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LEPTIN AND THE DEVELOPMENT OF OBESITY.

by

KEN WALDER, BSc (HONS)

AUGUST 1997

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AUSTRALIA, 3217.

SUBMITTED AS REQUIRED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
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I dedicate this work to Lauren.
ABSTRACT

The focus of this thesis was leptin and its role in the development of obesity and non-insulin-dependent diabetes mellitus (NIDDM). Studies in Psammomys obesus, a polygenic animal model of obesity and NIDDM, showed that ob gene expression and plasma leptin concentration correlated significantly with body weight, percentage body fat and plasma insulin concentration. In addition, plasma leptin concentrations were significantly elevated in insulin resistant Psammomys obesus independent of body weight. Dietary energy restriction from weaning in Psammomys obesus prevented excessive body weight gain, hyperleptinemia and hyperglycemia compared with ad libitum fed animals. Interestingly, 19% of the energy-restricted animals still developed hyperinsulinemia and tended to have increased plasma leptin compared with normoinsulinemic energy-restricted Psammomys obesus. Fasting for 24 hours significantly reduced plasma leptin concentration in lean, insulin-sensitive but not obese, insulin-resistant P. obesus, suggesting a dysregulation in the response of leptin to acute caloric deprivation in these animals.

The effects of leptin administration to P. obesus were also investigated. Single daily intraperitoneal injection of 5 mg leptin/kg body weight for 14 days had no significant effect in lean or obese P. obesus. This dose had previously been shown to rapidly and significantly reduce food intake and body weight in ob/ob and wild-type mice, suggesting relative leptin resistance in P. obesus. Acute (8 hour) effects of administration of 5 mg leptin/kg body weight were also investigated. No significant effects on food intake or plasma insulin were detected, however blood glucose concentrations were significantly elevated in obese, glucose intolerant P. obesus, suggesting an exacerbation of insulin resistance in susceptible animals. Treatment of lean, healthy P. obesus with 45 mg leptin/kg body weight/day for 7 days resulted in significant decreases in food intake and percentage body fat, showing that the leptin resistance observed in this species could be overcome by the administration of very large doses of leptin.

In another study, leptin was shown to significantly inhibit maximal insulin binding to isolated adipocytes, suggesting that leptin may represent an important link between obesity and NIDDM. Links between aspects of obesity and NIDDM and polymorphisms in the ob and β3-adrenergic receptor genes were also investigated in two human populations.
PUBLICATION STATUS

A total of six separate manuscripts have been prepared and each has constituted a separate chapter of this thesis. The publication status of each chapter is described below:

Chapter 3  *Ob* (obese) Gene Expression and Leptin Levels in *Psammomys obesus*. *Biochimica et Biophysica Acta* (In Press)

Chapter 4  Association Studies of an *Ob* Gene Polymorphism and a β3-Adrenergic Receptor Polymorphism in Two Distinct Human Populations. Manuscript in Preparation.


Chapter 6  Dysregulation of Leptin in Response to Fasting in Insulin-Resistant *Psammomys obesus* (Israeli Sand Rats). *Metabolism* (In Press)


Chapter 8  Leptin Inhibits Insulin Binding in Isolated Rat Adipocytes. *Journal of Endocrinology* (In Press)
CHAPTER 1: INTRODUCTION

1.1 OBESITY

1.1.1 OBESITY: DEFINITION AND MEASUREMENT

Obesity is defined as a pathological excess of body fat and is the result of an imbalance between energy intake and energy expenditure for a sustained period of time, often years. A simple measure of body mass does not give an accurate measure of the degree of adiposity, and therefore associated health risks, so a range of techniques have been developed to measure body fatness and the distribution of body fat.

Anthropomorphic measurements such as height and weight, waist and hip circumference, and skinfold thicknesses are the cheapest and easiest ways to gain an estimate of adiposity and body fat distribution. Height and weight can be used to calculate the Body Mass Index (BMI) or Quetelet's Index, where BMI is equal to body weight/height\(^2\) in kg/m\(^2\). In large populations, BMI is strongly associated with the degree of body fat and its related morbidity and mortality, so BMI is widely used as an estimate of body fatness (Bray 1976; Benn 1970). In Australia, BMI is used to define overweight and obesity as shown in Table 1.1 (WHO Expert Committee 1995).

<table>
<thead>
<tr>
<th>BMI</th>
<th>CLASSIFICATION</th>
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<tr>
<td>&lt;20</td>
<td>underweight</td>
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<tr>
<td>20-25</td>
<td>ideal weight</td>
</tr>
<tr>
<td>25-30</td>
<td>overweight</td>
</tr>
<tr>
<td>&gt;30</td>
<td>obesity</td>
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</table>

**TABLE 1.1**: Australian definitions using BMI

These classifications are based on large-scale, population-based studies which have shown an increasing morbidity and mortality as BMI increases above 25 kg/m\(^2\) (Leow and Garfinkel 1979; Bray 1985; WHO Study Group 1990). Some other countries define obesity differently, for example in Canada an individual with a BMI over 27 kg/m\(^2\) is regarded as obese, whereas in the USA, the National Institutes of Health National Task Force on Prevention and Treatment of Obesity concluded that individuals up to 34 years of age should be considered to be obese if they had a BMI over 25 kg/m\(^2\), and those aged over 34 years if they had a BMI over 27 kg/m\(^2\) (NIDDK 1993). Still other classification systems take gender-specific differences in BMI into account. These differences in the classification of obesity make comparisons between studies
performed in different countries difficult. The major criticism of the use of BMI to define obesity is that it fails to distinguish between weight that is due to muscle and weight that is due to fat, and therefore its relationship with obesity is likely to vary in individuals and populations who differ in body build (for example athletes, body builders) and body proportions (for example individuals with unusually long or short legs; WHO Expert Committee 1995). In addition, BMI fails to account for the distribution of body fat, an important determinant of the health risks associated with obesity (see Section 1.1.4). Despite these potential shortcomings, BMI remains the most widely used measure of adiposity due to its low cost, ease of use and generally satisfactory accuracy.

In addition to measuring total body fatness, the distribution of that body fat has very important health implications (see Section 1.1.5). Anthropomorphic measurements commonly used to estimate central or visceral adiposity are the waist-to-hip circumference ratio (WHR) and various ratios of skinfold thicknesses. Cutoff points for WHR used to define visceral obesity are 0.9-1.0 for men, and 0.8-0.9 for women. In Australia, the recommended cutoff points are 0.9 for men and 0.8 for women (National Health and Medical Research Council 1996). These values are derived from studies of all cause mortality and represent the points above which risk is significantly increased (Bray 1987; Bjornktop 1985). Unfortunately, different studies have used different cutoff values over the years, once again making comparisons between some studies very difficult. Studies have shown that the measurement of waist girth, or of WHR are good indicators of visceral obesity, as validated by the use of more sophisticated technologies such as computerised tomography (Lemieux et al. 1996a,b). Obesity has also been defined using skinfold thicknesses as follows: sum of triceps and subscapular skinfold thicknesses in males of over 45 mm, and in females of over 69 mm (Bray 1985). Various ratios of skinfold thicknesses have been used to estimate body fat distribution such as trunk to extremity ratio, or subscapular to triceps ratio.

In addition to anthropomorphic measurements, there are many other methods used to determine body fatness and fat distribution. Isotope or chemical dilution techniques use fat-soluble substances such as cyclopropane, whose dilution in the body can be related to total body fat. However, the dilution time is long and special equipment is required (Bray 1985). Another approach is to calculate body fat from measurements of body water, by the distribution of tritiated water or antipyrine which equilibrates with body water (Bray 1985).
Body density can also be used to measure body fat. By measuring body weight in and out of water it is possible to separate the body into fat and fat-free components. However, special facilities are required and this method is primarily used in research.

Other techniques which measure body fat include ultrasound, electromagnetic conductivity, computerised tomography, absorptiometry, nuclear magnetic resonance and neutron activation (Bray 1985). The use of double X-ray absorptiometry (DEXA) has been compared with anthropometry in some studies, showing that BMI and WHR give very good estimates of the actual body fat content and distribution (Van Loan et al. 1992; Svendsen et al. 1993). In general, these techniques are relatively expensive and difficult to use as dedicated facilities are often required with trained personnel, and are therefore not as widely used as anthropometry. When body fat is measured directly, obesity is defined as a percentage of body fat over 25% in males, or over 35% in females (Bouchard 1994).

In summary, body fat can be measured in many ways, but the most accurate methods are not widely available and tend to be expensive. Therefore, it is more practical in most cases to use the anthropomorphic measurements described above to estimate adiposity and body fat distribution.

1.1.2 EPIDEMIOLOGY OF OBESITY

Obesity is the most common metabolic disease found in both affluent ("Western") societies and 'developing' nations. The prevalence of obesity in these nations is alarmingly high, ranging from 10% to upwards of 50% in some subpopulations (Bouchard 1994). Of particular concern is the fact that the prevalence of obesity appears to be rising consistently in affluent societies, and increasing rapidly in less prosperous nations as they change to a more "Western" culture (Zimmet 1992). Reported prevalence rates must be examined carefully to ascertain the methods and cutoff points used to define obesity in that particular population.

In Australia, two recent studies using the definition of obesity of BMI>30 have found similar results. The National Heart Foundation Risk Factor Prevalence Study of 1989, where measurements of 9,300 respondents aged 20-69 years were made by trained nurses, found prevalence rates for obesity of 9.3% in men and 11.1% in women (Risk Factor Prevalence Study Management Committee 1990; Waters and Bennett 1995). The 1989-1990 National Health Survey, where self-reported height and weight were used to calculate BMI for 41,500 respondents aged over 18, results suggested that 8.2% of men and 9.1% of women were obese (Waters and Bennett 1995). The professionally
measured results are likely to be more accurate, as obese individuals often underestimate their body weight and food intake (Bray 1976).

The Australian prevalence rates for obesity are similar to those found in Canada (males 12%, females 15%; Gurney and Gorstein 1988) and the United Kingdom (males 8%, females 13%; Sports Council 1992), while rates in other nations vary considerably, with the lowest rates reported in Central American countries, and the highest rates in South Africa, the Seychelles and Trinidad (Gurney and Gorstein 1988; Tappy et al. 1991; Steyn et al. 1990).

The prevalence rates for obesity are increasing in Australia, as they are in many affluent societies. Bennett and Magnus (1994) found that the mean weight of Australian females aged 20-69 increased by 3.1 kg (from 61.7 to 64.8 kg) from 1980 to 1989, while the corresponding increase in males was 1.8 kg (from 77.0 to 78.8 kg). No change in height was observed during this period. Accordingly, the crude prevalence rates of obesity increased from 8.0 to 13.2% in females, and from 9.3 to 11.5% in males (Bennett and Magnus 1994). All of the above changes were statistically significant (p<0.05).

Similar increases in the prevalence of obesity during the 1980s have been observed in the United Kingdom (from 6 to 8% in males, 8 to 13% in females; Knight 1984; Sports Council 1992). In the United States, the prevalence of obesity has also increased dramatically over recent decades, with both males and females of the same height becoming 1-3.5 kg heavier over a ten-year period up to 1983 (Abraham et al. 1983), and a steady increase has been observed throughout the whole of this century (Bray 1985). The combined data from several affluent societies suggests that the prevalence of obesity is rising steadily, and so far there has been no sign of a reduction in this trend.

In addition, very high prevalence rates for obesity have been recorded in developing nations during the process of "modernisation" or "Westernisation". Examples include the Pima Indians of Arizona, Australian Aborigines and Pacific Islanders (Knowler et al. 1991; Zimmet et al. 1979; Coventry et al. 1986; O'Dea et al. 1988; Hodge et al. 1993). For example, the Pima Indians of Arizona have the highest known prevalence rates of obesity and non-insulin dependent diabetes mellitus (NIDDM), with over half of the individuals over 35 years of age classified as obese (BMI>30 kg/m²; Knowler et al. 1991). As energy intake increases and energy expenditure falls, the prevalence rates of both obesity and NIDDM have been shown to be greatly increased (Zimmet 1982); these conditions have reached epidemic proportions in many newly industrialised nations such as Nauru (Hodge et al. 1993; Zimmet 1995).
In addition to overall obesity prevalence rates, data regarding the distribution of body fat is also very important (see Section 1.1.5). The recent Australian data available for estimation of visceral adiposity is in the form of WHR measurements from the National Heart Foundation Risk Factor Prevalence Study of 1989 (Risk Factor Prevalence Study Management Committee 1990; Waters and Bennett 1995). The mean WHR for men was 0.89, and 0.76 for women. 41% of men had a WHR of greater than 0.9, while 23% of women had a WHR over 0.8. Using the higher cutoff points for WHR (1.0 for men, 0.9 for women), 5.2% of men and 2.6% of women had a WHR above these levels (Waters and Bennett 1995). These data indicate that visceral obesity is highly prevalent and is likely to be a significant health problem in Australian adults.

Various epidemiological factors have consistently been associated with obesity both in Australia and internationally:

1) Age - In Australian men, obesity prevalence increased steadily from age group 35-39 years (7.9%) to a peak at 55-59 years (16.0%), and levelled out to about 11% in older age groups. In women, the prevalence of obesity increased rapidly after age group 40-44 years and reached a peak at age group 55-59 years (23%; Waters and Bennett 1995). International studies have yielded similar results, including a large Swedish study (Kuskowska-Wolk and Rossner 1990) and a Hawaiian study (McGarvey 1991).

2) Sex - In Caucasians, females have a greater prevalence of obesity than males, especially in the older age groups (Bouchard 1994), and this is reflected in the Australian data (Waters and Bennett 1995).

3) Ethnic Factors - Many studies have shown different prevalence rates of obesity among different racial groups within the one community. In Australia, individuals born in Greece and Italy have significantly higher mean BMI’s than those born in Australia, while people from South-East Asia and other Asian countries have significantly lower mean BMI values (Waters and Bennett 1995). Similarly, Aboriginal and Torres Strait Islanders have almost double the prevalence rate of obesity found in the general Australian community (Risk Factor Prevalence Study Management Committee 1990). Similar results have been found elsewhere, with blacks and Hispanics having higher rates of obesity than whites in the United States (Bouchard 1994), and extremely high rates of obesity prevalence have consistently been found in Native Americans (Ellis and Campos-Outcalt 1994). The Pima Indians of Arizona have been extensively studied over a long period of time because of their extremely high prevalence rates of obesity and non-insulin-dependent diabetes mellitus (NIDDM). Table 1.2 summarises the findings from one such study (Knowler et al. 1981), indicating very high prevalence of obesity in this ethnic group:
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</tbody>
</table>

**TABLE 1.2:** Prevalence rates of obesity among Pima Indians.

4) Socioeconomic Status - The unemployed and low income groups have significantly higher rates of obesity in Western cultures. Australian males employed in "blue-collar" employment had a 2.4 times higher risk of obesity than those employed in "professional" occupations, while in women the risk was increased by a factor of 2.9 (Waters and Bennett 1995). Education is also an important determinant for risk of obesity in both sexes, with those who never attended high school having a 2.2-fold increased risk, and those who attended some high school a 1.6-fold increased risk of developing obesity than individuals who were tertiary educated (Waters and Bennett 1995). International studies have found similar results, with excess body weight found to be 7-12 times more frequent in women from lower compared to upper socioeconomic groups (Goldblatt et al. 1965). It should be noted that it is difficult to delineate socioeconomic from ethnic factors in some cases, but controlled studies have consistently shown a significant contribution of both of these factors in the predisposition to obesity (Bray 1985).

5) Other Factors - Some of the other variables which have been implicated in epidemiological studies of obesity include marital status (obesity may be more prevalent in the married), parity (BMI increases with increasing number of children), smoking (inverse relationship), and moderate alcohol intake, which has been associated with obesity (Scidell 1995).

1.1.3 **OBESITY AND HEALTH**

The high and increasing prevalence of obesity has significant implications for the health of Australians. Obesity has been identified as a key risk indicator of preventable morbidity and mortality due to diseases such as NIDDM and cardiovascular disease (National Health and Medical Research Council 1996). The annual costs of obesity associated with these diseases and others have been conservatively estimated at $810 million (in 1992-93 dollar terms) in Australia (National Health and Medical Research Council 1996). $510 million of the costs were directly within the health system, while $300 million were accounted for by indirect costs such as worker absenteeism and loss
of earnings due to premature death (National Health and Medical Research Council 1996).

In 1992-93 there were 3,512 obesity-related surgical procedures performed in Australian hospitals, of which 84% were performed on women (National Health and Medical Research Council 1996). The most common procedure was adipose tissue removal (77%), with gastric stapling/stomach plication contributing 15% of cases. In 1994, 336,838 prescriptions were written for appetite suppressants (predominantly dexfenfluramine, phenteramine or diethylpropion) for a total cost of $16.8 million (National Health and Medical Research Council 1996). In addition, it has been estimated that up to 300,000 consumers spend in excess of $500 million annually on weight loss programs. The total health costs of obesity are impossible to accurately determine, the precision being dependent on epidemiological assumptions which have to be made regarding the precise relationships between obesity and various diseases, and the accuracy of quantifying health costs. The Australian National Health and Medical Research Council has recently estimated that the total economic burden to the health system for the treatment and prevention of obesity is in excess of $1.5 billion annually (National Health and Medical Research Council 1996).

In the United States, it has been estimated that obesity is responsible for approximately 5% of the nation's total health care costs (Colditz 1992). Based on 1986 figures, the total health cost of obesity for the year was about US$40 billion (Bouchard 1994).

Given the rapidly escalating prevalence rates of obesity in developing nations such as those in the Pacific Islands (Zimmet 1982, 1995), the potential costs on the fledgling health systems found in these nations are likely to have severe consequences.

1.1.4 HEALTH RISKS ASSOCIATED WITH OBESITY

Obesity is associated with a significantly higher all-cause mortality rate (Bray 1976; Black et al. 1983). A deviation of 10% above average weight is accompanied by an 11% decrease in life expectancy in males, and a 7% reduction in females. The corresponding figures for a body weight 20% above average are 20% for males and 10% for females (Bray 1985). The major causes of this mortality are cardiovascular disease, NIDDM, hypertension, gallbladder disease and certain types of cancer.

In the large-scale, long-term Framingham Heart Study, body weight was found to be the third most important risk factor for coronary heart disease in males (behind age and dyslipidemia), with a 10% increase in body weight associated with a 13% increase in
the prevalence of coronary heart disease in males, and an 8% increase among females (Hubert et al. 1983). Statistical analysis of this and other data have revealed both a univariate and multivariate relationship between obesity and cardiovascular disease (including myocardial infarction and stroke), indicating that obesity is an independent risk factor (Hubert et al. 1983; Manson et al. 1991a). For these reasons, weight loss is a primary treatment strategy in individuals who have cardiovascular disease, and also in preventative medicine.

Epidemiological studies have also revealed a significant relationship between obesity and hypertension (Ferrannini 1995). In addition, normotensive, nondiabetic obese individuals have higher average blood pressure than lean individuals, regardless of age or sex, and there is a linear relationship between BMI and both systolic and diastolic blood pressure (Ferrannini 1995). Loss of weight has been shown to be associated with a reduction in blood pressure (Reisin et al. 1983).

The influence of obesity on the cardiovascular system may be simply due to the increased cardiac output and work rate required to adequately circulate blood through the increased body mass. Conversely, a complicated relationship is likely to exist whereby some of the pathophysiological changes observed in obesity also have direct or indirect effects on the cardiovascular system, such as autonomic nervous output. Some of these factors will be discussed in more detail below (see Section 1.5).

Obesity has also consistently been shown to be closely related to insulin resistance and NIDDM. The incidence of NIDDM increases with increasing BMI, and those in the highest weight groups have an 8-fold increase in the risk of dying from NIDDM-related causes (Bray 1985). The physiological relationship between obesity and NIDDM will be discussed in further detail later (see Section 1.3.2).

Obesity is also a significant risk factor for choledocholithiasis (gallstones). In one study, 33% of cholecystectomies in females were performed on individuals with a BMI greater than 29. The study concluded that approximately 90% of these cases were directly attributable to obesity (Colditz 1992).

Individuals with obesity also have an increased susceptibility to various forms of cancer. A study of 750,000 individuals with a twelve-year follow-up period found an increased risk of cancer of the endometrium, cervix, ovary and breast in females with obesity, while obese males had an increased incidence of colorectal and prostate cancer (Garfinkel 1985). Another large epidemiological study of obesity and cancer was the Danish Record Linkage Study, which followed 44,000 subjects over an eleven-year
period. Results from this study indicated an increased risk of cancer of the oesophagus, liver and pancreas in obese males and females (Moller et al. 1994). In addition, obese females had a higher incidence of certain endocrine tumours, such as endometrial cancer, and gastrointestinal cancers, including colorectal and gall bladder cancer, as well as renal carcinoma (Moller et al. 1994). Increased circulating oestrogen levels are found in obese patients, and this may partly explain the increased incidence of endometrial and breast cancers in these patients. Oestrogen levels rise with increasing BMI and, because adipose tissue contains high levels of aromatase, the enzyme that converts androgens into oestrogens, the increased fat mass contributes directly to the high oestrogen levels (Levi et al. 1992). The increased incidence of gastrointestinal cancers found in obese subjects could possibly be due to the increased propensity of obese individuals to habitually consume a high-fat, low-fibre diet, however this relationship is difficult to measure and remains highly speculative (Deslypere 1995).

Some of the other health problems identified in obese individuals include arthritis, respiratory problems such as sleep apnoea and snoring, increased anaesthetic risk, increased risk of accidental injury, prostatic hypertrophy and menstrual problems (Bray 1976).

It is clear that obesity is associated with very great health costs to the community, both in terms of economic cost, and in morbidity and mortality from a range of associated diseases.

1.1.5 BODY FAT DISTRIBUTION

It is now known that the distribution of the body fat stores, more so than the absolute amount, determines the associated health risks of obesity (Bjorntop 1991a,b). Excess adipose tissue stored deep in the abdomen, around the visceral organs, carries a far higher risk of developing the diseases listed above, as well as a higher mortality independent of obesity per se (Hartz et al. 1983, Ohlson et al. 1985, Larsson et al. 1984, Lapidus et al. 1984, Ducimetiere et al. 1986, Despres et al. 1990, Despres 1991, Despres 1993). In particular, a strong correlation has been found between visceral fat mass and insulin resistance (Bjorntop 1989), a key factor in the pathophysiology of obesity. Several possible mechanisms have been suggested to explain the relationship between visceral obesity and insulin resistance (Bjorntop 1991a).

Plasma free fatty-acid (FFA) turnover is increased in generalised obesity, and especially in visceral obesity (Kisschah and Peiris 1989, Jensen et al. 1989). This may be due to the elevated sensitivity of enlarged visceral adipocytes to lipolytic stimuli.
(Bjorntop and Ostman 1971) and decreased sensitivity to insulin-mediated suppression of lipolysis (Fraze et al. 1985; Swislocki et al. 1987). The elevation of circulating FFA may interfere with muscle insulin sensitivity by the mechanisms described in the Randle Cycle (Randle et al. 1963). Briefly, there are two steps by which circulating FFA may interfere with insulin sensitivity. Firstly, elevated levels of fatty acid oxidation results in increased ratios of acetyl-CoA/CoA, NADH2/NAD and ATP/ADP, which stimulate pyruvate dehydrogenase kinase, phosphorylating pyruvate dehydrogenase (PDH) to its inactive form (Randle et al. 1963, 1964; Taylor et al. 1973, 1975). In the second stage, elevated levels of acetyl-CoA result in excessive production of citrate, which has been shown to inhibit the kinase activity of the bifunctional enzyme phosphofructo-2-kinase/fructo-2,6-bisphosphatase (Randle et al. 1963, 1964), resulting in stimulation of the gluconeogenic pathway (Randle et al. 1988).

This sequence of events suggests that excessive rates of FFA oxidation can inhibit glycolysis indirectly by inhibiting PDH and phosphofructokinase while at the same time stimulating gluconeogenesis. A continuation of this process would result in a build up of glucose-6-phosphate which inhibits hexokinase and therefore limits the rate at which glucose is taken up into the cell. It has been speculated that this would lead to inhibition of not only glucose oxidation but glycogen synthesis secondary to reduced glucose transport (DeFronzo et al. 1988).

One of the major functions of insulin is to reduce circulating FFA concentrations. Therefore the systemic elevations of FFA in visceral obesity in the presence of hyperinsulinemia suggests that this visceral adipose tissue has a relative resistance to the antilipolytic effects of circulating insulin (Fraze et al. 1985; Swislocki et al. 1987).

Visceral adipocytes are 2-4-fold more sensitive to lipolytic stimuli than subcutaneous fat cells (Rebuffe-Scrive et al. 1983a,b) and less sensitive to the antilipolytic actions of insulin (Bolinder et al. 1983a), apparently due to a reduced density of insulin receptors (Bolinder et al. 1983b). Given that visceral fat constitutes about 20% of total fat mass in abdominally obese individuals (Kvist et al. 1988), it has been estimated that visceral adipose tissue contributes more than 50% of the FFA in the systemic circulation (Bjorntop 1991a,b).

Although portal FFA concentrations have not been measured in humans, it is assumed that this concentration would be significantly increased in subjects with visceral obesity, directly exposing the liver, via the portal vein, to excess FFA. This would be likely to result in an increase in hepatic gluconeogenesis by altering the NADPH:NADP ratio, which affects the activities of several key enzymes to favour gluconeogenesis.
over glycogenolysis, leading to increasing hepatic glucose production and eventually resulting in insulin resistance in the liver (Ferrannini et al. 1983; Reaven 1995).

Interestingly, the fact that visceral adipocytes have an increased lipolytic activity has implications for the sympathetic nervous system, which is the main stimulatory factor for lipolysis in humans (Bjorntorp and Ostman 1971). The possible role of sympathetic nervous system in the pathophysiology of obesity will be discussed in greater detail below (see Section 1.5.4.3).

The association between visceral obesity and insulin resistance forms an important component of the clinical cluster known as the "Metabolic Syndrome" or "Syndrome X".

1.2 \textbf{THE METABOLIC SYNDROME}

Syndrome X was first described by Reaven (1988) as a cluster of cardiovascular risk factors which tended to occur together and significantly increased the individual’s risk of coronary heart disease (Reaven 1988). These risk factors included hypertension, abnormal glucose tolerance, dyslipidemia (increased very-low density lipoprotein (VLDL) triglylyceride and decreased high density lipoprotein (HDL) cholesterol levels) and hyperinsulinemia/insulin resistance. Visceral obesity was later added to this group (Zimmet 1989), which is now known as the Metabolic Syndrome (Stern 1995).

This cluster of risk factors has regularly been associated with a significantly increased risk of death from cardiovascular disease (Haffner et al. 1992; Laws and Reaven 1993; Stern 1995). In addition, it has been noted that these risk factors tend to occur in clusters to a very significant degree. For example, in the San Antonio Heart Study of 2930 subjects, the major finding was that a combination of 3 or more of these risk factors was significantly more prevalent than the occurrence of each of the risk factors in isolation, and even more prevalent than a combination of any pair of the factors (Ferrannini et al. 1991).

Although the mechanisms underlying the development of the Metabolic Syndrome are as yet undefined, the favoured explanation proposes a central role for hyperinsulinemia (Reaven 1988). The proposal that hyperinsulinemia plays a central role in the Metabolic Syndrome is supported by a body of evidence implicating hyperinsulinemia in the pathogenesis of the other risk factors (reviewed by Walker and Alberti 1993).
Briefly, it has been proposed that hyperinsulinemia may be involved in the development of hypertension via stimulation of the sympathetic nervous system (Rowe et al. 1981) or by direct effects on the proximal renal tubule, where insulin promotes sodium absorption, which could lead to increased blood pressure (DeFronzo et al. 1975). With respect to dyslipidemia, insulin may stimulate VLDL-TG production (Gibbons 1990) and, if insulin resistance is present, secretion (Walker and Alberti 1993). Insulin may also have an effect on the clearance of HDL cholesterol by the liver (Abbott et al. 1987).

Some authors have proposed that the Metabolic Syndrome is simply a subset of NIDDM, which has been associated with hypertriglyceridemia and low HDL levels (Howard 1987), and excess cardiovascular risk. However, NIDDM is a highly heterogeneous disorder, and a significant proportion of NIDDM patients have normal lipid profiles and are normotensive. Therefore NIDDM and the Metabolic Syndrome probably represent separate conditions but with significant and important areas of overlap (Walker and Alberti 1993).

The role of obesity and in particular adipose tissue insulin resistance in the pathogenesis of NIDDM and dyslipidemia was recently reviewed (Reaven 1995). It was suggested that the development of adipose tissue insulin resistance was associated with increased circulating FFA and glycerol concentrations which stimulated hepatic VLDL-TG secretion and eventually resulted in hypertriglyceridemia (Reaven 1995). It is likely that further studies may reveal the importance of obesity in the pathogenesis of the Metabolic Syndrome.

1.3 NON-INSULIN DEPENDENT DIABETES MELLITUS (NIDDM)

The major focus of this dissertation is obesity, and the role of leptin in this disease. However, because of the close association between obesity and NIDDM, a brief description of NIDDM will be included.

NIDDM is one of the most prevalent chronic diseases worldwide, and represents a major public health problem in both developed and developing countries. The World Health Organisation (WHO) has suggested that the number of individuals with NIDDM in the world may exceed 100 million by the year 2000 (WHO Study Group 1994).
1.3.1 **NIDDM: DEFINITION AND EPIDEMIOLOGY**

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 diagnosis of diabetes mellitus is based on fasting and/or post-glucose load hyperglycemia, assessed by oral glucose tolerance test (OGTT) according to Table 1.3:

<table>
<thead>
<tr>
<th></th>
<th>Fasting</th>
<th>2-hr post load*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Glucose Tolerance</td>
<td>&lt;7.8 AND &lt;7.8</td>
<td></td>
</tr>
<tr>
<td>Impaired Glucose Tolerance</td>
<td>&lt;7.8 AND 7.8-11.0</td>
<td></td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>&gt;7.8 OR &gt;11.0</td>
<td></td>
</tr>
</tbody>
</table>

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

**TABLE 1.3**: WHO diagnostic criteria for diabetes mellitus.

These diagnostic criteria are based on the bimodal frequency distributions found in high-risk populations (Bennett et al. 1976; Zimmet and Whitehouse 1978). The bimodality of these distributions provide the strongest evidence that NIDDM constitutes a distinct disease entity, rather than simply the tail of a skewed distribution (Knowler et al. 1990). The category of impaired glucose tolerance (IGT) was defined by WHO as a risk category for NIDDM, but with prognostic uncertainty due to the difficulty in establishing where "normality" ended and diabetes began (WHO Study Group 1994). Many of these subjects progress to NIDDM at a rate of about 1.5-6% per year, but about a third return to normal glucose tolerance and a third remain glucose intolerant after a 5-10 year follow-up (Niskanen et al. 1995).

NIDDM is often undiagnosed due to its insidious nature and late age of onset. Studies have shown that there are as many undiagnosed cases of NIDDM in the USA as diagnosed cases (Cowie et al. 1994). While many cases of NIDDM are asymptomatic, the disease often causes great suffering through a number of debilitating complications including:

1) Cataracts, glaucoma and diabetic retinopathy leading to blindness
2) Diabetic nephropathy causing end-stage renal disease
3) Coronary artery disease, stroke, hypertension, peripheral vascular disease
4) Increased susceptibility to infection
5) Increased rates of periodontal disease
6) Increased rates of perinatal mortality and congenital abnormalities
7) Neuropathy (Joe and Young 1994).

The information gathered from many epidemiological studies of NIDDM has revealed several important risk factors for the development of the disease including:

1) Family history of NIDDM in a parent or sibling
2) Obesity - age-sex-adjusted incidence rates of NIDDM increase with increasing BMI, and duration of obesity is also correlated with NIDDM prevalence (Knowler et al. 1990)
3) Race/ethnicity - NIDDM prevalence is often quite varied in different ethnic groups within the same country (Zimmet 1992)
4) Age - in the general population, NIDDM incidence is relatively uncommon in the first three decades of life, increasing thereafter to a peak around 50 years of age, then declining slightly after age 70 (Warram et al. 1994)
5) Diet and physical inactivity - especially in individuals changing from a traditional to a modern lifestyle (Zimmet 1992).

Most epidemiological data suggest that the rates of NIDDM are rising in general throughout both the developed and developing world. However, temporal differences in the prevalence of NIDDM are difficult to establish because of differing diagnostic methods used (Knowler et al. 1990). Also, increases in prevalence rates would be expected as the quality of health care is improved due to the declining death rate from the disease (Harris et al. 1987). Table 1.4 shows a slight increase in the prevalence of NIDDM in Australia over time, however this data must be viewed with caution in view of the non-representative nature of the population samples and the non-identical ascertainment methods used (Welborn et al. 1995):

<table>
<thead>
<tr>
<th>Survey</th>
<th>Year</th>
<th>Age group</th>
<th>Prevalence ratesa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Busselton</td>
<td>1966</td>
<td>&gt;20</td>
<td>1.3</td>
</tr>
<tr>
<td>Cunderdin</td>
<td>1967</td>
<td>&gt;20</td>
<td>1.6</td>
</tr>
<tr>
<td>Busselton</td>
<td>1981</td>
<td>&gt;19</td>
<td>2.5</td>
</tr>
<tr>
<td>NHF b 8 cities</td>
<td>1983</td>
<td>&gt;19</td>
<td>2.6</td>
</tr>
<tr>
<td>NHSe</td>
<td>1989-90</td>
<td>&gt;19</td>
<td>2.7</td>
</tr>
</tbody>
</table>

aAge-standardised to the Australian population of 1989-90.

bNational Heart Foundation
cNational Health Survey (54 241 people)(Welborn et al. 1995).

**TABLE 1.4**: Prevalence rates of NIDDM in Australia
Studies in high-risk populations have shown very large increases in NIDDM prevalence over time. The age-standardised death rates from diabetes mellitus in Australian Aborigines has risen from 36 per 100,000 person-years in 1985-6 to 82 deaths per 100,000 person-years in 1991-2 (Bhatia et al. 1995). The prevalence of NIDDM in Pima Indians was 0.9% in 1954 (Joe and Young 1994), 14.4% in 1965 (Joe and Young 1994) and 25.5% in 1988 (Grabauskas 1988).

1.3.2 RELATIONSHIP BETWEEN OBESITY AND NIDDM

The relationship between obesity and NIDDM is complex, and is confounded by the heterogeneity of both conditions and the close interaction between obesity and other NIDDM risk factors such as hyperinsulinemia and physical inactivity (Zimmet 1992). Obesity is associated with insulin resistance, and has been shown to be an independent strong predictor of NIDDM in both males and females (Cook and Turner 1993; Spelsberg and Manson 1993). Evidence for these conclusions comes from numerous cross-sectional epidemiological studies in high-risk populations (Zimmet et al. 1992) as well as Caucasian studies (Baird 1982). A long term Swedish study showed that the relative risk of developing NIDDM after 13.5 years was 21.7 among those in the highest BMI quintile compared with those in the lowest BMI quintile (Olsson et al. 1988). Similarly, in nurses from the USA a strong continuous relationship was demonstrated between obesity and NIDDM; those with a BMI > 29 kg/m² had a 20-60-fold increased risk of developing NIDDM compared with those people with a BMI < 22 kg/m² (Spelsberg and Manson 1993). In most studies, NIDDM incidence was related to both current weight and history of obesity. In addition, weight loss has been shown to improve glucose tolerance and insulin resistance in obese NIDDM patients (Doar et al. 1975; Henry et al. 1986a,b; Henry and Gumbiner 1991). For example, in nurses from the USA with a BMI > 27 kg/m², those who lost 5-20 kg over a four year period had a 30% reduction in the risk of developing NIDDM compared with those whose weight did not change by more than 1 kg during that time (Spelsberg and Manson 1993). However, despite the fact that most NIDDM sufferers are obese, only a very small percentage of obese subjects are glucose intolerant.

A further factor complicating the interaction between obesity and NIDDM is the effect of body fat distribution. Many epidemiological studies have shown that abdominal (visceral) obesity is an independent predictor of NIDDM regardless of BMI (Spelsberg and Manson 1993), as discussed in detail previously. Obesity may be best viewed as an independent variable resulting in impaired insulin sensitivity in both normal and diabetic individuals. Its impact on glucose tolerance varies with the duration of obesity,
body fat distribution, and with the individual's insulin sensitivity and beta-cell secretory reserve.

Weight loss associated with physical activity has been repeatedly demonstrated to reduce basal insulin concentrations and improve insulin sensitivity in both 'normal' and diabetic individuals (Devlin and Horton 1985; Krokiewski et al. 1985). Obese subjects were shown to have lower insulin levels and improved glucose tolerance after moderate exercise, even without weight loss (Bjorntop et al. 1970; Soman et al. 1979). Exercise training reduced insulin resistance in skeletal muscle (Mondon et al. 1980; Dahm et al. 1987), and improved glycemic control in both diabetic and non-diabetic obese individuals (Schneider et al. 1984; Holloszy et al. 1987). Also, prospective studies have consistently shown a marked decrease in NIDDM risk in physically active individuals compared with their sedentary peers (Helmrich et al. 1991; Manson et al. 1991b; Manson et al. 1992). It has been theorised that skeletal muscle may be the primary site of insulin resistance (DeFronzo 1988; DeFronzo et al. 1992), and stimulation of muscle blood flow has been shown to be an important determinant of glucose uptake in the muscles after administration of insulin (DeFronzo et al. 1992). Regular exercise augments muscle blood flow and may thereby improve the insulin sensitivity of muscle tissue (Spelsberg and Manson 1993), as well as facilitating weight loss.

The precise effect of diet in the pathogenesis of NIDDM is unclear. Zimmet (1992) concluded that "no specific dietary component has been shown to be linked etiologically to NIDDM on a consistent basis" (Zimmet 1992). One early study found a positive correlation between total fat intake and NIDDM prevalence, as well as an inverse correlation between total carbohydrate consumption and NIDDM (West and Kalbfleisch 1971). These results have not been confirmed in subsequent studies. The potential for confounding, especially by total caloric intake, BMI or level of physical activity makes these studies very difficult. However, high fat diets produce obesity and NIDDM in several animal models. Despite the apparent lack of evidence linking specific dietary elements with NIDDM pathogenesis, it is likely that dietary factors may significantly influence the development of NIDDM through the promotion of obesity. In addition, dietary control is the first choice of treatment for most cases of NIDDM (Ilarde and Tuck 1994), suggesting a significant contribution to the disease process.

1.4 THE PATHOGENESIS OF OBESITY

Both genetic and environmental influences are thought to be involved in the etiology of obesity. A wealth of evidence from studies of twins, adoption studies and population-based analyses suggests that genetic effects account for 25-80% of the variation in body
weight in the general population (Bouchard 1994; Kopelman et al. 1994; Ravussin 1995). Genetics may also play a greater role in the development of central obesity than lower body obesity (Bouchard et al. 1990). There is no doubt however that the environment also plays a major role in the development of obesity, as the provision of sufficient food to exceed daily energy expenditure is critical to the possibility of weight gain.

The main evidence to suggest an important role for the environment in the development of obesity in the general population comes from changes in the prevalence of obesity over time. For example, in Copenhagen the incidence of obesity remained stable between 1925 and 1942 at 0.1% (Sorensen and Price 1990), however since 1942 there has been a dramatic and steady increase in the incidence of obesity. Similar observations from developing countries suggest that changes in environmental factors, especially those involved in the transition from a traditional to a "Westernised" lifestyle, can rapidly and significantly increase the prevalence of obesity, in such a manner that genetic changes in the population could not possibly account for the rise in obesity (Zimmet 1992; Bouchard 1994). Some of the factors implicated in these changes include increases in both dietary energy intake and fat intake, and decreases in physical activity associated with motorised transport, the advent of television and changing work practices.

The Pima Indians of Arizona, who have the highest prevalence rates for obesity and NIDDM in the world (Knowler et al. 1991), have often been cited as support for genetic influences on obesity. Ironically, recent studies of another tribe of Pima Indians from Mexico suggest a significant role for the environment. These two groups are genetically similar, but the Mexican Pima Indians have, until recently, been isolated and living a traditional lifestyle. The BMI of the Mexican group was significantly lower than that of age- and sex-matched Pima Indians living in Arizona, and the observed difference in BMI between the two groups was attributed to environmental influences (Ravussin et al. 1994).

It is currently believed that genes determine the possible range of body weight in an individual, and that the environment influences the point within this range where the individual is located at any given time (Bouchard 1994). Obesity is a complex and heterogeneous disorder, and it is likely that any inherited effects are polygenic in nature. A summary of some of the evidence implicating genetics in the etiology of obesity is presented below.
1.4.1 **TWIN STUDIES**

Monozygotic (MZ) twins are genetically identical, whereas dizygotic (DZ) twins share, on average, 50% of their genes. If the twins are raised together, the difference between members of MZ pairs compared with DZ pairs represents the extent to which a trait is genetically determined (Bouchard 1994). Conversely, in MZ twins reared apart, differences can be attributed to environmental influences.

Several twin studies in children found a greater similarity in MZ compared to DZ twin pairs for skinfold thicknesses and BMI (Brook et al. 1975; Borjeson 1976), with estimated heritabilities ranging from 22-44% for DZ, and 74-84% for MZ twins.

In adult twin pairs reared together, numerous studies have found high heritabilities for BMI (ranging from 40-90%; Bouchard 1994). A major study of 4071 pairs found that between 77 and 84% of the variation in BMI was attributable to inherited factors (Stunkard et al. 1986a), and that the change in BMI over a 25-year period had a heritability of 60-71% (Fabsitz et al. 1980). Other findings from large studies of twins reared together include a higher heritability of BMI in women than men (75 vs 69%) and interestingly, the opposite-sex correlation for DZ twins was lower than the same-sex correlation, raising the possibility that different genes might influence BMI across gender (Neale and Gordon 1992). In addition, the heritability estimates for BMI appear to decrease slightly with increasing age, suggesting a gradual increase in the influence of the environment (Fabsitz et al. 1980; Korkeila et al. 1991). In contrast, a recent study found an increase in heritability of BMI with increasing age in men, but not in women (Herskind et al. 1996).

In studies investigating twins reared apart, a remarkable similarity was found between twin pairs reared together and those reared apart, indicating that the rearing environment has at most a very modest effect on BMI (Stunkard et al. 1990). Under both rearing conditions, a high heritability of BMI was found. Other studies have found similar results, adding to the evidence that genetic influences are a major determinant of BMI (MacDonald and Stunkard 1990; Price and Gottesman 1991).

Factors other than BMI have also been shown to have significant heritability in twin studies, including skinfold thicknesses, % body fat and fat mass. In general, correlations for MZ twins range from 60-90%, and are usually double those for DZ pairs, suggesting a relative absence of family environmental effects (Bouchard et al. 1988a, Selby et al. 1990). In addition, twin studies have shown a high heritability for basal metabolic rate
(BMR) independent of fat-free mass, which indicated a significant genetic influence on BMR (Fontaine et al. 1989; Bouchard et al. 1989).

More recent studies have concentrated on genotype-environment interactions, such as when genotypes differ in their sensitivity to environmental conditions, which cannot be assessed using traditional methods. In one study, 12 pairs of MZ twins were overfed by 1000 kcal/day for 84 days (Bouchard et al. 1990; Mauriege et al. 1992). The authors found a high heritability for body composition, abdominal fat and adipose tissue lipolysis, ranging from 40-80%. In addition, a higher heritability was found for visceral compared to subcutaneous fat (Bouchard et al. 1990; Borecki et al. 1994; Borecki et al. 1995).

In summary, twin studies have consistently and clearly shown that measures of body fatness are more closely correlated between MZ twin pairs compared to DZ pairs, indicating that genetics play a major role in the predisposition to obesity. Heritability estimates from twin studies suggest that 40-80% of the observed variability in body weight may be genetically determined.

1.4.2 ADOPTION STUDIES

Adoption studies have provided further information on the influence of genetic and environmental factors on the etiology of obesity. Excluding very early environmental effects, adoptees share their genes with their biological parents and their environment with their adopted parents. Trait similarities can then be examined between adoptees and their biological parents, indicating genetic influence, and the adoptee and adoptive parents, suggesting shared environmental influences. Adoption studies may be "complete", in which the adoptee is compared to both his adoptive and biological parents, or "partial", where adoptive families are compared to natural families (not the adoptee's biological family; Bouchard 1994).

Partial adoption studies have found that correlations between natural parents and offspring are usually approximately double those of adoptive families for such measures as skinfold thicknesses, BMI and fat mass (Garn et al. 1976; Garn et al. 1977; Hartz et al. 1977; Biron et al. 1977; Annest et al. 1983; Bouchard et al. 1985; Bouchard et al. 1988a).

There have been three major complete adoption studies into the etiology of obesity. An extensive Danish study found that the BMI of biological parents, full and half siblings increased significantly as the BMI of the adoptee increased. By contrast, there was no
change in the BMI of adoptive parents or siblings (Stunkard et al. 1986b; Sorensen et al. 1989). The data was analysed using a comprehensive path model incorporating additive effects of both genetic and environmental factors (Vogler et al. 1995). The model could be reduced to one in which genetic effects alone accounted for the familial resemblance, the heritability estimate was 34%. Other studies have yielded similar results (Price et al. 1987; Cardon and Fulker 1994), suggesting a significant role for genetic influences in the development of obesity.

In summary, numerous adoption studies have found that the family resemblance in body fatness is mainly due to shared genes. All of the complete and most of the partial adoption studies found that the shared family environment had little or no effect on measures of body fatness. Although the design of partial adoption studies is questionable, the complete adoption studies add considerable weight to the hypothesis that obesity has a significant genetic component.

1.4.3 FAMILY STUDIES

Studies of families show a clear correlation of body weight between parents and children, and a still higher correlation between siblings. These studies imply that genetics play a role in the development of obesity, although the shared family environment is a complicating factor. The data from many studies have been combined and analysed, revealing significant correlations for skinfold thicknesses in over 8000 parent-child pairs (r=0.23), and in more than 3300 full sibling pairs (r=0.28; Bouchard 1994). Family correlations for BMI range from 0.20-0.23 for parent-offspring, and 0.24-0.34 for full sibling pairs (Bouchard 1994), with a broad heritability estimated for BMI of 40%.

Body fat distribution has also been shown to have a significant genetic component. The across-generation transmissible effect reached about 40% for trunk skinfolds, limb skinfolds and the trunk-to-limb ratio, and 28% for the WHR (Perusse et al. 1988). The authors concluded that the pattern of body fat distribution was partly determined by genotype, and that for a given level of fatness, it is the genotype which influences whether an individual will store more fat centrally (Perusse et al. 1988). In support of this data, a large scale study found that 37% of the variation in the trunk-to-extremity skinfold ratios was attributable to a "recessive major gene determinant" (Borecki et al. 1995).
1.4.4  CANDIDATE GENE STUDIES

The studies summarised above provide strong evidence for a major genetic component in the pathogenesis of obesity. However, the precise genes involved are not known at this time. A number of approaches have been used in an attempt to elucidate these specific genes.

The candidate gene approach has been used to investigate possible factors involved in the etiology of obesity. These studies have generally been based on relatively small sample sizes, and have therefore been criticised for their potential to yield both false positive and false negative results (Bouchard 1994). Relatively strong associations (p<0.01 or better) have been described between BMI or body fat phenotypes and several genes including apolipoprotein-B (Rajput-Williams et al. 1988), apolipoprotein-D (Vijayaraghavan et al. 1993), tumor necrosis factor-α (Norman et al. 1995), the low density lipoprotein receptor (Zee et al. 1992) and the dopamine D2-receptor (Comings et al. 1993). Of these, only the association with apolipoprotein-B has been independently confirmed by another laboratory (Saha et al. 1993). A further study failed to confirm the association between the dopamine D2-receptor and BMI (Noble et al. 1994). Many other genes have been examined which appear not to be associated with the development of obesity including the glucocorticoid receptor (Weaver et al. 1992b), insulin and the insulin receptor (Weaver et al. 1992a).

A number of genetic linkage studies have also been performed in an attempt to define chromosomal regions associated with obesity phenotypes. Of these, evidence for strong linkage was reported for the following markers: ACP1 (chromosomal region 2p25; Lucarini et al. 1990), TNFα (6p21.3; Norman et al. 1995), KEL (7q33; Borecki et al. 1994), and ADA (20q12; Borecki et al. 1994). It should be remembered that these studies linked the obesity phenotype with a gene in the region of these markers, and not necessarily the gene after which the markers were named.

While there is little doubt that genes are influential in the development of obesity, the heterogeneity of human obesity suggests a polygenic disorder, with several or many susceptibility genes likely to be involved in its etiology. A susceptibility gene is defined as one that increases the risk of developing the disease, but is not absolutely necessary for the disease development (Greenberg 1993). An allele at a susceptibility gene is therefore not sufficient by itself to explain the occurrence of the disease, but it does increase the likelihood of the carrier becoming obese (Bouchard 1994).
As technology continues to progress and new molecular techniques are devised and used for research into complex disease phenotypes such as obesity, it is likely that additional genes and chromosomal regions will be linked with the obesity phenotype.

1.4.5 **ETIOLOGY OF NIDDM**

There is a multitude of scientific evidence demonstrating that the metabolic defects found in NIDDM result from a complex interaction between genetic and environmental factors. Strong data supporting a genetic component come from familial aggregation and twin studies, genetic admixture studies and prevalence data in high risk populations (Zimmet 1992; Bouchard 1994).

Although it is generally accepted that NIDDM is a genetic disease, the relative importance of genetic predisposition and environmental factors are keenly debated in the literature (Hales 1994; McCarthy et al. 1994). Following many years of research, basic issues such as mode of inheritance, number of loci involved and pathogenesis of the disease remain significant future scientific challenges. Major efforts world-wide are now concentrated on a search for "diabetes genes" and results of these studies are certain to improve our understanding of the physiological events leading to NIDDM, and the development of more effective therapies.

A number of studies of NIDDM in MZ versus DZ twins have shown greater concordance in identical twins (Barnett et al. 1981; Newman et al. 1987; Rotter and Rimoin 1987), however the degree of concordance varied from 28 to 91 per cent in MZ twins, and was generally much lower than the often-cited concordance rate (approximately 100 per cent) of the early studies in the United Kingdom (Barnett et al. 1981). The less than 100 per cent concordance may be related to inadequate follow-up in a number of these studies. For example, in a study by Newman et al. (1987) the initial concordance in MZ twins was 58 per cent; however after a 6-year follow-up, most pairs initially discordant became concordant (Newman et al. 1993). This illustrates an inherent problem in genetic studies of NIDDM as the age of onset of the disease varies and is often quite late in life. In addition, the presence of discordance among MZ twins implies that environmental factors, including diet and exercise, may contribute significantly to disease development (Collier and Walder 1995).

In studies examining the familial aggregation of NIDDM the association was striking. For example, studies examining Pima Indian families in North America have demonstrated that age- and obesity-adjusted incidence of NIDDM was two to three times higher in subjects with one diabetic parent and up to four times higher in subjects
with two diabetic parents than in those with two non-diabetic parents (Knowler et al. 1981). The severity of diabetes, insulin resistance and obesity also aggregate into families in Pima Indians (Knowler et al. 1981). In addition, family studies in a number of other populations have confirmed these results and demonstrate an increase in NIDDM in siblings and first degree relatives of diabetic parents compared with non-diabetic parents (Simpson 1968; Kobberling 1971; Baird 1973; Keen et al. 1982; Cheta et al. 1990). Interestingly, an interaction between familial aggregation of insulin sensitivity and the impact of obesity on severity of insulin resistance has been demonstrated. These studies suggested that offspring of diabetic parents became more insulin resistant for every increment in body weight than individuals with no family history of diabetes (Warram et al. 1990).

Further evidence supporting the genetic basis of NIDDM comes from population studies. Within a given environment and adjusted for obesity, it is clear that diabetes is far more prevalent in certain ethnic groups. For example, a high prevalence rate of NIDDM in South Pacific islanders such as Nauruans and other isolated populations has been extensively reported (Zimmet and O'Dea 1993). Additional support for the genetic basis of NIDDM is found in population studies examining genetic admixture. Serjeaston et al. (1983) reported that prevalence of NIDDM in Nauruans over 60 years of age was 83 per cent in full-blooded, but only 17 per cent in Nauruans demonstrated to have a genetic admixture. This has been further supported by other genetic admixture studies in Pima Indians (Knowler et al. 1988).

One of the major difficulties in determining the genes responsible for the development of NIDDM is the intrinsic heterogeneity of the disease. Genetic studies are further complicated by the gaps in our knowledge of the pathophysiology of NIDDM and our ignorance regarding the primary defect. This has resulted in research groups studying populations or families with discrete types of diabetes such as maturity-onset diabetes of the young (MODY). Consequently, no single genetic approach is the 'correct' one and, in fact, two fundamentally different approaches have been used; the 'candidate gene' approach, and positional cloning (see Collier and Walder 1995 for review).

As with obesity, the precise etiology of NIDDM is unclear at this time, although it is widely accepted that both diseases have significant genetic contributions. Two interesting theories put forward to explain the etiological basis of obesity and NIDDM in human populations will be briefly discussed below.
1.4.6  THE THRIFTY GENOTYPE

Over 30 years ago, J. Neel proposed the "thrifty genotype" hypothesis to explain how such a debilitating and prevalent disease as NIDDM could exist for such a long duration throughout evolution despite expected strong genetic selection against the condition (Neel 1962). To explain this apparent paradox, he contended that individuals with a particular genetic makeup engineered to be exceptionally efficient in the uptake and/or utilisation of food would have, throughout history, provided significant survival advantage during times of inconsistent food supply (Neel 1962). During the first 99% or more of man's time on earth he existed as a hunter/gatherer, and the food supply could be described as feast or famine. That is, periods of gorging following a successful hunt would have been regularly interspersed with short periods of greatly reduced food intake (Neel 1962; Zimmet and O'Dea 1993). Thus a genetic composition allowing an individual to rapidly store large amounts of energy during times of plentiful food supply would provide a significant survival advantage during subsequent famines. Neel suggested that this genotype may feature hyperresponsiveness of pancreatic islets in response to circulating substrates (Neel 1962), later termed a "quick insulin trigger" (Wendorf and Goldfine 1991; Zimmet and O'Dea 1993). Thus, a pancreatic response which "minimised post-prandial glycosuria" and promoted fat deposition could represent the expression of the thrifty genotype (Neel 1962). It was later proposed that insulin resistance may be the phenotypic expression of the thrifty genotype (Wendorf and Goldfine 1991). This was further refined to "selective insulin resistance" on the basis that a highly efficient system for converting large amounts of dietary protein (for example, from a big game animal kill) into glucose and fat for energy storage could be achieved by a high capacity for gluconeogenesis (which was not suppressed by insulin) plus a high capacity for hepatic lipogenesis (stimulated by insulin; Dowse and Zimmet 1993; Zimmet and O'Dea 1993).

