach the brain parenchyma (Banks et al., 1996). Autoradiographic data have shown binding of $^{125}$I-leptin in the arcuate nucleus-eminence complex, while outside this area uptake of $^{125}$I-leptin is diffuse (Banks et al., 1996). It is likely that leptin reaches the cells in the arcuate nucleus via the median eminence as this area was stained with $^{125}$I-leptin and is a structure known to belong to the circumventricular organs, regions known to lack a BBB. Hakansson et al. (1998a) therefore postulated that leptin secreted from the adipose tissue is transported to the brain via the general circulation and acts on leptin receptors located
in the ventromedial arcuate nucleus devoid of BBB. Leptin may also traverse through
the median eminance via specialised ependymal cells into the third ventricle and
thereafter reach other brain regions including the choroid plexus. Bjorback et al.
(1998) demonstrated expression of leptin receptors in the microvessels of the brain in
addition to the choroid plexus and leptomeninges, further supporting the hypothesis
that the choroid plexus is not the only site for leptin to cross the BBB.

1.6.5.1 Leptin Signalling
Leptin's ability to regulate body weight has been shown to be mediated by OB-RB in
the hypothalamus. The homology of OB-R to members of the class 1 cytokine
receptor family provided initial indications as to the possible intracellular mediators
of leptin receptor activation (Kishimoto et al., 1994; Tartaglia et al., 1995; Heldin et
al., 1995). Class 1 cytokine receptors lack intrinsic tyrosine kinase activity and are
activated by ligand-induced receptor homo- or heterodimerisation. In most cases, the
event which triggers signalling requires receptor-associated kinases of the Janus
family (JAKs; Ihle et al., 1994). In response to ligand binding and receptor
aggregation, a series of phosphorylation events occur that include
autophosphorylation of the associated JAK kinase. The activated JAK proteins then
phosphorylate the receptor intracellular domain at specific tyrosine residues which
provides docking sites for members of the signal transducer and activator of
transcription (STAT) family of transcription factors (Ihle et al., 1994). The receptor
associated JAKs then phosphorylate and activate the STAT proteins. Subsequently,
the activated STAT proteins then dimerise and translocate to the nucleus where they
bind DNA and activate transcription (Ihle et al., 1994).

Upon closer examination of OB-R it was found to be most related to gp130 and LIRF
(Tagga et al., 1989; Gearing et al., 1992). The extracellular domain of gp130, LIFR
and OB-R is composed of either six or eight fibronectin type III modules and contains
4 conserved cysteine residues and a trp-ser-X-trp-ser motif (Tagga and Kishimoto,
1992; Tartaglia et al., 1995). The cytoplasmic regions of all three receptors possess
three conserved motifs, box 1, box 2 and box 3 in the membrane-proximal-to-distal
order. Mutational analyses conducted with gp130 have indicated that the box 1 motif
is important for the association of JAKs, while the box 2 motif plays a critical role, in
concert with box 1, in inducing DNA synthesis (Stahl et al., 1994; Narazaki et al.,
Box 3 contains a tyrosine-X-X-glutamine (Y-X-X-Q) motif which is proposed as a docking site for STAT 3 (Stahl et al., 1995). STAT 3 is a transcription factor originally characterised to bind to the IL-6-responsive element and induce transcription (Yuan et al., 1994; Zhong et al., 1994). It is recruited to the phosphorylated Y-X-X-Q motif through its Scr homology (SH) 2 domain and this recruitment leads to tyrosine phosphorylation of STAT3 by JAKs, with subsequent transcriptional activation (Heim et al., 1995; Stahl et al., 1995). In addition to STAT3, other STATs including STAT1 may also be tyrosine phosphorylated, however their role is less clear.

There have been several investigations into STAT activation by leptin, both in vivo and in vitro, which demonstrate that only OB-RB, the long leptin receptor, is capable of activating these transcription factors. In addition OB-RB has been shown to activate STAT3 and STAT 5, however there are conflicting reports regarding activations of STATs 1 and 6 (Baumann et al., 1996; Ghilardi et al., 1996; Takahashi et al., 1996b). Intracellular domain mutagenesis of OB-RB has demonstrated that STAT protein activation and transcriptional regulation are dependent upon at least two distinct regions of the OB-RB molecule.

![Figure 1.14 Activation of the Jak/Stat pathway by Leptin binding to its receptor (Auwerx and Staels, 1998)](image_url)
Activation of STAT3 requires a functional box 3 motif (Y-X-X-Q) located near the extreme C-terminus of OB-RB (Baumann et al., 1996; White et al., 1997a). This box does not appear to be necessary for STAT5 activation by OB-RB and is dependent rather on transmembrane adjacent sequences of the intracellular domain, including the JAK box1 and box2 interaction motifs (White et al., 1997a). Therefore, there appear to be multiple distinct signals emanated by OB-RB following receptor activation. OB-RB activation of STAT3 tyrosine phosphorylation has been shown to stimulate c-fos gene transcription in vitro (Björnback et al., 1997). Two in vivo studies have demonstrated a time-dependent stimulation of STAT3 in response to leptin (Vaisse et al., 1996; McCowen et al., 1998). Surprisingly however, leptin administration does not activate any JAK proteins or MAP kinase, indicating that in vivo propagation of the leptin signal within the hypothalamus may involve novel signaling intermediates (McCowen et al., 1998).

db/db mice exhibit morbid obesity and severe endocrine abnormalities phenotypically identical to ob/ob mice. The db gene was subsequently identified as the gene for the leptin receptor and found to encode the OB-R protein. In addition, a mutation was found within this gene which results in an insertion within OB-RB which ultimately truncates the protein so that the intracellular domain is identical to the short forms of OB-R (OB-R3) (Lee et al., 1996; Chen et al., 1996a). This inability to produce OB-RB provided strong evidence that this receptor isoform was required for bodyweight regulation. Further studies demonstrated that db/db mice had defective STAT signaling by the leptin receptor, with leptin treatment of db/db mice unable to activate STATs within the hypothalamus (Vaisse et al., 1996; Ghilardi et al., 1996).

Based on the structural similarity between gp130 and OB-R, and their common ability to activate STAT1 and STAT3 proteins, it was initially thought that OB-R utilises gp130 for signaling (Nakashima et al., 1997a). Several groups have investigated the OB-R signaling complex and early reconstitution assays demonstrated that signaling by OB-RB was unaltered in the presence of gp130, indicating that this subunit is not required for signaling by OB-RB. Further studies examining the role of gp130 in the OB-R signaling process demonstrated that while gp130 and OB-RB are closely related molecules, they mediate overlapping but distinct cytoplasmic signals within the Ras-ERK signaling cascade (Nakashima et al., 1997a). Examination of the
induction of p42\textsuperscript{ERK2} activation in COS7 cells by both OB-R and gp130, demonstrated that after activation of p42\textsuperscript{ERK2} gp130 is able to recruit the SHP-2 adapter which is subsequently tyrosine-phosphorylated. OB-RB, however stimulates p42\textsuperscript{ERK} but cannot induce tyrosine-phosphorylation of SHP-2 (Nakashima et al., 1997a). This study and a subsequent study by the same group further confirmed that gp130 is not involved in OB-R signaling, as it does not form a heterodimer with OB-R, and in fact it was shown that OB-R dimerises with itself to form a homodimer (Nakashima et al., 1997a, Nakashima et al., 1997b). The interaction of other accessory binding chains, including G-CSFR, has also been investigated using chimeric receptor complexes. However, these too have been shown not to be involved in OB-R signaling, indicating that aggregation of two intracellular OB-R domains, and thus homodimerisation or homo-oligomerisation, is sufficient to initiate receptor signaling (Nakashima et al., 1997b).

Early transient co-transfection studies demonstrated that OB-RB but not OB-R\textsubscript{s} was capable of activating the STAT proteins required for signal transduction, although both forms of the receptor were found to bind leptin with the same affinity. Subsequent studies have further characterised OB-RB signal transduction and also revealed that OB-R\textsubscript{s} has weak signaling capabilities. Using transient transfection models, Bjorbaek et al. (1997) demonstrated that in addition to inducing tyrosine phosphorylation of itself and STAT3, OB-RB can also mediate leptin-dependent tyrosine phosphorylation of JAK2 and IRS-1 and activate mitogen-activated protein kinase (MAPK) via JAK (Takahashi et al., 1997; Bjorbaek et al., 1997). Activation of STAT-dependent transcription involves activation of c-fos.

The proto-oncogene c-fos is expressed in neurons in response to direct stimulation by growth factors and neurotransmitters (Sagar et al., 1988). The c-fos gene is the normal cellular counterpart of the viral oncogene v-fos, and is rapidly and transiently expressed in many tissues in response to growth factor stimulation (Sambucetti and Curran, 1986; Curran and Morgan, 1987; Renz et al., 1987; Sagar et al., 1988). It encodes a nuclear phosphoprotein (fos) that exhibits both non-specific and sequence specific DNA binding properties. Although the exact function of c-fos is unknown, it is thought to act as a third messenger molecule in signal transduction systems, where it would couple short-term intracellular signals elicited by a variety of extracellular
stimuli to long-term responses by altering gene expression (Curran and Morgan, 1987). As the basal level of *fos* is relatively low in most CNS regions, *fos* expression has been suggested to provide a useful technique for identification of activated neurons and thereby metabolic mapping of functional pathways (Sagar et al., 1988).

In comparison to OB-RB, OB-Rs were found to also possess signaling potential. Although unable to activate tyrosine phosphorylation of STAT3 or STAT-dependent transcriptional activation, OB-Rs is able to mediate leptin-dependent tyrosine phosphorylation of JAK2 and IRS-1 and activation of MAPK activity. This finding has been confirmed in other systems (Takahashi et al., 1997).

Intracellular domain mutagenesis of OB-R has demonstrated two distinct regions capable of generating intracellular signals (White et al., 1997a). A mutant receptor lacking the most C-terminal 50 amino acids lost the ability to stimulate transcription via the IL-6 response element. However this 50-amino acid C-terminal truncation did not alter the ability of OB-R to stimulate transcription via a hematopoietic receptor response element (White et al., 1997a). In addition, when the C-terminal tyrosine was mutated to phenylalanine similar results were seen. Furthermore, this single amino acid substitution differentially effected the ability of OB-R to activate different STAT proteins, with the ability to activate STAT1 and STAT3 strongly affected and the ability to activate STAT5 only minimally affected. It has been proposed by Tartaglia (1997) that the mutational separation of distinct activities emanating from OB-R suggests that multiple signals may be propagated by OB-R triggering.

The finding that OB-RB signals through homodimerisation or homo-oligomerisation raised the possibility that overexpression of OB-Rs may poison the signaling capability of OB-RB through OB-RB dimerising with a partner that is signaling deficient (White et al., 1997a). Further studies revealed that when the short and long forms of OB-R are co-expressed, OB-Rs can in fact modulate receptor signaling by OB-RB in a form of dominant negative repression. However OB-RB signaling has been shown to be only modestly susceptible to this (White et al., 1997a).

Chemical cross-linking studies determined that both OB-RB and OB-Rs expressed on COS-1 cells exist as homodimer or homo-oligo complexes (Devos et al., 1997). The
observation that the expressed extracellular domain of OB-R is a dimer suggested that the membrane-bound receptor complex may be preformed in the absence of leptin as dimerisation of OB-R was found to be ligand-independent (Devos et al., 1997; Nakashima et al. 1997a; Nakashima et al., 1997b). These studies also found no evidence of the existence of heterodimers between OB-RB and OB-Rs. In addition, the secreted extracellular domain of the leptin receptor also exhibits self-association into dimers or oligomers in the absence of leptin and is fully competent at binding leptin. This study further determined the stoichiometry for leptin binding to its receptor to be one-to-one, i.e. one molecule of leptin binds to one receptor extracellular domain (Devos et al., 1997).

These studies have provided strong evidence that leptin signaling occurs via leptin receptors which have preformed homodimers or homo-oligomers, probably in the absence of leptin. Leptin appears to then bind to the receptor complex in a 1:1 relationship, and initiate a series of signal transduction events, which ultimately alters transcription. Only OB-RB has been shown to activate STAT proteins, and thereby alter transcription, and animals such as the db/db mouse, which lack functional OB-RB receptors, are unable to activate STAT proteins upon leptin binding. In fully functional systems, the pathways subsequently activated by leptin in the brain are detailed below.

1.6.5.2 Leptin and Neurotransmitters
Leptin receptors have been found to be co-localised with a number of neurons known to be involved with feeding behaviour in various regions of the hypothalamus. Studies have concentrated on the interaction between NPY and leptin within the brain, with leptin treatment found to inhibit NPY expression (Schwartz et al., 1996a; Stephens et al., 1995; Schwartz et al., 1996b) and secretion (Stephens et al., 1995). In addition, leptin administration has been reported to reduce the starvation induced increases in NPY mRNA levels in ob/ob mice (Ahima et al., 1996).

NPY is known to be a potent stimulator of feeding behaviour in mice and one proposed mechanism for leptin’s actions centrally are via NPY-mediated pathways in the brain. This pathway has been suggested because double-labeling experiments demonstrated that OB-R- and NPY-like immunoreactivity (LI) were co-localised in
the same arcuate neurons (Mercer et al., 1996b; Hakansson et al., 1996) and several studies have now shown that leptin treatment results in reduced NPY gene expression (Schwartz et al., 1996a; Stephens et al., 1995; Cusin et al., 1996). NPY has been shown to be increased with fasting, however Schwartz et al. found that ICV leptin administration during a 40-hour fast also results in decreased levels of both OB-R and NPY mRNA in the ARC (by 24%) (Schwartz et al., 1996a). These results suggest that leptin may mediate its ability to decrease food intake via reduced NPY expression.

NPY however appears not to be the sole mediator of leptin action, as an early study by Schwartz et al. (1996a) revealed that ICV administration of leptin during a 40-hour fast resulted in decreased levels of NPY mRNA in the arcuate nucleus (-24%) and increased levels for CRH (30%) in the paraventricular nucleus. This study suggested that the effects of leptin were two-fold: to decrease food intake via reduced NPY expression and to increase inhibition of feeding via enhanced CRH (Schwartz et al., 1996a). The use of ob/ob mice with an additional mutation resulting in NPY-deficiency (NPY-knock-out mice) provides further evidence that NPY is not the sole mediator of leptin action as these NPY knockout mice reduce food intake and decrease body weight with leptin administration (Erickson et al., 1996a,b).

Other potential mediators of leptin action include pro-opiomelanocortin (POMC) and the recently identified cocaine- and amphetamine-regulated transcript (CART). Products of the POMC gene are known to affect feeding behaviour and POMC neurons have been shown to share a similar distribution with leptin receptor mRNA in the arcuate nucleus. OB-R is also present in POMC neurons of the arcuate nucleus and POMC-derived peptides such as α-MSH have been shown to be effectors of feeding and therefore may be important mediators of leptin action within the CNS. Similar to the NPY neurons, POMC-containing neurons of the ARC show rich projections to the PVN (Everitt et al., 1986). That both NPY and POMC neurons project to the PVN has been taken to indicate that the arcuate neurons, which may be located outside the BBB, both operate via the PVN to reduce food intake (Hakansson et al., 1998a).
Boston et al. (1997) cross bred the agouti mouse (defective POMC signaling) with the ob/ob mouse (leptin deficient) in an attempt to uncover the signaling pathways for both of these regulators of body-weight. Interestingly, the deletion of the OB gene restored leptin sensitivity to the agouti mouse, although the animals were still obese. Leptin treatment resulting in decreased body weight and normalisation of glucose and insulin concentrations in this previously leptin-resistant strain of mice, although the ability of these mice to increase body weight irrespective of leptin sensitivity, implies that the POMC neurons act independently of leptin to alter energy homeostasis and that the obesity seen in the agouti mouse results from desensitisation of leptin signaling (Boston et al., 1997). This study also demonstrates that leptin signaling does not occur via POMC as leptin was effective at reducing obesity in these mice with defective POMC signaling.

ICV leptin administration of either leptin or glucagon-like peptide-1 (GLP-1) amide has been shown to lead to a significant reduction in food intake in rodents. GLP-1 is thought to be a short-term regulator of feeding and may be involved in meal termination, as GLP-1 levels in the periphery have been shown to rise after ingestion. Both leptin and GLP-1 elevated c-fos-like immunoreactivity (c-FLIR) in the PVN and central amygdala after leptin administration, however only leptin elevated c-FLIR in the DMN, while only GLP-1 elevated c-FLIR in the NTS, AP and lateral parabrachial nucleus (Elmqquist et al., 1997; Elmqquist et al., 1998a). These results suggest that the two peptides may have different roles in the regulation of food intake and body weight.

Hypothalamic CART is a newly characterised satiety factor that is closely associated with the actions of leptin and NPY (Kristensen et al., 1998). Energy-restriction has been found to reduce CART mRNA levels in the arcuate nucleus, while ICV administration of the CART peptide to rats inhibits both normal and starvation-induced feeding. Interestingly CART administration also completely blocks the feeding response induced by NPY (Kristensen et al., 1998). Initial studies have demonstrated that peripheral administration of leptin to obese animals increases the expression of CART mRNA, while CART mRNA is almost absent from the arcuate nucleus of animal models known to have defective leptin signaling (Kristensen et al.,
The authors suggest that leptin-mediated suppression of food intake is controlled by a balanced reduction and induction of NPY and CART, respectively.

OB-R-like immunoreactivity (OB-R-LI) has also been demonstrated in some parvocellular neurons of the dorsomedial and ventrolateral arcuate nucleus (Elmquist et al., 1997; Elmquist et al., 1998a). These neurons are known to project into the external layer of the median eminence and to influence secretion of anterior pituitary hormones (Hakansson et al., 1998a). These neurons have been shown to co-contain a large number of neurotransmitters and peptides, including GAL and GHRH, two peptides known to affect feeding behaviour (Meister, 1993). OB-R-LI has also been demonstrated in CRH-containing neurons in the parvocellular part of the PVN. Leptin administration directly into the third ventricle causes an increase in CRH gene expression in the PVN of lean rats (Schwartz et al., 1996b). ICV administration of CRH has been shown to inhibit feeding (Kaiyala et al., 1995; Schwartz et al., 1994). Therefore it is possible that the weight-reducing effect of leptin may be partly mediated via an increased CRH expression (Hakansson et al., 1998a). In addition, OB-R-LI was found to be co-localised to neurons containing GAL in the parvocellular PVN, and GAL and CRH have previously been demonstrated to occur in the same cells in this region (Ceccatelli et al., 1989).

Melanin-concentrating hormone (MCH) is another neurotransmitter found to be involved in feeding regulation. Fasting has been shown to increase MCH mRNA in both normal and obese animals (Qu et al., 1996), while ICV injection of MCH increases food consumption (Qu et al., 1996). The neurons found to contain MCH are located exclusively in the zona incerta and lateral hypothalamus (Skofitsch et al., 1985), regions known to be involved in the regulation of ingestive behaviour. In addition, OB-RL1 was recently demonstrated in many MCH-containing neurons in the lateral hypothalamus. These findings suggest that MCH is also a mediator of leptin’s actions within the CNS to induce reduction in bodyweight (Hakansson et al., 1998a).

Two recent studies by Sahu have further examined the regulation of neuropeptides by leptin. Central administration of leptin to Sprague-Dawley rats for three days resulted in decreased food intake and decreased body weight gain in association with decreased hypothalamic galanin (by 30%), MCH (by 29%), POMC (18%) and NPY
(22%) gene expression, and an increase in neurotensin (33%; NT) gene expression. The changes in gene expression of galanin, MCH, POMC and NT were found not to be the result of decreased food intake, as the same changes were not seen in pair-fed animals. However, pair-fed animals did have increased NPY mRNA levels when compared to the non-pairfed PBS control group (Sahu, 1998a). Overall, these results suggest that leptin inhibits gene expression of those neurons that are excitatory and stimulates gene expression of those neurons that are inhibitory to feeding behaviour.

In a subsequent study, Sahu demonstrated that administration of NPY, MCH or GAL stimulated food intake in saitied rats. A prior ICV injection of leptin to these rats completely inhibited the NPY-, GAL- and MCH-induced food intake, demonstrating leptin's ability to directly antagonise the action of these neuropeptides (Sahu, 1998b). Although leptin inhibited the actions of MCH at all doses, leptin was unable to completely prevent the effects of the highest dose of either GAL or NPY. This indicates that the orexigenic effect of MCH may be mediated solely via a leptin sensitive pathway, however the effects of NPY and GAL may involve multiple neuronal systems, some of which may not be sensitive to leptin (Sahu, 1998b).

The co-localisation of leptin receptors with neurotransmitters thought to play important regulatory roles in food intake within the hypothalamus provides further evidence that leptin's actions within the brain may be mediated by several alternative pathways. Overall, leptin has been shown to activate pathways involved in inhibiting food intake, and suppressing expression of neuropeptides known to increase food intake. Within the brain, the expression of leptin receptors has been measured in both rodents and humans, and the level of expression compared between lean and obese subjects. In addition, the effects of different stimuli on leptin receptor gene expression has also been investigated in a limited number of studies.

1.6.6 OB-R mRNA EXPRESSION WITH THE CNS
1.6.6.1 Rodents

In db/db and C57BL/BJ ob/ob mice there is at least a 2-fold higher level of OB-R mRNA in the arcuate nucleus compared to lean +/-ob controls (Fci et al., 1997; Huang et al., 1997; Baskin et al., 1998). The overexpression of OB-R/B in these mice is thought to be a result of decreased leptin signaling because of either no leptin
circulating (ob/ob) or decreased receptor functioning (db/db). As stated above C57BL/BJ ob/ob mice have higher levels of OB-mRNA in their arcuate nucleus compared to lean +/-ob controls, which is thought to suggest a hypersensitivity to leptin and there is no ligand-induced downregulation of receptor numbers as endogenous circulating leptin levels increase. Leptin has been shown to suppress the expression of mRNA encoding pre-proNPY and increase POMC mRNA levels in the arcuate nucleus. Therefore overexpression of OB-RB mRNA in the arcuate nucleus may contribute to leptin hypersensitivity of mice deficient in leptin (ob/ob) or NPY (NPY-knock out) (Fei et al., 1997; Baskin et al., 1998).

In Zucker fatty rats, which have a base substitution in the extracellular domain of the OB-R gene, Iida et al. (1996a) reported that the level of OB-RB mRNA in total brain was higher than seen in lean litter mates. A subsequent study by Bennett et al. (1998) demonstrated that there was significantly lower expression of OB-R mRNA in the choroid plexus (CP), thalamus (TH) and PC of Zucker compared to lean control rats, although the level of OB-RB mRNA is increased in the TH, PC and ARC. They hypothesise that differential regulation of isoforms of OB-R may indicate an attempt to alter the balance of OB-R transcripts in favour of OB-RB (Bennett et al., 1998).

1.6.6.2 Humans

OB-R mRNA levels have been examined in human hypothalamus by Considine et al. It was found that there was no difference in the level of OB-R mRNA between lean and obese subjects, although this study was performed using total hypothalamus and did not specifically measure OB-RB mRNA (Considine et al., 1996). Studies using human hypothalami are rare, and this is the only study published to date examining the level of OB-R mRNA in the hypothalamus of lean and obese humans. The usefulness of this study is limited however, as OB-R mRNA was measured in only a small number of lean (n=7) and obese (n=8) subjects, and this study measured total OB-R mRNA, with no differentiation between the different isoforms of the receptor. As several rodent models appear to have different levels of OB-RB gene expression within the brain of lean and obese animals, further examination is needed to confirm the finding that lean and obese humans do not have differing levels of expression.
1.6.7 REGULATION OF HYPOTHALAMIC OB-R GENE EXPRESSION

The level of expression of OB-R has been suggested to be sensitive to physiological interventions that result in changes in circulating leptin concentrations (Baskin et al., 1998). In addition, OB-R gene expression in the hypothalamus may also be under direct hormonal or nutritional regulation, independent of circulating leptin concentrations. Several different groups have examined the regulation of OB-R gene expression within the hypothalamus, and changes in expression in response to dietary or hormonal changes.

1.6.7.1 Dietary Regulation-Fasting

The effect of fasting on OB-RA and OB-RB has been investigated in mice and Baskin et al. (1998) demonstrated that a 48-hour fast resulted in an increase in OB-RB mRNA in the arcuate nucleus of normal and NPY-knock out mice to 2.5- and 4-times baseline, respectively. A study by Lin and Huang (1997) also demonstrated that although lean mice show increased expression of hypothalamic OB-RB with fasting, this is not seen in ob/ob mice.

In normal Wistar rats, a 48-hour fast resulted in an increase in OB-RB mRNA levels by 40% in the arcuate nucleus and by 75% in the VMH compared with fed controls (Baskin et al., 1998). A short-term fast in Sprague Dawley rats resulted in a decrease in OB-R mRNA in all areas examined, with changes being greatest in the ARC and VMH. In these same animals however, OB-RB mRNA increased in the thalamus (TH) as a result of fasting (Bennett et al., 1998), indicating a differential regulation of OB-RB and OB-R_5 in different regions of the brain.

A study conducted by Dyer et al. (1997) demonstrated the presence of an OB-R cDNA in ewes with 84% and 78% nucleotide homology to human and mouse OB-RB, with mRNA detected in the hypothalamus, anterior pituitary and adipose tissue of these animals. It was found that expression of OB-RB mRNA was higher in the arcuate nucleus and VMH of ewes that had been feed-restricted for 3 weeks compared to well-fed animals (Dyer et al., 1997).

These studies demonstrate that fasting increases OB-RB gene expression in a number of animal models and provide evidence of a feed-back loop, by which a reduction in
circulating leptin (as with fasting) results in up-regulation of leptin receptor gene expression as part of a regulatory mechanism. The study by Lin and Huang also showed that in ob/ob mice, which lack circulating leptin, fasting does not increase leptin receptor gene expression, providing further support for the hypothesis that circulating leptin concentrations are important regulators of OB-R expression. These findings however, do not rule out the possibility that factors other than leptin may play a role in the regulation of leptin receptor gene expression, particularly in polygenic models of obesity, which more closely mimic the situation of human obesity.

1.6.7.2 Hormonal Regulation-Leptin

Leptin administration to ob/ob mice has been found to decrease OB-RB gene expression in a number of studies. A study by Mercer et al. demonstrated a reduction of about 30% in OB-RB mRNA expression in the arcuate nucleus of ob/ob mice after twice daily systemic leptin administration (Mercer et al., 1997). Several other studies have also shown a decrease in OB-RB mRNA after leptin administration, when compared to both control (saline-treated) and pair-fed animals (Stephens et al., 1995; Schwartz et al., 1996a; Baskin et al., 1998). These results are consistent with the hypothesis that OB-R gene expression in the hypothalamus, particularly the arcuate nucleus, is sensitive to physiological interventions that cause a change in circulating leptin concentrations. Baskin et al. (1998) have further suggested that binding of leptin to OB-RB at the surface of the arcuate nucleus neurons may initiate intracellular signal transduction events that result in reduced transcription of the OB-RB receptor.

Although both the fasting and leptin-treatment studies favour the idea of direct regulation of OB-RB gene expression by leptin concentrations, the data do not rule out the possibility that other metabolic or hormonal conditions. In addition to alterations in leptin concentrations may also contribute. Only a limited number of studies have examined the effects of altered environments or different hormones on OB-R gene expression within the hypothalamus.

Cold exposure of mice has been used as a method of altering energy balance, and has been shown to rapidly decrease OB gene expression and plasma leptin concentrations.
in adipose tissue in lean mice (Trayhurn et al., 1995a; Hardie et al., 1996). Subsequently, exposure of lean mice to cold (4°C) for 24 hours has been reported to result in increased OB-R gene expression within the arcuate nucleus, an effect which is completely normalised when the mice are returned to the warm (24°C) (Mercer et al., 1997). This study provides further evidence of regulation of hypothalamic leptin receptors by circulating leptin concentrations, although the authors do not rule out an indirect effect of cold exposure on leptin receptor gene expression, such as via the stress axis (Mercer et al., 1997).

1.6.7.3 Growth Hormone

Transgenic mice constructed to produce human growth hormone-releasing hormone (hGHRH) were found to have a 5-fold higher level of OB-RB mRNA expression in the anterior pituitary compared to normal mice (Cai and Hyde, 1998). In addition, OB-Rs, which is undetectable in the anterior pituitary of normal mice was detectable in hGHRH transgenics. These results suggest that OB-R mRNA within the anterior pituitary is regulated, either directly or indirectly by growth hormone or GHRH (Cai and Hyde, 1998). The reasons for this regulation are unclear, however these results suggest that the anterior pituitary, in addition to the hypothalamus, may be a target site for leptin action within the brain.

1.6.7.4 Oestrogen

The effect of oestrogen on brain OB-R expression was investigated recently in normal female rats with and without ovariectomy (OVX) by Bennet et al. (1998). It was reported that the abundance of OB-R mRNA was highly sensitive to oestrogen and that this sensitivity displayed regional differences within the brain. Oestrogen to normal female rats for two weeks caused a marked reduction in OB-R expression in all regions measured, however the level of OB-RB mRNA was decreased by a smaller degree than total OB-R mRNA while these decreases were seen in the ARC and VMH but not in the periform cortex (PC) or the thalamus. Ovariectomy had no significant effect on the abundance of OB-RB mRNA although there was a significant increase in the abundance of OB-R mRNA in all areas measured except the PC. It appears therefore that the different receptor isoforms display differential sensitivity to oestrogen, with OVX only increasing expression of OB-Rs (Bennett et al., 1998).
Ovariectomy in the rat has previously been shown to result in weight gain, due partly to increased food intake (Richter et al., 1954). In this study, ovariectomy resulted in a decrease in the OB-RB/OB-R$_S$ ratio, which may cause a reduction in sensitivity to leptin, which has been suggested to allow for increased food intake and weight gain (Bennett et al., 1998). In contrast, administration of oestrogen, which is known to suppress food intake and reduce body weight (Mook et al., 1972), resulted in an increased OB-RB/OB-R$_S$ ratio, thought to increase leptin sensitivity, leading to a subsequent reduction in food intake and body weight (Bennett et al., 1998).

The presence and distribution of leptin receptors within the brain is well characterised in several animal models of obesity, although regulation of leptin receptor gene expression needs further examination in animal models other than the ob/ob mice, particularly polygenic models of obesity. Leptin receptors have been detected in a large number of tissue outside of the brain and both in vitro and in vivo studies have been performed in an effort to determine the role of these receptors in the regulation of body weight and energy balance. This section will discuss some of the peripheral effects of leptin and the potential role of peripheral leptin receptors.

1.6.8 PERIPHERAL LEPTIN ACTION

This section will discuss the possible roles of leptin in the pancreas, liver, muscle, adipose tissue, gastrointestinal tract, sympathetic nervous system and reproductive system, as well as leptin receptor gene expression in these various tissues.

1.6.8.1 Pancreas

In the pancreas, leptin receptors have been identified on pancreatic β-cells (Kieffer et al., 1996; Leclercq-Meyer et al., 1996; Emilsson et al., 1997; Fehmann et al., 1997) and leptin has been found to directly inhibit insulin secretion (Emilsson et al., 1997; Fehmann et al., 1997; Pallett et al., 1997). This inhibition can be seen in both the basal and glucose-stimulated states (Emilsson et al., 1997; Fehmann et al., 1997). Although inhibition of insulin secretion by leptin is not seen in all studies. One possible mechanism for leptin-induced inhibition of insulin secretion has recently been found to involve phosphoinositide 3-kinase (PI 3-kinase)-dependent activation of cyclic nucleotide phosphodiesterase 3B (PDE3B) (Zhao et al., 1998). In pancreatic
β-cells, leptin was found to activate PDE3B which in turn lead to marked inhibition of GLP-1 stimulated insulin secretion.

1.6.8.2 Liver

High amounts of OB-R gene expression have been detected in the liver, although the direct effects of leptin on hepatocytes are still unclear. Cohen et al. (1997) demonstrated that incubation of cells from a hepatoma cell line with physiological concentrations of leptin, results in leptin antagonising insulin signaling by attenuation of insulin-induced tyrosine phosphorylation of IRS-1 and inhibition of expression of the gene encoding PEPCK, the enzyme catalysing the rate limiting step in gluconeogenesis (Cohen et al., 1997). This study indicated that leptin may modulate insulin action in vitro, providing a mechanism whereby increased adiposity leads to insulin resistance. In addition, these findings provided evidence of ‘cross-talk’ between the signaling pathways downstream from insulin and leptin receptors (Cohen et al., 1997).

Barzilai et al. (1997) examined the in vivo effects of leptin administration on hepatic glucose production during physiologic hyperinsulinemia (insulin clamp). Under these condition, leptin markedly enhanced insulin action on both inhibition of hepatic glucose production and stimulation of glucose uptake, with an approximately 2-fold increase in glycogen synthesis, while the rate of glycolysis was not altered (Barzilai et al., 1998). In addition, leptin altered hepatic gene expression of key metabolic enzymes, with a marked decrease in the hepatic abundance of glucokinase mRNA and several-fold increases in hepatic abundance of PEPCK and glucose-6-phosphate mRNAs (Barzilai et al., 1998). These changes in gene expression are similar to those seen in fasting and are thought to suggest that leptin plays a role in intrahepatic partitioning of metabolic fluxes which may represent a defense against excessive storage of energy in adipose tissue (Barzilai et al., 1998). Similar results have been reported by other groups (Rosetti et al., 1997; Kamohara et al., 1997), and Sivitz et al. (1997) in Sprague-Dawley rats, in which leptin was administered during a 48-hour fast. In this study leptin increased insulin sensitivity under fasting conditions and in the presence of hyperinsulinemia at clamped glucose, with an increase in whole body glucose utilisation by about 30% during the glucose clamp (Sivitz et al., 1997).
Together these studies suggest that leptin enhances insulin action within the liver, with decreased hepatic glucose production, increased glucose turnover and uptake, and increased glycogen synthesis.

1.6.8.3 Muscle

In muscle cells, leptin appears to have no effect on basal or insulin-stimulated glucose metabolism in cultured rat or human skeletal muscle cells (Furnsinn et al., 1998; Ranganathan et al., 1998; Zierath et al., 1998). In mouse C2C12 myotubes however, leptin appears to exert an insulin-like effect on glucose uptake and glycogen synthesis within 1-3 hours of exposure and this effect is thought to occur through PI 3-kinase activation (Berti et al., 1997). Kellerer et al. (1997) demonstrated that the leptin signal to PI 3-kinase occurs via IR2 and not IRS-1, suggesting that leptin most likely activates JAK-2 which induces tyrosine phosphorylation of IRS-2 leading to activation of PI 3-kinase (Kellerer et al., 1997).

A study by Muoio et al. (1997) demonstrated that leptin may directly alter lipid partitioning in skeletal muscle, channeling metabolic fuels toward utilisation and away from storage. Ex vivo studies on the soleus and EDL muscles from mice demonstrated that leptin increased soleus muscle fatty acid oxidation by 42% and decreased fatty acid incorporation into triglyceride by 35% in a dose-dependent manner. In contrast to leptin, insulin was found to decrease soleus muscle fatty acid oxidation (by 40%) and increase its incorporation into triglyceride (by 70%). When both hormones were present, leptin attenuated both the antioxidative and lipogenic effects of insulin (by 50%), however throughout the study leptin did not alter insulin-stimulated muscle glucose metabolism (Muoio et al., 1997). These results imply that skeletal muscle also plays an important role in mediating leptin’s effects on fuel homeostasis.

Recently, Wang et al. demonstrated that glucosamine rapidly activates OB gene expression in skeletal muscle (Wang et al., 1998). The hexosamine biosynthetic pathway is considered a cellular ‘sensor’ of energy availability, and increased tissue concentrations of the end product of this pathway, UDP-N-acetylglucosamine (UDP-GlcNAc) result in rapid and marked increases in OB mRNA expression and leptin levels (Wang et al., 1998). This regulation of OB gene expression was also apparent.
in adipose tissue and myocytes and is reproduced by either hyperglycemia or hyperlipidemia, which both increase tissue levels of UDP-N-acetylglucosamine in conscious rodents (Wang et al., 1998). This study provided another mechanism whereby leptin gene expression and secretion may be regulated by altered states of energy.

1.6.8.4 Gastrointestinal Tract

Recently, the gastrointestinal tract has been identified as a potential site of leptin action, with leptin receptors identified in the small intestine and OB mRNA detected in the rat stomach (Bado et al., 1998). Within the stomach, OB mRNA and leptin were found in rat gastric epithelium, while the cells in the glands of the gastric fundic mucosa were immunoreactive for leptin (Bado et al., 1998). The role of gastric leptin is unknown, however a role in CCK-mediated regulation of gastrointestinal function has been hypothesised. Both feeding and administration of CCK-8 result in a rapid and large decrease in both leptin cell immunoreactivity and the leptin content of the fundic epithelium, with a concomitant increase in plasma leptin concentration (Bado et al., 1998). This finding implies the existence of a gastric leptin store (found to be ~1.4 ng) which has a potential role in satiety (Bado et al., 1998).

In the small intestine, leptin receptors have been identified in the intestinal mucosa and leptin has been reported to have a direct, inhibitory effect on sugar absorption. Lostao et al. (1998) recently demonstrated that leptin inhibited approximately 30% of D-galactose uptake by rat small intestinal rings, with the inhibition found to be effective within 5 mins of administration. In addition, Morton et al. (1998) have identified leptin receptors in the jejunum and demonstrated that intravenous leptin administration resulted in a significant, 2-fold reduction in apolipoprotein AIV mRNA expression in jejunum 90 mins after a fat load. The APO-AIV system is thought to serve as a conduit for transport of triglycerides as chylomicrons into the circulation and their transfer to acceptor membranes in various tissues. In addition, APO-IV synthesis in the jejunum is known to be stimulated by a fat load. The authors suggest that this novel, peripheral action of leptin may represent an adipose-enteric loop providing a negative-feedback signal from fat stores to the intestine to regulate lipid handling (Morton et al., 1998).
1.6.8.5 Sympathetic Nervous System

Leptin administration to ob/ob mice has been reported to induce increases in energy expenditure, hyperactivity and a rise in core temperature (Pellymouter et al., 1995). and it became apparent that the changes in body weight seen after leptin administration in ob/ob mice could not be accounted for simply by decreased food intake, as smaller decreases in body and fat-depot weights were seen in vehicle treated mice fed the same amount of food as that consumed by leptin-treated mice (Levin et al., 1996). Leptin’s role in regulating fat mass has been hypothesised to involve signaling the sympathetic nervous system (SNS) to increase thermogenesis and energy expenditure in BAT as fat mass increases (Collins et al., 1996a). Collins et al. (1996a) demonstrated that leptin treatment results in increased sympathetic outflow without changes in food intake. A selective increase in norepinephrine turnover to BAT was detected, however this effect was not seen in WAT, indicating that leptin may regulate body composition by altering thermogenesis in BAT (Collins et al., 1996a). Interestingly, OB gene expression has been shown to decrease rapidly after treatment with β3-agonists (Collins et al., 1996b; Kosaki et al., 1996; Trayhurn et al., 1996; Mantzoros et al., 1996).

