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LIVER FAT METABOLISM, OBESITY
AND DIABETES IN
PSAMMOMYS OBESUS

by
Paul Lewandowski, Bsc (Hons)

Submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

Deakin University 1999
DEAKIN UNIVERSITY

CANDIDATE DECLARATION

I certify that the thesis entitled

LIVER FAT METABOLISM OBESITY AND DIABETES IN PSAMMOMYS OBESUS

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ABSTRACT

Defects in fat metabolism are central to the aetiology and pathogenesis of obesity and type II diabetes. The liver plays a central role in these disease states via its regulation of glucose and fat metabolism. In addition, accumulation of fat within the liver has been associated with changes in key pathways of carbohydrate and fat metabolism. However a number of questions remain. It is hypothesised that fat accumulation within the liver is a primary defect in the aetiology and pathogenesis of obesity and type II diabetes. Fat accumulating in the liver is the result of changes in the gene expression of key enzymes and proteins involved with fat uptake, fat transport, fat oxidation, fat re-esterification or storage and export of fat from the liver and these changes are regulated by key lipid responsive transcription factors.

To study these questions *Psammomys obesus* was utilised. This polygenic rodent model of obesity and type II diabetes develops obesity and diabetes in a similar pattern to susceptible human populations. In addition dietary and environmental changes to *Psammomys obesus* were employed to create different states of energy balance, which allowed the regulation of liver fat gene expression to be examined. These investigations include: 1) Measurement of fat accumulation and fatty acid binding proteins in lean, obese and diabetic *Psammomys obesus*. 2) Characterisation of hepatic lipid enzymes, transport protein and lipid responsive transcription factor gene expression in lean, obese and diabetic *Psammomys obesus*. 3) The effect of acute and chronic energy restriction on hepatic lipid metabolism in *Psammomys obesus*. 4) The effect of sucrose feeding on the development of obesity and type II diabetes in *Psammomys obesus*. 5) The effect of nicotine treatment in lean and obese *Psammomys obesus*. 6) The effect of high dose leptin administration on hepatic fat metabolism in *Psammomys obesus*.

The results of these studies demonstrated that fat accumulation within the liver was not a primary defect in the aetiology and pathogenesis of obesity and type II diabetes. Fat accumulating in the liver was not the result of changes in the gene expression of key enzymes and proteins involved in hepatic fat metabolism. However changes in the mRNA level of the transcription factors PPARα and SREBP-1C was associated with the development of diabetes and the gene expression of these two transcription factors was associated with changes in diabetic status.
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Ego ipse alyd alci dedicare qui ad tute.
At time of submission the results of two chapters within this thesis have been published in international scientific journals and two more manuscripts have been submitted for publication and are currently under review.

**Published Papers**
The data contained within this publication form all of chapter three of this thesis.

The data contained within this publication form the metabolic data presented in chapter eight of this thesis.

**Manuscripts Submitted For Publication**
The data contained within this publication form all of chapter four of this thesis.

The data contained within this publication form all of chapter eight of this thesis.

The remaining chapters have been prepared as manuscripts and are to be submitted for review and publication.
In addition, laboratory techniques, tissue samples and portions of metabolic data from animals that were used in the various studies contained within this dissertation has lead to the publication or submission for review of the following collaborative papers.


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Chapter 1

INTRODUCTION

1.1 TYPE II DIABETES

1.1.1 Definition And Diagnosis
Type II diabetes, also known as non-insulin dependent diabetes mellitus is one of the most pressing health problems the world currently faces. The World Health Organisation (WHO) defined diabetes mellitus as “characterised by hyperglycemia and disturbances of carbohydrate, fat and protein metabolism that are associated with absolute or relative deficiencies in insulin action and/or insulin secretion” (WHO 1994). The diagnosis of diabetes mellitus is based on fasting and/or post-glucose load hyperglycemia according to Table 1.1.

Table 1.1: WHO Diagnostic Criteria For Diabetes Mellitus.

<table>
<thead>
<tr>
<th></th>
<th>PLASMA GLUCOSE (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fasting</td>
</tr>
<tr>
<td>Normal Glucose Tolerance</td>
<td>&lt;7.8 and</td>
</tr>
<tr>
<td>Impaired Glucose Tolerance</td>
<td>&lt;7.8 and</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>&gt;7.8 or</td>
</tr>
</tbody>
</table>

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

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

It has been recently been recommended by the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus of the American Diabetes Association (ADA) that a modification to the WHO cutpoint for fasting plasma glucose (FPG) be lowered from 7.8 to 7.0 mmol/L for the diagnosis of diabetes. In addition it was recommend that a new category of intermediate glucose metabolism, termed impaired fasting glucose (IFG) for FPG values between 6.1-7.0 mmol/L. The ADA suggest that these new criteria will reduce the number of oral glucose tolerance tests (OGTT) that need to be performed, reduce the complications derived from it and reduce the economic cost, yet preserving the diagnostic efficiency. The acceptance of these new diagnostic criteria has been met with varied support. In two retrospective studies in Spanish populations and one in an Italian population the new ADA FPG values would have resulted in a diminished number of people with undiagnosed diabetes and may have avoided some OGTT (Conge 1998; Falip 1998; Vaccaro 1998). In contrast to these findings, a retrospective investigation in a population of Mauritians found that the current WHO criteria using OGTT provided a more sensitive predictor of diabetes, especially in women (Shaw 1998). In Australia at the current time the WHO diagnostic criteria are currently used and the majority of the studies reviewed in the following discussion have used the WHO values (WHO 1994).