While this "thrifty genotype" would provide a survival advantage during periods of feast and famine, it could be detrimental in times of plentiful, high energy food supply, where it could result in obesity and/or NIDDM (Neel 1962; Zimmet and O'Dea 1993). Several populations have been cited as examples of the thrifty genotype. For example, American Indians of several tribes were known to rely on unpredictable big game species, such as bison, as a major food source, which would favour selection of the thrifty genotype (Wendorf and Goldfine 1991). With the transition to more constant and high energy food supply seen in the past 50 years, American Indians have some of the highest prevalence rates of obesity and NIDDM in the world (Knowler et al. 1981; Knowler et al. 1991; Wendorf and Goldfine 1991).
Another example could be the Pacific Islanders, who faced regular periods of caloric deprivation during long, migratory canoe voyages, as well as the effects of droughts and hurricanes, favouring the selection of the thrifty genotype. In addition, food productivity was limited on the often barren coral atolls (Zimmet and O'Dea 1993). Pacific Islanders have been shown to have extremely high prevalence rates of obesity and NIDDM after transition to a "Westernised" lifestyle (Zimmet 1992; Zimmet and O'Dea 1993; Hodge et al. 1993; Zimmet 1995).

It has been proposed that the "thrifty genotype" represents a major cause of obesity and NIDDM in specific populations, and there is also the possibility that aspects of the thrifty genotype contribute to many or most cases of human obesity.

1.4.7 THE THRIFTY PHENOTYPE

A novel, alternative hypothesis concerning the etiology of NIDDM was published in 1992 (Hales and Barker 1992). The "thrifty phenotype" hypothesis contends that poor fetal and early post-natal nutrition "imposes mechanisms of nutritional thrift upon the growing individual" (Hales and Barker 1992). That is, severe inadequacies of early nutrition are proposed to impair the development of the endocrine pancreas and increase susceptibility to the development of NIDDM, and other constituents of the Metabolic Syndrome, later in life (Hales and Barker 1992).

Evidence to support this theory comes from numerous studies showing a significant association between low birth weight and the development of NIDDM (Hales et al. 1991; Barker et al. 1993; Phillips 1996), ischaemic heart disease (Barker et al. 1989; Barker 1991), and blood pressure (Barker et al. 1990). It has been proposed that components and combinations of the Metabolic Syndrome are late outcomes of abnormal growth and development processes occurring during crucial phases of fetal and early infant life (Hales and Barker 1992; Barker et al. 1993). These phases of undernutrition during development promote metabolic adaptations from which immediate benefits result in the form of increased fuel availability, but these adaptations become permanently "programmed" and persist throughout life, predisposing the individual to the components of the Metabolic Syndrome when a constant, high energy food supply is available (Phillips and Barker 1993).

However, criticism of this hypothesis has revolved around the fact that low birth weight can result from a number of diverse causes independent of fetal nutritional status, and the fact that there may exist etiologic factors which contribute to both low birth weight and predisposition to elements of the Metabolic Syndrome (Purdy and Metzger 1996). The use of birth weight as a proxy for intrauterine nutritional status in retrospective data
collection is fraught with difficulty, since many confounding factors could not be controlled for (Purdy and Metzger 1996). These may include maternal prepregnancy BMI and pregnancy weight gain, maternal nutritional status and diet during pregnancy, smoking, drug use, infection and many other factors. Therefore it is "precarious to attribute the NIDDM seen in these studies to intrauterine undernutrition" (Purdy and Metzger 1996). In addition, it has been pointed out that in some studies, birth weights were not adjusted for gestational age, making interpretation of intrauterine nutritional status very difficult (Purdy and Metzger 1996).

Although the data suggest that intrauterine nutrition has a major role in the development of adult disease, well controlled prospective studies are necessary to delineate the magnitude of the putative contribution, and to elucidate other confounding factors which may interact with unknown genetic determinants during fetal life and later development (Purdy and Metzger 1996).

1.4.8 ENERGY BALANCE

The concept of energy balance is inherent to any discussion about body weight homeostasis and the development of obesity. It is currently accepted that most cases of human obesity are the result of a relatively small excess in energy intake compared to energy expenditure maintained over a significant period of time. Energy balance may be defined as the absolute difference between metabolisable energy intake and total energy expenditure.

In a given individual, there are likely to be substantial daily variations in energy intake and expenditure which are not closely synchronised. Despite the daily variations, body weight and body composition remain particularly stable in most individuals for very long periods of life. This is because over a period of time, energy balance will be restored. An example of this phenomenon is found in individuals exposed to acute overfeeding, which causes increased energy expenditure by various mechanisms. After a period of adaptation and weight gain, energy expenditure will match energy intake and body weight will stabilise at a higher level. This same relationship holds true for weight loss. Energy intake and expenditure will be discussed in more detail below.

1.4.8.1 ENERGY INTAKE

The control of eating is complex, and involves many learned responses and physiological drives as well as psychosocial factors. Two components of energy intake
have been investigated with respect to their contribution to the pathogenesis of obesity: Total energy intake and the macronutrient composition of the diet.

The hypothesis that obese individuals eat more than their lean counterparts has been extensively investigated with few conclusive results. The methodology for assessing food intake is poor and it is known that all subjects underestimate their consumption, the obese by more than those of normal weight (Prentice and Black 1986; Bandini and Schoeller 1990; Lightman and Pisarska 1996). It is likely that some cases of obesity result from significant hyperphagia and vastly excessive food intake. However, other cases may result from periodic excesses in energy intake, for example, repeated binges during stressful times (Ganley 1989). In other obese individuals, although the dietary intake may be normal by community standards, it may exceed the genetically determined energy needs of that person (George and Tremblay 1989).

Apart from total dietary energy intake, the macronutrient composition of the diet, as well as the substrate utilisation rates by the individual, are important in determining energy balance. To preserve homeostasis, the absorbed carbohydrate and fat must be stored and mobilised later at rates appropriate to bring about the oxidation of a fuel mix matching, on average, the macronutrient composition of the diet. Overall substrate oxidation rates are determined by the need to maintain temperature, to regenerate ATP necessary for metabolic function, and for locomotion and physical activity (Flatt 1995). The fuel mix oxidised, as well as the overall oxidation rate, vary considerably, but are carefully controlled to minimise changes in body protein and glycogen content (Abbott et al. 1988), rather than regulating overall fat or energy balances. From a survival advantage point of view this is logical, considering the functional importance of proteins, and the need to maintain a constant supply of glucose to the brain (Flatt 1995). This means that any daily variations in energy balance are accommodated by gains or losses of body fat.

The composition of the fuel mix being oxidised at any time is primarily regulated by circulating hormone and substrate concentrations, especially insulin (Flatt and Blackhurn 1974), and, especially in the post-prandial phase, this is significantly influenced by the degree of replenishment of glycogen stores and the size of the adipose tissue mass (Flatt 1995). If the oxidation rate for a substrate does not match its intake, body composition will be altered until the composition of the fuel mixture oxidised matches, on average, the macronutrient composition of the diet (Flatt 1988; Flatt 1993). This is achieved via complex endocrine and enzymatic regulation which is thought to be controlled by the hypothalamus. The eventual body composition for which this
match occurs is subject to great variability between individuals, and depends on the interactions between dietary and environmental factors (Flatt 1995).

Carbohydrate oxidation is very carefully matched to ingestion in humans (Mayer and Thomas 1967; Russek 1981), which is not surprising given the importance of the body's glycogen stores in maintaining adequate glucose supply to the brain, and ensuring muscular responses for sudden demands (Flatt 1995). Precise regulation of the body's glycogen stores is critical, since the storage capacity (200-400 g in adults) is not much larger than the amount of carbohydrate consumed daily (Bjorntop and Sjostrom 1978). Therefore the oxidation of glucose is precisely and acutely regulated in response to fluctuations in dietary carbohydrate intake, irrespective of the amount of fat consumed (Acheson et al. 1984; Flatt et al. 1985).

Maintenance of stable amounts of stored glycogen would be greatly facilitated by signals which regulated food intake when glycogen reserves rose or declined excessively (Mayer and Thomas 1967). The search is on in earnest for the elusive (and hypothetical) "Factor X" which communicates the amount of glycogen stored to the brain, thereby resulting in appropriate adjustments in food intake. While such a signal has not yet been identified, it is clear that the regulation of food intake is much more closely linked to the maintenance of carbohydrate than fat balance (Mayer and Thomas 1967; Russek 1981; Flatt 1993; Flatt 1995).

Quantitatively significant conversion of carbohydrate into fat does not occur in humans (Hellerstein et al. 1991), probably due to the high energy cost involved compared to simple storage of ingested fat (Flatt 1995). Even in conditions of significant overconsumption of carbohydrates, where glycogen stores are considerably expanded, glucose is utilised for fuel almost exclusively until the glycogen stores return to normal levels (Acheson et al. 1982).

Dietary fat is absorbed relatively slowly, and is almost all targeted for direct deposition in adipose tissue, with negligible effect on postprandial substrate oxidation (Flatt et al. 1985). Ingested fat has a very poor ability to stimulate fat oxidation following a mixed meal, and even the consumption of a high-fat meal is followed by a period of reduced fat oxidation (Griffiths et al. 1994). Therefore, the oxidation rate of fat is primarily determined by consumption of carbohydrate and protein, not by the fat content of the diet (Flatt 1988). These data provide an explanation for the association between consumption of a high fat diet and expansion of the adipose tissue mass over time.
Consumption of a diet high in fat will eventually produce a balance when the interactions between an individual's body fat content and their genetic make-up leads to adjustments in fat oxidation rates to the fat content of the diet (Flatt 1995). Importantly, the adjustment of adipose tissue mass so that fat oxidation on average matches fat ingestion leads to maintenance of the new body weight. For these reasons, the diet, and especially its fat content, are vitally important in the determination of body weight and composition (Flatt 1993; Flatt 1995).

Studies have shown that high-fat foods cause an inappropriate metabolic response in individuals with a susceptibility to obesity (Heitmann et al. 1993; Rolls et al. 1994). These studies led to the suggestion that subjects with a predisposition to obesity may have a combination of deficient fat oxidation and enhanced fat deposition after ingestion of high-fat foods.

Investigators have used the respiratory quotient (RQ), calculated from carbon dioxide production divided by oxygen consumption, to estimate the relative proportion of lipid to carbohydrate oxidised. When fat oxidation is increased at the expense of carbohydrate oxidation, the RQ is decreased. Conversely, when carbohydrate oxidation is favoured, the RQ is increased. The usual range of RQ is between 0.7 and 1.0. Several studies have found a correlation of RQ with weight gain over a period of time (Zurlo et al. 1990; Eckel 1992; Seidell et al. 1992). In Pima Indians, a high RQ (low ratio of fat:carbohydrate oxidation) predicted a 2.5-times increased risk of excessive weight gain compared to those with a low RQ (Zurlo et al. 1990). It appears that lean subjects have an increased ability to stimulate fat oxidation in response to a high-fat diet compared with obese subjects (Thomas et al. 1992). In addition, obese subjects tend to consume diets with a higher fat content than lean individuals (Miller et al. 1990). A number of studies have shown that increasing the fat content of the diet is associated with increased total energy intake (Kendall et al. 1991; Shah et al. 1994). Other studies have shown that the satiating effect of energy derived from fat is significantly less than that from carbohydrate (Walls and Koopmans 1989; Lawton et al. 1993; Rolls et al. 1994), and dietary fat is less able than carbohydrate to stimulate its own oxidation (Flatt et al. 1985; Tremblay et al. 1985).

Dietary intervention studies have consistently shown that the dietary fat:carbohydrate ratio per se has a significant effect on energy balance and body weight (Astrup and Raben 1992). Subjects alter their dietary intake according to its fat content more so than the total energy content (Lissner et al. 1987; Kendall et al. 1991). In several studies aimed at reducing blood lipids without a focus on body weight, the change in weight
was best predicted by the reduction in percentage dietary energy from fat, rather than from the change in total energy intake (Sheppard et al. 1991; Prewitt et al. 1991).

Overall, the available data suggest that both the total amount of energy intake, and the composition of the diet are important factors in the energy balance equation, and both of these factors are implicated in the pathogenesis of obesity.

1.4.8.2 ENERGY EXPENDITURE

Total energy expenditure has three principal components. Basal metabolic rate (BMR), which constitutes 60-70% of total energy expenditure, the thermic effect of food (TEF; 10%) and spontaneous physical activity (SPA; 20-30%). These percentages are averages, and show wide variation between individuals. BMR is determined by the amount and activity of lean body mass, and varies considerably with total body mass, age and gender. TEF is the increase in thermogenic activity after eating and depends on the amount and type of food consumed, and also on the state of energy balance. SPA is highly variable and difficult to measure accurately in humans, but is thought to be affected by body mass and composition, and the intensity and duration of exercise. All three components are increased in obesity, and decrease with weight loss.

Another aspect of energy expenditure which has received considerable attention concerns 'futile cycles' (metabolic processes not associated with any specific function other than the use of energy per se and the generation of heat; Newsholme 1978). One example of a futile cycle is the uncoupled mitochondrial respiration observed in brown adipose tissue (BAT; Nicholls and Locke 1984), which is thought to represent an important thermogenic mechanism in rodents (Himms-Hagen 1989). The contribution of futile cycles and in particular BAT thermogenesis to energy expenditure has been extensively debated (MacDonald 1995; Flier and Lowell 1997). The amount of BAT in adult humans appears to be minimal, although it has been very difficult to quantify, suggesting that this tissue does not play a major role in human energy expenditure (see also section 1.5.4.3; MacDonald 1995).

The increasing sedentariness in modern societies has been implicated in the escalating prevalence rates for obesity and other diseases. Prospective studies in Britain have shown that there has been an increase in adiposity over the past decade but no change in dietary intake, either in energy or composition. This increased adiposity has therefore been attributed to decreased physical activity (Prentice 1994; Prentice and Jebb 1995). American population studies have also shown a strong relationship between a lack of physical activity and obesity (Di Pietro and Williamson 1993; Williamson and Madans
Modern appliances designed to make life easier, such as the motor car, and the pervasiveness of television watching are probably contributing to the apparent reduction in exercise in modern societies. Indeed, proxy measures of physical inactivity such as car ownership and television/video viewing have been found in some studies to be more closely related to temporal changes in obesity than any dietary measures (Prentice and Jebb 1995).

Conversely, increasing physical activity has been shown to reduce body weight (Despres and Pouliot 1991; Despres and Lamarche 1993), and is associated with marked improvements in other cardiovascular risk factors (Despres 1994). Exercise regimes are most effective when combined with a reduction in dietary intake (Despres 1994).

In Pima Indians, a population with a very high prevalence rate of obesity (Knowler et al. 1991), several components of energy expenditure have been implicated in the pathogenesis of obesity. Firstly, a prospective study of 126 subjects showed that a low resting BMR, measured with a ventilated-hood indirect calorimeter after an overnight fast, predicted significantly higher body weight gain over a four-year follow-up period (Ravussin et al. 1988). In addition, 24-hour energy expenditure, measured in 95 Pima Indians using a respiratory chamber, also correlated inversely with the rate of weight gain during a two-year follow-up (Ravussin et al. 1988). 24-hour energy expenditure was also shown to aggregate in families, suggesting a significant genetic component (Ravussin et al. 1988).

Subsequent studies in Pima Indians have also shown a strong familial pattern of spontaneous physical activity (Zurlo et al. 1992), suggesting a significant genetic influence on various aspects of energy expenditure. Pima Indian children, who have a strong predisposition to obesity, were found to have significantly reduced physical activity and increased time spent watching television compared with Caucasian controls (Fontvieille et al. 1993). In addition, obesity was associated with lower levels of physical activity in adult Pima Indians, compared to Caucasian controls (Rising et al. 1994). Cumulatively, the results obtained in Pima Indians over the past decade strongly suggest that energy expenditure is genetically determined to a significant degree, and that reduced energy expenditure is predictive of excessive body weight gain over time.

The energy content of an organism at any point in time represents only a minute fraction of the total energy consumed during the life of that organism. Therefore, the factors controlling energy intake and energy expenditure are less important for body weight homeostasis than the mechanisms responsible for adjusting one to the other.
regardless of the overall state of energy turnover. Some of the factors thought to be important in the control of energy balance are discussed below.

1.5 CONTROL OF ENERGY BALANCE

The hypothalamus has long been recognised as a key brain area in the regulation of energy intake. Early studies led to the dual-centre hypothesis (summarised in Figure 1.1), which proposed that two opposing centres in the hypothalamus were responsible for the initiation and termination of eating; the lateral hypothalamus (LHA; "hunger centre") and ventromedial hypothalamus (VMH; "satiety centre", Stellar 1954).

\[\text{VMH} \rightarrow \text{HYPERPHAGIA} \rightarrow \text{OBESITY}\]
\[\text{LHA} \rightarrow \text{ANOREXIA} \rightarrow \text{WEIGHT LOSS}\]

*Figure 1.1:* The dual centre hypothesis

This hypothesis was based on the observations that electrical stimulation of the VMH led to reduced food intake, while electrical stimulation of the LHA caused an increase in feeding (Stellar 1954; Mayer 1953). In addition, lesions of the VMH led to hyperphagia and obesity (Hetherington and Ranson 1940), while LHA lesions caused anorexia and severe weight loss (Anand and Brobeck 1951).

Further studies revealed that glucose caused depolarisation of VMH neurons and reduced the firing rate of LHA neurons (Oomura 1983). Because decreased brain glucose utilisation is a powerful stimulus of food intake (Smith and Epstein 1969) it was proposed that during fasting the fall in blood glucose and subsequent decrease in brain glucose utilisation rate caused refeeding hyperphagia via stimulation of the LHA and inhibition of VMH neurons (Schwartz et al. 1995). However, plasma glucose concentrations normalise within minutes of refeeding, whereas food intake may be
elevated for hours or days following starvation. Therefore, other factors must contribute to the control of energy intake.

The dual-centre hypothesis has been repeatedly modified to accommodate the increasing information about the roles played by various other brain regions, neurotransmitter systems, and hormonal and neural signals originating in the gut on the regulation of food intake. In addition to the LHA and VMH, the paraventricular nucleus (PVN) is now considered to have an important integrative function in the control of energy intake. PVN-lesioned rats develop a hyperphagic obesity syndrome similar to VMH-lesioned animals, but, unlike VMH-lesioned rats they do not increase their food intake in response to starvation (Shor-Posner et al. 1985). The PVN receives afferent neuronal processes from a variety of areas involved in food intake, including the brainstem, VMH and LHA, and several of the neurotransmitters discussed below (including neuropeptide Y, galanin and cholecystokinin) are at their most potent when injected directly into the PVN (Frankish et al. 1995).

Other hypothalamic areas implicated in feeding behaviour include the dorsomedial nucleus, suprachiasmatic nucleus and the medial preoptic area. Many interconnections are known to exist between these areas and those described above.
A large number of neurotransmitters have been investigated as possible hypothalamic regulators of feeding behaviour. Some of these neurotransmitters stimulate food intake, some act in an anorexigenic manner, and some have diverse effects on energy intake depending on the site of administration. For example, gamma-aminobutyric acid (GABA) inhibits food intake when injected into the LHA, but stimulates eating when injected into the VMH or PVN (Leibowitz 1985). Feeding behaviour is thought to be greatly influenced by the interaction of stimulatory and inhibitory signals in the hypothalamus, some of which are detailed below.

1.5.1 NEUROPEPTIDE Y (NPY)

NPY is found in the mammalian brain in higher concentrations than all other peptides and is a potent orexigenic agent after intracerebroventricular (ICV) administration in rodents (Clark et al. 1984). In rats, prolonged ICV NPY caused hyperphagia and a rapid and marked expansion of fat stores (Stanley et al. 1986; Stanley 1993). NPY is synthesised primarily in the arcuate nucleus (ARC) of the hypothalamus (O'Donohue et al. 1985; Stanley 1993), with extensive neuronal projections to the PVN. Fasting in rodents caused increases in NPY messenger ribonucleic acid (mRNA) in the ARC and NPY content in the PVN and its extracellular fluid (Sahu et al. 1988; White and Kershaw 1989; Brady et al. 1990; Kalra et al. 1991; Schwartz et al. 1993a).
NPY also acts within the brain to alter peripheral metabolism in a way that promotes energy storage as fat. For example, ICV NPY in rats resulted in stimulation of lipogenic enzymes in liver and adipose tissue (Billington et al. 1991; Zarjevski et al. 1993), and decreased the activity of the sympathetic nervous system (Billington et al. 1991; Bray 1992; Stanley 1993), leading to reduced thermogenesis. NPY administration also stimulated insulin release from the pancreas and increases adipose tissue lipoprotein lipase content (Zarjevski et al. 1993). Therefore, NPY acted centrally to facilitate a constellation of peripheral anabolic responses that favour energy storage by fat deposition.

The control of NPY biosynthesis appears to be primarily mediated by circulating levels of insulin and glucocorticoids. ARC neurons which synthesise NPY contain receptors for both of these factors (McEwen et al. 1986; Schwartz et al. 1992a,b), suggesting that their central effects may be at least partially mediated via regulation of NPY synthesis and release (Dallman et al. 1993). In addition, states of systemic insulin deficiency in rodents, such as uncontrolled insulin-dependent diabetes mellitus and starvation, were associated with increased hypothalamic NPY gene expression (Williams et al. 1989; Brady et al. 1990; Sahu et al. 1992) that were reversed by either adrenalectomy (Ponsalle et al. 1993; Strack et al. 1995) or ICV insulin infusion (Schwartz et al. 1991a; Sipols et al. 1995). Interestingly, a glucocorticoid-responsive element has been identified upstream of the gene for the NPY precursor (Dean and White 1990). The roles of insulin and glucocorticoids in the control of energy balance will be discussed in more detail later.

Evidence to support the specificity of endogenous NPY in the control of food intake in rats has been provided by a study in which NPY biosynthesis was inhibited in the ARC by direct application of antisense oligonucleotides, causing a significant reduction in food intake (Akahayashi et al. 1994a). In addition, studies in which NPY signalling was greatly reduced by immunoneutralisation showed similar reductions in energy intake in rats (Morley 1987; Stanley et al. 1992; Shibasaki et al. 1993).

The ARC-PVN pathway of NPY synthesis and release has been shown to be overactive in animal models of obesity and NIDDM including ob/ob mice (Williams et al. 1991; Wilding et al. 1993), fa/fa (Beck et al. 1990; Sanacora et al. 1990; Abc et al. 1991) and JCR:LA cp/cp rats (Williams et al. 1992). It has been proposed that some animal models of obesity may be due to hypothalamic insensitivity to insulin, which would cause overactivity of the ARC NPY neurons leading to hyperphagia, decreased energy expenditure and ultimately, obesity (Schwartz et al. 1995).
At least five receptors for NPY have been described, each with varying inherent properties and reactivity with NPY and its fractions (Arakanis et al. 1996). The interaction of NPY with these receptors and their physiological importance in the control of energy balance is complex and unclear at this time (Dryden and Williams 1996). The potential of NPY antagonists as possible anti-obesity drugs is currently an area of intense research. These include peptides, such as the decapeptide ([D-Tyr
\textsuperscript{25,36}, D-Thr
\textsuperscript{32}] NPY
\textsubscript{25-36}) which when injected ICV inhibited both spontaneous feeding and the hyperphagia induced by subsequent NPY administration in rats (Myers et al. 1995), and non-peptides including BIBP3226, a selective NPY-Y
\textsubscript{1} receptor antagonist which inhibited food intake (Haddo et al. 1992).

A further NPY receptor subtype was recently described (Y5), and appeared to mediate the NPY-induced feeding response in Wistar rats (O’Shea et al. 1997). The substantial interest in NPY receptors and the potential of NPY antagonists as anti-obesity agents will ensure continued studies in this area of physiology.

However, crossbreeding experiments recently generated a mouse which failed to produce endogenous NPY (‘NPY knockout’ mice; Erickson et al. 1996a). Interestingly, these animals exhibited normal food intake, body weight and adiposity, and became appropriately hyperphagic after fasting (Erickson et al. 1996a). These results suggest significant redundancy in the physiological mechanisms which regulate food intake, which is not unexpected in the control of such a vital system for the survival of the organism. Some or all of the factors discussed below may be involved in the regulation of energy intake in 'NPY knockout' mice.

Overall, there is a large amount of experimental data to support the theory that NPY released in the hypothalamus, especially from the ARC-PVN projection, is a key molecule involved in the hypothalamic regulation of energy balance.

1.5.2 GLUCAGON-LIKE PEPTIDE-1 (GLP-1)

GLP-1 is an incretin (a gut-derived peptide which stimulates insulin secretion) released from enteroendocrine cells which is thought to also act centrally to inhibit food intake (Turton et al. 1996). GLP-1 acts on the pancreas to stimulate insulin release following oral but not intravenous glucose administration (Mojsov et al. 1987; Holst et al. 1987; Kreymann et al. 1987; Fehmann et al. 1995), which is characteristic of incretin molecules. ICV administration of GLP-1 (1-10 µg) in rats inhibits feeding (Tang-Christensen et al. 1996; Turton et al. 1996; Van Dijk et al. 1996; Van Dijk et al. 1997), while administration of a GLP-1 antagonist, exendin, doubled food intake after ICV
injection and potentiated the NPY-mediated stimulation of food intake (Turton et al. 1996).

GLP-1 appears to have a significant role in carbohydrate metabolism, via stimulation of insulin secretion and inhibition of glucagon secretion, which reduces hepatic glucose production and leads to a reduction in blood glucose concentration in both healthy subjects and those with NIDDM (Gutniak et al. 1992; Nauck et al. 1993). Mice with targeted disruption of the GLP-1 receptor gene ate and gained weight normally, but exhibited both fasting hyperglycemia and impaired glucose tolerance (Scrocchi et al. 1996).

Specific GLP-1 receptors have been demonstrated in the hypothalamus, especially the PVN (Kanse et al. 1988; Turton et al. 1996). In addition, ICV administration of GLP-1 significantly inhibited food intake in rats (Schick et al. 1992; Turton et al. 1996), an effect that could be blocked by the GLP-1 antagonist exendin (Tang-Christensen et al. 1996). These findings have led to the suggestion that GLP-1 may play an important physiological role in the regulation of food intake.

1.5.3 MELANIN-CONCENTRATING HORMONE (MCH)

Recent studies have implicated MCH in the control of energy balance. MCH was found to be overexpressed in the hypothalamus of obese ob/ob mice (Qu et al. 1996), while fasting led to an increase in MCH mRNA in both normal and obese animals (Qu et al. 1996). In addition, ICV MCH administration doubled food intake in normal rats (Qu et al. 1996).

In particular the relationship between MCH and Melanin-Stimulating Hormone (MSH) has been the subject of intense research efforts. Anti-MSH antibodies cross-react with MCH, suggesting that these two molecules share a common epitope (Cechetto and Saper 1988). MCH antagonises the action of MSH in fish skin (Rance and Baker 1979), whereas high concentrations of MCH have an MSH-like action in reptiles and amphibians (Matsunaga et al. 1989), presumably as a result of interaction with one or more of the melanocortin receptors (Cone et al. 1993).

The possible importance of MCH and MSH in the regulation of energy balance was highlighted by the discovery that the obesity syndrome in the A/ry (agouti) mouse was due to ectopic production of the agouti protein which enters the circulation and binds to melanocortin receptors, antagonising the actions of MSH (Lu et al. 1994). It was postulated that one mechanism by which this ectopic production of the agouti protein
may cause obesity would be to mimic the orexigenic actions of MCH in the hypothalamus (Qu et al. 1996). However, recent studies have suggested that it is more likely that the agouti protein may cause obesity by inhibiting the anorexigenic actions of MSH by competitive binding at its hypothalamic receptor (Fan et al. 1997).

The agouti protein was found to be a high-affinity antagonist of the melanocortin-4 receptor (MC4-R; Gantz et al. 1993; Lu et al. 1994; Mountjoy et al. 1994), through which MSH is thought to act. Synthesis of specific agonists and antagonists of this receptor and their administration in rodents showed that the melanocortinergic neurons exhibit a tonic inhibition of feeding behaviour mediated primarily by the actions of MSH on the MC4-R, and that the agouti protein causes obesity by chronic disruption of this inhibitory signal (Fan et al. 1997).

The importance of MCH and MSH in human energy balance is currently unknown, but these compounds are certain to receive much attention in the near future.

1.5.4 OTHER REGULATORS OF ENERGY BALANCE

As mentioned earlier, there is a long list of factors thought to influence energy balance. Some of these will be discussed briefly below.

Serotonin, or 5-hydroxytryptamine (5-HT) is a neurotransmitter which appears to have a significant inhibitory effect on food intake. Indeed, the commercially available appetite suppressant dextenfluramine acts by stimulating the production and release of 5-HT in the brain. Pharmacological stimulation of 5-HT receptors has been shown to markedly reduce spontaneous food intake, while blockade of these receptors with specific antagonists leads to enhancement of food intake (Blundell 1984).

In humans, dextenfluramine treatment reduces the perception of hunger, reduces meal size and decreases snacking (Blundell 1984). 5-HT is synthesised in the brain from its essential amino acid precursor tryptophan, and obese humans have been shown to exhibit a plasma amino acid profile which is indicative of diminished 5-HT biosynthesis (Ashley 1985).

Apart from a potential role in the development of obesity, the importance of 5-HT in dieting to achieve stable long-term weight loss has also been proposed. Dieting is very common but remarkably ineffective in the long-term (Brownell & Rodin 1994). Because dieting significantly lowers plasma tryptophan levels and the ratio of tryptophan to branch-chain amino acids (leucine, isoleucine and valine), it is likely that
the availability of tryptophan for 5-HT biosynthesis is reduced by dieting. This has been shown to lead to increased responsiveness of 5-HT receptors involved in food intake (Cowen et al. 1995). Thus 5-HT is likely to have significant importance not only in the regulation of energy balance, but also particularly in the difficulty experienced by most individuals in achieving long-term weight loss (Cowen et al. 1995).

Cholecystokinin (CCK) and especially its sulphated octapeptide CCK-8 are assumed to be important in controlling satiety (Silver and Morley 1991). CCK is released from the gut after consumption of a meal (Langhans and Scharrer 1992) and has a rapid and potent satiety effect mediated primarily by the vagus nerve (Smith and Gibbs 1992). When injected peripherally, CCK lowers meal size, but a compensatory increase in meal frequency leads to no change in overall caloric intake (West et al. 1984). In addition, exogenous administration of CCK to elicit a reduction in meal size requires supraphysiological doses (several-fold greater than that found in plasma after a meal). Nevertheless, 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.

Recently the membrane-bound isoform of tripeptidyl peptidase II was shown to be a CCK-inactivating enzyme found in neurons responding to CCK (Rose et al. 1996). The authors designed a specific inhibitor of this enzyme which had a significant satiety effect when administered to fasted rats, and significantly reduced spontaneous food intake at the beginning of the dark period, during which time most feeding usually occurred, in another group of rats (Rose et al. 1996). These studies highlighted the possibilities associated with manipulation of the CCK pathways, and will continue the considerable interest in this area of physiology.

Galanin is a 29 amino acid neuropeptide which stimulates feeding when injected into the PVN of rats, and is overexpressed in obese fa/fa Zucker rats (Beck et al. 1993). Furthermore, galanin gene expression in rat PVN has been positively correlated with spontaneous fat intake, while injections of antisense oligonucleotides into the PVN targeted at galanin mRNA significantly reduced fat intake (Akabayashi et al. 1994b). However, although galanin administration led to acute hyperphagia, this was followed by a compensatory hypophagia, and long term, repeated ICV administration of galanin for 14 days had no net effect on either body weight or food intake (Smith et al. 1994).

It is therefore considered unlikely that galanin has a significant physiological role in the long-term control of fat intake and energy balance (Dryden and Williams 1996). Other studies suggest that the major role of galanin is in neurally induced hyperglycemia and
hypoinsulinemia, as it inhibits basal insulin secretion (McDonald et al. 1985; Manabe et al. 1986) and stimulates basal glucagon secretion (Manabe et al. 1986; Dunning et al. 1986).

Future interest in galanin is likely to concern the proposed specificity of its effects on patterns of fat consumption and signals related to fat oxidation (Akabayashi et al. 1994b; Leibowitz 1995).

1.5.4.1 THE HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS

The HPA axis involves a series of hormones which are thought to be very important in the control of energy balance. The primary role of the HPA axis is in controlling the response of the organism to stress. In rodents, administration of Corticosterone-Releasing Hormone (CRH) into the PVN, its principal site of biosynthesis (Beyer et al. 1988) elicited a constellation of effects which strongly favored a state of negative energy balance including marked suppression of food intake (Arase et al. 1988; Glowa and Gold 1991) and stimulation of sympathetic outflow (Arase et al. 1988; Egawa et al. 1990) which increased lipolysis and energy expenditure (Egawa et al. 1990) and inhibited insulin secretion (Brown et al. 1982; Rothwell 1989).

Chronic ICV CRH caused a significant and sustained decrease in food intake and body weight in both normal and obese rodents (Arase et al. 1988; Arase et al. 1989a,b), and was shown to inhibit NPY gene expression (Van Huijsduijnen et al. 1993). In addition, administration of a CRH antagonist or pretreatment with glucocorticoids, which inhibit CRH biosynthesis, both potentiated the feeding response elicited by ICV NPY administration in rats (Heinrichs et al. 1993). Conversely, adrenalectomy, which results in deficiency of circulating glucocorticoids, increased CRH biosynthesis and impaired refeeding in rats after a fast (Green et al. 1992). Adrenalectomy also prevented most experimental forms of obesity in rodents (Bray et al. 1990a,b), as well as causing a complete inhibition of the increase in NPY gene expression usually seen during fasting (Ponsalle et al. 1993).

These data support the hypothesis that CRH is a very important factor in the control of energy balance. One of the main functions of CRH is to cause an increase in the synthesis and release of Adrenocorticotrophic Hormone (ACTH) from the pituitary gland, which then acts on the adrenal gland to increase the circulating levels of glucocorticoids (GC; cortisol in humans, corticosterone in rodents). Relatively few studies have investigated the role of ACTH in obesity, however Slavnov and Epshtein (1977) found a moderate increase in circulating ACTH concentrations in obese humans.
Other studies have confirmed an increase in ACTH release from the pituitary in obesity (Weaver et al. 1993) and an altered pituitary responsiveness to CRH in obese compared with lean individuals (Stafford et al. 1989).

The levels of circulating GCs, which tend to directly oppose the effects of insulin in various organs, are likely to play a significant role in energy balance. GCs have catabolic activity in all tissues except liver and brain. For example, they cause protein breakdown in muscle and adipose tissue lipolysis, yet cause hepatic lipogenesis and protein synthesis in the liver (York 1993). Overall, GCs are thought to have a permissive effect on fat deposition and the development of obesity because, as mentioned above, adrenalectomy prevents or impairs further development of obesity in animal models, while replacement of GCs restores some, if not all, of the obesity (Bray et al. 1990a,b; Sainsbury et al. 1996).

One of the consequences of increased circulating GCs appears to be decreased sympathetic and increased parasympathetic outflow, which may partly account for the resulting hyperinsulinemia and hyperphagia (Bray et al. 1990a,b). The role of the autonomic nervous system in the development of obesity will be discussed in more detail below.

GCs also act centrally to regulate metabolism, feeding behaviour and stress responses (Macco et al. 1980; Bohus et al. 1982; Funder and Sheppard 1987). It is possible that GCs act through their ability to regulate the production and secretion of other peptides including CRH and the pro-opiomelanocortin (POMC) family of hormones (Drouin et al. 1987). The importance of GCs in obesity has recently been illustrated by studies which showed that the GC receptor antagonist RU486 can prevent, or even reverse, the obesity of young fa/fa rats (Okada et al. 1992) and diet-induced obese rats (Langley and York 1990), while having no effect in lean controls (Langley and York 1990; Okada et al. 1992). Animal models of obesity including fa/fa rats and ob/ob mice have been shown to have increased central nervous system (CNS) numbers of GC receptors (Langley and York 1990), which could explain the increased responsiveness to GCs in these animals. Furthermore, most animal models of obesity have some degree of hyperactivity of the HPA axis (York 1993).

Interestingly, hyperactivity of the HPA axis and increased GC secretion is at odds with other observations in obesity which imply a decreased central CRH activity, including hyperphagia and decreased sympathetic drive (Brown and Fisher 1985; Arase et al. 1988; Holt and York 1989; Stubbs and York 1991). Obese humans have a normal circulating plasma GC (cortisol) concentration, but have an accelerated rate of
degradation, which is compensated for by an increase in cortisol production and secretion (Migcon et al. 1963; Galvao-Tales et al. 1976). It is currently unclear whether the effects found in experimental ICV administration of CRH represent a pharmacological effect or a restoration of normal physiological function (York 1993).

1.5.4.2 INSULIN

Insulin's effects in the central nervous system (CNS) promote a state of negative energy balance, in sharp contrast to its anabolic effects in the periphery (Kaiyala et al. 1995). Interest in the role of CNS insulin in the regulation of energy balance is understandable, given that plasma insulin levels vary with changes in energy balance and adiposity (Bagdade et al. 1978) and a dose-dependent entry of insulin into the brain has been demonstrated in several species (Steffens et al. 1988; Schwartz et al. 1991b). Hypothalamic neurons express high concentrations of insulin receptors (Baskin et al. 1987) and both central (Woods et al. 1984; McGowan et al. 1990) and peripheral (Vanderweele et al. 1982) administration of exogenous insulin caused marked reductions in food intake, while increasing sympathetic output and thermogenesis (Rothwell and Stock 1988).

A feedback loop has been proposed whereby an increase in adiposity leads to elevated plasma, and therefore CNS, insulin levels which in turn causes a reduction in food intake and increased energy expenditure to restore normal adiposity (Schwartz et al. 1992b). In addition, insulin deficiency (for example, in uncontrolled diabetes mellitus or starvation) is associated with hyperphagia (Harris et al. 1986; Leedom and Meehan 1989). However, the opposing actions of insulin centrally and in the periphery must be considered. This is particularly evident in the treatment of previously uncontrolled insulin-dependent diabetes mellitus (IDDM), where the restoration of normoglycemia with exogenous insulin causes weight gain despite a reduction in food intake (DCCT Study Group 1988).

One mechanism by which insulin plays a role in the central control of energy balance is via inhibition of the hypothalamic synthesis and secretion of NPY (Schwartz et al. 1991a; Schwartz et al. 1992a,b; Schwartz et al. 1993b; Sipols et al. 1995). Interestingly, central insulin also potentiates the satiety effect of centrally administered CRH (Kaiyala et al. 1995) and peripherally administered CCK (Figlewicz et al. 1986). Thus the role of CNS insulin may be characterised by the promotion of a state of negative energy balance by inhibition of the synthesis and release of central anabolic factors such as NPY and by increasing the hypothalamic sensitivity to peripheral satiety signals such as CCK and central catabolic factors including CRH (Kaiyala et al. 1995).
The Autonomic Nervous System (ANS)

Both insulin and GC's, as well as most of the centrally acting factors described above, are thought to have effects on energy expenditure via the autonomic nervous system (Bray 1992). For example, NPY acts to inhibit sympathetic outflow, while CRH appears to stimulate sympathetic outflow. The fate of ingested energy is significantly influenced by sympathetic tone (Bray et al. 1990a). For example, sympathetic drive is stimulated by feeding (Young and Landsberg 1977a) and contributes to TEF (Ravussin and Swinburn 1992), whereas fasting inhibits sympathetic outflow (Young and Landsberg 1977b). Therefore sympathetic activity is increased during states of positive energy balance, leading to a compensatory increase in energy expenditure, whereas decreased sympathetic tone is a component of the adaptation to negative energy balance, such as in starvation (Kaijala et al. 1995).

The sympathetic nervous system (SNS) is thought to participate in the regulation of adaptive changes in thermogenesis, and therefore energy expenditure (Landsberg and Young 1984). Alterations in SNS activity or in thermic responses to SNS stimulation have been proposed as contributing factors to the energy imbalance found in obesity. However, plasma noradrenaline levels, the principal index of SNS activity in humans, and urinary noradrenaline excretion have been shown in various studies to be lower, higher or not different in obese subjects compared with lean controls (reviewed by Young and MacDonald 1992), and the putative existence of an intrinsic thermogenic abnormality in human obesity is controversial (Kush et al. 1986).

Several studies have suggested that low SNS activity may be a risk factor for excessive body weight gain in humans (Spraul et al. 1993; Ravussin and Tataranni 1996). These include prospective studies showing that low BMR (Ravussin et al. 1988), low spontaneous physical activity (Zurlo et al. 1992) and high 24-hour RQ (Zurlo et al. 1990), all factors which are significantly controlled by the SNS (Ravussin and Tataranni 1996), predict excessive body weight gain in Pima Indians compared with controls. In addition, Pima Indians have low sympathetic nervous system activity compared with weight-matched controls, which suggests that the ANS may be involved in the pathogenesis of obesity in this genetically predisposed population (Spraul et al. 1993).

However, a further group of studies have suggested that central obesity may in fact be associated with increased SNS activity (Spraul et al. 1993; Jones et al. 1996). The contradictory nature of many of the published results regarding the role of the SNS in the pathogenesis of obesity make definitive conclusions very difficult at this time. Large-scale, carefully controlled studies are needed to clarify this relationship.
preferably prospective studies in pre-obese and obese individuals if a cause and effect relationship is to be elucidated.

An impaired response of the SNS in response to feeding has been demonstrated in many animal models of obesity including ob/ob mice (Zaror-Behrens and Himms-Hagen 1983; Knehans and Romsos 1982), gold thioglucoce-treated (GTG) mice (Young and Landsberg 1980) and VMH-lesioned rats (Vandertuig et al. 1982). It has been proposed that obesity in some rodents may stem from a deficiency in the thermogenic components of the SNS in response to food intake (Bray et al. 1990b).

A decreased brown adipose tissue (BAT) thermogenic capacity is the earliest defect shown in 2 day old preobese fa/ta rat pups (Bazin et al. 1984). At 10 days of age, the fa/ta pups showed a significant decrease in the P32000 uncoupling protein mRNA levels, the protein responsible for the energy dissipation as heat (Ricquier et al. 1986). While BAT thermogenic capacity is undoubtedly important in rodent obesity syndromes, its importance in human obesity is disputed. Infant humans have relatively small amounts of BAT, and this rapidly disappears in childhood so that adult humans have very little detectable BAT. Therefore it has been proposed that the role of BAT thermogenesis in human energy balance is minimal (MacDonald 1995; Flier and Lowell 1997).

1.5.4.4 β3-ADRENERGIC RECEPTOR (β3AR)

In spite of the lack of BAT thermogenesis in adult humans, the available evidence suggests that SNS activity may be an important determinant of resting and daily energy expenditure, and individuals with low energy expenditure and SNS activity may be at increased risk of weight gain and obesity (Ravussin et al. 1988; Zarlo et al. 1992). The effects of the SNS on energy expenditure in humans is likely to be mediated by stimulation of β-adrenergic receptors in target tissues, as propranolol (a β-adrenergic receptor antagonist) administration has been shown to reduce basal energy expenditure and the thermogenic response to a high carbohydrate test meal (Astrup et al. 1989; Saad et al. 1991). In addition, two weeks of propranolol treatment caused a significant accumulation of body fat consistent with a decrease in energy expenditure in circumstances of a maintained energy intake, whereas there was a loss of fat with the β-adrenergic receptor agonist, terbutaline (Acheson et al. 1988). The markedly opposite effects of the β-adrenergic receptor agonist and antagonist provide strong evidence to support the hypothesis that the SNS acts on energy balance through these receptors in humans (MacDonald 1995).
Further studies revealed that the principal β-adrenoceptor found in human adipose tissue is the β3AR (formerly known as the "atypical β-adrenoceptor"; Giacobino 1995). Therefore, catecholamines are thought to act on adipose tissue via β3ARs to stimulate lipolysis, increase fatty acid β-oxidation and heat production via the uncoupling protein in BAT.

A recent study of the β3AR gene in 642 Pima Indians revealed a missense mutation in the gene which resulted in the replacement of tryptophan by arginine (Trp64Arg) in the first intracellular loop of the receptor (Walston et al. 1995). The allelic frequencies were 0.31 in Pima Indians, 0.13 in Mexican Americans, 0.12 in African Americans and 0.08 in Caucasians. Pima Indians homozygous for the Trp64Arg mutation tended to have a lower adjusted resting metabolic rate, and had a significantly lower age of onset of NIDDM compared with heterozygotes and normal homozygotes (Walston et al. 1995). The authors concluded that this mutation may accelerate the development of NIDDM in Pima Indians by altering the balance of energy metabolism in visceral adipose tissue (Walston et al. 1995).

Subsequent studies have tended to confirm and extend these findings. A study in 335 Finns found no difference in allele frequency between subjects with NIDDM and controls (0.11 and 0.12 respectively), but found that the mean age of onset of NIDDM was significantly lower in those with the mutation, as well as an increase in mean WHR and reduced glucose tolerance (Widen et al. 1995). A French study (n=278) found that heterozygotes for the mutation were significantly heavier than normal homozygotes, despite no difference in allele frequency between obese (0.08) and non-obese (0.10) subjects, and concluded that the mutation is associated with an increased capacity to gain weight (Clement et al. 1995). However, a number of other studies have found no association between the mutatin and aspects of obesity or NIDDM (for example Oksanen et al. 1996, Sipilainen et al. 1997).

A number of further studies have revealed that the Trp64Arg mutation is associated with significantly increased BMI (Kadowaki et al. 1995; Fujisawa et al. 1996; Urhammer et al. 1996; Kurabayashi et al. 1996), impaired glucose tolerance or NIDDM (Kadowaki et al. 1995; Fujisawa et al. 1996; Urhammer et al. 1996), increased serum cholesterol and triglycerides (Urhammer et al. 1996) and a reduced basal metabolic rate (Sipilainen et al. 1997). Alternatively, other studies have failed to find an association between the Trp64Arg mutation and BMI, glucose tolerance or age of onset of NIDDM (Oksanen et al. 1996, Sipilainen et al. 1997). In summary, the Trp64Arg mutation of the β3AR gene is prevalent in diverse populations and in several distinct cohorts has
been associated with various features of the Metabolic Syndrome including central obesity, hyperinsulinemia/insulin resistance, and hypertension. Subjects with this mutation may also have an earlier onset of NIDDM compared with those who don't. Further studies of this gene are required to elucidate the physiological effects of the mutation, and to further clarify its importance in the pathophysiology of obesity and NIDDM (Emorine et al. 1994; Mauriege and Bouchard 1996).

1.5.4.5 SEX HORMONES

Hyperactivity of the HPA axis causes not only increased GC production, but also suppression of sex steroid and growth hormone synthesis and release. While these changes are not considered to be important etiological factors in the development of obesity, they may be important in determining body fat distribution (Bjorntop 1995). Women with central or abdominal obesity had increased plasma testosterone and decreased circulating progesterone levels (Kissebah and Peiris 1989), while centrally obese men had reduced circulating testosterone (Seidell et al. 1990). In obese men, the increased circulating cortisol and insulin both promote lipid accumulation and diminish fat mobilization, while growth hormone and testosterone, both of which promote lipid metabolism, are decreased (Bjorntop 1995).

In obese women, serum estradiol and estrogen were correlated with body fat mass (Meldrum et al. 1981), while reduced circulating steroid-hormone binding globulin levels suggested an increase in the free fraction of circulating oestradiol (Kopelman et al. 1980; Evans et al. 1983). Ovariectomy caused an increase in food intake and adiposity in several mammalian species and this response was reversed by estrogen replacement (Wade and Gray 1979). In addition, estradiol administration stimulated CRH production in the PVN (Swanson and Simmons 1989) and inhibited hypothalamic NPY release (Bonavera et al. 1994). These data support the hypothesis that sex hormones may influence the amount and distribution of an individual's body fat (Kopelman 1994). Interestingly, some of the animal models of obesity and NIDDM discussed below exhibit gender-specific penetrance of the diabetic phenotype including cp/cp rats (Russell et al. 1994), Aβ mice (Klebig et al. 1995) and OLETF rats (Kawano et al. 1992), suggesting a significant role for sex hormones in these diseases.

In summary, many factors have been implicated in the pathogenesis of obesity, some of which are represented schematically in Figure 1.2. There are likely to be many interconnected pathways which contribute to the control of energy balance, and elucidation of the precise mechanisms involved is sure to be very difficult. Leptin, which is shown in Figure 1.2, will be discussed in some detail below (Section 1.6).
FIGURE 1.2: Some aspects of the pathophysiology of obesity
1.5.5 THE LINK BETWEEN OBESITY AND NIDDM

As discussed in section 1.3.2 above, a significant epidemiological and pathophysiological link has been established between obesity and NIDDM. The precise mechanisms which are involved in this relationship have been investigated, and are thought to involve insulin, glucocorticoids and the HPA axis. However, causal relationships have been difficult to establish between these factors and the pathogenesis of obesity and NIDDM, leading some researchers to look elsewhere for the link between obesity and NIDDM.

1.5.5.1 TUMOR NECROSIS FACTOR-α (TNFα)

One factor which may represent an important link between obesity and NIDDM is the adipocyte-derived cytokine TNFα. Like other cytokines, TNFα has diverse functions in both immune and extra-immune systems (Hotamisligil and Spiegelman 1994). TNFα is paradoxically associated with extreme states of both catabolism (wasting/cachexia) and anabolism (obesity; Spiegelman and Hotamisligil 1993). The role of TNFα in cachexia is likely to be only at very high circulating concentrations and in conjunction with other cytokines in the context of an expanding tumor mass (Spiegelman and Hotamisligil 1993).

TNFα has profound effects on whole body lipid metabolism (Grunfeld and Feingold 1991; Feingold and Grunfeld 1992). Administration of TNFα increases serum triglycerides and very low density lipoproteins in rats and humans, probably by stimulation of hepatic lipogenesis (Feingold and Grunfeld 1992), and inhibits insulin-stimulated glucose uptake in fat and muscle (Douglas et al. 1991; Lang et al. 1992). The net effect of TNFα administration is therefore an increase in basal glucose turnover and a decrease in insulin-stimulated peripheral glucose utilisation (Hotamisligil and Spiegelman 1994) by diverse actions affecting insulin action (Hotamisligil et al. 1993; Feinstein et al. 1993; Hotamisligil et al. 1996a) and liver gluconeogenesis (Lang et al. 1992), as well as altering the pituitary-adrenal axis (Grunfeld and Feingold 1991). In adipose tissue, the major action of TNFα appears to be suppression of lipogenic enzymes, including lipoprotein lipase (Torti et al. 1985; Hotamisligil and Spiegelman 1994).

TNFα expression is increased in adipocytes of obese animals (Hotamisligil et al. 1993) and humans (Hotamisligil et al. 1995; Kern et al. 1995). Neutralisation of TNFα with a soluble TNFα receptor-IgG fusion protein for three days caused a 2-3-fold increase in insulin sensitivity in obese Zucker (fa/фа) rats, while no change was detected in hepatic
glucose output (Hotamisligil et al. 1993). Chronic exposure of 3T3-L1 adipocytes to low concentrations of TNFα strongly inhibited insulin-stimulated glucose uptake, caused a moderate decrease in the insulin-stimulated phosphorylation of the insulin receptor and resulted in a dramatic reduction in the phosphorylation of IRS-1 (Hotamisligil et al. 1994). The authors concluded that TNFα directly interferes with the signalling of insulin through its receptor and consequently blocks the biological actions of insulin (Hotamisligil et al. 1994). These results suggested a role for TNFα in the development of insulin resistance associated with obesity (Hotamisligil et al. 1993). In addition, TNFα administration to humans (Van Der Poll et al. 1991), lambs (Douglas et al. 1991) and rats has been associated with the induction of varying degrees of insulin resistance (Hotamisligil and Spiegelman 1994). Conversely, reduction of insulin resistance in obese mice with either pharmacotherapy or energy restriction decreased TNFα synthesis in muscle but not fat (Hofmann et al. 1994).

Interestingly, mice with a null mutation in aP2 (the gene encoding the adipocyte fatty acid binding protein), developed dietary-induced obesity but, unlike control mice, they did not express TNFα in adipose tissue (Hotamisligil et al. 1996b). The authors concluded that fatty acid binding proteins such as aP2 are critical components of the pathway linking obesity and insulin resistance, possibly by linking fat metabolism to expression of TNFα (Hotamisligil et al. 1996b).

In obese humans TNFα expression in adipocytes is increased 2.5-fold compared with lean controls (Hotamisligil et al. 1995; Kern et al. 1995). Strong correlations have been documented between TNFα mRNA in adipose tissue and plasma insulin concentrations (Hotamisligil et al. 1995), BMI and percent body fat (Kern et al. 1995). Body weight reduction through dieting was associated with an increase in insulin sensitivity and a concomitant reduction in TNFα expression (Hotamisligil et al. 1995). The association of TNFα with human obesity led to investigation of the TNFα gene. Norman et al. (1995) utilised sib-pair analysis on 3 polymorphic dinucleotide repeat loci adjacent to the TNFα gene in Pima Indians, and found that one of these markers was significantly linked to BMI and percent body fat. A separate study found that the genetic variability in the TNFα promoter was not associated with NIDDM in both Caucasians and Pima Indians (Hamann et al. 1995).

A very recent study showed that the TNFα receptor subtype known as TNFR2 was overexpressed in adipose tissue of obese women, and there was also approximately 6-fold more soluble TNFR2 in their circulation relative to lean control subjects (Hotamisligil et al. 1997). TNFR1, another subtype, was not different in expression or
circulating levels between lean and obese subjects. TNFR2 expression levels correlated with BMI, degree of hyperinsulinemia and level of expression of TNFα in adipose tissue. It was postulated that the actions of TNFα in obesity, where it possibly causes or exacerbates insulin resistance, may be modulated by TNFR2 (Hotamisligil et al. 1997).

Taken together, the data suggest that TNFα synthesis and secretion may be increased in obesity, and that, through effects on adipocytes, muscle and pancreatic β-cells (Zhang and Kim 1995), TNFα may contribute to insulin resistance and NIDDM (Hotamisligil and Spiegelman 1994). Another adipocyte-derived factor which may represent an important link between obesity and NIDDM is leptin, which is the focus of this dissertation and will be discussed in some detail below.