The effect of leptin administration on NE turnover in tissues other than fat was further investigated by Haynes et al. (1997). In vivo, leptin treatment resulted in increases sympathetic nerve activity in BAT (by ~290%), hindlimb (~290%) and adrenal gland (~390%), while a dose-dependent increase in sympathetic nerve activity was detected in the kidney (~230%; Haynes et al., 1997). Despite the overall increase in sympathetic nerve activity, leptin treatment did not increase arterial pressure or heart rate in this study. Similarly, in humans a positive correlation has been reported between muscle sympathetic nervous activity and leptin (Snitker et al., 1997). β-adrenergic stimulation is known to decrease leptin secretion in vivo and in vitro, therefore it is possible that leptin acts centrally by stimulating sympathetic outflow, which in turn decreases leptin secretion, thereby effecting a feedback, regulatory loop between adipose tissue and the brain (Tataranni, 1998).

1.6.8.6 Adipose Tissue

The identification of leptin receptors in adipose tissue supported the idea that leptin may be having direct actions on adipose tissue (Fei et al., 1997; Louh et al., 1997). In
adipose tissue leptin over-expression resulted in increased UCP-2 gene expression in adipose tissue, with an increase of more than 10-fold in epididymal, retroperitoneal and subcutaneous adipose tissue (Zhou et al., 1997). Other studies have demonstrated changes in several other enzymes involved in lipolysis, with leptin-treatment found to increase malic enzyme and lipoprotein lipase approximately 2-fold in cultured brown adipocytes (Siegrist-Kaiser et al., 1997) and increase UCP-2 gene expression up to 2-fold in adipose tissue (brown and white) (Sariemento et al., 1997). Leptin treatment has also been found to have age-specific effects in adipose tissue. ICV leptin treatment was found to increase expression of PPAR-γ by 70-80% in both young (3 months old) and old (8 months old) rats, whereas TNF-α expression was decreased by 40% with leptin treatment in only old rats (Qian et al., 1998b). UCP-2 expression was also found to decrease with leptin treatment in young rats, but was increased with treatment in old rats (Qian et al., 1998b). PPAR-γ has been implicated in apoptosis and increased expression of this gene after leptin administration is thought to signify the involvement of PPAR-γ in leptin-induced apoptosis. The changes in UCP-2 and TNF-α expression after leptin treatment further support the hypothesis that these genes are involved in a leptin-induced lipolysis pathway (Qian et al., 1998b). Scarpace et al. demonstrated that leptin increased UCP expression more than two-fold in BAT from both ad-libitum fed and food restricted rats. Body temperature and whole body oxygen consumption were also measured and found to increase, suggesting a leptin-induced increase in energy-expenditure via increased thermogenesis (Scarpace et al., 1997).

Campbell et al. have demonstrated in vitro that human leptin binds FFAs. This binding was found to be reversible and showed positive co-operativity. In addition, leptin binding to FFAs increased the electrophoretic mobility of the protein in native polyacrylamide gels in a manner that was dependent upon the chain length and number of double bonds within the fatty acid (Campbell et al., 1998). The authors of this study suggest that leptin may function in a manner similar to fatty acid binding protein (FABP) to traffic intracellular FFAs to different organelles and to protect enzymes or membranes from the deleterious effects of high FFA concentrations (Campbell et al., 1998).
Leptin has now been shown to directly inhibit insulin-stimulated glucose incorporation into lipid, stimulate glucose decarboxylation in isolated adipocytes and disrupt the kinetics of insulin-binding in isolated adipocytes (Ceddia et al., 1998, Mueller et al., 1997; Walder et al., 1997a), although several groups have not found these effects (Ranganathan et al., 1998; Zierath et al., 1998). These findings suggest that leptin may be involved in energy partitioning in adipocytes, by moving away from glucose metabolism to increased fat metabolism, with increased lipolysis and thermogenesis.

Barzilai et al. (1997) have reported that leptin administration to rats resulted in a specific and marked reduction (by 62%) in visceral adiposity which was independent of its effect on food intake, suggesting that leptin may play a role in the distribution of body fat. Although the actions of leptin are thought to be direct, leptin is known to rapidly decrease fat mass when administered ICV. An interesting report by Qian et al. (1998a) suggests that the process of adipose tissue loss after leptin treatment involves adipocyte apoptosis. Rats treated ICV with leptin were found to have dramatically reduced fat pad weight, and the adipose tissue of leptin treated animals demonstrated characteristic features of apoptosis including internucleosomal fragmentation of genomic DNA, elevated levels of DNA strand breaks and a reduction in total DNA content and cellular volume (Qian et al., 1998a). This report suggests that leptin treatment induces the deletion of adipocytes in addition to increasing lipolysis.

In adipose tissue therefore, leptin appears to increase lipolysis, and disrupt insulin action. These effects may be involved in energy partitioning within the adipocyte, and alter metabolic fluxes in such a way as to increase adipose tissue breakdown.

Although leptin is thought to play a primary role in energy balance, the discovery of leptin also generated considerable excitement in the area of reproductive biology, as many believe that leptin may be the long awaited indicator of nutritional status that allows reproductive processes to proceed. Leptin is produced in the adipose tissue, which responds to metabolic and nutritional changes, and the production of leptin increases with feeding and body fat content. In addition, leptin receptors are found in hypothalamic regions identified to be involved with the control of appetite and
reproductive neuroendocrine function, as well as on reproductive organs. Thus leptin may provide an accurate, circulating signal of nutritional status, and will be discussed in this context below.

1.6.9 LEPTIN AND REPRODUCTION
Adequate nutritional intake is known to be important for maintenance of reproductive function (Asdell, 1949; Short and Bellows, 1971; Armstrong and Britt, 1987). A metabolic signal has been hypothesised to transmit information regarding energy stores to the reproductive system, although the signaling metabolic has not been clearly elucidated. Attention has now turned towards leptin as a possible modulator of the productive axis as increasing evidence becomes available regarding its role in reproduction.

The majority of the evidence implicating a role for leptin as a reproductive hormone has come from studies of the ob/ob mouse, which are leptin deficient. Female ob/ob mice are sterile and are constantly in a prepubertal stage of development. Ovarian weights, sex steroid concentrations and pituitary gonadotropin concentrations have been found to be low in these animals (Barash et al., 1996) while administration of recombinant leptin to female ob/ob mice completely restores gonadotropin secretion, secondary sex organ weight and function, and fertility (Barash et al., 1996; Chehab 1996). Male ob/ob mice similarly demonstrate low levels of fertility with low gonadotropin secretion and hypogonadism reported (Swerdloff et al., 1978; Mounzih et al., 1997). Similar to female ob/ob mice, leptin administration to male ob/ob mice also restores full fertility.

Low circulating leptin levels are characteristic of starvation or energy restriction in animals, including humans. The starvation-induced delay in ovulation in non-obese female mice was shown by Ahima et al. (1996) to be prevented by leptin treatment. Furthermore, leptin administration to fasted male mice increases serum LH and testosterone levels (Ahima et al., 1996). Cheung et al. (1997) demonstrated that mice energy-restricted to 80% of ad libitum food intake had over 50% reduction in ovarian and uterine weights, in addition, this reduction was completely prevented by twice-daily injections of leptin (Cheung et al., 1997).
Leptin receptors have been detected in the ovary, testis, uterus, hypothalamus and pituitary gland in both rodents and humans (Cioffi et al., 1996; Schwartz et al., 1996c; Zamorano et al., 1997) which indicates that leptin may act at multiple sites within the reproductive system. To date, leptin has been shown to enhance gonadotropin secretion (Barash et al., 1996), inhibit insulin-induced progesterone and oestrodial production by granulosa cells, (Spicer and Francisco 1997), and increase ovarian side-chain cleavage and 17α-hydroxylase mRNA levels (Zamorano et al., 1997). It has been suggested that the overall leptin-induced stimulation of reproductive function occurs secondarily to gonadotropin secretion (Houseknecht et al., 1998) and increased gonadotropin secretion is seen consistently in leptin-treated ob/ob mice as well as in undernourished animals. In addition, leptin directly stimulates gonadotropin secretion from cultured rat, steer and pig pituitary cells (Barb et al., 1997; Liou et al., 1997; Yu et al., 1997). Yu et al. (1997) have also demonstrated a direct stimulatory effect of leptin on gonadotropin releasing hormone (GnRH) concentration in cultured median-eminence-arcuate explants from rats.

The effects of leptin on hypothalamic GnRH are possibly mediated by NPY, as NPY is known to inhibit gonadotropin secretion in addition to its role as a potent stimulator of feeding (Houseknecht et al., 1998). Leptin administration is known to decrease NPY expression in the arcuate nucleus (Campfield et al., 1996; Schwartz et al., 1996a), thereby removing inhibition of GnRH release. In support of this hypothesis, leptin receptors have been found to be co-localised with NPY neurons within the hypothalamus (Mercer et al., 1996a; Mercer et al., 1996b), ob/ob mice who lack circulating leptin are deficient in hypothalamic GnRH (Swerdlow et al., 1978; Batt et al., 1982) and ob/ob mice that have been bred to lack NPY in addition to OB gene expression are found to be less obese and more fertile than ob/ob mice with intact NPY production (Erickson et al., 1996b).

In humans, there is also evidence that leptin is important for normal reproductive function. Strobel et al. (1998) have recently characterised a Turkish family in which a missense mutation within the OB gene results in low levels of circulating leptin and morbid obesity in three affected members. Two of the affected individuals were adults (22 year old, male; 34 year old female). The male was found not to have gone
through puberty, and had endocrine measurements which indicated impaired hypothalamic activation of the pituitary gonadotroph. The affected female was reported never to have menstruated (Strobel et al., 1998). The effect of leptin treatment in these individuals will help to further characterise the role of leptin in human reproductive function.

Leptin levels have regularly been found to be higher in women than in men, even after accounting for different degrees of adiposity. The role of sex hormones in this gender difference in leptin secretion has been examined in various studies. Murakami et al. (1995a) demonstrated that estradiol increases OB gene expression about 2-fold in cultured rat adipocytes, while ovariectomised rats were found to have similar leptin levels compared to sham-treated rats, despite the reduced level of estradiol recorded in the ovariectomised group (Yoneda et al., 1998). A study in humans by Paolissio et al. (1998) reported that after adjustment for age, gender, adiposity, WHR and fasting plasma insulin, circulating leptin levels were found to be associated with plasma estradiol and total testosterone (negative association) in males, and with fasting plasma estradiol in women. The negative relationship between leptin and testosterone in men has also been demonstrated in boys (Wabitsch et al., 1997), with both testosterone and its biologically active metabolite dihydrotestosterone found to suppress OB gene expression in cultured human adipocytes (Wabitsch et al., 1997).

The effect of oestrogen on leptin levels in women has also been examined. Havel et al. (1996a) demonstrated that oestrogen replacement does not affect plasma leptin concentrations in postmenopausal women, with similar findings reported by Kohrt et al. (1998). Rosenbaum et al (1996) however, demonstrated lower levels of plasma leptin in postmenopausal women compared to premenopausal women, raising the possibility of a stimulatory effect of oestrogen on leptin secretion. In addition, several studies have found that leptin levels in healthy, normally ovulating women depends on the phase of the menstrual cycle (Hardic et al., 1997; Lukaszuk et al., 1998; Mannucci et al., 1998) and a relationship between leptin and oestrogen has been found, with both hormones showing similar patterns of fluctuation during the menstrual cycle.
There is considerable evidence therefore, that leptin plays a role in the regulation of reproductive function in neuroendocrine, and possibly reproductive tissues, particularly in rodents and humans which are leptin deficient. The importance of leptin as a regulator of reproductive function in polygenic animal models of obesity, and the general human population, who do not lack circulating leptin, still needs to be examined, with further work necessary to determine the site and mechanism of leptin action.
Despite the wealth of knowledge presented in this overview, several key areas remain poorly understood. At this time, little is known about the importance of lifestyle factors on circulating leptin concentrations or the role of leptin receptor sequence polymorphisms in obesity in humans. In addition, little is known about the role of leptin receptors in polygenic animal models of obesity and/or type 2 diabetes, such as *Psammomys obesus*.

A number of studies detailed in this dissertation have been undertaken to investigate the role of leptin and leptin receptors in the pathogenesis of obesity in humans and *Psammomys obesus*. The following areas will be examined in detail and each will constitute a separate chapter of this dissertation.

### 1.7 AIMS

1. **The relationship between circulating leptin concentrations, obesity and lifestyle factors in Australian women:** The relationship between circulating leptin concentrations and metabolic parameters and lifestyle factors such as alcohol intake, physical activity level, smoking habits and reproductive history will be examined in a population of Caucasian Australian women.

2. **Associations between leptin receptor polymorphisms and obesity in Australian women:** The relationship between the Gln223Arg and PRO1019pro leptin receptor sequence polymorphisms and obesity and metabolic parameters in Australian women will be examined in cross-sectional analysis, and associations between these polymorphisms and longitudinal changes in body mass and composition in this same population will also be examined.

3. **Associations between leptin receptor polymorphisms and obesity in Nauruan males:** The relationship between the Gln223Arg and PRO1019pro leptin receptor sequence polymorphisms and an OB gene polymorphism with phenotypic measures of obesity and type 2 diabetes in Nauruan males will be examined in cross-sectional analysis. This population has previously been shown to have very high prevalence rates of obesity and type 2 diabetes and associations between these polymorphisms and the development of obesity and type 2 diabetes over a 12-year period will also be examined.
4. **Leptin concentrations in *Psammomys obesus***: Associations between circulating plasma leptin concentrations and various metabolic and phenotypic parameters will be examined.

5. **Leptin receptor gene expression in *Psammomys obesus***: The levels of expression of OB-RA, OB-RB and the transcription factor, c-fos will be measured in the hypothalamus, liver and suprascapular white adipose tissue of *Psammomys obesus* with a range of body weight, blood glucose and plasma insulin concentrations. The gene expression will be examined with respect to body weight, body fat and a range of metabolic parameters.

6. **The effects of short-term fasting on leptin receptor gene expression in *Psammomys obesus***: The effects of fasting for 24 hours on OB-RA, OB-RB and c-fos gene expression, as well as the effects of fasting on other biochemical parameters, will be investigated in *Psammomys obesus*.

7. **The effects of sucrose-feeding on dysregulation of energy balance and leptin receptor gene expression in *Psammomys obesus***: The effects of the addition of a 5% sucrose solution to the diet of lean and obese *Psammomys obesus* will be investigated, with particular attention on alterations in OB-RA, OB-RB and c-fos gene expression in the hypothalamus, liver and adipose tissue.
CHAPTER 2

MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

2.1.1 PSAMMOMYS OBESUS

A colony of *Psammomys obesus* is maintained at Deakin University using the San Poiley outbreeding method (Poiley, 1960). This breeding technique provides for maximum genetic variation in subsequent generations and is based on a systematic rotation of breeders. The original *Psammomys obesus* breeders came from the Hebrew University-Hadassah Medical School in Jerusalem, courtesy of Professor Johnathon Adler and in collaboration with Professor Eleazar Shafrir (Hadassah University Hospital, Jerusalem, Israel) and Professor Paul Zimet (International Diabetes Institute, Caulfield, Australia). These first animals were used to establish a breeding colony at Deakin University, Geelong, and the major criterion for selection of breeding pairs was the absence of cataracts, which has previously been shown to predict reduced breeding potential (Adler et al., 1985).

At Deakin University, the breeding pairs were maintained on a diet consisting of standard laboratory chow (Barastoc, Pakenham, Victoria, Australia) and lucerne. The chow was in pellet form and the energy components were: 12% energy from fat, 63% energy from carbohydrates and 25% energy from protein. Lucerne supplementation of the diet of breeding pairs reduced the energy density of the diet, which has previously been shown to maximise breeding (Adler et al., 1985).

*Psammomys obesus* to be used as experimental animals were weaned at 4 weeks of age and housed in family, sex-specific groups of between 2 and 4 and given free access to water and laboratory chow. For certain dietary studies (fasting and sucrose-feeding) experimental animals were placed into individual cages at approximately 16 weeks of age prior to commencement of the study in order to measure individual food and water intakes. As discussed earlier, when *Psammomys obesus* are maintained on an *ad libitum* diet of laboratory chow a significant proportion of the animals develop varying degrees of obesity and glucose intolerance. All animals were maintained in a temperature controlled room at 22±1°C with a 12/12-hour light-dark cycle (light 0600-1800; dark 1800-0600). Each cage was lined with saw dust, with animals transferred to fresh cages twice weekly and all
animals were maintained in accordance with the Code of Practice of the National Health and Medical Research Council.

2.2 BIOCHEMICAL ANALYSES

2.2.1 PLASMA GLUCOSE

Plasma glucose concentrations were determined using a colorimetric method (Boehringer Mannheim, Mannheim, Germany) on an automatic analyser (Hitachi 705-0013, Tokyo Japan). 5ul of sample was added to 100ul water and 700ul of a reagent solution containing phosphate buffer (100 mmol/l, pH 7.0), phenol (11 mmol/l), 4-aminophazone (0.77 mmol/l), phenol oxidase (0.9 U/ml) and glucose oxidase (13 U/ml). Incubation at 25°C for 1 hour allowed the conversion of glucose to gluconate and hydrogen peroxide by glucase oxidase, and the oxidation of phenol with the substrates hydrogen peroxide and 4-aminophenazone via phenol oxidase. This reaction produced 4-(p-benzoquinone-meno-imino)phenazone which has maximal absorption at 540 nm. The amount of glucose in the plasma sample therefor was determined from the optical density measured at 540 nm. At this wavelength, a linear relationship existed between the optical density and the original glucose concentration. A linear curve was thus developed using a calibration serum specifically designed for automated systems (glucose concentration 7.16 mmol/l; Boehringer Mannheim, Mannheim, Germany). External standards were also incorporated into each run and measured every 40 samples (Boehringer Mannheim, Mannheim, Germany). The two standards used were Precinorm U (normal physiological, 6.55 mmol/l, acceptable range: 5.90-7.20 mmol/l) and Precipath U (pathological, 13.5 mmol/l, acceptable range: 12.2-14.8 mmol/l).

2.2.2 WHOLE BLOOD GLUCOSE

Whole blood glucose measurements were made using a YSI enzymatic glucose analyser (YSI 2300 STAT Plus, Yellow Springs Instrument Company, Ohio, USA). The instrument was calibrated using a 10 mmol/l glucose standard every 5 samples. The analyser utilised an enzymatic reaction to generate a measurable redox potential. Glucose oxidase immobilised on a polycarbonate/cellulose membrane, converted D-glucose to gluconic acid, thereby generating hydrogen peroxide as a by-product. The current produced was dependent on the amount of hydrogen peroxide produced. The relationship between the current produced and the concentration of glucose in the sample remained linear in the range of the calibration standard.
2.2.3 PLASMA INSULIN

Plasma insulin concentrations were determined using a commercially available radioimmunoassay (RIA) kit (Phadseph, Pharmacia Diagnostics AB, St Louis, USA). This kit utilised a double antibody solid phase technique whereby the insulin in the plasma competed with $^{125}$Iodine-labelled insulin for binding sites on highly specific antibodies. In the procedure, 20 µl of *Psammomys obesus* plasma, or 50 µl of human plasma, was added to 50 µl of $^{125}$I-labelled insulin (tracer) and 50ul of guinea-pig anti-insulin antibody ('first antibody') and left to bind for 2 hours at room temperature. A 1 ml aliquot of sheep anti-guinea pig antibody ('second antibody') was then added, which was highly specific for the 'first antibody' and served as a carrier. Binding proceeded at room temperature for a further 30 mins, after which the tubes were centrifuged at 3500 g for 20 mins (Beckman Centrifuge, Model J6B, Beckman Instruments Australia, Gladesville, Australia) to separate the bound from the free insulin. Each tube was then aspirated, leaving the bound insulin in a pellet in the bottom of the tube. The radioactivity of the solid phase pellet was determined using a gamma counter (Multigamma Counter Model LKB Wallac 1261, Turku, Finland). The concentration of each unknown sample was then computed by comparing the competitive capacity of the plasma insulin with standards of known concentrations (0, 3, 10, 30, 100 and 240 mU/l). A spline-shaped curve described the relationship between the ratio of bound to unbound $^{125}$I-labelled insulin and the log of the insulin concentration. Under standard conditions, the gradient and linearity of the curve were maximal in the central region of the curve (approximately 10-100 mU/l). Samples of high insulin concentration were diluted further and re-assayed where necessary to enable measurement in the linear region of the curve. Three external standards (Lymphochek Immunoassay Control Serum, Bio-Rad, Anahcim, USA) were incorporated into each assay and comprised low (15.9±0.4 mU/l), medium (52.3±3.0 mU/l) and high (146.9±4.0 mU/l) quality controls.
2.2.4 PLASMA LEPTIN

2.2.4.1 Human

Leptin concentrations in human plasma were measured using a commercially available human leptin RIA kit (Linco Research Inc., St Louis, USA). This kit utilised $^{125}$I-labeled leptin and a human leptin antiserum to determine the level of leptin in plasma by the double antibody technique. In the procedure 50 µl of human plasma was added to 50 µl of $^{125}$I-human leptin (tracer) and 50 µl of rabbit anti-human leptin antiserum ('first antibody') and left to bind overnight (20-24 hours) at 4°C. A 0.5 ml ml aliquot of cold (4°C) goat anti-rabbit IgG antibody ('second antibody') was then added, which was highly specific for the 'first antibody' and served as a carrier. Binding proceeded at 4°C for 20 mins, after which the tubes were centrifuged at 3500 g for 30 mins (Beckman Centrifuge, Model J6B, Beckman Instruments Australia, Gladesville, Australia) to separate the bound from the free leptin. Each tube was then aspirated, leaving the bound leptin in a pellet in the bottom of the tube. The radioactivity of the solid phase pellet was determined using a gamma counter (Multigamma Counter Model LKB Wallac 1261, Turku, Finland). The concentration of each unknown sample was then computed by comparing the competitive capacity of the plasma leptin with standards of known concentrations (0, 0.5, 1, 2, 5, 10, 20, 50, and 100 ng/ml). A spline-shaped curve described the relationship between the ratio of bound to unbound $^{125}$I-labelled leptin and the log of the leptin concentration. Under standard conditions, the gradient and linearity of the curve were maximal in the central region of the curve (approximately 10-100 ng/ml). Samples of high leptin concentration were diluted further and re-assayed where necessary to enable measurement in the linear region of the curve. Two quality controls (Linco Research Inc, St Louis, USA) were incorporated into each assay with low (expected range: 2.1-3.9 ng/ml), and high (expected range: 16.4-24.6 ng/ml) concentrations.

2.2.4.2 Animal

Measurement of leptin in plasma from Psammomys obesus was performed using a commercially available RIA kit (Linco Research Inc, St Louis, USA). This kit utilised $^{125}$I-labeled leptin and a human leptin antiserum to determine the level of leptin in plasma by the double antibody technique. In the procedure 50 µl of Psammomys obesus plasma was added to 50 µl of multispecies leptin antibody (produced in guinea pig, 'first antibody') and left to bind overnight (20-24 hours) at 4°C. 50 µl of $^{125}$I-human leptin (tracer) was then added to each tube and again left to bind overnight (20-24 hours) at 4°C. A 0.5 ml aliquot
of cold (4°C) normal guinea pig IgG antibody ("second antibody") was then added, which was highly specific for the "first antibody" and served as a carrier. Binding proceeded at 4°C for 20 mins, after which the tubes were centrifuged at 3500 g for 30 mins (Beckman Centrifuge, Model J6B, Beckman Instruments Australia, Gladesville, Australia) to separate the bound from the free leptin. Each tube was then aspirated, leaving the bound leptin in a pellet in the bottom of the tube. The radioactivity of the solid phase pellet was determined using a gamma counter (Multigamma Counter Model LKB Wallac 1261, Turku, Finland). The concentration of each unknown sample was then computed by comparing the competitive capacity of the plasma leptin with human leptin standards of known concentrations (0, 1, 2, 5, 10, 20, and 50 ng/ml). As the multispecies leptin antibody was raised against human leptin, human leptin standards were used in this assay and the recommended units are thus ng/ml Human Equivalent leptin. A spline-shaped curve described the relationship between the ratio of bound to unbound 125I-labelled leptin and the log of the leptin concentration. Under standard conditions, the gradient and linearity of the curve were maximal in the central region of the curve (approximately 10-100 ng/ml). Samples of high leptin concentration were diluted further and re-assayed where necessary to enable measurement in the linear region of the curve. Two quality controls (Linco Research Inc, St Louis, USA) were incorporated into each assay with low (expected range: 3-5 ng/ml HE), and high (expected range: 21-37 ng/ml HE) concentrations.

2.2.5 PLASMA TRIGLYCERIDES
A commercially available enzymatic calorimetric method (Boehringer Mannheim, Mannheim, Germany) was used to determine plasma triglyceride concentrations on an automatic analyser (Hitachi Model 705-0013, Tokyo, Japan). A 5ml plasma sample was mixed with a reagent solution and incubated at 25°C for 10 mins. During this incubation the triglycerides in the plasma were hydrolysed to form glycerol, which was then converted to glucose-3-phosphate by glycerol kinase using ATP as a substrate. Glucose-3-phosphate was then oxidised by glycerolphosphate oxidase to form dihydroxyacetone phosphate and hydrogen peroxide. The substrates 4-aminophenazone and 4-chlorphenol were oxidised by hydrogen peroxide to form 4-(O-benzoquinone-mono-imino)phenazone, which has maximal absorbance at 540 nm. The amount of triglyceride in the plasma sample therefore was determined by measuring the optical density at 540 nm. A standard (triglyceride concentration 1.77 mmol/l, Boehringer Mannheim, Mannheim, Germany) was incorporated initially to calibrate the assay, and two external
standards were measured every 40 samples (Precinorm U, normal physiological triglyceride concentration, 1.62 mmol/l, acceptable range: 1.39-1.85 mmol/l; and Precipath U, pathological triglyceride concentration, 4.29 mmol/l, acceptable range: 3.69-4.89 mmol/l).

2.2.6 PLASMA CHOLESTEROL
A commercially available enzymatic colorimetric method (Boehringer Mannheim, Mannheim, Germany) was used to determine plasma cholesterol concentrations on an automatic analyser (Hitachi Model 705-0013, Tokyo, Japan). In a 5ml plasma sample cholesterol esterase hydrolysed the cholesterol ester to yield free cholesterol which was subsequently oxidised by cholesterol oxidase to yield \( \delta^\text{4} \)-cholesterone and hydrogen peroxide. The phenol group was then oxidised by hydrogen peroxide which subsequently bound to 4-aminophenazone generating 4-(p-benzoquinone-mono-imino)phenazone, of which the absorbance was measured at 540 nm. A standard (cholesterol concentration 4.07 mmol/l; Boehringer Mannheim, Mannheim, Germany) was incorporated at the commencement of each assay to calibrate the analyser. Two external standards (Precinorm U, normal physiological cholesterol concentration, 4.61 mmol/l, acceptable range: 3.96-5.26 mmol/l; and Precipath U, pathological cholesterol concentration, 8.81 mmol/l, acceptable range: 7.57-10.10 mmol/l) were measured every 40 samples.

2.2.7 PLASMA FREE FATTY ACIDS
A commercially available enzymatic colorimetric method (Wako NEFA C Test Kit; Wako Pure Chemical Industries, Ltd, Osaka, Japan) was used to determine non-esterified (or free) fatty acids in serum. A 17\( \mu \)l serum sample was mixed with a reagent solution and incubated at 37°C for 10 mins. During this incubation the free-fatty acids (FFA) in the sample were treated with acyl-CoA synthase (ACS) and formed the thiol esters of CoA, acyl CoA, and the by-products AMP and pyrophosphate. The acyl-CoA was then oxidised by acyl-CoA oxidase to produce 2,3-trans-Enoyl-CoA and hydrogen peroxide which, in the presence of added peroxidase, allowed the oxidative condensation of 3-methyl-N-ethyl-N-(b-hydroxyethyl)-aniline with 4-aminoantipyrine to form a purple solution with maximum absorption at 550 nm. The absorbance of each sample at 550 nm was measured using a spectrophotometer (Model DU640B UV/VIS-Spectrophotometer, Beckman Instruments, Australia, Sydney, Australia) and the concentration of FFA determined
against a standard curve made from the Standard Solution provided (Wako Pure Chemical Industries, Ltd. Osaka, Japan).

2.3 MOLECULAR TECHNIQUES
2.3.1 RNA EXTRACTION
Animals were killed by lethal overdose of barbiturates (pentobarbitone, 120 mg/kg) between 10:00 am and 12:00 pm and the required tissues immediately removed, weighed, snap frozen in liquid nitrogen and stored at -80°C. Tissues examined included: hypothalamus, liver, heart, pancreas, skeletal muscle (gastrocnemius), testis, small intestine, kidney, lung, various fat depots, including suprascapular, perirenal, mesenteric, intramuscular (from the hind leg) and epididymal (male animals only). For total RNA extraction 100 mg of tissue was homogenised in 2 ml of RNAzol B (Bresatec, Adelaide, Australia) and 200 µl of chloroform was added to the homogenate, briefly vortexed and incubated on ice (5 min). The suspension was centrifuged (12000 g, 15 min, 4°C) and the aqueous (upper) phase was transferred to a fresh tube. An equal volume of isopropanol containing 0.2M NaCl was added, mixed briefly and stored at -20°C overnight to precipitate the RNA. The RNA was pelleted by centrifugation (12000g, 20 mins, 4°C) and the supernatant decanted. The RNA pellet was washed with 75% ethanol then dried at room temperature before being resuspended in an appropriate volume of water containing 1% diethylpyrocarbonate (DEPC).

The absorption of a 250-fold dilution of the RNA was measured at 260 nm using a spectrophotometer (Model DU640B UV/VIS-Spectrophotometer, Beckman Instruments, Australia, Sydney, Australia) and the RNA was then quantitated using the following equation: 

\[
[RNA (\mu g/ml)] = Abs_{260} \times \text{dilution factor} \times \text{constant},
\]

where Abs\text{260} was the absorbance measured above, the dilution factor was 250 and the constant used for quantitation of RNA was 40. An absorbance reading of 0.05 units thus corresponded to an RNA concentration of 0.03 x 250 x 40 = 300 µg/ml.

2.3.2 cDNA SYNTHESIS
After the RNA was quantitated, 1 µg of RNA was reverse transcribed to generate cDNA using a commercially available Reverse Transcription System (Promega, Madison, USA). Briefly, the RNA was added to a mixture containing 10 mM Tris-HCL (pH 8.8), 50 mM
KCL, 1% Triton X-100, 2.5 mM MgCl₂, 10 μM each dNTP, 0.5 mg/ml oligo(dT) primer, recombinant RNasin ribonuclease inhibitor (25 U) and AMV reverse transcriptase (15U). After the addition of the enzyme the mixture was incubated at 42°C for 1 hour, before the reaction was terminated by incubation at 95°C for 5 mins. The cDNA generated was stored at -20°C for further use.

2.3.3 POLYMERASE CHAIN REACTION (PCR)
Amplification of OB-RA, OB-RB and c-fos was performed using oligonucleotide primers selected from published sequences (OB-RA: Lei et al., 1996; OB-RB: Leclercq-Meyer et al., 1996; c-fos: Kambe et al., 1996).

- OB-RA:
  - forward: 5'-acactgttaatttcaacaccagag-3'
  - reverse: 5'-agtattacaaaccattagttagg-3'

- OB-RB:
  - forward: 5'-tatggaagggagtggaaccac-3'
  - reverse: 5'-taacttagggttgactctgac-3'

- c-fos:
  - forward: 5'-gccagacggggagttggtaa-3'
  - reverse: 5'-ggcttgggtcaggctcatt-3'

For amplification of each specific gene, PCR was performed by adding 1.0 μg of cDNA to a reaction mixture containing 20 mM Tris-Cl (pH 8.4), 50 mM KCl, 1.25 mM (0.5 mM for c-fos) MgCl₂, 200 μM each dNTP, 10 pmol each specific forward and reverse oligonucleotide primer and 1.25 U Taq DNA Polymerase (Life Technologies, Gibco, Gaithersburg MD, USA). The PCR consisted of 94°C for 5 mins, then 35 cycles of denaturation at 94°C for 30 sec, annealing at either 55°C (OB-RA and OB-RB) or 60°C (c-fos) for 30 sec and extension at 72°C for 30 sec, followed by a final extension step of 72°C for 5 mins. PCR was performed in a GeneAmp PCR System 9700 Thermal Cycler (Perkin Elmer Applied Biosystems, Foster City, USA) in a total volume of 20 μl in 0.2 ml microcentrifuge tubes (Perkin Elmer, Foster City, USA).

2.3.4 DNA SEQUENCING
Prior to DNA sequencing a total of 40 μl of each PCR product was fractionated by agarose gel electrophoresis in a TAE gel containing 0.5 μg/ml ethidium bromide at 6 V/cm in a
DNA SubCell system (Bio-Rad, Anahiem, USA). The PCR products were then excised from the gel and purified using Wizard® PCR Preps (Promega, Madison, USA). After purification 150 ng of PCR template was added to a mixture containing 3.2 pmol of the specific forward primer and a premix stock of Terminator Ready Reaction Mix (containing A-Dye Terminator, C-Dye Terminator, G-Dye terminator, T-Dye terminator, dITP, dATP, dCTP, dTTP, Tris-HCL (pH 9.0), MgCl₂, thermal stable phosphatase and AmpliTaq DNA polymerase, FS) to a final volume of 15 μl (Perkin Elmer Applied Biosystems, Foster City, USA). The sequencing reaction was then carried out in a GeneAmp PCR System 9700 Thermal Cycler (Perkin Elmer Applied Biosystems, Foster City, USA) with the following reaction conditions: 25 cycles of 96°C for 10 secs, 50°C for 5 secs and 60°C for 4 mins, then held at 4°C. To remove excess dye terminators and purify the extension products the DNA was precipitated with ethanol and 3M sodium acetate and centrifuged for 30 mins at 4°C to pellet the precipitated DNA. The pellet was washed twice with 75% ethanol and air dried. The DNA sequence was determined using an ABI PRISM™ Applied Biosystems 373 automated DNA sequencer (Perkin Elmer Applied Biosystems, Foster City, USA).

2.3.5 SEMI QUANTITATIVE RT-PCR
To examine the relative quantitation of the level of expression of specific genes the Taqman ‘Real Time’ system was utilised (Perkin Elmer Applied Biosystems, Foster City, USA). This system utilised the 5′nuclease activity of Taq polymerase first described by Holland et al. (1991) to cleave a non-extendable oligonucleotide hybridisation probe during the extension phase of the PCR reaction. The probe is labelled with a reporter fluorescent dye [FAM (6-carboxy-fluorescein) for OB, OB-RA, OB-RB and c-fos; VIC (confidential; Perkin Elmer Applied Biosystems, Foster City, USA) for ribosomal RNA] at the 5′ end and a quencher fluorescent dye [TAMRA (6-carboxy-tetramethyl-rhodamine)] at the 3′ end. When the probe is intact, the reporter emission is quenched owing to the physical proximity of the reporter and quencher fluorescent dyes. During the extension phase of the PCR cycle however, the 5′-3′ nucleyolytic activity of the DNA polymerase cleaves the hybridisation probe and releases the reporter dye from the probe. The fluorescence of the reporter dye is no longer absorbed by the quencher dye and the relative increase in reporter fluorescent dye emission is monitored in real time during PCR amplification using a sequence detector (ABI Prism 7700 Sequence Detector, Perkin Elmer Applied Biosystems, Foster City, USA).
Figure 2.1  The Basis of 'Real Time' PCR

The 'Real Time' PCR reaction is based on the cleavage of a fluorescent labelled target probe during PCR by the 5' nuclease activity of Taq DNA polymerase.

Polymerisation

\[ \text{Forward Primer} \quad 5' \rightarrow 3' \]

\[ \text{Reverse Primer} \quad 3' \rightarrow 5' \]

Two fluorescent dyes, a Reporter (R) and a Quencher (Q) are attached to the probe.

Strand Displacement

\[ \begin{align*}
5' & \quad R \quad Q \\
3' & \quad 5' \\
5' & \quad 3' \quad 5'
\end{align*} \]

When both are attached to the probe, reporter dye emissions is quenched.

Cleavage

\[ \begin{align*}
5' & \quad R \quad Q \\
3' & \quad 5' \\
5' & \quad 3' \quad 5'
\end{align*} \]

During each extension cycle, the Taq DNA polymerase cleaves the reporter dye from the probe.

Polymerisation Complete

\[ \begin{align*}
5' & \quad R \quad Q \\
3' & \quad 5' \\
5' & \quad 3' \quad 5'
\end{align*} \]

Once separated from the quencher, the reporter dye emits its characteristic fluorescence.
The sequence detector is a combination thermal cycler, laser, and detection and software system that automates the 5’ nuclease-based detection and quantitation of nucleic acid sequences. The instrument uses a charge coupled device (CCD camera) to measure the fluorescence emission spectra from 500 to 650 nm and each PCR tube was monitored sequentially for 25 msec with continuous monitoring throughout the amplification. A computer algorithm compares the amount of reporter dye emission (R) with the quenching dye emission (Q) every 8.5 seconds during the PCR amplification, generating a ΔRn (or ΔRQ) value (R/Q). The ΔRn value reflects the amount of hybridisation probe that has been degraded as the emission intensity of the quencher dye remains relatively constant during amplification (Livak et al., 1995). The algorithm fits an exponential function to the mean ΔRn values of the last three data points of every PCR extension cycle, generating an amplification plot. A relative fluorescent emission threshold is set, based on the baseline ΔRn during the first 10-15 cycles. The algorithm calculates the cycle at which each PCR amplification reaches a significant (ie usually 10 times the standard deviation of the baseline) threshold (C_T). The calculated C_T is proportional to the number of target copies present in the sample (Heid et al., 1996) and is the point (cycle number) at which the amplification plot crosses the threshold. Thus the C_T value is a quantitative measurement of the quantity of input target found in any sample and the C_T value has been shown to decrease linearly with increasing target quantity (ie, if there is more input target found in the sample it will cross the threshold at an earlier cycle, thus a higher C_T).
Please Set the Threshold Value on All Reporter Layers.

Click OK to Continue.
Prior to analysis all sets of primers and fluorescent probes were designed from *Psammomys obesus* sequences for OB, OB-RA, OB-RB and *c-fos* using Primer Express software (Perkin Elmer Applied Biosystems, Foster City, USA). This software is specifically designed to generate primers and probes to be used under the standard conditions of the TaqMan 'Real Time' PCR system. The primers and probes used for analysis were as follows:

**OB gene:**
forward primer 5' - tgcgggtcatccacactc-3’  
reverse primer 5' - tcctattggatgcttcga-3’  
probe 5' - FAM tagcagcctgccctccccgaat TAMRA-3’

**OB-RA:**
forward primer 5' - gctgttcgcctggaactc-3’  
reverse primer 5' - cttttttgaataacttttg-3’  
probe 5' - FAM atggcataaccccacaaggtctcgg TAMRA-3’

**OB-RB:**
forward primer 5' - aagcctgaaacatttgacatct-3’  
reverse primer 5' - gccatgcactccattctct-3’  
probe 5' - FAM atcagttgatatttgggcccttcctcggagcc TAMRA-3’

**c-fos:**
forward primer 5' - tgaagagagaagaaaaactgtagttta-3’  
reverse primer 5' - cccggtgagccacagaca-3’  
probe 5' - FAM tggcagcccccagcctgc TAMRA-3’

In addition, the TaqMan Ribosomal RNA kit was (Perkin Elmer Applied Biosystems, Foster City, USA) used for quantitation of the ‘housekeeping’ gene ribosomal RNA in all samples. The sequence of the primers and probe used to amplify this gene were as follows:  
forward: 5' - gctggaattaccgcgct-3’  
reverse: 5' - cggctacccacatccaggaa-3’  
probe: 5' - VIC tgctggcaccagacttggctc TAMRA-3’

Each set of primers was initially optimised to provide the best amplification conditions according to a specific optimisation protocol. Briefly, different concentrations of primer were tested in various combinations within the PCR reaction with subsequent examination
of the individual amplification plots used to determine the primer concentrations necessary for generation of an optimal ΔRn for each gene. A similar protocol was used to determine the optimal concentration of the probe and cDNA to be used in the PCR reaction with the following final primer and probe concentrations determined to be optimal for each gene.