1.1.2 Prevalence

The prevalence of diagnosed diabetes mellitus in the Australian population aged 25 years and over is estimated to be 3.0% (Welborn 1995) compared with 6.6% in the USA (Zimmet 1996). The prevalence rises to at least 8.0% over the age of 75 years. Of known diabetic subjects 80-85% have type II diabetes (Welborn 1995) and the rate of undiagnosed type II diabetes in Australia is probably equal to the rate of known cases (Guest 1992). Globally, type II diabetes out numbers all other types of diabetes. In Western countries it constitutes approximately 80-85% of all cases of diabetes, as in Australia.

Most epidemiological data suggest that the rates of type II diabetes are rising in general throughout both the developed and developing world. However, temporal differences in the prevalence of type II diabetes are difficult to establish because of differing diagnostic methods used (Knowler 1990). Increases in prevalence rates might be
expected as the quality of health care is improved due to improved detection and the declining death rate from the disease (Harris 1987). In Australia the prevalence rate of type II diabetes has shown a slight increase over time, from 1.3% (males) and 1.2% (females) in 1966 to 2.7% (males) and 2.8% (females) in 1990 (Welborn 1995). 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 1995).

Increasing prevalence of type II diabetes is not unique to Australia, studies in high-risk populations have shown very large increases in type II diabetes prevalence over time. The age-standardised death rates from diabetes mellitus in Australian Aborigines have risen from 36 per 100,000 person-years in 1985-6 to 82 deaths per 100,000 person-years in 1991-2 (Bhatia 1995). The prevalence of type II diabetes in Pima Indians was 0.9% in 1954 (Joe 1994), 14.4% in 1965 (Joe 1994) and 25.5% in 1988 (Grabauskas 1988). Similarly in Fijians the overall prevalence of type II diabetes rose from 0.6% in 1967 to greater than 6% in 1985 (Zimmet 1990).

1.1.3 Health Risks And Mortality Related To Type II Diabetes

Patients with type II diabetes are at a high risk of developing a range of debilitating complications that can lead to premature disability and death. These complications include:

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.

Largely due to the development of complications such as those listed above (Pirart 1978) diabetes represents the seventh most common cause of death in the United States, accounting for 6.8% of total mortality (Huse 1989). An other study conducted in the USA using a national cohort of persons with diabetes demonstrated age-adjusted death rates were twice those for persons without diabetes (Kleinman 1988). The predominant cause of excess mortality was found to be due to cardiovascular disease (Kleinman 1988).
As well as excess mortality, people with diabetes experience significant morbidity, particularly if complications develop. It was shown that people with diabetes were significantly more likely to report the presence of long term conditions compared to those without diabetes (Aust. Bureau of Stats. 1991). Persons with diabetes also suffer in ways that are difficult to quantitate, they experience pain and suffering resulting from disease, anxiety, stress on individuals and their families associated with living with the disease and loss of independence (Dunn 1992).

Despite the trend away from inpatient services towards less costly outpatient services (NHMRC 1991), the cost associated with diabetes, in the public and private health sectors are high (NHMRC 1991). Based on calculations of costs associated with diabetes in the USA, including lost productivity resulting from morbidity and premature mortality, disease related costs in Australia have been estimated to exceed $600 million dollars per year (Zimmet 1985; Huse 1989). It is difficult to calculate diabetes related costs in Australia because no population data on diabetes are available (Songer 1990).

1.2 AETIOLOGY OF TYPE II DIABETES

1.2.1 Genetic Factors
There is strong evidence of a significant genetic component in type II diabetes. 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).

A number of studies of type II diabetes in identical versus non-identical twins have shown greater concordance in identical twins (Barnett 1981; Rotter 1987; Newman 1993), however the degree of concordance varied from 28 to 91 per cent in identical 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 1981). The less than 100 per cent concordance may be related to inadequate follow-up in a number of these studies. For example, in one study the initial concordance in identical twins was 58 per cent; however after a 6-year follow-up, most pairs initially discordant became concordant (Newman 1993). The presence of discordance among identical twins implies that environmental factors, including diet and exercise, may contribute significantly to disease development (Collier 1995).
The familial aggregation of type II diabetes the association is striking. For example, studies examining Pima Indian families have demonstrated that age- and obesity-adjusted incidence of type II diabetes are 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 1981). The severity of diabetes, insulin resistance and obesity also aggregate into families in Pima Indians (Knowler 1981). In addition, family studies in a number of other populations have confirmed these results and demonstrate an increase in type II diabetes in siblings and first degree relatives of diabetic parents compared with non-diabetic parents (Simpson 1968; Kobberling 1971; Baird 1973; Keen 1982; Cheta 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 1990).

Further evidence supporting the genetic basis of type II diabetes comes from population studies. Within a given environment, adjusted for obesity, diabetes is far more prevalent in certain ethnic groups. For example, a high prevalence rate of type II diabetes in South Pacific islanders such as Nauruans and other isolated populations has been extensively reported (Zimmet 1993). Additional support for the genetic basis of type II diabetes is found in population studies examining genetic admixture. Serjeanston et al. (1983) reported that prevalence of type II diabetes 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 1988).

1.2.1.2 Thrifty Genotype

The marked susceptibility to type II diabetes of traditional populations, including Pima Indians (Knowler 1983), Australian Aborigines (O'Dea 1992) and Nauruans (Zimmet 1990), has been proposed to be due to the presence of a thrifty gene, the exact nature of which is unknown (Neel 1962; Zimmet 1993). The "thrifty genotype" hypothesis contends 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, this involved periods of gorging following a successful hunt that were regularly interspersed with short periods of greatly reduced food intake (Neel 1962; Zimet 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 hyper-responsiveness of pancreatic islets in response to circulating substrates (Neel 1962), later termed a "quick insulin trigger" (Wendorf 1991; Zimet 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 1991; Zimet 1993). This was further refined to "selective insulin resistance" on the basis that a highly efficient system for converting large amounts of dietary protein 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 1993; Zimet 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 type II diabetes (Neel 1962; Zimet 1993). Several populations have been cited as examples of the thrifty genotype. These include American Indians of several tribes that 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 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 type II diabetes in the world (Knowler 1981; Knowler 1991; Wendorf 1991).