1.6 THE LEPTIN PHENOMENON

1.6.1 THE LIPOSTAT HYPOTHESIS

It is well known that the amount of stored fat in the body is homeostatically regulated by a system that responds to both increases and decreases in adiposity with powerful behavioural and physiological drives which strongly favor the return of body fat to the regulated level. This system in humans is thought to consist of a slow feedback pathway in which total fat mass is the variable which is sensed and regulated (Rink 1994).

In 1953, G. C. Kennedy proposed the lipostat model to explain this homeostasis, suggesting that a circulating factor was released from adipose tissue into the circulation, and then acted on the brain (hypothalamus) to regulate energy balance (Kennedy 1953). Scientific evidence to support this theory came mainly from a series of parabiosis experiments over the next 20 years. In parabiosis, the circulation of two animals are surgically joined using skin anastomoses, so that the animals effectively share the same circulation. When an ob/ob mouse was joined to a normal, wild-type mouse, the ob/ob mouse reduced its food intake and lost body weight (Hausberger 1959). When a db/db mouse was joined to a normal mouse, the normal mouse rejected food and starved to death (Coleman and Hummel 1969). Ob/ob mice made parabiotic with db/db mice dramatically reduced their food intake and lost body weight (Coleman 1973; Coleman 1978). The pioneering studies of Coleman led to the conclusion that a circulating satiety factor was inadequately produced by ob/ob mice, and was present in sufficient amounts but ineffective in db/db mice (Coleman 1973; Coleman 1978).
These studies, when considered along with other experiments which showed that overfeeding one of a pair of parabiotic mice reduced food intake and caused weight loss in the other, and that a VMH lesion in one caused obesity in that mouse but reduced food intake and caused weight loss in the unlesioned parabiont, appeared to support the lipostat hypothesis (Hervey 1958). These experiments indicated that a circulating factor had a profound effect on energy balance, and experts contended that this was likely to be derived from adipose tissue (as shown in Figure 1.3). The nature of this 'lipostat' was to remain elusive until a remarkable discovery was made in New York in 1994.

![Diagram of the lipostat hypothesis]

**Figure 1.3:** The lipostat hypothesis

1.6.2 **The Ob Gene, Leptin and Obesity**

Over eight years of painstaking research including both classical and molecular genetics culminated in the discovery of the gene responsible for the phenotype observed in *ob/ob* mice. Dr. Friedman and his team from the Rockefeller University isolated the *ob* gene and characterised its expression in mice and humans (Zhang *et al.* 1994). Eventually narrowing the search down to a 650-kilobase region on mouse chromosome six, they then used the method of exon-trapping to finally isolate a gene expressed exclusively in adipose tissue. Further experiments proved that the gene responsible for the phenotype of *ob/ob* mice had been isolated (Zhang *et al.* 1994). The *ob* (obese) gene was shown to encode a 167 amino acid product with a putative signal sequence in wild-type mice, but a C->T mutation was detected in codon 105 of the gene in C57B6/J *ob/ob* mice, which changed an arginine codon to a stop codon (Zhang *et al.* 1994). The
net result of this mutation is a truncated, dysfunctional protein which is unable to be secreted from the adipocytes which express it. In addition, a polymorphism was detected upstream of the ob gene in SM/Ckc-+Dewob21/ob21 mice which presumably disrupts the promoter region of the gene, resulting in no ob gene expression in this strain (Zhang et al. 1994). The authors concluded that the lack of a functional, circulating product of the ob gene resulted in the ob/ob phenotype. The coding regions of the ob gene were shown to be highly homologous between mouse and human, predicting an amino acid homology of 84% (Zhang et al. 1994), suggesting a highly conserved protein with an important physiological function. This protein is now known as leptin, named from the Greek root leptos, meaning thin (Halaas et al. 1995).

Apart from the potential importance of leptin in human physiology, this discovery highlighted the importance and validity of the use of animal models to investigate human diseases (Lindpainter 1995). This research showed clearly that the disease phenotypes observed in various animal models of human diseases should be investigated as fully as possible, as new discoveries, such as leptin, may be the result, and the impact on our understanding of the etiology and pathophysiology of the human disease in question could be very significant.

The potential of leptin as a satiety factor led to a large number of studies following the initial publication described above. Three groups simultaneously reported the effects of administration of leptin to various mice. Friedman's group tested leptin at a dose of 5 mg/kg/day, delivered by intraperitoneal (IP) injection, in ob/ob, db/db, and wild-type mice (Halaas et al. 1995). The ob/ob mice treated with leptin lost a staggering 40% of their initial body weight after 33 days of treatment, with food intake reduced significantly after 2 days, and stabilizing at about 40% of the intake of control animals at all subsequent time points (Halaas et al. 1995). Interestingly, paired ob/ob mice lost significantly less weight than the leptin treated animals, suggesting that leptin affected energy expenditure as well as food intake (Halaas et al. 1995). No effect of leptin treatment was observed in db/db mice, supporting the suspicion that these animals have a defective leptin receptor, while the effects in wild-type mice were somewhat equivocal. A small but significant decrease in body weight was observed up until the twelfth day of treatment, but there was no significant difference to control animals after that time point (Halaas et al. 1995). When the leptin dosage was increased to 25 mg/kg/day, significant decreases in food intake (by 8%), body weight (by 12%) and percent body fat were seen in wild-type mice (Halaas et al. 1995). The decrease in body weight in all animals was almost exclusively accounted for by reduced body fat, with no significant difference in lean body mass between treated animals and controls. In
addition, treatment of mice with either human or mouse leptin had equal effects in mice (Halaas et al. 1995).

The two other reports published simultaneously concurred with and extended the results of Halaas and coworkers. One group showed a dose- and time-dependent effect of leptin in young ob/ob mice, but less conclusive results in wild-type animals (Pellemounter et al. 1995). In fact, no significant difference in food intake or body weight were observed in wild-type mice given daily IP injections of leptin at doses of 0.1, 1 or 10 mg/kg. Continuous infusion of 0.3 mg leptin/kg body weight/day in wild-type mice for two weeks caused a small (about 5%) but significant drop in body weight, which was partly attributed to a decreased food intake (Pellemounter et al. 1995). Leptin treatment also corrected the decreased oxygen consumption found in ob/ob mice to the level of lean mice, raised their body temperature to that of lean mice, reversed the characteristic hypoactivity of ob/ob mice, and reduced the hyperglycemia and hyperinsulinemia normally found in these animals (Pellemounter et al. 1995). Leptin had no effect on any of these parameters when administered to wild-type mice (Pellemounter et al. 1995).

The other study showed that IP leptin at a dose of 6 mg/kg/day reduced food intake by 48% and body weight by 7% after 5 days in ob/ob mice (Campfield et al. 1995). In db/db mice this dose had no measurable effect, while 12 mg/kg/day had no significant effect in wild-type mice (Campfield et al. 1995). In mice with diet-induced obesity (DIO), leptin at 6 mg/kg/day caused no significant change compared with saline-treated control animals. However, in a small group (n=3) of DIO mice, leptin given IP at a dose of 30 mg/kg/day significantly reduced food intake (by 39%) and body weight (by 9%; Campfield et al. 1995). In addition, leptin was shown to be more effective in wild-type and ob/ob mice when administered ICV, suppressing food intake for 7 hours, which suggested a central site of action (Campfield et al. 1995).

These studies collectively strengthened the hypothesis that leptin represented the satiety factor first postulated by Kennedy, and investigated by Coleman. Many subsequent studies were generated by the reports described above, which will be summarised briefly as follows.

The ob gene was shown to be expressed exclusively in mature adipocytes and not in any other tissues tested (Zhang et al. 1994; Maffei et al. 1995b; Ogawa et al. 1995; Rentsch et al. 1995). In addition, the ob gene was not expressed in the stromal-vascular cells contained in adipose tissue (Maffei et al. 1995b; Ogawa et al. 1995; Rentsch et al. 1995), nor in undifferentiated preadipocytes (Masuzaki et al. 1995a). Indeed, ob gene
expression and leptin secretion has been measured in vitro following differentiation from rat fibroblastic preadipocytes to mature adipocytes (Wabitsch et al. 1996; Mitchell et al. 1997).

Ob gene expression was increased (relative to controls) in adipocytes from massively obese humans (Hamilton et al. 1995; Lonngqvist et al. 1995), and in many animal models of obesity including db/db mice (Maffei et al. 1995a,b), fa/fa rats (Maffei et al. 1995b, Ogawa et al. 1995; Igel et al. 1996), mice and rats with hypothalamic lesions caused by gold thioglucose, monosodium glutamate or electrolytic lesions (Funahashi et al. 1995; Maffei et al. 1995a), DIO mice and rats (Frederich et al. 1995a; Masuzaki et al. 1995a; Mizuno et al. 1996) and JCR:LA corpulent (cp/cp) rats (Vydelingum et al. 1995). Levels of ob mRNA were correlated with body weight and/or BMI in both rodents and humans (Considine et al. 1995; Hamilton et al. 1995; Considine et al. 1996a; Igel et al. 1996; Vidal et al. 1996).

The development of a radioimmunoassay for leptin led to many studies of levels of this protein in both humans and rodent models of obesity. Relative to controls, leptin concentrations are increased 5-10-fold in db/db mice (Frederich et al. 1995a; Maffei et al. 1995b), 50-fold in fa/fa rats (Maffei et al. 1995a), 5-15-fold in hypothalamic-lesioned obese mice (Frederich et al. 1995a; Maffei et al. 1995a), 25-fold in DIO mice, 10-fold in Av (yellow agouti) mice, 5-fold in fa/fat (fatty) mice and 2-fold in tub/tub (rubby) mice (Maffei et al. 1995a). Once again, leptin levels were shown to correlate with the amount of body fat (Maffei et al. 1995a), as predicted by the lipostat hypothesis.

It was hoped that in human obesity leptin levels may be deficient, so that administration of this satiety factor would then offer a relatively simple therapeutic approach to the correction of this disease. However, in humans, leptin levels have been shown to be increased in obesity, relative to lean controls. Some of these studies are summarised in Table 1.5 (below).
<table>
<thead>
<tr>
<th>Reference</th>
<th>Lean (n)</th>
<th>Leptin (ng/ml)</th>
<th>Obese (n)</th>
<th>Leptin (ng/ml)</th>
</tr>
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<td>Considine et al. 1996a</td>
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<td>31.3 ± 24.1</td>
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<tr>
<td>Caro et al. 1996</td>
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<td>9.6 ± 1.5</td>
<td>8</td>
<td>40.2 ± 8.6</td>
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<tr>
<td>Hosoda et al. 1996</td>
<td>10</td>
<td>5.6 ± 1.3</td>
<td>10</td>
<td>43.0 ± 9.4</td>
</tr>
<tr>
<td>Sinha et al. 1996b</td>
<td>6</td>
<td>12.0 ± 4.4</td>
<td>11</td>
<td>41.7 ± 9.0</td>
</tr>
<tr>
<td>Boden et al. 1996</td>
<td>5</td>
<td>11 ± 3</td>
<td>5</td>
<td>31 ± 12</td>
</tr>
</tbody>
</table>

**TABLE 1.5**: Plasma leptin concentrations in lean and obese humans

In addition, leptin levels have repeatedly been shown to be highly correlated with BMI and the percent body fat (Maffei et al. 1995a; Considine et al. 1996a; Dua et al. 1996; Haffner et al. 1996a,b; Hosoda et al. 1996; Ma et al. 1996; McGregor et al. 1996; Pratley et al. 1996; Zimmet et al. 1996). This correlation holds true in both lean and obese individuals (Maffei et al. 1995a), and even in those with extremely low body fat including sufferers of anorexia nervosa (Hebebrand et al. 1995; Grinspoon et al. 1996) and elite endurance athletes (Hickey et al. 1996). There have been reports of higher leptin levels in females compared to males for a given BMI (Haffner et al. 1996a), but this was generally accounted for by differences in percent body fat (Maffei et al. 1995a; Considine 1996a; Considine and Caro 1996). The elevated levels of leptin found in obese humans led to the suggestion that these individuals must have a reduced sensitivity to the actions of this hormone (Meier 1996; Rohner-Jeanrenaud and Jeanrenaud 1996; Zimmet and Collier 1996).

Factors which bring about a change in body fat content over a period of time have been associated with parallel changes in plasma leptin concentration. For example, long-term overfeeding which resulted in a 10% increase in body weight was associated with a parallel increase in circulating leptin levels (Kolaczinski et al. 1996c), while caloric restriction or exercise training to reduce body fat content were also associated with falls in plasma leptin concentrations (Maffei et al. 1995a; Considine et al. 1996a; Kohrt et al. 1996; Fesus et al. 1997).

Interestingly, plasma leptin concentrations were found to be reduced in cigarette smokers, when compared to controls of similar BMI who didn't smoke (Hodge et al. 1997). These results have implications for individuals who give up smoking, as this is often associated with weight gain. The authors proposed that nicotinic mechanisms may modify the sensitivity of receptors to leptin, subsequently modulating leptin synthesis.
and body weight (Hodge et al. 1997). These findings were recently confirmed independently in a population of Mexican-Americans, and also in Caucasians (Wei et al. 1997). This area of research is one worthy of further study to elucidate the connection between smoking/nicotine, body weight/composition and the leptin system.

The association of leptin with body fat distribution is still unclear at this time. One study found positive correlations of leptin with BMI, WHR, waist circumference, hip circumference, triceps skinfold and subscapular skinfold and concluded that leptin concentration was associated with all adipose tissue depots and not disproportionately with central obesity (Haffner et al. 1996b). Further studies detailed a strong correlation between plasma leptin and total body fat, but no correlation with visceral fat mass independent of total body fat (Dua et al. 1996; Solin et al. 1997). However, other reports suggest a relationship between leptin and body fat distribution. For example, two studies of regional ob gene expression in humans found higher levels of ob mRNA in subcutaneous compared with omental adipocytes in all patients (total of 49) tested (Hube et al. 1996; Montaguc et al. 1997), leading the authors to propose a role for leptin in the control of body fat distribution. Strangely, there was no correlation between leptin expression and BMI in the study of Hube et al., casting some doubt over these results. In a larger study measuring plasma leptin concentrations and fat areas using computerised tomography, leptin levels correlated with BMI and subcutaneous fat at the umbilicus level, but not visceral fat area in obese (n=51) and non-obese (n=41) subjects (Takahashi et al. 1996a). In addition, a study of 23 pairs of Finnish twins discordant for obesity revealed that intrapair differences in leptin concentrations correlated with percent body fat in female pairs but not males, but positively correlated with visceral fat area in both genders, leading the authors to suggest that visceral fat may be of special importance in the regulation of leptin in males (Ronnemaa et al. 1997).

Studies of regional ob gene expression in rodents have yielded inconsistent results. In general higher levels of expression have been found in abdominal fat depots (Masuzaki et al. 1995a; Ogawa et al. 1995; Trayhurn et al. 1995; Zheng et al. 1996), however several studies noted highly variable expression from animal to animal, and even from within the same depot in the same individual (Maffei et al. 1995b; Masuzaki et al. 1995a; Hayase et al. 1996). The influence of leptin on body fat distribution is one area of research which is sure to generate considerable interest in the near future.

Other notable findings regarding leptin levels in humans include the lack of a change in leptin concentration postprandially (Dagogo-Jack et al. 1996) or following acute exercise (Hickey et al. 1996; Perusse et al. 1997), and the lack of effect of diabetic

In humans, diurnal variation of plasma leptin concentrations has been demonstrated (Sinha et al. 1996a). The highest levels were found between midnight and the early morning, with the nadir in the early afternoon (Sinha et al. 1996a). In addition, 1-7 ultradian oscillations were reported, with a mean of 3.25 pulses per 24-hour period (Sinha et al. 1996b). The physiological relevance of these variations is currently unknown, although it was proposed that the decline in plasma leptin overnight may suppress hunger during sleep (Sinha et al. 1996a). In rodents, a putative diurnal rhythm was observed with a maximum at 4 am, and minimal leptin concentrations late in the afternoon, almost directly opposite to that found in humans (Saladin et al. 1996; Ahima et al. 1996). These changes in leptin were linked with feeding, and no variation was observed in fasted rats (Saladin et al. 1995). Another group noted a "reciprocal temporal relationship" with plasma corticosterone (Ahima et al. 1996), and suggested a possible regulatory role for leptin on corticosterone production via the HPA axis.

1.6.3 The relationship between leptin and insulin

Although ob gene expression and serum leptin concentrations correlate strongly with BMI and percent body fat, there is considerable variation in leptin levels at any given BMI. This suggests that circulating leptin concentrations are determined in part by factors other than body fat mass (Lonnqvist 1996). Due to the established relationship between hyperinsulinemia, insulin resistance and obesity, the interaction between insulin and the ob gene and/or leptin has been extensively investigated.

Fasting plasma insulin concentration and ob gene expression or plasma leptin levels have been shown to be significantly correlated in humans (Boden et al. 1996; Dagogo-Jack et al. 1996; Malmstrom et al. 1996; Zimmet et al. 1996) and lean and obese rodents (Mizuno et al. 1996). In several cases, plasma insulin and leptin concentrations were positively correlated even after adjusting for percent body fat (Larsson et al. 1996; Malmstrom et al. 1996; Zimmet et al. 1996). In addition, plasma leptin concentrations were found to be significantly lower in lean insulin-sensitive men relative to insulin-resistant men despite similar body composition (Segal et al. 1996).

Potential effects of insulin on ob gene expression and plasma leptin concentrations have been investigated both in vitro and in vivo, for both short- and long-term studies. In isolated rat adipocytes, the addition of insulin (10 nM) caused a 50% increase in ob gene expression after 2 hours (Saladin et al. 1995). A similar study found a 2-3-fold
increase in \textit{ob} gene expression in response to the addition of 10 nM insulin (Rentsch \textit{et al}. 1995). However, in another study on isolated rat adipocytes, the addition of insulin had no effect on \textit{ob} gene expression (Murakami \textit{et al}. 1995). Long-term \textit{in vitro} studies on human adipocytes have shown that the addition of insulin (100 nM) had no effect on \textit{ob} gene expression for the first 72 hours of culture, but significantly increased expression and leptin concentration in the medium after this time point (Kolaczynski \textit{et al}. 1996a; Nolan \textit{et al}. 1996; Wabitsch \textit{et al}. 1996). The effect of insulin was potentiated by the addition of cortisol in long-term culture of human adipocytes (Wabitsch \textit{et al}. 1996).

\textit{In vivo}, a single insulin injection in fasted rats was shown to normalise \textit{ob} gene expression to the level of fed controls after 4 hours (Saladin \textit{et al}. 1995). The technique of the hyperinsulinemic-euglycemic clamp (HEC), which induces large increases in circulating insulin while maintaining normal blood glucose levels, has been used to further investigate the relationship between insulin and leptin. In lean rats, a 48 hour HEC caused an 88\% increase in \textit{ob} gene expression (Cusin \textit{et al}. 1995), while in fasted lean rats, a 2.5 hour HEC significantly increased (2-3-fold) \textit{ob} mRNA levels in adipose tissue (Zheng \textit{et al}. 1996).

In humans, short-term (3-5 hour) HEC studies have generally found no acute effect of supraphysiological hyperinsulinemia on \textit{ob} gene expression or plasma leptin concentrations (Dagogo-Jack \textit{et al}. 1996; Kolaczynski \textit{et al}. 1996a; Larsson \textit{et al}. 1996; Pratley \textit{et al}. 1996; Vidal \textit{et al}. 1996). However, one study found a significant increase in plasma leptin after a 4 hour HEC in 8 overweight subjects (mean BMI 26.3 \pm 0.6 kg/m\textsuperscript{2}), and after 8.5 hours in 7 patients with NIDDM (mean BMI 27 \pm 0.9 kg/m\textsuperscript{2}; Malmstrom \textit{et al}. 1996). Longer studies utilising the HEC have shown a 1.7-fold increase in \textit{ob} gene expression after 24 hours (Sainsbury \textit{et al}. 1996), and a significant increase in plasma leptin after 48 hours (Kolaczynski \textit{et al}. 1996a).

Collectively the human studies tend to indicate that insulin has no acute effect on \textit{ob} gene expression or plasma leptin concentrations, but that a long-term regulatory effect is likely. In rodents insulin does appear to acutely regulate leptin production (Saladin \textit{et al}. 1995; Zheng \textit{et al}. 1996), and this could reflect the different metabolism of rodent compared with human adipocytes, or possibly the increased metabolic rate in rodents. Either way, a relationship between insulin and leptin has been clearly established. One possible explanation of the human results was that the trophic effects of insulin on adipose tissue leads to the increase in leptin production (Kolaczynski \textit{et al}. 1996a), however this is not in keeping with other results (Malmstrom \textit{et al}. 1996), and further studies are needed to clarify this relationship.
Studies aimed at decreasing plasma insulin in rodents by using streptozotocin to prevent insulin secretion or a 3-day fast consistently found 40-90% decreases in ob gene expression (Cisin et al. 1995; Igel et al. 1996; Sivitz et al. 1996), which was partially restored by insulin administration (Sivitz et al. 1996).

All of the data presented above support a role for leptin in the long-term regulation of energy balance, with significant relationships existing between leptin, body fat content and plasma insulin. A further group of studies has implicated leptin in the short-term response to perturbations in energy balance, such as fasting.

### 1.6.4 LEPTIN AND THE RESPONSE TO STARVATION

In wild-type mice, fasting for 16 hours caused an 85% fall in ob mRNA levels (MacDougald et al. 1995), for 24 hours caused a decrease of 72-90% (Frederich et al. 1995b; Trayhurn et al. 1995), while a 48 hour fast reduced ob gene expression by 90% (Mizuno et al. 1996). Similarly, in lean control rats, fasting for 12-72 hours reduced ob gene expression by 50-90% (Frederich et al. 1995b; MacDougald et al. 1995; Moinat et al. 1995; Saladin et al. 1995; Igel et al. 1996; Sivitz et al. 1996; Zhang et al. 1996). The fall in ob gene expression caused by fasting could be rapidly reversed upon refeeding (Frederich et al. 1995b; MacDougald et al. 1995; Saladin et al. 1995; Trayhurn et al. 1995).

While fasting clearly reduced leptin biosynthesis in lean animals, the results in animal models of obesity were less clear. Fasting for 24 hours caused a significant (60%) reduction in ob mRNA levels in mildly obese KK mice, and a 40% drop in severely obese KKA mice (Hayase et al. 1996). In db/db mice fasted for 15 days there was no change in plasma leptin concentration, despite a 25% decrease in BMI (Maffei et al. 1995b). However, fasting for a further 13 days, which caused body weight to drop 35% from baseline, also caused leptin to fall to undetectable levels (Maffei et al. 1995b). However, the length of this study and the decrease in body weight mean that the results don’t reflect a short-term response to caloric deprivation. Studies of fasting for 24 hours in fa/fa rats (Igel et al. 1996), for 16- (MacDougald et al. 1995) or 24-hours (Trayhurn et al. 1995a) in ob/ob mice, and for 48 hours in DIO mice (Mizuno et al. 1996) resulted in no change in ob gene expression. Hence it appears that the response to fasting observed in lean animals is not generally seen in their obese counterparts.

Several small studies have investigated the effects of fasting on plasma leptin concentrations in humans. In the first study, a 24-hour fast was found to have a significantly greater effect on plasma leptin concentration in lean (n=3; 15.8 ± 1.5 to
4.6 ± 0.3 ng/ml) than obese humans (n=3; 34.6 ± 8.9 to 19.9 ± 4.9 ng/ml; Sinha et al. 1996b). Another study in 5 obese and 5 lean individuals fasted for 52 hours found significant decreases in plasma leptin in both groups (Boden et al. 1996). A further study in 11 individuals ranging in BMI from 20-36 (mean 24.4 ± 8.6 kg/m²) found a fall in plasma leptin after 12 hours of fasting, which continued to drop until the 36th hour of fasting to 35% of baseline levels (Kolaczynski et al. 1996b). Interestingly, these authors did not look at lean and obese individuals separately, so any difference in the response of these groups to fasting, as found in rodents and suggested by the study of Sinha et al. (1996), was not addressed by this study. Further studies on the effects of fasting on plasma leptin concentrations are required to investigate possible differences in the response between lean and obese humans.

In this context it is worth noting a study of “semi-fasting” in 7 morbidly obese individuals given a hypocaloric diet (1045 kJ/day) for 5 days, which only marginally affected body weight (-3.7 ± 2.1%; Vidal et al. 1996). No significant change in ob gene expression was observed in this study, with the average change measured being +17%, and the range -46% to +69% (Vidal et al. 1996).

Despite this possible discrepancy between lean and obese humans, it appears that, at least in lean animals and humans, leptin may have a dual role in the physiology of energy balance (Kolaczynski et al. 1996b). In addition to its (principal?) role as a long-term sensor of the amount of triglycerides stored in adipose tissue and afferent signal to the hypothalamus, leptin may also act as an acute sensor of energy balance. In this context, leptin may be acutely regulated by perturbations of normal energy balance independent of changes in body fat mass. It should be noted that insulin is also markedly decreased in fasted individuals, a further aspect of this intriguing relationship which deserves further investigation.

1.6.5 OTHER FACTORS AFFECTING LEPTIN PRODUCTION

A range of other factors have been shown to influence ob gene expression and/or plasma leptin concentrations. The administration of β3AR agonists such as CL316243, Ro16-8714, BRL 35135A, ZD2079 and isoprenaline have been shown to inhibit ob gene expression and reduce plasma leptin concentrations in lean mice and rats (Moinat et al. 1995; Mantzoros et al. 1996; Trayhurn et al. 1996; Mitchell et al. 1997). In contrast, these compounds had minimal effects in ob/ob mice (Trayhurn et al. 1996), and no effect in β3AR knockout mice (Mantzoros et al. 1996) or fafia rats (Moinat et al. 1995). Interestingly, the administration of CL316243 to wild-type mice fasted for 24 hours reduced ob mRNA and leptin levels to 20% of baseline, yet also acutely
suppressed food intake. The authors concluded that β3AR agonists acutely suppress leptin production and simultaneously suppress food intake via a mechanism that operates downstream of leptin (Mantzoros et al. 1996). Further interest to the relationship between leptin and the β3AR was recently added by a report of a genome wide scan of human DNA which identified significant linkage between fat mass/leptin levels and a marker adjacent to the β3AR gene on chromosome 8 (Comuzzie et al. 1997).

It may be concluded that β3AR agonists directly inhibit leptin expression, independent of changes in adipocyte lipid content (Giacobino 1996), suggesting that leptin expression is under the control of the SNS, whose effects are known to be mediated by β3AR in adipose tissue (Giacobino 1996). Thus a pathway has been proposed whereby leptin acts on the hypothalamus to produce satiety, but also stimulates the SNS which leads to β3AR-mediated lipolysis in white adipose tissue, and thermogenesis in brown adipose tissue, and this SNS stimulation also results in reduced leptin expression (Giacobino 1996). In this highly plausible model, leptin exerts a retroregulatory inhibition of its own expression via the SNS, which contributes to the maintenance of a constant energy balance. This hypothesis is consistent with the "set-point" theories of energy balance, including the lipostat hypothesis, and also with the fact that leptin is greatly overexpressed in ob/ob mice, due to the lack of this feedback regulation via the SNS. In this model it easy to envisage how a defect in the β3AR could contribute to leptin resistance in humans, and therefore to obesity (Giacobino 1996).

Given the fact that adrenalectomy can prevent or even reverse the obesity syndrome in ob/ob mice (principally by abolishing the characteristic hyperphagia; Feldkircher et al. 1996), various groups have investigated a possible relationship between glucocorticoids and leptin. In vitro, dexamethasone (a synthetic glucocorticoid), rapidly and significantly induced ob gene expression in isolated adipocytes (Murakami et al. 1995; Mitchell et al. 1997). The observation that adrenalectomy in NPY-treated mice prevents both obesity and the expected NPY-induced rise in leptin expression suggests complex interactions between leptin, glucocorticoids and NPY in the pathophysiology of obesity (Rohner-Jeanrenaud and Jeanrenaud 1996). Several glucocorticoid response element consensus binding sites have been identified in the proximal promoter region of the ob gene, suggesting a direct transcriptional effect of glucocorticoids on leptin production (Saladin et al. 1996). In addition, cortisol potentiated (3-fold) the action of insulin to stimulate leptin production in a long-term culture of human adipocytes (Wabitsch et al. 1996). In vivo, adipose tissue ob gene expression was rapidly induced by various glucocorticoids (hydrocortisone, dexamethasone or triamcinolone) in albino rats, which
was followed by a concordant decrease in body weight gain and food intake (De Vos et al. 1995).

In humans, dexamethasone administration resulted in a rapid and sustained increase in plasma leptin levels (Miell et al. 1996). This increase was associated with parallel increases in plasma insulin concentrations, reinforcing the link between these two hormones. The fact that dexamethasone administration has been associated with stimulation of appetite and increases in circulating leptin levels is contrary to the proposition that leptin acts as a satiety factor, and suggests that the regulation of both appetite and leptin levels are likely to be multifactorial and involve alternate pathways and neurotransmitters (Miell et al. 1996). In patients with Cushing's disease, which is associated with chronically increased circulating glucocorticoids and obesity, increased plasma leptin concentrations were observed compared with controls matched for BMI (Leal-Cerro et al. 1996). These results suggest that the chronic increase in circulating glucocorticoids may cause an increase in plasma leptin concentrations, presumably by reducing the action(s) of circulating leptin.

Furthermore, the effects of ICV leptin administration on food intake and body weight were greatly increased and prolonged in adrenalectomised rats compared with controls (Zakrewska et al. 1997). In addition, glucocorticoid supplementation in adrenalectomised animals inhibited the effects of exogenous leptin administration in a dose-dependent manner, suggesting that glucocorticoids play a significant inhibitory role in the action(s) of leptin.

A theory recently proposed to explain the above results suggests that chronic oversecretion of glucocorticoids in response to a variety of "stress paradigms" could result in reduced activity of leptin, generating a state of leptin resistance similar to that observed in fa/fa rats and db/db mice (Ur et al. 1996). This natural leptin resistance could lead to obesity in the context of unlimited food supply and reduced energy expenditure with the removal of sources of stress. This theory could explain the higher incidence of obesity in lower socioeconomic groups, in which hypersecretion of cortisol has been demonstrated (Ur et al. 1996). The hypothesis that oversecretion of glucocorticoids leads to leptin resistance is also compatible with the observation that obese humans have hyperleptinemia, and predicts a significant relationship between glucocorticoids, adiposity and appetite (Ur et al. 1996).

Another group of compounds known to significantly influence ob gene expression and leptin biosynthesis are the insulin-sensitising agents known collectively as thiazolidinodiones (TDs). TDs enhance the actions of insulin by increasing glucose-
disposal and inhibiting hepatic glucose production (HGP), and are widely regarded as potential therapeutic agents for NIDDM (Saltiel and Olefsky 1996). TDs directly regulate gene expression by interactions with a family of nuclear receptors known as the peroxisome proliferator-activated receptors (PPARs).

In vitro studies in human adipocytes have shown that TDs inhibit leptin production by up to 40% (Kallen and Lazar 1996; Nolan et al. 1996), and also abolish the stimulatory effect of insulin on leptin production (Nolan et al. 1996). TDs administered to rodents reduced ob gene expression by up to 67% in wild-type rats, 70% in fa/fa rats, and 78% in db/db mice (Zhang et al. 1996). However, in obese humans treated with TDs for 12 weeks, which reduced plasma insulin concentrations by 40-50% (indicative of increased insulin sensitivity), no change was detected in plasma leptin levels (Nolan et al. 1996). Interestingly, a positive correlation between the change in insulin sensitivity and plasma leptin was detected in these subjects. That is, plasma leptin increased in subjects with the greatest increase in insulin sensitivity, while leptin was unchanged or slightly decreased in those with smaller changes in insulin sensitivity (Nolan et al. 1996).

Interpretations of this data are complicated by the increased insulin sensitivity accompanied by decreased circulating insulin concentrations, and further studies are likely to be forthcoming in this area.

The administration of the endotoxin lipopolysaccharide (LPS), TNFα or interleukin-1 to hamsters caused anorexia and weight loss, and induced ob gene expression in adipose tissue (Grunfeld et al. 1996). These results suggest that induction of leptin production during the host response to infection, which is usually associated with elevated levels of TNFα and interleukin-1, and, in the case of gram-negative bacterial infections, LPS, may contribute to the characteristic anorexia of infection.

Catecholamines, which are known to affect lipid metabolism, were investigated in vitro in isolated adipocytes. The addition of 100 nM noradrenaline or isoproterenol caused an 80% fall in ob mRNA levels (Kosaki et al. 1996). These effects were partially reversed by propanolol but not phenolamine, while cholera toxin (100 ng/ml) and dibutyryl cAMP (100 μM) almost completely abolished ob gene expression (Kosaki et al. 1996). The authors concluded that a signalling pathway which results in the activation of protein kinase A regulates ob gene expression (Kosaki et al. 1996).

Analysis of the 3'- and 5'-flanking regions of the ob gene revealed several binding sites for the adipocyte-specific transcription factor CEBPα, which is induced during adipocyte differentiation and maintained in mature fat cells. Addition of CEBPα to
isolated adipocytes significantly enhanced \textit{ob} gene expression (Hwang \textit{et al.} 1996; Miller \textit{et al.} 1996).

Some of the factors which can affect \textit{ob} gene expression/leptin biosynthesis are summarised in Figure 1.4. These factors, along with others yet to be elucidated, are likely to be the focus of intensive research as agents capable of altering the leptin/obesity axis are sought.

\textbf{FIGURE 1.4:} Some factors affecting \textit{ob} gene expression/leptin biosynthesis

\subsection{MOLECULAR BIOLOGY OF THE HUMAN \textit{OB} GENE}

The isolation of the human \textit{ob} gene resulted in a series of studies which unsuccessfully searched for mutations in the coding region of the gene in obese individuals (Considine \textit{et al.} 1995; Hamilton \textit{et al.} 1995; Considine \textit{et al.} 1996c; Maffei \textit{et al.} 1996; Niki \textit{et al.} 1996). Only one conservative nucleotide substitution was found which did not appear to alter the phenotype in that individual, and all authors concluded that mutations in the human \textit{ob} gene probably account for very few, if any, cases of human obesity (Considine \textit{et al.} 1996c; Considine and Caro 1996). This is not surprising given the heterogeneous nature of human obesity, and its likely polygenic etiology, both factors making it distinct from the obesity syndrome found in \textit{ob/ob} mice. However, these studies do not preclude the possibility that a small proportion of cases of human obesity could result from genetic mutations in the \textit{ob} gene.

There are at least 8 polymorphic microsatellite markers located in close proximity to the human \textit{ob} gene at the chromosomal position 7q31.3 (Clement \textit{et al.} 1996). Linkage
analysis in this region for extreme obesity (BMI > 35 kg/m²) in sib-pairs revealed suggestive evidence for linkage to three markers (D7S514, D7S680 and D7S530) in 101 obese French families (Clement et al. 1996). A further study of haplotypes generated in 78 families noted significant allele disequilibrium in extreme obesity, suggesting a role for the ob gene (or a nearby gene) in familial morbid obesity (Reed et al. 1996).

However, several other comprehensive studies have yielded conflicting results to these reports. An extensive study of 716 Pima Indians (217 families) utilising two of these polymorphic microsatellite markers for sib-pair linkage revealed no significant associations with BMI, percent body fat, BMR, 24-hour energy expenditure or 24-hour RQ (Norman et al. 1996). Another group identified a novel tetranucleotide repeat polymorphism very close to the ob gene in its 3'-flanking region. Although a tendency for increased body weight was associated with this polymorphism, no significant differences in allele frequency between lean and obese subjects were found (Shintani et al. 1996). Similarly, a study in 346 Mexican-American sib-pairs revealed no evidence of linkage or association between markers adjacent to the ob gene and obesity/NIDDM (Stirling et al. 1995).

1.6.7 THE LEPTIN RECEPTOR

The lack of coding mutations in the human ob gene, and the lack of linkage to obesity in most studies, combined with the elevated leptin levels in obese humans, have led researchers to investigate the role of the leptin receptor (OBR), and the possibility that human obesity may be characterised by a reduced sensitivity to the hypophagic and hypermetabolic actions of endogenous leptin (Meier 1996; Rohner-Jeanrenaud and Jeanrenaud 1996; Zimmert and Collier 1996).

The OBR was first detected in mouse choroid plexus (Tartaglia et al. 1995), the part of the brain where macromolecules such as leptin (16 kD in size) are actively transported across the blood-brain barrier into the cerebrospinal fluid, which gives these molecules access to the brain parenchyma.

The OBR identified was a single membrane-spanning receptor most closely related to the gp130 signal-transducing component of the interleukin-6 receptor, the granulocyte-colony stimulating factor receptor, and the leukemia inhibitory factor receptor (Tartaglia et al. 1995). In addition, the OBR contained several conserved motifs consistent with a class I cytokine receptor (Tartaglia et al. 1995). Apart from the choroid plexus, the OBR was also detected in lung and kidney, the normal sites of
disposal of polypeptide hormones, and the hypothalamus, leptin’s putative site of action (Tartaglia et al. 1995). Indeed subsequent studies have shown that urine is the major route of disposal of radiolabelled leptin in rodents (Cumin et al. 1996; Van Heek et al. 1996) and humans (Iida et al. 1996).

Genetic mapping studies suggested that the mouse db mutation, the rat fa mutation and OBR were in fact the same gene (Chua et al. 1996a). Subsequent studies revealed that the db mutation is caused by a point mutation (G->C) in the OBR gene which generates a mutant donor splice site in the gene, causing a 106 nucleotide insertion which prematurely terminates the intracellular (IC) domain of the receptor (Chen et al. 1996; Lee et al. 1996). This is thought to result in a receptor defective in signal transduction, which could account for the phenotype observed in db/db mice. Once again, the theory of Kennedy and the conclusions of Coleman appear to have been proven correct.

The rat fa mutation results from a nucleotide substitution (A->C) at position 880 of the OBR gene, which results in an amino acid substitution (Gln269Pro), reducing the receptor’s affinity for leptin and resulting in the fa/fa phenotype (Chua et al. 1996b; Iida et al. 1996a; Takaya et al. 1996). It was also suggested that the fa mutation may inhibit the ability of the OBR to form homo-dimers, which may be important in the bioactivity of leptin (Phillips et al. 1996). Interestingly, a study of ICV administration to fa/fa rats and lean heterozygotes revealed that leptin, at a dose of 36 μg (10 times that required in lean rats), could reduce body weight gain and food intake after fasting in fa/fa rats, despite the receptor mutation (Cusin et al. 1996). The results suggest that the decreased leptin sensitivity in fa/fa rats, caused by the OBR mutation and resulting in the obese phenotype, could be overcome by high enough doses of leptin administered ICV (Cusin et al. 1996).

A recent study also showed that a Tyr763Stop mutation in the OBR of the obese Koletskey (f) rat was responsible for the phenotype found in this animal model of obesity (Wu-Peng et al. 1997). None of the OBR mutations described above have been detected in humans (Considine et al. 1996b), although this does not eliminate the possibility that OBR mutations could be the primary cause of obesity in some individuals.

Studies in animals have revealed the existence of at least six alternatively spliced forms of the OBR, with IC domains of varying lengths and sequence composition (Lee et al. 1996; Takaya et al. 1996). The major isoforms appear to be OBRb, a 1162 amino-acid receptor with a long cytoplasmic domain; OBRa which has a very short IC domain; and OBRc (805 amino acids), which lacks both the IC and transmembrane components of the receptor, and probably represents a soluble isoform (Lee et al. 1996; Takaya et al. 1996).
1996). The various isoforms of the leptin receptor are represented schematically in Figure 1.5a, and OBR defects known in animal models of obesity are shown in Figure 1.5b.

The long form of the receptor (OBRb) contains several sites thought to be involved in signal transduction, including two (Janus Kinase) JAK boxes. In class 1 cytokine receptors, such as OBR, signal transduction usually involves ligand-induced homo- or hetero-dimerisation and involves receptor-associated kinases of the Janus family (JAKs). These JAKs autophosphorylate, then phosphorylate the IC domain of the receptor at specific tyrosine residues, which provides docking sites for the 'signal transducers and activators of transcription' (STAT) family of transcription factors (White and Tartaglia 1996). The receptor associated JAKs then activate the STATs by phosphorylation, promoting their dimerisation and translocation to the nucleus where they activate transcription of specific genes (White and Tartaglia 1996). It is assumed that the short form of the receptor (OBRa) lacks signal transduction capabilities due to its lack of JAK binding sites in the IC domain.

Leptin has variable effects on STATs in the studies published to date. In vitro, in human renal carcinoma cells, leptin caused phosphorylation of STAT-1, but not STAT-3 or STAT-5 (Takahashi et al. 1996b). In another in vitro transfection study, the hypothalamic OBR from wild-type mice was shown to activate STAT-3, STAT-5 and STAT-6, however the OBR of db/db mice exhibited defective STAT signalling (Ghilardi et al. 1996). In vivo, leptin treatment activated STAT-3 but no other STAT in the hypothalami of ob/ob and wild-type, but not db/db mice (Vaisse et al. 1996). These studies confirm that STATs probably represent part of the signal transduction system activated by leptin, and that this system is defective in db/db mice due to an OBR mutation. The next major question is what genes are transcribed in response to leptin in the hypothalamus?
**FIGURE 1.5:** A) OBR isoforms. B) OBR mutations in some animal models of obesity.
1.6.7.1 **THE PHYSIOLOGICAL EFFECTS OF LEPTIN**

The relationship between NPY and leptin has been investigated extensively. As discussed previously, NPY is produced and acts in the hypothalamus as a potent stimulant of food intake. *Ob/ob* (leptin-deficient) mice have 3-fold increased NPY mRNA in ARC compared with wild-type mice, and NPY has been implicated in the hyperphagia and obesity of these animals (Wilding *et al.* 1993). The administration of leptin to wild-type mice fasted for 48 hours significantly reduced the increase in NPY mRNA observed in untreated animals, suggesting that the effects of starvation may be mediated by leptin's regulation of NPY (Ahima *et al.* 1996). In addition, a single bolus of 11 μg leptin administered in fasted rats reduced NPY by 20-50% in ARC, PVN and dorsomedial hypothalamus (DMH), and significantly decreased NPY mRNA levels in ARC by 22% (Schwartz *et al.* 1996a; Wang *et al.* 1997).

Conversely, the ICV administration of NPY significantly increased *ob* gene expression and plasma leptin concentrations in mice (Sainsbury *et al.* 1996; Wang *et al.* 1997). It should be remembered that ICV NPY increases both circulating insulin and corticosterone in rodents, and if chronic, leads to increased body weight and body fat content, suggesting that this relationship is likely to be a complex one involving divergent pathways (Rohner-Jeanrenaud and Jeanrenaud 1996). However, in animals which were paired to prevent the rise in body weight normally seen after ICV NPY, *ob* gene expression was still significantly increased, and this was reversed after cessation of NPY infusion, indicating a possible direct effect of NPY on *ob* gene expression (Sainsbury *et al.* 1996).

Together these data support the hypothesis that leptin acts directly on the hypothalamus to inhibit the biosynthesis of NPY, which may mediate at least part of leptin's hypophagic and thermogenic actions. Conversely, NPY-induced obesity, mediated at least in part by hyperinsulinemia and hypercorticosteronemia in rodents (Rohner-Jeanrenaud and Jeanrenaud 1996), results in increased circulating leptin concentrations. It is therefore possible to hypothesise that leptin and the NPY-ergic ARC-PVN projection may interact in a homeostatic loop to regulate energy balance and body fat mass.

However, the generation of mice deficient in NPY (NPY knockout mice) has cast some doubt on the importance of NPY in the effects of leptin. Unexpectedly, NPY knockout mice have normal food intake and body weight, and become appropriately hyperphagic after caloric deprivation (Erickson *et al.* 1996a). In addition, leptin treatment was as effective (or actually more effective in the short term) in these animals as wild-type
mice, which implied that NPY was not required for the hypophagic and body fat reducing effects of leptin (Erickson et al. 1996a). Furthermore, backcrossing generated NPY-deficient ob/ob mice which were phenotypically intermediate between ob/ob and wild-type mice. These mice exhibited significantly reduced hyperphagia and obesity compared to ob/ob mice which have NPY (Erickson et al. 1996b). Taken together, these studies suggest that leptin can suppress feeding and promote body weight loss via mechanism(s) independent of, or additional to, those involving NPY, and that NPY is involved in the central mechanisms of leptin deficiency (Erickson et al. 1996a,b).

Other prime candidates implicated in the pathways through which leptin alters food intake include MCH and GLP-1. At the time of writing, no reports linking leptin and GLP-1 were published, and only one implicated a relationship between leptin and the MCH/MSH system. A genome-wide scan of human DNA revealed a significant association between plasma leptin concentration, fat mass and a marker adjacent to the pro-opiomelanocortin (POMC) gene on chromosome 2 (Comuzzie et al. 1997), suggesting a relationship between the POMC system, including MSH, and leptin.

In addition to its hypothalamic effects on food intake, leptin is also believed to alter energy expenditure via the HPA axis. For example, leptin treatment in ob/ob mice selectively increased noradrenaline turnover (sympathetic outflow) in BAT, suggesting that thermogenesis may be another important mechanism by which leptin regulates energy balance in rodents (Collins et al. 1996). Once again however, the relevance of BAT thermogenesis in human physiology remains to be proven (MacDonald 1995). In another study, a single ICV injection of 0.01-1 μg leptin in ob/ob mice increased energy expenditure and reduced the RQ in a dose-dependent manner, indicative of a decrease in the fraction of energy derived from carbohydrate oxidation and a corresponding increase in energy from the oxidation of fat (Hwa et al. 1996).

1.6.7.2 LEPTIN RESISTANCE

The observations that plasma leptin concentrations are significantly increased in human obesity, in proportion to body fat content, suggests that the hypothalamic effects of leptin detailed above do not function correctly to maintain "normal" body weight. Therefore, the concept that obese individuals have a relative 'leptin resistance' has been postulated.

The apparent reduced sensitivity to leptin in obese humans may not occur at the level of the hypothalamus however. Inadequate leptin transport across the blood-brain barrier via the choroid plexus OBR (Tartaglia et al. 1995; Caro et al. 1996; Schwartz et al.)
1996b) represents another possible mechanism which could lead to "leptin resistance". Binding and internalisation of leptin has been demonstrated in isolated human brain capillaries, an in vitro model of the human blood-brain barrier (Golden et al. 1997). It appears that leptin enters the cerebrospinal fluid via a saturable system described by a two-site binding model (Banks et al. 1996; Caro et al. 1996; Schwartz et al. 1996b; Golden et al. 1997). Thus, the efficiency of transport across the blood-brain barrier is lower in obese, compared with lean, individuals, suggesting a possible regulating point for leptin resistance (Caro et al. 1996; Schwartz et al. 1996b). However, cerebrospinal fluid leptin concentrations still correlated positively with BMI, and further data is required to determine the physiological relevance of the decreased transport across the blood-brain barrier in obese individuals.

Another interesting phenomenon is the possible existence of a leptin binding protein in the human circulation, which may be the OBRe isoform described above which lacks both the IC and transmembrane domains (Lee et al. 1996; Takaya et al. 1996). Studies have shown binding of leptin to serum macromolecules which is specific, reversible and displaceable with unlabelled leptin (Houseknecht et al. 1996; Sinha et al. 1996b). Interestingly, it was suggested that a higher proportion of leptin circulates in the bound form in lean compared with obese individuals (and rodents), with possible effects on bioactivity yet to be elucidated (Houseknecht et al. 1996; Sinha et al. 1996b).

While the lipostat hypothesis and many of the studies described above strongly suggest that leptin's principal target tissue is the hypothalamus, several studies have revealed significant expression of OBR in tissues outside the brain (Lee et al. 1996; Takaya et al. 1996; Emilsson et al. 1997). Interestingly, full-length OBRs have been detected in pancreas, liver, kidney, heart, lung, small intestine, testes and adipose tissue (Iida et al. 1996a; Lee et al. 1996; Takaya et al. 1996; Emilsson et al. 1997). The presence of receptors for leptin in peripheral tissues led researchers to investigate possible functions of leptin alternative, or additional to, its hypothalamic effects.

1.6.7.3 EXTRAHYPOTHALAMIC EFFECTS OF LEPTIN (?)

The role of leptin in the reproductive process has received considerable attention. Female ob/ob mice, like a significant proportion of obese women (Hamilton 1996), are infertile, and have deranged reproductive hormone profiles (Chehab et al. 1996). Treatment with leptin in ob/ob mice (10 μg/g/day for 30 days) restored fertility, resulting in ovulation, pregnancy and parturition (Chehab et al. 1996). Significantly, animals which were pairfed to reduce their food intake and body weight in parallel with leptin-treated mice did not regain fertility (Chehab et al. 1996), suggesting that it was a
direct effect of leptin, and not a result of weight loss, which restored reproductive
capacity. This is supported by the fact that adrenalectomy, which corrects obesity in
ob/ob mice, does not restore fertility in these animals (Shimizu et al. 1993). In a
separate study, leptin treatment (50 μg twice daily for 14 days) in female ob/ob mice
significantly increased serum luteinizing hormone, increased uterine and ovarian
weights and stimulated aspects of ovarian and uterine histology compared with controls
(Barash et al. 1996). In male ob/ob mice, increased follicle-stimulating hormone,
testicular and seminal vesicle weights, and elevated sperm counts have been detected
after leptin treatment (Barash et al. 1996).

Furthermore, leptin administration reversed the prolonged dioestrus and delayed
vaginal oestrus found in 48-hour fasted mice (Ahima et al. 1996), and partially reversed
the fasting-induced drop in serum testosterone, luteinizing hormone and serum
thyroxine (Ahima et al. 1996). These results suggest that, despite the presence of OBR
in reproductive organs, the actions of leptin on reproduction may be mediated primarily
via the hypothalamic-pituitary system.

In addition, daily IP injections of leptin (2 μg/g/day) in normal weight, prepubertal
female mice accelerated the onset of puberty as determined by vaginal opening, oestrus
and cyclicity (Ahima et al. 1997). Importantly, the effect of leptin on the acceleration of
puberty occurred in the absence of any effect on body weight (Ahima et al. 1997).

The studies detailed above suggest an alternative hypothesis for the actions of leptin.
That is, leptin may act as the signal from fat to the brain communicating the adequacy
and readiness of body fat stores to undergo the physiological trial of reproduction (Bray
1996). This putative function of leptin may be especially important at low leptin
concentrations. It is known that menstruation occurs only if fat stores are adequate
(Frisch and McArthus 1974), and female endurance athletes who have fat levels below
this threshold cease menstruating. Conversely, massively obese women have menstrual
irregularities which can be corrected by weight loss. It is possible that these effects are
related to the function of leptin, which may alter the reproductive system via its actions
on NPY expression (Bray 1996). The precise physiological role of leptin in the control
of the reproductive system remains to be elucidated, and new studies to investigate this
hypothesis are likely to be forthcoming.

A role for leptin in hematopoiesis has also been postulated. A novel hemopoietin
receptor (B219) expressed in various isoforms in very primitive hematopoietic stem
cells was found to be an isoform of OBR, with almost identical ligand binding domains
(Cioffi et al. 1996; Bennett et al. 1996), suggesting a regulatory role for leptin in
hematopoiesis. Interestingly, the B219 receptor was also expressed at high levels in adult reproductive organs, further evidence for a role of leptin in reproduction (Cioffi et al. 1996). In one study, leptin was suggested to have a proliferative effect on multilincage progenitor cells, resulting in increased myelopoiesis and lymphopoiesis (Bennett et al. 1996). Analysis of db/db mice, which have a defective leptin receptor (as discussed above) revealed dramatically reduced steady-state circulating lymphocytes, suggesting that leptin plays a vital role in lymphopoiesis (Bennett et al. 1996).

In addition, structure prediction algorithms were used to construct a detailed molecular model of the human leptin molecule, and suggested that leptin is a bona fide member of the hematopoietic cytokine family (Rock et al. 1996). Furthermore, after administration of 125I-labelled leptin to ob/ob and db/db mice, significant amounts of leptin were detected in bone marrow, the major site of hematopoiesis (Van Heek et al. 1996). The role of leptin in the normal physiological regulation of hematopoiesis in humans is unknown at this time, and specifically designed studies to investigate this relationship are required.

1.6.7.4  **IS LEPTIN THE LINK BETWEEN OBESITY AND NIDDM?**

As discussed previously, a strong relationship appears to exist between obesity and NIDDM. Factors such as insulin, glucocorticoids and TNFα have been investigated in attempts to elucidate the physiological mechanisms resulting in this relationship. Recent studies into leptin have suggested that it may also represent a possible link between obesity and NIDDM. As discussed above, leptin is secreted in proportion to the body fat mass, and epidemiological studies have suggested a relationship between plasma leptin concentration and insulin resistance. In support of this hypothesis, several studies have investigated the relationship between leptin and insulin in peripheral tissues.

Exposure of isolated hepatocytes to leptin at concentrations similar to those observed in obese individuals, resulted in attenuation of several insulin-induced activities (Cohen et al. 1996). These effects included impairment of tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), reduced association of the adaptor molecule growth factor receptor bound protein-2 with IRS-1, and downregulation of gluconeogenesis (Cohen et al. 1996). In contrast, leptin increased the activity of IRS-1 associated phosphatidylinositol 3-kinase. In combination, these factors suggest that high plasma leptin concentrations may modulate insulin activities in some obese individuals (Cohen
et al. 1996), and it is possible that leptin, in a manner similar to that proposed for TNFα, may provide a link between obesity and insulin resistance.

Pancreatic β-cells also express the full length OBR, and binding of 125I-labelled leptin to β-cells has been demonstrated in vitro which was significantly displaced by unlabelled leptin (Kieffer et al. 1996). In one study of perfused pancreatic islets, leptin significantly inhibited insulin release in a dose-dependent manner, and also inhibited the stimulatory effects of 16.7 mM glucose on insulin secretion (Emilsson et al. 1997). These effects were found to be half-maximal at a leptin concentration of 10 nM (Emilsson et al. 1997). Interestingly, while these effects were found in isolated islets from wild-type and ob/ob mice, similar results were not found in islets from db/db mice or fa/fa rats, suggesting that the effects were mediated via the functional OBR (Emilsson et al. 1997). However, in another study, 1 nM leptin failed to affect the release of either insulin or glucagon from islets of Wistar rats (Leclercq-Meyer et al. 1996). At this stage it is unclear whether this last study failed to find an effect due to the low dose used. It is interesting to speculate that the relative insulin deficiency found in late-stage, obesity-associated NIDDM could be due, at least in part, to the inhibitory effects of hyperleptinemia on the pancreatic β-cells. At any rate, these studies provide a physiological basis for the formulation of hypotheses linking leptin and insulin secretion, as has been suggested by epidemiological studies.