<table>
<thead>
<tr>
<th>Table 2.1</th>
<th>Optimal concentrations of each primer used for RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OB gene</td>
</tr>
<tr>
<td>forward</td>
<td>300 nM</td>
</tr>
<tr>
<td>reverse</td>
<td>900 nM</td>
</tr>
<tr>
<td>fluorescent</td>
<td>100 nM</td>
</tr>
</tbody>
</table>

PCR was performed by adding 300 ng of cDNA to a tube containing: 1xTaqMan Universal PCR Master Mix (AmpIiTaq Gold™, AmpErase® Uracil N-glycosylase (UNG), dNTPs, 5x TaqMan Buffer A (with ROX passive reference) 12.5 mM MgCl₂ and glycerol; Perkin Elmer Applied Biosystems, Foster City, USA), the appropriate concentration of primers and probe (above) in a final volume of 25 µl in a MicroAmp® Optical 96-well Reaction Plate covered with MicroAmp® Optical caps (Perkin Elmer Applied Biosystems, Foster City, USA). Amplification was performed using the following conditions: 50°C for 2 mins, 95°C for 10 mins and 40 cycles of 95°C for 15 sec and 60°C for 1 min.

After amplification, the data generated during the run was collated and the threshold set at between the first 10-15 cycles. A computer generated \( C_T \) value was then assigned to each tube of the 96-well plate which was exported from the Sequence Detection System into an Excel spreadsheet. The comparative \( C_T \) method was used for relative quantitation of the gene of interest in each sample. This method uses arithmetic formulas for relative quantitation with the amount of target normalised to an endogenous reference (rRNA in this case) given by: \( 2^{ΔC_T} \), where \( C_T \) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold and \( ΔC_T \) is the difference in threshold cycles for target and reference. An example is given below:
Table 2.2 Example of calculations involved in quantitation using Real Time PCR system

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>OB-RB mean C&lt;sub&gt;r&lt;/sub&gt;*</th>
<th>rRNA mean C&lt;sub&gt;r&lt;/sub&gt;</th>
<th>ΔC&lt;sub&gt;r&lt;/sub&gt;** (OB-RA-rRNA)</th>
<th>OB-RA&lt;sub&gt;N&lt;/sub&gt; (2&lt;sup&gt;ΔCTx1000&lt;/sup&gt;)^</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29.06</td>
<td>18.29</td>
<td>10.77</td>
<td>0.57</td>
</tr>
<tr>
<td>2</td>
<td>28.71</td>
<td>18.30</td>
<td>10.41</td>
<td>0.73</td>
</tr>
<tr>
<td>3</td>
<td>29.7</td>
<td>19.63</td>
<td>10.07</td>
<td>0.93</td>
</tr>
<tr>
<td>4</td>
<td>27.84</td>
<td>19.79</td>
<td>8.06</td>
<td>3.76</td>
</tr>
<tr>
<td>5</td>
<td>27.10</td>
<td>18.55</td>
<td>8.55</td>
<td>2.67</td>
</tr>
</tbody>
</table>

*All samples were analysed in duplicate and the mean C<sub>r</sub> for each sample was used in calculations of gene expression.

**The ΔC<sub>r</sub> is generated by subtracting the rRNA C<sub>r</sub> from the C<sub>r</sub> of the gene of interest.

^As the numbers generated at this step were often small they were multiplied by a constant (1000) for ease of interpretation.

2.3.6 OB and OB-R POLYMORPHISMS (Gln223Arg and PRO1019pro)

2.3.6.1 Subjects

The Australian population consisted of 373 women aged between 20-91 years that were drawn from a larger, on-going, population-based study initiated in 1994, the Geelong Osteoporosis Study and access to this population was through a collaboration with Professor Geoff Nicholson at Geelong Hospital. All subjects were randomly selected from within the Barwon Region of Victoria, Australia using an Australian Electoral Commission Roll and were healthy. Of the 373 women, 188 were post-menopausal while 185 were premenopausal. Fourteen of the women had a fasting plasma glucose greater than 7.8 mmol/l and were classified as having type 2 diabetes mellitus. Fifty-four women were currently taking the contraceptive pill.

A population of Nauruan men was also studied in these experiments through a collaboration with the International Diabetes Institute and Professor Paul Zimmet. Nauru is a small central Pacific island located just south of the equator, with an indigenous population consisting of approximately 6000 individuals of Micronesian ancestry. A series of cross-sectional and longitudinal studies have been performed in Nauru since 1975 with
all Nauruan adults eligible to participate (Zimmet et al., 1977). Subjects for the current study (n=342) were males aged over 24 years of age covering a wide range of body weight and BMI. The Nauruan population has previously been shown to have very highly prevalence rates of obesity and type 2 diabetes and are thought to have a genetic background which significantly predisposes them to the development of these diseases (Zimmet et al., 1977; Zimmet et al., 1984; Dowse et al., 1991). Previously, large-scale epidemiological studies have reported age-standardised prevalence rates of type 2 diabetes (glucose \(>7.8 \text{ mmol/l}) \) of 28% in 1975-6, 25% in 1982 and 24% in 1987 (Zimmet et al., 1977; Zimmet et al., 1984; Dowse et al., 1991). Similarly, prevalence rates of obesity in Nauru have also been reported. The mean BMI of the population was 33.3 in 1975-6, 33.8 in 1982 and 34.5 in 1987; and in 1996 Dowse et al. (1996) reported the prevalence rate of obesity to be 64% in males and 69% in females. The Nauruan population is considered to be representative of the 'thrifty genotype' hypothesis (Neel et al., 1962; Dowse et al., 1991).

2.3.6.2 Genomic DNA Extraction

All subjects were required to fast overnight (from 10 pm the previous evening) and blood specimens were collected in heparinised tubes the next morning. 1 ml of whole blood was used for DNA extraction and the remainder was centrifuged and the plasma stored at -70°C for later biochemical analyses. DNA was extracted from all blood samples using Wizard™ Genomic DNA Purification Kits (Promega, Madison, USA). Briefly, the kit is based on a four-step process:

1. Erythrocyte lysis, leaving the leukocytes intact
2. Lysis of white blood cells and their nuclei
3. Precipitation of proteins using the supplied salt solution
4. Concentration and desalting of genomic DNA by isopropanol precipitation

The DNA was then re-suspended in the supplied DNA Rehydration Solution and quantitated by absorption spectrophotometry at 260 nm. Yields of 5-15 μg of DNA per 300 ml of whole blood were consistently obtained and the DNA was diluted to a final concentration of 100 ng/μl.
2.3.6.3a OB Gene Polymerase Chain Reaction (PCR)

The OB gene polymorphism is a highly polymorphic tetranucleotide repeat polymorphism close to the 3'-end of the last exon of the OB gene. This polymorphism consists of two classes of products after PCR analysis: a shorter (class I) form with fewer tetranucleotide repeats, and a longer (class II) form. Oligonucleotide primers for PCR amplification of this region of the OB gene were those published by Shintani et al. (1996) with the following sequences:

- forward: 5'-agttcaaataggtcctaatca-3'
- reverse: 5'-ttctgagttgtctactgcac-3'

PCR was performed by adding 100 ng of DNA to a reaction mixture containing: 10mM Tris-HCl (pH 8.3 AT 20°C), 50 mM KCl, 2 mM MgCl₂, 200 μM each dNTP, 100 pmol each primer and 1.25U Taq DNA polymerase (Life Technologies, Gaithersburg, USA). After an initial denaturation step of 94°C for 3 min, the reaction conditions consisted of 35 cycles of 94°C for 30 sec (denaturation), 60°C for 1 min (annealing) and 72°C for 1 min, followed by a final extension step of 72°C for 10 mins. PCR was performed in a Hybaid OmniGene Thermal Cycler (Teddington, England) in a total volume of 20 μl in 0.5 ml microcentrifuge tubes (Perkin Elmer, Norwalk, USA).

Electrophoresis And Analysis

The PCR products were separated by agarose gel electrophoresis in a 3% agarose gel (2:2 agarose:Nuseive) containing 0.5 μg/ml ethidium bromide at 6V/cm for 120 mins in a DNA SubCell system (Bio-Rad, Anaheim, USA). The gels were then photographed using a Polaroid DS34 camera with a DSH-7 Hood on Polaroid 665 positive/negative film (Sigma Chemical Company, St Louis, USA) under ultraviolet transillumination (model 4000, Stratagene, LaJolla, USA) at 302 nm. The genotypes were determined visually based on DNA fragment sizes and determined to be either 121-145 base pairs in length (short form), or 197-221 base pairs in length (long form), with subjects characterised as either: homozygous for the short form (class I/class I), heterozygous (class I/class II), or homozygous for the long form (class II/class II).
2.3.6.3b  **OB-R PCR (Gln223Arg and PRO1019pro)**

The oligonucleotide primers used for amplification of the appropriate region of the OB-R gene were those published by Gotoda *et al.* (1997), with the sequences shown below.

Gln223Arg:

forward: 5'-aaactcaagacactctcctt-3'
reverse: 5'-tgaactgacattagaggtgac-3'

PRO1019pro

forward: 5'-cagatctgtgaaaaaggtct-3'
reverse: 5'-tcccatgagctattagaggaatctctca-3'

PCRs were performed by adding 250 ng of DNA to a reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.25 mM MgCl₂, 200 mM each dNTP, 5 pmol each specific forward and reverse oligonucleotide primer and 1.25 U Taq DNA Polymerase (Life Technologies, Gaithersburg, USA). The PCR consisted of 94°C for 5 mins, then 35 cycles of denaturation at 94°C for 30 sec, annealing at either 52°C (PRO1019pro) or 54°C (Gln223Arg) for 30 sec and extension at 72°C for 30 sec, followed by a final extension step of 72°C for 5 mins. PCR was performed in a GeneAmp PCR System 9700 Thermal Cycler (Perkin Elmer Applied Biosystems, Norwalk, USA) in a total volume of 15 ul in 0.2 ml microcentrifuge tubes (Perkin Elmer, Norwalk, USA).

*Restriction Enzyme Digestion*

For the Gln223Arg polymorphism the total PCR product was digested by the addition to each sample of 10 μl of a mixture containing: 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol (DTT) (pH 7.9) and 10 units of *Hae III* (New England Biolabs, Beverly, USA) followed by incubation for 2 hours at 37°C to enable complete digestion. For the PRO1019pro polymorphism the total PCR product was digested by the addition to each sample of 10 μl of a mixture containing: 50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, 1 mM DTT (pH 7.9) and 10 units of *Nco I* (New England Biolabs, Beverly, USA) followed by incubation for 2 hours at 37°C to enable complete digestion. *Hae III* is a restriction enzyme that specifically cleaves DNA at the sequence 5'...GG'CC...3' and *Nco I* is a restriction enzyme that specifically cleaves at the DNA sequence 5'...C'CATGG...3'.
Electrophoresis And Analysis

The restriction digested PCR products were separated by agarose gel electrophoresis in a 3% agarose gel (2:2 agarose:Nuseive) containing 0.5 μg/ml ethidium bromide at 6V/cm for 120 mins in a DNA SubCell system (Bio-Rad, Anaheim, USA). The gels were then photographed using a Polaroid DS34 camera with a DSH-7 Hood on Polaroid 665 positive/negative film (Sigma Chemical Company, St Louis, USA) under ultraviolet transillumination (model 4000, Stratagene, LaJolla, USA) at 302 nm. The genotypes were determined visually based on DNA fragment sizes (table 2.3).

Table 2.3 Fragment sizes generated after restriction enzyme digestion of the PCR products for each of the OB-R polymorphisms.

<table>
<thead>
<tr>
<th>Gln223Arg polymorphism:</th>
<th>Fragment sizes (base pairs):</th>
<th>PRO1019pro polymorphism:</th>
<th>Fragment sizes (base pairs):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gln223/Gln223</td>
<td>91bp</td>
<td>PRO1019/PRO1019</td>
<td>220bp</td>
</tr>
<tr>
<td>Gln223/Arg223</td>
<td>91bp, 60bp, 31bp</td>
<td>PRO1019/pro1019</td>
<td>220bp, 200bp, 20bp</td>
</tr>
<tr>
<td>Arg223/Arg223</td>
<td>60bp, 31bp</td>
<td>pro1019/pro1019</td>
<td>200bp, 20bp</td>
</tr>
</tbody>
</table>

2.4 STATISTICAL ANALYSES

All statistical analyses were performed using a commercially available statistical software package (SPSS for Windows, version 8.0.2; USA). Dependent variables were tested for normality using the one-sample Kolmogorov-Smirnov Test. If variables were non-normally distributed (p-value<0.05 for the Kolmogorov-Smirnov test) either non-parametric tests were used for comparisons or the variable was transformed using the natural logarithm, as detailed in each chapter. Where non-parametric tests were used, comparison of differences in means between >2 independent groups was performed using the Kruskal-Wallis test (Kruskal and Wallis, 1992) with an appropriate Bonferroni correction applied for the use of multiple comparisons. When the means of 2 independent groups were compared the Mann-Whitney U test (Mann and Whitney, 1947) was used to test for significant differences. When dependent variables were found to be normally distributed (p-value>0.05 for the Kolmogorov-Smirnov test) an independent samples t-test was used to test for significant differences between the means of two independent groups.
or an analysis of variance (ANOVA) was used to test for significant differences between the means of more than two groups (Armitage and Berry, 1987). ANOVA was also used to examine differences in means between groups while adjusting for covariates (e.g. fasting insulin) as necessary. Linear associations between variables were determined using either the Pearson (for normally distributed variables) or the Spearman (for non-normally distributed variables) bivariate correlation coefficient. When examining linear associations between variables while adjusting for a covariate (e.g. bodyweight) the partial correlation coefficient was used. Linear regression analysis was used to determine the best predictors of plasma leptin.
CHAPTER 3

CIRCULATING LEPTIN CONCENTRATIONS, OBESITY AND LIFESTYLE FACTORS IN AUSTRALIAN WOMEN

3.1 SUMMARY

To assess the relationship between circulating leptin concentrations, metabolic parameters and lifestyle factors such as alcohol intake, physical activity level, smoking habits and reproductive history, a cohort of 359 women was drawn from a population-based study conducted in Victoria, Australia. The parameters measured included body weight, percent body fat, body mass index (BMI), waist and hip circumference, blood pressure, and fasting glucose, insulin, triglyceride, cholesterol and leptin concentrations. In addition, a self-administered questionnaire was used to assess reproductive history, physical activity level, alcohol intake and smoking habits. Our results demonstrated that percent body fat, BMI, body weight, waist circumference and hip circumference were all strongly correlated with circulating leptin concentrations in this population (r>0.50, p<0.001 in all cases). Waist/hip ratio, triglycerides, insulin, glucose and cholesterol were also associated with leptin, however, there was no association between leptin and age or blood pressure. After adjustment for percent body fat, only fasting plasma insulin and triglyceride concentrations remained significantly associated with leptin concentrations, although there was evidence of an independent relationship between leptin concentrations and waist circumference in this population. We also examined leptin concentrations across a number of behavioural measures which have been associated with energy balance, however there were no relationships between leptin and the level of physical activity, smoking habits or alcohol intake, independent of percent body fat and insulin in this population. In addition, several measures of reproductive history in these women were not associated with circulating leptin concentrations. Overall, these result suggest that in Australian Caucasian women, fasting plasma leptin concentrations are determined predominantly by the level of adiposity.
3.2 INTRODUCTION

Leptin, the OB gene product is a hormone that is secreted predominately from adipose tissue, and studies conducted in ob/ob mice (who lack circulating leptin) suggest that leptin acts as an afferent signal controlling energy balance and ultimately fat mass (Campfield et al., 1995; Halaas et al., 1995; Zhang et al., 1995). Studies in both humans and other rodent models of obesity have consistently demonstrated a strong positive correlation between leptin and body fat mass (Considine et al., 1995; Maffei et al., 1995b). In addition to body fat, leptin concentrations have been associated with circulating insulin concentration, a relationship which has been found to be independent of adiposity in several human populations (Considine et al., 1996c; Havel et al., 1996b; Larsson et al., 1996; Zimmet et al., 1996; Bertin et al., 1998; Zimmet et al., 1998). Circulating leptin has also been correlated with other measures of insulin sensitivity, as well as glucose concentrations and glucose metabolism in humans (Haffner et al., 1997; Mueller et al., 1998; Saad et al., 1998). Another common finding in human studies has been the higher leptin concentrations in females compared to males, even after adjustment for percent body fat (Rosenbaum et al., 1996; Bennett et al., 1997; Havel et al., 1997). This gender difference has been linked to different amounts of androgens (Wabitsch et al., 1997), insulin (Couillard et al., 1997) and OB gene expression (Lonnqvist et al., 1995) between males and females, although the underlying cause of this difference remains unclear.

In addition to the potential role of leptin in the regulation of body fat mass, there have been several reports suggesting that leptin may play a role in the control of reproduction. Prevention of the starvation-induced fall in circulating leptin in fasted ob/ob mice substantially blunted the starvation-induced disruption in fertility seen in these animals and also reduced the neuroendocrine response to fasting, with leptin appearing to alter the concentrations of gonadal, thyroid and adrenal hormones (Ahima et al., 1996). Leptin administration to female ob/ob mice also corrected the infertility associated with the OB genetic mutation, with animals experiencing weight loss and restoration of fertility with leptin treatment (Chehab et al., 1996).

Although the importance of leptin in energy balance and the regulation of reproduction has been examined, the relationship between leptin and other factors known to be important in the development of obesity have not been studied extensively. Environmental factors such as diet (Danforth, 1985), physical activity (Despres et al., 1995) and smoking habits (Wolk and
Rossner, 1995) all appear to impact on the level of obesity within a population, and leptin concentrations have been associated with all of these variables in humans (Hodge et al., 1997; Wei et al., 1997; Mantzoros et al., 1998). The aim of this study was to investigate the relationship between plasma leptin concentrations and factors such as lifestyle (alcohol intake, smoking habits and physical activity levels), reproductive history and metabolic parameters in a population of Caucasian Australian women independent of adiposity.
3.3 METHODS AND MATERIALS

3.3.1 SUBJECTS
373 women aged 20-91 years were initially drawn from a larger, ongoing population-based study begun in 1994, the Geelong Osteoporosis Study. All subjects were randomly selected using an Australian Electoral Commission roll and 14 subjects were excluded from further analyses as they were found to have a fasting blood glucose concentration greater than 7.8mmol/L, indicative of type 2 diabetes. Of the 359 healthy, non-diabetic women included in the study, 187 were post-menopausal and 172 were pre-menopausal, while 50 women were taking the contraceptive pill.

3.3.2 DATA COLLECTION
All subjects were required to fast overnight (from 10pm the previous evening) and blood specimens were collected the next morning, centrifuged and the plasma collected and stored at -80°C. Height, weight, waist circumference and hip circumference were measured and used to calculate body mass index (BMI, body weight (kg)/height (m²)) and the waist-to-hip ratio (WHR). All subjects underwent a dual-energy x-ray absorptiometry (DEXA) scan which was used to determine body fat mass and calculate percent body fat. Systolic and diastolic blood pressure were measured with an automated digital analyser with the subjects sitting. Data on physical activity, smoking habits, alcohol intake and reproductive history were collected using a self-administered questionnaire.

3.3.3 ANALYSES OF SAMPLES
All measurements were made in plasma stored at -80°C for less than 3 years. Leptin and insulin were measured using radioimmunoassays (RIA; Linco, St. Charles, MO, and Phadeseph, Kabi Pharmacia Diagnostics, Sweden, respectively). Glucose, cholesterol and triglycerides were measured using colorimetric methods (Boehringer Mannheim, Mannheim Germany) on an automatic analyser (Hitachi, 705-0013, Tokyo, Japan).

3.3.4 STATISTICAL ANALYSES
Logarithmic transformations were used to normalise distributions of leptin, triglycerides, glucose and insulin, and geometric means are presented in the text. One-way analysis of variance (ANOVA) was used to determine significant differences in leptin concentrations between the groups described below and a simple factorial model was used to adjust for covariates (percent body fat and insulin concentration), with a Bonferroni correction for
multiple comparisons. Differences between groups were assessed by the F-statistic, with p-values <0.05 considered significant. Multiple regression analysis was also conducted. All statistical calculations were performed using SPSS 8.0.

3.3.4.1 Physical activity
Women were grouped as either having a low, medium or high physical activity level based on their physical activity 'score'. This score was derived by summing the value given for the level of activity at work/home and that given for sport/recreation. "Low", "medium" and "high" physical activity levels were thus defined as physical activity scores of 1-2 (81 subjects), 3 (205) and 4-6 (87), respectively.

3.3.4.2 Alcohol
Subjects were grouped according to their usual alcohol intake and three groups were defined: 1-never drink alcohol (83 subjects), 2-drink less than once a week (158), and 3-drink more than once a week (118).

3.3.4.3 Smoking
Subjects were classified as either never-smokers (204 subjects), ex-smokers (106) or smokers (49). Subjects were then grouped according to how many years they had been regular smokers: up to 10 years (5), 10-25 years (25), and over 25 years (19); and also how many cigarettes they smoked each day: up to 10 per day (7 subjects), 10-20 per day (20) and over 20 per day (22).

3.3.4.4 Reproductive history
Subjects were divided into groups based on the number of pregnancies conceived: none (55 subjects), 1-2 (101), 3-4 (136), and more than 4 (67); the number of actual births: none (64 subjects), 1-2 (115), 3-4 (147) and more than 4 (33); the number of miscarriages reported: none (274 subjects), one (68), and more than one (17); and whether they had (11 subjects) or had not (348) ever experienced a stillbirth.
3.4 RESULTS
3.4.1 METABOLIC AND ANTHROPOMETRIC VARIABLES

Table 3.1 Anthropometric and metabolic variables in a population of Australian Caucasian women, and partial and bivariate associations between these variables and circulating leptin concentration.

<table>
<thead>
<tr>
<th>Variable:</th>
<th>Mean ± sem</th>
<th>r</th>
<th>p-value</th>
<th>p-adjusted for % body fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>49.1 ± 0.78</td>
<td>0.10</td>
<td>0.06</td>
<td>0.67</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>73.46 ± 0.85</td>
<td>0.59</td>
<td>&lt;0.0001</td>
<td>0.17</td>
</tr>
<tr>
<td>Percent body fat (%)</td>
<td>38.48 ± 0.38</td>
<td>0.73</td>
<td>&lt;0.0001</td>
<td>—</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>28.2 ± 0.31</td>
<td>0.61</td>
<td>&lt;0.0001</td>
<td>0.10</td>
</tr>
<tr>
<td>Waist-hip ratio</td>
<td>0.82 ± 0.004</td>
<td>0.23</td>
<td>&lt;0.0001</td>
<td>0.16</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>88.6 ± 0.72</td>
<td>0.58</td>
<td>&lt;0.0001</td>
<td>0.06</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>108.5 ± 0.68</td>
<td>0.60</td>
<td>&lt;0.0001</td>
<td>0.19</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.99 ± 0.05</td>
<td>0.11</td>
<td>0.04</td>
<td>0.64</td>
</tr>
<tr>
<td>Triglycerides* (mmol/L)</td>
<td>1.26 ± 1.03</td>
<td>0.24</td>
<td>&lt;0.0001</td>
<td>0.02</td>
</tr>
<tr>
<td>Insulin* (μU/ml)</td>
<td>8.51 ± 0.12</td>
<td>0.33</td>
<td>&lt;0.0001</td>
<td>0.05</td>
</tr>
<tr>
<td>Glucose* (mmol/L)</td>
<td>5.06 ± 0.03</td>
<td>0.23</td>
<td>&lt;0.0001</td>
<td>0.10</td>
</tr>
<tr>
<td>Systolic BP</td>
<td>122.82 ± 1.28</td>
<td>0.02</td>
<td>0.69</td>
<td>0.28</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>75.45 ± 0.70</td>
<td>0.02</td>
<td>0.71</td>
<td>0.94</td>
</tr>
<tr>
<td>Leptin* (ng/ml)</td>
<td>17.49 ± 1.03</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* geometric means; p-values bases on a two-tailed test using a Pearson correlation.
Table 3.1 describes the characteristics of the 359 women and the associations between circulating leptin concentrations and the anthropometric and metabolic variables measured. This table also shows positive associations between fasting plasma leptin concentrations and body weight ($r=0.58$, $p<0.0001$), percent body fat ($r=0.73$, $p<0.0001$), body mass index ($r=0.61$, $p<0.0001$), waist circumference ($r=0.58$, $p<0.0001$), hip circumference ($r=0.60$, $p<0.0001$), waist/hip ratio ($r=0.23$, $p<0.0001$), fasting plasma triglycerides ($r=0.24$, $p<0.0001$), fasting plasma insulin ($r=0.33$, $p<0.0001$), fasting plasma glucose ($r=0.23$, $p<0.0001$) and fasting plasma cholesterol ($r=11$, $p=0.04$). There was no relationship between leptin concentrations and either systolic ($r=0.02$, $p=0.69$) or diastolic ($r=0.02$, $p=0.71$) blood pressure, while there was evidence of a relationship between leptin concentrations and age ($r=0.10$, $p=0.06$). The relationship between fasting plasma leptin concentrations and percent body fat is shown in figure 3.1. The association was best described by a non-linear equation ($y=1.004x10^{0.031}$) and was highly significant ($r=0.73$, $p<0.0001$). In an effort to examine these relationships independent of the strong effect of adiposity, we performed partial correlations, with the correlations adjusted for percent body fat. With this adjustment, only the relationships between leptin and insulin, and leptin and triglycerides remained significant ($p=0.05$ and $p=0.02$, respectively), indicating that the associations between plasma leptin and the other metabolic variables were largely dependent on the correlation between leptin and percent body fat.
Figure 3.1. Association between fasting plasma leptin concentration and percent body fat ($y=1.004x10^{0.031x}$, $r=0.73$).
Table 3.2  Multiple regression analysis in Caucasian Australian women with leptin concentration as the dependent variable

<table>
<thead>
<tr>
<th>Variable</th>
<th>B</th>
<th>s.e. (B)</th>
<th>t</th>
<th>p-value</th>
<th>r (r² %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.73 (52.7%)</td>
</tr>
<tr>
<td>Constant</td>
<td>0.001</td>
<td>0.067</td>
<td>0.022</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>percent body fat</td>
<td>0.031</td>
<td>0.002</td>
<td>19.11</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Model 2:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.73 (53.3%)</td>
</tr>
<tr>
<td>Constant</td>
<td>-0.05</td>
<td>0.07</td>
<td>-0.67</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Percent body fat</td>
<td>0.03</td>
<td>0.002</td>
<td>17.16</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>0.11</td>
<td>0.054</td>
<td>1.98</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Model 3:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.73 (53.5%)</td>
</tr>
<tr>
<td>Constant</td>
<td>0.027</td>
<td>0.067</td>
<td>0.40</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>Percent body fat</td>
<td>0.03</td>
<td>0.002</td>
<td>18.099</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>132</td>
<td>0.056</td>
<td>0.09</td>
<td>0.018</td>
<td></td>
</tr>
</tbody>
</table>

Leptin was entered as a dependent variable and the following variables were included in the various regression models: percent body fat, age, hip circumference, waist circumference, waist/hip ratio, glucose, insulin, cholesterol and triglycerides.

Multiple regression analysis was used to determine the best predictors of plasma leptin concentrations in this population of Caucasian women (table 3.2). Using step wise regression, the best predictor of leptin concentrations was clearly percent body fat ($r^2=52.7\%$, $p=0.0001$), with fasting plasma insulin and triglycerides also adding significantly to the regression analysis. All other variables were excluded from these models, as they did not significantly influence leptin concentrations independently of percent body fat and insulin or triglycerides. They included age, hip circumference, waist circumference, glucose and cholesterol. The influence of triglycerides and insulin on leptin concentrations after adjustment for percent body fat were similar in both models, predicting approximately 53% of the variability in leptin concentrations within this population. For subsequent analyses, leptin concentrations were adjusted for percent body fat and insulin concentrations.
3.4.2 LIFESTYLE FACTORS; PHYSICAL ACTIVITY LEVEL, SMOKING LEVELS AND ALCOHOL INTAKE

Figure 3.2 shows that mean leptin concentrations were not different between women with different activity levels, adjusted for percent body fat and plasma insulin concentration (low: 17.38±1.74, medium: 18.11±1.45, high: 18.28±1.70; figure 3.2a). Leptin concentrations were also not different between women who were smokers (18.66±1.08) compared to women who had never smoked (17.82±1.04) or those who were ex-smokers (18.54±1.05), when adjusted for percent body fat and insulin concentrations (figure 3.2b). Of the women who smoked, those who smoked up to 10 cigarettes per day tended to have lower leptin concentrations (16.52±1.20) compared to women who smoked between 10 and 20 cigarettes per day (20.46±1.12) and those who smoked more than 20 cigarettes each day (17.78±1.11), although this difference was not significant (p=0.55). There was also a tendency for women who smoked for either 10-25 years (18.62±1.10) or over 25 years (20.09±1.12) to have higher leptin concentrations than women who smoked for a shorter time, less than 10 years (13.77±1.25), although these differences did not reach statistical significance (p=0.32).

Figure 3.2c shows that alcohol intake did not alter fasting leptin concentrations independently of percent body fat and insulin in this group of Australian women. Women who drank alcohol several times per week had a similar mean leptin concentration (17.82±1.06) as women who either never consumed alcohol (17.38±1.06) or those who drank only once per week (18.49±1.04).
Figure 3.2. Estimated marginal mean plasma leptin concentrations across different a) activity, b) smoking and c) alcohol levels in Australian women, adjusted for percent body fat and insulin concentration.
3.4.3 REPRODUCTIVE VARIABLES

In this study we found that women currently using the oral contraceptive pill did not have different leptin concentrations from those not currently using the pill, although there was a tendency for those on the pill to have higher leptin concentrations (19.50±1.07 vs 17.78±1.03, on pill vs not on pill; figure 3.3a). In addition, pre-menopausal women tended to have lower leptin concentrations than post-menopausal women, although this difference did not reach significance (17.34±1.04 vs 18.62±1.04, premenopausal vs postmenopausal; p=0.16; figure 3.3b).
Figure 3. Estimated marginal mean plasma leptin concentrations in a) subjects who are and are not taking the contraceptive pill, and b) pre- and post-menopausal subjects, adjusted for percent body fat and insulin concentration.
Figure 3.4. Estimated marginal mean plasma leptin concentrations across different reproductive parameters, a) number of births, b) number of miscarriages and c) number of still births, adjusted for percent body fat and insulin concentration.
Women who had never been pregnant tended to have lower leptin concentrations (16.90±1.07) than women who had conceived 1-2 times (19.10±1.05), 3-4 times (17.22±1.04), or >4 times (19.28±1.06), although this difference was not significant (p=0.2). Leptin concentrations also appeared to be similar between women who had never given birth (17.86±1.09), and women who had experienced 1-2 (19.68±1.05), 3-4 (16.94±1.05) or more than 4 (17.82±1.12) births (figure 3.4a). Of the women who had been pregnant, the women who reported more than one miscarriage had significantly higher leptin concentrations (22.34±1.14) than women who reported never having a miscarriage (18.62±1.03; p=0.01) or only one miscarriage (15.14±1.06; P=0.02; figure 3.4b). Women who reported experiencing a stillbirth also tended to have higher leptin concentrations than women who had never experienced a stillbirth (19.23±1.16 vs 17.99±1.03; figure 3.4c). For these analyses leptin concentrations were adjusted for number of pregnancies in addition to percent body fat and insulin.
3.5 DISCUSSION

Within this group of non-diabetic Caucasian women, leptin was strongly correlated with several measures of obesity, including percent body fat, body weight, BMI and waist and hip circumferences, and a number of metabolic variables, including fasting glucose, insulin, cholesterol and triglyceride concentrations. The data is consistent with other studies (Considine et al., 1996c; Maffei et al., 1995b), and again confirms the relationship between leptin concentrations and adiposity in humans. When associations were adjusted for percent body fat, only fasting insulin and triglycerides remained significantly correlated with leptin, while there was evidence of an association between fasting leptin concentrations and waist circumference after adjustment for percent body fat. In addition to these phenotypic variables, we assessed differences in leptin concentrations across a number of different lifestyle-related variables (physical activity level, smoking habits and alcohol intake) and reproductive history. Overall, these variables were not associated with plasma leptin concentrations independently of insulin and percent body fat.

In this population of Australian women, fasting plasma leptin concentrations correlated strongly with percent body fat, determined by DEXA scan. This strong relationship is consistent with other studies in various human populations and supports the hypothesis that the strongest predictor of leptin concentrations in humans is adiposity. After adjustment for percent body fat, both fasting plasma insulin and triglyceride concentrations were still significantly associated with leptin concentrations. The independent association between leptin and insulin concentrations is again consistent with other human studies (Saad et al., 1998; Couillard et al., 1997; Zimmet et al., 1998), although this relationship has not been found to be independent of body fat across all populations (Hickey et al., 1996). Insulin does not regulate leptin acutely in vivo, however leptin concentrations have been found to be elevated in hyperinsulinemic insulin resistant states, and also increased as a result of chronic insulin administration (an 8.5-hr hyperinsulinemic clamp) in humans (Malmstrom et al., 1996). These results highlight the possibility that differences in the degree of insulin resistance may explain some of the residual variability observed in leptin concentrations in humans.

We examined several measures of body fat distribution in this study and found that leptin concentrations correlated with waist circumference, hip circumference and WHR in this population. After adjustment for percent body fat, these relationships were no longer
significant, although there was evidence of an independent relationship between waist circumference and leptin concentrations. Waist circumference is considered a useful measure of central obesity and has been shown to predict visceral adipose tissue mass in both normal weight and overweight subjects (Lemieux et al., 1996). This suggests that leptin may be associated with a central distribution of body fat separate to the overall degree of adiposity. Associations between regional body fat distribution and circulating leptin concentrations have been investigated in several human populations, with conflicting results reported (Haffner et al., 1996a; Magada et al., 1996; Takahashi et al., 1996a; Bennett et al., 1997; Perry et al., 1997; Solin et al., 1997). Overall it appears that leptin concentrations are associated more strongly with total body fat mass than individual fat depots.

Differences in adiposity and metabolic characteristics did not fully explain the variation observed in circulating leptin concentrations. We endeavoured therefore, to examine a range of lifestyle factors that may help explain some of the variation in leptin between individuals. The relationship between physical activity and leptin concentrations, independent of percent body fat and insulin, was examined using physical activity levels both at home/work and leisure/sport. The results presented are consistent with several studies, including that by Zimmet et al. in Western Samoans, in which indirect measures of physical activity were also used and shown not to be associated with leptin concentrations once BMI was controlled (Zimmet et al., 1996). Several reports have suggested that exercise can reduce serum leptin concentrations however, this appears to occur only at the extremes of severity or duration of exercise, as in the case of an ultramarathon (Landt et al., 1997), or endurance exercise training (Kohrt et al., 1996). Landt et al. (1997) demonstrated that leptin concentrations decreased to 68% of pre-race levels with prolonged exercise. Interestingly, Perusse et al. (1997) demonstrated that after a standardised 20-week endurance program, leptin concentrations decreased significantly in men but not in women, however the decrease in leptin in men was accounted for by the training-induced changes in body fat mass.

In the current study we also examined the impact of smoking on leptin concentrations and found that leptin concentrations were not significantly different between women who smoked and those who did not smoke (both ex-smokers and never-smokers), independent of percent body fat and insulin concentrations. There was a tendency however for women who smoked more cigarettes per day, and those who had smoked for a longer period of time, to have higher leptin concentrations than women who smoked fewer cigarettes per day or had smoked
for a shorter length of time, although this was not significant after adjustment for percent body fat and insulin concentrations. The effects of smoking on leptin concentrations have been investigated in several other populations. In an epidemiological study, Hodge et al. found leptin concentrations in 3 populations to be lower in male smokers than in male non-smokers, even after adjusting for BMI or waist circumference (Hodge et al., 1997). Due to the low prevalence of smoking in women within these 3 populations this relationship was not confirmed in women. Mantzoros et al. (1998) also reported a negative relationship between fed leptin concentrations and smoking in a population of young males which was independent of body fat. Cigarette smoking is associated with lower body weight in humans (Wolk and Rossner, 1995) and despite the association between smoking and leptin concentrations, it has been suggested that the effects of smoking on energy balance and body weight occur independent of leptin, most likely via increased sympathetic nervous system activity, which may itself be responsible for the fall in leptin concentrations (Mantzoros et al., 1998).

Leptin concentrations have also been positively associated with alcohol intake, independent of adiposity in young males (Mantzoros et al., 1998). We did not find this relationship in the present study. One difference may be the different alcohol intakes of the study populations, with the young male population studied by Mantzoros et al. appearing to have a higher alcohol intake than the Australian women studied here. Another difference may be that in the present study we measured leptin concentrations in the fasted state, whereas the association was seen between alcohol intake and leptin concentrations collected in the fed state (Mantzoros et al., 1998).

In addition to its role in energy balance, leptin has been proposed as an indicator of nutritional status that allows reproduction to proceed. This suggestion stems from the knowledge that leptin is produced in the adipose tissue, which responds to metabolic and nutritional changes, and the production of leptin increases with feeding and body fat content. Thus leptin provides an accurate, circulating signal of nutritional status, and thereby, reproductive 'readiness'. Leptin treatment has been found to accelerate reproduction in ob/ob mice (Chehab et al., 1997), while in humans, congenital leptin deficiency, resulting from mutations in either the OB gene or the leptin receptor gene (OB-R), has been associated with failure to undergo puberty and decreased levels of growth hormone and thyroid hormone (Clement et al., 1998). In this population of Australian women, the several parameters of reproductive history measured were not associated with differences in leptin concentrations.
independently of percent body fat and insulin. Interestingly, women who reported more than one miscarriage had significantly higher leptin concentrations than women who reported never experiencing a miscarriage, or having only one miscarriage. This association is in contrast to the reproductive and endocrine changes associated with leptin deficiency previously observed in a limited number of humans, but further emphasises the possible role of leptin in reproductive functioning in humans.

In this cross-sectional survey of Australian Caucasian women, we found that leptin correlated strongly with adiposity and a number of important metabolic variables. Despite the strong associations found, the variability in leptin concentrations seen across this population could not completely be explained by these phenotypic measures. We examined leptin concentrations across a number of behavioural measures which have been associated with energy balance, however there were no relationships between leptin and the level of physical activity, smoking habits or alcohol intake, independent of percent body fat and insulin in this population. In addition, several measures of reproductive history in these women were not associated with circulating leptin concentrations. These result suggest that in Australian Caucasian women, fasting plasma leptin concentrations are determined predominately by the level of body fat.
CHAPTER 4

ASSOCIATIONS BETWEEN LEPTIN RECEPTOR POLYMORPHISMS AND OBESITY IN AUSTRALIAN WOMEN: A PROSPECTIVE STUDY.