Another example may be the Pacific Islanders, who faced regular periods of caloric deprivation during long, migratory canoe voyages, as well as the effects of droughts and cyclones, favouring the selection of the thrifty genotype. In addition, food productivity was limited on the often barren coral atolls (Zimet 1993). Pacific Islanders have been shown to have extremely high prevalence rates of obesity and type II diabetes after transition to a "Westernised" lifestyle (Zimet 1992; Zimet 1993; Zimet 1995).
1.2.1.3 The Thrifty Phenotype

An alternative hypothesis concerning the aetiology of type II diabetes is the "thrifty phenotype" hypothesis, which contends that poor foetal and early post-natal nutrition "imposes mechanisms of nutritional thrift upon the growing individual" (Hales 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 type II diabetes and other constituents of the Metabolic Syndrome, later in life (Hales 1992).

Evidence supporting this theory comes from numerous studies showing a significant association between low birth weight and the development of type II diabetes (Hales 1991; Barker 1993; Phillips 1996), ischaemic heart disease and blood pressure (Barker 1989). 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 foetal and early infant life (Hales 1992; Barker 1993). These phases of under-nutrition 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 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 foetal nutritional status, and the fact that there may exist aetologic factors which contribute to both low birth weight and predisposition to elements of the Metabolic Syndrome (Purdy 1996). The use of birth weight as an estimate for intrauterine nutritional status in retrospective data collection is fraught with difficulty, since many confounding factors cannot be controlled (Purdy 1996). These may include maternal prepregnancy BMI, pregnancy weight gain, maternal nutritional status and diet during pregnancy, smoking, drug use, infection and many other factors (Purdy 1996). Therefore it is "precarious to attribute the type II diabetes seen in these studies to intrauterine under-nutrition" (Purdy 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 1996).

A recent study by Hattersley et al (1998) add weight to the role of nutrition in utero and birth weight contributing to the development of diabetes latter in life. Their
investigations show that foetuses carrying mutations in the glucokinase (CGK) gene are approximately half a kilogram lighter at birth than their unaffected sibs (Hattersley 1998). The suggested mechanism behind this defect involves alterations in insulin release. Insulin is a crucial growth factor during foetal life, detectable in the pancreas from the tenth week of gestation (Hattersley 1998). As glucose, but not insulin, crosses the placenta, foetal insulin production is dictated by maternal glucose levels. The foetus who carries a CGK mutation, but is born to a normoglycemic mother will have endured substantially reduced foetal insulin throughout pregnancy. If, however, the mother also carries the mutation, maternal hyperglycemia during pregnancy will restore foetal insulin secretion to normal and with it interuterine growth (Hattersley 1998).

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 foetal life and later development (Purdy 1996).

1.2.2 Environmental Factors
The importance of foetal and early post-natal nutrition discussed in the context of the thrifty phenotype hypothesis demonstrates the important role the environment may play in the development of type II diabetes. Environmental influences that may also interact with an underlying genetic predisposition and result in the expression of the diabetic phenotype include; obesity, physical activity and nutrition.

1.2.2.1 Physical Activity
Exercise training, defined as repeated bouts of exercise, results in multiple physical and metabolic changes, including a significant increase in aerobic capacity (Goodyear 1998). The metabolic changes have important implications for individuals who have impaired glucose tolerance (IGT), gestational diabetes, and type II diabetes. Epidemiological studies show that physical inactivity may be a risk factor for type II diabetes, at least in some ethnic groups, especially among Mexican Americans (US Dept. Health 1996). In persons without diabetes, glucose and insulin levels after a meal are significantly higher in less-active compared with more-active persons (US Dept. Health 1996). Although cross-sectional studies do not find a consistent association between physical activity and type II diabetes in all populations, such an association has been shown in both case-control and cohort studies (US Dept. Health 1996). One
population-based study of women aged 55-69 showed that high levels of physical activity reduced the risk of developing type II diabetes by 50% compared with age-matched women with low levels of physical activity (Kaye 1991).

Prospective cohort studies of male college alumni (Helmrich 1991) and female nurses (Manson 1991) demonstrate a reduced risk of developing type II diabetes with increased physical activity. This is particularly evident among high-risk persons, defined as those with a high body mass index, high blood pressure, or a parent with type II diabetes (Helmrich 1991). In one study, each 500 kcal of additional leisure-time physical activity per week was associated with a 6% decrease in the risk of developing type II diabetes. In addition, in feasibility studies in Sweden and China, physical activity has been instituted as part of an intervention to prevent the development of diabetes among persons with impaired glucose tolerance (Eriksson 1991; Pan 1995). Both studies showed a 50% reduction in the number of subjects who developed diabetes over a five- to six-year follow-up in the group that exercised compared with the group that did not. All of these approaches indicate a significant beneficial effect of regular physical exercise to prevent type II diabetes among high-risk subjects (Goodyear 1998).

Although there is no strong evidence that regular physical activity, even in combination with dietary modification, is associated with significant reductions in over-weight or obesity, there are significant psychological benefits to be gained from regular exercise (Bray 1991). However, despite the substantial evidence that regular physical activity is associated with reduced morbidity and mortality, many Australians take little or no exercise. Bauman and colleagues found that 32.7% of Australians were completely sedentary (Bauman 1990) and the proportion of Australians who were physically inactive appears to have increased between 1987 and 1992 (Thomas 1992).