Many cytokines have significant autocrine and/or paracrine effects, so it is interesting to note the presence of both long- and short-form OBRS in rodent adipocytes (Iida et al. 1996a; Lee et al. 1996; Takaya et al. 1996). In isolated preadipocytes induced to differentiate with dexamethasone and insulin, expression of acetyl-CoA carboxylase (ACC), the rate-limiting enzyme in long chain fatty acid synthesis and a marker of lipid biogenesis, is normally induced. The addition of leptin or inducement of ob gene expression suppressed production of ACC, suggesting a paracrine role for leptin in the regulation of lipid metabolism (Bai et al. 1996).

A schematic summary of the known physiological effects of leptin is given in Figure 1.6.
**FIGURE 1.6:** Physiological effects of leptin.

1.6.8 **IS LEPTIN THE "MAGIC BULLET"?**

These possible peripheral actions of leptin, along with the fact that leptin apparently fails to reduce food intake and body fat content correctly in human obesity, have raised significant doubts that the primary role of leptin is in the regulation of energy balance (Bray 1996). Additional doubts surround the role of leptin in energy balance during times of plentiful food supply as evolutionary pressure would be expected to select for genes of value to the organism during times of food shortage, which have been more common during evolution than times of plenty (Flier and Elmquist 1997). In this context, it is noteworthy that the presence of the *ob* or *db* gene in heterozygote (lean) mice has been shown to confer a selective advantage over the wild type (lean) phenotype in mice fasted to starvation (Coleman 1979). Therefore, if leptin is involved in the regulation of energy balance, it is probably more important when body fat is low,
rather than in obesity, as starvation has been a greater and more immediate threat during human evolution than obesity (Flier and Elmquist 1997). In this regard it is interesting that leptin concentrations correlate closely with adiposity in individuals with very low body fat content, such as endurance athletes and sufferers of anorexia nervosa (Hebebrand et al. 1995; Grinspoon et al. 1996; Hickey et al. 1996).

Despite these apparently logical assumptions, the discovery of leptin has led to a frenzy of research activity, with the hope that an antiobesity drug may be the net result. Indeed, the pharmaceutical giant Amgen Inc. apparently paid US$20 million for the rights to market leptin or its analogues (Keightley 1995). In this context it is worth noting the results from more recent studies of leptin administration in rodents. In support of the earlier studies of Halaas et al., Pelleymounter et al. and Campfield et al. (all 1995) described above, other studies have also shown significant dose-dependent reductions in food intake, body weight, blood glucose and plasma insulin and corticosterone, as well as decreasing NPY mRNA levels in ob/ob, but not db/db mice (Stephens et al. 1995; Weigle et al. 1995; Schwartz et al. 1996a). In paired ob/ob mice not treated with leptin, no change in NPY mRNA levels was detected, and much smaller decreases in circulating glucose and insulin concentrations were reported (Schwartz et al. 1996a). In another study, a single intravenous injection of leptin reduced refeeding after a fast in albino mice (Rentsch et al. 1995). These studies confirm that leptin has hypophagic effects which are partially mediated by regulation of NPY gene expression, and that leptin may have direct effects on blood glucose and/or plasma insulin concentrations independent of its effects on food intake.

Continuous infusion of leptin (0.1 \( \mu g/g/day \) for 14 days) in ob/ob mice resulted in a 35% decrease in body weight compared with saline-infused controls (Levin et al. 1996). Other changes observed included a 50% reduction in fat pad weight, a 38% fall in blood glucose, a 27% drop in plasma insulin, and 58% and 24% reductions in plasma cholesterol and triglycerides respectively (Levin et al. 1996). No significant effect was seen in db/db mice, while lean heterozygotes (ob/+) had small but significant changes in food intake and body weight (Levin et al. 1996). Paired ob/ob mice lost significantly less body weight than leptin-treated animals, confirming that leptin exerts effects on energy balance in excess of those induced by reductions in food intake (Levin et al. 1996). This study confirms that subcutaneous infusion of leptin is a very effective form of administering this hormone to rodents.

Several recent studies have shown that small doses of leptin administered ICV can have substantial effects. The significantly lower dose required, when compared to peripheral administration, strongly suggested a central (hypothalamic) site of action for leptin.
(Stephens et al. 1995). For example, the administration of 3.5 μg recombinant leptin to Long-Evans rats significantly suppressed food intake when administered ICV, but had no effect when given IP (Seeley et al. 1996). In addition, leptin had significant effects on food intake and body weight when administered directly into the VMH, but not when injected into the dorsal raphe or lateral ventricle (Jacob et al. 1997). In another study, after ICV leptin (3.5 μg) administration, which reduced food intake by 53%, elevated c-Fos-like immunoreactivity was detected in the PVN, central amygdala and DMH (Van Dijk et al. 1996). Other studies have shown that, in addition to significantly reducing food intake, ICV leptin increased uncoupling protein mRNA in BAT (by 70%), and reduced NPY levels by 20-50% in ARC, PVN and DMH, and NPY mRNA in ARC by 22% (Schwartz et al. 1996a; Wang et al. 1997). These studies confirm that, despite the possible peripheral actions of leptin discussed above, the principal site of action of exogenous leptin appears to be the hypothalamus.

If leptin is the "magic bullet" which will successfully treat obesity (Zimmet and Collier 1996), it seems clear that relatively high doses may need to be administered to achieve sufficient levels at the hypothalamus. If so, the effects of leptin other than those on the hypothalamus may become vitally important in the overall welfare of the patient.

1.6.9 OVERVIEW

There is now a large quantity of evidence to support the theory that leptin represents a "lipostat" in human physiology. It is synthesised exclusively in adipocytes, and concentrations of leptin in plasma are strongly correlated with body fat content. Studies in some animal models of obesity suggest that administration of leptin can reduce food intake and increase energy expenditure, leading to an overall reduction in body fat content. The degree to which leptin is effective appears to be related to the circulating levels of the hormone in the animals treated. That is, animals deficient in leptin (ob/ob mice) are highly sensitive, while increased concentrations of plasma leptin in db/db mice are associated with a severe insensitivity to exogenous leptin administration.

Between these extreme examples are lean, wild-type mice, which appear to have a sensitivity to leptin intermediate between these two, while DIO mice have reduced sensitivity compared with wild-type mice. In this spectrum, if it were extrapolated to humans, one would expect the typical case of human obesity, which is characterised by increased circulating leptin levels, to be associated with a relative insensitivity to exogenous leptin. If this is true, as will be at least partially answered by clinical trials currently underway (Saladin et al. 1996; Flier and Elmquist 1997), then relatively large doses of leptin may need to be administered to achieve significant weight loss. The effects of exogenous leptin administration in polygenic animal models of obesity and/or
NIDDM with hyperleptinemia are currently unknown, as no studies have yet been published. This type of study could provide considerable insight into what can be expected in similarly hyperleptinemic obese humans.

Many studies have shown that leptin has effects other than those on food intake and energy expenditure. Of particular importance is likely to be the relationship between leptin and insulin. The fact that leptin levels are highly correlated with plasma insulin concentrations, and the possibility that leptin may affect insulin action in the liver, and insulin secretion by the pancreas, has serious implications for the use of leptin in the treatment of obesity. Once again, studies of leptin treatment in polygenic animal models of obesity with varying degrees of hyperinsulinemia and insulin resistance could significantly advance our understanding of the potential of leptin as a therapeutic agent for obesity. If leptin was used to treat obesity, but had the unfortunate side effect of worsening insulin resistance and perhaps even precipitating the development of NIDDM, the health costs would surely outweigh the benefits of such a treatment in most cases. Careful studies will be needed to clarify this relationship before leptin could be widely accepted as a pharmacotherapeutic agent for obesity. In addition, studies designed to study effects of leptin in other tissues expressing the leptin receptor, such as adipose tissue, could provide further insight into the physiological effects of leptin.

In addition, more information is required to explain the short term response of leptin in fasting, and any possible relevance of this to the reproductive function of the organism. Alteration of reproductive hormonal balance would constitute another dire side effect of leptin treatment if it were to occur.

1.7 ANIMAL MODELS OF OBESITY/NIDDM

Studies seeking to elucidate the etiology and pathophysiology of obesity and NIDDM in human subjects are plagued with inherent difficulties. These include factors related to the diseases themselves, including the heterogeneity of these conditions in humans, age-dependent penetrance which make longitudinal studies very cumbersome, uncontrollable gene-environment interactions (for example diet, drug intake and activity level) and gene-gene interactions (that is, susceptibility genes interacting with disease genes; Nagy et al, 1995). In addition, ethical considerations prevent the use of many invasive techniques in humans, including determination of gene expression in, for example, the hypothalamus.

The use of animal models in the study of human diseases confers considerable experimental advantages for the dissection of heterogeneous and polygenic traits such
as obesity and NIDDM (Friedman and Leibel 1992). Elucidation of genes causing
obesity in animal models is likely to provide considerable insight into the molecular
mechanisms regulating physiological processes such as energy balance, and the
discovery of leptin provides evidence to support the role of animal models in obesity
research. Even if these animal models do not directly lead us to the genes responsible
for human obesity and/or NIDDM, they could identify common pathways that are
important in nutrient partitioning and energy balance (Naggert and Nishina 1995).

Many studies on animal models of diabetes have been performed in mice or rats treated
with alloxan or streptozotocin, which destroy the pancreatic β-cells, leading to insulin-
deficient diabetes mellitus. These animals, along with animal models of autoimmune
diabetes (BB rat, NOD mouse), will not be considered in detail here.

1.7.1 SINGLE GENE MODELS OF OBESITY/NIDDM

All of the commonly utilised strains of single-gene mutant obese rodents arose
spontaneously from animals inbred for many generations, and have distinctive phenotypes
which are "all or none" in nature. That is, they are either definitively obese
(homozygous mutants) or lean and healthy (heterozygotes and wild-type), although
rodents heterozygous for several of the obesity genes display increased susceptibility to
high-fat diets and other metabolic stresses. These animals offer important experimental
opportunities to elucidate genes and pathways which control energy balance.

The ob/ob (obese) mouse, which we now know is deficient for leptin (Zhang et al.
1994), exhibits a phenotype consisting of severe, early-onset obesity with insulin
resistance, hyperglycemia and hyperinsulinemia which is strain- and background-
dependent (Bray and York 1979). For example, C57BL/Ks ob/ob mice develop severe
diabetes and insulinopenia, while C57BL/6J ob/ob mice develop only transient insulin
resistance that is compensated by b-cell hypertrophy (Friedman and Leibel 1992).
Therefore, the severity of the diabetic state in ob/ob mice depends on which strain are
carrying the mutation (Wilkinson 1996).

The obesity in ob/ob mice is evident by 4 weeks of age, and is due to both hyperphagia
and hypometabolism (Friedman and Leibel 1992). Ob/ob mice exhibit a myriad of
associated metabolic and hormonal abnormalities including defective thermogenesis
and impaired gonadotrophin secretion, resulting in infertility (Friedman and Leibel
1992). With the knowledge that these mice are completely deficient in leptin, it is
interesting to revisit earlier studies in these animals, and interpret the results in this new
context. For example, the earliest metabolic defect described in ob/ob mice is
enlargement of epididymal adipocytes, which occurred between 12 and 14 days of age, and preceded the development of hyperinsulinemia by at least a week (Joosten and Van Der Kroon 1974). These authors rightly concluded that hyperinsulinemia and/or insulin resistance cannot be the primary cause of adipocyte enlargement in ob/ob mice, indeed it is almost certainly due to leptin deficiency. Interestingly, ob/ob mice are insulin sensitive and have normal circulating glucose and insulin concentrations prior to weaning, and hyperinsulinemia only develops when the animals are about 4 weeks old, and is followed later by hyperglycemia (Genuith et al. 1971). These results suggest minimal effects of leptin deficiency prior to weaning, and that the post-weaning diet in conjunction with leptin deficiency precipitates the development of obesity, hyperinsulinemia and presumably insulin resistance. The precise nature by which leptin deficiency causes the metabolic profile observed in ob/ob mice is not yet known.

Db/db (diabetes) mice develop a phenotype which is metabolically indistinguishable from that of ob/ob mice (Hummel et al. 1966), including the strain-dependent severity of diabetics described above (Friedman and Leibel 1992). It is now known that the db mutation results in a leptin receptor defective in signal transduction (Chen et al. 1996; Lec et al. 1996). Therefore it would be expected that the ob/ob and db/db mice would be phenotypically similar, as one cannot make functional leptin, and the other is insensitive to it, as predicted by the parabiosis experiments of Coleman in the early 1970s (Coleman 1973; Coleman 1978).

The Zucker fa/fa (fatty) rat (Zucker and Zucker 1961) has a different mutation in the leptin receptor which affects the extracellular, ligand-binding domain, resulting in greatly reduced affinity for leptin (Chua et al. 1996b; Iida et al. 1996; Phillips et al. 1996; Takaya et al. 1996). The metabolic profile of the fa/fa rat has been well characterised and features obesity resulting from hyperphagia and decreased energy expenditure, hyperglycemia, hyperinsulinemia and progressive insulin resistance after weaning (Mathe 1995). In addition, fa/fa rats exhibit hyperlipidemia, hypertriglyceridemia, hypercholesterolemia, hypoglycagomemia (Mathe 1995), and they exhibit overexpression of lipogenic enzymes including lipoprotein lipase (Dugail et al. 1992), resulting in increased hepatic and adipose tissue lipogenesis (Langley and York 1992), and polydipsia (Bray and York 1979). Hyperphagia is not necessary for the development of obesity in these animals (Cleary et al. 1980), suggesting a defect in the regulation of energy expenditure, but is marked in ad libitum fed animals compared with controls (Bray and York 1972). The fa/fa rat is likely to be important in the testing of high-dose leptin therapy and the testing of leptin analogs, as it is hyperleptinemic and markedly leptin resistant, a situation which is likely to be similar to many cases of human obesity. It is interesting that the three animal models of obesity discussed above,
which have been the most extensively studied over the past decades, all have defects in the leptin pathway, providing support for the importance of leptin in the regulation of energy balance.

The JCR:LA cp/cp (corpulent) rat (Hansen 1983) is another animal model believed to have a mutation in the leptin receptor resulting in no mRNA expression for OBR (Campfield 1996), although details are yet to be published. Cp/cp rats develop obesity, hyperinsulinemia and severe lipid abnormalities resulting in accelerated atherosclerosis and premature death from myocardial infarction (Frankish et al. 1995). Insulin resistance is profound in males, but much less severe in females, yet female cp/cp rats are much more hyperlipidemic than their male counterparts (Russell et al. 1994).

The agouti gene product is a 131 amino-acid protein normally produced only in hair follicles. However, several mutations at this locus including lethal yellow (A\(^Y\)), sienna yellow (A\(^y\)) and viable yellow (A\(^v\)) result in not only yellow coats, but also obesity, insulin resistance and premature infertility in affected mice (Carpenter and Mayer 1958; Dickie 1969). Both hyperphagia and reduced energy expenditure are present, but not hypercorticosteronemia (Rubin and Barsh 1996). A\(^y\) mice also have heightened susceptibility to mammary, hepatic and bladder tumors, and they are the only obese mutant known to have increased linear (skeletal) growth (Friedman and Leibel 1992). A moderate hyperglycemia is found in male but not female agouti mutants (Klebig et al. 1995). Molecular studies revealed that the genetic mutation resulting in obesity causes ectopic overexpression of the agouti protein in almost all tissues of the body, and it is presumed that the agouti promoter is somehow subsumed by a heterologous promoter (Bultman et al. 1992; Miller et al. 1993; Duhl et al. 1994; Michaud et al. 1994; Klebig et al. 1995). Transgenic mice ectopically expressing the agouti protein have an identical pleiotropic phenotype to the known agouti mutant mice, confirming that this is the cause of obesity in these animals (Klebig et al. 1995). The physiological mechanism leading to obesity in these animals is thought to be due to antagonism of a MSH at its receptor(s) by the ectopically produced agouti protein (Lu et al. 1994; Fan et al. 1997). At least two melanocortin receptors are present in the hypothalamus and it has been suggested that antagonism of these receptors in particular results in hyperphagia and obesity in mutant animals (Lu et al. 1994). The elucidation of the mechanism causing obesity in agouti mice has revealed a whole new area of physiology relating to energy balance, and proves again the usefulness of animal models in the study of complex human diseases. Interestingly, the human homolog of the agouti gene (ASP) has been identified and is expressed in testis, ovary and heart in humans (Wilson et al. 1995). This implies that the agouti protein has a function in humans not affecting hair color, and suggests a possible neuroendocrine function via hypothalamic melanocortin
receptors. This area of physiology is sure to receive considerable attention in the near future (see also section 1.5.3).

The fat/fat mouse is characterised by slowly-developing obesity from puberty onwards which is preceded by hyperinsulinemia (Roberts and Greenberg 1996). The gene defect in these animals has recently been shown to be a point mutation (Ser202Pro) in the carboxypeptidase E (CPE) gene (Naggert et al. 1995). CPE is involved in the processing of prohormone intermediates, including proinsulin, and the fat mutation results in a virtual absence of CPE in pancreas, pituitary and brain of affected animals (Naggert et al. 1995). Deficient processing of proinsulin, resulting in a partial defectiveness of circulating insulin explains the apparent hyperinsulinemia but insulin sensitivity found in fat/fat mice (Roberts and Greenberg 1996), however not all of these mice are hyperglycemic, indicating that defective insulin processing is not the only mechanism of this phenotype (Keightley 1995). Indeed, it is likely that defective processing of one or more of the neuroendocrine prohormones, including POMC (the precursor for MSH), involved in the regulation of energy balance is the primary cause of obesity in fat/fat mice (Naggert and Nishima 1995).

The tub mutation arose spontaneously in a C57BL/6J mouse colony, and causes slowly developing obesity and hyperinsulinemia after weight gain, but not hyperglycemia or hypercorticosteronemia (Coleman and Eicher 1990). The tub gene was recently cloned, and the mutation in obese animals identified (Noben-Trauth et al. 1996). A G->T transversion in the mutant gene abolishes a splice donor site resulting in a larger transcript containing the unspliced intron which is overexpressed in the hypothalamus (Noben-Trauth et al. 1996; Kleyn et al. 1996). The function of the tub gene is not known, although it is expressed in testis, brain, eyes, large intestine and ovary, and is hydrophilic in nature, suggesting a cytosolic site of action (Noben-Trauth et al. 1996; Kleyn et al. 1996). A fuller understanding of the physiology of the tub gene product may provide yet another avenue to research the mechanisms contributing to human obesity, and further results are eagerly awaited.

1.7.2 INBRED POLYGENIC MODELS OF OBESITY/NIDDM

Polygenic animal models of obesity and NIDDM could 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 very nature of the genetic interactions in these animals makes it much more difficult to isolate specific aspects of the milieu which are important in the development of these diseases. Few truly polygenic rodent models of obesity actually exist in outbred colonies, and of
those, only one, *Psammonys obesus*, which will be discussed in more detail below, exhibits the full phenotypic range from lean to grossly obese, and glucose tolerant to frankly diabetic, similar to that found in human populations.

The KK mouse develops age- and diet-dependent obesity, insulin resistance and mild hyperglycemia, as well as hypertrophy and degranulation of pancreatic β-cells (Ikeda 1994). NIDDM develops after obesity in appropriate dietary conditions, and is associated with polyphagia, polyuria and increased gluconeogenesis (Ikeda 1994). The mild obesity found in KK mice was exacerbated by crossing with *Aβ* mice, generating KKAβ mice which develop more severe obesity and diabetes which is not as diet-dependent (Ikeda 1994). Further studies are required in these animals to characterise the physiological and metabolic factors which result in obesity.

The New Zealand Obese (NZO) mouse is another example of an inbred strain which is prone to gradual, maturity-onset weight gain, with a concomitant increase in circulating insulin, presumably due to insulin resistance (Naggert and Nishina 1995). Despite no difference in body weight until 3-4 months of age compared with control animals, NZO mice had altered body composition from day 1, with higher lipid content and lower body nitrogen (Crofford and Davis 1965; Proietto and Larkins 1992). In adults, NZO mice consisted of about 75% (dry weight) lipid, compared to 45% in controls. This fat was stored primarily in the abdomen, with relative sparing of the limbs and neck (Proietto and Larkins 1992). Hyperphagia was present in young NZO mice (during the rapid growth phase), however hypophagia was reported in adult NZO mice (stable weight) compared with random breed albino mice of similar length (Crofford and Davis 1965). Further studies on the biochemical and genetic abnormalities in NZO mice could yield important insights into the pathogenesis of obesity.

The Otsuka Long-Evans Tokushima Fatty rat (OLETF) is a relatively new inbred obese strain with a late onset of hyperglycemia in male animals only (Kawano et al. 1992). These animals are innately hyperphagic and slowly accumulate abdominal visceral fat compared with controls (Ishida et al. 1995), which is followed by hyperinsulinemia, hypertriglyceridemia and hyperglycemia in males (Sato et al. 1995). Further investigations are warranted in these animals, which may be especially important for the study of body fat distribution and its relationship to insulin resistance.

### 1.7.3 Induced Mutants

Chemical or electrolytic destruction of the hypothalamus has long been known to induce obesity in rodents, and provides strong evidence for the hypothalamus as an
important site for the regulation of energy balance. For example, mice treated with gold thioglucose, which specifically causes cell destruction in the hypothalamus, become hyperphagic, obese, hyperinsulinemic and hyperglycemic (Marshall et al. 1955), and have defective BAT thermogenesis (Cooney et al. 1987) and increased fatty acid synthesis compared with controls (Cooney et al. 1989). Adrenalectomy reduced body weight and circulating insulin and glucose levels in these animals, as well as significantly improving glucose tolerance (Blair et al. 1994). Other methods for creating hypothalamic lesions include injections of monosodium glutamate, and direct electrolytic destruction.

The application of molecular biology has had a significant impact on the generation of animal models of obesity and NIDDM. Techniques such as induced mutagenesis and the creation of transgenic mice give researchers the ability to manipulate the genome by changing gene expression levels, addition of novel genes or deletion of specific genes of interest. Examples include the NPY knockout mice discussed earlier (Erickson et al. 1996a,b), transgenic mice overexpressing phosphoenolpyruvate carboxykinase (PEPCK; Valera et al. 1994), and many others. These techniques allow researchers to assess the role of specific genes in the development of the obese and/or diabetic phenotypes (Naggert and Nishina 1995).

1.7.4 Obesity and NIDDM in Primates

The use of primates, such as Macaque (Howard and Yasuda 1990) or Rhesus monkeys (Hamilton et al. 1972), which are known to be prone to obesity and NIDDM when kept in a laboratory setting, has both advantages and disadvantages compared with the use of rodents. Obviously, monkeys are taxonomically very similar to humans, and any findings in monkeys could be applied to human physiology more easily than those from rodents. However, it should be remembered that the differences between rodent and human physiology are relatively minor in a broader context (Friedman and Leibel 1992), and there are some difficulties involved in studying primates. For example, monkeys are large, free roaming animals in the wild, so any changes found when caged could be significantly affected by this reduction in energy expenditure, very much more so than for rodents, which are burrowing animals with a small home range. But perhaps the greatest disadvantage of studying Rhesus monkeys is that, like humans, these animals exhibit age-dependent penetrance of the disease phenotype. In the case of the monkeys, obesity and NIDDM may not be evident for many years (at least 10 in most cases), greatly increasing the cost and difficulty of research (Hansen and Bodkin 1986). Between the ages of 10 and 25 years, a proportion of Rhesus monkeys held in captivity become obese, hyperglycemic and hyperinsulinemic (Bodkin and Hansen 1986; Bodkin...
et al. 1989), with hypertriglyceridemia and altered insulin receptor splicing noted in some diabetic animals (Bodkin et al. 1993; Huang et al. 1994; Huang et al. 1996). These animals are likely to represent a very good animal model of human obesity and NIDDM, but the inherent difficulties in confining such large animals, coupled with the extensive time periods involved in the development of obesity and NIDDM make Rhesus monkeys a difficult prospect for the study of these diseases.

All of the animal models of obesity and NIDDM discussed above have contributed to the study of these diseases, and will probably continue to provide insight into specific physiological pathways involved in the development of obesity. One rodent model of obesity and NIDDM which exhibits a complete spectrum of phenotypic responses when held in captivity, *Psammomys obesus*, was the focus of several studies in this dissertation, and will be discussed in detail below.

### 1.7.5 *PSAMMOMYS OBEUS*

*Psammomys obesus* (the Israeli Sand Rat or Fat Sand Rat) is a unique animal model of obesity and NIDDM. Several aspects of metabolism in *Psammomys obesus* closely resemble those found in human obesity and NIDDM, and many studies performed in this animal have confirmed its status as an important animal model of obesity and NIDDM. In particular, *Psammomys obesus* are representative of those human populations which develop high prevalence rates of obesity and NIDDM 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).

The common name of "Sand Rat" is inaccurate as these animals actually belong to the sub-family Gerbillinae. The precise taxonomy of the sub-family Gerbillinae is disputed, being variously included in the family Muridae (Nowak 1991) or Cricetidae (Harrison and Bates 1991). In either case, *Psammomys obesus* are relatively large rodents (mean adult body weight approximately 180 g) native to the Saharo-Arabian deserts from Algeria to the Sudan in North Africa, and extending eastwards to Arabia (Harrison and Bates 1991). These arid environments support mainly halophytic vegetation, and *Psammomys obesus* in Israel apparently feed entirely on *Atriplex halimus* (saltbush) from the family Chenopodiaceae.

*Atriplex halimus* is highly tolerant of arid conditions and is usually readily available throughout the year (Degen 1993). It has a high moisture content, but is low in energy yield and organic matter content, with a very high ash and electrolyte composition (Frenkel and Kraicer 1972; Degen 1988; Kam and Degen 1988). For these reasons
Psammomys obesus scrape the leaves of Atriplex halimus with their teeth to remove electrolytes (Degen 1988; Kam and Degen 1988) which are highly concentrated on the leaf surfaces (Kenagy 1973). This leaf-scraping behaviour has little effect on either the energy content or the organic matter of the leaves consumed, reducing each of these factors by only 3.1% (Degen 1993).

Psammomys obesus have a relatively low basal metabolic rate (BMR), only 57-60% of that expected for a rodent of similar size (Pinshow 1993; Degen 1993). The average daily metabolic rate of Psammomys obesus was 88% of that expected for a eutherian mammal of its body mass (Degen 1993). This low metabolic rate is considered to be an adaptation to reduce internal heat load in hot environments (McNab and Morrison 1963; Shkolnik and Schmidt-Nielsen 1976). In addition, the efficiency of energy utilisation of Atriplex halimus was calculated to be 0.3, while the heat increment of feeding was 0.7 (Degen 1993). The low utilisation of Atriplex halimus and its low energy yield and high water content forces Psammomys obesus to consume large quantities of this food source to maintain a neutral energy balance. It has been estimated that, in the field, Psammomys obesus consume 50-65% of their body mass in plant material daily (Pinshow 1993), and up to 68% under laboratory conditions (Kam and Degen 1988). The advantages of consuming this diet are that it is a stable food source throughout the year, no other rodents in the area consume significant amounts of Atriplex halimus, and since Psammomys obesus often locate their burrows at the base of these plants, they can expend minimal energy on foraging (Degen 1993).

Psammomys obesus are unique among desert gerbillids in that they are active above ground during the daylight hours, as all other species are strictly nocturnal (Ilan and Yom-Tov 1990). In addition, most other gerbillid species are granivorous and live on high-energy diets (Kenagy 1972; Daly and Daly 1973), whereas Psammomys obesus subsist on a low-energy, herbivorous diet.

No evidence of obesity, hyperglycemia or hyperinsulinemia have ever been recorded in Psammomys obesus freshly trapped from the wild (Shafrir and Gutman 1993). However, it is now well established that when fed ad libitum laboratory chow, a significant proportion of Psammomys obesus become obese and develop NIDDM. Initially, investigators at Duke University studied Psammomys obesus because of interest in their very high tolerance to electrolytes and the exceptional concentrating capacity of their kidneys (Schmidt-Nielsen 1964). It was noted during the establishment of this Psammomys obesus colony that a number of the animals became obese and developed NIDDM (including hyperglycemia, glycosuria, ketonuria and cataract formation) when fed regular laboratory chow, but not on a diet of fresh vegetables.
These investigators found that newly trapped *Psammomys obesus* had a mean body weight of $141 \pm 11$ g, laboratory animals fed solely on fresh vegetables weighed $148 \pm 9$ g, while those fed laboratory chow had body weight of $251 \pm 11$ g (Schmidt-Nielsen 1964). *Psammomys obesus* was then clearly an animal model of obesity in response to a diet of laboratory chow, on which other rodents (including other gerbils) remain lean and normoglycemic.

It was noted very early that a significant proportion of *Psammomys obesus* were unaffected by a diet of laboratory chow (Schmidt-Nielsen *et al.* 1964; Hackel *et al.* 1966), indicating that some animals may have had a genetic predisposition to the deleterious effects of a relatively high-energy diet. Other findings of early studies in *Psammomys obesus* included the fact that plasma insulin was increased early in the course of disease development with a subsequent drop in severely diabetic animals (Hackel *et al.* 1966; Miki *et al.* 1967). The fact that only some *Psammomys obesus* develop obesity and/or NIDDM in response to a diet of laboratory chow is indicative of the heterogeneous nature of the disease in this animal model, which is analogous to that found in human population studies (Barnett *et al.* 1994a; DeFronzo 1988).

**1.7.5.1 Dietary Studies in *Psammomys obesus***

Early studies showed that *Psammomys obesus* developed a range of metabolic abnormalities in response to a diet of normal laboratory chow, on which many other rodents remain lean and free from diabetes. Increases in body weight and various signs of diabetes were noted including hyperglycemia, glycosuria, cataracts and reduced glucose tolerance (Hackel *et al.* 1965; Hackel *et al.* 1966). In addition, the authors noted that a range of responses to the laboratory chow diet occurred in various animals. Some progressed rapidly to marked glucose intolerance, hypoinsulinemia and death, whereas others were more resistant and remained glucose intolerant and hyperinsulinemic, while still other *Psammomys obesus* were apparently unaffected by the laboratory chow diet (Hackel *et al.* 1966).

A proportion of *Psammomys obesus* became hyperphagic when fed a laboratory chow diet, compared to their food intake on a vegetable diet (Hackel *et al.* 1966). Studies in which energy intake was restricted have shown that the development of obesity and NIDDM can be significantly reduced in *Psammomys obesus*. In animals fed a vegetable diet of 30 calories per day (cal/d), no evidence of hyperinsulinemia or abnormal glucose tolerance was detected (Hackel *et al.* 1967). Similarly, animals fed 30 cal/d of laboratory chow also remained normoinsulinemic and glucose tolerant compared with those given *ad libitum* laboratory chow, which consumed an average of 49 cal/d and
were found to have increased body weight, hyperinsulinemia and impaired glucose tolerance (Hackel et al. 1967). In addition, a diet with a high component of wheat straw, which was relatively low calorie compared to laboratory chow, caused a reduction in body weight, body fat, blood glucose and plasma insulin concentrations, and increased reproductive success in *Psammomys obesus* (Adler et al. 1985). In a further study, animals were fed 3 diets based on pellets composed of different ratios of salt bush and standard laboratory chow. Significant correlations were found between the level of energy intake and measures of obesity and NIDDM including percentage body fat, blood glucose and plasma insulin concentration (Adler et al. 1986).

Other studies have shown that *Psammomys* fed an unrestricted diet of laboratory chow gain weight at a greater rate when compared to animals fed low energy dense diets of salt bush or green cabbage ad libitum (Haines et al. 1965, Schafer et al. 1980). In addition, circulating levels of free fatty acids and triglycerides have been reported to increase following a green cabbage diet and increase further following an unrestricted diet of laboratory chow when compared to animals that had just been captured and maintained on their natural diet (Kohler et al. 1980).

Studies in our laboratory have previously shown that restriction of energy intake to 67% of *ad libitum* consumption, which did not cause a significant reduction in body weight over a two-week period, reduced blood glucose and plasma insulin concentrations in normoglycemic, hyperinsulinemic *Psammomys obesus*, and reduced blood glucose but not plasma insulin in hyperglycemic, hyperinsulinemic animals. In addition, plasma cholesterol and triglyceride levels were significantly reduced in hyperinsulinemic animals, and glucose tolerance was improved by caloric restriction (Shafrir 1988; Barnett et al. 1994b).

Cumulatively, these studies indicate that the increase in energy intake in the transition from a diet of salt bush to that of laboratory chow is vitally important in the pathogenesis of obesity and NIDDM in these animals.

1.7.5.2 CHARACTERISATION OF *PSAMMOMYS OBESUS*

In our laboratory, we have previously shown that *Psammomys obesus* offered an *ad libitum* diet of standard laboratory chow display a continuous range of phenotypic responses. A significant proportion of the animals become hyperphagic and obese, with increased body fat content (Barnett et al. 1994a,b). However, approximately half of the animals, when offered the same diet and environmental conditions, do not develop hyperphagia and remain lean and healthy relative to their obese littermates. These
results indicate that a proportion of *Psammomys obesus* may have a genetic predisposition to overeat whenever food is available, in keeping with the thrifty gene hypothesis (Collier *et al.* 1997).

Other abnormalities noted in obese *Psammomys obesus* include hypertriglyceridemia and hypercholesterolemia relative to lean littermates, and reduced spontaneous physical activity when housed in a cage with an exercise wheel (Collier *et al.* 1997). Energy restriction studies in adult *Psammomys obesus* led to body weight reduction and significant improvements in hyperglycemia and hyperinsulinemia, confirming the importance of obesity in the metabolic abnormalities observed in these animals (Barnett *et al.* 1994b; Barnett *et al.* 1995).

The heterogeneous response of *Psammomys obesus* to a laboratory chow diet has led to a system of classification of the animals into four groups for experimental purposes. Cutoff points for blood glucose and plasma insulin concentrations define the groups, and are based on experimental data showing stages in the development of the disease process. The cutoff for blood glucose is 8 mmol/L, while for plasma insulin it is 150 μU/ml. The *Psammomys obesus* groups are as follows: Group A animals are normoglycemic and normoinsulinemic, Group B are normoglycemic and hyperinsulinemic, Group C are hyperglycemic and hyperinsulinemic, and Group D are hyperglycemic and normoinsulinemic (Kalderon *et al.* 1986; Barnett *et al.* 1994a).

Cross-sectional analyses of populations of *Psammomys obesus* have shown that the distribution of blood glucose and plasma insulin levels forms a continuous U-shaped curve, similar to that found in human populations (DeFronzo *et al.* 1988). This is very important, as it makes *Psammomys obesus* the only rodent model of obesity and NIDDM known which mimics the pattern found in human populations. In addition, longitudinal studies in *Psammomys obesus* have shown that individual animals tend to progress around the curve in a clockwise direction over time (Barnett *et al.* 1994a).

Studies investigating these groups of *Psammomys obesus* have shown that the both Group B and Group C *Psammomys obesus* were hyperphagic and obese compared with Group A animals (Barnett *et al.* 1995; Collier *et al.* 1997). Analysis of a large number of animals showed that the degree of hyperphagia and obesity was greater in Group C than in Group B animals, however both were significantly different to Group A *Psammomys obesus* (unpublished data). Some of the other metabolic defects observed in *Psammomys obesus* are detailed below.
1.7.5.3 METABOLIC ABNORMALITIES IN *PSAMMOMYS OBEUS*

Degeneration of pancreatic β-cells was noted in early studies of *Psammomys obesus* fed a diet of laboratory chow (Schmidt-Nielsen et al. 1964). Subsequent studies, using progressively more elegant technologies have shown β-cell degranulation, vacuolation and glycogen nephrosis (Hackel et al. 1965), and infiltration of fibroblasts, fibrocytes and lymphocytes (Pekov et al. 1983).

A large number of earlier studies compared *Psammomys obesus* with other species, most notably the albino rat. The physiological relevance of these studies to the factors resulting in obesity in Group B and C but not Group A *Psammomys obesus* despite identical access to food and environmental conditions is highly questionable. However, these studies do provide a background of the animal and a physiological framework from which to consider *Psammomys obesus*.

*In vitro* studies have shown a lower threshold for glucose-stimulated insulin release and an increased responsiveness of *Psammomys obesus* pancreatic β-cells to various external stimuli compared to other species (Ziegler et al. 1975; Chajek-Shaul et al. 1988). In addition, hepatocytes from *Psammomys obesus* were found to exhibit a low number of insulin receptors compared with albino rats (Mandarino 1984; Kanety et al. 1994). The lack of insulin binding to isolated hepatocytes, adipocytes and erythrocytes from obese, diabetic *Psammomys obesus* was proposed to be sufficient to explain entirely the insulin resistance found in this species (Mandarino 1984). In addition, hepatic enzymes involved in insulin degradation, such as thio-protein disulfide oxidase and insulin proteinase, have substantially lower activity in *Psammomys obesus* compared to albino rats (Ansorge et al. 1977). These data have led to the suggestion that the hyperinsulinemia in *Psammomys obesus* may arise in part due to oversecretion in response to energy-rich nutrition in combination with a low hepatic capacity to bind and degrade insulin (Shafir and Gutman 1993; Shafir 1996).

Group A (normoglycemic, normoinsulinemic) *Psammomys obesus* have a relatively high VLDL-TG uptake and lipoprotein lipase (LPL) activity in adipose tissue compared with albino rats (Gutman et al. 1990; Kalderon et al. 1986). The VLDL-TG uptake by adipose tissue of *Psammomys obesus* was 5-fold increased, and LPL activity 10-fold that of 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), and 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). Interestingly, in fasted Psammomys obesus a decreased rate of fat store utilisation has been observed in comparison to albino rats (Frenkel and Kraicer 1972).

Other metabolic observations in Group A Psammomys obesus include increased liver gluconeogenesis and phosphoenolpyruvate carboxykinase (PEPCK) activity, increased lipid synthesis and activity of NADP-malate dehydrogenase, and decreased pyruvate kinase activity compared with 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 concomitantly increased 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).

Fatty acid synthesis in Psammomys obesus occurs almost entirely in the liver in contrast to other rodents, but consonant with higher mammals (Kalderon et al. 1983). Psammomys obesus adipose tissue 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 labelled triglycerides in Psammomys obesus led to a slower rate of disappearance from plasma than that observed in albino rats, and an increased recovery in adipose tissue was found in Psammomys obesus (Gutman et al. 1990). These factors indicate a state of readiness in Psammomys obesus to store lipids whenever available.

Further studies found that hepatic pyruvate dehydrogenase, a key enzyme in glucose oxidation, had decreased activity in Psammomys obesus compared with albino rats (Nakai et al. 1997). In addition, the activity of hepatic 3-hydroxy-CoA dehydrogenase was 4-fold higher in Psammomys obesus compared with albino rats. These results suggest a low capacity for glucose oxidation, but a high capacity for fatty acid oxidation in the liver of Psammomys obesus (Nakai et al. 1997).

In addition to possible defects in insulin secretion in Psammomys obesus, several studies have investigated the role of the insulin receptor in this animals. Insulin receptors from hepatocytes of Group A Psammomys obesus had normal tyrosine kinase activity, but a higher $K_m$ for ATP in the phosphorylation of exogenous substrates. In contrast, hyperinsulinemic animals had a dramatic reduction in insulin receptor tyrosine kinase activity in hepatocytes which could be reversed after dietary manipulation to correct the hyperinsulinemia (Kanety et al. 1994), leading to the suggestion that hyperphagia may lead to the development of hyperinsulinemia in these animals by
inefficient extraction of excess insulin by the scarce liver receptors. Hyperinsulinemia, in turn, could then cause a reversible reduction in the activity of the receptor tyrosine kinase which could cause insulin resistance (Kanety et al. 1994).

A pronounced resistance to the actions of insulin has been documented in muscle and adipose tissue of *Psammomys obesus*, as indicated by low glucose transport and a low conversion of glucose to carbon dioxide, triglycerides and glycogen, even in the presence of added insulin (Schafer et al. 1977; Kohler and Knosp 1980). Hepatic insulin resistance has also been noted, as attested by an increased rate of gluconeogenesis (Shafrir and Guzman 1993; Ziv and Shafrir 1995).

As mentioned previously, the studies described above compared *Psammomys obesus* with other species, and cannot explain why some animals become hyperphagic and obese when given access to a diet of laboratory chow, yet others under the same conditions remain lean and healthy. To address this question, studies need to compare obese (Group B and C) animals with their lean (Group A) littermates.

One such study found that the development of hyperinsulinemia in *Psammomys obesus* (Group B) was associated with several metabolic changes including hyperphagia and obesity (Barnett et al. 1995; Collier et al. 1997). Uptake of glucose by muscle and adipose tissue was only about 40% of that found in Group A animals, indicating severe insulin resistance, which was compensated by a large increase in adipose tissue mass (Kalderon et al. 1986). The lack of hyperglycemia in Group B *Psammomys obesus* despite the reduction in peripheral glucose uptake was possibly attributable to a fall in hepatic glucose production in this group compared with Group A animals, as inferred from a reduced PEPCK activity (Kalderon et al. 1986).

Although the reduction in peripheral glucose utilisation in Group B *Psammomys obesus* may be regarded as resulting from insulin resistance in these animals, the activity of adipose tissue LPL (known to be insulin dependent; Garfinkel et al. 1976) was maximal in this group and the uptake of VLDL-TG was similar to that in Group A (Kalderon et al. 1986). The coexistence of elevated LPL activity and insulin resistance has also been noted in obese humans (Boscolo et al. 1984), and is recognised as part of the phenomenon known as 'selective insulin resistance' (Jeanrenaud 1994).

In Group C *Psammomys obesus*, the emergence of hyperglycemia in the face of hyperinsulinemia has been associated with a further decrease in peripheral glucose uptake and an increase in gluconeogenesis (determined by an increase in PEPCK activity and an increased conversion rate of alanine to glucose by isolated hepatocytes),
indicating a worsening of insulin resistance (Kalderon et al. 1986). The discrepancy noted above in the effects of hyperinsulinemia on adipose tissue was no longer observed, and glucose uptake, LPL activity and VLDL-TG uptake were all lower than in Group B animals (Kalderon et al. 1986). Despite these observed changes in adipose tissue metabolism, Group C Psammomys obesus had the highest levels of adipose tissue mass, and were clearly obese relative to Group A animals (Kalderon et al. 1986; Barnett et al. 1994a,b).

In contrast to the reduced VLDL-TG uptake in adipose tissue of Group C compared with Group B Psammomys obesus, the hepatic lipogenesis of Group C animals was markedly higher, as measured by the activities of the lipogenic enzymes acetyl-CoA carboxylase and NADP-malate dehydrogenase (Kalderon et al. 1986). It has been estimated that the increase in fat mass in Group C animals is equivalent to the decrease in VLDL-TG uptake per gram of adipose tissue in these animals, suggesting that the total VLDL-TG uptake by adipose tissue is similar in both Group A and Group C Psammomys obesus (Kalderon et al. 1986). The additional triglyceride synthesis by the liver would then be directed towards muscle, and to a lesser extent, retained in the liver. Because both glucose uptake and utilisation were decreased in Group C muscle (Kalderon et al. 1986), it is assumed that at least part of the increased uptake of VLDL-TG by muscle was utilised for energy production to overcome the insulin resistance present. Whether the elevated levels of lipogenesis and LPL activity in Group C animals is contributing significantly to the insulin resistance observed in these animals remains to be determined.

Hepatic glucose production was also shown to be elevated in Group C Psammomys obesus compared with Group A, while the metabolic clearance rate of labelled glucose tended to be lower in Group C animals (Habito et al. 1995). In addition, Group C animals had an increased glucose metabolic index (indicating increased glucose uptake) in adipose tissue, which may be due to the increased driving force of hyperglycemia forcing glucose into the cells by mass action (Habito et al. 1995). Interestingly, glucose uptake was increased in the soleus and gastrocnemius muscles of Psammomys obesus from Group B compared with group A despite no difference in glycaemia (Habito et al. 1995).

The role of increased fatty acid oxidation in the pathogenesis of obesity and NIDDM in Psammomys obesus was examined by administration of etomoxir, a fatty acid oxidation inhibitor. In Group C animals, etomoxir treatment for 5 days reduced plasma glucose and hepatic glucose production, as well as glucose uptake in several tissues (Barnett et al. 1996). In addition, etomoxir treatment reduced plasma insulin levels in
hyperinsulinemic animals (Barnett et al. 1996). These results imply that the elevated fatty acid oxidation levels found in *Psammomys obesus* may contribute significantly to the development of NIDDM in these animals.

Recent immunocytochemical studies suggest that the β-cells of Group C *Psammomys obesus* are in a chronic hypersecretory state during which impaired processing of proinsulin appears to take place (Bendayan et al. 1995). Similarly, findings in plasma and pancreas of marked increases in proinsulin levels have been associated with a 90% reduction in the storage capacity of Group C *Psammomys obesus* pancreata for insulin (Gadot et al. 1994). These abnormalities were partially corrected by fasting, which reduces the demand on insulin secretion. The authors concluded that Group C *Psammomys obesus* may have a limited pancreatic storage capacity for insulin, and that the metabolic consequences of this are exacerbated by an increased secretory demand secondary to peripheral insulin resistance. Thus the conditions are established for the development of hyperglycemia, which in turn may exacerbate the pancreatic defect (Gadot et al. 1994).

Collectively, the numerous metabolic studies in *Psammomys obesus* offered a diet of laboratory chow have unmasked various defects in both carbohydrate and lipid metabolism. The extent to which these metabolic anomalies contribute to the obese state and the development of NIDDM is unclear, but it appears that in a proportion of these animals, an underlying genetic predisposition to the development of obesity and NIDDM may be unmasked by a diet of *ad libitum* laboratory chow.

The data summarised above indicate that *Psammomys obesus* is a good polygenic model of obesity displaying a range of body weight and food intake in response to a diet of laboratory chow. In addition, obese *Psammomys obesus* display hypertriglyceridemia, hypercholesterolemia and reduced spontaneous physical activity compared with their lean littermates. At the time of writing, no studies had been published regarding the role of leptin in the pathogenesis of obesity in *Psammomys obesus*.
1.8 **AIMS**

Despite the wealth of information presented in this overview, several key areas remain poorly understood. At this time little is known regarding the role of leptin in polygenic animal models of obesity and/or NIDDM such as *Psammomys obesus*. A number of studies detailed in this dissertation have been undertaken to investigate the role of leptin in the pathogenesis of obesity and NIDDM in *Psammomys obesus*. In addition, an *ob* gene polymorphism was investigated in both Caucasians and Nauruans, a population with a high prevalence rate of obesity and NIDDM.

Given the possibility that leptin may be considered as a potential therapeutic agent for obesity, several studies were undertaken to investigate the effects of leptin treatment, in various doses and routes of administration, in both lean and obese *Psammomys obesus*. The following areas will be examined in detail and each will constitute a separate chapter of this study.

1. *Ob* (obese) gene expression in *Psammomys obesus*: The levels of *ob* gene expression will be measured in both lean and obese *Psammomys obesus*. In addition, the basic molecular biology of the *Psammomys obesus* *ob* gene will be investigated. Both *ob* gene expression and plasma leptin concentrations will be examined with respect to body weight, plasma insulin concentrations and other parameters.

2. *Ob* gene polymorphism association studies: A recently characterised polymorphism adjacent to the human *ob* gene will be examined in both a Caucasian population, and in Nauruans, a population previously shown to have very high prevalence rates of obesity and NIDDM. Associations of the various alleles of this polymorphism with aspects of obesity and NIDDM will be assessed.

3. The effects of energy restriction on the development of obesity and NIDDM in *Psammomys obesus*: The role of hyperphagia in the pathogenesis of obesity and NIDDM in *Psammomys obesus* will be investigated. In particular, the effects of the prevention of hyperphagia on body weight gain, plasma leptin concentrations and the development of NIDDM will be assessed.

4. The effects of short-term fasting on plasma leptin concentrations in *Psammomys obesus*: The effects of fasting for 24 hours on plasma leptin concentration and other biochemical parameters will be investigated in *Psammomys obesus*. 
5. **Leptin administration in *Psammomys obesus***: The effects of leptin administration to *Psammomys obesus* will be investigated, including acute (8 h) effects of intraperitoneal injection, effects of continuous infusion or daily intraperitoneal injections for a 2-week period, and effects of administration of supraphysiological doses of leptin for 7 days. The collective aim of these studies was to investigate as fully as possible any effects of leptin administration in *Psammomys obesus*.

6. **Effects of leptin on insulin binding in isolated rat adipocytes**: The aim of this study was to investigate the effects of exogenous leptin on insulin binding and uptake by isolated rat adipocytes.
CHAPTER 2: MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

2.1.1 PSAMMOMYS OBEUS

The Deakin University colony of *Psammomys obesus* was maintained using the San Poiley outbreeding method (Poiley 1960). This breeding technique is based on a systematic rotation of breeders that provides for maximum genetic variation in subsequent generations. The animals came originally 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 Zimmet (International Diabetes Institute, Caulfield, Australia). These animals were used to establish a breeding colony at Deakin University, Geelong. The major criterion for selection of breeding pairs was the absence of cataracts, which has been previously shown to predict reduced breeding potential (Adler et al. 1985).

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

Experimental animals were weaned at 4 weeks of age and, except for the energy restricted animals detailed in Chapter 5, were housed in groups of 2-3 and given free access to water and laboratory chow (Barastoc, Pakenham, Victoria, Australia). As discussed in detail previously (section 1.7.5), a significant proportion of *Psammomys obesus* develop varying degrees of obesity and glucose intolerance when fed *ad libitum* laboratory chow. In experiments where food and water intakes were measured, the animals were housed individually. All animals were maintained in a temperature controlled room (22 ± 1°C) with a 12-12-hour light-dark cycle (light 0600-1800; dark 1800-0600). Each cage was lined with sawdust and was cleaned out twice weekly. All animals were maintained in accordance with the Code of Practice of the National Health and Medical Research Council.
2.1.2 RATTUS NORVEGICUS

Male Sprague-Dawley rats were obtained from a colony maintained by the Deakin University Animal House. These animals were also maintained at 22 ± 1°C with a 12-12-hour light-dark cycle and had free access to chow and water. All procedures conformed 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). 5 µl of sample was added to 100 µl H₂O and 700 µl of a reagent solution containing phosphate buffer (100 mmol/l, pH 7.0), phenol (11 mmol/l), 4-aminophanazine (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 glucose oxidase, and the oxidation of phenol with the substrates hydrogen peroxide and 4-aminophenazine via phenol oxidase. This reaction produced 4-(p-henzoquinone-meno-imino) phenazone which has a maximal absorption at 540 nm. The amount of glucose in the plasma sample was therefore 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. Hence, a linear curve was developed using a calibration serum specifically designed for automated systems (glucose concentration 7.16 mmol/l; Boehringer Mannheim, Mannheim, Germany). External standards were incorporated into each run and measured every 40 samples (Boehringer Mannheim, Mannheim, Germany). The 2 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 Sidekick 1500 glucose analyser (Yellow Springs Instruments Co., 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, which was immobilised on a polycarbonate/cellulose membrane, converted D-glucose to gluconic acid, 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 implemented calibration standard.

2.2.3 **PLASMA INSULIN**

Concentrations of plasma insulin were determined using a commercially available radioimmunoassay (RIA) kit (Phadeseph, Pharmacia Diagnostics AB, St. Louis, USA), which utilised a double antibody solid phase technique where the insulin in the sample competed with $^{125}$I-labelled insulin for binding sites on highly specific antibodies. In the procedure 20 µl of plasma sample was added to 50 µl of $^{125}$I-labelled insulin (tracer) and 50 µl of guinea-pig anti-insulin antibody ('first antibody') and left to bind at room temperature for 2 hours. A 1 ml aliquot of sheep anti-guinea pig antibody ('second antibody') was added, which was highly specific for the 'first antibody', and served as a carrier. Binding was allowed to proceed at room temperature for 30 min, then the tubes were centrifuged at 3500 g for 20 min (Beckman Centrifuge, Model J6B, Beckman Instruments Aust., Gladesville, Australia) to separate the bound from the free insulin. Each tube was then aspirated, leaving the bound insulin in a pellet at 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 determined by comparing the competitive capacity of the plasma insulin with standards of known concentrations (0, 3, 10, 30, 100 and 240 µU/ml). 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 the linearity of the curve were maximal in the central region of the graph (approximately 10-100 µU/ml). 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, Anaheim, USA) were incorporated into each assay and comprised low (15.9 ± 0.4 µU/ml), medium (52.3 ± 3.0 µU/ml) and high (146.9 ± 4.0 µU/ml) quality controls.

2.2.4 **PLASMA TRIGLYCERIDES**

A commercially available enzymatic colorimetric method (Boehringer Mannheim, Mannheim, Germany) was used to determine plasma triglyceride concentrations on an automatic analyser (Hitachi model 705-0013, Tokyo, Japan). 5 µl of plasma sample was mixed with a reagent solution and incubated at 25°C for 10 min. During this incubation the triglycerides 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-chlorophenol were oxidised by hydrogen peroxide to form 4-(O-benzoquinone-mono-imino) phenazone, which has maximal absorbance at 540 nm. The amount of triglycerides in the plasma sample was therefore 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, while 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; Precipath U, pathological triglyceride concentration, 4.29 mmol/l, acceptable range 3.69-4.89 mmol/l).

2.2.5 PLASMA CHOLESTEROL

Plasma cholesterol concentrations were determined on an automatic analyser (Hitachi model 707-0013, Tokyo, Japan) using a commercially available enzymatic colorimetric method (Boehringer Mannheim, Mannheim, Germany). In a 5 µl aliquot of plasma, cholesterol esterase hydrolysed the cholesterol ester to yield free cholesterol which was subsequently oxidised by cholesterol oxidase to yield 8α-cholesterol 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 were measured every 40 samples (Precinorm U, normal physiological cholesterol concentration, 4.61 mmol/l, acceptable range 3.96-5.26 mmol/l; Precipath U, pathological cholesterol concentration, 8.81 mmol/l, acceptable range 7.57-10.10 mmol/l; Boehringer Mannheim, Mannheim, Germany).