4.1 SUMMARY

Leptin, the protein product of the OB gene, has been shown to be an important regulator of energy balance in several rodent models of obesity, with defects in either the OB gene or the leptin receptor gene (OB-R) shown to be associated with the development of obesity in both mouse and rat models of obesity. In humans the role of leptin and its receptor is less clear. Several studies have examined sequence variation within the OB-R gene and demonstrated the presence of a number of polymorphic areas within the coding region of this gene. Cross-sectional studies conducted in a variety of populations have failed to show associations between polymorphisms in OB-R and obesity, or obesity-related measures. In the present study we examined the presence of the Gln223Arg and PRO1019pro OB-R polymorphisms in a population of Australian Caucasian women and investigated associations between genotypes at these polymorphic sites and a number of anthropometric and metabolic measures related to obesity. These polymorphisms were also examined in relation to changes in body mass and composition in this population over a two year period. Overall, the Gln223Arg and PRO1019pro polymorphisms were not associated with obesity, or obesity related phenotypic markers, including body weight, percent body fat, BMI, waist circumference or circulating leptin concentrations in this population of Caucasian women, with no differences seen across the three genotypes for either polymorphism. Furthermore, there was no association between the genotypes at either polymorphism and changes in body fat mass, fat-free mass or percent body fat over a two year period, although there was evidence of an association between the PRO1019pro polymorphism and increased body weight gain (Δbody weight: 1.63±0.47 vs -0.58±0.57, PRO/PRO vs PRO/pro genotypes; P₁=0.01), however this difference was seen in the subjects with the PRO/pro genotype, and is therefore difficult to explain. Together, the cross-sectional and longitudinal data presented in this study suggest that the Gln223Arg and PRO1019pro OB-R polymorphisms are unlikely to play a role in the development of obesity or regulation of energy balance in a majority of Caucasian women.
4.2 INTRODUCTION

Leptin, the protein product of the OB gene, has been demonstrated to play an important role in the regulation of energy balance in several monogenic rodent models of obesity, although a similar role has not been demonstrated as clearly in non-genetic or polygenic animal models (Hallas et al., 1995; Campfield et al., 1995; Cusin et al., 1996; Levin et al., 1996; Halaas et al., 1997; Igel et al., 1997; Van Heek et al., 1997; Widdowson et al., 1997; Walder et al., 1999). In humans circulating leptin concentrations have consistently been shown to increase with increasing body weight and adiposity (Maffei et al., 1995b; Considine et al., 1995), however a role for leptin in the regulation of energy balance in the majority of humans is yet to be unequivocally established. An extensive number of studies have examined the OB gene in humans and found that in the majority of obese individuals, there does not appear to be a defect in this gene which can be linked to the obese phenotype (Maffei et al., 1995b; Clement et al., 1996; Considine et al., 1996a; Niki et al., 1996; Shintani et al., 1996).

The receptor for leptin, OB-R, has also received much attention, as in several rodent models of obesity and a small number of humans, a mutation in the OB-R gene has been shown to result in defective leptin action and subsequently, morbid obesity (Chen et al., 1996a; Iida et al., 1996a; Takaya et al., 1996; Lee et al., 1996; Ghiardi et al., 1997; Clement et al., 1998). Extensive genetic analysis of the OB-R gene has revealed several areas in which sequence variation exists between human subjects (Considine et al., 1996b; Chung et al., 1997; Gotoda et al., 1997; Thompson et al., 1997; Rolland et al., 1997). These areas lie both within and between exons and could potentially alter leptin action at the receptor level. Several studies have been conducted examining the presence of these polymorphisms and associations with obesity-related variables, however in these cross-sectional analyses, sequence alteration in this gene did not appear to be related to the obese phenotype. These studies have been performed in a variety of human populations, including subjects of Pima Indian (Thompson et al., 1997), British (Gotoda et al., 1997), Japanese (Matsuoka et al., 1997), Danish (Echwald et al., 1998), French (Rolland et al., 1999) and American (Silver et al., 1997; Chung et al., 1997; Considine et al., 1996b) descent.

In the present study we aim to examine the relationship between two of the first described OB-R polymorphisms (Gln223Arg and PRO1019pro) and obesity-related variables in a
large population of Australian Caucasian women and, further, examine associations between these polymorphisms and longitudinal changes in body mass and composition over a two year period.
4.3 SUBJECTS, MATERIALS AND METHODS

4.3.1 SUBJECTS
The women participating in this study (n=335) were drawn randomly from a larger, population-based osteoporosis study (the Geelong Osteoporosis Study) undertaken in the Barwon region of Victoria, Australia in 1994. All subjects in the Geelong Osteoporosis Study were randomly selected from the region using an Australian Electoral Roll. Of the 335 subjects, 161 were lean (BMI<25), 56 were overweight (BMI: 25-30) and 118 were obese (BMI>30). In addition, 8 subjects were classified as type 2 diabetic (glucose>7.8mmol/L) and 327 subjects were classified as non-diabetic (glucose<7.8mmol/L).

4.3.2 DATA COLLECTION
At baseline, all subjects were required to fast overnight (from 2200 h the previous evening) and blood specimens were collected the next morning, with a proportion of the blood set aside for DNA extraction, and the remainder centrifuged and the plasma stored at -80°C. Height, weight, waist circumference and hip circumference were measured at baseline and two years later and used to calculate body mass index (BMI; body weight [kg]/height [m]²). In addition, all subjects underwent a dual-energy x-ray absorptiometry (DEXA) scan to determine body composition at baseline and two years later.

4.3.3 BIOCHEMICAL ANALYSES
All measurements were performed using blood collected after an overnight fast. Fasting plasma insulin and leptin concentrations were determined using commercially available radioimmunoassay kits (Phadescph Human Insulin RIA kit, Pharmacia Diagnostics AB, St Louis, USA; Human Leptin RIA kit, Linco Research Inc., St Charles, Missouri, USA). Plasma glucose, cholesterol and triglyceride concentrations were measured using colorimetric kits (Boehringer-Mannheim, Mannheim, Germany) on an automatic analyser as described in detail in Chapter 2.

4.3.4 POLYMERASE CHAIN REACTION (PCR)-RESTRICTION FRAGMENT-LENGTH POLYMORPHISM (RFLP)
Genomic DNA was extracted from 1 ml of whole blood stored at the time of collection and used for subsequent analyses. DNA was extracted using Wizard® Genomic DNA
Purification Kit (Promega, Madison, USA). Briefly, the kit is based on a four-step process:

1. Erythrocyte lysis, leaving the leukocytes intact
2. Lysis of white blood cells and their nuclei
3. Precipitation of proteins using the supplied salt solution
4. Concentration and desalting of the genomic DNA by isopropanol precipitation.

The DNA was resuspended in the supplied DNA Rehydration Solution and quantitated by absorption spectrophotometry at 260 nm. The DNA was diluted to 100 ng/μl and used for subsequent analyses.

4.3.4.1 Gln223Arg PCR

The Gln223Arg sequence variation results from an A to G base change in the second position of codon 223. This alteration changes the amino acid encoded at position 223 from CAG (Gln) to CCG (Arg). Oligonucleotide primers for PCR amplification of this region of OB-R were those published by Gotoda et al. (1997) with the following sequences:

- forward: 5′-aaactcaacgacatctccttt-3′
- reverse: 5′-tgaactgtatagaggtgac-3′

PCR was performed by adding 200 ng of DNA to a reaction mixture containing: 20mM Tris-HCl (pH 8.4), 50 mM KCl, 1.25 mM MgCl₂, 200 μM each dNTP, 5 pmol each primer and 1.25U Taq DNA polymerase (Life Technologies, Gibco, Gaithersburg, MD, USA). After an initial denaturation at 94°C for 5 min, the reaction conditions consisted of 35 cycles of 94°C for 30 sec (denaturation), 54°C for 30 sec (annealing) and 72°C for 30 sec, followed by a final extension at 72°C for 5 mins. PCR was performed in a GeneAmp PCR System 9700 Thermal Cycler (Perkin Elmer Applied Biosystems, Foster City, USA) in a total volume of 15 μl in 0.2 ml microcentrifuge tubes (Perkin Elmer Applied Biosystems, Foster City, USA).

4.3.4.2 PRO1019pro PCR

The PRO1019pro polymorphism is the result of a G to A base change in codon 1019 of the OB-R gene. This alteration does not change the amino acid encoded at position 1019, CCG (proline) to CCA (proline). Oligonucleotide primers for PCR amplification of this
region of OB-R were those published by Gotoda et al. (1997) with the following sequences:

forward: 5'-cagatcttgaaaggtct-3'
reverse: 5'-tcccatgagcattagagaagatctctc-3'

PCR was performed by adding 200 ng of DNA to a reaction mixture containing: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.25 mM MgCl₂, 200 μM each dNTP, 5 pmol each primer and 1.25 U Taq DNA polymerase (Life Technologies, Gibco, Gaithersburg, MD, USA). After an initial denaturation at 94°C for 5 min, the reaction conditions consisted of 35 cycles of 94°C for 30 sec (denaturation), 52°C for 30 sec (annealing) and 72°C for 30 sec, followed by a final extension at 72°C for 5 mins. PCR was performed in a GeneAmp PCR System 9700 Thermal Cycler (Perkin Elmer Applied Biosystems, Foster City, USA) in a total volume of 15 μl in 0.2 ml microcentrifuge tubes (Perkin Elmer Applied Biosystems, Foster City, USA).

4.3.4.3 Restriction Enzyme Digestion

For the Gln223Arg polymorphism the total PCR product (15 μl) was digested by the addition of 10 μl of a mixture containing: 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol (DTT) (pH 7.9) and 10 units of Hae III (New England Biolabs, Beverly, USA) followed by incubation for 2 hours at 37°C to enable complete digestion. For the PRO1019pro polymorphism the total PCR product was digested by the addition of 10 μl of a mixture containing: 50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, 1 mM DTT (pH 7.9) and 10 units of Nco I (New England Biolabs, Beverly, USA) followed by incubation for 2 hours at 37°C to enable complete digestion. Hae III is a restriction enzyme that specifically cleaves DNA at the sequence 5'...GG'CC...3' and Nco I is a restriction enzyme that specifically cleaves at the DNA sequence 5'...C'CATGG...3'.

4.3.4.4 Electrophoresis and Analysis

The digested PCR products were separated by agarose gel electrophoresis in a 3% agarose gel (1:2 agarose:Nuseive) containing 0.5 μg/ml ethidium bromide at 6V/cm for 120 mins in a DNA SubCell system (Bio-Rad, Anaheim, USA). The gels were then photographed using a Polaroid DS34 camera with a DSH-7 Hood on Polaroid 665 positive/negative film (Sigma Chemical Company, St Louis, USA) under ultraviolet transillumination (model

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4000, Stratagene, LaJolla, USA) at 302 nm. The genotypes were determined visually based on DNA fragment sizes (table 4.1).

**Table 4.1** Fragment sizes generated after restriction enzyme digestion of the PCR products for each of the OB-R polymorphisms.

<table>
<thead>
<tr>
<th>Gln223Arg polymorphism:</th>
<th>Fragment sizes (base pairs):</th>
<th>PRO1019pro polymorphism:</th>
<th>Fragment sizes (base pairs):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gin/Gln</td>
<td>91 bp</td>
<td>PRO/PRO</td>
<td>220 bp</td>
</tr>
<tr>
<td>Gin/Arg</td>
<td>91 bp, 60 bp, 31 bp</td>
<td>PRO/pro</td>
<td>220 bp, 200 bp, 20 bp</td>
</tr>
<tr>
<td>Arg/Arg</td>
<td>60 bp, 31 bp</td>
<td>pro/pro</td>
<td>200 bp, 20 bp</td>
</tr>
</tbody>
</table>

Of the 335 subjects genotyped, 100% were successful for the Gln223Arg polymorphisms (n=335), and 91% were successful for the PRO1019pro polymorphism (n=306).

**4.3.5 STATISTICAL ANALYSIS**

All statistical analyses were performed using SPSS (version 8.0.2) and results are expressed as mean±standard error of the mean (SEM). Comparison of mean differences in baseline anthropometry and metabolic variables, and mean changes in body mass or composition across the three genotypes was performed using the Kruskal-Wallis test (Kruskal and Wallis, 1952). Change in body mass or composition over time in relation to genotypes while adjusting for covariates (baseline insulin and age) was accomplished using analysis of variance (Armitage and Berry, 1987), with Bonferroni correction for the use of multiple comparisons ($P_c$-value given). In all cases a $p$-value <0.05 was considered significant.
4.4 RESULTS

Table 4.2 shows the baseline anthropometric and metabolic characteristics of the women participating in this study. They can be seen to encompass a range of body weights, as well as a range of glucose and insulin concentrations.

Table 4.2 Baseline anthropometry and metabolic variables in the Australian women examined (mean ± S.E.M.).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean ± sem</th>
<th>range (minimum-maximum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>45.2 ±0.69</td>
<td>20 - 83</td>
</tr>
<tr>
<td>Bodyweight (kg)</td>
<td>71.6 ±0.9</td>
<td>42.3 - 121.20</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>38.0 ±0.4</td>
<td>14.9 - 54.2</td>
</tr>
<tr>
<td>Fat-free mass (kg)</td>
<td>43.43 ±0.31</td>
<td>30.32 - 65.57</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.3 ±0.3</td>
<td>18.2 - 46.4</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>85.3±0.7</td>
<td>61.9 - 128.0</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>106.4±0.6</td>
<td>82.9 - 157.6</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.37 ±0.11</td>
<td>3.11 - 17.70</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>10.96 ±0.43</td>
<td>2.60 - 43.17</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>20.19 ±0.79</td>
<td>1.77 - 72.93</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.47 ±0.05</td>
<td>0.31 - 5.80</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.99 ±0.06</td>
<td>2.16 - 8.76</td>
</tr>
</tbody>
</table>
Table 4.3 shows the allele and genotype frequencies of the Gln223Arg and PRO1019pro polymorphisms in this population of Australian women. For the Gln223Arg polymorphism, the Gln allele was the most frequent (frequency: 0.60) in this population, while the most frequent genotype for this polymorphism was the Gln/Arg (0.50), showing that most subjects had at least one Gln allele. The most frequent allele for the PRO1019pro polymorphism was the PRO allele (0.66) while there was an equal representation of the PRO/PRO and PRO/pro genotypes (0.44) in this population. The genotype frequencies of both polymorphisms were found to be in Hardy-Weinberg equilibrium, however the polymorphisms were not in linkage disequilibrium ($\chi^2=3.065$, p=0.55).

<table>
<thead>
<tr>
<th>Polymorphism:</th>
<th>Allele Frequency:</th>
<th>Genotype Frequency:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gln223Arg</td>
<td>Gln</td>
<td>Arg</td>
</tr>
<tr>
<td></td>
<td>0.60</td>
<td>0.40</td>
</tr>
<tr>
<td>PRO1019pro</td>
<td>PRO</td>
<td>pro</td>
</tr>
<tr>
<td></td>
<td>0.66</td>
<td>0.34</td>
</tr>
</tbody>
</table>
Table 4.4. Baseline anthropometry and metabolic variables in Australian women in relation to the Gln223Arg polymorphism (mean ± S.E.M.).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Gln/Gln (n=116)</th>
<th>Gln/Arg (n=170)</th>
<th>Arg/Arg (n=49)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>42.8±1.3</td>
<td>41.9±0.9</td>
<td>41.7±1.7</td>
</tr>
<tr>
<td>Bodyweight (kg)</td>
<td>72.6±1.5</td>
<td>71.6±1.2</td>
<td>69.7±2.1</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>38.7±0.7</td>
<td>38.0±0.6</td>
<td>36.7±1.2</td>
</tr>
<tr>
<td>Fat-free Mass (kg)</td>
<td>43.49±0.53</td>
<td>43.44±0.46</td>
<td>43.25±0.76</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.94±0.59</td>
<td>27.20±0.45</td>
<td>25.95±0.82</td>
</tr>
<tr>
<td>Waist circum. (cm)</td>
<td>85.9±1.3</td>
<td>85.7±1.0</td>
<td>81.8±1.9</td>
</tr>
<tr>
<td>Hip circum. (cm)</td>
<td>106.6±1.1</td>
<td>107.0±0.9</td>
<td>104.1±1.4</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.3±0.2</td>
<td>5.3±0.2</td>
<td>5.7±0.3</td>
</tr>
<tr>
<td>Insulin (μU/ml)</td>
<td>10.4±0.6</td>
<td>11.1±0.6</td>
<td>11.7±1.1</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>20.7±1.3</td>
<td>20.4±1.2</td>
<td>18.5±1.8</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.4±0.09</td>
<td>1.5±0.07</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.9±0.1</td>
<td>5.1±0.09</td>
<td>4.8±0.2</td>
</tr>
</tbody>
</table>

Table 4.4 shows differences in means for a variety of baseline anthropometric and metabolic measurements across the three genotypes of the Gln223Arg polymorphism. While there were no differences which reached statistical significance, compared to subjects with the Gln/Gln genotype, those with the Arg/Arg genotype tended to have lower bodyweight (69.7±2.1 vs 72.6±1.5), BMI (25.9±0.8 vs 27.9±0.6), waist circumference (81.8±1.9 vs 85.9±1.3), hip circumference (104.1±1.4 vs 106.6±1.1) and fasting leptin concentration (18.5±1.8 vs 20.7±1.3).
Table 4.5  Baseline anthropometry and metabolic variables in Australian women in relation to the PRO1019pro polymorphism (mean ± S.E.M.).

<table>
<thead>
<tr>
<th>Variable:</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PRO/PRO (n=133)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>39.5±1.0*</td>
</tr>
<tr>
<td>Bodyweight (kg)</td>
<td>71.2±1.2</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>37.97±0.68</td>
</tr>
<tr>
<td>Fat-free Mass (kg)</td>
<td>43.24±0.44</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.8±0.5</td>
</tr>
<tr>
<td>Waist circum. (cm)</td>
<td>83.7±1.1</td>
</tr>
<tr>
<td>Hip circum. (cm)</td>
<td>105.1±1.0</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.2±0.1</td>
</tr>
<tr>
<td>Insulin (μU/ml)</td>
<td>10.4±0.7</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>22.0±1.5</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.5±0.09</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>5.1±0.1</td>
</tr>
</tbody>
</table>

* P<0.03, significantly different from PRO/pro

Table 4.5 shows differences in means for a variety of baseline anthropometric and metabolic measurements across the three genotypes of the PRO1019pro polymorphism. Although subjects with the PRO/PRO genotype were younger than subjects with the PRO/pro genotype (39.5±1.02 vs 44.2±1.1; p=0.01; P<0.03) no other significant differences were seen across the three groups. Compared to subjects with the PRO/PRO genotype, subjects with the pro/pro genotype tended to have increased body weight (74.2±2.9 vs 71.2±1.2), waist circumference (86.7±2.07 vs 83.7±1.1) and hip circumference (107.8±2.0 vs 105.1±1.0), although these differences did not reach statistical significance.
Table 4.6 Estimated marginal means (±SEM) of change in body weight, BMI, fat mass, fat free mass and percent body fat over a two-year follow-up in Australian women in relation to the Gln223Arg and PRO1019Pro polymorphisms, after adjustment for age and baseline insulin concentration.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Δ Body Weight (kg)</th>
<th>Δ BMI (kg)</th>
<th>Δ Fat Mass (kg)</th>
<th>Δ Fat free mass (kg)</th>
<th>Δ % body fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gln223Arg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gln/Gln</td>
<td>0.46 ±0.57</td>
<td>0.12 ±0.31</td>
<td>1.23 ±0.54</td>
<td>-0.60 ±0.33</td>
<td>1.25 ±0.44</td>
</tr>
<tr>
<td>Gln/Arg</td>
<td>0.91 ±0.46</td>
<td>0.60 ±0.25</td>
<td>1.55 ±0.45</td>
<td>-0.58 ±0.28</td>
<td>1.54 ±0.36</td>
</tr>
<tr>
<td>Arg/Arg</td>
<td>-0.10 ±0.92</td>
<td>-0.02 ±0.49</td>
<td>0.67 ±0.82</td>
<td>-0.70 ±0.51</td>
<td>0.78 ±0.67</td>
</tr>
<tr>
<td>PRO1019Pro</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRO/PRO</td>
<td>-0.58 ±0.57</td>
<td>0.27 ±0.43</td>
<td>0.38 ±0.52</td>
<td>-1.05 ±0.34</td>
<td>0.96 ±0.43</td>
</tr>
<tr>
<td>PRO/pro</td>
<td>1.63 ±0.47*</td>
<td>0.65 ±0.36</td>
<td>1.90 ±0.47</td>
<td>-0.21 ±0.31</td>
<td>1.55 ±0.39</td>
</tr>
<tr>
<td>pro/pro</td>
<td>0.11 ±0.97</td>
<td>1.00 ±0.73</td>
<td>2.37 ±0.96</td>
<td>-0.99 ±0.64</td>
<td>2.20 ±0.80</td>
</tr>
</tbody>
</table>

*P<0.01, significantly different from PRO/PRO

Table 4.6 shows associations between the genotypes for both polymorphisms and changes in body mass and composition over a two-year follow-up in the population of Australian women. There were no significant differences in the mean changes in fat mass, fat-free mass or percent body fat across the three genotypes for either polymorphism, although the mean change in body weight over two years was significantly higher in subjects with the PRO/pro genotype when compared to those with the PRO/PRO genotype, after adjustment for age and baseline insulin (1.63±0.47 vs -0.58±0.57, PRO/pro vs PRO/PRO, P<0.01). In addition, there was a tendency for subjects with the PRO/PRO genotype to have a smaller increase in fat mass over the two years compared to subjects with either the PRO/pro or pro/pro genotypes (Δfat mass: 0.38±0.52 vs 1.90±0.47 and 2.37±0.96; p=0.03, P<0.09). For the Gln223Arg polymorphism, subjects with the Arg/Arg genotype tended to have a smaller increase in body weight and BMI over the two year period then subjects with the Gln/Arg genotype (Δbody weight: -0.10±0.92 vs 0.91±0.46; ΔBMI: -0.02±0.49 vs 0.60±0.25; Arg/Arg vs Gln/Arg genotypes), although these differences did not reach statistical significance.
4.5 DISCUSSION

In this population of Australian Caucasian women, the Gln223Arg and PRO1019pro polymorphisms in the OB-R gene were not associated with baseline age, body weight, percent body fat, fat-free mass, body mass index, waist circumference, hip circumference, or fasting concentrations of glucose, insulin, leptin, triglycerides or cholesterol. In addition, these polymorphisms were not associated with changes in body weight, fat mass, fat-free mass or percent body fat, over a two-year period in this population of women.

The allele frequency of the Arg allele of the Gln223Arg polymorphism was found to be 0.34 in this population of Australian women. This is similar to allele frequencies reported in other Caucasian populations, with two separate studies in American Caucasians reporting frequencies of the Arg allele to be 0.14 (Silver et al., 1997) and 0.34 (Chung et al., 1997), and a study in white British males reporting a frequency of the Arg allele of 0.44 (Gotoda et al., 1997).

The PRO1019pro polymorphism has only previously been examined in three populations, a population of white British males (Gotoda et al., 1997), a Pima Indian population (Thompson et al., 1997) and a Japanese population (Matsuoka et al., 1997). In the British Caucasian population, an allele frequency of approximately 0.36 was reported for the pro allele, which is similar to that seen in this Australian population of Caucasian women (0.34).

In this study we did not find any associations between a large number of anthropometric and metabolic variables measured in this population and either the Gln223Arg or PRO1019pro polymorphisms. Similar results have been reported in several different populations, including British (Gotoda et al., 1997), American (Considine et al., 1996b; Chung et al., 1997; Silver et al., 1997), Japanese (Matsuoka et al., 1997) and Danish (Echwald et al., 1997), and the only associations seen with these polymorphisms and obesity-related variables were reported by Thompson et al. (1997) in Pima Indians. In this study, the Gln223Arg polymorphism was found to be present only in all but one of the obese Pima Indians, and not in lean subjects, while the PRO1019pro polymorphism was found to be associated with percent body fat in this population.
In the present study, the polymorphisms in OB-R were also found not to be associated with longitudinal changes in body mass or body composition in Australian Caucasian women. As leptin and its receptor are thought to play a role in energy balance and body weight regulation we hypothesised that a prospective study may provide more information regarding the effects of these sequence changes in regulation of energy balance in humans. This type of analysis for these polymorphisms has not been reported in any other populations to date, despite the fact that many cross-sectional reports have been published. In this population of Australian women, the genotypes for both polymorphisms were not associated with changes in body mass or body composition over a two year period. This finding, coupled with the cross-sectional data, provides strong evidence that the Gln223Arg and PRO1019pro polymorphisms are unlikely to be involved in the regulation of energy balance in a majority of Caucasian women.
CHAPTER 5

COMBINATION OF POLYMORPHISMS IN OB-R AND THE OB GENE ASSOCIATED WITH INSULIN RESISTANCE IN NAURUAN MALES.

5.1 SUMMARY

Leptin, the protein product of the OB gene has been shown to be an important regulator of energy balance in several rodent models of obesity, and defects in either the OB gene or the leptin receptor gene (OB-R) have been shown to be associated with the development of obesity in several rodent models. In humans the role of leptin and its receptor is less clear. Many studies have been conducted examining sequence variation with the human OB and OB-R genes, with a number of polymorphic areas identified. Several of these polymorphisms have been examined in different populations with respect to associations between specific genotypes and cross-sectional data on obesity- or diabetes-related phenotypic markers. The aim of the present study was to investigate the relationship between polymorphisms in the OB-R and OB genes and metabolic markers for obesity and glucose intolerance in a population of Nauruan men. In addition, we examined the effect of the simultaneous presence of the three polymorphisms on the phenotype of individuals in this population, and also looked for associations between these polymorphisms and the development of obesity and type 2 diabetes over a twelve year period. Cross-sectional analysis was conducted with 232 non-diabetic male subjects with a mean age of 31 years and a mean body weight of 104 kg. In this population, there was no association between several phenotypic measures of body fatness and fat distribution (anthropometry), fasting plasma insulin, glucose or leptin concentrations, blood pressure or 2hr plasma glucose concentration and genotypes for the Gin223Arg and OB gene polymorphisms. There was an association between the pro/pro genotype of the PRO1019pro polymorphism and hyperinsulinemia and increased diastolic blood pressure ($P_e=0.04$), however this polymorphism was not associated with the other phenotypic variables examined. In this study, we also examined the development of obesity and type 2 diabetes in approximately 100 Nauruan men. In this longitudinal analysis we found that neither of the three polymorphisms were associated with a higher prevalence of obesity and diabetes. In addition, individuals found to simultaneously exhibit all three polymorphisms (genotypes: classII/classII, Arg/Arg, pro/pro) exhibited significantly elevated fasting insulin levels.
($P_c=0.03$) compared to subjects without this genotype combination, while subjects with this genotype combination tended to develop a higher proportion of diabetes over a twelve year period. Pacific Island populations exhibit a remarkably high prevalence rate of obesity and type 2 diabetes and represent a unique population for genetic studies of obesity. In the present study we have revealed that a specific combination of alleles in OB and OB-R, two candidate genes for obesity, may confer an increased risk for the development of insulin resistance and type 2 diabetes in Nauruan males.
5.2 INTRODUCTION

Leptin, the protein product of the OB gene, has been demonstrated to play an important role in the regulation of energy balance in several monogenic rodent models of obesity, although a similar role has not been demonstrated as clearly in non-genetic or polygenic animal models (Campfield et al., 1995; Halaas et al., 1995; Cusin et al., 1996; Levin et al., 1996; Halaas et al., 1997; Igel et al., 1997; Van Heek et al., 1997; Widdowson et al., 1997; Walder et al., 1998). In humans, circulating leptin concentrations have consistently been shown to increase with increasing body weight and adiposity (Maffei et al., 1995b; Considine et al., 1995), however the role of leptin in the regulation of energy balance in the majority of humans is yet to be unequivocally established. In addition, circulating leptin concentrations are also associated with insulin concentrations, independent of body mass (Considine et al., 1996c; Havel et al., 1996b; Zimmet et al., 1996; Zimmet et al., 1998).

An extensive number of studies have examined the OB gene in humans and found that in most obese individuals there does not appear to be a simple mutation in this gene which can be linked to the obese phenotype (Maffei et al., 1995b; Considine et al., 1996c; Clement et al., 1996; Niki et al., 1996). A novel highly polymorphic marker within the OB gene has been discovered however, which may provide an informative tool for association and linkage studies of the OB gene with obesity and type 2 diabetes (Shintani et al., 1996).

The receptor for leptin, OB-R, has also received much attention, as in several rodent models of obesity and a small number of humans, a mutation in the OB-R gene has been shown to result in defective leptin action and subsequently, morbid obesity (Chen et al., 1996a; Lee et al., 1996; Iida et al., 1996a; Takaya et al., 1996; Ghilardi et al., 1997; Clement et al., 1998). Extensive genetic analysis of the OB-R gene has revealed several areas in which sequence variation has been detected between human subjects (Considine et al., 1996b; Gotoda et al., 1997; Chung et al., 1997; Rolland et al., 1997; Thompson et al., 1997). These areas lie both within and between exons and could potentially alter leptin action at the receptor level. Several association studies have been conducted examining the presence of these polymorphisms and obesity-related variables, however in these cross-sectional analyses, sequence alteration in this gene was related to the obese phenotype in only one population (Thompson et al., 1997). These studies were performed in a variety of human populations, including subjects of Pima Indian (Thompson et al., 1997), British (Gotoda et al., 1997), Japanese (Matsuoka et al., 1997), Danish (Echwald et al., 1998),
French (Rolland et al., 1998) and American (Silver et al., 1997; Chung et al., 1997; Considine et al., 1996b) descent.

This study investigates subjects from the Pacific island of Nauru. Obesity is extremely common in Nauru, with 64% of men and 69% of women having a BMI greater than 30kg/m² (Dowse et al., 1996). Furthermore, approximately one-third of adult Nauruans also develop type 2 diabetes (Dowse et al., 1996). In addition to the remarkably high prevalence rates of these two diseases, Nauru is one of the most isolated of Pacific islands. This geographic isolation is thought to have played a role in the rise to epidemic proportions of debilitating diseases on the Island, and thus Nauru offers a unique population for genetic studies into the underlying causes of obesity and type 2 diabetes. A previous study examining the role of leptin in obesity and type 2 diabetes in Nauru demonstrated that, as in other populations, circulating leptin levels were strongly associated with adiposity (Hodge et al., 1997). In the present study, we examined two OB-R polymorphisms (Gln223Arg and PRO1019pro) and a polymorphic microsatellite marker in the OB gene to investigate associations of these candidate genes with metabolic markers of obesity and glucose intolerance in a population of men from Nauru. Furthermore, we have investigated the presence of any additive effect of these markers on the phenotypes examined and also looked longitudinally, at associations between genotypes for each of the polymorphisms and the development of obesity and type 2 diabetes in a proportion of the men studied.
5.3 SUBJECTS, MATERIALS AND METHODS

5.3.1 SUBJECTS
The subjects examined in this study were Nauruan males (n=342). 110 of the subjects were type 2 diabetic and were excluded from the cross-sectional analyses. The remaining non-diabetic Nauruan males (n=232) had a mean age of 31 years, a mean body weight of 104 kg (range: 59-200 kg) and a mean BMI of 37 kg/m² (range: 22-61). A proportion of the total male population were represented in data collected over a 12-year period (n=97) and were the basis of the longitudinal analyses of the development of diabetes and obesity in this population. Subjects included in the longitudinal analyses had a mean age of 23.8 years (range: 20-29), a mean body weight of 93.7 kg (range: 55.2-144.6) and a mean BMI of 33.6 kg/m² (range: 19.3-51.8) at baseline (1982).

5.3.2 DATA COLLECTION
Blood samples were collected as part of a population-based survey conducted in Nauru in 1994 which formed the basis of a 7-year follow-up to a survey conducted in 1987 (Dowse et al., 1991). This work is a continuation of a study originating in 1975/1976 (Zimmet et al., 1977; Serjeantson et al., 1983). The survey protocol was approved by the Alfred Healthcare Group Ethics Committee (Melbourne, Australia) and a written agreement with the Republic of Nauru has been reached for the genetic analysis of samples.

5.3.3 BIOCHEMICAL ANALYSES
Measurements of insulin and leptin were performed using plasma collected after an overnight fast. Fasting plasma insulin and leptin concentrations were determined using commercially available radioimmunoassay kits (Phadeseph Human Insulin RIA kit, Pharmacia Diagnostics AB, St Louis, USA; Human Leptin RIA kit, Linco Research Inc., St Charles, Missouri, USA). Glucose concentration was measured in plasma collected before and 2 hours after an oral glucose load (75 g). Plasma glucose was measured on-site using a YSI Glucose Analyser (Yellow Springs Instrument Co., Ohio, USA).

5.3.4 POLYMERASE CHAIN REACTION (PCR)-RESTRICTION FRAGMENT-LENGTH POLYMORPHISM (RFLP)
Genomic DNA was extracted from 1 ml of whole blood, stored at the time of collection. DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega, Madison, USA). Briefly, the kit is based on a four-step process:
1. Erythrocyte lysis, leaving the leukocytes intact
2. Lysis of white blood cells and their nuclei
3. Precipitation of proteins using the supplied salt solution
4. Concentration and desalting of the genomic DNA by isopropanol precipitation.
The DNA was resuspended in the supplied DNA Rehydration Solution and quantitated by absorption spectrophotometry at 260 nm. The DNA was diluted to 100 ng/μl and used for subsequent analyses.

5.3.4.1 OB gene PCR, Electrophoresis and Analysis
The OB gene polymorphism is a highly polymorphic tetranucleotide repeat polymorphism close to the 3’-end of the last exon of the OB gene. This polymorphism consists of two classes of products after PCR analysis: a shorter (class I) form with fewer tetranucleotide repeats, and a longer (class II) form. Oligonucleotide primers for PCR amplification of this region of the OB gene were those published by Shintani et al. (1996) with the following sequences:

forward: 5’-agtctaaataggttcataatca-3’
reverse: 5’-tctgtaggttgctcactggca-3’

PCR was performed by adding 100 ng of DNA to a reaction mixture containing: 10mM Tris-HCl (pH 8.3 at 20°C), 50 mM KCl, 2 mM MgCl₂, 200 μM each dNTP, 100 pmol each primer and 1.25U Taq DNA polymerase (Life Technologies, Gaithersburg, USA). After initial denaturation at 94°C for 3 min, the reaction conditions consisted of 35 cycles of 94°C for 30 sec (denaturation), 60°C for 1 min (annealing) and 72°C for 1 min, followed by a final extension at 72°C for 10 mins. PCR was performed in a Hybaid OmniGene Thermal Cycler (Teddington, England) in a total volume of 20 μl in 0.5 ml microcentrifuge tubes (Perkin Elmer, Norwalk, USA).

The PCR products were separated by agarose gel electrophoresis in a 3% agarose gel (1:2 agarose:Nuseive) containing 0.5 μg/ml ethidium bromide at 6V/cm for 120 mins in a DNA SubCell system (Bio-Rad, Anaheim, USA). The gels were then photographed using a Polaroid DS34 camera with a DSH-7 Hood on Polaroid 665 positive/negative film (Sigma Chemical Company, St Louis, USA) under ultraviolet transillumination (model 4000, Stratagene, LaJolla, USA) at 302 nm. The genotypes were determined visually based on DNA fragment sizes and determined to be either 121-145 base pairs in length (short form),
or 197-221 base pairs in length (long form), with subjects characterised as either: homozygous for the short form (class I/class I), heterozygous (class I/class II), or homozygous for the long form (class II/class II).

5.3.4.2 Gln223Arg PCR
The Gln223Arg sequence variation results from an A to G base change in the second position of codon 223. This alteration changes the amino acid encoded at position 223 from CAG (Gln) to CGG (Arg). Oligonucleotide primers for PCR amplification of this region of OB-R were those published by Gotoda et al. (1997) with the following sequences:

- forward: 5'-aaactcaacgacacctctccttt-3'
- reverse: 5'-tgacgtgcattagaggigac-3'

PCR was performed by adding 200 ng of DNA to a reaction mixture containing: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.25 mM MgCl₂, 200 μM each dNTP, 5 pmol each primer and 1.25U Taq DNA polymerase (Life Technologies, Gaithersburg, USA). After initial denaturation at 94°C for 5 min, the reaction conditions consisted of 35 cycles of 94°C for 30 sec (denaturation), 54°C for 30 sec (annealing) and 72°C for 30 sec, followed by a final extension at 72°C for 5 mins. PCR was performed in a GeneAmp PCR System 9700 Thermal Cycler (Perkin Elmer Applied Biosystems, Foster City, USA) in a total volume of 15 μl in 0.2 ml microcentrifuge tubes (Perkin Elmer Applied Biosystems, Foster City, USA).

5.3.4.3 PRO1019pro PCR
The PRO1019pro polymorphism is the result of a G to A base change in codon 1019 of the OB-R gene. This alteration does not change the amino acid encoded at position 1019, CCG (proline) to CCA (proline). Oligonucleotide primers for PCR amplification of this region of OB-R were those published by Gotoda et al. (1997) with the following sequences:

- forward: 5'-cagatcttggaattcritcttc-3'
- reverse: 5'-tcccctggctttgattatatcacca-3'

PCR was performed by adding 200 ng of DNA to a reaction mixture containing: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.25 mM MgCl₂, 200 μM each dNTP, 5 pmol each primer and 1.25U Taq DNA polymerase (Life Technologies, Gaithersburg, USA). After
initial denaturation at 94°C for 5 min, the reaction conditions consisted of 35 cycles of
94°C for 30 sec (denaturation), 52°C for 30 sec (annealing) and 72°C for 30 sec, followed
by a final extension at 72°C for 5 mins. PCR was performed in a GeneAmp PCR System
9700 Thermal Cycler (Perkin Elmer Applied Biosystems, Foster City, USA) in a total
volume of 15 µl in 0.2 ml microcentrifuge tubes (Perkin Elmer Applied Biosystems, Foster
City, USA).
5.3.4.4 Restriction Enzyme Digestion

For the Gln223Arg polymorphism, the total PCR product (15 μl) was digested by the addition of 10 μl of a mixture containing: 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol (DTT) (pH 7.9) and 10 units of Hae III (New England Biolabs, Beverly, USA) followed by incubation for 2 hours at 37°C to enable complete digestion. For the PRO1019pro polymorphism, the total PCR product was digested by the addition of 10 μl of a mixture containing: 50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, 1 mM DTT (pH 7.9) and 10 units of Nco I (New England Biolabs, Beverly, USA) followed by incubation for 2 hours at 37°C to enable complete digestion. Hae III is a restriction enzyme that specifically cleaves DNA at the sequence 5'...GG'CC...3' and Nco I is a restriction enzyme that specifically cleaves at the DNA sequence 5'...C'CATGG...3'.

5.3.4.5 Electrophoresis and Analysis

The digested PCR products were separated by agarose gel electrophoresis in a 3% agarose gel (1:2 agarose:Nuseivc) containing 0.5 μg/ml ethidium bromide at 6V/cm for 120 mins in a DNA SubCell system (Bio-Rad, Anaheim, USA). The gels were then photographed using a Polaroid DS34 camera with a DSH-7 Hood on Polaroid 665 positive/negative film (Sigma Chemical Company, St Louis, USA) under ultraviolet transillumination (model 4000, Stratagene, LaJolla, USA) at 302 nm. The genotypes were determined visually based on DNA fragment sizes (table 5.1).