1.2.2.2 Nutrition

Although there is limited evidence of the relationship between diet and the onset of diabetes, nutrition is often an important factor in the development and reduction of obesity. The diet of many 'westernized' populations is associated with an increase in the prevalence of chronic diseases such as diabetes, although studies have not yet revealed a direct relationship between dietary content and type II diabetes. However, there is evidence of low prevalence of type II diabetes among rural, traditional living populations and relatively high prevalence in urban, 'westernized' populations of the
same ethnic origin (Zimmet 1986). The low fat diet containing plant foods, which is common among non-urbanised populations, may be protective (Zimmet 1986).

Studies of populations that have undergone rapid changes in their way of life, such as Australian Aborigines (O'Dea 1992), Japanese migrant populations (Kawate 1979) and Pima Indians (Ravussin 1994), provide indirect evidence that dietary fat induces a higher prevalence of diabetes. In these populations the shift from traditional diets that were high in carbohydrates and fibre to diets containing large quantities of fat (Bennett 1984) is associated with a higher prevalence of diabetes. Data examined in prospective study analysing the diet of Pima Indian women suggested an association between the incidence of diabetes and both fat and total calorie intake (Bennett 1984) and a diet high in saturated fat and cholesterol was positively associated with fasting glucose in normoglycemic men (Feskens 1990). A number of other prospective studies in Dutch (Feskens 1995), male health professionals (Chan 1994), subjects in the San Luis Valley Diabetes Study (Marshall 1994) and Japanese Americans (Fujimoto 1994) add support for the implication of dietary fat as a potentiator of diabetes.

Overall the Australian diet still includes a number of components that have been linked closely to ill health, including fats, sugars and alcohol (Council 1992). The over consumption of foods high in fat and sugar may contribute to nutritional imbalance in some individuals and communities and contribute to the development of type II diabetes (Feskens 1990).

1.2.3 Obesity

In comparison to physical activity and nutrition, both of which are also important in treating patients in which type II diabetes already exists, the environmental factor which has the greatest impact on the development of type II diabetes is obesity.

1.2.3.1 Definition & Diagnosis

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. A simple measure of body mass fails to provide 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 thickness 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), 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 (Benn 1970; Bray 1985). In Australia, BMI is used to define overweight and obesity as shown in Table 1.2 (WHO 1995).

<table>
<thead>
<tr>
<th>Classification</th>
<th>BMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Under Weight</td>
<td>Less than 20</td>
</tr>
<tr>
<td>Ideal Weight</td>
<td>20-25</td>
</tr>
<tr>
<td>Over Weight</td>
<td>25-30</td>
</tr>
<tr>
<td>Obese</td>
<td>Greater than 30</td>
</tr>
</tbody>
</table>

Table 1.2: 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$^2$ (Lew 1979; Bray 1985; WHO 1995). 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 1995). In addition, BMI fails to account for the distribution of body fat, an important determinant of the health risks associated with obesity (WHO 1995). 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 (WHO 1995).

In addition to measuring total body fatness, anthropomorphic measurements are used to determine the distribution of that body fat. Such measurements commonly used to estimate central or visceral adiposity are the waist-to-hip circumference ratio (WHR) and various ratios of skinfold thickness. 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 (NHMRC 1996). These values are
derived from studies of all cause mortality and represent the points above which risk is significantly increased (Bjorntorp 1987; Bray 1987). Studies have shown that the WHR, or even simply the measurement of waist girth, are very good indicators of visceral obesity, as validated by the use of more sophisticated technologies such as computerised topography (Lemieux 1996). Obesity has also been defined using skinfold thickness 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 (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). Other techniques which measure body fat include ultrasound, electromagnetic conductivity, computerised topography, absorpiometry, 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 1992, Svendsen, 1993 #2865). 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).

1.2.3.2 Prevalence Of Obesity

The prevalence of obesity in these western 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).

In Australia, three studies have examined the prevalence of obese persons, with a cutpoint of BMI>30. The National Heart Foundation Risk Factor Prevalence Study of 1989, found the prevalence rates for obesity of 9.3% in men and 11.1% in women.
(Waters 1995). Whereas the 1989-1990 National Health Survey, results suggested that 8.2% of men and 9.1% of women were obese, based on self reported data (Waters 1995). Most recently the 1994-95 National Nutrition Survey found the self reported rate of obesity had risen to 18% (National Nutrition Survey 1997). The first study had measurements of BMI carried out by health professionals and is more likely to be 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 the Canada; males 12%, females 15% (Gurney 1988) and the United Kingdom; males 8%, females 13% (NHMRC 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 1988; Steyn 1990; Tappy 1991).

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 1994). Whereas 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 1983) and a steady increase has been observed throughout the whole of this century (Bray 1985).

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 (Zimmet 1977; Coventry, 1986; Knowler 1991; O'Dea 1992; Hodge 1993). For example, the Pima Indians have the highest known prevalence rates of obesity and type II diabetes, with over half of the individuals over 35 years of age classified as obese (Knowler 1991). As energy intake increases and energy expenditure falls, the prevalence rates of both obesity and type II diabetes have been shown to be greatly increased (Zimmet, 1992), these conditions have reached epidemic proportions in many newly industrialised nations such as Nauru (Zimmet 1992; Hodge 1993).
1.2.3.3 Health Risks & Mortality Related To Obesity
Obesity is associated with a number of co-morbid conditions, these included type II diabetes, coronary heart disease, hypertension and cancer.