2.2.6 PLASMA TESTOSTERONE

Plasma testosterone/dihydrotestosterone concentrations were measured using a commercially available RIA (Amersham, Buckinghamshire, England). As testosterone circulates in the blood bound to protein, the plasma samples (200 µl) were extracted twice with 3 ml diethyl ether to liberate the testosterone prior to the assay. For the assay, the extracts were added to 200 µl tritiated dihydrotestosterone solution and 200 µl antiserum, and incubated at room temperature for 1 hour. The tubes were cooled in an ice/water bath for 15 min, then 200 µl charcoal suspension was added and mixed
briefly, and the tubes returned to the ice/water bath for 10 min. The samples were centrifuged at 3500 g for 15 min at 4°C to sediment the charcoal, and 500 μl of the supernatant was added to 5 ml of liquid scintillant and counted using a beta-counter (Wallac, Turku, Finland). Standards were included for both testosterone (12.5, 25, 50, 100, 200 and 400 pg/ml) and dihydrotestosterone (25, 50, 100, 200, 400 and 800 pg/ml), allowing the construction of linear standard curves from which the concentrations in plasma samples were determined (Amersham, Buckinghamshire, England).

2.2.7 PLASMA LEPTIN

Plasma leptin levels were measured using a solid phase double enzyme immunoassay (EIA) with affinity purified polyvalent antibodies. Concentrations were calculated from standard curves generated with recombinant murine leptin. The limits of detection for the leptin EIA were found to be 20 pg/ml of serum or plasma. The inter-assay coefficient of variation was 7.7% for the high standard and 10.5% for the low standard.

2.3 SURGERY

Osmotic minipumps (Alzet model 2002, Alza, California, USA) were surgically implanted for studies involving continuous infusion of leptin in Psammomys obesus. The pumps used delivered a constant dose of 0.5 μl/h for 14 days. These pumps were 3.0 cm in length with a diameter of 0.7 cm, weighed 1.1 g and displaced a total of 1.0 ml. The outer membranes of the pumps was composed of cellulose ester blend and was designed to be inert. No evidence of inflammation was observed at the implantation site of any animals after a 2 week period.

For implantation of the pumps, the animals were anesthetised with halothane, then a 1 cm transverse incision was made over the interscapular region. The pumps were digitally inserted to lie subcutaneously above the scapulae and the wound immediately sutured closed. The duration of anaesthesia was only approximately 10 min for this procedure, and the animals were observed closely for at least the following hour. In general, the animals did not appear to act differently following the surgery, and most resumed eating and/or drinking within this time. No weight loss or any other indicators of stress were observed in the animals following the surgery.
2.4 MOLECULAR TECHNIQUES

2.4.1 MEASUREMENT OF OB GENE EXPRESSION

2.4.1.1 RNA EXTRACTION

Animals were killed by lethal overdose of barbiturates (pentobarbitone 120 mg/kg) and the required tissues removed immediately, weighed, and either snap frozen in liquid nitrogen or RNA extracted without delay. Tissues examined included liver, heart, spleen, kidney, pancreas, reproductive organs (testis or ovary), brain, small intestine, various fat depots including suprascapular, perirenal, intramuscular, epididymal (male animals only) and mesenteric, and various skeletal leg muscles including gastrocnemius, soleus, plantaris and extensor digitalis longus (EDL). For RNA extraction, 100 mg of tissue was homogenised in 2 ml RNAzol B (Bresatec, Adelaide, Australia). 200 µl of chloroform was added to the homogenate, vortexed briefly and incubated on ice for 5 min. The suspension was centrifuged at 12000 g (4°C) for 15 min and the aqueous (upper) phase 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 at 12000 g for 20 min at 4°C, and the supernatant decanted. The pellet was washed with 75% ethanol, then dried at room temperature before being resuspended in an appropriate volume of water containing 1% diethylpyrocarbonate (DEPC).

RNA was treated with 20U RNAse-free DNase I (Boehringer Mannheim, Mannheim, Germany) at 37°C for 3 h according to the manufacturer's instructions to remove chromosomal DNA contaminants. The RNA was then repurified by extraction with 200 µl chloroform and reprecipitated with an equal volume of isopropanol containing 0.2M NaCl, and resuspended in 20 µl of DEPC water.

The absorption of a 200-fold dilution of the RNA was measured at 260 nm using a spectrophotometer (Model DU640B UV/VIS-Spectrophotometer, Beckman Instruments Aust., Sydney, Australia). The RNA was then quantitated using the following equation:

\[ \text{Concentration (µg/ml)} = (\text{Abs}_{260} \times \text{dilution factor} \times \text{constant}) \]

where \( \text{Abs}_{260} \) was the absorbance measured above, the dilution factor was 200, and the constant used for quantitation of RNA was 40. For example, an absorbance reading of 0.02 units corresponded to an RNA concentration of 0.02 x 200 x 40 = 160 µg/ml.
2.4.1.2  **cDNA SYNTHESIS**

After the RNA was quantitated, 1 µg of RNA was then reverse transcribed to generate cDNA using the enzyme AMV Reverse Transcriptase (Boehringer Mannheim, Mannheim, Germany). Briefly, the RNA was added to a mixture containing 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM MgCl₂, 0.5 mM spermidine and 10 mM dithiothreitol (Boehringer Mannheim, Mannheim, Germany). After the addition of 10 U AMV Reverse Transcriptase (Boehringer Mannheim, Mannheim, Germany), the mixture was incubated at 42°C for 1 h before the reaction was terminated by incubation at 99°C for 10 min. The cDNA generated was stored at -70°C.

2.4.1.3  **POLYMERASE CHAIN REACTION (PCR)**

Oligonucleotide primers for the *ob* gene PCR were chosen from the published sequence (Zhang et al. 1994). Primers were also selected for the β-actin gene by comparing mRNA sequences from various mammals to identify highly conserved regions. The primer sequences used were:

- **β-actin** - forward 5'-ggc tac agc ttc acc acc ac-3'
  reverse 5'-get tgc tga tcc aca tct gc-3'
- **ob** - forward 5'-cac tgg ctt gga ctt cat tc-3'
  reverse 5'-ctg cta gta gag tga ggc tc-3'

PCR was performed by adding 300 ng of cDNA to a reaction mix containing 10 mM Tris-HCl (pH 8.3 at 20°C), 2 mM MgCl₂, 50 mM KCl, 200 µM each dNTP, 100 pmol each primer and 1.25 U Taq DNA Polymerase (Boehringer Mannheim, Mannheim, Germany). Standard PCR consisted of 30 cycles of 94°C for 1 min (denaturation), 58°C (β-actin) or 56°C (*ob*) for 1 min (annealing) and 72°C for 1 min (extension), with a final extension step of 72°C for 5 min. PCR's were 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).

2.4.1.4  **ELECTROPHORESIS**

10 µl of each PCR product was fractionated by agarose gel electrophoresis in a 1% gel containing 0.5 µg/ml ethidium bromide at 6 V/cm for 90 min in a DNA SubCell system (Bio-Rad, Anaheim, USA). The gels were then photographed using a Polaroid D534 camera with a DS-17 Hood on Polaroid 665 positive/negative film (Sigma Chemical Co., St. Louis, USA) under ultraviolet transillumination (model 4000, Stratagene,
LaJolla, USA) at 302 nm. An example of a gel showing PCR products for both β-actin and the ob gene is shown in Figure 2.1.

\[\text{FIGURE 2.1: Electrophoresis of PCR products for both β-actin and the ob gene of Psammomys obesus. Lane 1-perirenal fat, 2-epididymal fat, 3-interscapular fat, 4-intramuscular fat, 5-heart, 6-liver, 7-spleen, 8-pancreas, 9-kidney, 10-lung, 11-small intestine, 12-testis, 13-skeletal muscle, 14-negative control.}\]

2.4.1.5 DNA SEQUENCING

For sequencing of the Psammomys obesus ob gene, PCR products were excised from the gel and purified using Wizard PCR Preps (Promega, Madison, USA) and their sequence determined by fluorescent DyeDeoxy Terminator chemistry (Perkin Elmer, Norwalk, USA) using an Applied Biosystems 373A automated DNA sequencer (Perkin Elmer, Norwalk, USA). Sequencing was performed on both strands of the PCR product for confirmation. The Psammomys obesus ob gene sequence was aligned with that of other species using the Blast program on the Genbank database.

2.4.1.6 SEMI QUANTITATIVE RT-PCR

To enable semi-quantitation of RT-PCRs, the linear phases of both the ob and β-actin PCRs had to be determined. This was achieved by setting up 10 duplicate PCRs for both the ob gene and β-actin as described above, and consecutively removing tubes after each group of 4 cycles. After removal the tubes were cooled immediately in an ice-water bath, then stored at 4°C before agarose gel electrophoresis as described above.

The PCR products were quantitated using computerised densitometry (Eagle Eye II System, Stratagene, LaJolla, USA). Basically, this system utilised a CCD still video camera which was focussed on a transilluminator contained in a built-in benchtop darkroom. The image captured was saved and integrated into a computer system featuring Eagle Sight™ Windows densitometry software, which gave numerical data for each point analysed. This data was expressed graphically using Cricket Graph III™
software (Figure 2.2), and the linear phase of each PCR determined visually to be 20 cycles for the \( ob \) gene PCR and 24 cycles for the \( \beta \)-actin PCR.

**OB GENE**

**BETA ACTIN**

![Graphs showing density against number of cycles for OB GENE and BETA ACTIN](image)

**FIGURE 2.2:** Determination of linear phases for \( ob \) gene and \( \beta \)-actin PCRs.

To quantitate the expression of the \( ob \) gene, PCRs for both the \( \beta \)-actin and the \( ob \) gene were conducted on the same cDNA samples. The conditions were as described in section 2.4.1.3 except that the \( ob \) gene PCR was performed for 20 cycles, and the \( \beta \)-actin PCR for 24 cycles. After electrophoresis (as described in Section 2.4.1.4), the PCR products were quantitated using the Eagle Eye II system as described above. \( Ob \) gene expression was determined as the ratio of densities of \( ob \) to \( \beta \)-actin PCR products from the same tissues.

2.4.2 **OB GENE POLYMORPHISM**

2.4.2.1 **SUBJECTS**

The Australian cohort consisted of 373 women aged 20-91 years and were drawn from a larger, ongoing population-based study initiated in 1994, the Geelong Osteoporosis Study. All subjects were randomly selected using an Australian Electoral Commission roll and were healthy. Of the 373 women, 188 were post-menopausal while 185 were pre-menopausal. Fourteen women had a fasting glucose greater than 7.8 mmol/L, and were classified as having NIDDM. Fifty-four women were currently taking the contraceptive pill.
The other subjects studied in these experiments were from Nauru, 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 being eligible (Zimmet et al. 1977). Subjects for the current study (n=342) were men aged over 24 years, who had high prevalence rates of obesity (BMI>30 kg/m²; 82.7%) and NIDDM (15.2%; WHO Study Group 1994). The Nauruan population have previously been shown to have very high prevalence rates of obesity and NIDDM 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 found age-standardised prevalence rates of NIDDM (based on WHO criteria) of 28% in 1975-6, 25% in 1982 and 24% in 1987 (Zimmet et al. 1977; Zimmet et al. 1984; Dowse et al. 1991). The mean BMI of the population was 33.3 kg/m² in 1975-6, 33.8 kg/m² in 1982 and 34.5 kg/m² in 1987, suggesting very high prevalence rates of obesity in this population (Zimmet et al. 1977; Zimmet et al. 1984; Dowse et al. 1991). The Nauruan population is thought to be representative of the 'thrifty genotype' hypothesis (Neel 1962; Dowse et al. 1991).

2.4.2.2 DNA EXTRACTION

All subjects were required to fast overnight (from 10pm the previous evening) and blood specimens were collected in heparinised tubes the next morning. 1 ml of whole blood was used for DNA extraction, while the remainder was centrifuged and the plasma was stored at -70°C for later biochemical analyses. Height, weight, waist circumference and hip circumference were measured and used to calculate BMI and WHR. Systolic and diastolic blood pressure were measured with an automated digital analyser with the subjects sitting.

DNA was extracted from all of the 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 the genomic DNA by isopropanol precipitation. The DNA was resuspended in the supplied DNA Rehydration Solution and then 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.

2.4.2.3 PCR

Primers used were those published by Shintani et al. (1996), and comprised of the forward primer (HOBF), 5'-agt tea at ga ggt cca aat ca-3', and the reverse primer (HOBRI), 5'-ttc tga ggt tgt gtc act ggc a-3'. PCR's were performed by adding 100 ng of each DNA to a reaction mix containing 10 mM Tris-HCl (pH 8.3 at 20°C), 2 mM MgCl₂, 50 mM KCl, 200 μM each dNTP, 100 pmol each primer and 1.25 U Taq DNA Polymerase (Boehringer Mannheim, Mannheim, Germany). After an initial denaturation step of 94°C for 3 min, the tubes were subjected to 35 cycles of 94°C for 30 sec (denaturation), 60°C for 1 min (annealing) and 72°C for 1 min (extension), with a final extension step of 72°C for 10 min. PCR's were 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).

2.4.2.4 ELECTROPHORESIS AND ANALYSIS

The PCR products (10 μl) were separated by agarose gel electrophoresis in a 3% gel containing 0.5 μg/ml ethidium bromide at 6 V/cm for 120 min 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 Co., St. Louis, USA) under ultraviolet transillumination (model 4000, Stratagene, LaJolla, USA) at 302 nm. The size of the PCR products was determined visually to be either 121-145 base pairs in length (short form), or 197-221 base pairs (long form). Individual subjects were therefore placed into 1 of 3 groups: a) homozygotes for the short form, b) heterozygotes, or c) homozygotes for the long form. Examples of PCR products for these three groups are illustrated in Figure 2.3.
**FIGURE 2.3:** Human 

ob gene polymorphism. (Lane 1-molecular weight marker; lanes 2, 4 and 6-homozygous for the long form; lanes 3, 8, 9 and 12-heterozygotes; lanes 5, 7, 10 and 11-homozygous for the short form).

### 2.4.3 β3-ADRENERGIC RECEPTOR POLYMORPHISM

#### 2.4.3.1 PCR

Primers used were those published by Widen *et al.* (1995), and were composed as follows:

- Forward primer 5'-cgc cca ata ccg cca aca c-3'
- Reverse primer 5'-cca cca gga gtc cca te-3'.

PCR's were performed by adding 100 ng of each DNA to a reaction mix containing 10 mM Tris-HCl (pH 8.3 at 20°C), 1.5 mM MgCl₂, 50 mM KCl, 200 μM each dNTP, 100 pmol each primer and 1.25 U Taq DNA Polymerase (Boehringer Mannheim, Mannheim, Germany). After an initial denaturation step of 94°C for 3 min, the tubes were subjected to 35 cycles of 94°C for 30 sec (denaturation), 61°C for 1 min (annealing) and 72°C for 1 min (extension), with a final extension step of 72°C for 10 min. PCR's were 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).

#### 2.4.3.2 RESTRICTION ENZYME DIGESTION

The PCR products were digested by the addition of 10 μl of a mixture containing 30 mM Tris-Cl (pH 7.9), 30 mM MgCl₂, 150 mM NaCl and 10 units of *Bst*O1 (Promega, Madison, USA), a restriction enzyme which specifically cleaves DNA at the sequence CC(A/T)GG. This mixture was incubated for 2 hours at 60°C to enable complete digestion.
2.4.3.3 ELECTROPHORESIS AND ANALYSIS

The restriction digested PCR products were separated by agarose gel electrophoresis in a 3% gel containing 0.5 μg/ml ethidium bromide at 6 V/cm for 120 min 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 Co., St. Louis, USA) under ultraviolet transillumination (model 4000, Stratagene, LaJolla, USA) at 302 nm. The genotypes were determined visually as follows. DNA from individuals homozygous for the wild type (Trp64) yielded fragments of 99, 62, 30, 12 and 7 base pairs; homozygotes for the Trp64Arg mutation generated fragments of 161, 30, 12 and 7 base pairs; while heterozygotes yielded fragments of 161, 99, 62, 30, 12 and 7 base pairs (Widen et al. 1995). The smallest fragments (30, 12 and 7 base pairs) could not be resolved on the gel, so genotyping was based on the 161, 99 and 62 base pair fragments (see Figure 2.4).

![Image of gel electrophoresis](image)

**FIGURE 2.4:** Human β3-AR polymorphism. (Lanes 1, 3-8-homozygous wild-type (Trp64/Trp64); lane 2-heterozygote (Trp64/Arg64); lane 8-molecular weight marker)

2.5 INSULIN BINDING

2.5.1 ISOLATED ADIPOCYTE PREPARATION

Adipocytes were isolated from epididymal fat pads according to the method of Vinten et al. (1976). Briefly, the adipocytes were isolated by incubation in Krebs Ringer HEPES buffer (NaCl 154 mM, KCl 6 mM, CaCl₂ 2.5 mM, H₂PO₄ 6 mM, MgSO₄·7H₂O 2.5 mM, NaHCO₃ 5 mM, HEPES (4-(-2-hydroxyethyl)-1piperazinedithane-sulfonic acid) 25 mM, 1% BSA; pH 7.4) containing 0.5% (w/vol) collagenase (Worthington Type V, Seimar, Melbourne, Australia) for 45 min at 37°C in a shaking waterbath (100 orbitals/min). The cells were strained and washed 5 times with Krebs
Ringer HEPES buffer to remove any residual collagenase. Cells were quantitated using a hematocytometer in a phase-contrast microscope, and adjusted to a concentration of $4 \times 10^6$ cells/ml.

2.5.2  **INSULIN BINDING ASSAY**

The insulin binding assay was performed as previously published (Vinten *et al.* 1976). Briefly, $2 \times 10^6$ adipocytes were incubated in Krebs Ringer HEPES buffer (5% BSA; pH 7.4) containing (A14)$^{125}$I-labelled insulin (final concentration 40 pM; Amersham, Buckinghamshire, England) and varying concentrations of unlabelled insulin (0, 0.4, 2, 8 nM, 2 µM; Actrapid HM human monocomponent insulin, Novo Nordisk, Copenhagen, Denmark) in a total volume of 1 ml for 45 min at room temperature. Incubations were terminated by the oil flotation method (Vinten *et al.* 1976), and incorporation of radiolabelled insulin by adipocytes measured using a gamma counter (Multigamma Counter, model LKB Wallac 1261, Turku, Finland). The standard insulin binding assay results are shown below (Figure 2.5).

![Graph showing specific insulin binding](image)

**FIGURE 2.5:** Standard insulin binding in isolated rat adipocytes.
2.6 STATISTICAL ANALYSES

All statistical comparisons were made using commercially available statistical software (Minitab Version 8.1, Minitab Inc. Enterprises, State College, USA). Where data were not normally distributed appropriate transformations were performed, as detailed in each chapter. Statistical comparisons between two groups of data were determined using a paired Student t-test for dependent data, and a two-sample unpaired Student t-test for independent data. For the analysis of one or more groups of data, statistical differences were assessed using a one-way analysis of variance (ANOVA). Two modifications of ANOVA were utilised depending on the type of comparisons being made. Where only one control group existed, significant differences were determined using the Dunnet's multiple range test. In this instance, the number of statistical comparisons conducted was equal to n-1, where n represents the total number of groups being compared. When all groups were compared on an equal basis, significant differences were established using Tukey's test. In this instance, the number of statistical comparisons conducted was equal to (n-1)!, where n represents the total number of groups being compared. In all instances, statistically significant differences were accepted for p<0.05. All experimental data have been expressed as mean ± SEM.
CHAPTER 3:

**OB (OBESE) GENE EXPRESSION AND LEPTIN LEVELS IN **

**PSAMMOMYS OBESUS.**

3.1 **SUMMARY**

In this study we investigated *ob* gene expression and plasma leptin levels in *Psammomys obesus* (the Israeli Sand Rat), a polygenic animal model of obesity and non-insulin dependent diabetes mellitus. The *ob* gene was expressed exclusively in adipocytes of *Psammomys obesus*. DNA sequencing revealed a high degree of homology with other species (90% with mouse, 88% with rat and 79% with human). No *ob* gene sequence differences were found between lean and obese *Psammomys obesus*, and the codon 105 mutation found in *ob/ob* mice was not detected.

*Ob* gene expression in *Psammomys obesus* correlated with body weight (*r*=0.436, *p*<0.001), percent body fat (*r*=0.645, *p*<0.001) and plasma insulin concentration (*r*=0.651, *p*<0.001). This is the first time that *ob* gene expression has been shown to increase steadily over a continuous wide range of body weight or plasma insulin in an animal model of obesity. *Ob* gene expression was significantly elevated in obese compared with lean *Psammomys obesus* (*p*<0.05). No significant difference in *ob* gene expression was found between the 4 adipose tissue depots tested.

*Psammomys obesus* plasma leptin levels correlated with body weight (*r*=0.36, *p*<0.05), percent body fat (*r*=0.702, *p*<0.01) and plasma insulin concentration (*r*=0.735, *p*<0.001). Plasma leptin concentrations were significantly increased in insulin-resistant animals independent of body weight, suggesting a significant physiological relationship between leptin and insulin in these animals.

These results show that *Psammomys obesus* is an excellent animal model in which to study the *ob* gene and leptin, and confirm the importance of insulin as a significant factor in the regulation of leptin and *ob* gene expression.
3.2 INTRODUCTION

An *ob* (obese) gene mutation was recently shown to be the cause of morbid obesity in the *ob/ob* mouse (Zhang et al. 1994). Subsequent studies have demonstrated *ob* gene homologues in humans and rats (Zhang et al. 1994; Considine et al. 1995; Funahashi et al. 1995). The *ob* gene encodes a protein secreted from adipose tissue which is thought to act in a hormone-like manner through receptors in the hypothalamus, and its potential regulatory action on other leptin-sensitive neuromodulators, such as neuropeptide Y (Zhang et al. 1994; Considine et al. 1995; Maffei et al. 1995a). In the *ob/ob* mouse the protein product of this gene is truncated and dysfunctional resulting in a phenotype characterised by massive obesity, hyperphagia, hypometabolism, hypothermia, diabetes and infertility (Zhang et al. 1994). Several independent groups have shown that daily injections of the 16-kilodalton OB protein (known as leptin) in a range of dosages from 0.1 to 10 mg/kg/day rapidly reduced food intake and body weight in obese *ob/ob* mice in a dose-dependent and time-dependent manner, as well as increasing energy expenditure (Campfield et al. 1995; Halaas et al. 1995; Pelleyeumouter et al. 1995).

Several animal models of obesity have increased *ob* gene expression in adipose tissue. These include single-gene models such as *ob/ob* and *db/db* mice (Zhang et al. 1994; Maffei et al. 1995a), *fa/fa* and JCR:LA-cp rats (Maffei et al. 1995a; Ogawa et al. 1995). *Ob* gene expression is also increased in obese humans (Considine et al. 1995; Hamilton et al. 1995; Lonnqvist et al. 1995).

Various factors increase *ob* gene expression in animal models including hypothalamic lesions in mice (Frederich et al. 1995a; Maffei et al. 1995a) and rats (Funahashi et al. 1995), high-fat diets (Maffei et al. 1995b; Masuzaki et al. 1995a) and infusions of insulin (Cusin et al. 1995) or glucocorticoids (DeVos et al. 1995; Murakami et al. 1995). Fasting (Cusin et al. 1995; Moinat et al. 1995), streptozotocin treatment (MacDougald et al. 1995; Sivitz et al. 1996) and β2-adrenergic receptor agonists (Moinat et al. 1995; Mantzoros et al. 1996) have all been shown to suppress *ob* gene expression.

*Psammomys obesus* (Israeli Sand Rat) is a unique animal model of obesity and NIDDM. Its natural habitat is the desert regions of the Middle East, where it subsists on a diet composed almost entirely of saltbush, a very low energy food source (Shafir and Gutman 1993; Ziv and Shafir 1995; Shafir 1996). In this setting *Psammomys obesus* remain lean and normoglycemic. However, when taken into the laboratory and fed *ad libitum* chow, a relatively energy dense food, the animals exhibited a range of
pathophysiological responses. Approximately half of the animals became obese, whilst about one third developed NIDDM. Other phenotypes found include hyperglycemia, hyperinsulinemia, dyslipidemia and impaired glucose tolerance (Barnett et al. 1994a,b; Barnett et al. 1995). *Psammomys obesus* exhibited a range of bodyweight, and blood glucose and insulin concentrations which formed a continuous curve, and closely resembled the pattern found in human populations, that is, the inverted U-shaped relationship known as “Starling’s curve of the pancreas” (DeFronzo 1988; Barnett et al. 1994a). It is the heterogeneity of the phenotypic response of *Psammomys obesus* which make it a unique model to study the etiology and pathophysiology of obesity and NIDDM.

The aim of this study was to investigate *ob* gene expression and plasma leptin levels in *Psammomys obesus*, a polygenic model of obesity and NIDDM.
3.3 MATERIALS AND METHODS

3.3.1 EXPERIMENTAL ANIMALS

A *Psammomys obesus* colony is maintained at Deakin University, with the breeding pairs fed *ad libitum* a diet of lucerne and chow. Experimental animals were weaned at four weeks of age and given a diet of standard laboratory chow from which 12% of energy was derived from fat, 63% from carbohydrate and 25% from protein (Barastoc, Pakenham, Australia). Animals were housed in a temperature controlled room (22 ± 1°C) with a 12-12-hour light-dark cycle (light 0600-1800; dark 1800-0600).

*Ob* gene expression was measured in 31 animals with a wide range of body weight, percent body fat, blood glucose and plasma insulin levels.

For plasma leptin studies, a total of 69 animals from all groups were separated into two groups matched for age, sex, body weight and blood glucose: Group 1 - insulin-sensitive (plasma insulin < 150 μU/ml; n=33) and Group 2 - insulin-resistant (plasma insulin > 150 μU/ml; n=36).

3.3.2 RNA PREPARATION AND cDNA SYNTHESIS

Animals were killed by lethal overdose of pentobarbitone (120 mg/kg) and the following tissues were removed: liver, spleen, kidney, heart, skeletal muscle (gastrocnemius), and adipose tissue from the suprascapular, perirenal, intramuscular and mesenteric fat depots. After homogenisation, RNA was purified from 100 mg of each tissue using RNAzol B (Brestatec, Adelaide, Australia). RNA was treated with 20U RNAse-free DNase I (Boehringer Mannheim, Mannheim, Germany) at 37°C for 3 h according to the manufacturer's instructions to remove chromosomal DNA contaminants. The RNA was repurified by extraction with 200 μl of chloroform and precipitated with an equal volume of isopropanol. RNA was quantitated by spectrophotometry at 260 nm, and 1 μg of RNA was then reverse transcribed at 42°C for 1 h with 10U of AMV Reverse Transcriptase (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions.

3.3.3 POLYMERASE CHAIN REACTION (PCR)

Oligonucleotide primers for the *ob* gene PCR were chosen from the published sequence (Zhang *et al.* 1994). Primers were also selected for the β-actin gene by comparing
mRNA sequences from various mammals to identify highly conserved regions. The primer sequences used were:

\[
\begin{align*}
\beta\text{-actin} & \quad \text{forward } 5'\text{-ggctacaggtcaccacacca}-3' \\
& \quad \text{reverse } 5'\text{-gcttgctgatccacatctg}-3' \\
ob & \quad \text{forward } 5'\text{-cacttgctggaactcactc}-3' \\
& \quad \text{reverse } 5'\text{-tgctcgagtagtagtgaggtc}-3'
\end{align*}
\]

PCR was performed by adding 300 ng of cDNA to a reaction mix containing 10 mM Tris-HCl (pH 8.3 at 20°C), 2 mM MgCl₂, 50 mM KCl, 200 μM each dNTP, 100 pmol each primer and 1.25 U Taq DNA Polymerase (Boehringer Mannheim, Mannheim, Germany). Standard PCR consisted of 30 cycles of 94°C for 1 min (denaturation), 58°C (β-actin) or 56°C (ob) for 1 min (annealing) and 72°C for 1 min (extension), with a final extension step of 72°C for 5 min. 10 μl of each PCR product was fractionated by agarose gel electrophoresis in a 1% gel containing 0.5 μg/ml ethidium bromide at 6 V/cm for 90 min and photographed under ultraviolet transillumination at 302 nm.

3.3.4 DNA SEQUENCING

PCR products were excised from the gel and purified using Wizard PCR Preps (Promega, Madison, USA) and their sequence determined by fluorescent DyeDeoxy Terminator chemistry (Perkin Elmer, Norwalk, USA) using an Applied Biosystems 373A automated DNA sequencer (Perkin Elmer, Norwalk, USA). Sequencing was performed on both strands of the PCR product for confirmation. The \textit{Psammomys obesus} ob gene sequence was aligned using the Blast program on the Genbank database.

3.3.5 SEMI-QUANTITATIVE RT-PCR

For quantitation of ob gene expression, the linear phases of both of the above PCR's were determined empirically as 20 cycles for β-actin and 24 cycles for ob. PCR's and electrophoresis were conducted as above (for the appropriate number of cycles) and gene expression quantitated by computerised densitometry (Eagle Eye II System, Stratagene, LaJolla, USA). Ob gene expression was determined as the ratio of densities of ob to beta-actin PCR products from the same tissues.
3.3.6 **BIOCHEMICAL ANALYSES**

Whole blood glucose was measured using an enzymatic glucose analyser (Model 27, Yellow Springs Instruments, Ohio). Plasma insulin concentrations were determined using a double antibody solid phase radioimmunoassay (Phadeseb, Kabi Pharmacia Diagnostics, Sweden).

Plasma leptin levels were measured in collaboration with Amgen Inc. (Thousand Oaks, USA) in a solid phase double enzyme immunoassay (EIA) with affinity purified polyvalent antibodies. Concentrations were calculated from standard curves generated with recombinant murine leptin. The limits of detection for the leptin EIA were 20 pg/ml of serum or plasma. The inter-assay CV was 7.7% for the high standard and 10.5% for the low standard.

3.3.7 **STATISTICAL ANALYSES**

Where data were not normally distributed, normalisation was achieved using log transformation. All data are expressed as mean ± s.e.m. Data were analysed using unpaired Student's T-tests, results were considered significant at p<0.05.
3.4 RESULTS

3.4.1 TISSULAR OB GENE EXPRESSION

As shown in Figure 3.1, the ob gene was expressed exclusively in adipose tissues of *Psammomys obesus*. β-actin gene expression was detected in all tissues examined, showing that the cDNA used in all ob gene PCRs was valid.

![Image of gel showing expression of β-actin and ob gene](image)

**FIGURE 3.1:** Adipose tissue specific expression of the ob gene in *Psammomys obesus*. β-actin was expressed in all tissues examined. Lane 1-perirenal fat, 2-epididymal fat, 3-intersecapular fat, 4-intramuscular fat, 5-heart, 6-liver, 7-spleen, 8-pancreas, 9-kidney, 10-lung, 11-small intestine, 12-testis, 13-skeletal muscle, 14-negative control.

3.4.2 OB GENE SEQUENCE

DNA sequencing of the ob gene RT-PCR product revealed the nucleotide sequence shown in Figure 3.2. The *Psammomys obesus* ob gene sequence was aligned with the mouse (*Mus musculus*), rat (*Rattus norvegicus*) and human (*Homo sapiens*) ob genes, and differences in the sequence of the ob gene in these other species are highlighted in Figure 3.2. No differences were detected when the ob gene sequences of lean and obese *Psammomys obesus* were compared, which suggests that a mutation in the coding sequence of the ob gene is unlikely to explain the phenotypic differences observed in these animals. The codon 105 mutation found in ob/ob mice (Zhang *et al.* 1994) was not found in any of the *Psammomys obesus* tested.
**Psammomys obesus:** 5'-ctgacctccc gcgaattgtct gcgaatattc aagacaacctag agaactctcg

**Mus musculus:** 5'-ctgacctccc gaaatagtgt gcgaagatagc aatggacctgg agaactctcg

**Rattus norvegicus:** 5'-ctgacctccc gaaatagtgt gcgaagatagc ctgacctgg agaactctcg

**Homo sapiens:** 5'-atgcctccca gagaactgtgat ccaaatatcc aagacaacctggagaactctcg

**Psammomys obesus:** agaacttcct cactgtgtcgg cctctctccaa gcagctgtcc ctgccttcga

**Mus musculus:** agaacttcct cactgtgtcgg cctctctccaa gcagctgtcc ctgccttcga

**Rattus norvegicus:** agaacttcct cactgtgtcgg cctctctccaa gcagctgtcc ctgccttcga

**Homo sapiens:** ggaaccttct cactgtgtcgg cctctctccaa gcagctgtcc ctgccttcga

**Psammomys obesus:** ccagtggccct gcagaagcccg ggagacgtcct acggcgctct ggaagcctca

**Mus musculus:** ccagtggccct gcagaagcccg ggagacgtcct atgcctgtcc ggaagcctca

**Rattus norvegicus:** ccagtggccct gcagaagcccg ggagacgtcct atgcctgtcc ggaagcctca

**Homo sapiens:** ccagtggccct ggaagcctgc ggagacgtcct acggcgctct ggaagcctca

**FIGURE 3.2:** Partial DNA sequence of *Psammomys obesus* ob gene, aligned with the ob gene sequences of mouse, rat and human. Bases highlighted in colors are different to the corresponding nucleotide in the *Psammomys obesus* ob gene sequence.

### 3.4.3 **OB GENE EXPRESSION IN PSAMMOMYS OBESUS**

Total expression of the ob gene in fat depots from *Psammomys obesus* was positively correlated with body weight (r=0.435, p<0.001), percentage body fat (r=0.645, p<0.001) and plasma insulin concentration (r=0.651, p<0.001; Figure 3.3). Ob gene expression did not correlate with blood glucose, plasma triglyceride or cholesterol concentration. No significant difference was found in ob gene expression between the 4 adipose tissue sites tested (suprascapular, perirenal, intramuscular and mesenteric fat depots; Figure 3.4).
**FIGURE 3.3:** Correlations of *ob* gene expression in *Psammomys obesus* with A) body weight, B) estimated percent body fat and C) plasma insulin concentration.
3.4.4 **OB GENE EXPRESSION IN LEAN AND OBESE *P. OBESUS***

Two groups of *Psammomys obesus* were selected based on estimated percentage body fat above and below 3.5% and designated obese and lean respectively. The animals characterised as obese (body weight 221 ± 7 g; percentage body fat 4.2 ± 0.4%) had 2.3-fold increased expression of the *ob* gene (relative to β-actin) compared with lean animals (176 ± 8 g; 2.4 ± 0.2%; Figure 3.5). The difference in *ob* gene expression between obese and lean *Psammomys obesus* was statistically significant (p=0.015, Figure 3.5). The obese animals were also hyperinsulinemic and hyperglycemic compared with the lean animals (Table 3.1).
FIGURE 3.5: Increased ob gene expression in obese Psammomys obesus compared with lean animals (* p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>LEAN</th>
<th>OBESE</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>176 ± 8</td>
<td>221 ± 7*</td>
</tr>
<tr>
<td>% Body Fat</td>
<td>2.4 ± 0.2</td>
<td>4.2 ± 0.4*</td>
</tr>
<tr>
<td>Blood Glucose (mmol/l)</td>
<td>4.5 ± 0.2</td>
<td>6.0 ± 0.8*</td>
</tr>
<tr>
<td>Plasma Insulin (μU/ml)</td>
<td>63 ± 16</td>
<td>432 ± 85*</td>
</tr>
<tr>
<td>Food Intake (g/day)</td>
<td>13.4 ± 0.9</td>
<td>16.5 ± 0.8*</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>2.5 ± 0.2</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.0 ± 0.3</td>
<td>1.7 ± 0.5</td>
</tr>
</tbody>
</table>

* Significantly greater than the lean group (p<0.05)

TABLE 3.1: Characteristics of obese and lean Psammomys obesus groups.

3.4.5 PLASMA LEPTIN LEVELS IN PSAMMOMYS OBESUS

Plasma leptin levels in Psammomys obesus were significantly correlated with body weight (r=0.36, p<0.05), percentage body fat (r=0.702, p<0.01) and plasma insulin concentration (r=0.735, p<0.001; Figure 3.6).
**FIGURE 3.6:** Correlation of A) body weight, B) percentage body fat and C) plasma insulin with plasma leptin concentration in *Psammomys obesus*.
3.4.6 RELATIONSHIP BETWEEN LEPTIN AND INSULIN IN *PSAMMOMYSES OBEDES*

A total of 69 *Psammomys obesus* with a range of body weight, blood glucose and plasma insulin concentrations were separated into two groups matched for sex, age and body weight: Group 1 - insulin-sensitive (plasma insulin < 150 μU/ml; n=33) and Group 2 - insulin-resistant (plasma insulin > 150 μU/ml; n=36). Plasma leptin levels were significantly increased in insulin-resistant (hyperinsulinemic) animals compared to those with normoinsulinemia, despite the fact that there was no difference in body weight between the two groups (Figure 3.7). In addition, no difference in blood glucose concentration was found between the two groups.
**FIGURE 3.7:** Body weight, blood glucose, plasma insulin and leptin levels in insulin-sensitive (Group 1) and insulin-resistant (Group 2) *Psammomys obesus*. Data are expressed as mean ± sem, significant differences were determined by unpaired t-test (*p<0.05).
3.5 DISCUSSION

In these studies we have shown adipocyte-specific expression of the \textit{ob} gene in \textit{Psammomys obesus}, with no sequence difference between lean and obese animals, and significantly increased \textit{ob} gene expression in the obese state. \textit{Ob} gene expression correlated significantly with body weight, estimated percentage body fat and plasma insulin concentration. We also found that plasma leptin levels were correlated with body weight, estimated percentage body fat and plasma insulin concentration, and that leptin levels were elevated in insulin-resistant animals independent of body weight or percent body fat.

As shown in Figure 3.1, the \textit{ob} gene was expressed exclusively in adipose tissues of \textit{Psammomys obesus}. This is the same pattern of expression as that seen in mice and humans (Zhang et al. 1994; Masuzaki et al. 1995a). The \textit{ob} genes of mice, rats and humans have been sequenced (Zhang et al. 1994; Funahashi et al. 1995; Masuzaki et al. 1995a). Analysis of the sequences revealed a high degree of homology between these species: mouse-rat 95\%, mouse-human 83\%, rat-human 83\%. The predicted leptin amino acid sequences also have very high homology: mouse-rat 96\%, mouse-human 84\%, rat-human 83\% (Zhang et al. 1994; Funahashi et al. 1995; Masuzaki et al. 1995a). DNA sequence analysis of the \textit{Psammomys obesus ob} gene (Figure 3.2) revealed a high degree of homology with the published sequences from other species: 90\% with mouse, 88\% with rat and 79\% with human. The predicted amino acid sequence of the \textit{Psammomys obesus ob} gene product had 89\% homology with that of mouse, 87\% with that of the rat and 74\% with the human product. The high degree of homology of the \textit{ob} gene suggests that it has been conserved throughout evolution and encodes a protein of significant physiological importance. No differences were observed between the \textit{ob} gene sequences of lean and obese \textit{Psammomys obesus}. This suggests that an \textit{ob} gene mutation is unlikely to be responsible for the phenotypic differences observed in these animals. These findings correspond with those observed in similar studies in obese humans, which found no evidence of an association between coding mutations in the human \textit{ob} gene and obesity phenotypes (Considine et al. 1995; Hamilton et al. 1995; Considine et al. 1996c; Maffei et al. 1996; Niki et al. 1996).

The reported mutation in the \textit{ob} gene of \textit{ob/ob} mice (Zhang et al. 1994) was not found in this study of the \textit{Psammomys obesus ob} gene. However this does not exclude the possibility that this mutation may be present in a subpopulation of this animal. The mouse codon for arginine 105 (CGA) requires only a single nucleotide substitution to mutate into a stop codon, as found in the \textit{ob/ob} mouse, but the arginine 105 codon in
Psammomys obesus is encoded by CGG and would require two base substitutions for the same stop codon to arise, and is therefore significantly less likely to occur.

Ob gene expression in Psammomys obesus correlated significantly with body weight and percent body fat (Figure 3.3). Increased expression of the ob gene has previously been shown in various animal models of obesity (Zhang et al. 1994; Maffei et al. 1995a; Ogawa et al. 1995) and obese humans (Considine et al. 1995; Hamilton et al. 1995; Lonnqvist et al. 1995) compared with lean controls. However, a continuous relationship between body weight/body fat and ob gene expression over a wide range of body weight has not previously been demonstrated in rodents.

Ob gene expression also correlated with plasma insulin concentration (Figure 3.3), a relationship not previously shown in animal models of obesity. The relationship between insulin and leptin in body weight homeostasis could prove vital to the understanding of the physiological role of leptin. Fasting plasma insulin and leptin concentrations have been shown to be significantly correlated in human populations even after adjusting for body fat content (Larsson et al. 1996; Malmsstrom et al. 1996; Zimmet et al. 1996; de Courten et al. 1997). In vitro, insulin acutely increased ob gene expression in rodents (Rentsch et al. 1995; Saladin et al. 1995), and caused an increase in ob gene expression in isolated human adipocytes after 72 hours (Kolaczynski et al. 1996a; Nolan et al. 1996; Wabitsch et al. 1996). In vivo, hyperinsulinemic-euglycemic clamps caused rapid increases in ob gene expression in rodents (Cusin et al. 1995; Zheng et al. 1996), and similar effects after 24–48 hours in humans (Kolaczynski et al. 1996a; Sainsbury et al. 1996). Collectively, these results suggest a significant role for insulin in the long term regulation of ob gene expression in humans, and possibly also in the short-term in rodents.

Obese Psammomys obesus exhibited significant overexpression of the ob gene compared with their lean littermates (Figure 3.5). This is consistent with findings in single-gene animal models of obesity (Zhang et al. 1994; Maffei et al. 1995a; Ogawa et al. 1995) and in human obesity (Considine et al. 1995; Hamilton et al. 1995). The obese group of animals had significantly higher mean body weight, blood glucose and plasma insulin concentrations, and percent body fat compared to the lean animals (Table 3.1). No significant difference was found in ob gene expression between the adipose tissue sites tested (suprascapular, perirenal, intramuscular and mesenteric fat depots; Figure 3.4). Several studies in humans have found no difference in ob gene expression between adipose tissue sites (Lonnqvist et al. 1995; Segal et al. 1996). In addition, plasma leptin concentrations have been consistently associated with total body fat, not disproportionately with central obesity (Dua et al. 1996; Haffner et al. 1996).
1996h). Some studies in single-gene animal models of obesity have found that intraabdominal fat depots have higher levels of ob gene expression relative to subcutaneous adipose tissue (Maffei et al. 1995a; Ogawa et al. 1995). However, other authors noted highly variable expression between adipose tissue depots from animal to animal, and even from within the same depot in a given individual (Masuzaki et al. 1995a; Hayase et al. 1996).

Although we have clearly demonstrated overexpression of the ob gene in obese Psammomys obesus, the precise role of the ob gene and leptin in the disease process is unknown. The obese group of animals were hyperinsulinemic and had a higher body fat content relative to their lean counterparts (Table 3.1), and both of these factors have been linked with ob gene expression. Our findings regarding ob gene expression in Psammomys obesus appear to be more analogous to the human data than that found in single-gene animal models of obesity (Zhang et al. 1994; Considine et al. 1995; Hamilton et al. 1995; Lonkqvist et al. 1995; Maffei et al. 1995a).

We then measured plasma leptin levels in animals with a range of body weights and glucose and insulin levels. A weak relationship was found between plasma leptin levels and body weight ($r=0.36$, $p<0.05$). Circulating plasma leptin correlated more closely with percent body fat ($r=0.702$, $p<0.01$), while a strong relationship was found between plasma leptin and insulin levels ($r=0.641$, $p<0.001$, Figure 3.6). These correlations in Psammomys obesus are similar to those previously found in human studies. Correlations between BMI and plasma leptin have ranged from 0.51 to 0.85 (Maffei et al. 1995a; Considine et al. 1996a; Dua et al. 1996; Haffner et al. 1996a; Ma et al. 1996; McGregor et al. 1996; Zimmert et al. 1996; de Courten et al. 1997). In addition, human plasma leptin concentrations have been strongly correlated with percentage body fat (range $r=0.59$ to $r=0.86$; Maffei et al. 1995a; Considine et al. 1996a; Dua et al. 1996; Ma et al. 1996) and plasma insulin levels (range $r=0.55$ to $r=0.64$; Considine et al. 1996a; Zimmert et al. 1996; de Courten et al. 1997). Therefore the findings in Psammomys obesus resemble closely the results published from human studies, strengthening the hypothesis that Psammomys obesus represents a very good model of obesity and NIDDM.

Animals were then separated into two groups matched for age, sex, body weight and blood glucose: Group 1 - insulin-sensitive (plasma insulin < 150 μU/ml; $n=33$) and Group 2 - insulin-resistant (plasma insulin > 150 μU/ml; $n=36$). The body weight, blood glucose, plasma insulin and leptin data for insulin-sensitive (Group 1) and insulin-resistant (Group 2) are shown in Figure 3.7. Leptin levels were significantly increased in the insulin-resistant animals independent of body weight or body fat.
content. The correlation between leptin and insulin concentrations was found in both sexes, and within both the insulin-sensitive and insulin-resistant groups. A relationship between insulin and leptin has previously been demonstrated in humans (Zimmet et al. 1996; de Courten et al. 1997), with insulin resistance associated with hyperleptinemia in men independent of body fat mass (Segal et al. 1996). To our knowledge this is the first time that this relationship has been demonstrated in an animal model of obesity, providing a unique opportunity to further explore this potentially important interaction.

The existence of a relationship between leptin and insulin raises important questions regarding the pathogenesis of obesity and NIDDM, and may have significant implications for the potential use of leptin as a therapeutic agent for the treatment of obesity. Obesity in *Psammomys obesus* and humans is associated with hyperleptinemia and hyperinsulinemia. As discussed above, hyperinsulinemia may increase *ob* gene expression in the long-term (Kolaczynski et al. 1996a; Sainsbury et al. 1996). *In vitro* studies have shown that leptin attenuates the actions of insulin in isolated rat hepatocytes (Cohen et al. 1996). Therefore it is possible to envisage the following paradigm: as obesity develops and body fat is deposited, plasma leptin increases in parallel with the accumulation of adipose tissue. The increase in circulating leptin could precipitate or exacerbate hepatic insulin resistance, resulting in hyperinsulinemia which in turn stimulates *ob* gene expression in the long-term, worsening the hepatic insulin resistance. Leptin may therefore represent an important physiological link between obesity and NIDDM. This has serious implications for the use of leptin to treat obesity, as one possible side-effect could be worsening of insulin resistance and possibly even the development of NIDDM in susceptible individuals. Further aspects of this relationship will be examined in the subsequent chapters.
CHAPTER 4:

ASSOCIATION STUDIES OF AN OB GENE POLYMORPHISM AND A β3-ADRENERGIC RECEPTOR POLYMORPHISM IN TWO DISTINCT HUMAN POPULATIONS

4.1 SUMMARY

In this study we examined polymorphisms in two putative candidate genes for obesity and NIDDM, the ob gene and the gene encoding the β3-adrenergic-receptor (β3AR). We have demonstrated that the ob gene polymorphism studied was associated with aspects of insulin resistance including elevated fasting blood glucose (p=0.031) and plasma insulin concentrations (p=0.046) in Nauruans but not Australians. In addition, the class II allele of this polymorphism was found more frequently in hyperinsulinemic compared with normoinsulinemic Nauruans, suggesting that this gene may be implicated in the 'thrifty genotype' of the Nauruan population. The β3AR Trp64Arg mutation was associated with reduced plasma leptin concentrations in Nauruans (p=0.031), which has previously been associated with an increased propensity to gain weight in Pima Indians, another population thought to be representative of the 'thrifty genotype' hypothesis. The Trp64Arg mutation of the β3AR was associated with increased body weight (p=0.035), hip circumference (p=0.042) and obesity (allele frequency 0.11 vs. 0.06; p<0.05) in the Australian population studied. Interestingly, a combination of the two mutations was observed in 19 Australians, who exhibited significantly greater body weight (p=0.05) and waist circumference (p=0.033), and therefore visceral obesity, compared with those without this genotype. We propose that both of these genetic mutations may be implicated in the pathogenesis of obesity, insulin resistance and NIDDM, and that further studies are required to investigate the possible interaction between these genes in the disease process.
4.2 INTRODUCTION

Obesity and NIDDM are important public health problems both in Western societies, and in nations changing from a more traditional to a Western lifestyle, such as Pacific Islanders (Dowse et al. 1991; Knowler et al. 1991; Zimmet 1992; ). Many genetic studies have revealed a significant inherited component in the etiology of these diseases, although the specific genes involved are not yet known (Zimmet et al. 1990; Bouchard 1994).

The discovery of the ob gene (Zhang et al. 1994) and its product leptin has provided considerable insight into the genetic factors which influence energy balance and body weight homeostasis. However, several studies have failed to show mutations in the coding region of the ob gene in obese humans (Considine et al. 1995; Maffei et al. 1996; Niki et al. 1996), strongly suggesting that mutations within the coding region of this gene are unlikely to account for the majority of cases of obesity in the general population. Two previous studies have found suggestive evidence for association of polymorphisms in the region of the ob gene with extreme obesity (Clement et al. 1996; Reed et al. 1996), while others have found no significant association (Stirling et al. 1996; Norman et al. 1996).

A highly polymorphic tetranucleotide repeat polymorphism was recently identified very close to the 3'-end of the last exon of the ob gene (Shintani et al. 1996). This polymorphism consisted of two classes of products after polymerase chain reaction analysis: a shorter (class I) form with fewer tetranucleotide repeats, and a longer (class II) form. The authors found a tendency for higher body weight in those homozygous for the class I allele, however statistical significance was not achieved in this population, which had a relatively low prevalence of obesity (11.9%; Shintani et al. 1996).

Another putative candidate gene for obesity and aspects of the Metabolic Syndrome is the β3-adrenergic-receptor (β3AR; Emorine et al. 1989). The adrenergic system is thought to be important in the regulation of energy balance through regulation of both thermogenesis and lipolysis in adipose tissues of various mammalian species (Arch and Kaumann 1993; Lafontan 1994). Stimulation of the β3AR by agonists activated adenylate cyclase, which increased the intracellular concentration of cyclic AMP and resulted in increased thermogenesis and lipolysis in rodents (Emorine et al. 1989; Krief et al. 1993; Van Spronsen et al. 1993).
The β3AR was expressed at significant levels in human visceral adipose tissue (Krief et al. 1993), but only minor expression was found in subcutaneous fat. Therefore the β3AR was implicated in the increased lipolysis of visceral fat and the increased delivery of free fatty acids to the portal circulation (Lonnqvist et al. 1995). It has been proposed that the β3AR could be important in the link between visceral obesity and insulin resistance (Widen et al. 1995; Mauriege and Bouchard 1996). Evidence to support an association between the β3AR and obesity includes a marked decrease in the expression of the β3AR in rodent models of obesity (Muzzin et al. 1991; Collins et al. 1994), and greatly reduced lipolysis in response to β-agonists in β3AR-knockout transgenic mice (Susulic et al. 1994). In addition, β3-specific agonists had potent anti-obesity and anti-diabetic effects in both animals and humans (Mitchell et al. 1989; Connachter et al. 1992; Himms-Hagen et al. 1994).

In 1995, several groups simultaneously published reports of a T → C nucleotide substitution at position 190 of the gene encoding the β3AR which resulted in a tryptophan (TGG) to arginine (CGG) mutation at amino acid number 64 of the receptor (Trp64Arg; Clement et al. 1995; Walston et al. 1995; Widen et al. 1995). This mutation has been associated with earlier onset of NIDDM in Pima Indians (Walston et al. 1995), Finns (Widen et al. 1995) and Japanese (Fujisawa et al. 1996), features of insulin resistance in Finnish and Japanese study populations (Kadowaki et al. 1995; Widen et al. 1995), and rapid weight gain in morbidly obese French patients (Clement et al. 1995).

The aim of this study was to investigate the relationship between the β3AR Trp64Arg and ob gene polymorphisms and aspects of obesity and NIDDM in two distinct populations, Australians and Nauruans.
4.3 MATERIALS AND METHODS

4.3.1 STUDY SUBJECTS

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 being eligible (Zimmet et al. 1977). Subjects for the current study (n=342) were aged over 24 years, who had high prevalence rates of obesity (BMI>30 kg/m²; 82.7%) and NIDDM (15.2%; WHO Expert Committee 1985).

The Nauruan population have previously been shown to have very high prevalence rates of obesity and NIDDM and are thought to have a genetic background which significantly predisposes them to the development of these diseases. Previously, large scale epidemiological studies have found age-standardised prevalence rates of NIDDM (based on WHO criteria) of 28% in 1975-6, 25% in 1982 and 24% in 1987 (Zimmet et al. 1977; Zimmet et al. 1984; Dowse et al. 1991). The mean BMI of the population was 33.3 kg/m² in 1975-6, 33.8 kg/m² in 1982 and 34.5 kg/m² in 1987, suggesting very high prevalence rates of obesity in this population (Zimmet et al. 1977; Zimmet et al. 1984; Dowse et al. 1991). The Nauruan population is thought to be representative of the 'thrifty genotype' hypothesis (Neel 1962; Dowse et al. 1991).

In addition 322 Australian subjects were selected from a large prospective study of osteoporosis (the Geelong Osteoporosis Study, GOS), and consisted of women aged over 19 years who responded to a mailed questionnaire relating to osteoporosis. 39% (125/322) of the selected subjects had a BMI>30 kg/m² and were classified as obese, 30% (95/322) were overweight (BMI 25-30 kg/m²), and a further 32% (102/322) had a BMI<25 kg/m². Of the individuals selected from the Geelong population, 3.4% had NIDDM (WHO Expert Committee 1985).

4.3.2 SUBJECT CHARACTERISTICS

For all subjects, height was measured to the nearest centimeter and weight to the nearest kilogram in subjects without shoes and wearing only light clothing. Body mass indices (BMI) were calculated as kilograms per meter². In addition, waist and hip circumference were measured to the nearest centimeter and used to calculate the waist-to-hip ratio (WHR). Systolic and diastolic blood pressure were measured with an automated digital analyser with the subjects sitting.
4.3.3 DNA EXTRACTION

All subjects were required to fast overnight (from 10pm the previous evening) and blood specimens were collected in heparinised tubes the next morning. 1 ml of whole blood was used for DNA extraction, while the remainder was centrifuged and the plasma was stored at -70°C for later biochemical analyses.

DNA was extracted from all of the 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 the genomic DNA by isopropanol precipitation. The DNA was resuspended in the supplied DNA Rehydration Solution and then 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.