Table 5.1 Fragment sizes generated after restriction enzyme digestion of the PCR products for each of the OB-R polymorphisms.

<table>
<thead>
<tr>
<th>Gln223Arg polymorphism:</th>
<th>Fragment sizes (base pairs):</th>
<th>PRO1019pro polymorphism:</th>
<th>Fragment sizes (base pairs):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gln/Gln</td>
<td>91 bp</td>
<td>PRO/PRO</td>
<td>220 bp</td>
</tr>
<tr>
<td>Gln/Arg</td>
<td>91 bp, 60 bp, 31 bp</td>
<td>PRO/pro</td>
<td>220 bp, 200 bp, 20 bp</td>
</tr>
<tr>
<td>Arg/Arg</td>
<td>60 bp, 31 bp</td>
<td>pro/pro</td>
<td>200 bp, 20 bp</td>
</tr>
</tbody>
</table>
As the numbers of subjects for some genotypes were limited, subjects with the I/I (n=5) and I/II (n=38) genotypes were pooled and compared to subjects with the II/II genotype (n=189) for the OB gene polymorphism. Similarly, subjects with the PRO/PRO (n=8) and PRO/pro (n=79) genotypes were pooled and compared to subjects with the pro/pro (n=145) genotype. For the Gln223Arg, there were no non-diabetic subjects with the Gln/Gln genotype, so subjects with the Gln/Arg (n=52) genotype were compared to those with the Arg/Arg (n=180) genotype.

4.3.5 STATISTICAL ANALYSIS

Logarithmic transformations were used to normalise distributions of leptin, glucose and insulin and used in all analyses for these variables. The unpaired t-test was used to examine differences between the groups with a Bonferroni correction to determine the level of significance after adjusting for multiple tests (Pc-corrected P-value). A chi-squared test was used to test for the presence of Hardy-Weinberg equilibrium amongst the genotypes and the presence of linkage disequilibrium between the OB-R polymorphisms.
5.4 Results

Table 5.2. Allele and genotype frequencies of the Gln223Arg, PRO1019pro and OB gene polymorphisms in a population of Nauruan males.

<table>
<thead>
<tr>
<th>Polymorphism:</th>
<th>Allele Frequency:</th>
<th>Genotype Frequency:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gln223Arg</td>
<td>Gln 0.11, Arg 0.89</td>
<td>Gln/Gln 0.00, Gln/Arg 0.22, Arg/Arg 0.78</td>
</tr>
<tr>
<td>PRO1019pro</td>
<td>PRO 0.20, pro 0.80</td>
<td>PRO/PRO 0.03, PRO/pro 0.34, pro/pro 0.63</td>
</tr>
<tr>
<td>OB Gene</td>
<td>I 0.10, II 0.90</td>
<td>I/I 0.02, I/II 0.16, II/II 0.82</td>
</tr>
</tbody>
</table>

The allele and genotype frequencies determined using PCR-RFLP analysis of the Gln223Arg and PRO1019pro OB-R polymorphisms and the OB gene microsatellite polymorphism are shown in Table 5.2. The genotype frequencies for all polymorphisms were found to be in Hardy-Weinberg equilibrium and the two OB-R polymorphisms were found to be in strong linkage disequilibrium (p=0.002).
Table 5.3. Associations between the OB gene polymorphism and risk factors for obesity and type 2 diabetes assessed using an unpaired t-test (mean ± standard deviation).

<table>
<thead>
<tr>
<th>Variable</th>
<th>I/I (n=189)</th>
<th>I/II + II/II (n=43)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>31.5 ± 3.8</td>
<td>30.3 ± 4.5</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>37.0 ± 8.2</td>
<td>35.2 ± 7.5</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>104.2 ± 24.8</td>
<td>100.3 ± 22.1</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>9.6 ± 2.2</td>
<td>7.8 ± 2.3</td>
</tr>
<tr>
<td>Fasting Glucose (mmol/l)</td>
<td>5.7 ± 1.1</td>
<td>5.5 ± 1.1</td>
</tr>
<tr>
<td>2hr Glucose (mmol/l)</td>
<td>5.9 ± 1.3</td>
<td>6.1 ± 1.3</td>
</tr>
<tr>
<td>Fasting Insulin (μU/ml)</td>
<td>14.2 ± 1.8</td>
<td>12.4 ± 1.7</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>126.5 ± 16.6</td>
<td>128.0 ± 22.5</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>73.9 ± 13.6</td>
<td>75.5 ± 17.3</td>
</tr>
</tbody>
</table>

* back transformed geometric mean ± standard deviation.
Table 5.4  Associations between the Gln223Arg polymorphism and risk factors for obesity and type 2 diabetes assessed using an unpaired t-test (mean ± standard deviation).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Gln/Gln + Gln/Arg (n=52)</th>
<th>Arg/Arg (n=180)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>30.5 ± 3.5</td>
<td>31.5 ± 4.1</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>36.8 ± 7.0</td>
<td>36.6 ± 8.0</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>103.1 ± 21.2</td>
<td>103.6 ± 25.2</td>
</tr>
<tr>
<td>Leptin (ng/ml)a</td>
<td>9.5 ± 2.0</td>
<td>9.2 ± 2.2</td>
</tr>
<tr>
<td>Fasting Glucose (mmol/l)a</td>
<td>5.6 ± 1.1</td>
<td>5.6 ± 1.1</td>
</tr>
<tr>
<td>2hr Glucose (mmol/l)a</td>
<td>6.1 ± 1.3</td>
<td>5.9 ± 1.3</td>
</tr>
<tr>
<td>Fasting Insulin (μU/ml)a</td>
<td>12.9 ± 1.7</td>
<td>14.1 ± 1.8</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>128.8 ± 17.2</td>
<td>126.2 ± 18.0</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>75.3 ± 16.0</td>
<td>73.9 ± 13.8</td>
</tr>
</tbody>
</table>

*a back transformed geometric mean ± standard deviation

Tables 5.3 and 5.4 show that the Gln223Arg and OB gene polymorphisms were not significantly associated with obesity (body weight, BMI or plasma leptin concentration) or parameters of risk of type 2 diabetes (fasting plasma glucose, 2hr glucose or fasting insulin concentration) in this population. In addition, genotypes for these polymorphisms were not associated with systolic or diastolic blood pressure.
Table 5.5. Associations between the PRO1019pro polymorphism and risk factors for obesity and type 2 diabetes assessed using an unpaired t-test (mean ± standard deviation).

<table>
<thead>
<tr>
<th>Variable</th>
<th>PRO/PRO (n=87)</th>
<th>+ pro/pro (n=145)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>30.9 ± 3.9</td>
<td>31.5 ± 4.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>36.1 ± 8.2</td>
<td>37.2 ± 7.4</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>101.0 ± 24.0</td>
<td>105.7 ± 22.9</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>8.0 ± 2.2</td>
<td>10.1 ± 2.1</td>
</tr>
<tr>
<td>Fasting Glucose (mmol/l)</td>
<td>5.6 ± 1.1</td>
<td>5.7 ± 1.1</td>
</tr>
<tr>
<td>2hr Glucose (mmol/l)</td>
<td>5.7 ± 1.3</td>
<td>6.1 ± 1.3</td>
</tr>
<tr>
<td>Fasting Insulin (µU/ml)</td>
<td>12.4 ± 1.7</td>
<td>14.8 ± 1.8*</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>126.5 ± 15.5</td>
<td>127.0 ± 19.1</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>71.5 ± 14.9</td>
<td>75.9 ± 13.8*</td>
</tr>
</tbody>
</table>

* back transformed geometric mean ± standard deviation
* P<0.04

Table 5.5 shows that compared to all other subjects, individuals with the pro/pro genotype had a higher fasting insulin concentration (14.8±1.8 vs 12.4±1.7; p=0.02, P<0.04), a higher fasting leptin concentration (10.1±2.1 vs 8.0±2.2; p=0.03, P<0.09), and increased diastolic blood pressure (75.9±13.8 vs 71.5±14.9; p=0.02, P<0.04). There was no association between the genotype for the PRO1019pro polymorphism and BMI, body weight, fasting glucose, two-hour glucose or systolic blood pressure in this population.
Table 5.6 Association of genotype combinations and risk factors for obesity and type 2 diabetes assessed using an unpaired t-test (mean ± standard deviation).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Subjects homozygous for the 3 polymorphisms(^a) (n=97)</th>
<th>All others (n=135)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Years)</td>
<td>31.7 ± 3.9</td>
<td>31.0 ± 4.0</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>37.8 ± 8.1</td>
<td>36.1 ± 7.4</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>107.1 ± 24.7</td>
<td>101.7 ± 22.1</td>
</tr>
<tr>
<td>Leptin (ng/ml)(^a)</td>
<td>10.6 ± 2.2</td>
<td>8.4 ± 2.2*</td>
</tr>
<tr>
<td>Fasting Glucose (mmol/l)(^a)</td>
<td>5.7 ± 1.1</td>
<td>5.6 ± 1.1</td>
</tr>
<tr>
<td>2hr Glucose (mmol/l)(^a)</td>
<td>6.0 ± 1.3</td>
<td>5.9 ± 1.3</td>
</tr>
<tr>
<td>Insulin (μU/ml)(^a)</td>
<td>15.9 ± 1.8</td>
<td>12.5 ± 1.7**(^*)</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>126.7 ± 17.9</td>
<td>126.8 ± 17.8</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>75.5 ± 13.4</td>
<td>73.3 ± 14.9</td>
</tr>
</tbody>
</table>

\(^a\) back transformed geometric mean ± standard deviation

\#genotypes: II/II, Arg/Arg, pro/pro

*P=0.03, **P=0.002;

\(^*\)P\(_c\)=0.032

Table 5.6 shows that when individuals homozygous for the three polymorphisms (genotypes: II/II, pro/pro and Arg/Arg) were combined, this group had significantly higher fasting insulin concentrations (15.9±1.8 vs 12.5±1.7; p=0.002, p\(_c\)=0.032) and a tendency for higher fasting leptin concentrations (10.6±2.2 vs 8.4±2.2; p=0.03, P\(_c\)=0.48), compared to subjects with all other genotype combinations (figure 5.1). There was no association between this genotype combination and BMI, body weight, fasting glucose, two-hour glucose, systolic blood pressure, or diastolic blood pressure in this population of Nauruan males.
Figure 5.1. Comparison of insulin and leptin concentrations in individuals homozygous for the three polymorphisms (genotypes: Arg/Arg, pro/pro, II/II) (■) and subjects with all other genotype combinations (□). *P<0.03.
Figures 5.2-5.5 show the development of obesity and diabetes in a smaller group of Nauruan males (n=97), followed longitudinally from 1982 to 1994. As the number of subjects for each genotype is limited in some cases, this analysis provides only a preliminary examination of the relationship between the development of these diseases and the three polymorphisms investigated in this population. As displayed in figures 5.2 and 5.3, a large proportion of the subjects studied had a BMI>30 by 1994, however, there does not appear to be any relationship between the development of obesity and any particular genotype for either the Gln223Arg, PRO1019pro or OB gene polymorphism. Figures 5.3b and 5.4(a,b) show that within the 12 years, approximately 30% of the subjects examined had developed type 2 diabetes, however again, the development of this disease does not appear to be associated with a particular genotype for any of the three polymorphisms studied.
Figure 5.2: Longitudinal association between transition to obesity and genotype for (a) PRO1019pro and (b) CHRNA5 A59G polytomous in a population of 12-year-old males over a 12-year period (1982–1994).
A population of Naxi males over a 12 year period (1982-1994). Figure 5.3: Longitudinal associations between association in obesity and (transmission 10) type 2 diabetes and obesity for the OB Gene microsatellite polymorphism in 1994, 1987, and 1982.
Figure 5A. Logistic regression analysis between transition to PTEs and genotypes for (a) PRO101990 and (b) GII.2334 05A. R-Participants in a population.

1994

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1976

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Figure 5.5: Longitudinal associations between transition to obesity and transition to type 2 diabetes and genotype combination in a population of Pima males.

1994

1987

1982

27/7
5.5 DISCUSSION

Individually, the OB gene and Gln223Arg polymorphisms were not associated with the obese or pre-diabetic phenotype in this population of Nauruan males. There was an association between the PRO1019pro polymorphism and elevated insulin concentrations and diastolic blood pressure. In addition, individuals homozygous for all three polymorphisms exhibited significantly higher fasting insulin concentrations than individuals without this genotype combination.

The allele frequencies for the three polymorphisms investigated were compared to frequencies observed in several other populations. In a Japanese cohort, the longer, class II alleles for the OB gene microsatellite polymorphism were found to be present at a frequency of approximately 74% (Shintani et al., 1996). In the Nauruan population the frequency of this group of alleles was 90%.

A frequency of 0.89 was observed for the Arg allele of the Gln223Arg polymorphism in the Nauruan subjects. This is not dissimilar from previous studies conducted in Japanese and Pima Indian populations where the frequency of this allele was found to be approximately 0.85 and 0.75, respectively (Matsuoka et al., 1997; Thompson et al., 1997). It is however, somewhat higher than the frequency reported for British males, 0.44 (Gotoda et al., 1997). The frequency of the pro allele for the PRO1019pro polymorphism, approximately 0.80, also appears to be similar in the Nauruan population to that seen in several other populations. Frequencies of 0.88 and 0.85 were observed for this allele in Japanese and Pima Indian populations, respectively (Matsuoka et al., 1997; Thompson et al., 1997), however, once again the frequency in the Nauruan population was higher than that reported for the British population, 0.36 (Gotoda et al., 1997).

The lack of association between the Gln223Arg polymorphism and obesity or risk factors for diabetes is consistent with earlier studies in British males, Caucasians and Japanese subjects (Gotoda et al., 1997; Matsuoka et al., 1997; Silver et al., 1997). In white British males, no association was observed between the genotype at polymorphic sites in OB-R and obesity or obesity-related phenotypes (Gotoda et al., 1997). A similar lack of association was demonstrated in a cohort of Caucasians and Japanese subjects (Matsuoka et al., 1997; Silver et al., 1997).
The PRO1019pro polymorphism was found to be associated with insulin concentrations and diastolic blood pressure in this Nauruan population. Several earlier studies failed to find an association between this polymorphism and obesity (Gotoda et al., 1997; Matsuoka et al., 1997), however, one study conducted in Pima Indians found that the pro allele was associated with differences in percent body fat (Thompson et al., 1997). Similar to the Nauruan population, Pima Indians have been shown to have remarkably high prevalence rates of obesity and type 2 diabetes. It was surprising to find an association between these variables and the PRO1019pro polymorphism but not the Gln223Arg polymorphism, as these polymorphisms were found to be in linkage disequilibrium, and the same associations would have been expected for both. The reason for this anomaly is unclear from this study, and is the basis of further investigations.

The OB and OB-R genes have been mapped to human chromosomes 7q31.3 and 1p32, respectively. These genes lie in regions identified through genome-wide scans as containing loci important in the development of obesity and type 2 diabetes. Strong evidence of linkage has been demonstrated for insulin precursors and the OB region (Duggirala et al., 1996). In addition, several studies have also reported linkage between other genetic markers and obesity in humans. In the Quebec Family Study, suggestive linkage was found between markers on human 1p32-p22 and body fat and insulin (Chagnon et al., 1997). In the present study, the microsatellite marker in the OB gene was not associated with obesity or risk of diabetes in the Nauruan population. An earlier study in Japanese subjects also failed to find a significant relationship between this region of the OB gene and variables associated with obesity (Shintani et al., 1996).

When we examined the development of diabetes and obesity in approximately 100 of the Nauruan male subjects, there did not appear to be any association between a particular genotype and development of either obesity or diabetes, with any of the three polymorphisms examined. Although the number of subjects in some groups is limited, this preliminary study reveals that approximately the same proportion of men develop diabetes and obesity throughout the twelve year period, irrespective of their genotype at either the OB gene, Gln223Arg or PRO1019pro polymorphisms.

The etiologies of diseases such as obesity and diabetes in humans are complex and often involve a combination of both genetic and environmental risk factors. It has been
suggested that common polymorphisms may represent genetic risk factors that alone are neither necessary nor sufficient for a disease to develop, however, in combination these genes may act to predispose an individual to developing the disease (Morton and Lio, 1997).

A novel study involving the use of a knock-out mouse model of type 2 diabetes has demonstrated that insulin resistance and diabetes can progressively develop as a result of modest genetic defects (Bruning et al., 1997). These defects, present at birth, are thought to alter the expression of molecules involved in the insulin signaling cascade (Bruning et al., 1997; O’Rahilly, 1997). Although the alterations occur in different genes, the combination of these gene defects is thought to act synergistically to initiate a series of metabolic disturbances leading to diabetes through effects on the key components of insulin action (O’Rahilly, 1997).

In addition, four loci have been identified that determine obesity in the BSB mouse, a multifactorial model (Warden et al., 1995). Individually, these loci have varying effects on the level and distribution of body fat in the animal, however the effect is dependent on the genotypes at the other loci. The four loci did not appear to act in a simple additive manner, but rather represented complex interactions among a variety of genes (Warden et al., 1995).

In this study we have demonstrated that Nauruan subjects who have all three sequence polymorphisms investigated exhibit significantly elevated fasting insulin concentrations when compared to individuals in this population without this genetic combination. A recent study in obese Finns demonstrated that subjects with polymorphisms in both uncoupling protein-1 (UCP-1) and β3-adrenergic receptor genes had significantly lower basal metabolic rates than subjects with only one of these polymorphisms (Valve et al., 1998). This study again highlights the observation that complex gene interactions may manifest themselves physiologically in humans.

The major complication of obesity in Pacific Island populations, type 2 diabetes, is known to affect approximately one-third of the adult population of Nauru and is associated with much morbidity and mortality (Dowse et al., 1996). Our study suggests that a specific combination of genotypes may increase the risk of subsequently developing type 2 diabetes in a proportion of Nauruan males. This genotype combination is associated with an
elevated fasting insulin concentration, suggesting the presence of insulin resistance. This study has revealed this association in a unique population, characterised by one of the highest prevalence rates for obesity and type 2 diabetes ever reported.

Longitudinal analysis of approximately 100 of the Nauruan males revealed that overall the individual polymorphisms were not associated with an increased rate of development of obesity of type 2 diabetes. In addition, subjects homozygous for the three polymorphisms did not appear to develop obesity at an increased rate compared to subjects with all other genotype combinations, and if anything, there appeared to be a tendency for a reduced percentage of subjects with all three polymorphisms to develop obesity. The development of type 2 diabetes was also compared between the subjects with all three sequence polymorphisms and those with all other genotype combinations. There was a tendency for the group homozygous for the three polymorphism to have an increased proportion of diabetics, compared to subjects with all other genotype combinations.

The relationship described may be absent in other populations either because of different haplotype combinations at the OB-R gene or because the markers tested may not themselves be functional polymorphisms. Whilst the functionality of these polymorphisms needs to be investigated, the lack of physiological functions at these polymorphic sites would not necessarily rule out the possibility of the presence of linkage disequilibrium between these markers and closely linked functional polymorphisms. This may be particularly important for the PRO1019pro polymorphism, in which the nucleotide substitution does not result in an amino acid change. In addition, any other population differences revealed may in part arise through the effects of distinct selection pressures operating historically in Nauru.

It has been suggested that inconsistent results observed in linkage studies are a consequence of alleles being either too rare or too prevalent in a second population for linkage to be demonstrated (Chagnon et al., 1997). In addition, allele frequencies reported for these same polymorphisms in different populations are known to differ with ethnicity (Shintani et al., 1996, Gotoda et al., 1997; Matsuoka et al., 1997; Silver et al., 1997; Thompson et al., 1997). The causes of obesity and type 2 diabetes in the Nauruan population, therefore may be uniquely different to the causes of these diseases in other populations. Alterations in leptin concentrations or leptin action may be involved in the
development of obesity of type 2 diabetes in this population, and epidemiological evidence from several populations shows a strong relationship between leptin concentrations and body mass as well as insulin resistance (Havel et al., 1996b; Larsson et al., 1996; Zimmet et al., 1996; Bertin et al., 1998; Zimmet et al., 1998).

Nauruans have been demonstrated to have an extraordinarily high prevalence rate of obesity and type 2 diabetes, and the unique geographic isolation of the island further compounds genetic predispositions existing in this population. Genetic studies may uncover novel causes of obesity and type 2 diabetes in the Nauruan population and the present study has revealed that insulin resistance in this population may partly be a consequence of alterations in leptin and the OB-R system. The polymorphisms studied may not themselves be functional, however confirmation of an interaction between OB and OB-R polymorphisms in other populations would add strength to these findings.
CHAPTER 6

CROSS-SECTIONAL CHARACTERISATION OF PLASMA LEPTIN IN *PSAMMOMYS OBESUS*

6.1 SUMMARY
Leptin, the protein product of the OB gene has been shown to be involved in the regulation of body weight in several rodent models of obesity. In most animal models, as well as in humans, leptin concentrations increase with increasing adiposity and have been found to be associated with circulating insulin, and in some cases, sex and age. The role of leptin in polygenic models of obesity and in humans remains unclear, and in this study we examined associations between circulating leptin concentrations and various markers of obesity and diabetes in *Psammomys obesus*, a polygenic rodent model of obesity and type 2 diabetes. *Psammomys obesus* display a range of glucose, insulin and leptin concentrations, similar to those seen in various human populations, and obese *Psammomys* have previously been demonstrated to have increased OB gene expression and circulating plasma leptin concentrations compared to their lean littermates. In this study we have found that leptin concentrations are associated with percent body fat ($r=0.25$, $p=0.006$), insulin ($r=0.36$, $p=0.001$) and glucose concentrations ($r=0.19$, $p=0.03$), in a population of *Psammomys obesus* ($n=130$), however there was no relationship between leptin and age ($r=0.08$, $p=0.36$). The association between leptin and insulin was found to be independent of percent body fat ($r=0.23$, $p=0.01$). We also examined the influence of sex, obesity and diabetes on plasma leptin concentrations, however differences in plasma leptin between these groups were found to be dependent on adiposity, as after adjustment for percent body fat there was no difference in plasma leptin concentrations between male and female, lean and obese, or diabetic and non-diabetic, *Psammomys obesus*. Linear regression analysis revealed that in this group of *Psammomys obesus*, circulating insulin concentrations alone were the best predictor of leptin concentrations, however this regression model only accounted for approximately 13% ($R^2=0.359$) of the variability in leptin in this polygenic rodent model of obesity, indicating that the major determinants of leptin concentrations in *Psammomys obesus* may not have been measured in this study and remain to be determined.
6.2 INTRODUCTION

Leptin is the protein product of the OB gene (Zhang et al., 1994) and administration of exogenous leptin to several rodent models of obesity has been shown to result in reduced food intake, increased thermogenesis and subsequently decreased body weight and body fat (Halaas et al., 1995; Pellymouther et al., 1995; Levin et al., 1996; Scarpace and Matheny, 1997). The role of leptin in regulation of energy balance was first demonstrated in the ob/ob mouse, a model found to have a defect in the OB gene which results in a failure to produce functional leptin (Zhang et al., 1994; Campfield et al., 1995; Pellymouther et al., 1995). In most other animal models of obesity, circulating leptin concentrations have been shown to increase with increasing adiposity (Maffei et al., 1995a,b; Frederick et al., 1995b; Considine et al., 1996c) with leptin levels found to be increased 10-fold in db/db and ugouti mice, 5-fold in fat mice and 2-fold in tubby mice, relative to lean littersmates (Maffei et al., 1995b). The results of this early study by Maffei et al. have subsequently been supported by similar studies of mice and additionally in several rat models including the falfa and Koletsky rat (Hiroaka et al., 1997). Non-genetic rodent models of obesity also show increased leptin levels in obese animals, as do polygenic mice models such as the diet-induced obese mouse (Maffei et al., 1995a) and Psammomys obesus (Walder et al., 1997b). In addition to the strong relationship between leptin concentrations and body weight and body fat, leptin has also been shown to correlate with circulating insulin concentrations and age in rodents (Ahren et al., 1997; Collier et al., 1997c; Li et al., 1997; Li et al., 1998b).

In humans and non-human primates, there is also a strong relationship between leptin concentrations and body weight or percent body fat. In humans, leptin concentrations are increased in obese individuals (Considine et al., 1995; Maffei et al., 1995b) and there is a strong relationship between circulating insulin and leptin concentrations, although the association is not always seen to be independent of adiposity (Maffei et al., 1995b; Zimmet et al., 1996). In humans, females have also been found to have higher circulating leptin concentrations than males, although again this relationship does not always persist when the leptin levels are adjusted for body fat mass (Havel et al., 1996a; Bennett et al., 1997). Rhesus monkeys are a useful primate model of human obesity and similar relationships are seen in these animals between circulating leptin and body weight, body fat and plasma insulin concentrations as those seen in human populations (Bodkin et al., 1996).
Psammomys obesus is a polygenic rodent model of obesity and type 2 diabetes mellitus that is unique, because when held in captivity on a diet of standard laboratory chow, a proportion of animals remain lean and healthy, while approximately half the animals develop obesity, and about one-third develop diabetes (Shaffir and Gutman, 1993; Barnett et al., 1994a). Psammomys obesus also exhibit a range of circulating insulin, glucose and leptin concentrations, similar to those seen in human populations (Shaffir and Gutman, 1993; Barnett et al., 1994a). Initial studies in Psammomys obesus indicated that OB gene expression and plasma leptin concentrations were associated with body weight, body fat and circulating insulin concentrations (Collier et al., 1997b; Walder et al., 1997b). In addition, a short-term longitudinal study (8 weeks) demonstrated increases in plasma leptin with increasing age in Psammomys obesus between the ages of 4 and 12 weeks, although the increase was only seen in animals who were developing obesity during the same period of time (Collier et al., 1997c). The present study aims to further examine the relationships between plasma leptin concentrations and phenotypic measures of obesity and diabetes in a large group of Psammomys obesus who are over 15 weeks of age. Associations between leptin concentrations and circulating metabolites, as well as body weight and body fat are examined in an effort to determine the best predictors of plasma leptin concentrations in older Psammomys obesus.
6.3 MATERIALS AND METHODS

6.3.1 EXPERIMENTAL RESULTS

A *Psammomys obesus* colony is maintained at Deakin University, with breeding pairs fed *ad libitum* a diet of lucerne and chow. Experimental animals were weaned at 4 weeks of age and given a diet of standard laboratory chow (12% energy from fat, 63% from carbohydrates, 25% from protein; Barastoc, Pakenham, Australia). Animals were housed in a temperature-controlled room (22°C±1°C) with a 12-hour/12-hour light/dark cycle.

6.3.2 CHARACTERISATION OF ANIMALS

A total of 130 *Psammomys obesus* aged over 15 weeks (15-23 weeks of age) were used in this study. Of the 130, 47 animals were female and 83 were male. Where animals are divided into lean and obese, lean animals are classified as those animals with a body weight below 200g (n=50) and obese animals as those animals with a body weight greater than 200g (n=80). Where animals are divided into diabetic and non-diabetic, non-diabetic animals are classified as those with a blood glucose <8.0 mmol/l (n=27) and diabetic animals as those with a blood glucose ≥8.0 mmol/l (n=103). When animals were classified as lean and healthy, obese, non-diabetic and obese, diabetic the following classifications were used, lean, healthy: plasma insulin <150 μU/ml, blood glucose <8.0 mmol/l (n=64); obese, non-diabetic: plasma insulin ≥150 μU/ml, blood glucose <8.0 mmol/l (n=40); obese, diabetic: blood glucose ≥8.0 mmol/l; plasma insulin ≥150 μU/ml (n=26).

6.3.3 BIOCHEMICAL ANALYSES

Whole blood glucose was measured using an enzymatic glucose analyser (Model 2300 STAT Plus, Yellow Springs Instruments, Ohio). Plasma insulin concentrations were determined using a commercially available double antibody solid phase radioimmunoassay (Phadeseph, Kabi Pharmacia Diagnostics, Sweden) and plasma leptin concentrations were measured using a commercially available radioimmunoassay kit (Multispecies leptin assay; Linco Reaserch, St Charles, MO, USA). A multispecies leptin antibody (produced in guinea pig) was used in this kit, with a 125I-human leptin tracer and a normal guinea pig IgG antibody added as the second antibody. The concentration of leptin in plasma from *Psammomys obesus* was determined using human leptin standards of known concentrations and as the multispecies leptin antibody was raised against human leptin and human leptin standards were used, the units of measurement are ng/ml Human Equivalent (H.E.) leptin.
6.3.4 ESTIMATED PERCENT BODY FAT
When *Psammomys obesus* were between 15 and 23 weeks of age the animals were euthanased by lethal overdose of pentobarbitone (120 mg/kg) and adipose tissue from several depots removed. The weight of selected fat depots (suprascapular, perirenal, epididymal, intramuscular and mesenteric) was used to provide an estimate of body fat content and the weights of the various depots were combined and divided by total body mass to provide an estimate of percent body fat.

6.3.5 STATISTICAL ANALYSES
The one-sample Kolmogorov-Smirnov test was used to test variables for normality and non-normal variables were log-transformed (natural log) to normalise the distribution, and subsequently used in all analyses. Differences in mean leptin concentrations between different groups of *Psammomys obesus* were tested for significance using a univariate analysis of variance, with estimated percent body fat as a covariate, and pair-wise comparisons used in analyses as stated. Bivariate and partial correlations between variables were assessed for significance using the Pearson correlation coefficient. In all statistical tests a p-value less than 0.05 was considered significant. All statistical analyses were performed using SPSS (version 8.0.2).
6.4 RESULTS

**Table 6.1** Metabolic characteristics of *Psammomys obesus* (n=130)

<table>
<thead>
<tr>
<th></th>
<th>Mean±SEM</th>
<th>Range</th>
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<tbody>
<tr>
<td>Age (weeks)</td>
<td>18.3±0.17</td>
<td>15-23</td>
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<tr>
<td>Body weight (g)</td>
<td>206.6±3.1</td>
<td>126.0-278.0</td>
</tr>
<tr>
<td>Estim. Percent Body Fat (%)</td>
<td>4.31±0.16</td>
<td>1.35-8.93</td>
</tr>
<tr>
<td>Blood Glucose (mmol/l)</td>
<td>6.6±0.37</td>
<td>2.6-22.2</td>
</tr>
<tr>
<td>Plasma Insulin (µU/ml)</td>
<td>228.6±17.1</td>
<td>13.9-837.4</td>
</tr>
<tr>
<td>Plasma Leptin (ng/ml H.E.)</td>
<td>53.9±3.02</td>
<td>2.3-160.6</td>
</tr>
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</table>

Table 6.1 shows the metabolic characteristics of the population of *Psammomys obesus* used in this study.
Figure 6.1 shows that plasma leptin concentrations correlate significantly with estimated percent body fat \((r=0.25, p=0.006)\) and insulin concentrations \((r=0.36, p=0.001)\) in *Psammomys obesus*.

**Figure 6.1.** Associations between plasma leptin in *Psammomys obesus* \((n=130)\) and a) estimated percent body fat, and b) plasma insulin (Pearson correlation coefficient).
Table 6.2  Bivariate and partial Pearson correlation coefficients for associations between plasma leptin and phenotypic measures in *Psammomys obesus*.

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>Bodyweight</th>
<th>Estimated % Body Fat</th>
<th>Blood Glucose*</th>
<th>Plasma Insulin*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bivariate</strong></td>
<td>r=0.08</td>
<td>r=0.17</td>
<td>r=0.25</td>
<td>r=0.19</td>
<td>r=0.36</td>
</tr>
<tr>
<td><strong>Correlation:</strong></td>
<td>p=0.36</td>
<td>p=0.056</td>
<td>p=0.006</td>
<td>p=0.03</td>
<td>p=0.001</td>
</tr>
<tr>
<td><strong>Partial</strong></td>
<td>r=0.11</td>
<td>r=0.06</td>
<td>-</td>
<td>r=0.091</td>
<td>r=0.23</td>
</tr>
<tr>
<td><strong>Correlation:</strong></td>
<td>p=0.23</td>
<td>p=0.53</td>
<td>p=0.36</td>
<td>p=0.011</td>
<td></td>
</tr>
</tbody>
</table>

**adjacent for estimated percent body fat
*log transformed

Similar to figure 6.1, table 6.2 shows that circulating leptin concentrations are significantly correlated with estimated percent body fat (r=0.25, p=0.006) and insulin concentrations (r=0.36, p=0.001). Table 6.2 also shows that leptin concentrations correlated significantly with blood glucose (r=0.19, p=0.03), while there was evidence of an association between leptin concentrations and body weight (r=0.17, p=0.06). In an effort to examine relationships between leptin and metabolic variables independent of the effect of adiposity, partial correlations were examined, with the correlation adjusted for estimated percent body fat. The relationship between body weight and leptin concentrations appeared to be dependent on percent body fat (adj.r=0.06, p=0.53), as was the relationship between blood glucose concentration and leptin (adj.r=0.09, p=0.36). The relationship between leptin and plasma insulin concentration was found to persist, even after adjustment for percent body fat (adj.r=0.23, p=0.01).
Figure 6.2. Estimated marginal mean plasma leptin concentrations in a) female and male, b) diabetic and non-diabetic and c) lean and obese Psammomys obesus, after adjustment for estimated percent body fat.
We also examined the effect of sex, type 2 diabetes and obesity on plasma leptin concentrations in *Psammomys obesus*. Male animals tended to have higher plasma leptin concentrations compared to female animals (44.39±1.08 vs 36.6±1.11, male vs female) although after adjustment for estimated percent body fat, the plasma leptin concentrations tended to be higher in female animals (50.60±1.13 vs 42.52±1.08, female vs male; figure 6.2a). Diabetic *Psammomys obesus* had significantly higher leptin concentrations compared to non-diabetic animals (54.98±1.15 vs 39.81±1.07, diabetic vs non-diabetic; p=0.05), although after adjusting for estimated percent body fat, this difference was no longer significant (43.77±1.18 vs 45.29±1.08, diabetic vs non-diabetic; figure 6.2b). Plasma leptin concentrations were also compared between lean and obese *Psammomys obesus* and it was found that obese animals had significantly higher levels of circulating leptin compared to lean animals (48.33±1.08 vs 35.66±1.11, obese vs lean; p=0.02). However, after adjusting for adiposity, the difference in leptin was no longer significant, although obese animals still tended to have higher leptin concentrations (48.38±1.09 vs 41.10±1.13, obese vs lean; figure 6.2c).
Figure 6.3. Estimated marginal mean plasma leptin concentrations in lean, obese, non-diabetic and obese, diabetic *Psammomys obesus*, after adjustment for estimated percent body fat.

In many studies conducted in our laboratory with *Psammomys obesus*, the animals are classified as lean, obese, or obese and diabetic. This study also examined differences in leptin concentrations between these groups of animals. Leptin concentrations in lean *Psammomys obesus* were significantly lower than leptin concentrations in obese (34.36±1.09 vs 51.68±1.11, lean vs obesc; p=0.005) and obesc, diabetic (34.36±1.09 vs 55.37±1.14, lean vs obese, diabetic; p=0.005) animals. After the leptin concentrations were adjusted for estimated percent body fat, the differences in plasma concentrations largely disappeared (41.89±1.13 vs 50.91±1.11 vs 45.02±1.19; lean vs obese vs obese diabetic animals; figure 6.3).
Table 6.3  Multiple regression analysis in *Psammomys obesus* with plasma leptin concentration as the dependent variable.

<table>
<thead>
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<tbody>
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<td>constant</td>
<td>2.339</td>
<td>0.335</td>
<td>6.98</td>
<td>0.0001</td>
</tr>
<tr>
<td>Insulin*</td>
<td>0.279</td>
<td>0.065</td>
<td>4.26</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Leptin was entered as the dependent variable and the following predictors were in the regression model: age, body weight (or percent body fat), insulin and glucose. $R^2=(0.359)^2=12.9\%$.

*log transformed

Table 6.3 shows the result of step-wise linear regression analysis. In an attempt to determine the best predictors of plasma leptin concentrations in *Psammomys obesus* we performed regression analysis with leptin concentration as the dependent variable and the following predictors included in the model: age, glucose, estimated percent body fat or body weight, plasma insulin and blood glucose concentrations. Step-wise regression results showed plasma insulin to be a significant predictor of plasma leptin, (p=0.0001) with all other predictors excluded as they did not significantly add to the model (age p=0.69, glucose p=0.89, bodyweight p=0.47). This model (plasma insulin) predicted approximately 12.9% of the variability in plasma leptin seen in this group of *Psammomys obesus*.
6.5 DISCUSSION

In this study we have examined associations between plasma leptin concentrations in *Psammomys obesus* and various markers of the obese and diabetic phenotype. Plasma leptin concentrations were found to be associated with body weight, percent body fat, plasma insulin concentrations and blood glucose concentrations, while leptin was not associated with age in this group of animals. After adjusting for percent body fat, the relationships between leptin and glucose, and leptin and body weight, were no longer significant, although the relationship between leptin and insulin persisted. There was no difference in mean plasma leptin concentrations between male and female, diabetic and non-diabetic or lean and obese *Psammomys obesus* after adjusting for percentage body fat.

Several studies in rodents have demonstrated a relationship between leptin concentrations and age (Ahren et al., 1997; Collier et al., 1997c; Li et al., 1997; Li et al., 1998b) although there was no association between these variables in the present study. The studies conducted in mice demonstrated that the age-dependent increase in leptin was largely accounted for by increasing body weight, although Ahren et al. (1997) demonstrated a relationship between leptin and age that was found to be independent of adiposity in wild-type mice. In a short term longitudinal study conducted in *Psammomys obesus*, Collier et al. (1997c) found that age was associated with plasma leptin concentrations however the relationship was seen only in animals which developed obesity and type 2 diabetes, and not in animals that remained lean and healthy during the 8-week study period. During the 8 weeks of study, both groups of animals increased bodyweight significantly, in accordance with this phase of development, however the increase in bodyweight was not accompanied by increases in leptin concentration in lean animals, as it was in obese animals (Collier et al., 1997c). At the end of the study (at 12 weeks of age) plasma leptin concentrations were significantly elevated in the obese animals compared to the lean animals, although it is unclear if the higher plasma leptin levels in the obese animals were accounted for by their significantly higher body weight. It is known that *Psammomys obesus* than develop obesity become hyperphagic between 4 and 10 weeks of age (Barnett et al., 1995) and it appears that obese animals also develop leptin resistance during this time (Collier et al., 1997c). The animals used in the present study were between 15 and 23 weeks of age and there was no relationship between age and circulating leptin concentrations in these animals. It may be that leptin plays a role in the regulation of energy balance in *Psammomys obesus* during an early stage of development, and this effect is not seen in
older animals, which have already become obese. Alternatively it may be that a cross-sectional study is insufficient to determine the effect of age on leptin in *Psammomys obesus*.

In this group of *Psammomys obesus* there was no difference in plasma leptin concentrations between male and female animals after adjustment for body fat mass. Many reports examining leptin levels in mouse models of obesity use either all male or all female animals, and the effect of sex on leptin levels in mice has not been widely reported. In several rat models, Landt *et al.* (1998) demonstrated a reversal of the sexual dimorphism in leptin concentrations seen in humans, with leptin concentrations found to be higher in male rats compared to female rats, consistent with male rats actually having a larger fat mass that females. In humans, leptin concentrations have been found to be higher in females compared to males, and in most studies this relationship is independent of adiposity (Havel *et al.*, 1996a; Rosenbaum *et al.*, 1996; Bennett *et al.*, 1997). In *Psammomys obesus*, we have demonstrated that the effect of sex on plasma leptin is minor and appears to be dependent on fat mass in these animals.