1.2.3.3.1 Type II Diabetes & Obesity
The most important environmental factor in the development of type II diabetes is obesity. The incidence of type II diabetes increases with increasing BMI (Colditz 1992). In one study, 59% of individuals diagnosed as having type II diabetes had a BMI greater than 28.3 kg/m² (Melton 1983). Similarly, in nurses from the USA a strong continuous relationship was demonstrated between obesity and type II diabetes; those with a BMI >29 kg/m² had a 20-60-fold increased risk of developing type II diabetes compared with those people with a BMI <22 kg/m² (Colditz 1992). In addition, obese type II diabetic patients have a further decrease in hepatic insulin sensitivity and glucose oxidation rates compared with lean type II diabetics (Groop 1991). Thus it was suggested that the main effect of obesity in type II diabetic patients was to increase insulin resistance (Walker 1995) and obesity accelerates the progression from insulin resistance to type II diabetes (DeFronzo 1988).

Studies in Pima Indians have shown that adiposity is the most important predictor for the development of type II diabetes in children with one diabetic parent (McCance 1994). It was suggested that by preventing the development of obesity in these children, it could be possible to reduce their risk of developing type II diabetes (McCance 1994). Similar studies in Caucasian families with a history of type II diabetes have also shown that obesity appears to be an important early predictor for the development of type II diabetes (Walker 1995). In addition, weight loss has been shown to improve glucose tolerance and insulin resistance in obese type II diabetic patients (Doar 1975; Henry 1985; Henry 1986; Henry 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 type II diabetes compared with those whose weight did not change by more than 1 kg during that time (Colditz 1992).

1.2.3.3.2 Coronary Heart Disease & Obesity
The Framingham Heart Study identified obesity as an independent risk factor for the development of coronary heart disease (Hubert 1983). Body weight was ranked as the third most important risk factor for coronary heart disease in men, preceded by age and
dyslipidemia (Hubert 1983). Body weight was found to be a greater risk factor that systolic blood pressure and smoking (Hubert 1983). In the same study a 10% increase in weight was associated with a 13% increase in coronary heart disease among men and an 8% increase in coronary heart disease among women (Hubert 1983).

1.2.3.3 Hypertension & Obesity
The effects of BMI on blood pressure are greater in women that men (Ferrannini 1995). Epidemiological data have shown that both systolic and diastolic blood pressure increase with BMI (Ferrannini 1995). Data from the San Antonio Heart showed that nondiabetic obese patients had a higher average blood pressure than lean individuals, regardless of age and sex (Ferrannini 1995). A BMI of >25 kg/m² results in a linear increase in blood pressure, both systolic and diastolic, until a BMI of 40 kg/m² is reached (Ferrannini 1995).

Cardiac output increases in proportion to the expansion of body mass (Messcril 1983). Expanding adipose tissue would be expected to be associated with a decrease in blood pressure due to the increase in vascular volume. However, these feed back mechanism compensats for the expansion of body tissues by increasing cardiac output. The increase in cardiac output is greater than required and pushes the blood pressure up (Ferrannini 1995). Individuals who lose weight show a reduction in both cardiac output and blood pressure (Reisin 1983).

1.2.3.4 Cancer & Obesity
A number of studies have been performed in large groups of patients to assess the long term effects of obesity on the risk of developing cancer. The American Cancer Society Study included more than 750,000 individuals with a 12 year follow up period (Garfinkel 1985). The study showed that obese men and women have an increased risk of some types of cancer. In obese women there was an increased risk of cancer of the endometrium, cervix, ovary and breast (Garfinkel 1985). In obese men the incidence of colorectal and prostate cancer was increased (Garfinkel 1985). The Danish Record Linkage Study, which consisted of 44,000 individuals and an 11 year follow up period (Moller 1994), found a higher risk of cancer of the oesophagus, liver and pancreas in obese men and women. There was also an increased risk of some endocrine cancers, such as endometrial cancer, gastrointestinal cancers and kidney cancer, in obese women (Moller 1994).
1.2.4 Summary Of Type II Diabetes Aetiology

In summary, the information presented thus far it can be seen that type II diabetes is a prevalent disease in both western populations and newly industrialised populations. Associated with type II diabetes are a number of health risks which have considerable impact on an individual’s quality of life who suffers from the disease as well as imposing a considerable socioeconomic burden on the community at large. The development of type II diabetes is under the influence of an individual's genetic make up and the environment in which they live. The influence of a diabetic genetic predisposition is more prevalent in certain populations, which may be considered to express a 'thrifty genotype'. The environment has a major impact on the development of type II diabetes. The major environmental factor associated with the development of diabetes is obesity. The prevalence of obesity is increasing world wide as are the health risks associated with obesity which contribute to the mortality and morbidity of person suffering from it as well as those with type II diabetes.
1.4 DEFECTS IN CARBOHYDRATE METABOLISM IN TYPE II DIABETES

We shall now move to examine the mechanisms which are thought to contribute to the development of type II diabetes as well as examining specific alterations in metabolism that occur after the onset of the disease.

1.4.1 Normal Carbohydrate and Lipid Metabolism

The phenotypic expression of type II diabetes is characterised by persistent hyperglycemia accompanied by dyslipidemia. The underlying biochemical basis that results in these two conditions is complex and interrelated, and it is appropriate to consider the metabolism of carbohydrates and lipids in the postabsorptive and postprandial states.