4.3.4 OB GENE PCR

Primers used were those published by Shintani et al. (1996), and comprised of the forward primer (HOBF), 5'-agt tca aat aga gtt cca aat ca-3', and the reverse primer (HOBR), 5'-ttc tga ggt tgg gtc act ggc a-3'. PCR's were performed by adding 100 ng of each DNA to a reaction mix containing 10 mM Tris-HCl (pH 8.3 at 20°C), 2 mM MgCl₂, 50 mM KCl, 200 μM each dNTP, 100 pmol each primer and 1.25 U Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany). After an initial denaturation step of 94°C for 3 min, the tubes were subjected to 35 cycles of 94°C for 30 sec (denaturation), 60°C for 1 min (annealing) and 72°C for 1 min (extension), with a final extension step of 72°C for 10 min. PCR's were performed in a Hybaid OmniGene Thermal Cycler (Tedddington, England) in a total volume of 20 μl in 0.5 ml microcentrifuge tubes (Perkin Elmer, Norwalk, USA).

4.3.4.1 ELECTROPHORESIS AND ANALYSIS

The PCR products (10 μl) were separated by agarose gel electrophoresis in a 3% gel containing 0.5 μg/ml ethidium bromide at 6 V/cm for 120 min in a DNA SubCell system (Bio-Rad, Anaheim, USA). The gels were then photographed using a Polaroid DS34 camera with a DSIH-7 Hood on Polaroid 665 positive/negative film (Sigma Chemical Co., St. Louis, USA) under ultraviolet transillumination (model 4000,
Stratagene, LaJolla, USA) at 302 nm. The size of the PCR products was determined visually to be either 121-145 base pairs in length (short form), or 197-221 base pairs (long form). Individual subjects were therefore placed into 1 of 3 groups: a) homozygotes for the short form, b) heterozygotes, or c) homozygotes for the long form. Examples of PCR products for these three groups are illustrated in Figure 2.3.

4.3.5  **β3AR PCR**

Primers used were those published by Widen *et al.* (1995), and were composed as follows:

- forward primer 5’-cgc cca ata ceg cca aca c-3’
- reverse primer 5’-cca cca gga gtc cca tca cc-3’

PCR's were performed by adding 100 ng of each DNA to a reaction mix containing 10 mM Tris-HCl (pH 8.3 at 20°C), 1.5 mM MgCl₂, 50 mM KCl, 200 μM each dNTP, 100 pmol each primer and 1.25 U Taq DNA Polymerase (Boehringer Mannheim, Mannheim, Germany). After an initial denaturation step of 94°C for 3 min, the tubes were subjected to 35 cycles of 94°C for 30 sec (denaturation), 61°C for 1 min (annealing) and 72°C for 1 min (extension), with a final extension step of 72°C for 10 min. PCR's were performed in a Hybird OmniGene Thermal Cycler (Teddington, England) in a total volume of 20 μl in 0.5 ml microcentrifuge tubes (Perkin Elmer, Norwalk, USA).

4.3.5.1  **Restriction Enzyme Digestion**

The PCR products were digested by the addition of 10 μl of a mixture containing 30 mM Tris-HCl (pH 7.9), 30 mM MgCl₂, 150 mM NaCl and 10 units of *BsrQ1* (Promega, Madison, USA), a restriction enzyme which specifically cleaves DNA at the sequence CC(A/T)GG. This mixture was incubated for 2 hours at 60°C to enable complete digestion.

4.3.5.2  **Electrophoresis and Analysis**

The restriction digested PCR products were separated by agarose gel electrophoresis in a 3% gel containing 0.5 μg/ml ethidium bromide at 6 V/cm for 120 min 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 Co., St. Louis, USA) under ultraviolet transillumination (model 4000, Stratagene, LaJolla, USA) at 302 nm. The genotypes were determined visually as follows. DNA from individuals homozygous for the wild type (Trp64) yielded
fragments of 99, 62, 30, 12 and 7 base pairs; homozygotes for the Trp64Arg mutation generated fragments of 161, 30, 12 and 7 base pairs; while heterozygotes yielded fragments of 161, 99, 62, 30, 12 and 7 base pairs (Widen et al. 1995). The smallest fragments (30, 12 and 7 base pairs) could not be resolved on the gel, so genotyping was based on the 161, 99 and 62 base pair fragments (see Figure 2.4).

4.3.6 BIOCHEMICAL ANALYSES

Whole blood glucose was measured after an overnight fast using a glucose analyser (Yellow Springs Instrument Co., Yellow Springs, Ohio, USA), and fasting plasma insulin and leptin concentrations were determined using commercially available radioimmunoassay kits (Phadebeph Human Insulin RIA kit, Pharmacia Diagnostics AB, St. Louis, USA; Human Leptin RIA kit, Linco Research Inc., St. Charles, Missouri, USA). Plasma cholesterol and triglycerides were measured using colorimetric kits (Boehringer Mannheim, Mannheim, Germany) on an automatic analyser as described in detail in Chapter 2.

4.3.7 STATISTICAL ANALYSIS

All results are expressed as mean ± SEM. Differences between group means were tested by Student's t-test. Chi-square test was used to compare allele frequencies.
4.4 RESULTS

The two populations varied significantly in all parameters which were measured in this study (Table 4.1). The distribution of the short (class I) and long (class II) forms of the ob gene tetranucleotide repeat polymorphism (Table 4.2), and the presence of the Trp64Arg mutant allele were also markedly different in the two populations studies (Table 4.3). Therefore the two populations were not directly compared in any of the analyses described below.

<table>
<thead>
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<th>AUSTRALIAN</th>
<th>p-value</th>
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<td>322</td>
<td></td>
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<tr>
<td>Age (y)</td>
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<tr>
<td>Body weight (kg)</td>
<td>103.1 ± 1.3</td>
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</tr>
<tr>
<td>Height (cm)</td>
<td>166.9 ± 0.8</td>
<td>161.4 ± 0.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>36.5 ± 0.4</td>
<td>28.9 ± 0.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
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<td>5.40 ± 0.10</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>16.60 ± 0.68</td>
<td>10.17 ± 0.35</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>12.1 ± 0.5</td>
<td>22.7 ± 0.8</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

**TABLE 4.1:** Characteristics of the two selected populations.

<table>
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<tr>
<th>GENOTYPE</th>
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<td>NAURUAN</td>
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<td>AUSTRALIAN</td>
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<td>TOTAL</td>
<td>78</td>
<td>194</td>
<td>392</td>
<td>664</td>
</tr>
</tbody>
</table>

**TABLE 4.2:** Genotype frequencies according to the ob gene polymorphism in the Nauruan and Australian populations studied ($\chi^2=157.2$, $p<0.05$).

<table>
<thead>
<tr>
<th>GENOTYPE</th>
<th>Trp/Trp</th>
<th>Trp/Arg</th>
<th>Arg/Arg</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAURUAN</td>
<td>332</td>
<td>10</td>
<td>0</td>
<td>342</td>
</tr>
<tr>
<td>AUSTRALIAN</td>
<td>272</td>
<td>47</td>
<td>3</td>
<td>322</td>
</tr>
<tr>
<td>TOTAL</td>
<td>604</td>
<td>57</td>
<td>3</td>
<td>664</td>
</tr>
</tbody>
</table>

**TABLE 4.3:** Genotype frequencies according to the β3AR Trp64Arg mutation in the Nauruan and Australian populations studied ($\chi^2=32.41$, $p<0.05$).
4.4.1 *OB GENE POLYMORPHISM*

In the Nauruan population, those homozygous for the long (class II) form of the *ob* gene polymorphism had significantly increased fasting blood glucose (7.13 ± 0.21 vs. 6.24 ± 0.26 mmol/l; p=0.031) and plasma insulin concentrations (102 ± 4.8 vs. 88.2 ± 0.5 pmol/l; p=0.046) compared with individuals with the other genotypes. Notably, there was no significant difference in any measures of body fatness between these groups, nor was there a significant difference in plasma leptin concentrations (Table 4.4). Although only 5 individuals were homozygous for the short form of this polymorphism, which restricts statistical analysis, they appeared to have a significantly reduced fasting blood glucose concentration (5.42 ± 0.18 mmol/l) compared with both heterozygotes (6.24 ± 0.26 mmol/l; p=0.0014) and long form (class II) homozygotes (7.13 ± 0.21 mmol/l; p<0.0001; Table 4.4).

Within the Nauruans with the highest 20% of fasting plasma insulin concentrations (>22.5 μU/ml), there was a significant difference in the class II allele frequency compared with those with fasting plasma insulin less than 22.5 μU/ml ($\chi^2$=8.18; p<0.05). This finding adds weight to the suggestion that this genotype was associated with elevated fasting insulin concentrations in the Nauruan population studied.
<table>
<thead>
<tr>
<th>GENOTYPE</th>
<th>I/I</th>
<th>I/II</th>
<th>II/II</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>59</td>
<td>278</td>
</tr>
<tr>
<td>Age (y)</td>
<td>27.2 ± 1.0</td>
<td>31.7 ± 0.6</td>
<td>31.8 ± 0.2</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>94.2 ± 11.8</td>
<td>102.7 ± 2.6</td>
<td>104.1 ± 1.4</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>166.4 ± 4.2</td>
<td>168.6 ± 0.7</td>
<td>167.8 ± 0.4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>33.8 ± 3.5</td>
<td>36.1 ± 0.9</td>
<td>36.9 ± 0.5</td>
</tr>
<tr>
<td>SYS BP¹ (mm Hg)</td>
<td>132.4 ± 4.0</td>
<td>127.1 ± 2.8</td>
<td>126.2 ± 1.0</td>
</tr>
<tr>
<td>DIA BP² (mm Hg)</td>
<td>67.2 ± 7.5</td>
<td>76.3 ± 2.0</td>
<td>74.3 ± 0.8</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.42 ± 0.18*†</td>
<td>6.24 ± 0.26</td>
<td>7.13 ± 0.21‡</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>16.15 ± 6.69</td>
<td>14.74 ± 0.87</td>
<td>17.04 ± 0.81‡</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>8.89 ± 3.56</td>
<td>11.61 ± 1.05</td>
<td>12.26 ± 0.59</td>
</tr>
</tbody>
</table>

¹ Systolic Blood Pressure  
² Diastolic Blood Pressure  
* Significantly lower than genotype I/II (p<0.05)  
† Significantly lower than genotype II/II (p<0.05)  
‡ Significantly greater than genotypes I/I and I/II (p<0.05).

**TABLE 4.4:** Characteristics of the Nauruan population according to *ob* gene polymorphism genotype.
No significant associations were found between aspects of the Metabolic Syndrome studied and the \textit{ob} gene polymorphism in the Australian population studied (Table 4.5). No difference in allele frequency was detected between lean and obese Australians in this study, however within the lean group (BMI<30 kg/m²), those homozygous for the class II allele had a tendency for increased fasting plasma insulin concentration (8.01 ± 0.42 μU/ml) compared with lean Australians with either the class I/class I or heterozygote genotypes (7.03 ± 0.40; p=0.093).

<table>
<thead>
<tr>
<th>GENOTYPE</th>
<th>I/I</th>
<th>I/II</th>
<th>II/II</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>73</td>
<td>135</td>
<td>114</td>
</tr>
<tr>
<td>Age (y)</td>
<td>47.6 ± 1.7</td>
<td>51.4 ± 1.2</td>
<td>50.0 ± 1.3</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>75.9 ± 1.9</td>
<td>74.3 ± 1.3</td>
<td>75.9 ± 1.6</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>161.6 ± 0.7</td>
<td>161.1 ± 0.5</td>
<td>161.5 ± 0.6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.0 ± 0.7</td>
<td>28.6 ± 0.5</td>
<td>29.1 ± 0.6</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>90.2 ± 1.6</td>
<td>89.7 ± 1.2</td>
<td>90.9 ± 1.3</td>
</tr>
<tr>
<td>Hip (cm)</td>
<td>110.0 ± 1.5</td>
<td>109.1 ± 1.0</td>
<td>110.9 ± 1.3</td>
</tr>
<tr>
<td>WHR</td>
<td>0.82 ± 0.01</td>
<td>0.82 ± 0.01</td>
<td>0.82 ± 0.01</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.44 ± 0.18</td>
<td>5.27 ± 0.11</td>
<td>5.53 ± 0.21</td>
</tr>
<tr>
<td>Insulin (μU/ml)</td>
<td>10.34 ± 0.72</td>
<td>9.87 ± 0.50</td>
<td>10.42 ± 0.67</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>22.74 ± 1.87</td>
<td>22.75 ± 1.19</td>
<td>22.57 ± 1.33</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>4.90 ± 0.12</td>
<td>4.99 ± 0.08</td>
<td>5.07 ± 0.10</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.39 ± 0.08</td>
<td>1.52 ± 0.08</td>
<td>1.45 ± 0.07</td>
</tr>
</tbody>
</table>

\textit{Table 4.5}: Characteristics of the Australian population according to \textit{ob} gene polymorphism genotype.
4.4.2 **β3AR TRP64ARG MUTATION**

In the Nauruan population studied the Trp64Arg mutation of the β3AR was associated with significantly lower plasma leptin concentrations (p=0.031; Table 4.6). No other significant differences were detected between wild-type homozygotes and those heterozygous for the Trp64Arg mutation.

<table>
<thead>
<tr>
<th>GENOTYPE</th>
<th>Trp/Trp</th>
<th>Trp/Arg</th>
<th>Arg/Arg</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>332</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Age (y)</td>
<td>31.8 ± 0.2</td>
<td>31.0 ± 1.3</td>
<td>-</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>103.3 ± 1.3</td>
<td>98.6 ± 4.7</td>
<td>-</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>166.8 ± 0.8</td>
<td>169.5 ± 1.5</td>
<td>-</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>36.6 ± 0.4</td>
<td>34.3 ± 1.6</td>
<td>-</td>
</tr>
<tr>
<td>SYS BP (mm Hg)</td>
<td>125.1 ± 1.1</td>
<td>132.4 ± 10.2</td>
<td>-</td>
</tr>
<tr>
<td>DIA BP (mm Hg)</td>
<td>73.7 ± 0.8</td>
<td>80.4 ± 5.3</td>
<td>-</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>6.97 ± 0.18</td>
<td>6.40 ± 0.63</td>
<td>-</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>16.66 ± 0.70</td>
<td>15.67 ± 2.16</td>
<td>-</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>12.20 ± 0.53</td>
<td>8.64 ± 1.40*</td>
<td>-</td>
</tr>
</tbody>
</table>

*Significantly less than genotype Trp/Trp (p<0.05)

**TABLE 4.6:** Characteristics of the Nauruan population according to β3AR Trp64Arg mutation genotype.
In the Australian population the Trp64Arg mutation of the β3AR was associated with significantly increased body weight (p=0.035) and, in heterozygotes, significantly greater hip circumference (p=0.042) compared with wild-type homozygotes (Trp/Trp; Table 4.7). In addition, those with the Trp64Arg mutation tended to have increased BMI (p=0.064), waist circumference (p=0.078) and plasma leptin concentrations (p=0.084; Table 4.7). These differences may have become statistically significant if more subjects were included in the study. Although only 3 individuals were homozygous for the β3AR mutation, there was a slight tendency for higher body weight (p=0.15), waist circumference (p=0.12), hip circumference (p=0.14), BMI (p=0.14), WHR (p=0.16) and plasma leptin concentration (p=0.12) in this group compared with heterozygotes (Table 4.7). Once again, if more Australian Trp64Arg homozygotes were available for study it is possible that we may have found a significant difference between individuals heterozygous and homozygous for this mutation for some of these parameters. However, from the available data, where p>0.05 we conclude that there was no difference between the groups.

In the Australian population studied the Trp64Arg allele was found more frequently in obese (BMI>30 kg/m²) individuals (0.11 in obese vs. 0.06 in lean; χ²=6.90; p<0.05). This finding further suggests a relationship between the β3AR Trp64Arg mutation and obesity in this Australian population.
<table>
<thead>
<tr>
<th>GENOTYPE</th>
<th>Trp/Trp</th>
<th>Trp/Arg</th>
<th>Arg/Arg</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>272</td>
<td>47</td>
<td>3</td>
</tr>
<tr>
<td>Age (y)</td>
<td>50.3 ± 0.9</td>
<td>49.8 ± 2.0</td>
<td>43.7 ± 4.1</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>74.2 ± 0.9*</td>
<td>79.0 ± 2.6</td>
<td>96.5 ± 9.7</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>161.1 ± 0.4</td>
<td>162.2 ± 1.0</td>
<td>163.4 ± 2.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.5 ± 0.3</td>
<td>30.1 ± 1.0</td>
<td>36.0 ± 3.1</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>89.5 ± 0.8</td>
<td>92.6 ± 2.1</td>
<td>107.1 ± 6.6</td>
</tr>
<tr>
<td>Hip (cm)</td>
<td>108.9 ± 0.7</td>
<td>113.6 ± 2.2†</td>
<td>123.8 ± 6.4</td>
</tr>
<tr>
<td>WHR</td>
<td>0.82 ± 0.00</td>
<td>0.81 ± 0.01</td>
<td>0.87 ± 0.01</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.35 ± 0.10</td>
<td>5.58 ± 0.25</td>
<td>5.35 ± 0.44</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>10.18 ± 0.38</td>
<td>11.08 ± 1.03</td>
<td>13.10 ± 2.71</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>22.49 ± 0.89</td>
<td>23.51 ± 1.95</td>
<td>28.40 ± 3.46</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.01 ± 0.06</td>
<td>5.00 ± 0.12</td>
<td>5.96 ± 0.86</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.44 ± 0.05</td>
<td>1.63 ± 0.18</td>
<td>1.31 ± 0.44</td>
</tr>
</tbody>
</table>

* Significantly lower than Trp/Arg and Arg/Arg (p<0.05)
† Significantly greater than Trp/Trp (p<0.05)

**TABLE 4.7:** Characteristics of the Australian population according to β3AR Trp64Arg mutation genotype.
4.4.3 Interaction Between the Two Polymorphisms

After finding indications of associations between these polymorphisms and aspects of obesity and NIDDM, we investigated possible interactions between them by analysing subgroups of the two populations. In the Australian population studied, those with the class II/class II/Trp/Arg genotype had significantly greater body weight (p=0.05) and waist circumference (p=0.033) compared with individuals without this genotype (Table 4.9). This group also exhibited tendencies for increased hip circumference (p=0.06) and BMI (p=0.12) compared with those with other genotypes.

<table>
<thead>
<tr>
<th>GENOTYPE</th>
<th>II/II, Trp/Arg</th>
<th>ALL OTHERS</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>19</td>
<td>303</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>51.2 ± 3.4</td>
<td>50.0 ± 0.83</td>
<td>0.74</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>83.8 ± 4.4</td>
<td>74.6 ± 0.9</td>
<td>0.05*</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>163.2 ± 1.5</td>
<td>161.3 ± 0.3</td>
<td>0.25</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>31.6 ± 1.8</td>
<td>28.6 ± 0.3</td>
<td>0.12</td>
</tr>
<tr>
<td>waist (cm)</td>
<td>97.5 ± 3.3</td>
<td>89.7 ± 0.8</td>
<td>0.033*</td>
</tr>
<tr>
<td>hip (cm)</td>
<td>117.2 ± 3.9</td>
<td>109.3 ± 0.7</td>
<td>0.060</td>
</tr>
<tr>
<td>W/H</td>
<td>0.83 ± 0.01</td>
<td>0.82 ± 0.00</td>
<td>0.31</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.6 ± 0.45</td>
<td>5.35 ± 0.09</td>
<td>0.60</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>11.22 ± 1.60</td>
<td>10.10 ± 0.37</td>
<td>0.49</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>24.30 ± 2.98</td>
<td>22.60 ± 0.84</td>
<td>0.58</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.02 ± 0.11</td>
<td>5.00 ± 0.06</td>
<td>0.88</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.41 ± 0.13</td>
<td>1.46 ± 0.05</td>
<td>0.71</td>
</tr>
</tbody>
</table>

*TABLE 4.8: Characteristics of the Australian population according to ob gene polymorphism class II/II and β3AR Trp/Arg genotype compared with those without this genotype.*
4.5 DISCUSSION

In this study we investigated polymorphisms of the ob and β3AR genes in populations from Australia and Nauru. In the Nauruans, the ob gene polymorphism was associated with fasting glucose and insulin concentrations and the β3AR mutation was associated with decreased fasting plasma leptin concentrations. In the Australian population studied, the β3AR Trp64Arg mutation was associated with increased body weight and hip circumference, and the mutation was more frequent in obese compared with lean individuals. In addition, a combination of the two polymorphisms in the Australian population was associated with elevated body weight and waist circumference, an indicator of visceral obesity. The possible involvement of these polymorphisms in the pathogenesis of obesity and NIDDM is discussed.

The Nauruan population, both in this study and previously, have been shown to have very high prevalence rates of obesity and NIDDM (82.7% and 15.2% respectively in this study), and are thought to have a genetic background which significantly predisposes them to the development of these diseases. Previously, large scale epidemiological studies have found age-standardised prevalence rates of NIDDM (based on WHO criteria; WHO Expert Committee 1985) of 28% in 1975-6, 25% in 1982 and 24% in 1987 (Zimmet et al. 1977; Zimmet et al. 1984; Dowse et al. 1991). The mean BMI of the population was 33.3 kg/m² in 1975-6, 33.8 kg/m² in 1982 and 34.5 kg/m² in 1987, suggesting very high prevalence rates of obesity in this population (Zimmet et al. 1977; Zimmet et al. 1984; Dowse et al. 1991). The Nauruan population is thought to be representative of the 'thrifty genotype' hypothesis (Neel 1962; Dowse et al. 1991).

Epidemiological data from several studies support a link between leptin and insulin resistance. In a study of 240 Western Samoans, plasma leptin concentration was positively correlated with plasma insulin both in the fasting state and two hours after a glucose load (75 g glucose as dextrose monohydrate) after controlling for body mass index (Zimmet et al. 1996; de Courten et al. 1997), suggesting a possible role for leptin in insulin resistance. In another study, plasma leptin concentrations were significantly lower in lean insulin-sensitive men compared with another group of men matched for percentage body fat and fat mass but with relative insulin resistance (Segal et al. 1996). These studies suggest a significant relationship between plasma leptin concentration and insulin resistance.

The first polymorphism investigated in this study was located only 157 bp downstream of the end of the last exon of the ob gene (Shintani et al. 1996). In a previous study in a
Japanese population, individuals homozygous for the class I genotype tended to have higher body weight compared to those without this genotype, however statistical significance was not achieved (Shintani et al. 1996). In addition, no significant difference in allele frequency was noted between individuals with NIDDM and controls with respect to this polymorphism (Shintani et al. 1996). In contrast, in the Nauruan population, individuals homozygous for the class II genotype had significantly elevated fasting blood glucose and plasma insulin concentrations compared to those without this genotype, however no significant associations were observed between this polymorphism and plasma leptin concentrations or measures of obesity such as BMI.

The results of the current study, together with the epidemiological data described above, suggest that a significant link may exist between leptin and insulin resistance. However, the current study does not directly link the ob gene with insulin resistance, only a gene in this region. To our knowledge there are no other genes in this vicinity which have previously been associated with insulin resistance, however this possibility cannot be excluded based on the results of this study. In addition, the polygenic nature of obesity and NIDDM mean that it is likely that other genes, apart from those investigated in this study, are likely to be involved in the physiological link between these diseases.

In the Australian population, no associations were found between the ob gene polymorphism and aspects of the Metabolic Syndrome. The reason that this polymorphism was associated with elevated fasting glucose and insulin in Nauruans but not in the Geelong population is unknown. However, the Geelong population contained only 3.4% of subjects with NIDDM, which was much lower than the Nauruan population (15.2%), and the Geelong population consisted of women, while the Nauruans studied were all male. In addition, these two populations would be expected to be genetically diverse, and the allelic frequency distributions of the polymorphism within the two groups supports this notion (Table 4.2). Interestingly, lean Australians homozygous for the class II form of the ob gene polymorphism tended to have increased plasma insulin concentrations relative to lean individuals without this genotype. This suggests that while this polymorphism had no obvious effects in Australians, it may have still subtly influenced circulating insulin levels in some individuals. The Nauruan population, both in this study and previously, have been shown to have very high prevalence rates of obesity and NIDDM (82.7% and 15.2% respectively in this study), and are thought to have a genetic background (‘thrifty genotype’) which significantly predisposes them to the development of these diseases. The results of this study suggest that homozygosity for the class II (longer) form of this ob gene polymorphism could form part of the ‘thrifty genotype’ in Nauruans.
In the limited number of Nauruans found to carry the β3AR Trp64Arg mutation we found significantly reduced plasma leptin concentrations compared with those without this allele (Table 4.6). We are not aware of any previous reports which have investigated plasma leptin and this mutation. It is interesting that these individuals expressing the Trp64Arg mutation, which has previously been associated with obesity (Clément et al. 1995; Kurabayashi et al. 1996) exhibited decreased plasma leptin levels. A recent report in Pima Indians (also thought to be representative of the 'thrifty genotype') showed that lower plasma leptin concentrations predicted excessive weight gain over a 3-year follow-up period (Ravussin et al. 1997). The Nauruan subjects in this study were relatively young (mean age 31.8 ± 0.2 years), so it would be interesting to follow these individuals prospectively to investigate whether the β3AR mutation and reduced plasma leptin concentrations predict excessive weight gain in this group.

A previous study of the Trp64Arg mutation in Nauruans found no mutant alleles in 39 individuals studied (Silver et al. 1996), while the allele frequency in this study was 0.02 in Nauruans. When the allele frequencies of the two studies were combined, the frequency of the Arg allele in Nauruans fell to 0.01. It is not clear why this mutation is so rare in Nauruans compared with other ethnic groups (Table 4.9).

In the Australian population studied the allele frequency of the Trp64Arg mutation was 0.08, which corresponds with a previous study in Australians (Kurabayashi et al. 1996). A summary of the frequency of the Trp64Arg allele in various populations is given in Table 4.9.
<table>
<thead>
<tr>
<th>POPULATION</th>
<th>ALLELE FREQUENCY</th>
<th># ALLELES TYPED</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pima Indians</td>
<td>0.31</td>
<td>1284</td>
<td>Walston <em>et al.</em> 1995</td>
</tr>
<tr>
<td>Japanese</td>
<td>0.20</td>
<td>700</td>
<td>Kadowaki <em>et al.</em> 1995</td>
</tr>
<tr>
<td></td>
<td>0.19</td>
<td>590</td>
<td>Fujisawa <em>et al.</em> 1996</td>
</tr>
<tr>
<td></td>
<td>0.23</td>
<td>556</td>
<td>Kim-Motoyama <em>et al.</em> 1997</td>
</tr>
<tr>
<td>TOTAL JAPANESE</td>
<td>0.20</td>
<td>1846</td>
<td></td>
</tr>
<tr>
<td>Mexican Americans</td>
<td>0.13</td>
<td>124</td>
<td>Silver <em>et al.</em> 1996</td>
</tr>
<tr>
<td>Finns</td>
<td>0.11</td>
<td>982</td>
<td>Widen <em>et al.</em> 1995</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>810</td>
<td>Oksanen <em>et al.</em> 1996</td>
</tr>
<tr>
<td></td>
<td>0.19</td>
<td>340</td>
<td>Sipilainen <em>et al.</em> 1997</td>
</tr>
<tr>
<td>TOTAL FINNS</td>
<td>0.12</td>
<td>2132</td>
<td></td>
</tr>
<tr>
<td>African Americans</td>
<td>0.12</td>
<td>98</td>
<td>Silver <em>et al.</em> 1996</td>
</tr>
<tr>
<td>Chinese</td>
<td>0.12</td>
<td>104</td>
<td>Silver <em>et al.</em> 1996</td>
</tr>
<tr>
<td>Chinese Americans</td>
<td>0.12</td>
<td>60</td>
<td>Silver <em>et al.</em> 1996</td>
</tr>
<tr>
<td>Indians (subcontinent)</td>
<td>0.09</td>
<td>88</td>
<td>Silver <em>et al.</em> 1996</td>
</tr>
<tr>
<td>Australians</td>
<td>0.08</td>
<td>1372</td>
<td>Kurabayashi <em>et al.</em> 1996</td>
</tr>
<tr>
<td></td>
<td>0.08</td>
<td>644</td>
<td>This study</td>
</tr>
<tr>
<td>TOTAL AUSTRALIANS</td>
<td>0.08</td>
<td>2016</td>
<td></td>
</tr>
<tr>
<td>USA Caucasians</td>
<td>0.08</td>
<td>96</td>
<td>Silver <em>et al.</em> 1996</td>
</tr>
<tr>
<td>Danes</td>
<td>0.07</td>
<td>760</td>
<td>Urhammer <em>et al.</em> 1996</td>
</tr>
<tr>
<td>French Caucasians</td>
<td>0.06</td>
<td>558</td>
<td>Clement <em>et al.</em> 1995</td>
</tr>
<tr>
<td>Western Samoans</td>
<td>0.06</td>
<td>84</td>
<td>Silver <em>et al.</em> 1996</td>
</tr>
<tr>
<td>Nauruans</td>
<td>0.00</td>
<td>78</td>
<td>Silver <em>et al.</em> 1996</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>684</td>
<td>This study</td>
</tr>
<tr>
<td>TOTAL NAURUANS</td>
<td>0.01</td>
<td>762</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.9:** Allele frequencies of the β3AR Trp64Arg mutation in various populations

In the Australian population studied, the Trp64Arg mutation was associated with elevated body weight and hip circumference (Table 4.7), and strong tendencies for increased BMI (p=0.064) and waist circumference (p=0.078) were also noted (Table 4.7). In addition, the Trp64Arg allele was found more frequently in obese (BMI>30 kg/m²) compared with lean Australians (0.11 vs. 0.06 respectively; p<0.05). Taken together, these results strongly suggest a role for the β3AR Trp64Arg mutation in the pathophysiology of obesity in this group of Australians.

Previous studies of the β3AR Trp64Arg mutation have found a significant association with increased body weight in Australians (Kurabayashi *et al.* 1996) and French
Caucasians (Clement et al. 1995). No association with obesity was found in Finns (Sipilainen et al. 1997), Swedes (Gagnon et al. 1996) or Utah Mormons (Elbein et al. 1996). The differences in these studies may be due to genetic differences between the populations studied, different numbers of subjects recruited, and variation in the characteristics of subjects in the populations studied. Similarly, increased Trp64Arg allele frequency in obese compared with lean individuals was found in Australian (Kurabayashi et al. 1996) and Japanese (Kadowaki et al. 1995; Kim-Motoyama et al. 1997) populations, but not in Finns (Oksanen et al. 1996), Danes (Urhammer et al. 1996) or French Caucasians (Clement et al. 1995).

It is difficult to assess the importance of the Trp64Arg mutation, or the meaning of the associations with obesity and NIDDM in some populations but not others, when the physiological relevance of the β3AR in humans remains controversial (Mauriege and Bouchard 1996). While β3AR stimulation resulted in increased thermogenesis in rodent brown adipose tissue, thus increasing overall energy expenditure, the relevance of this system in human adult physiology is not clear (MacDonald 1995; Flier and Lowell 1997). In humans, the enhanced sensitivity of visceral adipose tissue to catecholamine-induced lipolysis may be mediated primarily through effects on the β3AR (Lonnqvist et al. 1995). Therefore, the mutated receptor would be expected to reduce this lipolysis, resulting in fat accumulation. However, the molecular mechanism by which this amino acid substitution exerts an apparent predisposition to obesity and NIDDM in afflicted individuals needs to be elucidated before definitive pathophysiological conclusions can be reached.

It is known that the Trp64Arg substitution is located in the first intracellular loop of the β3AR, which is thought to be important for the correct movement of the receptor to the cell membrane and coupling to guanine-nucleotide (G) proteins (Walston et al. 1995; Widen et al. 1995). Therefore it was proposed that this mutation could result in defective expression of the β3AR at the cell surface or impaired intracellular signalling. This could cause reduced lipolysis in visceral adipose tissue of affected individuals which may contribute to visceral obesity, insulin resistance and NIDDM (Walston et al. 1995).

Studies of the β3AR mutation have been criticised for inconsistency of findings and, when positive, generally marginal levels of significance (Mauriege and Bouchard 1996). Specifically, the heterogeneity of allele frequencies in different populations (see Table 4.9) and the paucity of homozygous mutants have made conclusive interpretation of these studies difficult (Mauriege and Bouchard 1996). It should be noted that both
obesity and NIDDM are likely to be polygenic diseases, and it is possible that the β3AR could contribute to some degree to the disease processes in at least some ethnic groups. The general inconsistency of findings in different populations suggests that this mutation may have more or less important roles in the pathogenesis of obesity and NIDDM in different populations, depending on the presence or absence of other susceptibility genes for these diseases.

When considering interaction between susceptibility genes in the pathogenesis of obesity and NIDDM, we decided to investigate subgroups in our study populations with various combinations of the two polymorphisms described above. In the Australian population studied, individuals homozygous for the class II allele of the ob gene polymorphism and heterozygous for the β3AR mutation (Trp/Arg) had significantly greater body weight (p=0.05) and waist circumference (p=0.033) compared with those without this genotype (Table 4.8). Waist circumference has previously been shown to be a good indicator of visceral obesity, as determined by more sophisticated methods such as computerised tomography (Lemieux et al. 1996a,b). Previously we had shown no association of the ob gene polymorphism with body weight or waist circumference (Table 4.5), and that heterozygotes for the Trp64Arg mutation exhibited slight tendencies for increased body weight (p=0.09) and waist circumference (p=0.17) compared with wild-type heterozygotes (Table 4.7). When Trp/Arg heterozygotes with the class IV/class II genotype for the ob gene polymorphism were selected (n=19), they were found to have the greatest body weight and waist circumference of this group, with the most significant effect on waist circumference. Therefore in the Australian population studied it is possible that these two genes may have been interacting in the pathogenesis of obesity, and in particular visceral obesity, in individuals homozygous for the class II allele of the ob gene polymorphism and heterozygous for the Trp64Arg mutation of the β3AR. It does not seem unreasonable then to propose the ob and β3AR genes as likely susceptibility genes for the development of visceral obesity in Australians. This genotype was not significantly associated with any aspects of obesity or NIDDM studied in the Nauruan population, possibly due to the small number of individuals exhibiting this genotype. As more of the genetic factors contributing to the pathogenesis of obesity are discovered our understanding of the disease process should become clearer and hopefully new approaches for therapy may result.

In conclusion, we have demonstrated that the ob gene polymorphism studied was associated with aspects of insulin resistance in Nauruans but not Australians, suggesting that this gene may be implicated in the ‘thrifty genotype’ of the Nauruan population. The β3AR Trp64Arg mutation was associated with reduced plasma leptin
concentrations in Nauruans, and increased body weight, hip circumference and obesity in Australians. Interestingly, a combination of these mutations was observed in 19 Australians, who exhibited significantly greater body weight and waist circumference, and therefore visceral obesity, compared with those without this genotype. We propose that both of these genetic mutations may be implicated in the pathogenesis of obesity, insulin resistance and NIDDM.
CHAPTER 5:

THE EFFECT OF DIETARY ENERGY RESTRICTION ON BODY WEIGHT GAIN AND THE DEVELOPMENT OF NON-INSULIN-DEPENDENT DIABETES MELLITUS (NIDDM) IN PSAMMOMYS OBESUS

5.1 SUMMARY

Food intake was restricted to 75% of ad libitum levels in 37 male Psammomys obesus (Israeli Sand Rats) from the age of four weeks (weaning) to ten weeks. Energy restriction reduced the mean bodyweight at ten weeks by 29% compared with 44 ad libitum fed controls. Hyperglycemia was prevented completely in the food restricted group, and mean blood glucose concentrations were significantly reduced (3.8 ± 0.2 vs 5.5 ± 0.4 mmol/l; p<0.05) compared with control animals. Plasma insulin concentrations were also decreased significantly compared with ad libitum fed controls (105 ± 13 vs 241 ± 29 μU/ml; p<0.05). Whilst energy restriction prevented hyperglycemia from developing in ten week old Psammomys obesus, 19% of the food restricted animals still developed hyperinsulinemia. Plasma leptin concentrations were significantly reduced in the energy restricted group of animals compared with those which were fed ad libitum. Although plasma leptin concentrations tended to be higher in hyperinsulinemic energy-restricted animals compared with normoinsulinemic animals in this group, this effect was not statistically significant (p=0.28). We conclude that hyperphagia between four and ten weeks of age may be essential for the development of NIDDM in Psammomys obesus, but that hyperinsulinemia may still occur in the absence of hyperphagia and hyperglycemia, suggesting a significant genetic influence on the development of hyperinsulinemia in this animal model.
5.2 \hspace{0.5cm} \textbf{INTRODUCTION}

The pathophysiology of non-insulin-dependent diabetes mellitus (NIDDM) is poorly understood. The disease results from a complex interaction between genetic and environmental factors, and often forms part of the common metabolic cluster known as Syndrome X (Reaven 1988) or the Metabolic Syndrome (Zimmet 1993), which also includes obesity and other cardiovascular risk factors.

The primary defect in NIDDM is still under dispute, although hyperinsulinemia has been proposed to be the first metabolic sign of the disease (Zimmet 1993). It is possible that this hyperinsulinemia results from insulin resistance in muscle and represents a response to ensure adequate glycogen storage, with a side-effect of altering lipolytic enzyme activity in adipocytes which may lead to obesity (DeFronzo et al. 1992).

Alternatively, moderate sustained hyperinsulinemia may cause insulin resistance, and in turn obesity and/or NIDDM (Zimmet et al. 1991). Jeanrenaud (1995) has suggested that loss of normal oscillation of basal insulin levels, leading to insulin oversecretion in adulthood, may be the primary metabolic defect in obesity and/or NIDDM (Jeanrenaud 1995). Obesity, excess body fat, can be produced by different means, thus it is unlikely that a single metabolic defect is responsible for obesity.

There is a strong association between obesity and hyperinsulinemia, and either or both of these disorders may be induced by increased energy intake in susceptible individuals (Bjorntop 1988; Zimmet et al. 1989). Increased energy intake, without an increase in energy expenditure necessary to maintain a neutral energy balance, may therefore represent one possible mechanism in the etiology of NIDDM.

Plasma leptin concentrations were found to be significantly increased in obese humans (Maffei et al. 1995a; Considine et al. 1996a; Haffner et al. 1996a,b; Zimmet et al. 1996) and in most animal models of obesity (Frederich et al. 1995a; Maffei et al. 1995a). In all cases, plasma leptin concentrations correlated significantly with BMI and/or body fat mass, supporting the theory that leptin acts as a 'lipostat' to regulate energy balance via the hypothalamus, resulting in body weight homostasis (Kennedy 1953; Zhang et al. 1994). In addition, epidemiological studies have revealed a strong relationship between plasma leptin and insulin concentrations (Zimmet et al. 1996; de Courten et al. 1997), and plasma leptin concentrations were significantly elevated in lean, insulin-resistant men compared with insulin-sensitive men of similar body composition (Segal et al. 1996). Furthermore, in vitro studies showed that leptin attenuated insulin action in isolated hepatocytes (Cohen et al. 1996) and impaired insulin secretion from perfused pancreata (Emilsson et al. 1997). These studies suggest
the possibility of a physiological role for leptin in the development of insulin resistance and hyperinsulinemia, and implicate leptin as a possible link between obesity and NIDDM.

Previous studies which have utilised caloric restriction to reduce body fat content found parallel reductions in plasma leptin concentrations in humans (Maffei et al. 1995a; Considine et al. 1996a; Kohrt et al. 1996) and animal models of obesity (Cusin et al. 1995; Maffei et al. 1995b). Thus it appeared that leptin was acting as a 'lipostat' to reflect body fat mass, and that the long-term changes in body fat content induced by caloric restriction resulted in concomitant reductions in plasma leptin concentrations.

Studies in certain populations considered to have a heightened genetic predisposition to NIDDM, including Australian aborigines, Pima Indians and Pacific Islanders, have found a significant increase in the prevalence of NIDDM associated with the adoption of a "Westernised" lifestyle (Zimmet et al. 1979; Bogardus et al. 1984; Coventry et al. 1986; McKeigue et al. 1991). That is, a sedentary style of life with a steady supply of readily available energy-dense food. As energy intake increases and energy expenditure falls, the prevalence rates of both obesity and NIDDM have been shown to be greatly increased (Zimmet 1982), and these conditions have now attained epidemic proportions in many newly industrialised nations (Zimmet 1995).

Research into the etiology of NIDDM is made difficult due to the insidious nature, late onset and associated mortality of the disease. Studies in the USA have shown that there may be as many undiagnosed cases of NIDDM as diagnosed (Warram et al. 1994). Most cases of NIDDM are first diagnosed in subjects older than 40 years, so obtaining multigenerational affected family groups for study is very difficult. Animal models that develop NIDDM quickly and can be housed in a controlled environment provide an excellent opportunity to study the pathophysiology of the disease.

*Psammomys obesus* (the Israeli sand rat) is a unique animal model of dietary-induced obesity and NIDDM. In its natural desert habitat, an active lifestyle and saltbush diet ensure that they remain lean and normoglycemic (Shafrir and Gutman 1993; Shafrir 1996). However, in a laboratory setting on a diet of *ad libitum* chow (on which many other animal species remain healthy), a range of pathophysiological responses are seen (Kalderon et al. 1986; Barnett et al. 1994a). By the age of 16 weeks, more than half of the animals become obese, and approximately one third develop NIDDM. Only hyperphagic animals went on to develop hyperglycemia, highlighting the importance of excessive energy intake in the pathophysiology of NIDDM in *Psammomys obesus* (Barnett et al. 1995). Other phenotypes found include hyperinsulinemia, dyslipidemia
and impaired glucose tolerance (Barnett et al. 1994a; Barnett et al. 1995; Habito et al. 1995). *Psammomys obesus* exhibit a range of bodyweight and blood glucose and insulin levels which forms a continuous curve, and closely resembles the patterns found in human populations, including the inverted U-shaped relationship between blood glucose and insulin levels known as “Starling’s curve of the pancreas” (DeFronzo 1988; Barnett et al. 1994a). In Chapter 3 it was shown that plasma leptin concentrations were significantly correlated with body weight, percentage body fat and plasma insulin concentrations in *Psammomys obesus*. In addition, insulin resistance was associated with elevated plasma leptin concentration independent of body weight, similar to what has been found in human studies (Segal et al. 1996; Zimet et al. 1996; de Courten et al. 1997). Therefore we consider *Psammomys obesus* to be a good model in which to study the physiology of leptin and its role in the pathogenesis of obesity and NIDDM.

The aim of this study was to investigate the effect of energy restriction from weaning to ten weeks of age on bodyweight gain, plasma leptin concentrations and the development of NIDDM in *Psammomys obesus.*
5.3 MATERIALS AND METHODS

5.3.1 BREEDING OF THE COLONY

A *Psammomys obesus* colony is maintained at Deakin University, with the breeding pairs fed *ad libitum* a diet of lucerne and chow. Experimental animals were weaned at four weeks of age and given a diet of standard laboratory chow from which 12% of energy was derived from fat, 63% from carbohydrate and 25% from protein (Barastoc, Pakenham, Australia). Animals were housed in a temperature controlled room (22 ± 1°C) with a 12-12-hour light-dark cycle (light 0600-1800; dark 1800-0600).

5.3.2 EXPERIMENTAL PROTOCOL

The average daily food intake of 44 *Psammomys obesus* (20 males and 24 females) was calculated at four, eight and ten weeks of age. The animals were housed individually and food intake estimated by rate of disappearance over a 72 hour period (spillage was prevented by appropriate cage design). During the same period, bodyweight was recorded and blood samples obtained for determination of blood glucose and plasma insulin concentrations. Based on measurement of weekly food intake in *ad libitum* fed *Psammomys obesus*, 37 newly weaned animals (20 males and 17 females) were restricted to 75% of the average daily *ad libitum* intake levels for a period of six weeks. During this period, bodyweight measurements and blood samples were taken at four, eight and ten weeks of age. The food restricted animals were given their daily allocation of food each morning at 8 am. Blood was collected in heparinised tubes at 11 am for both groups of animals, ensuring that measurements for both groups were in the fed state. All experiments were carried out following the Australian NHMRC principles of laboratory animal care and approved by the Deakin University Animal Ethics Committee.

5.3.3 CLASSIFICATION OF ANIMALS

*Psammomys obesus* were categorised into three groups according to their blood glucose and plasma insulin concentrations, taken in the fed state at 11 am at ten weeks of age:

- **Group A**
  - normoglycemic (blood glucose < 8.0 mmol/l)
  - normoinsulinemic (plasma insulin < 150 µU/ml)

- **Group B**
  - normoglycemic (blood glucose < 8.0 mmol/l)
  - hyperinsulinemic (plasma insulin ≥ 150 µU/ml)
Group C  - hyperglycemic (blood glucose ≥ 8.0 mmol/l)
- hyperinsulinemic (plasma insulin ≥ 150 μU/ml)

The criteria for classification of animals into groups were based on those of Barnett *et al.* (1994a), who characterised the stages of development of the obesity/diabetes syndrome in this species (Barnett *et al.* 1994a).

### 5.3.4 ANALYTICAL METHODS

Whole blood glucose was measured using an enzymatic glucose analyser (Model 27, 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 in all animals at the completion of the study. Plasma leptin levels were measured in collaboration with Dr. Margery Nicolson at Amgen Inc., Thousand Oaks, USA using a solid phase double enzyme immunoassay (EIA) with affinity purified polyvalent antibodies. Concentrations were calculated from standard curves generated with recombinant murine leptin. The limits of detection for the leptin EIA were 20 pg/ml of serum or plasma. The inter-assay CV was 7.7% for the high standard and 10.5% for the low standard.

### 5.3.5 STATISTICAL ANALYSES

All experimental data are expressed as means ± s.e.m. A one-way analysis of variance in combination with a Tukey’s multiple comparison test was used to compare means between and within groups, and a two-sample unpaired t-test was used where appropriate. In all instances probability values of <0.05 were considered significant.
5.4 RESULTS

5.4.1 CALCULATION OF FOOD RESTRICTION

Average daily food intake was calculated in 46 *ad libitum* fed animals at four, eight and ten weeks of age (refer to Figure 5.1). From this data a line of best fit was calculated for daily food intake during this 6 week period. Daily food intake for the food restricted animals was calculated as 75% of the *ad libitum* levels using the line of best fit.
**FIGURE 5.1:** A) Mean *ad libitum* food intake in *Psammomys obesus* at weaning (4 weeks), 8 weeks and 10 weeks of age. B) Average food intake per day in *ad libitum* fed animals (upper curve), and calculated food intakes for restricted animals (lower curve), based on 75% of *ad libitum* intake. The line of best fit (food intake = 0.96(age) + 6.97; \( r^2 = 0.976; p < 0.05 \) was calculated using Minitab statistical software, and the daily food allocation for the food restricted animals was calculated from this equation.
5.4.2 CLASSIFICATION OF ANIMALS

Figure 5.2 shows the distribution to groups of *ad libitum* and food restricted animals. The *ad libitum* fed animals were classified as group A 45% (normoglycemic and normoinsulinemic), group B 41% (normoglycemic and hyperinsulinemic) and group C 14% (hyperglycemic and hyperinsulinemic). No food restricted animals were classified as group C, 19% were group B and 81% group A. Table 5.1 summarises the data found in each group of animals. No significant differences were found for any of the variables measured between males and females in either the food restricted or *ad libitum* fed animals.

**FIGURE 5.2:** Distribution into groups of *ad libitum* fed and food restricted *Psammomys obesus* based on classification at 10 weeks. Animals were classified as follows: Group A - normoglycemic (whole blood glucose < 8 mmol/l) and normoinsulinemic (plasma insulin < 150 mU/l), Group B - normoglycemic and hyperinsulinemic, Group C - hyperglycemic and hyperinsulinemic.
<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Bodyweight (g)</th>
<th>Insulin (µU/ml)</th>
<th>Glucose (mmol/l)</th>
<th>Leptin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group A ad libitum</strong></td>
<td>20</td>
<td>161 ± 6</td>
<td>76 ± 10</td>
<td>3.8 ± 0.1</td>
<td>58.7 ± 7.9</td>
</tr>
<tr>
<td><strong>Group B ad libitum</strong></td>
<td>17</td>
<td>172 ± 4</td>
<td>292 ± 31</td>
<td>4.5 ± 0.3</td>
<td>88.4 ± 10.0</td>
</tr>
<tr>
<td><strong>Group C ad libitum</strong></td>
<td>7</td>
<td>193 ± 5</td>
<td>589 ± 81</td>
<td>12.6 ± 1.3</td>
<td>82.3 ± 21.2</td>
</tr>
<tr>
<td><strong>Total ad libitum</strong></td>
<td>44</td>
<td>170 ± 5</td>
<td>241 ± 29</td>
<td>5.5 ± 0.4</td>
<td>75.1 ± 6.4</td>
</tr>
<tr>
<td><strong>Group A restricted</strong></td>
<td>30</td>
<td>120 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73 ± 7</td>
<td>3.8 ± 0.2</td>
<td>37.0 ± 3.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Group B restricted</strong></td>
<td>7</td>
<td>123 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>240 ± 40</td>
<td>3.8 ± 0.3</td>
<td>56.1 ± 16.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Total restricted</strong></td>
<td>37</td>
<td>121 ± 3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>105 ± 13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.8 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>40.8 ± 4.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> significantly less than Group A ad libitum (p<0.05)
<sup>b</sup> significantly less than Group B ad libitum (p<0.05)
<sup>c</sup> significantly less than Total ad libitum (p<0.05)

**Table 5.1:** Bodyweight, insulin, glucose and leptin data for each group of animals measured at ten weeks of age. All data expressed as mean ± sem, n = number of animals in that group.

### 5.4.3 Body Weight

After food restriction from four to ten weeks of age to 75% of *ad libitum* feeding, the mean body weight was decreased by 29% (170 ± 5 g *ad libitum* fed (n=44) vs. 121 ± 3 g food restricted (n=37); p<0.05). There was no significant difference in body weight between the two groups at weaning (4 weeks of age), but the *ad libitum* fed group had a significantly higher mean body weight at both 8 and 10 weeks of age (Figure 5.3). The change in body weight from week 4 to week 10 was also significantly reduced in the food restricted animals (63.4 ± 1.6 g) compared to the *ad libitum* fed animals (105.9 ± 3.3 g; p<0.05). After 6 weeks of food restriction, the mean body weight of group A restricted animals (n=30; 120 ± 2 g) was significantly lower than that of group A *ad libitum* fed animals (n=20; 161 ± 6 g; p<0.05). Similarly, the mean body weight of
group B energy restricted animals (n=7; 123 ± 5 g) was significantly less than group B ad libitum fed animals (n=17; 172 ± 4 g; p<0.05). Body weights were not significantly different between energy restricted group A and energy restricted group B animals. Within the ad libitum fed group of animals, body weight was significantly correlated with daily food intake (r=0.618, p<0.05).

**FIGURE 5.2:** Body weight of *Psammomys obesus* at weaning, 8 weeks and 10 weeks of age (mean ± s.e.m.). Ad libitum fed animals had significantly greater mean body weight at both 8 and 10 weeks of age. (*significantly greater than restricted group (p<0.01), calculated using an unpaired t-test).
There was no difference in mean whole blood glucose concentrations between the two groups at weaning, but the *ad libitum* fed group had significantly higher concentrations at both 8 and 10 weeks of age (Figure 5.4). After 6 weeks of food restriction, the mean whole blood glucose concentrations were significantly different between the two groups of *Psammomys obesus* (*ad libitum* fed 5.5 ± 0.4 mmol/l vs. food restricted 3.8 ± 0.2 mmol/l; *p*<0.05). None of the food restricted animals had a blood glucose level of greater than 8 mmol/l, the cutoff for hyperglycemia in our classification system for *Psammomys obesus*. Figure 5.5 shows the correlation between glucose and insulin concentrations in *ad libitum* fed and food restricted *Psammomys obesus*.

**Figure 5.4**: Whole blood glucose concentration of *Psammomys obesus* groups at weaning, 8 weeks and 10 weeks of age (mean ± s.e.m.). *Ad libitum* fed animals had significantly higher mean blood glucose concentration at both 8 and 10 weeks of age. (*â‡¥significantly greater than restricted group (p<0.01), calculated using an unpaired *t*-test).
**FIGURE 5.5:** Relationship between plasma insulin and blood glucose concentrations in *ad libitum* fed and food restricted *Psammomys obesus* measured at 10 weeks of age. The *ad libitum* fed animals displayed a range of glucose and insulin concentrations, whereas none of the restricted animals were hyperglycemic and only a small number were hyperinsulinemic.

### 5.4.5 PLASMA INSULIN

At weaning, no significant difference was found between the mean plasma insulin concentrations of the two groups, however the *ad libitum* fed group had significantly higher insulin concentrations at both 8 and 10 weeks of age (Figure 5.6). After 6 weeks of food restriction, mean plasma insulin concentrations were significantly reduced in the energy restricted animals (105 ± 13 μU/ml) compared with *ad libitum* fed animals (241 ± 29 μU/ml; *p*<0.05). The correlation between plasma insulin and blood glucose concentrations (Figure 5.4) demonstrates the significant reduction in insulin concentrations seen after food restriction. However it is notable that 7/37 (19%) of the food restricted animals still became hyperinsulinemic according to our system of classification (240 ± 40 mU/l) despite food restriction and in the absence of hyperglycemia. In the *ad libitum* fed group of animals plasma insulin concentrations
correlated significantly with both body weight ($r=0.363$, $p<0.05$) and daily food intake ($r=0.379$, $p<0.05$).

**FIGURE 5.6:** Plasma insulin concentration in *Psammomys obesus* groups at weaning, 8 weeks and 10 weeks of age (mean ± s.e.m.). *Ad libitum* fed animals had significantly elevated mean insulin concentrations at both 8 and 10 weeks of age. (*significantly greater than restricted group ($p<0.01$), calculated using an unpaired t-test).
5.4.6 **PLASMA LEPTIN**

Plasma leptin concentrations were significantly elevated in the *ad libitum* fed animals (75.1 ± 6.4 ng/ml) compared with the energy restricted group (40.8 ± 4.2 ng/ml; p<0.05; Table 1). Within the *ad libitum* fed group of animals, plasma leptin concentrations were significantly correlated with plasma insulin concentration (r=0.584, p<0.05), body weight (r=0.369, p<0.05) and daily food intake (r=0.562, p<0.05; Figure 5.7). Within the energy restricted group of animals, plasma leptin concentrations did not correlate with any of the other parameters measured.
**FIGURE 5.7:** Correlations of plasma leptin concentrations in the *ad libitum* group of animals with A) plasma insulin concentration, B) body weight and C) daily food intake.
5.5 DISCUSSION

In this study we have shown that restriction of food intake to 75% of *ad libitum* levels from four to ten weeks of age prevents excessive body weight gain and the development of NIDDM in *Psammomys obesus*. The data suggest that hyperphagia during this period is necessary for excessive bodyweight gain and NIDDM to occur, but that hyperinsulinemia may develop in the absence of excessive energy intake.