Circulating insulin concentrations have been reported to be strongly associated with leptin concentrations in rodents (MacDougald *et al.*, 1995; Saladin *et al.*, 1995; Igel *et al.*, 1996b; Ahren *et al.*, 1997), monkeys (Bodkin *et al.*, 1996) and humans (Considine *et al.*, 1996c; Havel *et al.*, 1996b; Larsson *et al.*, 1996; Bertin *et al.*, 1998). In some cases this relationship is independent of adiposity, however this is not always seen. The Rhesus monkey is a well characterised model of spontaneous adult-onset obesity that is very similar to human middle-age onset obesity. Bodkin *et al.* (1996) demonstrated that in Rhesus monkeys, plasma leptin concentrations were strongly and significantly related to fasting plasma insulin secretion and acute insulin response to glucose. In this study there was a strong relationship between plasma insulin and leptin concentrations which was independent of body fat. In two earlier studies in *Psammomys obesus*, plasma leptin concentrations were also found to be strongly associated with insulin concentrations (Collier *et al.*, 1997b; Walder *et al.*, 1997b), although this relationship was not examined independently of adiposity in either study. Walder *et al.* (1997b) did however compare leptin levels in weight-matched insulin-resistant and insulin-sensitive *Psammomys obesus* (n=8 in each group) and leptin concentrations were significantly higher in the insulin resistant animals in this study (Walder *et al.*, 1997b).
When circulating plasma leptin concentrations were examined in lean, obese and obese, diabetic *Psammomys obesus* there was no difference in plasma leptin concentrations after adjusting for percent body fat. The obese *Psammomys obesus* were also hyperinsulinemic and this classification of animals allows examination of the effects of insulin, separate to body fat. Normoinsulinemic (lean, healthy) *Psammomys obesus* had slightly lower leptin concentrations compared to hyperinsulinemic (obese and obese, diabetic) animals independent of adiposity, although this difference was not significant. In addition, in the obese animals only, there was no difference in leptin concentrations between obese, diabetic and obese, non-diabetic animals, thereby indicating that the presence of diabetes (hyperglycemia) does not further influence plasma leptin concentrations independently of body fat and insulin.

Linear regression analysis was performed in an attempt to determine which of the phenotypic measures examined best predicted plasma leptin concentrations in *Psammomys obesus*. In a stepwise regression containing body weight, plasma insulin, blood glucose and age the only variable to remain in the model was circulating insulin concentrations, with all other variables excluded as they did not significantly influence plasma leptin concentrations any further than insulin alone. Surprisingly however, the $R^2$ for this model was only 12.9%, indicating that the major predictor(s) of plasma leptin concentrations in this group of *Psammomys obesus* was not examined in this study and remains to be determined.

In Rhesus monkeys, body fat and insulin concentrations have been reported to account for approximately 63% of the variability in circulating leptin concentrations (Bodkin *et al.*, 1996). There was no increase in this value with the addition of body weight, β-cell acute insulin response to glucose, or whole body insulin-mediated glucose disposal rate, demonstrating that body fat and insulin concentrations were the best predictors of circulating leptin concentrations, independent of peripheral insulin sensitivity in monkeys (Bodin *et al.*, 1996). In the present study, the variability in plasma leptin concentrations in *Psammomys obesus* is best predicted by circulating insulin concentrations, which is similar to the results in non-human primates and humans, where insulin concentrations are significant predictors of leptin concentrations. These findings, coupled with earlier work in *Psammomys obesus*, demonstrate that this polygenic model is one of the best rodent
models of obesity and represents a very useful tool through which to investigate the role of the leptin regulatory pathway in energy balance in humans, as it so closely resembles the human situation.

In summary, we have found that plasma leptin concentrations are significantly associated with body weight, percentage body fat, circulating glucose and circulating insulin concentrations in a large group of *Psammomys obesus*, while there is no relationship between leptin concentrations and age. In addition, there was no difference in leptin concentrations between male and female animals, while differences in leptin concentrations between lean and obese and diabetic and non-diabetic *Psammomys obesus* were found to be dependent on adiposity. In this population of *Psammomys obesus*, circulating insulin concentration was found to be the best predictor of plasma leptin concentrations, although it accounted for roughly 13% of the variability in leptin concentrations seen in this polygenic model of obesity.
CHAPTER 7

CHARACTERISATION OF LEPTIN RECEPTOR GENE
EXPRESSION IN A POLYGENIC ANIMAL MODEL OF
OBESITY AND DIABETES, *PSAMMOMYS OBESUS*

7.1 SUMMARY

Leptin administration has been shown to decrease body weight and body fat in several animal models of obesity. Some animals respond to leptin administered peripherally, however others only show decreased food intake and body weight with central administration. Other animal models such as *db/db* mice do not respond to leptin at all. It has been postulated that the varying degrees of leptin resistance in these animal models are at least partly responsible for the development of obesity, although the importance of leptin resistance in polygenic animal models of obesity is not well characterised. *Psammomys obesus* is a polygenic rodent model of obesity and type 2 diabetes which exhibits a range of body weight and blood glucose and plasma insulin and leptin concentrations. We have previously demonstrated that *Psammomys* exhibit a range of responses to exogenous leptin administration, with lean animals found to be relatively leptin-sensitive compared to obese *Psammomys*, which do not respond to even very high doses of leptin. In several rodent models, leptin resistance has been found to result from defects within the gene for the leptin receptor, OB-R, with mutations identified which alter the functioning of this receptor. The leptin receptor has several alternatively spliced isoforms, and this study examined the sequence and tissue distribution of OB-RB (the longer, main signaling isoform of OB-R) and OB-RA (a short isoform of OB-R) in lean, obese and diabetic *Psammomys obesus*. OB-RA and OB-RB were found to be expressed in a variety of tissues in *Psammomys obesus*. In addition, this study demonstrates decreased expression of OB-RA, OB-RB and c-fos in the hypothalamus of group B (obese, non-diabetic) and group C (obese, diabetic) *Psammomys obesus* compared to group A (lean, healthy) controls (OB-RA: 0.08±0.02 and 0.06±0.02 vs 0.40±0.16; group B and C vs group A, respectively; OB-RB: 0.04±0.01 and 0.05±0.01 vs 0.22±0.07, group B and C vs group A, p<0.03 and p<0.07, respectively). In addition, the expression of OB-RB in the hypothalamus correlated inversely with plasma insulin concentration (r=-0.49, p=0.004) and was directly associated with c-fos activation (r=0.80, p=0.001). In the liver, group C *Psammomys obesus* had decreased levels of OB-RA (3.6±0.6 vs 40.0±10.1, p<0.003; group C vs group A) and
OB-RB (1.7±0.4 vs 7.6±1.15, p<0.003; group C vs group A) gene expression, compared to group A animals. After adjusting for the degree of adiposity, OB-RA and OB-RB gene expression in the liver was also found to be negatively associated with glucose and insulin concentrations. In suprascapular white adipose tissue no differences were found in OB-RA and OB-RB gene expression between the three groups of Psammomys obesus, however expression of OB-RB was found to be positively associated with percent body fat (r=0.43, p=0.03). The results suggest that the decreased level of leptin signaling in the hypothalamus of obese, hyperleptinemic Psammomys obesus may contribute to the apparent leptin resistance in these animals.
7.2 INTRODUCTION

Leptin, the protein product of the OB gene has consistently been shown to regulate energy balance in animal models of obesity and type 2 diabetes. Leptin administration to the ob/ob mouse, an animal found to have undetectable levels of circulating leptin, results in decreased food intake, increased thermogenesis and subsequently decreased body weight and body fat (Campfield et al., 1995; Halaas et al., 1995).

It is now apparent that obesity in several other rodent models results at least partly, from defects in signaling by the leptin receptor, OB-R. This receptor has several alternatively spliced isoforms which differ in the length of their intracellular domains (Lee et al., 1996; Tartaglia et al., 1996). OB-RB has a long intracellular domain that has been shown to activate signal transduction pathways within the cell and is thought to represent the main signaling form of the receptor (Bjorbaek et al., 1997; Takahashi et al., 1996b; White et al., 1997a; Elmquist et al., 1998a). OB-RA is a much shorter isoform and whilst it has some signaling capacity, the localisation of this receptor to the choroid plexus has led to the suggestion that OB-RA in the hypothalamus acts to transport leptin across the blood-brain barrier and into the cerebrospinal fluid (Tartaglia et al., 1996; Takahashi et al., 1997). In addition to the central nervous system, both OB-RA and OB-RB have been found to be expressed in a wide variety of peripheral tissues, including the liver, kidney, lung, heart, spleen, adipose tissue, reproductive organs, gastrointestinal tract, pancreas and skeletal muscle in a number of animal species, including humans (Lee et al., 1996; Fei et al., 1997; Hoggard et al., 1997; Lollman et al., 1997; Luoh et al., 1997; Zamerano et al., 1997), however the role of leptin receptors in these tissues remains to be determined.

Like other receptors of their type, leptin receptors lack intrinsic tyrosine kinase activity and require co-operation of associated JAK kinases for phosphorylation and activation of the signal transducing STAT proteins (Ihle et al., 1994; Nakashima et al., 1997b). Activation of STATs results in translocation to the nucleus where they bind DNA and thereby regulate transcription (Ihle et al., 1994). OB-RB is known to activate several STAT proteins, including STAT3 (Baumann et al., 1996; Ghilardi et al., 1996; Takahashi et al., 1996b). Tyrosine phosphorylation of STAT3 results in stimulation of c-fos gene transcription in vitro (Bjorbaek et al., 1997; Hakansson et al., 1998b). C-fos is a major component of the AP-1 transcription factor complex which also includes members of the JUN family (Ryseck and Bravo, 1991). Central administration of leptin has been reported to increase
c-fos-like immunoreactivity within several regions of the hypothalamus, including the paraventricular nucleus, the dorso-medial nucleus, the arcuate nucleus and the central amygdala (Bjorbaek et al., 1997; Elmquist et al., 1998a; Hakansson et al., 1998a).

Several groups have now demonstrated that fasting increases OB-RB gene expression several fold in different regions of the hypothalamus in wild-type mice and Sprague-Dawley, Wistar and Zucker (fa/fa) rats (Fei et al., 1997; Lin and Huang, 1997; Baskin et al., 1998; Bennett et al., 1998). These studies provide evidence of a feed-back loop, by which a reduction in circulating leptin (as with fasting) results in up-regulation of leptin receptor gene expression as part of a regulatory mechanism. The study by Lin and Huang also showed that in ob/ob mice, which lack circulating leptin, fasting does not increase leptin receptor gene expression, providing further support for the hypothesis that the circulating leptin concentration is an important regulator of OB-R expression. These findings however, do not rule out the possibility that other factors play a role in the regulation of leptin receptor gene expression, particularly in polygenic models of obesity, which more closely mimic the situation of human obesity.

The regulation of OB-RA and OB-RB gene expression in peripheral tissues is also complex and is not well characterised, with most work in this area focusing on the regulation of leptin receptor levels during pregnancy and lactation. Although the role of peripheral leptin receptors remains unclear, many studies have now demonstrated direct actions of leptin in several peripheral tissues, which are possibly mediated by OB-RA and OB-RB (Barazilai et al., 1997; Cohen et al., 1997; Muioi et al., 1997; Shimabukuro et al., 1997). To further examine the role of leptin and leptin receptors in energy balance, this study examined leptin receptor gene expression in the hypothalamus, liver and adipose tissue of Psammomys obesus with a range of body weight and blood glucose, plasma leptin and insulin concentrations.

Psammomys obesus is a polygenic rodent model of obesity and type 2 diabetes which exhibits a range of body weight and blood glucose and insulin concentrations (Shafrir and Gutman, 1993; Barnett et al., 1994a) which closely resemble that seen in human populations (DeFronzo, 1988). Obese Psammomys have been previously shown to have increased OB gene expression and elevated plasma leptin concentrations compared to their lean littermates (Walder et al., 1997b). In addition, studies involving peripheral leptin
administration to lean and obese *Psammomys obesus* have demonstrated that only lean animals are sensitive to the anorexigenic effects of leptin administration, with decreased food intake and body weight observed in these animals after peripheral leptin administration (Walder et al., 1999). Obese *Psammomys obesus* show marked leptin resistance, with even very high doses of leptin ineffective in these animals. In the present study, gene expression of OB-RA, OB-RB and c-fos is examined in *Psammomys obesus*, with associations between leptin receptor gene expression in the hypothalamus, liver and adipose tissue and circulating metabolites such as glucose, insulin and leptin also examined.
7.3 MATERIALS AND METHODS

7.3.1 EXPERIMENTAL ANIMALS

A Psammomys obesus colony is maintained at Deakin University, with breeding pairs fed ad libitum a diet of lucerne and chow. Experimental animals were weaned at 4 weeks of age and given a diet of standard laboratory chow (12% energy from fat, 63% from carbohydrates, 25% from protein; Barastoc, Pakenham, Australia). Animals were housed in a temperature-controlled room (22±1°C) with a 12-hour/12-hour light/dark cycle (See Chapter 2 for further details).

7.3.2 CHARACTERISATION OF ANIMALS

At 16 weeks of age animals were classified into one of three groups depending on their blood glucose and plasma insulin concentrations. Group A animals were those with a glucose concentration <8.0 mmol/L and an insulin concentration <150μU/ml (n=10). Group B animals had a glucose concentration <8.0 mmol/L and an insulin concentration ≥150μU/ml (n=11). Group C animals had a glucose concentration ≥8.0 mmol/L and an insulin concentration ≥150μU/ml (n=10), see table 6.1 for group characteristics.

7.3.3 RNA PREPARATION AND cDNA SYNTHESIS

Animals were killed by lethal overdosage of pentobarbitone (120mg/kg) and the following tissues were removed: hypothalamus, liver, pancreas, skeletal muscle (gastrocnemius), testis, small intestine, heart, kidney, lung and adipose tissue from the suprascapular, perirenal, intramuscular, epididymal and mesenteric fat depots. After homogenisation, RNA was purified from the whole hypothalamus or 100mg of each tissue using RNAzol B (Bresatec, Adelaide, Australia). The RNA was extracted with 200μl of chloroform and precipitated with an equal volume of isopropanol. RNA was quantitated by spectrophotometry at 260 nm and 1μg was then reverse transcribed at 42°C for 1 hr with 15U of AMV reverse transcriptase (Promega, Madison, USA) according to the manufacturer’s instructions.

7.3.4 POLYMERASE CHAIN REACTION (PCR) AND QUANTITATION

Comparative RT-PCR was performed using the Taqman ‘Real Time’ system (Perkin Elmer Applied Biosystems, Forster City, USA). This system utilises a fluorescent probe within the PCR reaction to detect the amplification of the appropriate region of the cDNA (Gibson et al., 1996). The level of fluorescence emitted is sampled every 7 seconds during the 40
cycles of amplification and is directly proportional to the amount of product generated (Gibson et al., 1996; Heid et al., 1996; See Chapter 2 for further details). All quantitation of gene expression reported is expressed relative to the ribosomal RNA house-keeping gene (Perkin Elmer Applied Biosystems, Forster City, USA). The sequences of the oligonucleotide primers and probes used for amplification of OB, OB-RA, OB-RB and c-fos were based on the *Psammomys obesus* sequence and lie in regions found to be highly conserved across several species including rat, mouse and human.

**OB gene:**
- **forward primer**: 5'-tcgcgggtctaccaacacat-3'
- **reverse primer**: 5'-ctcttagggcgtgatttga-3'
- **probe**: 5'- FAM tgaacagcctgctttccggaat TAMRA-3'

**OB-RA:**
- **forward primer**: 5'-tgctctgctgctggaaacact-3'
- **reverse primer**: 5'-ctctctctctctctctctgt-3'
- **probe**: 5'- FAM atgtccaaaaaacacaggttctg TAMRA-3'

**OB-RB:**
- **forward primer**: 5'-aagctggacatgctctctctct-3'
- **reverse primer**: 5'-gcgtcgcagctgctctctctct-3'
- **probe**: 5'- FAM atcagctgtatatggctctttctttcg TAMRA-3'

**c-fos:**
- **forward primer**: 5'-tgaagagagaagaaaaacgagttta-3'
- **reverse primer**: 5'-cgaggggagccacagca-3'
- **probe**: 5'- FAM tggcagccccacgacctgc TAMRA-3'

**Ribosomal RNA:**
- **forward primer**: 5'-cgctaccacatcagga-3'
- **reverse primer**: 5'-gctggaaattccgccgct-3'
- **probe**: 5'- VIC tgcgtggcaccagactggcct TAMRA-3'

PCR was performed by adding 50 ng of cDNA to a tube containing: 1xTaqMan Universal PCR Master Mix (AmpliTaqt Gold™, AmpErase® Uracil N-glycosylase (UNG), dNTPs, 5x TaqMan Buffer A (with ROX passive reference) 12.5 mM MgCl₂ and glycerol (Perkin Elmer Applied Biosystems, Foster City, USA), and the appropriate concentration of primers and probe (see chapter 2) in a final volume of 25 μl in a MicroAmp® Optical 96-
well Reaction Plate covered with MicroAmp® Optical caps (Perkin Elmer Applied Biosystems, Foster City, USA). Amplification was performed using the following conditions: 50°C for 2 mins, 95°C for 10 mins and 40 cycles of 95°C for 15 sec and 60°C for 1 min.

7.3.5 DNA SEQUENCING
PCR products were excised from a TAE agarose gel, purified using Wizard™ PCR preps (Promega, Madison, USA) and the sequence determined by fluorescent DyeDeoxy Terminator chemistry (Perkin Elmer Applied Biosystems, Foster City, USA) using an Applied Biosystems 373A automated DNA sequencer (Perkin Elmer Applied Biosystems, Foster City, USA). The Psammomys obesus DNA sequences were aligned using the Blast program on the Genbank database.

7.3.6 BIOCHEMICAL ANALYSES
Whole blood glucose was measured using an enzymatic glucose analyser (Model 2300 STAT Plus, Yellow Springs Instruments, Ohio). Plasma insulin concentrations were determined using a double antibody solid phase radioimmunoassay (Phadeseph, Kabi Pharmacia Diagnostics, Sweden). Plasma leptin concentrations were measured using a commercially available radioimmunoassay kit (Multispecies leptin assay) (Linco Research, St Charles, MO, USA). A multispecies leptin antibody (produced in guinea pig) was used in this kit, with a ^125^I-human leptin tracer and a normal Guinea pig IgG antibody added as the second antibody. The concentration of leptin in plasma from Psammomys obesus was determined using human leptin standards of known concentrations and as the multispecies leptin antibody was raised against human leptin, and human leptin standards were used, the units of measurement are ng/ml Human Equivalent (HE) leptin.

7.3.7 ESTIMATED PERCENT BODY FAT
The weight of selected fat depots (suprascapular, perirenal, epididymal, intramuscular and mesenteric) was used to provide an estimate of body fat content. The weights of the various depots were combined and divided by total body mass to provide an estimate of percent body fat.
7.3.8 STATISTICAL ANALYSES

All statistical analyses were performed using SPSS (version 8.0). The one-sample Kolmogorov-Smirnov test was used to test dependent variables for normality. If variables were non-normal they were log transformed (natural log) before use in analyses. For all variables, differences in means between group A, B and C *Psammomys obesus* were tested for significance using the Kruskal-Wallis test (Kruskal and Wallis, 1992) with p-values corrected for the use of multiple comparisons with a Bonferroni adjustment (p-corrected (p<sub>B</sub>) value). Bivariate and partial correlations between variables were assessed for significance using the Pearson correlation coefficient. In all statistical tests a p-value greater than 0.05 was considered significant.
7.4 RESULTS

7.4.1 SEQUENCES OF OB-RA AND OB-RB

The partial nucleotide and deduced amino acid sequences of *Psammomys obesus* OB-RA and OB-RB is shown in figure 7.1. The sequence presented encompasses the end of the transmembrane domain and the whole of the intracellular domain for OB-RA and approximately half of the intracellular domain of OB-RB. There were no differences in the nucleotide sequences of these regions of the OB-R gene between lean and obese *Psammomys obesus*. Figure 7.1b shows that the deduced amino acid sequence of *Psammomys obesus* OB-RA and OB-RB is highly homologous to the amino acid sequence seen in several other species. *Psammomys obesus* OB-RA was found to be 91%, 94% and 94% homologous to rat, mouse and human, respectively, while OB-RB was 88%, 86% and 73% homologous to rat, mouse and human, respectively.
a) *Psammomys obesus*: OB-RA

\[
\begin{align*}
\text{ATG AAAA ATT GTT TGG GAA ATG TTTT GGA AGA ATG TCC CCA CAA CCA CAA GA ATG TCC CCA CAA AAG CCG TGA} \\
\text{AAACCC AAA GATG TTG CCG ACCA AGGG CCAC ATGA CTG TCTG ATGATT TGG TCC} \\
\text{TF EH L FT K H AG SV IF GP} \\
\text{TC TCT TTG GAC CAC CG ACC ATTC CAG AAG AA ATC GAT GTC ATG CAG} \\
\text{LL EPI SE E IS V DAA} \\
\text{T TGA AAAA ATAA AGAT GAG ATG TAC CA GCAC ATG TCT CGG TCTTTT} \\
\text{W K N K DE MV PA AMV SL LL} \\
\text{G CAC ATCC GAG ACC CCGT GAGG GGT TTT TGT TGG GAT GGCG ATC TAA} \\
\text{TT P A P E R G S V G V G G QC N} \\
\text{CAG TGTCA ACTT TCT TGG GCT AGG TGA CTG GAT GAG TGA TGG} \\
\text{SAN FS GA QV AC ED E}
\end{align*}
\]

b) **OB-RA:**

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</tr>
<tr>
<td></td>
<td><strong>P. obesus</strong> SVIFGPLLLEPEIS EIS V DAA W NK D E MV PA AMV SL L T T P A  P E R G S V G G Q C N S A</td>
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<td>979</td>
</tr>
</tbody>
</table>

Figure 7.1a Partial nucleotide and deduced amino acid sequence of the intracellular domain of *Psammomys obesus* OB-RA and OB-RB (the translation stop codon is indicated by an asterisk; transmembrane domain is doubly underlined; Box 1 sequence is indicated by thick underline) and b) alignment of *Psammomys obesus*, mouse, rat and human amino acid sequences for the intracellular domain of OB-RA and OB-RB. Identical sequences are indicated by asterisks (The amino acid sequences for mouse, rat and human are from Lee et al., 1996; Takaya et al., 1996 and Cioffi et al., 1996, respectively).
7.4.2 TISSUE DISTRIBUTION OF OB-RA AND OB-RB IN LEAN *PSAMMOMYS OBEUS*

We have detected expression of OB-RA and OB-RB in pancreas, skeletal muscle, testis, small intestine, heart, kidney, lung, white suprascapular adipose tissue, hypothalamus and liver (figure 7.2). The highest level of expression of OB-RA was in the liver (40.0±10.1), followed by testis (3.2±0.09), adipose tissue (0.5±0.2), hypothalamus (0.4±0.16), and lung (0.2±0.1), and only low levels of OB-RA detected in pancreas (0.1±0.1), muscle (0.1±0.09), heart (0.02±0.002), kidney (0.02±0.001) and small intestine (0.007±0.001). As with OB-RA, the highest level of expression of OB-RB was detected in the liver (7.6±1.15), followed by the testis (5.7±0.9), pancreas (1.4±0.5), heart (1.2±0.03) and small intestine (1.1±0.01). Only low levels of expression of OB-RB were detected in hypothalamus (0.22±0.07), skeletal muscle (0.22±0.04) and kidney (0.01±0.003).
To examine the role of OB-RA and OB-RB gene expression in *Psammomys obesus*, animals were classified into three groups. The metabolic characteristics of these groups are shown in Table 7.1, with group A animals being lean and healthy, group B animals being obese and hyperinsulinemic and group C animals being obese and diabetic, with hyperglycemia and hyperinsulinemia.

**Table 7.1** Characteristics of Group A, B and C *Psammomys obesus* (mean±SEM)

<table>
<thead>
<tr>
<th></th>
<th>Group A (n=10)</th>
<th>Group B (n=11)</th>
<th>Group C (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>197.5 ± 8.5</td>
<td>206.2 ± 6.6</td>
<td>237.2 ± 4.7 *</td>
</tr>
<tr>
<td><strong>% Body fat</strong></td>
<td>2.9 ± 0.3</td>
<td><strong>4.4 ± 0.3</strong></td>
<td><strong>5.5 ± 0.3</strong> <em>^</em>*</td>
</tr>
<tr>
<td><strong>Blood glucosc (mmol/l)</strong></td>
<td>4.4 ± 0.3</td>
<td>4.6 ± 0.2</td>
<td>14.8 ± 1.0 *</td>
</tr>
<tr>
<td><strong>Plasma insulin (μU/ml)</strong></td>
<td>87.2 ± 10.8</td>
<td>341.1 ± 33.0 *</td>
<td>520.8 ± 58.2 *^</td>
</tr>
<tr>
<td><strong>Plasma leptin (ng/ml H.E.)</strong></td>
<td>45.0 ± 6.4</td>
<td>54.6 ± 4.3</td>
<td>63.3 ± 4.5 *#</td>
</tr>
</tbody>
</table>

* $p_c$ = 0.001, significantly different from group A
^ $p_c$ = 0.001, significantly different from group B
** $p_c$ = 0.01, significantly different from group A
^^ $p_c$ = 0.04, significantly different from group B
# $p_c$ = 0.06

$p_c$ = p-values adjusted for multiple comparisons (Bonferroni)
Figure 7.3. Hypothalamic OB-RA, OB-RB and c-fos gene expression (relative to ribosomal RNA in group A (lean), group B (obese, non-diabetic) and group C (obese, diabetic) Psammomys obesus. (**p<0.08, ^p<0.07 and *p<0.03; significantly different from group A Psammomys obesus)

7.4.3 HYPOTHALAMIC OB-R GENE EXPRESSION

Figure 7.3 shows that the level of OB-RB gene expression in the hypothalamus was lower in group B and C animals compared to group A animals. An approximately 5-fold lower level of OB-RB gene expression was detected in group B animals and 4-fold lower level in group C animals compared to group A animals (0.04±0.01 vs 0.22±0.07, p=0.01, p<0.03; and 0.05±0.01 vs 0.22±0.07, p=0.02, p<0.06, respectively). In addition, the level of c-fos expression was approximately 4-fold lower in group B and C animals compared to group A (0.19±0.05 vs 0.76±0.24, p=0.025, p<0.075; and 0.24±0.04 vs 0.76±0.24, p=0.095, p<0.29, respectively). OB-RA mRNA expression in the hypothalamus was found to be approximately 5-fold lower in group B compared to group A animals (0.08±0.02 vs 0.40±0.16, p=0.017, p<0.51) and 6-fold lower in group C compared to group A animals (0.06±0.02 vs 0.40±0.16, p=0.05, p<0.15), although these differences did not reach statistical significance.
Figure 7.4. Hepatic OB-RA, OB-RB and c-fos gene expression (relative to ribosomal RNA in group A (lean), group B (obese) and group C (obese, diabetic) Psammomys obesus. (*$P_c=0.003$, significantly different from group A; $^\wedge P_c=0.003$ and $^\#$ $P_c=0.02$, significantly different from group B Psammomys obesus)

7.4.4 HEPATIC OB-R GENE EXPRESSION

Figure 7.4 shows that the level of OB-RA mRNA expression in the liver is approximately 2-fold lower in group B compared to group A animals (25.0±6.6 vs 40±10.1) and 10-fold lower in group C compared to group A animals (3.6±0.6 vs 40.0±10.1, $p=0.001$, $P_c=0.002$). In addition, the level of OB-RA expression in group C animals was approximately 6.5 times lower than the level of expression in group B animals (3.6±0.6 vs 25.0±6.6, $p=0.001$, $P_c=0.002$). OB-RB expression was also lower in group C animals compared to group A animals, with approximately 4-fold lower OB-RB gene expression seen in group C animals compared to group A animals (1.7±0.4 vs 7.6±1.15, $p=0.001$, $P_c=0.003$). In addition, the level of OB-RB expression in group C animals was approximately 3.5 times lower than the level of expression in group B animals (1.7±0.4 vs 6.3±1.16, $p=0.007$, $P_c=0.021$). The level of c-fos expression in the liver was not different between the three groups.
7.4.5 GENE EXPRESSION IN ADIPOSE TISSUE

There was no difference in the level of OB-RA, OB-RB or c-fos gene expression between group A, B or C Psammomys obesus in white suprascapular adipose tissue (data not shown), although the level of OB gene expression in group C animals tended to be higher than group A (22.9±10.3 vs 5.09±1.5, p=0.04, p_c=0.12) and group B animals (22.9±10.3 vs 10.39±3.18, p=0.19, p_c=0.57).

7.4.6 CORRELATIONS OF LEPTIN RECEPTOR GENE EXPRESSION AND METABOLIC MARKERS IN *PSAMMOMYS OBEJUS*

Table 7.2 shows the correlation coefficients for bivariate and partial correlations between OB-RA and OB-RB gene expression and bodyweight, estimated percent body fat, plasma insulin, blood glucose and plasma leptin concentrations. This table shows a negative association between circulating insulin and OB-RB gene expression in the hypothalamus ($r=-0.49$, $p=0.004$) and also demonstrates that this relationship is independent of adiposity ($r=-0.61$, $p=0.001$). The level of hypothalamic OB-RA and OB-RB gene expression was not associated with body weight, percent body fat, blood glucose or plasma leptin concentrations.

In the liver, OB-RA gene expression was negatively associated with body weight ($r=-0.58$, $p=0.001$), percent body fat ($r=-0.65$, $p=0.001$), blood glucose ($r=-0.74$, $p=0.001$) and plasma insulin ($r=-0.62$, $p=0.001$). In addition, after controlling these associations for percent body fat, liver OB-RA was still associated with insulin ($r=-0.45$, $p=0.006$) and glucose ($r=-0.59$, $p=0.001$), and also tended to be associated with plasma leptin ($r=-0.31$, $p=0.09$) concentrations. OB-RB gene expression was also negatively associated with body weight ($r=-0.42$, $p=0.006$), percent body fat ($r=-0.51$, $p=0.001$), blood glucose ($r=-0.54$, $p=0.001$) and plasma insulin ($r=-0.57$, $p=0.001$). After controlling those associations for percent body fat, liver OB-RB was still associated with insulin ($r=-0.40$, $p=0.02$) and glucose ($r=-0.34$, $p=0.04$) concentrations.

The level of OB-RA gene expression in adipose tissue was not associated with body weight, percent body fat, plasma insulin, blood glucose or plasma leptin concentrations. OB-RB gene expression was positively associated with percent body fat ($r=0.43$, $p=0.03$) and tended to be associated with body weight ($r=0.33$, $p=0.07$). There was no association between OB-RB gene expression in adipose tissue and blood glucose or plasma insulin and leptin concentrations. OB gene expression in adipose tissue was positively associated with blood glucose concentration, ($r=0.50$, $p=0.01$) and after adjustment for percent body fat, OB gene expression was still positively associated with blood glucose ($r=0.69$, $p=0.01$), and additionally associated with plasma insulin ($r=0.49$, $p=0.03$) concentrations.
7.5 DISCUSSION

In this study, the partial nucleotide and amino acid sequences of OB-RA and OB-RB in *Psammomys obesus* are reported. The OB-RA sequence showed 94%, 91% and 94% amino acid homology to mouse, rat and human sequences, respectively, and OB-RB showed 86%, 88% and 73% amino acid homology to mouse, rat and human sequences, respectively. There is also evidence of expression of OB-RA and OB-RB in pancreas, skeletal muscle, testis, small intestine, heart, kidney, lung, white suprascapular adipose tissue, hypothalamus and liver in lean *Psammomys obesus*. In addition, it is demonstrated that obese animals exhibit decreased levels of OB-RA and OB-RB in the hypothalamus compared to lean *Psammomys obesus*. Examination of leptin receptor levels in the liver and white adipose tissue from lean, obese non-diabetic and obese diabetic also revealed decreased levels of OB-RA and OB-RB in the liver, while no differences were detected in the suprascapular white adipose tissue.

In this study, as with previous studies, obese *Psammomys obesus* were found to have higher circulating leptin levels than lean animals, which may be indicative of leptin resistance. These same animals had lower levels of leptin receptor gene expression and c-fos activation in the hypothalamus compared to lean animals. It is unclear whether leptin resistance in obese animals therefore develops as a consequence of decreased signaling by the receptors in the hypothalamus, or if there is a more distal defect somewhere along the leptin signaling pathway which alters leptin action in the brain. Regardless of the defect, decreased leptin signaling may lead to impaired leptin action and energy imbalance, which may in turn lead to increased adiposity and circulating leptin concentrations and consequently down-regulation of leptin receptor gene expression.

This study has shown that obese *Psammomys obesus* have between 1.2- and 1.4-times higher circulating leptin concentrations (group B and C, respectively) than lean (group A) animals. In contrast, obese animals have approximately 5-fold lower levels of OB-RA and OB-RB gene expression in the hypothalamus compared to lean animals. The reduced level of OB-R gene expression in the hypothalamus of obese animals therefore may not be solely accounted for by increased circulating leptin concentrations.

Hypothalamic expression of OB-RB has been suggested to be primarily controlled by circulating leptin concentrations, as physiological interventions which alter circulating leptin concentrations also result in changes in hypothalamic leptin receptor gene
expression (Fei et al., 1997; Lin and Huang, 1997; Mercer et al., 1997; Baskin et al., 1998; Bennett et al., 1998). The studies which have provided evidence supporting this hypothesis are from either non-obese or monogenic models of obesity, with no studies of this kind reported in polygenic models. The results from previous studies do not rule out the possibility that metabolic or hormonal conditions, in addition to changing leptin concentrations, contribute to the regulation of both central and peripheral OB-R expression, particularly in polygenic models of obesity.

In Psammomys obesus this study demonstrated a negative association between OB-RB gene expression in the hypothalamus and circulating plasma insulin concentrations, with the hyperinsulinemic animals found to have lower OB-R gene expression than normoinsulinemic animals. This association was found to be independent of body fat and indicates that insulin may play a role in the regulation of hypothalamic OB-RB gene expression in Psammomys obesus.

To explore the peripheral actions of leptin, leptin receptor gene expression was examined in the liver and adipose tissue of lean, obese and diabetic Psammomys obesus. In the liver, OB-RA and OB-RB gene expression were found to be lower in obese animals compared to lean Psammomys obesus. In addition OB-RA and OB-RB gene expression were negatively associated with body weight, percent body fat, blood glucose and plasma insulin concentrations, and after adjusting for body fat, negative associations were found between OB-RA and OB-RB gene expression and circulating insulin, glucose and leptin (only associated with OB-RA). In contrast, OB-RB gene expression in adipose tissue was found to be positively associated with body weight and percent body fat. It is unclear from this study if insulin or glucose play any role in the regulation of OB-RA and OB-RB gene expression in either the hypothalamus or periphery, however this study is the first to show associations between OB-RA and OB-RB gene expression and these metabolic variables in vivo in a polygenic model of obesity and type 2 diabetes.

In addition, there is evidence of an association between OB gene expression and circulating glucose and insulin concentrations in Psammomys obesus. Several studies have reported stimulatory effects of both glucose and insulin on OB gene expression in other rodent species (Cusin et al., 1995; Saladin et al., 1995; Hardie et al., 1996; Mizuno et al., 1996b). In the present study higher levels of OB gene expression are
seen in the animals with higher levels of glucose and insulin, independent of body fat mass.

In *Psammomys obesus*, obesity and diabetes develop in a proportion of animals for reasons that remain unclear. Resistance of these animals to the anorexigenic effects of leptin has previously been demonstrated (Walder et al., 1999) and here it is reported that leptin receptor gene expression within the hypothalamus is lower in the obese, leptin resistant animals. From this study it cannot be determined if leptin receptors in the brain are down-regulated in response to the increasing circulating leptin levels accompanying increasing adiposity, or if leptin levels increase in the periphery in an attempt to overcome some pre-existing defect in leptin signaling in obese animals. It is important to note however that circulating plasma leptin levels were only inversely associated with the level of OB-RA gene expression in the liver in the present study, and not the level of hypothalamic OB-RA or OB-RB gene expression.

In conclusion, this study demonstrates for the first time that leptin receptor gene expression in a polygenic model of obesity and diabetes is associated with body weight, body fat and circulating metabolites such as insulin, glucose and leptin. In obese, leptin resistant *Psammomys obesus*, hypothalamic and hepatic expression of both the long and short isoforms of the leptin receptor are markedly reduced compared to lean animals. In addition, leptin receptor gene expression in peripheral tissues is shown to be associated with insulin, glucose and leptin. The decreased levels of OB-RA and OB-RB expression in obese *Psammomys obesus* may contribute to the leptin resistance seen in these animals and further, in addition to leptin, other metabolites may be involved in the regulation of OB-R gene expression.
CHAPTER 8

REGULATION OF LEPTIN RECEPTOR GENE EXPRESSION WITH FASTING IN PSAMMOMYS OBESUS, A POLYGENIC MODEL OF OBESITY AND TYPE 2 DIABETES.

8.1 SUMMARY
Leptin, the protein product of the OB gene, has been found to play an important role in energy balance, with mutations in either the OB gene or the gene for the leptin receptor, OB-R, known to result in the development of obesity in several rodent models of obesity as well as humans. In both rodents and humans, leptin is secreted from adipocytes in proportion to body fat mass however, short-term fasting has been shown to cause a marked reduction in OB gene expression and circulating leptin in lean animals, despite no change in adiposity. The effects of fasting on OB gene expression and leptin in obese animals is less clear, with no effect seen in several obese rodent species. Psammomys obesus is a polygenic rodent model of obesity and type 2 diabetes which exhibits a range of bodyweight and plasma insulin, glucose and leptin concentrations. In Psammomys obesus, fasting has been shown to cause a reduction in leptin in lean animals but an increase in leptin in obese animals. The present study examined the effects of fasting on central and peripheral leptin receptors in the face of this apparent dysregulation of plasma leptin. The results demonstrate that OB gene expression tends to decrease with fasting in lean animals, which show a reduction in plasma leptin, while in obese animals there is no effect on OB gene expression, despite the increase in plasma leptin seen with fasting. In addition, OB-RB, the main signaling isoform of the leptin receptor was increased with fasting in the hypothalamus of lean (0.07±0.02 vs 0.18±0.09; fed vs fasted) and obese (0.02±0.01 vs 0.1±0.04; fed vs fasted) animals, although this increase only reached statistical significance in the obese group (p=0.04). In obese diabetic Psammomys obesus, fasting had no effect on hypothalamic OB-RB gene expression. As these animals are known to be severely leptin resistant, this finding may indicate some dysfunction in the regulation of leptin receptor gene expression. In the liver, OB-RA (a short leptin receptor) gene expression was increased with fasting in lean (0.6±0.5 vs 24.6±7.2, p=0.03; fed vs fasted) and obese (1.9±1.2 vs 43.1±10.8, p=0.001; fed vs fasted) Psammomys obesus, while OB-RB gene expression tended to be decreased. In adipose tissue, gene expression of both OB-RA and OB-RB tended to be increased in lean animals, while OB-RB gene
expression tended to be increased with fasting in obese *Psammomys obesus* (0.01±0.003 vs 0.05±0.01, p=0.07; fed vs fasted). Overall the changes in leptin receptor gene expression with fasting cannot be accounted for by changes in plasma leptin in *Psammomys obesus*, as in several tissues OB-RB gene expression increases with fasting in both lean and obese animals, despite differing effects of fasting on plasma leptin concentrations. From this study it is unclear what is regulating OB-RA and OB-RB gene expression in *Psammomys obesus*, however this is the first study to examine central and peripheral leptin receptor gene expression after fasting in a polygenic rodent model of obesity.
8.2 INTRODUCTION

The role of leptin in energy balance is well established in rodents, with leptin treatment shown to decrease body weight and body fat in several rodent models of obesity (Halaas et al., 1995; Pellymounter et al., 1995; Cusin et al., 1996; Halaas et al., 1997). Leptin is secreted from adipocytes in direct proportion to the adipose tissue mass (Frederich et al., 1995b; Maffei et al., 1995b), although changes in circulating leptin concentrations have been found to occur without changes in body fat mass.