1.4.1.1 Normal Postabsorptive State

In normal individuals, blood glucose homeostasis in the postabsorptive state is controlled by the endogenous production of glucose by the liver and the utilisation of glucose in peripheral tissues (Consoli 1989). Under steady state conditions glucose production is precisely matched by peripheral glucose utilisation and blood glucose levels remain relatively constant (De Fronzo 1992). Under these conditions a 60% of the blood glucose is taken up by the brain and nervous tissues (DeFronzo 1985). About 20-25% is utilised by the splanchnic bed, red blood cells and visceral organs (DeFronzo 1985). The insulin sensitive tissues, which are largely the skeletal muscles, account for approximately another 20-25% of the glucose clearance (Baron 1988). The low reliance of skeletal muscle on plasma glucose as a fuel source is due to the maintenance of low plasma insulin concentrations and increased circulating glucagon (De Fronzo 1992). This postabsorptive hormonal profile favours the oxidation of fatty acids and in the postabsorptive state fatty acids are the major fuel source for skeletal muscle, heart and lung tissues (Dinneen 1992).

1.4.1.2 Normal Postprandial State

Immediately after a meal the influx of glucose, lipids and amino acids into the circulation causes a transient increase in blood glucose concentration (Ferrannini 1988). This serves as a stimulus for insulin secretion into the bloodstream from pancreatic β-cells. Splanchnic and peripheral tissues uptake of glucose is enhanced and endogenous
production of glucose by the liver in inhibited by insulin, which maintains the blood glucose concentration within normal limits (DeFronzo 1985).

1.4.2 Diabetic Postabsorptive State
In type II diabetes the postabsorptive period is characterised by the maintenance of hyperglycaemia and elevated plasma lipid levels. The hyperglycaemia is due to the altered regulation of postabsorptive plasma insulin secretion, hepatic glucose output and peripheral glucose clearance.

1.4.2.1 Insulin Secretion
A number of studies involving individuals with type II diabetes have reported high, normal or low circulating insulin concentrations. The results of cross sectional studies in normal, impaired glucose tolerant and type II diabetic subjects with varying degrees of postabsorptive hyperglycaemia have shown that a complex relationship exists between plasma glucose and insulin concentrations (DeFronzo 1989; DeFronzo 1992). This relationship resembles an inverted ‘U’ shaped curve with insulin concentrations increasing as plasma glucose levels rose until a postabsorptive glucose concentration of approximately 9.8 mmol/L was reached. After which further elevations in plasma glucose concentrations were not matched by increased insulin levels, but are associated with a progressive decline in plasma insulin concentrations (Bogardus 1984; DeFronzo 1989). These data indicate that individuals with normal blood glucose concentrations have normal or increased circulating insulin levels. The high plasma insulin concentrations have been interpreted to be a compensatory mechanism where the pancreas hypersecretes insulin so that glucose levels are maintained within normal range (Reaven 1988). This implies that tissues become insensitive to normal amounts of insulin (Reaven 1988). However, the factors that initiate the increased insulin secretory response remain unclear.

The longitudinal evidence for progressive increase and subsequent fall in plasma insulin concentrations with elevations of plasma glucose concentration has been confirmed in several studies. In Pima Indians the sequential changes in plasma insulin concentrations as glucose tolerance declined demonstrated a sequence similar to that shown in the cross-sectional studies (Saad 1989). Plasma insulin concentrations were shown to rise initially with increasing plasma glucose concentrations before reaching a maximum and falling dramatically with any further increase in the plasma glucose concentration. A
similar developmental sequence has been in a primate model of type II diabetes (Bodkin 1989). These and subsequent studies (Eriksson 1989; Warram 1990) document that the earliest defect in the progression from normal to impaired glucose tolerance is the development of hyperinsulinemia and insulin resistance. The chronic hyperinsulinemia may lead to a down regulation of insulin receptors and insulin resistance (DeFronzo 1988). Furthermore hyperinsulinemia is an accurate predictor of the development of type II diabetes (Zimmet 1992).

1.4.2.2 Hepatic Glucose Output

In the postabsorptive state the plasma glucose levels are maintained by the endogenous production of glucose by the liver. Numerous studies have shown an increase in hepatic glucose output (HGO) in type II diabetic subjects, despite the presence of hyperinsulinemia (DeFronzo 1982; Bogardus 1984; DeFronzo 1989). In non-diabetic individuals hyperinsulinemia is a potent inhibitor of HGO, this insulin mediated inhibition of HGO is not present in type II diabetics, demonstrating the presence of hepatic insulin resistance is type II diabetic subjects (DeFronzo 1992). In type II diabetes the increased HGO is positively correlated with the degree of fasting hyperglycemia (DeFronzo 1982; DeFronzo 1989). However there is still debate as to whether in the earliest stages of type II diabetes, that fasting hyperglycemia results from excessive HGO. This question was addressed by DeFronzo et al. (1989) in 77 lean type II diabetic patients. In 33 subjects, with fasting glucose concentration 7.8 mmol/L, HGO was virtually identical to control subjects. However, this 'normal' basal rate of HGO was maintained at the expense of a two fold greater fasting plasma insulin concentration (DeFronzo 1989). However, with fasting glucose levels above 7.8 mmol/L, the restraining effect of hyperinsulinemia on the liver is lost and HGO increases progressively (DeFronzo 1989).

The accelerated rate of HGO maintained in type II diabetes can be due to elevated rates of gluconeogenesis and/or glycogenolysis. Indirect evidence, gained from isotopic infusion techniques, has demonstrated that significantly elevated rates of gluconeogenesis, through the phosphoenolpyruvate (PEP) pathway, is present in type II diabetes, correlating with the degree of hyperglycemia (Consoli 1990). In the same study the rate of glycogenolysis, determined as the difference between the total glucose output and the rate of glucose synthesised through the PEP pathway, was not significantly elevated in type II diabetic subjects. (Consoli 1990). In subsequent investigations the rate of gluconeogenesis was measured in-situ using $^{13}$C nuclear
magnetic resonance spectroscopy. Gluconeogenesis accounted for 70% of total glucose production in the first 23 hours of fasting in non-diabetic subjects and 88% in type II diabetic subjects (Magnusson 1992), indicating that increased gluconeogenesis was involved in the elevated HGO in type II diabetic subjects (Magnusson 1992).