Previously, we have demonstrated that food intake in mature diabetic *Psammomys obesus* is significantly higher than in matched normoglycemic animals (Barnett *et al*. 1994b), and that there is no difference in energy expenditure between groups A, B and C during the period from four to ten weeks of age as measured by spontaneous use of an exercise wheel (Collier *et al*. 1997). These data strongly suggest that hyperphagia from an early age is the main environmental factor contributing to the development of obesity and NIDDM in *Psammomys obesus*. We have also shown that energy restriction for two weeks (to 67% of *ad libitum* feeding) in mature (12-14 weeks old) obese, diabetic *Psammomys obesus* reduces hyperglycemia and improves both insulin and glucose responses to oral glucose loads (Barnett *et al*. 1994b). Similarly, energy restriction reduced insulin resistance and restored normal glucose tolerance in ob/ob mice (Duboc *et al*. 1984) and streptozotocin-treated mice (Hasegawa *et al*. 1990). However the current study was aimed at assessing the role of dietary restriction in the prevention of excessive body weight gain and NIDDM.

The results of the present study conclusively demonstrated that dietary restriction from four to ten weeks of age had a significant effect on bodyweight gain in *Psammomys obesus*, with a mean reduction of 29% compared with control animals at ten weeks of age. It is reasonable to conclude that none of the food restricted animals would have developed obesity on a diet of 75% of the mean *ad libitum* food intake. Whilst none of the animals in the wild become obese and/or diabetic, approximately half of the animals in the laboratory (by 16 weeks of age) become obese, and about one third diabetic, whereas a third of the animals do not become hyperphagic and remain lean and non-diabetic (Barnett *et al*. 1994a,b). It appears that in some animals there is a strong genetic influence causing them to overeat whenever food is available, consistent with Neel’s “thrifty genotype” hypothesis (Neel 1962). That is, in the wild natural selection in *Psammomys obesus* would be expected to favor those animals which are best able to survive and reproduce on the meagre diet provided by their natural environment. When taken into the laboratory and fed relatively energy dense laboratory chow, these animals with the ‘thrifty gene’ would be expected to consume, store and utilise this energy very efficiently, resulting in the development of obesity and NIDDM.
Hyperphagia is found in most animal models of obesity including the Zucker fatty rat (Bray and York 1972), ob/ob mouse (Ingalls et al. 1950), db/db mouse (Hummel et al. 1966), KK mouse (Ikeda 1994) and NZO mouse (Proietto and Larkins 1993). We have previously shown that adult obese Psammomys obesus are hyperphagic compared to lean controls (Barnett et al. 1994b), and found in this study that the trend to eat more begins immediately after weaning in animals that eventually become obese.

Studies in animal models have suggested that in the etiology of obesity a primary central nervous system abnormality could alter regulation of the autonomic nervous system with reduced sympathetic and increased parasympathetic activity (Jeanrenaud 1995). The central nervous system defect could involve dysregulation of neuropeptide Y, a powerful appetite stimulant, which is found to be increased in most genetically obese rodents (Jeanrenaud 1995). Alternatively, the recently discovered ob (obese) gene (Zhang et al. 1994), which encodes a protein (leptin), may be important in the regulation of food intake and bodyweight homeostasis (Maffei et al. 1995a). The ob gene has been shown to be overexpressed in obesity (Hamilton et al. 1995; Lonnerqvist et al. 1995), and administration of leptin to obese animals, deficient in functional leptin, caused a rapid decrease in food intake and bodyweight (Halaas et al. 1995; Pelleymouner et al. 1995). We have found that the ob gene is expressed in Psammomys obesus (see Chapter 3), and sequence analysis failed to reveal the stop codon mutation observed in obese ob/ob mice (Zhang et al. 1994). The hyperphagia and obesity observed in Psammomys obesus could be due to dysregulation of leptin and/or NPY in these animals, and requires further investigation.

Dietary energy restriction significantly reduced plasma leptin concentrations in Psammomys obesus when compared with ad libitum fed animals. The energy restricted group also had significantly lower body weight than the ad libitum fed animals (Figure 5.3). Although body fat content was not measured in this study it is reasonable to assume that body fat would have been significantly lower in the energy restricted animals. Therefore, leptin appeared to be acting as a 'lipostat' in Psammomys obesus, in concordance with results found in other animals (Frederich et al. 1995a; Maffei et al. 1995a) and humans (Maffei et al. 1995a; Considine et al. 1996a; Haffner et al. 1996a,b; Zimmet et al. 1996). In addition, plasma insulin concentrations, which have previously been strongly correlated with plasma leptin levels in humans (Zimmet et al. 1996; de Courten et al. 1997) were also significantly reduced in the energy restricted group of animals compared with ad libitum fed Psammomys obesus (Figure 5.6).

The Group B energy restricted animals were hyperinsulinemic and tended to be hyperleptinemic (56.1 ± 16 ng/ml) compared with Group A energy restricted animals
(37.0 ± 3.4 ng/ml, p=0.28). There was no difference in body weight or blood glucose concentration between these two groups (Table 5.1). Therefore it is possible that the hyperinsulinemia was associated with a tendency to increase plasma leptin in these animals independent of body weight, suggesting that insulin may be important in the long-term control of circulating leptin levels in *Psammomys obesus*. This hypothesis is consistent with results of studies in other rodents and humans (Cusin *et al.* 1995; Kolaczynski *et al.* 1996a; Sainsbury *et al.* 1996; Zheng *et al.* 1996), and with our previous results presented in Chapter 3.

It would be interesting to conduct future studies which investigate the significance of the elevated plasma insulin and leptin concentrations in energy restricted animals. For example, would this subgroup of animals be more or less prone to excessive body weight gain if placed on an *ad libitum* diet after energy restriction from weaning to 10 weeks of age? A recent prospective study in Pima Indians found that low plasma leptin levels were predictive of excessive weight gain (Ravussin *et al.* 1997). This suggests that the energy restricted animals that developed hyperinsulinemia and had increased plasma leptin would be expected to be resistant to the development of obesity, presumably due to reduced food intake and increased energy expenditure resulting from increased circulating leptin.

In the *ad libitum* fed group of *Psammomys obesus* plasma leptin concentrations were significantly correlated with body weight, plasma insulin concentrations and daily food intake (Figure 5.7). However, in the energy restricted group there was no correlation between plasma leptin concentration and any of these variables. Therefore, energy restriction from weaning to 10 weeks of age in *Psammomys obesus* resulted in significant dysregulation of circulating plasma leptin.

Although energy restriction completely prevented the development of hyperglycemia in this study, the influence on plasma insulin was not as pronounced (refer to Figure 5.5). Despite a significant reduction in the mean plasma insulin concentration of the energy restricted group, 19% of the animals developed hyperinsulinemia (240 ± 40 μU/ml) during the six weeks of food restriction, compared to 60% of *ad libitum* fed animals (379 ± 46 μU/ml). This suggests that while energy restriction significantly reduces the incidence of hyperinsulinemia, there are some animals in which genetic factors are sufficient to cause hyperinsulinemia even in the absence of deleterious environmental influences, such as hyperphagia. We have previously shown that plasma insulin concentration is positively associated with bodyweight in *Psammomys obesus* (Barnett *et al.* 1994a), however there was no significant correlation between hyperinsulinemia and bodyweight in the food restricted animals. In a similar study in Rhesus monkeys,
energy restriction to prevent obesity completely prevented the development of hyperinsulinemia (Hansen and Bodkin 1993). These data suggest that the genetic influence on the development of hyperinsulinemia is stronger in *Psammomys obesus*, while in the Rhesus monkey the effect of diet and other environmental factors may be relatively more important.

There is some debate over whether hyperinsulinemia or insulin resistance is the primary metabolic defect in the development of NIDDM (Zimmet 1993). One group argues that a small rise in insulin concentrations could eventually lead to insulin resistance in target tissues, thereby causing a further increase in insulin levels and creating a vicious circle which leads to NIDDM (Zimmet et al. 1991). Others suggest that the initial defect is peripheral insulin resistance, which necessitates an increase in circulating insulin to overcome it, and thus the same circle of events ensues, leading to NIDDM. Hyperinsulinemia may precede the development of NIDDM by many years (Zimmet et al. 1991), and studies in animal models such as rhesus monkeys (Hansen and Bodkin 1986; Hansen and Bodkin 1990) and ventromedial hypothalamic lesioned rats (Jeanrenaud et al. 1985) support the theory that primary hyperinsulinemia may result from a disruption of central nervous system homeostatic control (Zimmet 1992; Jeanrenaud 1995). Relative hyperinsulinemia has also been demonstrated in numerous populations at high risk for developing NIDDM (Savage et al. 1975; Zimmet et al. 1979; Haffner et al. 1988; O'Dea et al. 1988; Boyko et al. 1991; McKeigue et al. 1991). Together these data support the theory that a genetic influence causing hyperinsulinemia could be a primary causative factor leading to NIDDM. If this is true, our findings suggest that this genetic predisposition to developing hyperinsulinemia can cause that phenotype even in the absence of hyperphagia. Conversely, it is conceivable that insulin resistance is the primary defect in NIDDM, and that this can still occur without hyperphagia.

As the genetic causes of NIDDM are discovered, early genetic testing in high-risk groups, such as offspring of NIDDM patients, may enable targeted preventative intervention. Strategies will need to be developed for the implementation of measures designed to reduce the probability of NIDDM developing. Our results suggest that dietary control from an early age may be successful in significantly delaying or even preventing obesity and hyperglycemia, but additional measures may be necessary to prevent hyperinsulinemia and to alleviate insulin resistance over a greater period of time.

The results of this study suggest that long-term regulation of leptin in *Psammomys obesus* is similar to that observed in other rodents and humans in that it is controlled by
body fat mass and plasma insulin concentration. Long-term caloric restriction reduced body weight, plasma insulin and leptin concentrations in *Psammomys obesus*. Apart from the role of leptin as a long-term sensor of body fat mass and regulator of energy balance, several studies have suggested that leptin may be important in the physiological response to short-term perturbations of energy balance such as fasting (Maffei *et al.* 1995b; Ahima *et al.* 1996; Kolaczynski *et al.* 1996b). In the next part of this study we investigated the leptin response to a 24-h fast in *Psammomys obesus* with a range of body weight and blood glucose and insulin concentrations.
CHAPTER 6:

DYSREGULATION OF LEPTIN IN RESPONSE TO FASTING IN INSULIN-RESISTANT PSAMMOMYS OBESUS (ISRAELI SAND RATS)

6.1 SUMMARY

Leptin is thought to play a significant role in energy balance as an afferent signal to the hypothalamus which reflects body fat content. In addition, leptin may also act as an acute sensor of energy balance independent of body fat mass, as ob gene expression and plasma leptin concentrations are decreased in lean animals and humans in response to short-term caloric deprivation. However, in obese animals and humans the acute response of leptin to fasting is less clear.

We investigated the effects of a 24-hour fast on circulating plasma leptin concentrations in lean and obese Psammomys obesus (Israeli Sand Rats). In the lean, insulin-sensitive animals (n=25) a 24-hour fast caused a 44% decrease in plasma leptin, whereas in the obese, insulin-resistant group (n=24) plasma leptin increased by 18% after fasting (p<0.003). There was no difference between the two groups regarding the effect of a 24-hour fast on body weight, blood glucose or plasma insulin concentrations. Within the insulin-resistant group of animals, there was no difference in the response of leptin to fasting between hyperglycemic and normoglycemic animals. We conclude that there is a dysregulation of leptin in response to acute caloric deprivation in obese, insulin-resistant, but not lean, insulin-sensitive Psammomys obesus.
6.2 INTRODUCTION

Leptin, the product of the *ob* gene, is secreted exclusively from adipocytes and is thought to play an important role in the regulation of energy metabolism and body weight (Zhang *et al.* 1994). The hyperphagia, obesity and insulin resistance which characterise the *ob/ob* mouse phenotype were all rapidly reversed by leptin administration, which also caused significant weight loss in wild-type mice (Campfield *et al.* 1995; Halaas *et al.* 1995; Pelleymounter *et al.* 1995).

Numerous studies in both humans (Considine *et al.* 1995; Considine *et al.* 1996a; Hamilton *et al.* 1995; Lonnqvist *et al.* 1995) and animal models of obesity other than *ob/ob* mice (Campfield *et al.* 1995; Halaas *et al.* 1995; Murakami *et al.* 1995; Pelleymounter *et al.* 1995; Stephens *et al.* 1995) demonstrated that *ob* gene expression and plasma leptin concentrations reflect body fat mass, and suggest that leptin may act as an afferent signal to the brain influencing energy balance. Long-term energy restriction in both humans and rodents which reduced body fat content also resulted in parallel decreases in plasma leptin concentrations (Maffei *et al.* 1995a; Considine *et al.* 1996a).

The proposal that leptin plays an important role in the control of energy balance and utilisation has led to interest in the short-term regulation of *ob* gene expression and circulating plasma leptin concentration. In wild-type mice, fasting for 48-72 hours decreased plasma leptin levels by 60-70% (Maffei *et al.* 1995b; Ahima *et al.* 1996), while *ob* gene expression fell by 85% after a 16-hour fast (MacDougald *et al.* 1995) and 90% after a 48-hour fast (Mizuno *et al.* 1996). In lean control rats, fasts of 16-72 hours duration decreased plasma leptin levels by 50-80% (Cusin *et al.* 1995; MacDougald *et al.* 1995; Saladin *et al.* 1995; Sivitz *et al.* 1996). Similarly, in lean humans, fasting for 24-60 hours caused a 60-70% reduction in plasma leptin (Boden *et al.* 1996; Kolaczynski *et al.* 1996b; Sinha *et al.* 1996). It appears that fasting significantly reduces *ob* gene expression and plasma leptin in lean, healthy animals and humans.

In obesity the response of leptin to fasting is less clear. Fasting did not significantly reduce *ob* gene expression in *ob/ob* mice after 16 hours, in diet-induced obese mice after 48 hours, or in *fa/fa* rats after 72 hours (Cusin *et al.* 1995; MacDougald *et al.* 1995; Mizuno *et al.* 1996). However, several small studies in obese humans have shown that fasting for 24-60 hours reduced circulating plasma leptin concentration by 42-88% (Boden *et al.* 1996; Kolaczynski *et al.* 1996b; Sinha *et al.* 1996). In this study we investigated the response of leptin to a 24-hour fast in a polygenic, heterogeneous model of obesity and NIDDM, *Psammomys obesus* (the Israeli Sand Rat).
*Psammomys obesus* is a unique animal model of obesity and NIDDM. *Psammomys obesus* remains lean and normoglycemic in the wild on its natural low energy diet (Shafrir and Gutman 1993). However when taken into the laboratory and fed *ad libitum* chow, a relatively energy dense food, the animals exhibit a range of pathophysiological responses, with approximately half of the animals becoming obese, whilst about one third develop NIDDM (Barnett *et al.* 1994a; Barnett *et al.* 1995). *Psammomys obesus* exhibit a range of bodyweight, and blood glucose and insulin concentrations which form a continuous curve, and closely resembles the pattern found in human populations (DeFronzo 1988; Barnett *et al.* 1994a). We have previously shown that plasma leptin concentrations reflect body fat mass and plasma insulin concentrations in *Psammomys obesus*, and that long-term energy restriction reduced plasma leptin in these animals.

The aim of this study was to investigate the response of leptin to a 24-hour fast in *Psammomys obesus* with a wide range of body weights and circulating glucose and insulin concentrations.
6.3 MATERIALS AND METHODS

6.3.1 BREEDING OF THE COLONY

A *Psammomys obesus* colony is maintained at Deakin University, with the breeding pairs fed *ad libitum* a diet of lucerne and standard laboratory chow. Experimental animals were weaned at four weeks of age and sustained on a diet of standard laboratory chow from which 12% of energy was derived from fat, 63% from carbohydrate and 25% from protein (Barastoc, Pakenham, Australia). Animals were housed in a humidity and temperature controlled room (22 ± 1°C) with a 12-12-hour light-dark cycle. The animals used in the study were aged 12-14 weeks of age when fasted.

6.3.2 EXPERIMENTAL PROTOCOL

The animals (n=49) were weighed and blood collected from the tail vein in the fed state. They were then fasted for 24 hours before being weighed again, and blood was collected again in the fasted state. The animals were then followed for a further 28 days with *ad libitum* access to food and water before being weighed and bled again. All blood was collected into heparinised tubes. All experiments were carried out following the Australian NHMRC principles of laboratory animal care and approved by the Deakin University Animal Ethics Committee.

6.3.3 ANALYTICAL METHODS

Whole blood glucose was measured immediately using an enzymatic glucose analyser (Model 27, Yellow Springs Instruments, Ohio). Plasma insulin concentrations were determined using a double antibody solid phase radioimmunoassay (Phadeseph, Kabi Pharmacia Diagnostics, Sweden). Plasma leptin levels were measured in collaboration with Amgen Inc. (Thousand Oaks, USA) using a solid phase double enzyme immunoassay (EIA) with affinity purified polyclonal antibodies. Concentrations were calculated from standard curves generated with recombinant murine leptin. The limits of detection for the leptin EIA were 20 pg/ml of serum or plasma. The inter-assay CV was 7.7% for the high standard and 10.5% for the low standard.
6.3.4 **STATISTICAL ANALYSIS**

All experimental data are expressed as means ± s.e.m. A one-way analysis of variance in combination with a Tukey's multiple comparison test was used to compare means between and within groups, and a paired t-test was used where appropriate. In all instances probability values of <0.05 were considered significant.

6.4 **RESULTS**

Significant correlations were found between fasting plasma leptin concentration and fasting body weight ($r=0.502; p<0.001$) and fasting leptin and insulin concentrations ($r=0.737; p<0.001$; Figure 6.1A). The corresponding correlations in the fed state were also statistically significant; fed leptin and body weight ($r=0.545; p<0.001$), fed leptin and insulin ($r=0.665; p<0.001$; Figure 6.1B).
FIGURE 6.1: A) Correlations between fasting plasma leptin concentrations and body weight and plasma insulin concentrations in *Psammomys obesus* (n=49).

B) Correlations between plasma leptin concentrations in the fed state and body weight and fed plasma insulin concentrations in *Psammomys obesus* (n=49).
The animals were separated into two groups based on their fasting plasma insulin concentrations, as a surrogate measure of insulin sensitivity. The Insulin-Resistant group (n=24) had fasting plasma insulin concentrations of greater than 30 μU/ml, ranging up to 755 μU/ml. The corresponding values in the Insulin-Sensitive animals (n=25) ranged from 5-25 μU/ml.

The response to a 24-hour fast in the two groups is detailed in Table 6.1, while the percentage changes in variables are summarised in Figure 6.2. The fast caused small but significant decreases in body weight in both groups (-1.8% in both groups; the change in body weight induced by fasting was not significantly different between the two groups (p=0.621)). Blood glucose concentration also fell significantly in both groups (Table 1), by 12% in the Insulin-Sensitive group and by 27% in the Insulin-Resistant animals. Once again, the change in blood glucose was not significantly different between the two groups (p=0.057).
**FIGURE 6.2:** Percentage changes in body weight, blood glucose, plasma insulin and leptin concentrations in the Insulin-Sensitive (n=25) and Insulin-Resistant (n=24) groups of *Psammomys obesus* after a 24-hour fast (*p=0.003 compared with the insulin-sensitive group).*
TABLE 6.1: Effects of a 24-hour fast on body weight, blood glucose, plasma insulin and leptin concentrations in the Insulin-Sensitive (n=25) and Insulin-Resistant (n=24) groups of *Psammomys obesus*. Data are expressed as mean ± sem, probability (p) values are given for the change within groups.

<table>
<thead>
<tr>
<th></th>
<th>Bodyweight</th>
<th>Glucose</th>
<th>Insulin</th>
<th>Leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>INSULIN-</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SENSITIVE</td>
<td>fed</td>
<td>167.6 ± 7.1</td>
<td>4.01 ± 0.14</td>
<td>55.3 ± 15.4</td>
</tr>
<tr>
<td>(n=25)</td>
<td>fasted</td>
<td>164.5 ± 7.5</td>
<td>3.54 ± 0.12</td>
<td>15.1 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>0.002</td>
<td>0.005</td>
<td>0.015</td>
</tr>
<tr>
<td><strong>INSULIN-</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RESISTANT</td>
<td>fed</td>
<td>214.2 ± 5.7</td>
<td>7.44 ± 0.81</td>
<td>305.4 ± 55.4</td>
</tr>
<tr>
<td>(n=24)</td>
<td>fasted</td>
<td>210.3 ± 6.0</td>
<td>5.43 ± 0.83</td>
<td>143.3 ± 34.3</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>0.005</td>
<td>0.018</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Plasma insulin concentrations fell significantly in both groups, by 73% and 53% respectively (Figure 6.2). The absolute difference between the two groups was statistically significant (p=0.001), however the grouping of the animals ensured that the Insulin-Resistant group would have a far greater mean fed plasma insulin concentration. When the percentage changes in plasma insulin concentration was analysed there was not a significant difference between the two groups (Figure 6.2).
Plasma leptin concentrations decreased significantly after a 24-hour fast in the Insulin-Sensitive group. The 44% drop was highly significant (p<0.001), with all of the animals having a decrease in plasma leptin after the fast. Conversely, plasma leptin did not decrease in the Insulin-Resistant group of animals, in fact, they showed a tendency to increase their plasma leptin concentration after a 24-hour fast. Overall, the Insulin-Resistant group had an 18% increase in circulating leptin, which was significantly different to the Insulin-Sensitive group (p=0.003; Figure 6.2).

The animals were also investigated after 4 weeks of ad libitum access to food and water following this fast. No significant differences were found between the two groups in terms of body weight, glucose, insulin and leptin changes over this period. Additionally, there were no significant correlations evident between their leptin response to fasting and any of the changes in these parameters over the ensuing period.

The effect of hyperglycemia was also investigated within the Insulin-Resistant group of animals. Of this group, 11 animals had a fasting blood glucose concentration of greater than 8 mmol/l and were considered to be hyperglycemic relative to the other 13 from this group. These two sub-groups could be regarded as models of NIDDM and impaired glucose tolerance (IGT) respectively. No differences between the IGT and NIDDM animals were apparent in terms of fed or fasting leptin levels, or the change in leptin levels after a 24-hour fast (Figure 6.3; Table 6.2). The hyperglycemic, insulin-resistant animals had an increase in plasma leptin after a 24-hour fast of 11.7 ± 7.3 ng/ml, while the corresponding change in the normoglycemic, insulin-resistant animals was 10.9 ± 11.6 ng/ml.
**FIGURE 6.3:** Percentage changes in body weight, blood glucose, plasma insulin and leptin concentrations in the IGT (n=13) and NIDDM (n=11) groups of *Psammomys obesus* after a 24-hour fast.
<table>
<thead>
<tr>
<th></th>
<th>Bodyweight</th>
<th>Glucose</th>
<th>Insulin</th>
<th>Leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IGT</strong></td>
<td>fed</td>
<td>204.2 ± 8.1</td>
<td>4.11 ± 0.27</td>
<td>202.9 ± 37.6</td>
</tr>
<tr>
<td>(n=13)</td>
<td>fasted</td>
<td>200.7 ± 8.2</td>
<td>3.82 ± 0.28</td>
<td>60.6 ± 8.7</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>0.041</td>
<td>0.220</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>NIDDM</strong></td>
<td>fed</td>
<td>226.0 ± 6.9</td>
<td>11.38 ± 0.54</td>
<td>426.3 ± 103.3</td>
</tr>
<tr>
<td>(n=11)</td>
<td>fasted</td>
<td>221.7 ± 7.8</td>
<td>7.33 ± 1.64</td>
<td>241.1 ± 63.4</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>0.043</td>
<td>0.023</td>
<td>0.007</td>
</tr>
</tbody>
</table>

**TABLE 6.2:** Effects of a 24-hour fast on body weight, blood glucose, plasma insulin and leptin concentrations in the IGT (n=13) and NIDDM (n=11) groups of *Psammomys obesus*. Data are expressed as mean ± sem, probability (p) values are given for the change within groups.
6.5 DISCUSSION

In this study we have shown that obese, insulin-resistant *Psammomys obesus* have significantly elevated fasting and fed plasma leptin concentrations compared with their lean, insulin-sensitive littermates (Table 6.1). Using fasting plasma insulin concentrations as a marker of insulin sensitivity, we found that obese, insulin-resistant *Psammomys obesus* failed to reduce, and indeed tended to increase, their plasma leptin concentrations after a 24-hour fast (Figure 6.2). This is opposite to the results found in lean, insulin-sensitive *Psammomys obesus*, and other lean animals and humans (Cusin et al. 1995; MacDougald et al. 1995; Saladin et al. 1995; Boden et al. 1996; Kolaczyński et al. 1996b; Mizuno et al. 1996; Sinha et al. 1996; Sivitz et al. 1996), which have shown decreases of 40-80% in circulating plasma leptin after fasting from 1 to 3 days.

It is currently proposed that the principal physiological function of leptin is in the regulation of energy balance, especially in the control of food intake. Because circulating leptin levels appear to reflect the amount of body fat (Maffei et al. 1995a) and do not change after a meal (Ma et al. 1996), leptin is thought to act as a 'lipostat' in the long term control of body weight homeostasis. It has also been suggested that leptin may have a second function as an acute sensor of energy balance (Kolaczyński et al. 1996b), whereby circulating leptin concentration is regulated in an acute manner in response to departures from normal energy balance, including fasting. In this capacity, leptin appears to act independently of body fat stores, suggesting a dual role for this hormone in the control of energy balance (Kolaczyński et al. 1996b).

However, the effects of obesity and insulin resistance on the response of leptin to a short-term fast are still somewhat unclear. One small study (n=3) in obese subjects found a significant decrease in plasma leptin concentrations after a 24-hour fast (Sinha et al. 1996). However no data regarding plasma insulin concentrations was provided in this study. Two further studies, also with small numbers (n=3 and n=5) of obese subjects, found significant decreases in plasma leptin after a 52- or 60-hour fast (Boden et al. 1996; Kolaczyński et al. 1996b). In both of these studies the obese subjects did not appear to be significantly hyperinsulinemic relative to lean control subjects. Given the small numbers of subjects in these studies and the apparent lack of significant hyperinsulinemia and (probably) insulin resistance, we believe that these data do not exclude the possibility that a subgroup of obese humans with relatively severe insulin resistance may also have a dysregulation in the response of leptin to acute starvation, as seen in *Psammomys obesus* in this study.
Interestingly, acute dietary restriction (1045 kJ/d) for 5 days in 7 morbidly obese, NIDDM patients did not significantly alter ob gene expression, with the average change found to be a 17% increase (range -46% to +69%; Vidal et al. 1996). These subjects had NIDDM and presumably were considerably insulin-resistant, and the dietary restriction significantly reduced plasma insulin concentration, which is analogous to the findings in this study. In addition, in db/db mice fasted for 15 days, plasma leptin was unchanged despite a 25% drop in BMI, however it was reduced after 28 days (Maffei et al. 1995b). Similarly, in obese, hyperinsulinemic animal models such as ob/ob mice and fa/fa rats, fasting for 16-72 hours did not significantly change ob gene expression (Cusin et al. 1995; MacDougald et al. 1995; Mizuno et al. 1996).

The reduction in plasma leptin in the lean Israeli Sand Rats is almost certainly not related to a drop in body fat mass or body weight, as both groups had small but identical percentage decreases in body weight after the 24-hour fast (1.8%). Therefore it appears that plasma leptin concentration in this case was not simply reflecting body fat content, as previously suggested (Considine et al. 1996a).

Previous longitudinal studies in both Psammomys obesus and humans have indicated that individuals tend to progress from normal glucose tolerance to IGT to NIDDM (DeFronzo 1988; Barnett et al. 1994a). Within our Insulin-Resistant group of animals were representatives of both IGT and NIDDM, and it is likely that some of the IGT animals would progress to develop NIDDM if followed for a sufficient period of time (Barnett et al. 1994a). We were interested in whether the IGT animals already had the leptin dysregulation in response to fasting, or whether this developed concomitantly with, or as a consequence of, hyperglycemia. The results clearly show that the dysregulatory defect in the leptin response to fasting was already present in the IGT animals, and therefore appears to develop around the same time as hyperinsulinemia, and before hyperglycemia in these animals.

The normal physiological response to fasting is characterised by a reduction in blood glucose and insulin, increased gluconeogenesis and decreased glycogenolysis, and an increase in circulating fatty acids and ketones (Cahill et al. 1966; Cahill 1970; Saudek and Felig 1976). It is possible that one or more of the factors described above regulate the production of leptin in response to fasting, however it appears from a recent study that hyperketonemia, per se, does not directly inhibit leptin secretion (Kolaczynski et al. 1996b). We are unable to address this issue with the results of the current study, however it is interesting that the percentage decreases in blood glucose and plasma insulin after fasting were similar between the two groups of animals.
The previous studies in this dissertation have suggested a significant relationship between leptin and insulin, with insulin apparently having an effect on the long-term regulation of plasma leptin concentrations. In this study dysregulation of leptin in response to fasting was demonstrated in obese, insulin-resistant Psammomys obesus compared with lean, insulin-sensitive controls. The decrease in plasma insulin concentration was proportionally similar in both groups, and there was no difference in the effect of fasting on body weight or blood glucose in the two groups. Therefore it appears that some factor(s) other than insulin and body fat are involved in the short-term regulation of leptin in these animals.

It is possible that this unknown factor, which would result in a decrease in leptin biosynthesis and/or release in response to acute caloric deprivation could be defective in obese, insulin-resistant Psammomys obesus. Therefore these animals would not exhibit reduced plasma leptin in response to fasting. Alternatively, this factor could stimulate leptin production and an inappropriate failure to inhibit the production of this factor in response to fasting could explain the results presented here.

In summary, obese, insulin-resistant Psammomys obesus are hyperleptinemic relative to their lean, insulin-sensitive littermates and in response to a 24-hour fast there is a dysregulation of leptin in the obese animals. The mechanism(s) causing the failure of plasma leptin to decrease in fasted obese Psammomys obesus is unknown. Given the apparent dysregulation in plasma leptin in obese, insulin-resistant Psammomys obesus, we decided to investigate the effects of administration of exogenous leptin in both lean and obese animals. These studies are described in Chapters 7 and 8.
CHAPTER 7:

EFFECTS OF LEPTIN ADMINISTRATION IN A POLYGENIC, HYPERLEPTINEMIC ANIMAL MODEL OF OBESITY AND NIDDM: *PSAMMOMYS OBESUS*

7.1 SUMMARY

In these studies we investigated the effects of leptin treatment in *Psammomys obesus*. Treatment with 5 mg leptin/kg body weight/day for 14 days had no significant effect on food intake, body weight, blood glucose or plasma insulin concentrations in either lean or obese *Psammomys obesus*. This protocol had previously been shown to significantly reduce food intake and body weight in both wild-type and ob/ob mice. Continuous infusion of leptin (0.8 mg/kg/day for 14 days) also had no discernible effect on any of the parameters measured in *Psammomys obesus*. Interestingly, administration of 5 mg leptin/kg body weight resulted in increased circulating blood glucose after 8 hours in glucose intolerant (Group B) animals. The leptin resistance observed in *Psammomys obesus* was overcome in lean but not obese animals when the daily leptin dosage was increased to 45 mg/kg, as body weight, percentage body fat and food intake were all significantly reduced after 7 days in lean (Group A) leptin-treated animals compared with controls. However, in obese (Group B) animals, treatment with 45 mg leptin per kg per day had no significant effects after 7 days. In summary, the results of these studies confirm that *Psammomys obesus*, as a species, are leptin resistant relative to other rodents, and that obese *Psammomys obesus* are leptin resistant compared with their lean littermates, implicating leptin in the disease process in obese animals.
7.2 INTRODUCTION

The administration of daily intraperitoneal injections of exogenous leptin (5 mg/kg body weight) to ob/ob mice, which have no circulating endogenous leptin, resulted in rapid and significant reductions in body weight and food intake (Campfield et al. 1995; Halaas et al. 1995; Pelleymounter et al. 1995). A smaller but still significant decrease in body weight was noted in wild-type mice treated with the same leptin dose (Halaas et al. 1995). When wild-type mice were treated with 25 mg leptin/kg/day significant reductions in body weight, food intake and body fat content were observed (Halaas et al. 1995). In db/db mice, which have a defective hypothalamic leptin receptor and elevated circulating leptin levels (Lee et al. 1996), daily leptin administration of 5-10 mg/kg had no detectable effect on food intake or body weight (Halaas et al. 1995; Campfield et al. 1995). Campfield and coworkers administered leptin to diet-induced obese (DIO) mice and found that the dose required for a significant drop in bodyweight and food intake was five times that which produced similar results in the ob/ob mouse (Campfield et al. 1995).

Administration of leptin (5 mg/kg/day) to ob/ob mice also normalized their hyperglycemia and hyperinsulinemia to the levels of lean, control mice within several days (Pelleymounter et al. 1995). No significant effects on blood glucose or plasma insulin concentrations were detected in wild-type mice treated with leptin (Pelleymounter et al. 1995).

No data are available regarding the effects of leptin administration in polygenic animal models of obesity with elevated circulating leptin levels. In these studies we investigated the effects of leptin administration in Psammomys obesus, a unique animal model of obesity and non-insulin-dependent diabetes mellitus (NIDDM). As discussed previously, Psammomys obesus represent a good animal model of obesity and NIDDM, exhibiting a complete spectrum of body weights and blood glucose and plasma insulin concentrations when fed an ad libitum diet of normal laboratory chow (Barnett et al. 1994a,b). This heterogeneous response makes Psammomys obesus more analogous to the pattern of human obesity and NIDDM than the homogeneous single-gene models of obesity such as the ob/ob and db/db mice (Shafir and Gutman 1993). We have previously shown that the ob gene is expressed exclusively in adipose tissues of Psammomys obesus, and that the obese, diabetic animals have hyperleptinemia compared to their lean littermates (Chapter 3). In addition, we found an apparent dysregulation of leptin in the response to fasting in obese, insulin-resistant Psammomys obesus (Chapter 6). We considered Psammomys obesus a very good animal model in which to test the effects of leptin treatment, as obese, diabetic animals exhibit
hyperleptinemia and hyperinsulinemia similar to what is observed in human obesity and NIDDM.

The aim of this study was to determine the effects of various methodologies and dosages of leptin administration in *Psammomys obesus* with a range of body weights and blood glucose and plasma insulin concentrations.
7.3 MATERIALS AND METHODS

7.3.1 BREEDING OF THE COLONY

A *Psammomys obesus* colony is maintained at Deakin University, with the breeding pairs fed *ad libitum* a diet of lucerne and chow. Experimental animals were weaned at four weeks of age and given a diet of standard laboratory chow from which 12% of energy was derived from fat, 63% from carbohydrate and 25% from protein (Barastoc, Pakenham, Australia). Animals were housed in a temperature controlled room (22 ± 1°C) with a 12-12-hour light-dark cycle. The animals used in the study were aged 16-20 weeks during the pretreatment period.

7.3.2 CLASSIFICATION OF ANIMALS

*Psammomys obesus* were categorised into three groups according to their blood glucorc and plasma insulin concentrations, taken in the fed state at 11 am at twelve weeks of age:

Group A
- normoglycemic (blood glucose < 8.0 mmol/l)
- normoinsulinemic (plasma insulin < 150 µU/ml)

Group B
- normoglycemic (blood glucose < 8.0 mmol/l)
- hyperinsulinemic (plasma insulin ≥ 150 µU/ml)

Group C
- hyperglycemic (blood glucose ≥ 8.0 mmol/l)
- hyperinsulinemic (plasma insulin ≥ 150 µU/ml)

The criteria for classification of animals into groups were based on those of Barnett *et al.* (1994a), who characterised the stages of development of the obesity/diabetes syndrome in this species (Barnett *et al.* 1994a).

7.3.3 EXPERIMENTAL PROTOCOL

Four separate experimental protocols were used to investigate the effects of leptin treatment in *Psammomys obesus*, as described below. For each of the studies, a group of *Psammomys obesus* were housed individually and followed for at least a 7-day period with free access to food and water to establish baseline data for food intake (measured by the rate of disappearance), body weight, blood glucose and plasma insulin concentrations. The animals were characterised as described above into Groups A, B
and C (n=14-16 in each group). Within each of these groups, animals were paired into two treatment groups (leptin-treated and control) matched for body weight, sex, age, blood glucose and plasma insulin concentrations.

7.3.3.1 **SINGLE DAILY INJECTIONS**

Animals from Groups A (lean) and C (obese) were paired according to age, sex, body weight, blood glucose and plasma insulin concentrations into two groups (n=10), leptin-treated and controls. Throughout the 14-day study period the animals were given a single daily intraperitoneal injection of 5 mg leptin/kg body weight (kindly supplied by Dr. Margery Nicolson, Amgen Inc., California) or 5 ml phosphate-buffered saline/kg bodyweight (equivalent volume to leptin injection) at 10 am. Body weight was measured daily and blood was collected into heparinised tubes in the fed state twice weekly at 9 am. The animals were housed individually and food intake estimated daily by rate of disappearance (spillage was prevented by appropriate cage design). After the 14-day treatment period, selected body fat depots (interscapular, perirenal, epididymal and intramuscular) were removed and weighed to allow an estimate of body fat content. The weights of the various fat depots were combined and divided by total body mass to provide this estimate. In addition, several major muscles were removed to provide an estimate of muscle mass. All experiments were carried out following the Australian NHMRC principles of laboratory animal care and approved by the Deakin University Animal Ethics Committee. The experimental protocol is summarised in the timeline below:

![Timeline Diagram]

At each day, animals will be monitored for the following: Glucose, Insulin, Leptin
7.3.3.2 CONTINUOUS INFUSION STUDY

To study the effects of continuous infusion of leptin in *Psammomys obesus*, animals were surgically implanted with osmotic minipumps (Alzet model 2002, Alza, California, USA). The pumps delivered a constant dose of 0.5 μl/h for 14 days. The pumps implanted in leptin-treated animals (n=6 in each of Groups A, B and C) contained leptin with the concentration adjusted such that the animals received a dose of 0.8 mg leptin per kg body weight per day. Control animals (n=6 in each of Groups A, B and C) received an equivalent volume (0.5 μl/h) of saline for 14 days. The pumps used in the study were 3.0 cm in length with a diameter of 0.7 cm, weighed 1.1 g and displaced a total volume of 1.0 ml. The inert outer membrane of the pumps was composed of cellulose ester blend.

For surgical implantation of the pumps, the animals were anesthetised with halothane by inhalation, then a 1 cm transverse incision was made over the interscapular region. The pumps were placed in the animals to lie subcutaneously above the scapulae and the wound immediately sutured closed. The duration of anesthesia was only approximately 10 min for this procedure, and the animals were observed closely for the subsequent hour. All of the animals appeared to recover quickly from the surgery and no weight loss or other indicators of stress were observed in any animals.

During the two weeks of the study period, body weight and food intake were measured, and blood collected at 11 am in the fed state for biochemical analyses on days 4, 7, 11 and 14 as shown in the timeline below. At the end of the 14-day treatment period the animals were killed by anesthetic overdose (120 mg/kg pentobarbitone) and selected fat depots (interscapular, perirenal, epididymal, mesenteric and intramuscular) were removed and weighed to allow an estimate of body fat content. The weights of the various fat depots were combined and divided by total body mass to provide this estimate. In addition, several major muscles (soleus, gastrocnemius, plantaris and extensor digitalis longus) were removed and weighed to provide an estimate of leg muscle mass, a crude indicator of the animal’s lean body mass.
7.3.3.3 ACUTE STUDY

Blood was collected into heparinised tubes from all animals in the fed state just prior to injections given at 9 am. Leptin-treated animals (n=7 for each of Groups A, B and C) received an intraperitoneal injection of 5 mg leptin per kg body weight and the controls (n=7 for each of Groups A, B and C) were injected with an equivalent volume of saline. Food intake and body weight were measured and blood was collected at the timepoints 0, 1, 2, 4, 8 and 24 hours after injection. Following injections, all animals had free access to both food and water.

7.3.3.4 SUPRAPHYSIOLOGICAL STUDY

The effects of treatment with very large doses of leptin were investigated in a 'supraphysiological study'. After the baseline period, animals were given intraperitoneal injections three times per day (at 0800, 1600 and 2400) of 15 mg leptin per kg body weight (n=7 Group A, n=5 Group B), or equivalent volume of saline for control animals (n=7 Group A, n=5 Group B), for a total of 7 days. This dosage of leptin resulted in a total of 45 mg/kg/day. Body weight and food intake were measured daily throughout the study. In addition, as shown in the timeline below, blood was collected from the animals on days 2, 4 and 7 at midday (the midpoint between the morning and afternoon injections) for biochemical analyses. At the completion of the study (at midday on day 7, 4 hours after the final injection), the animals were killed by anesthetic overdose (120 mg/kg pentobarbitalone) and selected fat depots (interscapular, perirenal, epididymal, mesenteric and intramuscular) were removed and weighed to allow an estimate of body
fat content. The weights of the various fat depots were combined and divided by total body mass to provide this estimate. In addition, several major muscles (soleus, gastrocnemius, plantaris and extensor digitalis longus) were removed and weighed to provide an estimate of leg muscle mass, a crude indicator of the animal's lean body mass.

Day -7
Baseline

0

2

4

7

Treatment

Sacrifice

At each

animals will be monitored for the following: Glucose
Inulin

At each

animals will be monitored for the following: Leptin
Triglycerides
Cholesterol

All of the experiments described above were carried out following the Australian NHMRC principles of laboratory animal care and approved by the Deakin University Animal Ethics Committee.

7.3.4 BIOCHEMICAL ANALYSES

Whole blood glucose was measured using an enzymatic glucose analyser (Model 27, Yellow Springs Instruments, Ohio). Plasma insulin concentrations were determined using a double antibody solid phase radioimmunoassay (Phadeseq, Kabi Pharmacia Diagnostics, Sweden). Plasma leptin levels were measured in collaboration with Amgen Inc. (Thousand Oaks, California, USA) using a solid phase double enzyme immunoassay (EIA) with affinity purified polyvalent antibodies. Leptin concentrations in the plasma samples were calculated from standard curves generated with recombinant murine leptin. The limits of detection for the leptin EIA were 20 pg/ml of serum or plasma. The inter-assay CV was 7.7% for the high standard and 10.5% for the low standard. Plasma triglyceride and cholesterol concentrations were measured using colorimetric kits (Boehringer Mannheim, Mannheim, Germany) on an automatic analyser as described in detail in Chapter 2.
7.3.5 **STATISTICAL ANALYSIS**

All experimental data are expressed as means ± s.e.m. A one-way analysis of variance in combination with a Tukey's multiple comparison test was used to compare means between and within groups, and a two-sample unpaired t-test was used where appropriate. In all instances probability values of <0.05 were considered significant.
7.4 RESULTS

7.4.1 SINGLE DAILY INJECTION STUDY

7.4.1.1 PLASMA LEPTIN CONCENTRATION

Obese, diabetic (Group C) *Psammomys obesus* had significantly higher circulating plasma leptin concentrations at baseline (45.9 ± 5.6 ng/ml) compared with their lean, non-diabetic (Group A) littermates (25.6 ± 4.1 ng/ml; p<0.05; Figure 7.1). No change was found in either group after treatment with leptin (or saline) when measured throughout the study in blood collected just before the daily injections (Figure 7.1).
**FIGURE 7.1:** Plasma leptin concentrations in the A) obese (Group C) and B) lean (Group A) groups of *Psammomys obesus* at baseline and during the study. The experimental sample was taken just prior to the daily injection on day 10.
To assess plasma leptin concentrations achieved, levels were measured hourly for four hours following a single intraperitoneal injection of 5 mg/kg body weight in both lean (Group A) and obese (Group C) *Psammomys obesus* (Figure 7.2). There was no difference between the groups in the levels of plasma leptin achieved, nor in the rate of removal of leptin from the plasma over this time period, therefore the combined data is shown in Figure 7.2. In both groups, plasma leptin concentration rose within one hour to levels at least 40 times physiological levels, and remained this high for three hours before declining by approximately 50% during the fourth hour.

![Graph showing plasma leptin levels over time](image)

**FIGURE 7.2:** Plasma leptin concentration after intraperitoneal injection of 5 mg/kg body weight in *Psammomys obesus* (mean ± sem).

### 7.4.1.2 BODY WEIGHT

Leptin administration had no effect on body weight in either lean (Group A) or obese (Group C) *Psammomys obesus* (Figure 7.3). Within the lean and obese groups of animals there was no significant difference between the leptin-treated and control groups either before or after the treatment, and none of the groups had a significant change in body weight during the study.
**FIGURE 7.3:** Body weights (mean ± s.c.m.) of the leptin-treated and control groups throughout the pre-treatment (days -12 to 0) and treatment periods. (Legend: ——— Obese leptin-treated; ——— Obese control; ——— Lean leptin-treated; ——— Lean control).

In the obese group, the leptin-treated animals had an estimated body fat of $5.4 \pm 0.4\%$, compared to $4.8 \pm 0.6\%$ in obese control animals (Figure 7.4). There was no significant difference in estimated body fat percentage between the two groups, suggesting that leptin treatment had no effect on the obesity of these animals. Similarly, in the lean (Group A) animals, leptin-treated *Psammomys obesus* had an estimated body fat of $2.1 \pm 0.4\%$ compared with $1.9 \pm 0.5\%$ in lean control animals (Figure 7.4). As expected, obese (Group C) *Psammomys obesus* had a significantly higher estimated percent body fat compared with their lean (Group A) littermates ($p<0.05$).
**FIGURE 7.4:** Estimated percentage body fat in both lean (leptin-treated and control) and obese (leptin-treated and control) *Psammomys obesus*.

Leg muscle mass, as estimated by the weight of selected muscle groups, was also unaffected by leptin treatment in both obese (leptin-treated 1.6 ± 0.2%; controls 1.6 ± 0.1%) and lean animals (leptin-treated 1.6 ± 0.1%; controls 1.7 ± 0.2%; Figure 7.5). In addition, there was no difference in the estimated leg muscle mass between lean and obese *Psammomys obesus*. This confirmed our previous findings in these animals (Collier *et al.* 1997), and supported the contention that *Psammomys obesus* represent a good animal model of obesity, rather than simply a range of animals of different body size.
**FIGURE 7.5:** Estimated leg muscle mass (from the weight of selected muscle groups) in lean and obese *Psammomys obesus*, both leptin-treated and control animals.

### 7.4.1.3 FOOD INTAKE

In the obese, diabetic group of *Psammomys obesus* there was no difference in food intake between the leptin-treated and control groups in the pre-treatment period (leptin-treated 17.7 ± 5.4 g/day compared with control 17.3 ± 3.7 g/day), and neither group had a significant change in food intake during leptin treatment (leptin-treated 17.0 ± 6.4 g/day, controls 15.6 ± 4.7 g/day; Figure 7.6B). At the end of the treatment period there was no significant difference in food intake between the two groups. Similarly, in the lean animals, there was no significant difference in baseline food intake (leptin-treated 12.0 ± 1.7 g/day; controls 12.8 ± 0.6 g/day). There was no significant change in food intake in either group after the 14-day treatment period (leptin-treated 11.9 ± 0.6 g/day; controls 12.0 ± 0.5 g/day; Figure 7.6A).
FIGURE 7.6: Mean daily food intake during the baseline and treatment periods in A) lean (Group A), and B) obese (Group C) Psammomys obesus.
Leptin treatment also had no significant effect on the diabetes of the obese (Group C) animals. There was no difference in blood glucose concentrations (Figure 7.7) between the two groups at the start of the study, or after the treatment period, and neither group changed significantly during the study.

**FIGURE 7.7**: Whole blood glucose measurements of leptin-treated and control animals. Data are expressed as mean ± s.e.m. (Legend: □—Obese leptin-treated; ○—Obese control; ○—Lean leptin-treated; △—Lean control).

Similarly, the plasma insulin concentrations (Figure 7.8) of the two groups were not significantly different at the start or completion of the study, and neither group had a significant change in plasma insulin concentration during the study. In the lean group of *Psammomys obesus*, leptin treatment did not have a significant effect on either blood glucose or plasma insulin concentrations (Figures 7.7 and 7.8).
**FIGURE 7.8:** Plasma insulin levels of the leptin-treated and control groups (mean ± s.e.m.). (Legend: —□— Obese leptin-treated; —----○— Obese control; —----●— Lean leptin-treated; —----△— Lean control).

### 7.4.2 CONTINUOUS INFUSION STUDY

#### 7.4.2.1 PLASMA LEPTIN CONCENTRATION

Continuous infusion at a dose of 0.8 mg leptin/kg body weight/day for 14 days resulted in no significant changes in circulating plasma leptin concentrations in any group of *Psammomys obesus* (Figure 7.9), although plasma leptin tended to increase in both Group B and Group C animals.
**Figure 7.9:** Changes in plasma leptin concentrations from the baseline to experimental (continuous infusion) periods in A) Group A, B) Group B and C) Group C *Psammomys obesus.*
7.4.2.2 **BODY WEIGHT**

Continuous infusion of leptin had no effect on body weight in *Psammomys obesus* from Group A, B or C (Figure 7.10). Within each of these groups there was no difference in mean body weight between the leptin-treated animals and controls during the baseline or experimental periods.
**FIGURE 7.10**: Body weights (mean ± sem) of the leptin-treated and control animals from A) Group A, B) Group B and C) Group C *Psammomys obesus* throughout the baseline (days -7 to 0) and experimental periods.
In all three groups of *Psammomys obesus* (A, B and C) there was no significant
difference in estimated percentage body fat between the leptin-treated animals and
controls (Figure 7.11). The obese (Group B and C) animals had significantly greater
body fat than their lean (Group A) littermates (5.03 ± 0.25% vs 2.81 ± 0.77%;
p=0.004).

![Bar chart showing estimated body fat percentages](chart)

**FIGURE 7.11:** Estimated percentage body fat in leptin-treated and control
*Psammomys obesus* from Groups A, B and C.

Lev muscle mass (estimated from the weight of selected muscle groups) was also
unaffected by leptin treatment (Figure 7.12). There was no significant difference
between leptin-treated and control animals in any of the groups.
**FIGURE 7.12:** Estimated leg muscle mass in leptin-treated and control *Psammomys obesus* from Groups A, B and C.

**FOOD INTAKE**

Baseline food intake was significantly higher in Group C animals (17.31 ± 0.91 g/day) compared with both Group A (11.15 ± 0.91 g/day; p<0.001) and Group B (12.09 ± 0.76 g/day; p=0.001) *Psammomys obesus*. No significant changes in food intake were detected in any of the groups treated with leptin (Figure 7.13).
FIGURE 7.13: Mean food intake in leptin-treated and control *Psammomys obesus* from A) Group A, B) Group B and C) Group C.
7.4.2.4 BLOOD GLUCOSE AND PLASMA INSULIN

Continuous infusion of leptin had no significant effects on blood glucose (Figure 7.14) or plasma insulin (Figure 7.15) concentrations in any of the groups of *Psammomys obesus* investigated (Groups A, B and C).
**FIGURE 7.14:** Whole blood glucose concentrations (mean ± sem) of the leptin-treated and control animals from A) Group A, B) Group B and C) Group C *Psammomys obesus* throughout the baseline (days -7 to 0) and experimental periods.
FIGURE 7.15: Plasma insulin concentrations (mean ± sem) of the leptin-treated and control animals from A) Group A, B) Group B and C) Group C Psammonomys obesus.
7.4.3 ACUTE STUDY

7.4.3.1 PLASMA LEPTIN CONCENTRATION

Baseline plasma leptin concentrations for the various groups of *Psammomys obesus* are shown in Figure 7.16. Plasma leptin was significantly higher in obese (Groups B and C) *Psammomys obesus* compared with their lean littermates.

![Figure 7.16](image)

**FIGURE 7.16:** Baseline plasma leptin concentrations in the various groups of *Psammomys obesus* (mean ± sem). (* p<0.05 compared with Groups B and C).

No significant differences were observed between Groups A, B and C with respect to the maximal levels of circulating leptin achieved or the rate of disappearance of leptin from the plasma (Table 7.1). Therefore the data from the groups was combined, and is represented in Figure 7.17. Plasma leptin rose to 30 times baseline levels 1 hour after injection and remained at 26 times baseline 2 hours after the injection. Two hours later the mean plasma leptin concentrations in these animals had fallen to 12 times baseline, and 8 hours after the injection plasma leptin was 3 times baseline. 24 hours after the injection the plasma leptin concentrations were not different to pre-injection levels.
<table>
<thead>
<tr>
<th>TIME AFTER INJECTION (h)</th>
<th>GROUP A (ng/ml)</th>
<th>GROUP B (ng/ml)</th>
<th>GROUP C (ng/ml)</th>
<th>TOTAL (ng/ml)</th>
</tr>
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<tr>
<td>0</td>
<td>43.9 ± 9.8</td>
<td>76.6 ± 6.3</td>
<td>82.8 ± 7.7</td>
<td>70.3 ± 5.7</td>
</tr>
<tr>
<td>1</td>
<td>2206 ± 320</td>
<td>1978 ± 281</td>
<td>2055 ± 201</td>
<td>2066 ± 145</td>
</tr>
<tr>
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<td>1997 ± 203</td>
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</tr>
<tr>
<td>4</td>
<td>878 ± 109</td>
<td>893 ± 122</td>
<td>727 ± 65</td>
<td>824 ± 56</td>
</tr>
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<td>8</td>
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<td>282 ± 55</td>
<td>177 ± 26</td>
<td>224 ± 25</td>
</tr>
<tr>
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<td>47.5 ± 14.0</td>
<td>78.7 ± 5.5</td>
<td>82.4 ± 8.2</td>
<td>71.8 ± 6.0</td>
</tr>
</tbody>
</table>

**TABLE 7.1:** Plasma leptin concentrations after intraperitoneal injection of 5 mg/kg body weight in the various groups of *Psammomys obesus* (mean ± sem).
**FIGURE 7.17**: Plasma leptin concentrations after intraperitoneal injection of 5 mg/kg body weight in *Psammomys obesus* (mean ± sem). (*) Significantly different to both controls and baseline levels in leptin-treated animals; $p<0.05$.)
7.4.3.2 FOOD INTAKE

In the lean, healthy (Group A) *Psammomys obesus* injected with leptin there was a tendency for decreased mean cumulative food intake (grams/hour) during the 8 hours following leptin administration (0.45 ± 0.08 g/h) compared with both their baseline food intake (0.63 ± 0.06 g/h; p=0.25) and the food intake of the control animals (0.61 ± 0.09 g/h; p=0.18; Figure 7.18).