As a result of short term fasting, OB gene expression and circulating leptin concentrations have been shown to decrease markedly, despite no significant changes in body mass or adiposity. In wild-type mice, fasting for 48 to 72 hours was shown to decrease plasma leptin between 60% and 70% (Maffei et al., 1995b; Ahima et al., 1996), while OB gene expression was found to be decreased by 85% after a 16-hour fast (MacDougald et al., 1995) and by 90% after a 48 hour fast (Mizuno et al., 1996a). The dramatic effects of fasting on OB gene expression and plasma leptin concentrations in lean animals are not seen as consistently in obese animals, and there was no effect of fasting on OB gene expression in ob/ob mice after 16 hours (MacDougald et al., 1995), in diet-induced obese mice after 48 hours (Mizuno et al., 1996a) or in falfa rats after 72 hours (Cusin et al., 1995).

In humans, fasting has been shown to decrease circulating leptin concentrations in both lean and obese individuals, with fasting for 24 to 60 hours resulting in a 60% to 70% reduction in plasma leptin in lean subjects and a 42% to 88% reduction in plasma leptin in obese subjects (Boden et al., 1996; Kolaczynski et al., 1996). It is now clear however, that the acute changes in OB gene expression and leptin levels seen with fasting in both lean mice and humans are prevented or reversed by maintenance of normal glucose and insulin concentrations during the fasting period (Boden et al., 1996; Mizuno et al., 1996a; Muller et al., 1998).

The leptin receptor has been shown to play an integral part in mediating the actions of leptin in the central nervous system in both rodents and humans, and the leptin receptor (OB-R) is known to have several alternatively spliced isoforms which differ in the length of their intracellular domains (Lee et al., 1996; Tartaglia et al., 1996). OB-RB has a long intracellular domain that has been shown to activate signal transduction pathways within the cell and is thought to represent the main signaling form of the receptor (Bjorbeck et al., 1997; Takahashi et al., 1997; White et al., 1997a; Elmquist et
OB-RA is a much shorter isoform and although it has some signaling capacity, this receptor is thought to function predominately as a transporter of leptin into the cell (Tartaglia et al., 1996; Takahashi et al., 1997).

In the hypothalamus, leptin is thought to act via its receptors to decrease food intake and alter energy expenditure, and the effects of leptin and the role of its receptor in the hypothalamus have been examined in a number of animal models. Several groups have demonstrated co-localisation of leptin receptors with a number of neurons known to be involved with feeding behaviour, including neuropeptide Y (NPY) (Hakansson et al., 1996; Mercer et al., 1996a), melanin concentrating hormone (MCH) (Hakansson et al., 1998a) and pro-opiomelanocortin (POMC) (Hakansson et al., 1998a). The presence of leptin receptors in a large number of peripheral tissues also suggests that leptin may have direct actions on these tissues, however the role of leptin in peripheral tissues is not well characterised. In adipose tissue leptin appears to increase lipolysis and thermogenesis and may also be involved in shifting adipocyte metabolism away from glucose to fat (Muller et al., 1997; Siegrist-Kaiser et al., 1997; Zhou et al., 1997; Ceddia et al., 1998). In the liver leptin appears to enhance insulin action, with decreased hepatic glucose production and increased glucose turnover and uptake reported with leptin treatment (Barzilai et al., 1997; Rossetti et al., 1997; Sivitz et al., 1997). These studies suggest that leptin may be involved in energy partitioning, although the exact role of leptin in these tissues is unknown.

Several groups have now demonstrated that fasting increases OB-RB gene expression several fold in different regions of the hypothalamus in wild-type mice and Sprague-Dawley, Wistar and Zucker (fa/fa) rats (Fei et al., 1997; Lin and Huang, 1997; Baskin et al., 1998; Bennett et al., 1998). These studies provide evidence of a feed-back loop, by which a reduction in circulating leptin (as with fasting) results in up-regulation of leptin receptor gene expression as part of a regulatory mechanism. The study by Lin and Huang also showed that in ob/ob mice, which lack circulating leptin, fasting does not increase leptin receptor gene expression, providing further support for the hypothesis that circulating leptin concentrations are important regulators of OB-R expression. These findings however, do not rule out the possibility that other factors play a role in the regulation of leptin receptor gene expression, particularly in polygenic models of obesity, which more closely mimic the situation of human obesity.
We have previously demonstrated that in *Psammomys obesus*, a polygenic rodent model of obesity and type 2 diabetes, lean animals fasted for 24 hours show a 44% decrease in plasma leptin, while obese animals show an increase in leptin of approximately 20% when fasted for the same period of time (Walder et al., 1998). *Psammomys obesus* is a unique rodent model as these animals remain lean and healthy when maintained on their natural low-energy diet of salt bush, however when held in captivity and fed a relatively energy-dense diet of standard laboratory chow, the animals develop a range of pathophysiological responses, with approximately half of the animals developing obesity and about one third developing type 2 diabetes (Shafirir and Gutman, 1993; Barnett et al., 1994a). The heterogeneity of the metabolic response of *Psammomys obesus* to the energy dense diet closely resembles that seen in human populations (DeFronzo, 1988), making this a very useful model to study the etiology and pathophysiology of obesity and type 2 diabetes.

The aim of the present study was to further examine the response of *Psammomys obesus* to a 24-hour fast, with particular attention on the changes in leptin receptor gene expression in both the hypothalamus and periphery that accompany the changes in plasma leptin with fasting in animals with a wide range of body weight and circulating glucose and insulin concentrations.
8.3 MATERIALS AND METHODS
8.3.1 EXPERIMENTAL ANIMALS
A *Psammomys obesus* colony is maintained at Deakin University, with breeding pairs fed ad libitum a diet of lucerne and chow. Experimental animals were weaned at 4 weeks of age and given a diet of standard laboratory chow (12% energy from fat, 63% from carbohydrates, 25% from protein; Barastoc, Pakenham, Australia). Animals were housed in a temperature-controlled room (22°C±1°C) with a 12-hour/12-hour light/dark cycle (See chapter 2 for further details).

8.3.2 CHARACTERISATION OF ANIMALS AND EXPERIMENTAL PROTOCOL
For this study *Psammomys obesus* were classified into one of three groups at 16 weeks of age. Lean animals had a bodyweight <200g, were normoglycemic (whole blood glucose <8mmol/l) and normoinsulinemic (plasma insulin<150μU/ml); obese animals had a bodyweight ≥200g and were normoglycemic (whole blood glucose <8mmol/l) and hyperinsulinemic (plasma insulin ≥150μU/ml); obese diabetic animals had a bodyweight ≥200g and were hyperglycemic (whole blood glucose ≥8mmol/l) and hyperinsulinemic (plasma insulin ≥150μU/ml). The metabolic characteristics of the animals can be seen in table 6.1.

8.3.3 STUDY PROTOCOL
The animals were weighed and blood was collected from the tail vein in the fed state. Animals were then fasted for 24 hours after which they were re-weighed and blood was collected in the fasted state. All blood was collected into heparinised tubes.

8.3.4 RNA PREPARATION AND cDNA SYNTHESIS
At the end of the study animals were killed by lethal overdose of pentobarbitone (120mg/kg) and the hypothalamus, liver and adipose tissue from the suprascapular, perirenal, epididymal, intramuscular and mesenteric fat depots were removed. After homogenisation, RNA was purified from the whole hypothalamus or 100mg of liver and suprascapular white adipose tissue using RNAzol B (Bresatec, Adelaide, Australia). The RNA was extracted with 200μl of chloroform and precipitated with an equal volume of isopropanol. RNA was quantitated by spectrophotometry at 260nm and 1μg was then reverse transcribed at 42°C for 1 hr with 10U of AMV reverse transcriptase (Promega Madison, USA) according to the manufacturer's instructions.
8.3.5 POLYMERASE CHAIN REACTION (PCR) AND QUANTITATION

Comparative RT-PCR was performed using the Taqman 'Real Time' system (Perkin Elmer Applied Biosystems, Forster City, USA). This system utilises a fluorescent probe within the PCR reaction to detect the amplification of the appropriate region of the cDNA (Gibson et al., 1996). The level of fluorescence emitted is sampled every 7 seconds during the 40 cycles of amplification and is directly proportional to the amount of product generated (Gibson et al., 1996; Heid et al., 1996; See Chapter 2 for further details). All quantitation of gene expression reported is expressed relative to the ribosomal RNA house-keeping gene (Perkin Elmer Applied Biosystems, Forster City, USA). The sequences of the oligonucleotide primers and probes used for amplification of OB, OB-RA, OB-RB and c-fos were based on the Psammomys obesus sequence and lie in regions found to be highly conserved across several species including rat, mouse and human.
OB gene:
forward primer 5'-tgcctgctcctaccacacatc-3'
reverse primer 5'-ttctcattgtgcgatatttgc-3'
probe 5'-FAM tgaacgcctgtccctcccgaa TAMRA-3'

OB-RA:
forward primer 5'-tgctctgtctgtggacact-3'
reverse primer 5'-ctctcttggaaattaagctcttg-3'
probe 5'-FAM atcggtatatgttgtgtctctctctctgtgagcc TAMRA-3'

OB-RB:
forward primer 5'-aagccctgaaacatrgacatc-3'
reverse primer 5'-gcatcgcactctatttcctg-3'
probe 5'-FAM atcggtatatgttgtgtctctctctctgtgagcc TAMRA-3'

c-fos:
forward primer 5'-tgaaagagagagaactggagttta-3'
reverse primer 5'-ccagggagccacagaca-3'
probe 5'-FAM tgcagcagccacagactgc TAMRA-3'

Ribosomal RNA:
forward primer 5'-cgcctccatcagccagaaa-3'
reverse primer 5'-gcggaaattacccgsgct-3'
probe 5'-VIC tgcctgccaccagactggtc TAMRA-3'

PCR was performed by adding 50 ng of cDNA to a tube containing: 1xTaqMan Universal PCR Master Mix (AmpliTaq Gold™, AmpErase® Uracil N-glycosylase (UNG), dNTPs, 5x TaqMan Buffer A (with ROX passive reference) 12.5 mM MgCl₂ and glycerol (Perkin Elmer Applied Biosystems, Foster City, USA), the appropriate concentration of primers and probe (see Chapter 2) in a final volume of 25 µl in a MicroAmp® Optical 96-well Reaction Plate covered with MicroAmp® Optical caps (Perkin Elmer Applied Biosystems, Foster City, USA). Amplification was performed using the following conditions: 50°C for 2 mins, 95°C for 10 mins and 40 cycles of 95°C for 15 sec and 60°C for 1 min.

8.3.6 BIOCHEMICAL ANALYSES
Whole blood glucose was measured using an enzymatic glucose analyser (Model 2300 STAT Plus, Yellow Springs Instruments, Ohio). Plasma insulin concentrations were determined using a double antibody solid phase radioimmunoassay (Phadeseph, Kabi Pharmacia Diagnostics, Sweden). Plasma leptin concentrations were measured using a
commercially available radioimmunoassay kit (Multispecies leptin assay; Linco
Research, St Charles, MO, USA). A multispecies leptin antibody (produced in guinea
pig) was used in this kit, with a $^{125}$I-human leptin tracer and a normal guinea pig IgG
antibody added as the second antibody. The concentration of leptin in plasma from
Psammomys obesus was determined using human leptin standards of known
concentrations, and as the multispecies leptin antibody was raised against human leptin,
and human leptin standards were used, the units of measurement are ng/ml Human
Equivalent (HE) leptin.

8.3.7 ESTIMATED PERCENT BODY FAT
The weight of selected fat depots (suprascapular, perirenal, epididymal, intramuscular
and mesenteric) was used to provide an estimate of body fat content. The weights of
the various depots were combined and divided by total body mass to provide an
estimate of percent body fat.

8.3.8 STATISTICAL ANALYSES
The independent T-test was used to test for differences in means between the fed and
fasted animals within each group. A p-value greater than 0.05 was regarded as
significant in all cases and statistical analyses were performed using SPSS (version
8.0).
8.4 RESULTS

Table 8.1a Metabolic characteristics of lean Psammomys obesus before and 24-hours after either feeding or fasting

<table>
<thead>
<tr>
<th>Variables:</th>
<th>LEAN ANIMALS</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
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<tr>
<td></td>
<td>FED</td>
<td>Pre-</td>
<td>Post-</td>
<td>Fasted</td>
<td>Pre-</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td></td>
<td>175.8±6.0</td>
<td>175.3±6.2</td>
<td>173.8±7.8</td>
<td>169.6±9.2</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td></td>
<td>4.6±0.3</td>
<td>4.9±0.4</td>
<td>4.3±0.2</td>
<td>3.8±0.2*</td>
</tr>
<tr>
<td>Plasma Insulin (µU/ml)</td>
<td></td>
<td>59.9±8.8</td>
<td>63.5±11.3</td>
<td>57.4±8.6</td>
<td>19.1±2.7**</td>
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<tr>
<td>Plasma Leptin (ng/ml H.E.)</td>
<td></td>
<td>26.2±5.8</td>
<td>31.8±7.8</td>
<td>32.0±10.9</td>
<td>21.7±7.1</td>
</tr>
</tbody>
</table>

*p=0.03, **p=0.005; significantly different from fed-post mean

Table 8.1a shows the metabolic characteristics of the control (fed) and fasted groups of animals and demonstrates that there is no difference in the final bodyweight of the control group and the fasted group (175.3±6.2 vs 169.6±9.2), while there was a significant reduction in the final blood glucose (4.9±0.4 vs 3.8±0.2, control vs fasted, p=0.03) and plasma insulin concentrations (63.5±11.3 vs 19.1±2.7, control vs fasted, p=0.005) between these groups. Final plasma leptin levels also tended to be lower in the fasted group compared to the control group (31.8±7.8 vs 21.7±7.1, control vs fasted) however this difference did not reach statistical significance.
Table 8.1b Metabolic characteristics of obese *Psammomys obesus* before and 24-hours after either feeding or fasting.

<table>
<thead>
<tr>
<th>Variables:</th>
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<th>FASTED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>Pre- 221.1±7.0</td>
<td>Post- 221.4±7.0</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.8±0.4</td>
<td>4.8±0.5</td>
</tr>
<tr>
<td>Plasma Insulin (μU/ml)</td>
<td>302.4±56.3</td>
<td>268.5±34.0</td>
</tr>
<tr>
<td>Plasma Leptin (ng/ml H.E.)</td>
<td>40.2±5.5</td>
<td>36.5±7.2</td>
</tr>
</tbody>
</table>

*p=0.05, **p=0.006; significantly different from fcd-post mean

In obese *Psammomys obesus* there was again no difference in the final bodyweight of the control group and the fasted group (221.4±7.0 vs 217.0±10.2) but there was a significant reduction in the final blood glucose (4.8±0.5 vs 3.6±0.2, control vs fasted, p=0.05) and plasma insulin concentrations (268.5±34.0 vs 124.5±29.7, control vs fasted, p=0.006) between these groups. In contrast to lean *Psammomys obesus*, final plasma leptin levels tended to increase with fasting in obese animals (36.5±7.2 vs 55.8±8.8, control vs fasted) however this difference did not reach statistical significance (p=0.1).
### Table 8.1c

Metabolic characteristics of obese, diabetic *Psammomys obesus* before and 24-hours after either feeding or fasting.

<table>
<thead>
<tr>
<th>Variables:</th>
<th>OBESE DIABETIC ANIMALS</th>
<th>FED</th>
<th>FASTED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PRE-</td>
<td>POST-</td>
<td>PRE-</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>234.6±5.3</td>
<td>237.1±4.9</td>
<td>236.6±8.4</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>14.1±0.9</td>
<td>12.8±0.9</td>
<td>11.9±0.8</td>
</tr>
<tr>
<td>Plasma Insulin (μU/ml)</td>
<td>517.8±60.2</td>
<td>399.0±48.8</td>
<td>509.6±75.7</td>
</tr>
<tr>
<td>Plasma Leptin (ng/ml H.E.)</td>
<td>41.9±6.5</td>
<td>45.9±6.7</td>
<td>38.9±7.8</td>
</tr>
</tbody>
</table>

*p=0.001; significantly different from fed-post mean

In obese, diabetic *Psammomys obesus* there was no difference in the final bodyweight of the control group and the fasted group (237.1±4.9 vs 237.2±7.7; table 8.1c), however there were markedly decreased concentrations of final blood glucose (12.8±0.9 vs 4.4±0.3, control vs fasted, *p=0.001*) and plasma insulin (399.0±48.8 vs 107.0±7.5, control vs fasted, *p=0.001*) in the fasted group of animals. There was no difference in the final plasma leptin levels between the fasted and control group of animals (45.9±6.7 vs 38.3±8.5, control vs fasted).
Figure 8.1. Percentage changes in a) body weight and plasma leptin concentration, and b) blood glucose and plasma insulin concentrations, in after a 24-hour fast (*p=0.025, **p=0.001; significantly different from pre-fasting level).
When looking only at the fasted animals, figure 8.1a shows that a 24-hour fast resulted in a 28% decrease in plasma leptin in lean animals (p=0.025), a 24% increase in plasma leptin in obese animals (p=0.18) and no change in plasma leptin in obese, diabetic *Psammomys obesus*, despite no significant change in bodyweight in any of the groups of animals. The percentage change from baseline levels of glucose and plasma insulin after fasting is shown in figure 8.1b. Lean and obese, non-diabetic *Psammomys obesus* were normoglycemic prior to fasting and figure 8.1b shows that fasting does not significantly reduce glucose concentrations in these groups of animals. In contrast, obese diabetic *Psammomys obesus* show a marked decrease in blood glucose concentrations of 63% (p=0.001) with fasting. All three groups of animals show significant decreases in plasma insulin concentrations with fasting of approximately 62% in lean animals (p=0.001), 75% in obese animals (p=0.001) and 74% in obese, diabetic *Psammomys obesus* (p=0.001).
Figures 8.2 to 8.5 show the levels of gene expression for the OB gene, the leptin receptors OB-RA and OB-RB and the transcription factor *c-fos* between fed and fasted *Psammomys obesus*.

![Bar graph showing OB gene expression (relative to ribosomal RNA) in fed and fasted Psammomys obesus](image)

**Figure 8.2.** OB gene expression (relative to ribosomal RNA) in fed and fasted *Psammomys obesus*

Figure 8.2 shows that in both lean and obese, diabetic *Psammomys obesus*, fasting tends to decrease the level of OB gene expression in the adipose tissue (lean: 6.7±3.1 vs 2.1±0.5, and obese diabetic: 24.5±13.3 vs 5.6±1.4; fed vs fasted), although these differences did not reach statistical significance. In obese, non-diabetic *Psammomys obesus*, there was no difference in the level of OB gene expression between the fed and fasted groups (10.9±4.6 vs 10.4±2.7, fed vs fasted).
Figure 8.3. Hypothalamic expression of a) OB-RA, b) OB-RB and c) c-fos (relative to ribosomal RNA) in fed and fasted *Psammomys obesus* (*p=0.03, **p=0.04, #p=0.02; significantly different from fed group*)
Figure 8.3 shows that in lean *Psammomys obesus*, a 24-hour fast results in a tendency for decreased OB-RA gene expression (0.05±0.04 vs 0.01±0.01, fed vs fasted) but increased OB-RB (0.07±0.02 vs 0.18±0.09, fed vs fasted) and *c-fos* (0.24±0.08 vs 0.55±0.29, fed vs fasted) gene expression within the hypothalamus, however the differences did not reach statistical significance. In obese animals, fasting increased OB-RA (0.01±0.004 vs 0.03±0.008, p=0.04; fed vs fasted), OB-RB (0.02±0.009 vs 0.1±0.04, p=0.03; fed vs fasted) and *c-fos* (0.10±0.04 vs 0.25±0.05, p=0.02; fed vs fasted) gene expression within the hypothalamus, while in obese, diabetic *Psammomys obesus* there was no change in the level of expression of OB-RA (0.02±0.003 vs 0.02±0.005, fed vs fasted), OB-RB (0.04±0.009 vs 0.05±0.009, fed vs fasted) or *c-fos* (0.15±0.04 vs 0.19±0.03, fed vs fasted) in the hypothalamus with fasting.
Figure 8.4. Hepatic expression of a) OB-RA, b) OB-RB and c) C-FOS (relative to ribosomal RNA) in fed and fasted *Psammomys obesus* (#p=0.02, *p=0.03, **p=0.001; significantly different from fed group)
Figure 8.4 shows that in lean and obese *Psammomys obesus* there is increased expression of OB-RA in the liver (lean: 0.6±0.5 vs 24.6±7.2, p=0.03; obese: 1.9±1.2 vs 43.1±10.8, p=0.001; fed vs fasted) and evidence for decreased expression of OB-RB (lean: 9.4±6.3 vs 1.7±0.4, p=0.15; obese: 8.1±3.9 vs 1.7±0.6, p=0.46; fed vs fasted) and c-fos (22.8±8.3 vs 1.2±1.1, p=0.02; obese: 9.7±4.9 vs 1.9±0.9, p=0.32; fed vs fasted) in the liver after fasting. In obese, diabetic *Psammomys obesus*, fasting resulted in a tendency for increased OB-RA (0.6±0.2 vs 12.8±5.8, p=0.09; fed vs fasted) and c-fos (1.1±0.3 vs 17.4±6.7, p=0.1; fed vs fasted) gene expression, although these differences failed to reach statistical significance, while OB-RB gene expression was significantly increased with fasting (0.7±0.2 vs 10.3±4.3, p=0.02; fed vs fasted).
Figure 8.5. Adipose tissue expression of a) OB-RA, b) OB-RB and c) C-FOS (relative to ribosomal RNA) in fed and fasted *Psammomys obesus*.
Figure 8.5 shows that in adipose tissue, fasting tends to increase expression of OB-RA (0.02±0.009 vs 0.16±0.08, p=0.15; fed vs fasted) and OB-RB (0.02±0.008 vs 0.24±0.19, p=0.19; fed vs fasted) in lean *Psammomys obesus*, while there is no effect of fasting on c-fos gene expression in this group of animals (1.2±1.12 vs 1.5±0.67; fed vs fasted). In obese *Psammomys obesus*, both diabetic and non-diabetic animals show no effect of fasting on the level of OB-RA gene expression in adipose tissue (obese: 0.01±0.007 vs 0.02±0.005; obese, diabetic: 0.02±0.01 vs 0.02±0.006; fed vs fasted) while there is evidence for increased OB-RB gene expression in both groups of obese animals (obese: 0.01±0.003 vs 0.05±0.01, p=0.07; obese, diabetic: 0.07±0.03 vs 0.16±0.13; fed vs fasted). There was no significant difference in c-fos gene expression in adipose tissue between fed and fasted obese (1.3±1.0 vs 2.2±0.8, fed vs fasted) and obese, diabetic *Psammomys obesus*, although there was a tendency for decreased expression of c-fos after fasting in the obese, diabetic group (4.7±2.6 vs 1.4±0.90, p=0.12; fed vs fasted).
8.5 DISCUSSION

This study demonstrated that a 24-hour fast leads to a reduction in blood glucose and plasma insulin concentrations in lean, obese, and obese diabetic Psammomys obesus, despite no change in body weight in any of these groups of animals. In addition, plasma leptin levels decreased by approximately 30% in lean Psammomys obesus following fasting, however leptin levels increased by 24% with fasting in obese animals. In the hypothalamus, fasting led to an increase in OB-RB gene expression in both lean and obese Psammomys obesus, and in the liver OB-RA gene expression was increased with fasting.

The changes seen in circulating leptin concentrations with fasting in Psammomys are consistent with those seen in an earlier study, where the reported changes in plasma leptin were a reduction of 44% in lean animals and an increase of approximately 20% in obese animals (Walder et al., 1998). When looking only at the fasted animals, the level of OB gene expression appears to parallel circulating leptin concentrations, with lean animals having the lowest levels, obese animals having the highest level and obese, diabetic Psammomys obesus showing a disproportionately low level of OB gene expression for their adipose tissue mass. Fasting has been shown consistently to decrease OB gene expression in lean mice and rats, however this is not seen in obese animals, with no effect of fasting on OB gene expression reported for ob/ob, diet-induced obese mice or fa/fa rats (Cusin et al., 1995; MacDougald et al., 1995; Mizuno et al., 1996a). Consistent with this, obese Psammomys obesus do not show a reduction in OB gene expression with fasting. Interestingly, obese, diabetic Psammomys obesus demonstrated reduced levels of OB gene expression with fasting, although this group show no change in plasma leptin during this time.

Several previous studies have been conducted examining the regulation of hypothalamic leptin receptors after fasting. In Sprague Dawley rats fasting has been shown to result in a differential regulation of leptin receptors in the hypothalamus, with an overall decrease in leptin receptors in the hypothalamus, but a specific increase in OB-RB gene expression with fasting (Bennett et al., 1998). In Wistar rats fasting also increased OB-RB expression within the hypothalamus (in the arcuate nucleus) by 40% (Baskin et al., 1998). In mice, fasting has been shown to increase hypothalamic OB-RB gene expression, with a 48-hour fast found to increase expression of OB-RB in the arcuate nucleus of normal and NPY-knock-out mice to 2.5-times baseline and 4-times baseline respectively (Baskin et al., 1998). In Psammomys obesus, a 24-hour fast tends to
decrease expression of OB-RA and increase expression of OB-RB gene expression in the hypothalamus of lean animals, consistent with previous studies in lean animals. In obese animals, fasting increased expression of OB-RA and OB-RB, while in obese, diabetic Psammomys obesus, fasting had no effect on the level of expression of either leptin receptor isoform.

The changes in OB-RB gene expression previously seen with fasting in both mice and rats have been suggested to be a response to decreased circulating leptin as a result of fasting, with the level of expression of OB-RB suggested to be sensitive to physiological interventions that result in changes in circulating leptin levels (Baskin et al., 1998). Baskin et al. (1998) hypothesise that binding of leptin to OB-RB may initiate intracellular signal transduction events that result in reduced transcription of the OB-RB gene, and further, Huang et al. (1997) suggest that upregulation of OB-RB in the hypothalamus may result from suppressed inhibition, with reduced amounts of leptin binding to this receptor in the brain.

In the present study there was increased expression of OB-RB within the hypothalamus of lean and obese Psammomys obesus after fasting. The increase in OB-RB gene expression with fasting however cannot solely be explained by decreased circulating leptin, as although fasting led to a 30% reduction in circulating leptin in lean animals there was a 24% increase in circulating leptin in obese animals. We have previously shown that obese Psammomys are leptin resistant, with even very high doses of exogenous leptin ineffective at decreasing food intake or body weight in these animals (Walder et al., 1999). The effects of fasting on leptin concentrations and hypothalamic OB-RB gene expression in obese animals therefore, may indicate some dysregulation of the leptin regulatory pathway in these animals.

The role of peripheral leptin receptors has not been clearly defined in any species, however in the present study there is evidence for differential regulation of OB-RA and OB-RB gene expression with fasting in liver and adipose tissue. In all groups of Psammomys obesus there are large increases in the level of OB-RA gene expression in the liver after fasting, while in lean and obese, non-diabetic animals there is also a tendency for a reduction in OB-RB gene expression after fasting. In contrast, the level of OB-RB gene expression in the liver of obese, diabetic animals is increased with fasting. Several studies have now demonstrated that leptin may act in the liver to regulate intrahepatic energy partitioning, with leptin shown to decrease hepatic glucose
production and increase glucose turnover and uptake (Barzilai et al., 1997; Rossetti et al., 1997; Sivitz et al., 1997). The large increase in OB-RA gene expression in the liver of lean and obese *Psammomys obesus* with fasting suggests that hepatic leptin receptors may be involved in this regulation of energy partitioning by leptin. The actual signal for this increased OB-RA gene expression and the manner in which OB-RA is involved as the liver responds to fasting is worthy of further investigation.

This study also examined the regulation of leptin receptors with fasting in white adipose tissue. Interestingly, there appears to be no regulation of OB-RA gene expression in obese, diabetic or non-diabetic animals, while OB-RA tends to be increased with fasting in lean *Psammomys obesus*. OB-RB gene expression tends to be increased with fasting in all three groups of animals. The presence of leptin receptors in adipose tissue has been suggested to indicate the presence of an autocrine regulatory pathway, whereby the adipose tissue can 'sense' the amount of circulating leptin and adjust OB gene expression and leptin production accordingly (Lee et al., 1996; Fei et al., 1997; Hoggard et al., 1997; Zhang et al., 1997). However, again it is apparent that changes in plasma leptin concentrations are not necessarily followed by changes in leptin receptor gene expression, with obese animals showing an increase in OB-RB gene expression with fasting, despite an increase in plasma leptin at the same time. Surprisingly, there also appears to be a degree of differential regulation of peripheral leptin receptor gene expression, with different responses to fasting seen between the liver and adipose tissue.

An alternative view of the results of this study may be that lean animals show normal regulation of leptin homeostasis, while obese animals have a defect that results in dysregulation of this pathway and therefore obesity. In lean animals, fasting leads to decreased circulating leptin, which may be associated with decreased leptin binding to OB-RB in the hypothalamus. This may ultimately be a signal for the animal to eat, as there is co-localisation of neurons containing OB-RB and several neuropeptides involved in increasing food intake, such as NPY and POMC (Elmqquist et al., 1998b; Hakansson et al., 1998a). At the same time, in the periphery, lower leptin binding to OB-RB in adipose tissue also results in increased expression of OB-RB in adipose tissue. This increased expression of OB-RB may result in decreased OB gene expression (Zhang et al., 1997), which will further decrease circulating leptin and allow the animal to eat when food becomes available.
Obese *Psammomys obesus* develop obesity when maintained under the same conditions and fed the same diet as animals that remain lean and healthy (Shafrir and Gutman, 1993; Barnett *et al.*, 1994a). Obese animals have also been shown to be unresponsive to even very high doses of exogenous leptin (Walder *et al.*, 1999). These animals therefore are considered leptin resistant and the variable responses seen with fasting in circulating leptin and leptin receptor gene expression in the present study indicates a dysregulation of leptin homeostasis in these animals that needs further investigation.

In summary, this study has confirmed the previous finding that leptin levels are decreased with fasting in lean *Psammomys obesus*, but increased with fasting in obese animals. In addition, this study has found that the changes seen in hypothalamic leptin receptor gene expression may be explained by changes in circulating leptin in lean animals, but not in obese *Psammomys obesus*. In addition, this study provides evidence of regulation of leptin receptor gene expression in peripheral tissues with fasting, although there does not appear to be a clear explanation for the variable changes seen across the different tissues in the different phenotypic groups of *Psammomys obesus*.
CHAPTER 9

THE EFFECTS OF SUCROSE FEEDING ON ENERGY BALANCE IN *PSAMMOMYS OBESES*: THE ROLE OF LEPTIN AND LEPTIN RECEPTORS.

9.1 SUMMARY

Leptin is known to play an important role in the regulation of energy balance in rodents and humans, with changes in either leptin action or leptin receptor signaling found to perturb this regulatory pathway and result in obesity. Sucrose feeding is a useful method of inducing energy imbalance, obesity and type 2 diabetes in rodents and in this study we examined changes in leptin concentrations and the leptin receptors, OB-RA (short isoform) and OB-RB (long, signaling isoform), following sucrose feeding in lean and obese Psammomys obesus. Psammomys obesus is a polygenic rodent model of obesity and type 2 diabetes which exhibits a range of body weight and blood glucose and plasma insulin and leptin concentrations, similar to those seen in humans. In this study we demonstrated that in lean animals, sucrose feeding resulted in increased body weight but no change in percent body fat, or circulating glucose or insulin concentrations, however, in obese animals, sucrose feeding increased body weight and percent body fat and resulted in the development of type 2 diabetes, with increased glucose and insulin concentrations. In both groups of animals, despite the increased body weight, there was a tendency for reduced plasma leptin concentrations (p=0.19 and p=0.18 for lean and obese animals, respectively), although only lean animals showed evidence of a reduction in OB gene expression. In addition, in lean Psammomys obesus sucrose feeding tended to decrease OB-RA gene expression, but had no effect on OB-RB or c-fos gene expression in the hypothalamus. In contrast, in obese animals sucrose feeding tended to increase OB-RB and c-fos gene expression in the hypothalamus. There did not appear to be any effect of sucrose feeding on leptin receptor gene expression in suprascapular white adipose tissue, while in the liver there was a tendency for OB-RA and OB-RB gene expression to be decreased, and c-fos gene expression to be increased with sucrose feeding. From this study it is unclear how sucrose feeding decreased plasma leptin levels in both lean and obese Psammomys obesus or the role of leptin receptors in the dysregulation of energy balance and glucose homeostasis seen in obese animals with sucrose feeding, however it is interesting to speculate that the ability for animals to overeat on sucrose diets involves a direct effect of sucrose on the leptin signaling pathway.
9.2 INTRODUCTION

Leptin, the protein product of the OB gene has consistently been shown to be involved in the regulation of energy balance in animal models of obesity and type 2 diabetes. Leptin administration to the ob/ob mouse, an animal found to have undetectable levels of circulating leptin, resulted in decreased food intake, increased thermogenesis and subsequently decreased body weight and body fat (Campfield et al., 1995; Halaas et al., 1995).

The leptin receptor (OB-R) is also known to play a crucial role in energy balance with mutations in the OB-R gene found to result in obesity in several animal models. This receptor has several alternatively spliced isoforms which differ in the length of their intracellular domains (Lee et al., 1996; Tartaglia et al., 1996). OB-RB has a long intracellular domain that has been shown to activate signal transduction pathways within the cell and is thought to represent the main signaling form of the receptor (Bjorbaek et al., 1997; Takahashi et al., 1997; White et al., 1997a; Elmquist et al., 1998a). OB-RA is a much shorter isoform, and although it has some signaling capacity, this receptor is thought to function predominately as a transporter of leptin into the cell (Tartaglia et al., 1996; Takahashi et al., 1997).

Similar to other class I cytokine receptors, leptin receptors lack intrinsic tyrosine kinase activity and require co-operation of associated JAK kinases for phosphorylation and activation of the signal transducing STAT proteins (Ihle et al., 1994; Nakashima et al., 1997b). Activation of STATs results in translocation to the nucleus where they bind DNA and thereby regulate transcription (Ihle et al., 1994). OB-RB is known to activate several STAT proteins, including STAT3 (Baumann et al., 1996; Ghilardi et al., 1996; Takahashi et al., 1996b), and tyrosine phosphorylation of STAT3 results in stimulation of c-fos gene transcription in vitro (Bjorbaek et al., 1997; Hakansson et al., 1998b). C-fos is expressed in neurons in response to direct stimulation by growth factors and neurotransmitters (Sager et al., 1988) and central administration of leptin has been reported to increase c-fos-like immunoreactivity within several regions of the hypothalamus, including the paraventricular nucleus, the dorso-medial nucleus, the arcuate nucleus and the central amaygdala (Bjorbaek et al., 1997; Elmquist et al., 1998a; Hakansson et al., 1998b).

Several groups have now demonstrated that fasting increases OB-RB gene expression several fold in different regions of the hypothalamus in wild-type mice and Sprague-
Dawley, Wistar and Zucker (fa/fa) rats (Baskin et al., 1998; Lin and Huang, 1997; Fei et al., 1997; Bennett et al., 1998). These studies provide evidence of a feed-back loop, by which a reduction in circulating leptin (as with fasting) results in up-regulation of leptin receptor gene expression as part of a regulatory mechanism. The study by Lin and Huang also showed that in ob/ob mice, which lack circulating leptin, fasting does not increase leptin receptor gene expression, providing further support for the hypothesis that circulating leptin concentrations are important regulators of OB-R gene expression. These findings however, do not rule out the possibility that other factors play a role in the regulation of leptin receptor gene expression, particularly in polygenic models of obesity, which more closely mimic the situation of human obesity.

In addition to central effects, many studies have now demonstrated direct actions of leptin in a number of peripheral tissues (Barzilai et al., 1997; Cohen et al., 1997; Muioi et al., 1997; Shimabukuro et al., 1997). In adipose tissue leptin appears to increase lipolysis and thermogenesis and may also be involved in shifting adipocyte metabolism away from glucose to fat (Saricimento et al., 1997; Siegrist-Kaiser et al., 1997; Muller et al., 1997; Zhou et al., 1997; Ceddia et al., 1998). In the liver leptin appears to enhance insulin action, with decreased hepatic glucose production and increased glucose turnover and uptake reported with leptin treatment (Barzilai et al., 1997; Rossetti et al., 1997; Sivitz et al., 1997). These studies suggest that leptin may be involved in energy partitioning, although the exact role of the leptin receptor in these tissues is unknown.

The effects of leptin in peripheral tissues are possibly mediated by OB-RA and OB-RB, both of which are known to be expressed in the periphery as well as the brain (Lee et al., 1996; Fei et al., 1997; Hoggard et al., 1997; Lollman et al., 1997; Luoh et al., 1998). To further examine the role of leptin and leptin receptors in energy balance, this study examined the effect of sucrose feeding on leptin receptor gene expression in the hypothalamus, liver and adipose tissue of lean and obese Psammomys obesus. Sucrose feeding is a well established method of inducing the development of obesity and type 2 diabetes in rodents (Kanerek and Orthen-Gambill, 1982; Rattigan et al., 1986; Lombardo et al., 1996) and therefore represents an attractive model through which to study the role of environmental nutritional factors in the perturbation of energy balance and glucose homeostasis, and the subsequent effects of the dysregulation of energy balance on leptin and leptin receptors.
Psammomys obesus is a polygenic rodent model of obesity and type 2 diabetes which exhibits a range of body weight and blood glucose and insulin concentrations (Shafrir and Gutman, 1993; Barnett et al., 1994a) which closely resemble that seen in human populations (DeFronzo, 1988). Obese Psammomys obesus have previously been shown to have increased OB gene expression and elevated plasma leptin concentrations compared to their lean littermates (Walder et al., 1997b). In addition, studies involving peripheral leptin administration to lean and obese Psammomys obesus have demonstrated that only lean Psammomys obesus are sensitive to the anorexigenic effects of leptin administration, with decreased food intake and body weight observed in these animals after peripheral leptin administration (Walder et al., 1999). Obese Psammomys obesus show marked leptin resistance, with even very high doses of leptin ineffective in these animals. In the present study we examine the effects of sucrose feeding to lean and obese Psammomys obesus on leptin and leptin receptor gene expression in the hypothalamus, liver and adipose tissue.
9.3 MATERIALS AND METHODS

9.3.1 EXPERIMENTAL ANIMALS
A *Psammomys obesus* colony is maintained at Deakin University, with breeding pairs fed ad libitum a diet of lucerne and chow. Experimental animals were weaned at 4 weeks of age and given a diet of standard laboratory chow (12% energy from fat, 63% from carbohydrates, 25% from protein; Barastoc, Pakenham, Australia). Animals were housed in a temperature-controlled room (22°C±1°C) with a 12-hour/12-hour light/dark cycle (See Chapter 2 for details).