The primary factors responsible for the maintenance of the increased gluconeogenesis in type II diabetes are not clear. There are a number of proposed mechanisms, one of which is an increased supply of gluconeogenic substrate. The major gluconeogenic precursor is lactate, accounting for 60-80% of hepatic gluconeogenesis (Consoli 1990). Lactate supply from muscle has been shown to be regulated by the disposal rate of glucosc (Yki-Jarvinen 1990), which is increased in postabsorptive type II diabetics. The use of glycerol as a gluconeogenic substrate may also contribute to the excessive rate of gluconeogenesis found in type II diabetes (Nurjhan 1992; Puhakainen 1993). Increased glycerol is particularly important in promoting hyperglycemia as, unlike lactate, glycerol supplies carbon atoms for glucose from the triglyceride pool (Nurjhan 1992). In normal subjects the inhibition of lipolysis lowers HGO, due to a reduction in glycerol supply from triglycerides, which further supports the potential for increased glycerol supply in type II diabetes to stimulate HGO (Jahoor 1992).

Plasma glucagon has been found to be elevated in type II diabetic subjects in the postabsorptive state (Unger 1970; Claus 1984; Baron 1987). Elevated glucagon levels may significantly contribute to elevated rates of basal hepatic glucose production in type II diabetes through the activation of key enzymes in the gluconeogenic pathway (pyruvate carboxylase, fructose diphosphate) are stimulated by glucagon (Chiasson 1983; Cherrington 1986). Therefore it is postulated that hyperglucagonemia may be a possible factor that contributes to increased HGO in type II diabetes (Consoli 1992).

In addition to the previous mechanisms for maintaining HGO there are a number of intrahepatic factors involving lipid metabolism which are thought to contribute to elevated postabsorptive gluconeogenesis. An example of this is increased hepatic free fatty acid (FFA) supply and oxidation may further enhance the rate of gluconeogenesis (Willamsson 1966). As a result of increased β-oxidation of FFA within the liver, intracellular acetyl-CoA concentrations are elevated. The increased acetyl-CoA levels elevate the activity of the key gluconeogenic enzymes, pyruvate carboxylase and fructose-1, 6-diphosphatase (Foley 1992). Agius and Alberti (1985) reported that addition of FFA into the incubation medium increased the incorporation of 14C pyruvate
into glucose in isolated hepatocytes. Ferrannini et al (1983) demonstrated that infusion of exogenous FFA diminishes the ability of insulin to suppress hepatic glucose production. Increased FFA concentration and increased FFA rate of appearance have been reported in type II diabetes (Taskinen 1985; Groop 1989). It has been proposed that the increased FFA availability to the liver and the probable consequent increase in liver FFA oxidation might constitute a stimulus for increased gluconeogenesis in type II diabetes (Consoli 1992).

1.4.2.3 Peripheral Glucose Disposal

The maintenance of constant plasma glucose levels with increased HGO in type II diabetes necessitates increased peripheral glucose disposal (DeFronzo 1992). Significantly elevated rates of glucose uptake under postabsorptive conditions, using femoral vein catheterisation to measure glucose exchange across leg tissues, has been shown in type II diabetic subjects (DeFronzo 1985). This increase in skeletal muscle disposal in type II diabetes, during the postabsorptive period, was confirmed in isotope turnover studies (DeFronzo 1989). The maintenance of elevated rates of glucose disposal in type II diabetic subjects is dependent on the presence of hyperglycemia, which by mass action increases the amount of glucose entering skeletal muscle and other tissues (DeFronzo 1989). Evidence of the importance of the hyperglycemia in maintaining the increased rate of glucose clearance has been shown, in studies where overnight insulin infusions were maintained to normalise plasma glucose concentrations in type II diabetic subjects (DeFronzo 1989). Following the insulin infusion, plasma insulin concentrations were approximately two fold higher in the diabetic subjects than in non-diabetic controls. However, despite the elevated plasma insulin levels in the diabetic, the rate of whole body glucose uptake was reduced in the diabetic compared to the controls. When the same subjects were given another overnight insulin infusion designed to normalise the rate of HGO there was no change in plasma glucose levels, consistent with reduced glucose clearance (DeFronzo 1989). Therefore, in the hyperglycemic state the rate of whole body glucose uptake is greater in type II diabetics than that of normoglycemic controls, whereas under euglycemic conditions the rate of glucose disposal is lower in diabetics than non-diabetics.
1.4.3 Diabetic Postprandial State

After ingestion of a meal we see that type II diabetics have increased and sustained hyperglycemia (Ferrannini 1988). The increase in hyperglycemia in type II diabetes could result from an increased rate of appearance of oral glucose in circulation, a diminished inhibition of hepatic glucose output, impaired uptake of glucose in peripheral or splanchnic tissues or a combination of all factors (Ferrannini 1988). The first area we shall look at in the postprandial state is the response of the pancreas in type II diabetics following a meal or glucose load.

1.4.3.1 Glucose Induced Insulin Secretion

In cross sectional analysis of plasma insulin responses to an oral glucose load in subjects with varying degrees of glucose tolerance the relationship between plasma glucose and insulin concentrations following a meal resembles the inverted ‘U’ shape relationship demonstrated in the postabsorptive state (DeFronzo 1992). Insulin secretion in response to a glucose load increased with elevated plasma glucose levels. However, a critical point is reached beyond which further elevations in plasma glucose are not matched by increased insulin secretion, but rather show a progressive fall in plasma insulin levels (DeFronzo 1992). The functional change in insulin secretion with increasing plasma glucose levels is consistent with the progression from normal glucose tolerance to impaired glucose tolerance and to type II diabetes in humans (Lillioja 1988; Saad 1989) and primates (Bodkin 1989).