![Bar chart showing food intake over 8 hours for leptin-treated and control groups.]

**FIGURE 7.18:** Mean cumulative 8-hour food intake in leptin-treated and control *Psammomys obesus* from Group A.

There was no significant difference in the 24-hour food intake of the two groups following leptin injection (p=0.78), indicating that the 8-hour tendency to decrease food intake in the leptin-treated animals was compensated by a tendency to refeed (relative to control animals) in the subsequent 16 hours (Figure 7.19).
**FIGURE 7.19:** Mean cumulative 24-hour food intake in leptin-treated and control *Psammonys obesus* from Group A.

No acute effects of leptin administration on food intake were detected in either Group B or Group C animals at any of the timepoints tested.

### 7.4.3.3 BLOOD GLUCOSE AND PLASMA INSULIN

Leptin administration had no acute effect on blood glucose concentrations in lean, healthy (Group A) or obese, diabetic (Group C) *Psammonys obesus*. In obese, glucose intolerant (Group B) animals, leptin administration caused a significant increase in blood glucose concentration (11.38 ± 2.23 mmol/l) 8 hours after injection compared with baseline levels in these animals (5.34 ± 0.50 mmol/l; \( p = 0.04 \)) and the 8 hour glucose levels in control animals (5.84 ± 0.77 mmol/l; \( p = 0.05 \); Figure 7.20). Although this was the only statistically significant timepoint, blood glucose concentrations also tended to be higher after 2 and 4 hours (\( p = 0.13 \) and \( p = 0.10 \) respectively).
FIGURE 7.20: Whole blood glucose concentrations following leptin injections in A) Group A, B) Group B and C) Group C *P. papua*. (* p<0.05 vs control).
In both Group A and Group C *Psamnomys obesus* no difference was detected in plasma insulin concentrations between leptin-treated animals and controls (Figure 7.21). In Group B animals, which exhibited increased blood glucose levels after leptin injection, there was a tendency for elevated plasma insulin after 2 and 8 hours (p=0.19 and p=0.16 respectively; Figure 7.21).
Figure 7.21: Plasma insulin concentrations following leptin injections in A) Group A, B) Group B and C) Group C Psammomys obesus.
7.4.3.4 PLASMA TRIGLYCERIDES AND CHOLESTEROL

Plasma triglyceride concentrations were significantly reduced in leptin-treated animals from Groups A and C (p<0.05; Figure 7.22) compared with controls, and tended to be lower in leptin-treated Group B Psammomys obesus (p=0.28; Figure 7.22).

No differences were observed between leptin-treated animals and controls with respect to total plasma cholesterol concentrations in any of the Psammomys obesus groups tested (Figure 7.23).
FIGURE 7.22: Plasma triglyceride concentrations following leptin injections in A) Group A, B) Group B and C) Group C *Psammomys obesus* (*p < 0.05* vs. baseline)
**FIGURE 7.23:** Plasma cholesterol concentrations following leptin (or saline) injections in A) Group A, B) Group B and C) Group C *Psammomys obesus.*
7.4.4 SUPRAPHYSIOLOGICAL STUDY

7.4.4.1 PLASMA LEPTIN CONCENTRATION

As found previously in the 'acute' study, no significant differences were observed in the maximal leptin levels achieved in Group A animals compared with Group B, and the rate of clearance of leptin from the circulation was also not different between the groups. Therefore the data shown in Figure 7.24 is for a combination of Group A and Group B Psammomys obesus.

Figure 7.24A shows the effects of an individual injection of 15 mg leptin/kg body weight on plasma leptin concentrations for the period until the next dose. One hour after injection mean plasma leptin concentrations were increased by a factor of 314. Three hours later, at the midpoint between injections, plasma leptin was 84 times baseline levels, and 7 hours after the injection, just prior to the next dose, plasma leptin concentrations were still significantly increased (4 times) compared with baseline levels.

As shown in Figure 7.24B, plasma leptin concentrations at midday (midpoint between injection times) were significantly increased in the leptin-treated animals throughout the study (approximately 80 times baseline levels at days 4 and 7).
TABLE 7.24: A) Change in plasma leptin concentrations in leptin-treated and control *Psammomys obesus* after a single injection (mean ± sem). B) Plasma leptin concentrations in leptin-treated and control *Psammomys obesus* at baseline and throughout the 'supraphysiological' study (mean ± sem). (* Significantly greater than control group, p<0.05).
7.4.4.2  **BODY WEIGHT**

In Group A animals treated with leptin in the supraphysiologica study there was a significant difference in mean body weight change compared with control animals (change from baseline -4.2 ± 3.2 g vs 0.36 ± 1.5 g; p=0.0001; Figure 7.25). In Group B animals treated the same way no difference was observed (Figure 7.25).
**FIGURE 7.25:** Mean change in body weight of the leptin-treated and control animals from A) Group A and B) Group B *Psammomys obesus* throughout the experimental period.
The leptin-treated Group A animals had significantly reduced estimated percentage body fat compared with control animals (p=0.031; Figure 7.26). In Group B animals there was no significant difference in estimated percentage body fat between leptin-treated and control Psammomys obesus (Figure 7.26).

![Graph showing estimated percentage body fat comparison between leptin-treated and control groups](image)

**Figure 7.26**: Estimated percentage body fat (mean ± sem) in leptin-treated and control Psammomys obesus from Groups A and B. (* significantly lower than control Group A animals; p=0.031).

Leg muscle mass (estimated from the weight of selected muscle groups) was unaffected by leptin treatment in either Group A or B animals (Figure 7.27), suggesting that the body weight change in leptin-treated Group A animals was probably due to a reduction in body fat content.
FIGURE 7.27: Estimated leg muscle mass (mean ± sem) in leptin-treated and control *Psammomys obesus* from Groups A and B.

7.4.4.3 FOOD INTAKE

Leptin treatment significantly reduced food intake in Group A animals compared with their baseline consumption (9.67 ± 0.87 g/day vs 12.4 ± 0.66 g/day; p=0.03; Figure 7.28). Although food intake during the experimental period tended to be lower than baseline in the saline-treated control animals this was not statistically significant. Leptin treatment had no detectable effect on food intake in Group B animals (Figure 7.28).
**FIGURE 7.28:** Mean food intake in leptin-treated and control *Psammomys obesus* from A) Group A and B) Group B.
7.4.4.4 BLOOD GLUCOSE AND PLASMA INSULIN

Leptin treatment had no significant effect on blood glucose or plasma insulin concentrations in Group A *Psammomys obesus* compared with saline-treated control animals (Figures 7.29 and 7.30). In Group B animals no significant differences were found in blood glucose concentrations between leptin-treated and control *Psammomys obesus*, although in both groups a tendency toward hyperglycemia was observed (Figure 7.29). No significant difference in plasma insulin concentrations was noted in either leptin-treated or control Group B animals (Figure 7.30).
FIGURE 7.29: Whole blood glucose concentrations (mean ± scm) of the leptin-treated and control animals from A) Group A and B) Group B *Psammomys obesus* throughout the baseline (days -7 to 0) and experimental periods.
**FIGURE 7.20:** Plasma insulin concentrations (mean ± sem) of the leptin-treated and control animals from A) Group A and B) Group B *Psammomys obesus* throughout the baseline (days -7 to 0) and experimental periods.
7.4.4.5 PLASMA TRIGLYCERIDES AND CHOLESTEROL

Plasma triglyceride concentrations were not significantly different in leptin-treated animals from Groups A or B (p<0.05; Figure 7.31) compared with controls. No differences were observed between leptin-treated animals and controls with respect to total plasma cholesterol concentrations in either of the Psammomys obesus groups tested (Figure 7.32).

**FIGURE 7.31:** Plasma triglyceride concentrations following leptin injections in A) Group A and B) Group B Psammomys obesus.
**FIGURE 7.32:** Plasma cholesterol concentrations following leptin injections in A) Group A and B) Group B *Psammomys obesus.*
7.5 DISCUSSION

In the studies described in this chapter it was shown that leptin administration of 5 mg/kg/day by single daily intraperitoneal injection for 14 days was ineffective in *Psammomys obesus*. In addition, continuous infusion of leptin (0.8 mg/kg/day for 14 days) had no significant effect on any parameters measured in these animals. Both of these protocols were previously shown to significantly reduce food intake and body weight in both wild-type and *ob/ob* mice (Campfield *et al*. 1995; Halaas *et al*. 1995; Pellemounter *et al*. 1995). Administration of 5 mg leptin/kg body weight acutely (within 8 hours) tended to reduce food intake in lean (Group A) but not obese (Groups B and C) *Psammomys obesus*, and resulted in a significant increase in blood glucose concentrations in glucose intolerant (Group B) animals. These results suggest that obese *Psammomys obesus* may be more resistant to the anorexigenic actions of leptin than their lean littermates. Administration of very high doses of leptin (45 mg/kg/day) for 7 days significantly reduced food intake and percentage body fat in lean, healthy (Group A) *Psammomys obesus*, but had no effect on these parameters in glucose intolerant (Group B) animals. Together these results indicate that *Psammomys obesus* were insensitive to the effects of exogenous leptin administration relative to other rodents, and that obese animals were more leptin resistant than their lean littermates.

Leptin administration of 5 mg/kg body weight for 14 days had no significant effect on food intake, body weight, percentage body fat, blood glucose or plasma insulin concentration in lean, healthy (Group A) or obese, diabetic (Group C) *Psammomys obesus*. This dosage was sufficient to significantly reduce all of these parameters in *ob/ob* mice (Campfield *et al*. 1995; Halaas *et al*. 1995; Pellemounter *et al*. 1995), and in general it appeared that a daily dose of 5-10 mg leptin/kg body weight approximately halved food intake and caused a 10-40% reduction in body weight within 2-4 weeks in these animals (Halaas *et al*. 1995; Pellemounter *et al*. 1995). A smaller but still significant decrease in body weight was noted in wild-type mice treated with the same leptin dose (Halaas *et al*. 1995). This study suggested that *Psammomys obesus* were leptin resistant relative to other rodents, and further studies were conducted to investigate the effects of leptin administration in these animals.

In a previous study, continuous infusion of leptin at a dosage of 0.3 mg/kg/day in wild-type mice for 14 days caused a significant reduction in body weight which was partly attributed to a decrease in food intake (Pellemounter *et al*. 1995). We used the same methodology in a range of *Psammomys obesus* but increased the dose to 0.8 mg/kg/day after previously finding relative resistance to the effects of leptin administration in these animals. No significant changes were observed in food intake, body weight, percentage
body fat, blood glucose or plasma insulin concentrations in *Psammomys obesus* from Group A, B or C continuously infused with 0.8 mg leptin/kg/day for 14 days (Figures 7.10 to 7.15). These results confirmed the relative leptin resistance of this species compared with other rodents, and suggested that very high doses of leptin may be required to elicit significant long-term effects in *Psammomys obesus*.

The lack of change in circulating plasma leptin concentrations in this continuous infusion study from the baseline to experimental periods may partially explain these findings. It has been known for over thirty years that *Psammomys obesus* have unusual renal function, and are extremely efficient at concentrating their urine to prevent water loss (Schmidt-Nielsen *et al.* 1964). It is possible that these animals are also proficient at removing proteins such as leptin from their circulation, as suggested in Figure 7.17, and therefore the dose of leptin used in this study was insufficient to induce a sustained increase in circulating leptin concentrations. Alternatively, it must also be considered that this route of administration may be ineffective in this species and the leptin in the pumps never reached the circulation. Further studies investigating the clearance of leptin from the circulation in these animals are required to clarify these findings.

In the single daily injection study we found that plasma leptin levels were decreased to approximately 50% of maximal levels 4 hours after injection (Figure 7.2). We decided to investigate acute (within 8 hours) effects of leptin to see if any changes were occurring while circulating levels of leptin were very high. One hour after an injection of 5 mg leptin/kg body weight, plasma leptin concentrations rose to 30 times baseline levels, and gradually fell to 8 times baseline 8 hours after the injection (Figure 7.17). The plasma leptin concentrations were therefore significantly elevated for the 8-hour period following injection compared with baseline levels. A tendency for reduced food intake in the 8 hours following leptin administration was observed in lean, healthy (Group A) *Psammomys obesus* (Figure 7.18), however this was not statistically significant. There was no tendency for reduced 24-hour food intake following leptin administration (Figure 7.19), in concordance with the results described earlier. A similar tendency for reduced food intake in the 8 hours following leptin administration was not observed in obese (Group B and C) animals, giving the first indication that obesity in these animals may be associated with leptin resistance (relative to lean (Group A) *Psammomys obesus*).

Interestingly, leptin administration acutely increased blood glucose concentrations in obese, glucose intolerant (Group B) *Psammomys obesus* (Figure 7.20). This finding suggests that high circulating levels of leptin may be associated with a worsening of insulin resistance in animals with glucose intolerance. Epidemiological studies have
previously shown a significant relationship between insulin and leptin in humans (Zimmet et al. 1996; de Courten et al. 1997), and similar findings were observed in a cross-sectional analysis of *Psammomys obesus* (Chapter 3). *In vitro*, leptin was shown to inhibit insulin action in isolated hepatocytes (Cohen et al. 1996). Collectively these results raise the possibility that leptin treatment may exacerbate insulin resistance in susceptible individuals, which would represent a very dire side effect of leptin therapy for obesity.

The acute effect of leptin on blood glucose concentration was not observed in either lean, healthy (Group A) or obese, diabetic (Group C) animals (Figure 7.20), despite no difference in the plasma leptin concentrations achieved or the rate of clearance of leptin from the plasma of the three groups. This may be because the Group A animals did not have the genetic susceptibility for glucose intolerance present in Group B and C *Psammomys obesus*, and were able to compensate for any effects of leptin which reduced insulin sensitivity. The Group C animals were already profoundly hyperglycemic when treated with leptin, and this may have masked the effects seen in Group B *Psammomys obesus*.

Although plasma insulin concentrations were not significantly increased as hyperglycemia developed in Group B animals treated with leptin in the acute study, there was a tendency for circulating insulin levels to rise (Figure 7.21). These animals were already significantly hyperinsulinemic at the start of the study, so it is possible that their pancreatic insulin secretion was already maximal before leptin injection, and therefore significantly increased insulin output was not possible as hyperglycemia developed. Alternatively, it has been suggested that leptin may directly inhibit glucose-induced insulin secretion through actions on pancreatic β-cells (Emilsson et al. 1997). Hence, it is conceivable that the pharmacologic hyperleptinemia induced in this study may have directly resulted in reduced glucose-stimulated pancreatic insulin output. If this is the case then these animals would have been unable to respond to the leptin-induced hyperglycemia by increasing insulin secretion, thus preventing an elevation in circulating insulin levels.

Leptin treatment acutely decreased plasma triglyceride concentrations in *Psammomys obesus* (Figure 7.22). In a recent study, Shimabukuro and colleagues showed that hyperleptinemia induced by adenovirus gene transfer in wild-type rats depleted triglyceride content in various tissues, possibly by stimulating intracellular fat oxidation (Shimabukuro et al. 1997). Although plasma triglyceride levels were not reported in that study, it is conceivable that tissue triglyceride depletion could decrease circulating triglyceride concentrations by large-scale uptake into the tissues. Leptin treatment could
have reduced plasma triglyceride concentrations in our study by a similar mechanism. Future studies are planned in our laboratory to investigate the effects of leptin administration on aspects of fat oxidation in *Psammomys obesus*, including measurements of lipoprotein lipase and carnitine palmitoyl transferase activities, as well as measuring triglyceride content in various tissues.

To achieve very high circulating concentrations of leptin in *Psammomys obesus* for 7 days the 'supraphysiological' study was conducted. In this study both lean (Group A) and obese (Group B) animals were treated with 45 mg leptin/kg/day for 7 days. The plasma leptin concentrations achieved in this study are shown in Figure 7.24. One hour after injection in the leptin-treated group the plasma leptin levels rose to over 300 times baseline levels, and three hours later they were still approximately 80 times baseline concentrations. The plasma leptin concentrations remained significantly elevated (4 times baseline) just prior to the next injection, indicating that throughout this study plasma leptin was maintained at significantly increased ('supraphysiological') concentrations compared with baseline levels. By calculating the area under the curve shown in Figure 7.24a, the mean plasma leptin concentrations in these animals for the duration of the study was estimated to be 3062 ng/ml, or 125 times baseline levels.

In lean, healthy (Group A) *Psammomys obesus*, this method of leptin treatment reduced food intake (Figure 7.28), percentage body fat (Figure 7.26), and resulted in significantly different body weight change throughout the study compared with control animals (Figure 7.25). These findings showed that the murine leptin used in all of these studies was effective in *Psammomys obesus*, but that very high doses were required compared with those used in ob/ob and wild-type mice. In fact the dose used (45 mg/kg/day) was 9 times that shown to significantly reduce food intake and body fat content in ob/ob and wild-type mice (Halaas et al. 1995), suggesting significant leptin resistance in *Psammomys obesus*.

In obese, glucose intolerant (Group B) *Psammomys obesus* treatment with very large doses of leptin had no significant effect on food intake, body weight or body fat content, indicating that these animals were significantly leptin resistant relative to their lean (Group A) littermates. Therefore insensitivity to the anorexigenic and hypermetabolic actions of leptin was implicated in the pathophysiology of obesity and insulin resistance in these animals. It is conceivable that some genetic factor found in Group B *Psammomys obesus* renders these animals relatively insensitive to the actions of their endogenous leptin compared with Group A *Psammomys obesus*, and this defect could represent an expression of the thrifty genotype. That is, these animals could accumulate body fat more readily when food is available unhindered by the
anorexigenic effects of their circulating leptin. Conversely, the animals lacking this genotype would cease body fat accumulation when food is plentiful due to increases in circulating leptin which would result in decreased food intake and increased energy expenditure.

Very high doses of leptin had no effect on mean blood glucose or insulin concentrations in *Psammomys obesus* (Figures 7.29 and 7.30). Therefore the acute effect of leptin to increase circulating blood glucose in Group B animals was not observed in this longer term study. There are several possible explanations for this apparent discrepancy. One possibility is that hyperleptinemia does worsen insulin resistance acutely by direct actions on peripheral tissues such as liver (Cohen *et al.* 1996), but chronic hyperleptinemia results in down-regulation of hepatic leptin receptors to restore insulin sensitivity. In this regard it is interesting that studies in our laboratory have shown reduced expression of leptin receptor mRNA in livers of Group B *Psammomys obesus* compared with animals from Groups A and C (de Silva *et al.* 1997).

In addition, it is possible that leptin only exacerbates insulin resistance in a subpopulation of glucose intolerant *Psammomys obesus*. Obesity and NIDDM are thought to be polygenic diseases, with the number of genes involved in the disease processes unknown (Bouchard 1994). The heterogeneity of these disorders in both *Psammomys obesus* and humans suggests the involvement and interaction of several to many genes. Therefore the classification of *Psammomys obesus* into only 3 groups may be a little simplistic when investigating complex phenotypic traits. In 1 of the 5 glucose intolerant (Group B) animals treated in the 'supraphysiological' study, blood glucose concentration rose from a baseline of 5.34 mmol/l (normoglycemic) to 14.77 mmol/l (hyperglycemic) after 7 days of treatment. It is possible that treatment of more animals in this manner may reveal a subpopulation of Group B animals with a genetic predisposition to worsening of insulin resistance when treated with leptin. Further studies are required to investigate this hypothesis, including the ability of various animals to down-regulate leptin receptor numbers in tissues such as liver in response to pharmacologic hyperleptinemia. If such a subpopulation exists, it would be interesting to investigate similarities in human populations being treated with leptin (in clinical trials currently underway), as worsening of insulin resistance would constitute a substantial side-effect of obesity therapy with leptin.

Previous studies in *Psammomys obesus* have shown it to be a polygenic, heterogeneous model of obesity and NIDDM displaying a continuous range of metabolic responses to a normal chow diet. These animals develop hyperinsulinemia, hypertriglyceridemia, hypercholesterolemia and elevated leptin levels making it a unique model of obesity
and NIDDM (Shafrir and Gutman 1993; Barnett et al. 1994a,b; Barnett et al. 1995).
The phenotypic range of body weight and plasma leptin concentrations in *Psammomys obesus* make it more analogous to human obesity than the single-gene mutant models such as *ob/ob* mice. The results of these studies suggest that obese *Psammomys obesus* are leptin resistant relative to their lean littermates, implicating leptin resistance in the pathophysiology of obesity in these animals. If this situation is similar in human populations, as cross-sectional studies have suggested, then this would have significant implications for the potential use of leptin or leptin analogues in the treatment of human obesity.

Therefore, the findings of the studies described in this chapter may be particularly relevant to the use of leptin as a therapeutic agent for human obesity. If very high circulating levels of leptin are required to reduce food intake and body fat in human obesity, then potential side effects of this pharmacologic hyperleptinemia, including the potential to induce or exacerbate insulin resistance, will need to be extensively investigated. In the following chapter, we investigated the effects of leptin on insulin binding by isolated adipocytes.
CHAPTER 8:

LEPTIN INHIBITS INSULIN BINDING IN ISOLATED RAT ADIPOCYTES

8.1 SUMMARY

Leptin is secreted from adipose tissue, and is thought to act as a 'lipostat', signalling the body fat levels to the hypothalamus resulting in adjustments to food intake and energy expenditure to maintain body weight homeostasis. In addition, plasma leptin concentrations have been shown to be related to insulin sensitivity independent of body fat content, suggesting that the hyperleptinemia found in obesity could contribute to the insulin resistance. We investigated the effects of leptin on insulin binding by isolated adipocytes. Adipocytes isolated from Sprague-Dawley rats exhibited a dose-dependent reduction in the binding of $^{125}$I-labelled insulin when incubated with various concentrations of exogenous leptin. For example, addition of 50 nM leptin reduced total insulin binding in isolated adipocytes by 19% ($p<0.05$). Analysis of displacement curve binding data suggested that leptin reduced maximal insulin binding in a dose-dependent manner, but had no significant effect on the affinity of insulin for its binding site. We conclude that leptin directly inhibited insulin binding by adipocytes, and the role of leptin in the development of insulin resistance in obese individuals requires further investigation.
8.2 INTRODUCTION

Leptin is a recently discovered cytokine-like peptide secreted from adipose tissue (Zhang et al. 1994) which is thought to act on the hypothalamus to reduce food intake and increase energy expenditure. Leptin treatment of 5 mg/kg/day resulted in marked reductions in body weight and normalisation of circulating blood glucose and insulin concentrations when administered to leptin-deficient ob/ob mice (Campfield et al. 1995; Halaas et al. 1995; Pelleymounter et al. 1995), and had greatly reduced or marginal effects in wild-type mice (Campfield et al. 1995; Halaas et al. 1995; Pelleymounter et al. 1995). In hyperleptinemic db/db mice leptin treatment was ineffective at all doses tested (Campfield et al. 1995; Halaas et al. 1995; Pelleymounter et al. 1995), while in mice with diet-induced obesity (DIO) the dosage of leptin required to have a small but significant effect on food intake and body weight was greatly increased (30 mg/kg/day; Campfield et al. 1995).

We have previously shown that obese, diabetic Psammomys obesus, a polygenic animal model of obesity and NIDDM, are hyperleptinemic, with plasma leptin concentrations reflecting both body fat mass and plasma insulin concentrations (Chapters 3 and 5). In these animals very high concentrations of circulating leptin were required before any effect was detectable on food intake and body fat content (Chapter 7). Interestingly, in addition to its central role as a circulating 'lipostat' signalling the hypothalamus to the levels of fat storage, there is some evidence that leptin may have physiological effects outside the hypothalamus. As discussed previously, full length leptin receptors have been detected in a variety of tissues other than the hypothalamus. This study was conducted to investigate the possibility that high circulating leptin concentrations may have significant effects outside the hypothalamus in addition to its central anorexigenic and hypermetabolic functions.

Specific receptors for leptin have been shown to be expressed in various tissues apart from the hypothalamus including liver, pancreas and adipose tissue (Iida et al. 1996; Takaya et al. 1996). This pattern of expression has led to the suggestion that leptin may have significant physiological functions in addition to the control of energy balance (Cohen et al. 1996). In vitro studies have demonstrated that leptin may attenuate insulin activity in isolated hepatocytes (Cohen et al. 1996) and inhibit insulin secretion from perfused pancreatic islets (Emilsson et al. 1997). In addition, several studies have found a direct link between plasma leptin concentrations and measures of insulin sensitivity independent of the degree of body fatness (Zimmet et al. 1996; de Courten et al. 1997). That is, high circulating levels of leptin were associated with insulin resistance independent of BMI. Collectively, these findings raise the possibility that leptin may
have a role in contributing to the insulin resistance and hyperinsulinemia observed in some obese subjects with NIDDM. The aim of this study was to investigate the effects of exogenous leptin on insulin binding and uptake by isolated rat adipocytes.
8.3 MATERIALS AND METHODS

8.3.1 EXPERIMENTAL ANIMALS

Male Sprague-Dawley rats (Rattus norvegicus) aged 8 weeks, weighing 250-300 g were maintained on a 12-12-hour light-dark cycle at 22±1°C and fed on standard laboratory chow (Barastoc, Pakenham, Australia). Ethics approval was granted for these studies by the Deakin University Animal Experimental Ethics Committee, and all procedures were performed according to the animal handling Code of Practice of the Australian National Health and Medical Research Council.

8.3.2 ISOLATION OF ADIPOCYTES

Adipocytes were isolated from epididymal fat pads by incubation in Krebs Ringer HEPES buffer (NaCl 154 mM, KCl 6 mM, CaCl₂ 2.5 mM, H₃PO₄ 6 mM, MgSO₄·7H₂O 2.5 mM, NaHCO₃ 5 mM, HEPES 25 mM, 1% BSA; pH 7.4; Vinten et al. 1976) containing 0.5% (wt/vol) collagenase (Worthington Type V, Seimar, Melbourne, Australia) for 45 min at 37°C in a shaking waterbath (100 orbitals/min). The cells were strained and washed 5 times with Krebs Ringer HEPES buffer to remove any residual collagenase. Cells were quantitated using a hemocytometer in a phase-contrast microscope, and adjusted to a concentration of 4 x 10⁶ cells/ml.

8.3.3 INSULIN BINDING ASSAY

2 x 10⁶ adipocytes were incubated in Krebs Ringer HEPES buffer (5% BSA; pH 7.4) containing ¹²⁵I-labelled insulin (final concentration 40 pM; Amersham, Buckinghamshire, England) and varying concentrations of unlabelled insulin (0, 0.4, 2, 8 nM, 2 μM; Novo Nordisk, Copenhagen, Denmark) and murine leptin (0, 1, 50 nM, 2.5 μM; kindly provided by Dr. Margery Nicolson, Amgen Inc., Thousand Oaks, California, USA) in a total volume of 1 ml for 45 min at room temperature. Incubations were terminated by the oil flotation method (Vinten et al. 1976), and incorporation of radiolabelled insulin by adipocytes measured using a gamma counter (Multigamma Counter, model LKB Wallac 1261, Turku, Finland).

8.3.4 STATISTICAL ANALYSIS

All data are expressed as the mean ± sem. Statistical tests were unpaired Student t-tests, results were considered significant at p<0.05.
8.4 RESULTS

Figure 8.1 shows the dose-dependent effects of leptin on maximal $^{125}$I-insulin binding by isolated rat adipocytes. We found a non-significant tendency for a reduction in maximal $^{125}$I-insulin binding in the presence of 1 nM leptin. $^{125}$I-insulin binding was significantly reduced (by 19%) at a leptin concentration of 50 nM, and was further inhibited (24%) when the leptin concentration was 2.5 μM (Figure 8.1).

**FIGURE 8.1:** The effect of varying leptin concentration on specific $^{125}$I-insulin binding by isolated rat adipocytes. *Significantly different to no leptin and 1 nM leptin (p<0.05)

Displacement curves were generated for each of these leptin concentrations and are represented in Figure 8.2. These curves clearly show the dose-dependent effects of exogenous leptin on $^{125}$I-insulin binding by isolated rat adipocytes.
**FIGURE 8.2:** Displacement curves for specific $^{125}I$-insulin binding at various leptin concentrations in isolated rat adipocytes. (a significantly different to no leptin (p<0.05); b significantly different to no leptin and 1 nM leptin (p<0.05); c significantly different to no leptin, 1 nM and 50 nM leptin (p<0.05).
8.5 DISCUSSION

In this study we have demonstrated a dose-dependent inhibition of $^{125}$I-insulin binding by leptin in isolated rat adipocytes (Figure 8.1). Binding of labelled insulin tended to be lower in the presence of 1 nM leptin, was significantly reduced (by 19%) at a leptin concentration of 50 nM, and was further inhibited (24%) when the leptin concentration was 2.5 μM. Displacement curves were generated for each of these leptin concentrations and are represented in Figure 8.2. Analysis of displacement curve binding data suggested that leptin reduced maximal insulin binding in a dose-dependent manner, but had no significant effect on the affinity of insulin for its binding site.

Interestingly, a recent publication described long-term preincubation of isolated adipocytes which resulted in impairment of insulin action on metabolic processes including glucose transport, glycogen synthesis and lipogenesis (Muller et al. 1997). Specific insulin binding was not measured in this study, but it is possible that reduced binding of insulin by the adipocytes could account for part of the reduction in insulin action in these cells.

Previous studies by our group and others have clearly established a relationship between plasma leptin concentration and BMI/body fat content (Zimmet et al. 1996; de Courten et al. 1997). The degree of hyperleptinemia in obese individuals has been directly correlated with the extent of obesity. In addition, it was established that plasma leptin concentrations were positively correlated with plasma insulin, a surrogate measure of insulin sensitivity, independent of BMI (Zimmet et al. 1996; de Courten et al. 1997). In previous studies detailed in earlier chapters of this thesis we have shown a significant relationship between insulin and leptin in Psammomys obesus. In Chapter 3 plasma leptin was correlated with plasma insulin concentration in a cross sectional analysis, and insulin-resistant animals were hyperleptinemic relative to insulin-sensitive Psammomys obesus independent of body weight. These studies have led to the suggestion that leptin may be an important link between obesity and NIDDM.

Leptin is not the only adipocyte-derived peptide linked with insulin resistance. Tumor necrosis factor-alpha (TNFα) is a cytokine which, like leptin, is secreted from adipose tissue and circulates in plasma in concentrations proportional to body fat mass (Hotamisligil et al. 1993; Hotamisligil et al. 1995). TNFα has previously been linked with insulin resistance, as administration of TNFα causes insulin resistance both in vivo and in vitro (Douglas et al. 1991; Feingold and Grunfeld 1992; Lang et al. 1992). Chronic exposure of 3T3-L1 adipocytes to low concentrations of TNFα strongly inhibited insulin-stimulated glucose uptake, caused a moderate decrease in the insulin-
stimulated phosphorylation of the insulin receptor and resulted in a dramatic reduction in the phosphorylation of IRS-1 (Hotamisligil et al. 1994). The authors concluded that TNFα directly interfered with the signalling of insulin through its receptor (Hotamisligil et al. 1994). Although insulin binding by adipocytes was not measured directly in these studies, it is conceivable that a reduction in insulin binding may have occurred. Conversely, neutralisation of TNFα in vivo using a soluble TNFα receptor-IgG fusion protein ameliorated insulin resistance in Zucker fa/fa rats (Hotamisligil et al. 1993). The results of the current study suggest that leptin could also impact on insulin sensitivity, as incubation of isolated adipocytes with leptin significantly inhibited insulin binding in a dose-dependent manner. This may represent a mechanism whereby the elevated leptin concentrations associated with human obesity may have a paracrine effect in adipocytes limiting the activity of insulin-stimulated enzymes such as lipoprotein lipase, contributing to adipocyte insulin resistance as fat mass increases (Jeanrenaud et al. 1985; Jeanrenaud 1994).

Apart from adipocytes, leptin may have effects on insulin action in other tissues. For example, the recent publication of Cohen and colleagues showed that leptin significantly attenuated the actions of insulin on isolated hepatocytes (Cohen et al. 1996). We suggest that leptin (like TNFα) could be important in the relationship between obesity and reduced insulin sensitivity, and the role of leptin in the development of NIDDM in obese subjects needs further investigation.

In previous chapters we have shown that in Psammomys obesus plasma leptin concentrations correlate with body fat and plasma insulin, and that insulin resistance was associated with hyperleptinemia independent of body weight. Together with previous studies in these animals, we contend that Psammomys obesus represents a very good polygenic animal model of obesity and NIDDM, as almost all findings in these animals correspond closely with results from human populations. If this is true, and obese humans are shown to be relatively insensitive to the effects of exogenous leptin administration, as we showed in Psammomys obesus (Chapter 7), then it is possible that very high dosages of leptin may be required to elicit weight loss in human obesity. The results of the current study suggest that, if high doses of leptin were used to treat human obesity, then a possible side effect could be the precipitation or exacerbation of insulin resistance, possibly resulting in NIDDM.
CHAPTER 9:

CONCLUSIONS AND FUTURE DIRECTIONS

9.1 THE GENETICS OF OBESITY

Obesity and NIDDM are both heterogeneous diseases which are thought to be polygenic in etiology. Previous studies have identified several genes associated with obesity including apolipoprotein-B (Rajput-Williams et al. 1988), apolipoprotein-D (Vijayaraghavan et al. 1993), TNFα (Norman et al. 1995), the low density lipoprotein receptor (Zee et al. 1992) and the dopamine D2-receptor (Comings et al. 1993). In addition the β3AR mutation was associated with obesity in some studies (Clement et al. 1995; Kurabayashi et al. 1996), but not in several other studies.

In Chapter 4 of this dissertation we describe the results of investigations into the relationship between polymorphisms of the ob and β3-adrenergic-receptor (β3AR) genes and phenotypic features of obesity and NIDDM. The ob gene polymorphism was associated with elevated fasting blood glucose and insulin concentrations in Nauruans but not Australians. The β3AR mutation was associated with obesity in the Australian population studied. Interestingly, a combination of the two mutations was found in 19 Australians who exhibited increased body weight and waist circumference compared with those without this genotype. These results suggested that both the ob and β3AR genes may be implicated in the pathogenesis of obesity and/or NIDDM.

In this study we investigated 342 subjects from Nauru and 322 from Australia. The study was made difficult by a lack of Nauruan individuals with the class I allele of the ob gene polymorphism, and the low number in both populations who exhibited the β3AR Trp64Arg mutation. These factors meant that in the Nauruan population we investigated only 5 individuals homozygous for the ob gene polymorphism class I allele and none who were homozygous for the Trp64Arg allele. In addition only 10 Nauruans were heterozygous for this mutation. The Australian population studied contained only 3 Trp64Arg homozygotes.

Therefore the statistical power of analyses comparing the groups separated according to genotype was greatly reduced. In the future, the findings of this study could be strengthened considerably by the recruitment of additional subjects into the two groups. Despite these shortcomings, the study did clearly show a relationship between these two gene polymorphisms and aspects of the Metabolic Syndrome. The most interesting finding was the association of a combination of the two polymorphisms with body
weight and waist circumference, an indicator of visceral obesity (Lemieux et al. 1996a,b). This finding suggests that these two genes may interact in some way to predispose the afflicted individual to the development of visceral obesity, although further studies are required to clarify this relationship.

Firstly, the results reported here need to be independently confirmed in another laboratory to ensure that we did not detect a statistical anomaly, or an unrepresentative subgroup of the general population. In addition, studies are required which seek to explain the physiological and metabolic relevance of these findings and how they contribute to the disease phenotype. In particular, studies of how the tetranucleotide repeat polymorphism associated with the ob gene may affect the expression of the gene in vivo, and any effects of this polymorphism on adjacent promoters and enhancers need to be investigated.

The physiological relevance of the Trp64Arg mutation of the β3AR gene is also yet to be proven (Mauriege and Bouchard 1996). The putative functions of this receptor and how it could be implicated in the pathogenesis of obesity and/or NIDDM are discussed in detail in Chapter 4. Studies seeking to elucidate the metabolic functions of this receptor are required before its importance can be assessed. For example, how important is the β3AR in mediating the enhanced lipolysis observed in visceral adipose tissue (Lonnqvist et al. 1995)? In addition, the long-standing question of whether thermogenesis in brown adipose tissue is physiologically relevant in terms of overall energy expenditure in adult humans needs to be resolved. Only when more is known about the metabolic implications of these polymorphisms will we be able to interpret these results in a more definitive manner. At that time it is hoped that novel therapeutic approaches may result based on the physiology discovered.

One particularly interesting finding in this study was the significant association between the Trp64Arg mutation and reduced plasma leptin concentrations in the Nauruan population studied. A previous study in Pima Indians, who, like the Nauruans, are thought to be representative of the 'thrifty genotype', showed that lower plasma leptin concentrations were predictive of excessive body weight gain over time (Ravussin et al. 1997). Therefore future studies should be designed to prospectively follow these individuals to see whether they are more prone to body weight gain compared with individuals without the Trp64Arg mutation and with higher plasma leptin concentrations.

Once again, it would be difficult to explain the metabolic basis of such an association without further information regarding the physiological role of the β3AR in human
physiology. Administration of β3-agonists inhibited ob gene expression in vitro and resulted in decreased plasma leptin concentrations in lean mice and rats (Moinat et al. 1995; Mantzoros et al. 1996; Trayhurn et al. 1996; Mitchell et al. 1997). Interestingly, a genome wide scan of human DNA identified significant linkage between body fat mass/plasma leptin levels and a marker adjacent to the β3AR gene on chromosome 8, however it must be noted that other possible candidate genes in this region include POMC (Comuzzie et al. 1997). A model was recently proposed in which leptin inhibited its own expression via the sympathetic nervous system and the β3AR by stimulation of lipolysis in adipose tissue, reducing body fat and therefore ob gene expression (Giacobino 1996). In this model however, a β3AR defect such as the Trp64Arg mutation would be expected to result in reduced feedback inhibition of leptin biosynthesis and hyperleptinemia rather than reduced plasma leptin concentrations as we observed in Nauruans. As discussed above, further studies are required to explain the role of the β3AR in human physiology and the precise metabolic effects of the Trp64Arg mutation.

Future studies regarding the genetics of obesity could also include investigation of other published associations with obesity as listed above. In addition, scanning of other candidate genes for obesity and NIDDM could result in the identification of novel polymorphisms which could then be investigated in our study populations. These may include the genes encoding neuropeptide Y (NPY), melanin-concentrating hormone (MCH), melanocyte-stimulating hormone (MSH) and glucagon-like peptide-1 (GLP-1). Another gene which is currently receiving considerable attention is that encoding the leptin receptor. Preliminary studies in our laboratory have suggested an association between a polymorphism in the leptin receptor gene and insulin resistance in Nauruans (Andrea de Silva personal communication). In addition, combination of two polymorphisms in the leptin receptor gene with the polymorphisms examined in this study revealed a subgroup of the Nauruan population expressing all 4 mutations who were significantly obese compared with those not expressing this genotype. Further reports investigating the molecular biology of the leptin receptor gene will surely be forthcoming from our laboratory and others in the near future.

Other genes of interest which may be examined in humans include those involved in the pathogenesis of obesity in animal models, including carboxypeptidase E, the gene encoding the agouti protein and the gene responsible for obesity in tub/tub mice. Further studies should also be undertaken in an attempt to discover novel genes involved in the pathogenesis of obesity. Techniques are available such as differential display polymerase chain reaction (DDPCR) which would enable researchers to discover genes which are expressed at different levels in tissues of obese and lean
individuals. This methodology was previously used to find that the gene encoding MCH was overexpressed in hypothalami of ob/ob mice (Qu et al. 1996). Therefore it is possible that further investigations using DDPCR in both humans and animal models of obesity could lead to the discovery of novel genes and metabolic pathways involved in energy balance.

Many of the future studies suggested above could also be undertaken in appropriate animal models of obesity and NIDDM. That is, all of the genes listed above, and the technique of DDPCR could be used to investigate genetic factors which contribute to the disease phenotypes in these animals. More thorough studies are possible in animals due to the availability of tissues which should be examined such as the hypothalamus. One such animal which should be the subject of future genetic investigations is Psammomys obesus.

9.2 THE OB GENE AND LEPTIN IN PSAMMOMYS OBESUS

Previous studies by our group and others have shown Psammomys obesus to be a very good polygenic animal model of obesity and NIDDM (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 plasma insulin concentrations which is similar to the pattern of obesity and NIDDM which has been observed in cross-sectional analyses of human populations (DeFronzo 1988; Barnett et al. 1994a). When the ob gene was discovered in 1994 and shown to be the cause of morbid obesity in ob/ob mice (Zhang et al. 1994), we set about investigating the role of this gene, and its product leptin, in the pathogenesis of obesity and NIDDM in Psammomys obesus.

Initial studies identified overexpression of the ob gene in obese Psammomys obesus, similar to what has been reported in other obese rodents (Zhang et al. 1994; Maffuci et al. 1995a) and obese humans (Considine et al. 1995; Hamilton et al. 1995; Lonnqvist et al. 1995) compared with lean controls. As in humans, but not other rodents, a continuous relationship between ob gene expression and body weight or plasma insulin concentrations was found in Psammomys obesus. Therefore the pattern of ob gene expression in Psammomys obesus resembled that observed in humans, making Psammomys obesus a useful animal model in which to study factors which affect ob gene expression.

Future studies are planned to investigate factors which may alter ob gene expression in Psammomys obesus, and to determine the effects on energy balance and body weight of altering expression of the ob gene in vivo. Ob gene expression in Psammomys obesus
correlated with body fat content, and it has been suggested that leptin may act as a 'lipostat' (Kennedy 1953; Caro 1996). Therefore it is possible that factors which increase ob gene expression at a given level of body fat could 'trick' the hypothalamus and result in body weight loss in obese individuals.

The role of glucocorticoids (GCs) in ob gene expression in Psammomys obesus needs to be investigated. Such studies could examine the effects of adrenalectomy on body weight, ob gene expression and plasma leptin concentrations in these animals, and the effects of GC supplementation in adrenalectomised Psammomys obesus. GCs are thought to have a permissive effect on obesity as adrenalectomy prevented obesity in other animal models (Bray et al. 1990a,b; Sainsbury et al. 1996). Reports detailing the role of GCs in the obesity of Psammomys obesus have not been published to our knowledge, and the studies suggested above could simultaneously answer basic questions regarding the role of GCs in the obesity of these animals, as well as the relationship between GCs, the ob gene and leptin in Psammomys obesus.

Another factor which could be investigated in Psammomys obesus is CEBPα. CEBPα is an adipocyte-specific transcription factor which is induced during differentiation of adipocytes and was shown to induce ob gene expression in vitro (Hwang et al. 1996; Miller et al. 1996). If a compound were available which stimulated CEBPα production but not adipocyte differentiation, this may represent a method of elevating ob gene expression without increasing body fat content. To our knowledge no such compound exists, but basic characterisation of CEBPα expression in Psammomys obesus should be investigated anyway to expand our knowledge of adipose tissue metabolism in this species.

Additional studies should be conducted to investigate the role of PPARγ in the pathogenesis of obesity in Psammomys obesus. These investigations could be designed to elucidate the relationship between PPARγ and ob gene expression, and how this is affected by administration of thiazolidinediones (TDs). In addition, basic characterisation of the role of PPARγ in energy balance in Psammomys obesus could be investigated, including differences in expression of the gene between lean and obese animals.

Plasma leptin concentrations in Psammomys obesus correlated with body weight, percentage body fat and plasma insulin concentration. Once again these relationships were similar to those observed in human cross-sectional studies, confirming Psammomys obesus as a good animal model in which to study the pathogenesis of obesity and NIDDM. Indeed, to our knowledge Psammomys obesus is the only animal
model of obesity which exhibits a complete spectrum of plasma leptin concentrations which correlate with body fat content and plasma insulin levels. In addition, we showed that plasma leptin was significantly elevated in insulin-resistant *Psammomys obesus* compared with their insulin-sensitive littermates independent of body weight or body fat content, suggesting a significant physiological relationship between leptin and insulin in these animals. The relationship between leptin and insulin will be discussed in more detail below.

The basic characterisation of leptin in *Psammomys obesus* suggested that obese animals were relatively insensitive to the anorexigenic and hypermetabolic actions of their endogenous leptin, a paradigm which mirrored that observed in obese humans (Considine et al. 1996a; Caro et al. 1996). Future studies should concentrate on investigating the mechanisms involved in this apparent leptin resistance. Basic characterisation of the expression and function of leptin receptors in *Psammomys obesus* should be examined. This should include examination of the expression of the various receptor isoforms and their functional characterisation in various tissues including the hypothalamus in both lean and obese *Psammomys obesus*. Leptin receptor expression could be measured by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) specific for each of the receptor isoforms. The receptor isoforms should then be sequenced to search for differences between lean and obese *Psammomys obesus*, and for the published mutations in the leptin receptors of db/db mice, fa/fa rats, Koletsky (f) rats and JCR:LA cp/cp rats (Chen et al. 1996; Chua et al. 1996; Wu-Peng et al. 1997; Campfield et al. 1996).

Functional studies of the *Psammomys obesus* leptin receptor should also be undertaken in both lean and obese animals. These may include both in vitro and in vivo studies of the uptake of labelled leptin by various cells/tissues of *Psammomys obesus*, and the involvement of leptin receptors in the clearance of labelled leptin from the circulation. In addition, signalling from the leptin receptor should be investigated, such as the effects on STATs and other transcription factors and intracellular second messengers in cells exposed to leptin and in vivo in lean and obese *Psammomys obesus*.

We have shown that alteration of energy intake both in the long- and short-term affects plasma leptin in *Psammomys obesus*. Dietary energy restriction from weaning significantly reduced body weight, plasma insulin and leptin in *Psammomys obesus*. However a subgroup of animals (19%) still developed hyperinsulinemia and tended to have elevated plasma leptin concentrations compared with normoglycemic energy-restricted animals. This study showed that hyperphagia was essential for the development of obesity and NIDDM in *Psammomys obesus*, but some animals
developed insulin resistance and leptin resistance even in the absence of hyperphagia and obesity. In the short-term study, plasma leptin concentrations fell in lean, insulin-sensitive but not obese, insulin-resistant *Psammomys obesus* after a 24-hour fast. This suggested a significant dysregulation of leptin in obese, insulin-resistant *Psammomys obesus*.

These studies could be extended by examining the effects of caloric deprivation on actual *ob* gene expression in adipose tissue, and by measuring expression of the hypothalamic leptin receptor in these animals. In addition, parallel studies could be conducted to investigate the effects of increasing energy expenditure on leptin in *Psammomys obesus*, perhaps by using a motorised exercise wheel. To give a more complete picture of the control of energy balance in *Psammomys obesus*, we should also investigate other putative regulators of energy intake and energy expenditure including NPY, MCH/MSH and GLP-1, and the relationship of these factors with plasma leptin. The differences between lean and obese *Psammomys obesus* with respect to all of these factors along with leptin and its receptor should yield a clearer overview of the control of energy balance in *Psammomys obesus*, and the changes induced by alterations in energy intake and/or energy expenditure as described above.

Further examination of energy expenditure in *Psammomys obesus* could include investigation of the various uncoupling proteins (UCPs) in these animals. Several of these UCPs have been identified at this time, including one (UCP2) which appears to be expressed in various tissues (including skeletal muscle), other than brown adipose tissue. The role of these proteins, which are potentially important in determining the energy expenditure of the organism, needs to be investigated in *Psammomys obesus*.

In summary, *Psammomys obesus* is a very good animal model of obesity and NIDDM, and provides a great opportunity to investigate the role of leptin in these diseases. The studies described in this dissertation provide a solid basis from which to investigate leptin and other factors which influence energy balance in these animals.

### 9.3 LEPTIN TREATMENT IN *PSAMMOMYS OBESUS*

The similarities described above between *Psammomys obesus* and humans led us to investigate the effects of administration of leptin to *Psammomys obesus*, as leptin has been proposed to be a potential therapeutic agent for human obesity. Using a variety of experimental paradigms, we established that *Psammomys obesus*, considered as a species, were significantly leptin resistant relative to other rodents, with very high doses of leptin (45 mg/kg/day) required to reduce food intake and body fat content in these
animals. In addition, obese *Psammomys obesus* were shown to be leptin-resistant relative to their lean littermates, with no effects on food intake or body fat content detected at a dose of 45 mg leptin/kg body weight/day.

Plasma leptin concentrations were maintained at significantly elevated levels throughout this study, and when the animals were killed at the completion of the study the circulating leptin levels were approximately 80 times baseline concentrations. Therefore the tissues removed from these animals (and frozen) provide an opportunity to investigate the effects of pharmacologic hyperleptinemia on gene expression. Firstly, the expression of leptin receptors in various tissues should be examined. The results of this study were reminiscent of studies in Zucker fa/fa rats, in which the ICV dose of leptin required to affect food intake was approximately 10-fold higher than that which was effective in lean littermates (Cusin et al. 1996). It is now known that the phenotype observed in fa/fa rats results from a leptin receptor mutation, which may reduce the affinity of the receptor for leptin (Chua et al. 1996; Iida et al. 1996; Takaya et al. 1996). Therefore we should investigate fully the possibility that a leptin receptor abnormality may be involved in the apparent leptin resistance observed in *Psammomys obesus*. This should include studies on leptin receptor expression levels, as well as functional studies including the expression of STATs and other intracellular factors induced by leptin binding to its receptor. Other studies using these stored tissues should include the effects of leptin on other putative regulators of energy balance, such as the expression levels of NPY mRNA in the hypothalamus.

A recently published study utilised adenovirus gene transfer to induce hyperleptinemia in normal rats (Shimabukuro et al. 1997). This resulted in greatly reduced triglyceride content in various tissues, suggesting increased intracellular fat oxidation in response to hyperleptinemia (Shimabukuro et al. 1997). We showed in these studies that hyperleptinemia acutely decreased plasma triglycerides in *Psammomys obesus* (Chapter 7). It would therefore be interesting to investigate the effects of pharmacologic hypreleptinemia on aspects of fat metabolism in tissues from *Psammomys obesus*. Such investigations could include measurement of tissue triglyceride content in various tissues, as well as investigation of the expression and activities of key enzymes such as lipoprotein lipase and carnitine palmitoyl transferase in tissues including liver and adipose tissue.

One of the most interesting findings from our studies of leptin administration in *Psammomys obesus* was the suggestion that treatment with leptin may exacerbate insulin resistance in susceptible individuals (Chapter 7). As discussed previously, this suggests that a possible side-effect of leptin therapy for obesity could be worsening of
insulin resistance and possibly even the development of NIDDM in some patients. This would represent a very significant problem for individuals treated with leptin, and would probably override the health benefit of weight loss in most cases. For these reasons the effects of leptin treatment on insulin action should be extensively investigated.

Epidemiological studies have previously shown a significant relationship between plasma insulin and leptin in humans (Zimmet et al. 1996; de Courten et al. 1997). This relationship remained significant when corrected for BMI, showing a significant relationship between insulin and leptin independent of body fat content. In these studies we demonstrated a similar relationship between these hormones in Psammomys obesus (Chapter 3), with hyperleptinemia in insulin-resistant animals independent of body weight.

A recent in vitro study extended this relationship by showing that leptin inhibited insulin action in isolated hepatocytes (Cohen et al. 1996). We investigated this relationship by determining the effects of leptin on insulin binding by isolated rat adipocytes. Fat tissue represents another significant site of insulin resistance in humans with NIDDM (DeFronzo 1988). In this study we detected a significant, dose-dependent reduction in maximal insulin binding in adipocytes exposed to leptin.

This study, together with that of Cohen and coworkers, suggested that relatively high concentrations of leptin may significantly alter insulin sensitivity in some tissues. Further studies are required to investigate the precise mechanisms involved in these effects, including further in vitro studies on insulin binding by purified insulin receptors and adipocyte plasma membranes. In addition, these studies need to be extended by investigating the metabolic consequences of this reduced insulin binding, such as the effects on glucose uptake and metabolism by the cells, and effects on fat metabolism.

Further studies could also be designed to investigate the effects of leptin on insulin action in other cell culture systems such as myocytes/myotubes, as muscle is also a significant site of insulin resistance in affected individuals (DeFronzo 1988). Other investigations could include studies in cells cultured from Psammomys obesus, and direct comparisons between cells from lean, healthy animals and those with obesity and NIDDM. These studies could be conducted in hepatocytes and adipocytes from these animals, and could include investigations of both insulin binding and action in response to leptin.
9.4 OVERVIEW

In the studies detailed in this thesis, we have shown that ob gene expression and plasma leptin concentration correlated significantly with body weight, percentage body fat and plasma insulin concentration in *Psammomys obesus*, a unique animal model of obesity and NIDDM. In addition, plasma leptin concentrations were significantly elevated in insulin resistant *Psammomys obesus* independent of body weight, suggesting a significant physiological relationship between these two hormones in the control of energy balance. Dietary energy restriction from weaning in *Psammomys obesus* prevented excessive body weight gain, hyperleptinemia and hyperglycemia compared with *ad libitum* fed animals. Interestingly, 19% of the energy-restricted animals still developed hyperinsulinemia and tended to have increased plasma leptin compared with normoinsulinemic energy-restricted *Psammomys obesus*. Fasting for 24 hours significantly reduced plasma leptin concentration in lean, insulin-sensitive but not obese, insulin-resistant *Psammomys obesus*, suggesting a dysregulation in the response of leptin to acute caloric deprivation in these animals.

The effects of leptin administration to *Psammomys obesus* were also investigated. Single daily intraperitoneal injection of 5 mg leptin/kg body weight for 14 days had no significant effect in lean or obese *Psammomys obesus*. This dose had previously been shown to rapidly and significantly reduce food intake and body weight in ob/ob and wild-type mice, suggesting relative leptin resistance in *Psammomys obesus*. Acute (8 hour) effects of administration of 5 mg leptin/kg body weight were also investigated. No significant effects on food intake or plasma insulin were detected, however blood glucose concentrations were significantly elevated in obese, glucose intolerant *Psammomys obesus*, suggesting an exacerbation of insulin resistance in susceptible animals. Treatment of lean, healthy *Psammomys obesus* with 45 mg leptin/kg body weight/day for 7 days resulted in significant decreases in food intake and percentage body fat, showing that the leptin resistance observed in this species could be overcome by the administration of very large doses of leptin.

In another study, leptin was shown to significantly inhibit maximal insulin binding to isolated adipocytes, suggesting that leptin may represent an important link between obesity and NIDDM. Links between aspects of obesity and NIDDM and polymorphisms in the ob and β3-adrenergic receptor genes were also investigated in two human populations.
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