9.3.2 CHARACTERISATION OF ANIMALS AND EXPERIMENTAL PROTOCOL
At 16 weeks of age animals were classified as either lean or obese, with lean animals having a body weight <200g and obese animals having a body weight ≥200g and insulin concentration ≥150μU/ml. For this study we used 18 lean and 18 obese animals, with 9 animals from each group allowed *ad libitum* access to standard laboratory chow and water, and the other 9 animals from each group allowed *ad libitum* access to standard laboratory chow and a 5% sucrose solution for three weeks. During the three week study period body weight, blood glucose and plasma insulin concentrations were measured regularly (two times per week). At the end of the study animals were killed by lethal overdose.

9.3.3 RNA PREPARATION AND cDNA SYNTHESIS
Animals were killed by lethal overdose of pentobarbitone (120mg/kg) and the hypothalamus, liver and adipose tissue from the suprascapular, perirenal, epididymal, intramuscular and mesenteric fat depots were removed. After homogenisation, RNA was purified from the whole hypothalamus or 100mg of liver and suprascapular white adipose tissue using RNAzol B (Bresatec, Adelaide, Australia). The RNA was extracted with 200μl of chloroform and precipitated with an equal volume of isopropanol. RNA was quantitated by spectrophotometry at 260nm and 1μg was then reverse transcribed at 42°C for 1 hr with 10U of AMV reverse transcriptase (Promega, Madison, USA) according to the manufacturer’s instructions.
9.3.4 POLYMERASE CHAIN REACTION (PCR) AND QUANTITATION

Comparative RT-PCR was performed using the Taqman ‘Real Time’ system (Perkin Elmer). This system utilises a fluorescent probe within the PCR reaction to detect the amplification of the appropriate region of the cDNA (Gibson et al., 1996). The level of fluorescence emitted is sampled every 7 seconds during the 40 cycles of amplification and is directly proportional to the amount of product generated (Gibson et al., 1996; Heid et al., 1996; See Chapter 2 for details). All quantitation of gene expression reported is expressed relative to the ribosomal RNA house-keeping gene (Perkin Elmer Applied Biosystems, Forster City, USA). The sequences of the oligonucleotide primers and probes used for amplification of OB, OB-RA, OB-RB and c-fos were based on the *Psammomys obesus* sequence and lie in regions found to be highly conserved across several species including rat, mouse and human.

**OB gene:**

<table>
<thead>
<tr>
<th>Type</th>
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<tbody>
<tr>
<td>Forward primer</td>
<td>5’-tcgcggctaccaacacac-3’</td>
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<tr>
<td>Reverse primer</td>
<td>5’-ctctactgtctgtgatgta-3’</td>
</tr>
<tr>
<td>Probe</td>
<td>5’- FAM tgaacagctcctccgaat TAMRA-3’</td>
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**OB-RA:**

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<tr>
<td>Reverse primer</td>
<td>5’-ctcttttggaaataagcttg-3’</td>
</tr>
<tr>
<td>Probe</td>
<td>5’- FAM atgttccaaaccccagaatggctcg TAMRA-3’</td>
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**OB-RB:**

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<tbody>
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<td>Forward primer</td>
<td>5’-aagcttagaactggacttt-3’</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5’-tgcatgcactgattccttg-3’</td>
</tr>
<tr>
<td>Probe</td>
<td>5’-FAM atcaggtatatgtctttctcttgagg TAMRA-3’</td>
</tr>
</tbody>
</table>

**c-fos:**

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>5’-tgaagagaaggaactggagttta-3’</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5’-ccaggagggcagaca-3’</td>
</tr>
<tr>
<td>Probe</td>
<td>5’-FAM tggcagcaccacagctgc TAMRA-3’</td>
</tr>
</tbody>
</table>

**Ribosomal RNA:**

<table>
<thead>
<tr>
<th>Type</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>5’-cggctaccaatcagggua-3’</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5’-gcggaaattacacgctg-3’</td>
</tr>
<tr>
<td>Probe</td>
<td>5’-VIC tgcggcaccagacctggc TAMRA-3’</td>
</tr>
</tbody>
</table>
PCR was performed by adding 50 ng of cDNA to a tube containing: 1xTaqMan Universal PCR Master Mix (AmpliTaq Gold™, AmpErase® Uracil N-glycosylase (UNG), dNTPs, 5x TaqMan Buffer A (with ROX passive reference) 12.5 mM MgCl₂ and glycerol; Perkin Elmer Applied Biosystems, Foster City, USA), the appropriate concentration of primers and probe (See Chapter 2 for details) in a final volume of 25 μl in a MicroAmp® Optical 96-well Reaction Plate covered with MicroAmp® Optical caps (Perkin Elmer Applied Biosystems, Foster City, USA). Amplification was performed using the following conditions: 50°C for 2 mins, 95°C for 10 mins and 40 cycles of 95°C for 15 sec and 60°C for 1 min.

9.3.5 BIOCHEMICAL ANALYSES
Whole blood glucose was measured using an enzymatic glucose analyser (Model 2300 STAT Plus, Yellow Springs Instruments, Ohio). Plasma insulin concentrations were determined using a double antibody solid phase radioimmunoassay (Phadescoph, Kabi Pharmacia Diagnostics, Sweden). Plasma leptin concentrations were measured using a commercially available radioimmunoassay kit (Multispecies leptin assay; Linco Reaserch, St Charles, MO, USA). A multispecies leptin antibody (produced in guinea pig) was used in this kit, with a ¹²⁵I-human leptin tracer and a normal guinea pig IgG antibody added as the second antibody. The concentration of leptin in plasma from *Psammomys obesus* was determined using human leptin standards of known concentrations and as the multispecies leptin antibody was raised against human leptin, and human leptin standards were used, the units of measurement are ng/ml Human Equivalent (HE) leptin. Plasma free fatty acids, triglyceride and cholesterol concentrations were measured using colorimetric kits (Boehringer Mannheim, Mannheim, Germany) on an automatic analyser (for triglycerides and cholesterol, as described in detail in chapter 2) or by spectrophotometry at 550 nm (for free fatty acids, as described in detail in chapter 2).

9.3.6 ESTIMATED PERCENT BODY FAT
The weight of selected fat depots (suprascapular, perirenal, epididymal, intramuscular and mesenteric) was used to provide an estimate of body fat content. The weights of the various depots were combined and divided by total body mass to provide an estimate of percent body fat.
9.3.7 STATISTICAL ANALYSES
The independent t-test was used to test for differences in means between the control and sucrose-fed groups. A p-value greater than 0.05 was regarded as significant in all cases and statistical analyses were performed using SPSS (version 8.0).
9.4 RESULTS

Tables 9.1 and 9.2 show the metabolic characteristics of the lean and obese animals before and at the end of the study period, respectively.

Table 9.1 Comparison of metabolic characteristics (mean±sem) of lean and obese animals prior to the study.

<table>
<thead>
<tr>
<th>Metabolic Variable:</th>
<th>Lean</th>
<th>vs</th>
<th>Obese</th>
<th>vs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bodyweight (g)</td>
<td>Control</td>
<td>174.1±10.2</td>
<td>Sucrose fed</td>
<td>179.0±10.0</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.25±0.30</td>
<td>4.42±0.23</td>
<td>4.23±0.38</td>
<td>5.31±1.05</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>52.1±7.4</td>
<td>78.8±16.2</td>
<td>336.4±39.3</td>
<td>313.9±49.3</td>
</tr>
<tr>
<td>Plasma leptin (ng/ml H.E.)</td>
<td>50.5±4.8</td>
<td>60.7±9.8</td>
<td>88.4±7.5</td>
<td>91.7±6.8</td>
</tr>
</tbody>
</table>

*mean ± SEM

As a group, the obese animals were initially heavier, fatter and had higher plasma insulin concentrations than the lean animals. Obese animals also tended to have higher leptin and free fatty acid levels compared to lean animals.
**Table 9.2** Comparison of metabolic characteristics (mean±SEM) of lean and obese animals after three weeks ad libitum access to either chow (controls) or chow+sucrose (sucrose fed).

<table>
<thead>
<tr>
<th>Metabolic Variable</th>
<th>Lean Control vs Sucrose fed</th>
<th>Obese Control vs Sucrose fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bodyweight (g)</td>
<td>177.1±11.3 p=0.27</td>
<td>210.3±10.4 p=0.26</td>
</tr>
<tr>
<td>Estimated percent body fat</td>
<td>3.0±0.4 p=0.41</td>
<td>3.95±0.38 p=0.02</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.3±0.3 p=0.33</td>
<td>4.3±0.3 p=0.03</td>
</tr>
<tr>
<td>Insulin (μU/ml)</td>
<td>59.2±11.0 p=0.50</td>
<td>275.8±59.9 p=0.04</td>
</tr>
<tr>
<td>Plasma leptin (ng/ml H.E.)</td>
<td>42.7±6.2 p=0.34</td>
<td>79.8±10.8 p=0.12</td>
</tr>
<tr>
<td>Free fatty acids (mEq/ml)</td>
<td>0.43±0.05 p=0.01</td>
<td>0.24±0.04 p=0.07</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.0±0.16 p=0.32</td>
<td>1.62±0.2 p=0.91</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>2.3±0.2 p=0.95</td>
<td>2.4±0.2 p=0.90</td>
</tr>
</tbody>
</table>

*mean ± SEM

At the end of the study, sucrose-fed lean animals tended to be heavier and have lower plasma leptin levels than lean control animals, while sucrose-fed lean animals also had higher free fatty acids than lean controls (0.43±0.05 vs 0.22±0.04, p=0.01; sucrose-fed vs control) (table 9.2). Obese sucrose-fed animals also tended to be heavier, have lower plasma leptin levels and free fatty acids than obese control animals, while the sucrose fed obese group had significantly higher estimated percent body fat (5.4±0.39 vs 3.95±0.38, p=0.02; sucrose-fed vs control), blood glucose (10.7±2.4 vs 4.3±0.3, p=0.03; sucrose-fed vs control) and plasma insulin (526.9±92.8 vs 275.8±59.9, p=0.04; sucrose-fed vs control) concentrations compared to obese controls.
Figure 9.1. Effect of sucrose treatment on metabolic variables in lean and obese Psammomys obesus, percentage change in a) bodyweight and plasma leptin concentration, and b) blood glucose and plasma insulin concentrations (*p=0.002, **p=0.005, ^p=0.03; significantly different from pre-treatment level)
Figure 9.1a) shows that sucrose feeding resulted in an 8% and 9% increase in body weight in lean and obese animals respectively (lean $p=0.002$ and obese $p=0.005$; compared to control group). Despite this increase in bodyweight, plasma leptin concentrations tended to decrease in both groups with sucrose feeding. In lean animals there was a 23% decrease in circulating leptin concentration ($p=0.19$), while in obese animals there was a 30% decrease in leptin ($p=0.18$). Figure 9.1b) shows that while in lean animals there was no change in blood glucose or plasma insulin concentrations with sucrose feeding, in obese animals sucrose feeding resulted in a 94% increase in blood glucose and an 81% increase in plasma insulin concentrations ($p=0.05$ and $p=0.13$, respectively, compared to the control group).
Figure 9.2 Effects of sucrose-feeding on percent body fat and OB gene expression (relative to ribosomal RNA) in lean and obese Psammomys obesus (*p=0.02; significantly different from obese, chow-fed group).

Figure 9.2 shows that there was no difference in estimated percent body fat in control and sucrose-fed lean animals, despite the 8% increase in bodyweight. In addition, there was a tendency for OB gene expression to be reduced in the adipose tissue of the sucrose-fed lean animals. In the obese group, sucrose-fed animals had a significantly greater estimated percent body fat than obese control animals (3.95±0.38 vs 5.4±0.39, p=0.02; control vs sucrose-fed) while there did not appear to be any effect of sucrose feeding on the level of OB gene expression.
Figure 9.3 Hypothalamic gene expression of OB-RA, OB-RB and c-fos (relative to ribosomal RNA) in chow-fed and sucrose-fed, lean and obese *Psammomys obesus*.

Figure 9.3 shows that in lean animals, sucrose feeding had almost no effect on the level of hypothalamic OB-RB (31.5±13.0 vs 26.5±9.1; control vs sucrose fed) and c-fos (38.8±15.0 vs 41.2±20.6; control vs sucrose fed) gene expression, while there was a tendency for OB-RA gene expression to be decreased in the lean sucrose-fed group compared to the control group (16.3±6.6 vs 2.8±1.09; control vs sucrose fed). In obese animals, sucrose feeding tended to increase the level of expression OB-RA (6.1±2.2 vs 9.2±6.9; control vs sucrose fed), OB-RB (15.1±5.9 vs 34.9±11.9; control vs sucrose fed) and c-fos (9.95±3.9 vs 29.4±14.6; control vs sucrose fed) although these differences did not reach statistical significance.
Figure 9.4 Hepatic gene expression of OB-RA, OB-RB and c-fos (relative to ribosomal RNA) in chow-fed and sucrose-fed, lean and obese *Psammomys obesus*.

Figure 9.4 shows that in the liver of lean animals, the control group tended to have higher levels of OB-RA (12.2±6.4 vs 4.8±2.0; control vs sucrose fed) and OB-RB (1.6±0.7 vs 0.9±0.3), but lower levels of c-fos gene expression compared to the sucrose-fed animals (2.3±1.1 vs 4.3±1.5), although these differences were not statistically significant. In obese animals, similar results were seen, with a tendency for lower levels of gene expression of OB-RA (2.7±1.3 vs 1.3±0.4) and OB-RB (0.8±0.5 vs 0.3±0.06) seen in the sucrose fed group, and a tendency for increased c-fos gene expression in the sucrose fed group (2.2±0.9 vs 4.7±0.9; p=0.06)
Figure 9.5 Adipose tissue gene expression of OB-RA, OB-RB and c-fos (relative to ribosomal RNA) in chow-fed and sucrose-fed, lean and obese *Psammomys obesus*.

In adipose tissue, there were only low levels of OB-RA and OB-RB gene expression and these were not different between the control and sucrose-fed groups (lean-OB-RA: 0.06±0.04 vs 0.02±0.01 and OB-RB: 0.07±0.03 vs 0.15±0.12; obese-OB-RA: 0.02±0.01 vs 0.04±0.02 and OB-RB: 0.06±0.02 vs 0.05±0.01; control vs sucrose fed). The expression of c-fos was higher than OB-RA and OB-RB although again there was no difference in the level of expression between control and sucrose-fed animals (lean:1.04±0.4 vs 1.12±0.27; obese: 2.7±0.77 vs 2.36±1.05; control vs sucrose-fed).
9.5 DISCUSSION
In obese, non-diabetic *Psammomys obesus*, the addition of a 5% sucrose solution to the diet resulted in increased body weight, increased percent body fat, increased glucose and insulin concentrations, and the development of type 2 diabetes. In addition, despite the increase in adiposity, sucrose supplementation tended to decrease plasma leptin concentrations and increase OB-RB and *c-fos* gene expression in the hypothalamus of obese *Psammomys obesus*.

Within a colony of *Psammomys obesus* when animals from the same litter are weaned onto standard laboratory chow and maintained under identical conditions some animals will remain lean and healthy, while others will develop hyperphagia and subsequently obesity, which may or may not be accompanied by type 2 diabetes (Shafrir and Gutman, 1993; Barnett et al., 1994a). The animals which become obese are thought to be susceptible to the added environmental stress of a relatively high energy dense diet and in addition to being obese are also hyperinsulinemic. Initially this hyperinsulinemia is sufficient to maintain normal or impaired glucose homeostasis, however over time the animals may progress to a diabetic state. In this study we have demonstrated that obese, insulin resistant *Psammomys obesus* show increased body weight, increased percent body fat, and the development of diabetes when allowed access to sucrose in addition to their normal chow diet, indicating that the energy imbalance resulting with sucrose feeding increases body fat to a new 'set point' which eventually leads to diabetes. In addition, this study demonstrated that addition of only a 5% sucrose solution to the diet is enough to trigger this energy imbalance and the development of diabetes in these animals.

We have previously shown that obese *Psammomys obesus* are also leptin resistant, with exogenous leptin having no effect on body weight or body fat, even at very high doses (Walder et al., 1999). In the present study, the dysregulation of energy balance and glucose homeostasis with sucrose feeding in obese animals is accompanied by decreased circulating leptin concentrations, but no change in the level of OB gene expression in adipose tissue. In addition, within the hypothalamus obese animals show lower levels of OB-RA, OB-RB and *c-fos* gene expression when compared to lean *Psammomys obesus*, although there is a tendency for expression of these genes to be increased with sucrose feeding.
Within the liver, the development of diabetes in obese, sucrose-fed animals is associated with slightly lower levels of expression of OB-RA and OB-RB and increased expression of c-fos. The role of peripheral leptin receptors in the regulation of energy balance and glucose homeostasis is yet to be determined, however both in vivo and in vitro studies have demonstrated that leptin may enhance insulin action, with decreased hepatic glucose production and increased glucose turnover and uptake seen with leptin treatment (Barzilai et al., 1997; Rossetti et al., 1997; Sivitz et al., 1997). These studies have led to the suggestion that leptin may be involved in altering energy partitioning within the liver and leptin action in the liver may contribute to insulin resistance. In this study leptin levels decreased with only small changes in leptin receptor gene expression, which makes it difficult to speculate that leptin is having a direct action in the liver which would contribute to the development of diabetes.

Results of fasting and leptin-treatment studies in rodents have provided evidence of regulation of leptin receptor gene expression in accordance with changes in plasma leptin concentrations, with fasting found to decrease plasma leptin levels and increase expression of OB-RB in the hypothalamus (Huang et al., 1997; Baskin et al., 1998; Bennett et al., 1998), and leptin treatment shown to increase circulating leptin concentrations and decrease expression of OB-RB in the hypothalamus of ob/ob mice (Mercer et al., 1997; Schwartz et al., 1996a; Baskin et al., 1998). These studies led to the hypothesis that leptin receptor gene expression in the hypothalamus was altered as part of a negative feed-back regulatory pathway in a manner similar to the regulation of insulin receptor gene expression by circulating insulin concentrations. In the present study we show that in obese, leptin resistant Psammomys obesus, sucrose supplementation results in a differential regulation of OB-RA and OB-RB gene expression throughout the body, with a tendency for increased expression in the hypothalamus, decreased expression in the liver, and no change in gene expression in the adipose tissue. These differing responses are seen despite the decrease in circulating leptin concentrations with sucrose feeding, suggesting that in polygenic models of obesity, factors other than leptin concentrations may be involved in the regulation of leptin receptor gene expression throughout the body.

In lean animals, sucrose feeding increased body weight but did not effect percent body fat or glucose homeostasis, in addition these animals also showed evidence of a reduction in plasma leptin concentrations which was accompanied by a slight decrease in OB gene expression. Despite the reduced levels of leptin in the blood, the level of
hypothalamic OB-RA gene expression tended to be decreased and OB-RB and c-fos gene expression were not altered in lean, sucrose-fed Psammomys obesus. In addition, there was a tendency for a reduction in OB-RA and OB-RB gene expression in the liver, but no effect of sucrose feeding on adipose tissue leptin receptor gene expression in these animals. It appears that lean animals are resistant to the effects of increasing dietary sugar intake, and whether this difference is due to different effects of leptin metabolism is unclear from the current study.

In summary, we have demonstrated that sucrose supplementation to the diet of Psammomys obesus leads to energy imbalance, increased body fat and altered glucose homeostasis in susceptible animals only. The metabolic changes seen in obese animals are accompanied by decreased plasma leptin concentrations and evidence of increased leptin receptor gene expression in the hypothalamus, suggesting a disturbance of the leptin signalling pathway in these animals. In lean animals, sucrose feeding resulted in decreased plasma leptin concentrations, however it did not lead to increased adiposity or the development of diabetes. In addition, the decrease in leptin concentrations in lean animals was accompanied by evidence of decreased leptin receptor gene expression in the hypothalamus. Therefore, sucrose feeding leads to increased body fat and disturbances in leptin signaling, which may in part allow for the increased body fat to occur in susceptible animals.
CHAPTER 10

CONCLUSIONS AND FUTURE DIRECTIONS

10.1 CIRCULATING LEPTIN CONCENTRATIONS, OBESITY AND TYPE 2 DIABETES.

When the OB gene was first discovered in 1994, and subsequently shown to be the cause of morbid obesity in ob/ob mice, many laboratories, including our own, set out to investigate the role of the OB gene, and its protein product leptin, in the pathogenesis of obesity and type 2 diabetes. In humans, circulating leptin concentrations have now been shown to be associated with both adiposity and circulating metabolites such as insulin and glucose in various populations.

In a population of Caucasian Australian women, we demonstrated that fasting leptin concentrations were associated with both body mass and composition, as well as a range of circulating metabolites including insulin, glucose, triglycerides and cholesterol. In this population, the association between leptin and insulin concentrations was found to be independent of percent body fat, as was the relationship between leptin and triglyceride concentrations. Linear regression modelling, in fact revealed that the variability in circulating leptin concentrations across this population were best predicted by percent body fat and fasting insulin concentrations.

Environmental factors known to be important in the regulation of energy balance in humans were also examined. However there was no association between circulating leptin concentrations and environmental factors such as physical activity level, smoking habits or alcohol intake in this population. Leptin has also been suggested to play a role in the control of reproduction in both rodents and humans, however in this population of women, circulating leptin concentrations were not associated with several measures of reproductive history, including number of pregnancies, births or menopause status.

Although we have examined the relationship between circulating leptin concentrations and a variety of environmental variables in a cross-sectional study, cross-sectional studies are limited, as they do not take into account the dynamic nature of the phenotype or measure under investigation. There is a strong case implicating leptin in the regulation of reproductive function in rodents and a limited number of humans, and the role of leptin in reproductive processes warrants further study. It would be particularly valuable to conduct a longitudinal or prospective examination of this population and investigate further the associations between reproductive variables and circulating leptin.
concentrations. In addition, investigation of the relationship between leptin and more sensitive measures of reproduction function would also be useful, such as levels of a number of important sex hormones. It has also been suggested that a 24-hour profile of leptin is necessary to gain a true measure of leptin concentrations in an individual, given the fluctuating nature of this metabolite, and therefore it would be informative to look at associations between all of the variables mentioned and 24-hour leptin concentrations in this population and others.

10.2 LEPTIN CONCENTRATIONS IN PSAMMOMYS OBEUS, A POLYGENIC RODENT MODEL OF OBESITY AND TYPE 2 DIABETES.

Previous studies by our group and others have shown Psammomys obesus to be an excellent polygenic animal model of obesity and type 2 diabetes (reviewed in Section 1). In response to a diet of normal laboratory chow, these animals exhibit a complete spectrum of body weight and blood glucose and insulin concentrations, which is similar to the pattern of obesity and type 2 diabetes which have been observed in cross-sectional analysis of human populations.

Early studies conducted in Psammomys obesus demonstrated that obese Psammomys over-expressed the OB gene, and had higher circulating levels of leptin compared to lean, control animals, similar to obese humans. We have shown that circulating leptin concentrations are associated with percent body fat, insulin and glucose concentrations in a population of 130 Psammomys obesus. Similar to humans, the association between leptin and insulin was found to be independent of percent body fat. Plasma leptin concentrations were however not associated with age, sex, obesity or diabetes status independent of adiposity in Psammomys obesus.

These results further demonstrate that Psammomys obesus is an excellent animal model in which to examine the role of leptin in the regulation of energy balance and body weight homostasis, as it so closely resembles the metabolic characteristics associated with human obesity. The usefulness of Psammomys obesus as a model of human obesity and type 2 diabetes should be utilised in an effort to determine early predictors of obesity, including the development of leptin resistance, and the role of leptin resistance in the development of obesity.

Previous studies however, have demonstrated the difficulty associated with trying to determine whether leptin concentrations simply reflect changes in fat mass, and it is therefore important to examine other components of the leptin signaling pathway,
particularly the importance of the leptin receptor (OB-R), rather than simply circulating leptin concentrations.

10.3 SEQUENCE VARIATION IN THE HUMAN LEPTIN RECEPTOR GENE.

In 1995, Tartaglia et al. cloned the gene for the leptin receptor, OB-R, and subsequent studies demonstrated that mutations in this gene can result in the development of morbid obesity in rodents. Sequence analysis of the human OB-R gene in a number of populations revealed a variety of sequence polymorphisms in both the introns and exons of the gene, however these polymorphisms were found to be only very rarely associated with the development of obesity in humans.

We have examined two polymorphisms in the OB-R gene in two distinct populations; a population of Caucasian Australian women, and a population of Nauruan males. In the Australian population, the Gln223Arg and PRO1019pro polymorphisms in OB-R were not associated with obesity- or diabetes-related markers in cross-sectional analysis. In addition, the Gln223Arg polymorphism was not associated with changes in body mass or composition over a 2-year period. There was an association between changes in body mass and the PRO1019pro polymorphism, with subjects heterozygous for this polymorphism showing increased body weight gain over a two-year period. The meaning of this association is unclear, however this interesting finding warrants further investigation as this study is the first in which sequence variation in OB-R was examined in relation to longitudinal changes in body mass and composition in a human Caucasian population.

We also examined sequence variation in the OB and OB-R gene in a Nauruan male population. Nauru is an isolated island in the Pacific Ocean which contains a population which exhibits very high prevalence rates of obesity and type 2 diabetes. We examined the two OB-R polymorphisms in this population, as well as an OB gene microsatellite polymorphism. Cross-sectional analysis conducted in 232 non-diabetic male subjects showed no association between the Gln223Arg and OB gene polymorphisms and several phenotypic measures of body fatness and fat distribution, or a range of metabolic variables, including fasting glucose and insulin concentrations and 2hr glucose concentrations. Interestingly, there was an association between the PRO1019pro polymorphism and hyperinsulinemia and increased diastolic blood pressure in this population.

In a longitudinal analysis of the development of obesity and type 2 diabetes over a 12 year period in approximately 100 of these men, we found that the 3 polymorphisms
were not associated with an increased rate of development of either of these diseases. These cross-sectional and longitudinal results suggest that the polymorphisms in the OB-R and OB gene analysed probably do not play a role in the development of obesity of type 2 diabetes in the general population.

We also examined any relationship between polymorphisms in OB-R and the OB genes with phenotypic features of obesity and type 2 diabetes. As both obesity and type 2 diabetes are regarded as polygenic diseases, small sequence changes in multiple genes could possibly combine to exert an influence on the development of these diseases. We were in the unique position of being able to combine polymorphisms in two genes and examine associations with obesity and type 2 diabetes both cross-sectionally and longitudinally. This analysis revealed some interesting findings. Subjects found to be homozygous for all three sequence polymorphisms were found to have increased fasting insulin concentrations compared to subjects who were not homozygous for all three polymorphisms. Longitudinal analysis demonstrated that subjects with this particular genotype combination may show increased development of type 2 diabetes over a 12 year period, however, the numbers of subjects in this longitudinal analysis was limited for some genotypes. Hopefully in the future we will be able to further examine this relationship in a larger group of Nauruans, as well as in other populations.

10.4 LEPTIN RECEPTOR GENE EXPRESSION IN *PSAMMOMYX OBESUS*.

When the gene for the leptin receptor was first identified it was demonstrated that there were at least four isoforms of the receptor in both rodents and humans. Several investigators subsequently characterised the structure of the OB-R gene, and showed that the different isoforms of the receptor were generated by a process of alternative splicing. Two of the OB-R isoforms which have received much attention are OB-RA and OB-RB. OB-RB is the main signalling isoform of the leptin receptor, while OB-RA is one of a number of short isoforms of OB-R, thought to be involved in the transport of leptin into the cell, although this receptor does also have limited signaling capabilities. As *Psammomys obesus* have been shown repeatedly to be an excellent animal in which to study the development of obesity and type 2 diabetes, we have utilised this animal model in order to gain further understanding of the role of leptin receptors in the regulation of circulating leptin concentrations and the overall regulation of body weight homeostasis and energy balance.

OB-RA and OB-RB are thought to mediate the effects of leptin on energy balance and body weight, both in the hypothalamus and in peripheral tissues, and gene expression of OB-RA and OB-RB is thought to be regulated mainly by circulating leptin
concentrations. We initially examined the level of expression of OB-RA and OB-RB in the hypothalamus and a range of peripheral tissues in a cross-sectional study of a population of *Psammomys obesus*. These animals were divided into three groups based on body weight, blood glucose and plasma insulin concentrations and we demonstrated that in the hypothalamus of lean and healthy *Psammomys obesus* there were significantly higher levels of OB-RA, OB-RB and c-fos (a marker of neuronal activation) gene expression when compared to either obese, non-diabetic (group B) or obese, diabetic (group C) animals. The lower level of leptin signaling in the brain of obese *Psammomys obesus* raises the possibility that decreased signaling by the leptin receptor may result in leptin resistance, which may subsequently lead to an increased fat mass, which would further increase circulating leptin concentrations and exacerbate leptin resistance. However, the primary defect is unclear, as alternatively, increased circulating leptin concentrations may lead to down-regulation of leptin receptors.

Several groups have demonstrated that leptin receptor gene expression may be regulated by both leptin concentrations and dietary manipulations, although these studies have only examined hypothalamic leptin receptor expression. Leptin has also been shown to have a number of important peripheral actions in a range of tissues which may also be mediated by leptin receptors, which are widespread throughout the body. For example, in the liver, leptin appears to enhance insulin action and alter glucose metabolism. We have shown that both central and peripheral leptin receptor gene expression is regulated by dietary manipulations in *Psammomys obesus*.

**Fasting:**

When *Psammomys obesus* were fasted for 24 hours, a range of responses were observed. In lean animals, similar to other rodent models, OB gene expression and circulating leptin concentrations decreased, and hypothalamic OB-RB gene expression increased. In obese, leptin resistant *Psammomys obesus*, fasting increased circulating leptin concentrations, although there was no apparent rise in OB gene expression. In the hypothalamus of obese *Psammomys*, OB-RB gene expression was increased with fasting. As leptin levels increased with fasting in obese animals it is clear from this study that expression of leptin receptors in *Psammomys obesus* is not solely under the control of circulating leptin concentrations, as it appears to be in non-obese rodents, or monogenic rodent models of obesity. In polygenic obesity, regulation of leptin receptor gene expression appears to be more complicated and warrants further investigation.

With fasting, blood glucose and fasting insulin concentrations were normalised in all animals. In the liver, we saw that fasting resulted in a dramatic increase in OB-RA
gene expression in all animals. Leptin has previously been shown to have direct effects on the liver, with alterations in insulin action, hepatic glucose production and glycogen synthesis seen in vivo and in vitro. The changes in OB-RA gene expression in the liver of both lean and obese, non-diabetic Psammomys obesus with fasting suggests that hepatic leptin receptors may be involved in the regulation of glucose metabolism by leptin, and thereby energy partitioning. The signal for increased OB-RA gene expression with fasting is worthy of further investigation.

We examined the effects of fasting on leptin and leptin receptors in Psammomys obesus in an attempt to examine regulation of the leptin system with short-term perturbation of energy balance without changes in body weight or body fat. We also wanted to examine the role of leptin and leptin receptors in the development of obesity and type 2 diabetes with over-feeding in Psammomys obesus.

Over-feeding:
Sucrose-feeding is an established method of inducing energy imbalance and the development of obesity and type 2 diabetes in rodents, and we utilised this experimental paradigm in Psammomys obesus. Following the addition of a 5% sucrose solution to the diet of obese Psammomys, we saw increased body weight and percent body fat, as well as the development of diabetes, with increased blood glucose and plasma insulin concentrations. Despite the increased body weight in both lean and obese animals, there was evidence of decreased circulating leptin concentrations with sucrose feeding, and a range of responses were observed in leptin receptor gene expression. The decrease in circulating leptin concentrations may have been involved in allowing obese Psammomys to continue to overeat, highlighting a dysregulation of the leptin regulatory pathway in obese animals. It would be useful to determine if the effects of sucrose-feeding on leptin concentrations are a direct effect of sucrose, or if other carbohydrates, such as glucose or fructose, will also produce a similar effect.

In the hypothalamus, sucrose feeding did not appear to alter OB-RB gene expression in lean animals, however in obese animals, this treatment tended to increase OB-RB gene expression. The differing effects of sucrose-treatment in lean and obese animals warrants further investigation, as it may be related to defects in the leptin regulatory pathway, which results in the exacerbation of obesity and the development of type 2 diabetes in leptin resistant, obese Psammomys. In the liver, sucrose feeding tended to decrease OB-RA and OB-RB gene expression in both groups of Psammomys, however only the obese animals became diabetic. The possible role of leptin in this dysregulation of glucose metabolism in obese animals also warrants further investigation.
The effects of sucrose-feeding in Psammomys obesus on leptin and leptin receptors highlights again that leptin receptor gene expression is not regulated solely by circulating leptin concentrations in this polygenic rodent model of obesity and type 2 diabetes. More studies are required to examine the specific effects of sucrose on the leptin signaling pathway, and the influence of other hormones and neurotransmitters on leptin receptor gene expression in polygenic models also needs to be investigated more closely. In addition, it is important to determine if shorter term sucrose-feeding also has such profound effects on the development of obesity and type 2 diabetes in Psammomys obesus. The effects of other dietary manipulations, such as high fat feeding, on the leptin regulatory pathway also need to be examined.

If possible, it would be useful to try and separate the effects of sucrose (or specific dietary components) from the effects of over-feeding per se, and a pair-feeding study would help to determine which factor is having the major influence on the leptin pathway. We have demonstrated regulation of leptin and leptin receptors with dietary manipulations and it would also be interesting to examine the effects of exogenous leptin treatment on leptin receptor gene expression in Psammomys obesus, particularly in leptin resistant animals.

Future Directions:
The importance of leptin and leptin receptors in regulating energy balance and body weight in polygenic models of obesity has not clearly been demonstrated. It would be very interesting to alter the dynamics of leptin signaling by the leptin receptors with the use of agonists and/or antagonists, and examine the effects on body weight and energy balance in Psammomys obesus. In addition, it is also important to examine more closely the signaling pathways activated downstream of leptin receptors in Psammomys obesus and compare these pathways between lean and obese animals. The specific neurotransmitters involved in energy balance in Psammomys obesus are important to determine, and further, knowledge about which of these are leptin-sensitive may provide new avenues of investigation regarding the role of leptin resistance in the development of obesity in polygenic obesity. Neurotransmitter macroarrays are available which can detect expression of hundreds of genes involved in neurotransmission in a range of tissues. As the RNA has already been collected from Psammomys under each of the experimental conditions, this technology provides an avenue through which these studies could be expanded on immediately. One other area that needs to be examined more closely is the action of leptin in peripheral tissues, and the role of leptin receptors in mediating leptin-induced responses. It is obvious that leptin is not simply a marker of body fat mass, particularly in polygenic models of
obesity, and the importance of this hormone in the regulation of energy balance and body weight homeostasis in polygenic models, which more closely mimic human physiology, needs to be further elucidated.
10. 5. OVERVIEW

The focus of this dissertation was leptin and the leptin receptor, and the role of these genes (OB and OB-R) in the development of obesity and type 2 diabetes in humans and *Psammomys obesus*, a polygenic rodent model of obesity and type 2 diabetes.

In the studies conducted in this thesis, studies in humans showed that circulating leptin concentrations were positively associated with adiposity, and independently associated with circulating insulin and triglyceride concentrations. Analysis of two leptin receptor sequence polymorphisms in a Caucasian Australian population and a population of Nauruan males, with very high prevalence rates of obesity, showed no associations between sequence variation within the OB-R gene and obesity- or diabetes-related phenotypic measures. In addition, these two OB-R polymorphisms were not associated with longitudinal changes in body mass or composition in either of the populations examined. A unique analysis of the effects of multiple gene defects in the Nauruan population, demonstrated that the presence of sequence alterations in both the OB and OB-R genes were associated with insulin resistance.

*Psammomys obesus* is regarded as an excellent rodent model in which to study the development of obesity and type 2 diabetes in humans. Examination of circulating leptin concentrations in *Psammomys* revealed that, as in humans, leptin concentrations were associated with adiposity, and independently associated with circulating insulin concentrations. This animal model was utilised to examine expression of OB-R, and the regulation of expression of this gene after dietary manipulation.

OB-R is known to have several isoforms, and in particular, OB-RA and OB-RB gene expression were examined. OB-RB is the main signalling isoform of the leptin receptors. It has a long intracellular domain and has previously been shown to play an important role in energy balance and body weight regulation in rodents and humans. OB-RA is a much shorter isoform of OB-R, and although it lacks the long intracellular domain necessary to activate the JAK/STAT pathway, OB-RA is also capable of signalling, although to a lesser degree than OB-RB. OB-RA is found to be expressed almost ubiquitously throughout the body, and this isoform may be involved in transport of leptin into the cell, although its role remains unclear.

OB-RA and OB-RB were both found to be expressed in a large number of tissues in *Psammomys obesus*. Interestingly, obese *Psammomys* were found to have lower levels of expression of OB-RA and OB-RB in the hypothalamus, compared to lean animals. This finding raises the possibility that decreased leptin signalling in the brain
of obese, hyperleptinemic *Psammomys obesus* may contribute to the leptin resistance previously described in this animal model. However, the primary defect is unclear, as alternatively, increased circulating leptin concentrations may lead to down-regulation of leptin receptors.

The effect of fasting on leptin concentrations and gene expression of OB-RA and OB-RB was also examined. A 24-hour fast resulted in no change in body weight, but a reduction in circulating leptin concentrations, and an increase in hypothalamic OB-RB gene expression in lean *Psammomys*. In obese animals, fasting again did not alter body weight, but resulted in an increase in both circulating leptin concentrations and hypothalamic OB-RB gene expression. In the liver, fasting resulted in a large increase in OB-RA gene expression in both lean and obese animals. These results highlighted the fact that regulation of leptin receptor gene expression in polygenic models of obesity and type 2 diabetes is complex, and not solely under the control of circulating leptin concentrations. Sucrose-feeding is an established method of inducing obesity and type 2 diabetes in rodents, and this experimental paradigm was utilised to examine the effects of longer term perturbations of energy balance on the leptin signalling pathway in *Psammomys obesus*. Addition of a 5% sucrose solution to the diet of lean and obese *Psammomys* resulted in increased body weight in both groups of animals, however only obese *Psammomys* showed increased fat mass and the development of type 2 diabetes. The changes in body mass and composition with sucrose-feeding were accompanied by decreased circulating leptin concentrations in both groups of animals, as well as a range of changes in leptin receptor gene expression.

Sucrose-feeding increased hypothalamic OB-RB gene expression in obese *Psammomys* only, while in the liver there was evidence of a reduction in OB-RA and OB-RB gene expression in both lean and obese animals. The direct effects of sucrose on the leptin signalling pathway are unclear, however it is possible to speculate that the effect of sucrose to decrease leptin concentrations may have been involved in the exacerbation of obesity and the development of type 2 diabetes in obese *Psammomys*.

From these studies, it appears that sequence variation in the OB and OB-R genes is unlikely to be a major factor in the etiology of obesity in human populations. The ability to examine regulation of expression of these genes in *Psammomys obesus*, however, has demonstrated that the effects of nutritional modifications on leptin receptor gene expression need closer attention. In addition, the role of the OB and OB-R genes in metabolism and the development of type 2 diabetes also warrants further examination, with particular attention to the differential effects of dietary modifications on leptin receptor gene expression across a range of tissues.
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