The exact cause of this abnormal postprandial insulin secretion remains uncertain, however a number of mechanisms have been postulated. Insulin secretion is biphasic in a nature with a brief first phase release of insulin followed by a sustained second phase of insulin release (DeFronzo 1992). It has been shown that the brief and rapid rise in plasma insulin levels may be important in priming target tissues. Reduced or absent first phase insulin secretion results in reduced glucose disposal and reduced inhibition of HGO (Bruce 1988). Diminished first phase response has been shown in type II diabetic subjects and the restoration of this insulin phase lowers postprandial hyperglycemia (Bruce 1988). However, loss of first phase insulin secretion is not evident until type II diabetes is well established (DeFronzo 1992).

More recently it has been suggested that excessive accumulation of fat within the pancreas is contributing to the postprandial defect in insulin secretion. This concept has been termed ‘lipotoxicity’ (Unger 1995), the full details of which are covered in section
The basis of this hypothesis is the accumulation of fat within pancreatic islets has a detrimental effect on insulin secretion (Unger 1995). The key defects that are thought to occur postprandially are the accumulation of fat within the pancreatic islets causes a loss of glucose stimulated insulin secretion (Lee 1994). Further evidence comes from islets cultured in a medium high in FFAs, where first phase glucose stimulated insulin secretion is reduced (Lee 1994). These findings were then used to suggest that the accumulation of fat had a toxic effect in the pancreatic islets which manifest as defective insulin secretion.

1.4.3.2 Postprandial Hepatic Glucose Output

In the postprandial state hepatic glucose metabolism shifts from net glucose output to uptake, which acts to restore hepatic glycogen levels in non-diabetics (Firth 1986). In type II diabetics postprandial suppression of HGO is impaired (Firth 1986; Ferrannini 1988). During a subject’s ingestion of a carbohydrate meal or 75g glucose load, plasma insulin increases to a maximum of 50-70 mU/L after 1 hr and returns to a postabsorptive value in 3 to 4 hr (Campbell 1988). The average insulin concentration during this interval was ~30 mU/L (Campbell 1988). Analysis of the dose response curves for insulin action on suppression of HGO and stimulation of muscle glucose uptake shows that the value is close to the ED₃₀ for insulin suppression of HGO but far below the ED₃₀ for insulin stimulation of muscle glucose uptake (Campbell 1988). Thus it was suggested that potential abnormalities in insulin secretion and insulin sensitivity might affect postprandial suppression of HGO more than postprandial stimulation of muscle glucose uptake (Campbell 1988). This increase in postprandial HGO in type II diabetics is thought to account for approximately one third of the defect in whole body glucose metabolism (DeFronzo 1992).

Despite the presence of increased HGO the rate of hepatic glycogen repletion is comparable between non-diabetic and type II diabetic subjects (Giaccari 1992). This is because glycogen synthesis can occur via direct conversion of glucose or indirectly from three carbon precursors, primarily lactate and alanine, which originate in peripheral tissues (McGarry 1987). In normal subjects postprandial glycogen synthesis is predominantly via the indirect pathway and account for 55-65% of total glycogen repleted (Shulman 1988). However in type II diabetes glucose generated via gluconeogenesis is used for HGO and adequate rates of glycogen synthesis are achieved by synthesising glycogen directly from glucose (Shulman 1988; Giaccari 1992).
Separate from maintaining postprandial hepatic glycogen supplies, gluconeogenesis is also contributes to postprandial HGO. McMahon et al. (1989) observed that after ingestion of a mixed meal total postprandial systemic appearance of glucose derived from bicarbonate (a postabsorptive index of gluconeogenesis) was increased in type II diabetic patients compared with nondiabetic patients. In addition the contribution of gluconeogenesis from glycerol to HGO increased from 6% in the basal period to 8% after glucose ingestion, whereas in nondiabetic subjects it decreased from 3% to 2% (Nurjhan 1992). The results of these two studies suggest, as in the postabsorptive state, in the postprandial state gluconeogenesis is an important mechanism for maintaining increased HGO in type II diabetes (Consoli 1992). Interestingly, Mitreakou et al. (1990) observed that after glucose ingestion, lactate and alanine forearm release was increased in type II diabetes, whereas forearm glucose storage was decreased. It is therefore possible that increased availability of glucose precursors secondary to impaired muscle glucose storage may fuel increased gluconeogenesis in the postprandial state (Consoli 1992).

1.4.3.3 Postprandial Peripheral Glucose Disposal

In non-diabetic subjects following a meal, the postprandial elevation in plasma insulin stimulates glucose disposal in insulin sensitive tissues, primarily liver and skeletal muscle (Shulman 1990; Consoli 1992). Several studies have used a double-isotope technique to investigate glucose metabolism after a subject's ingestion of an oral glucose load (Ferrannini 1985; Firth 1986; Ferrannini 1988; Kelley 1988; McMahon 1989). These studies have demonstrated that whole body muscle glucose disposal measured during the 3.5-7 hr after glucose ingestion averages 33 g, or 50% of systemic glucose disappearance (Consoli 1992). In the same studies first pass splanchnic glucose extraction averages 16 g and can be calculated that an additional 24 g of glucose would have been produced by the liver if hepatic glucose production had not been suppressed after glucose ingestion (Consoli 1992). Glucose metabolism in the splanchnic bed during the 3.5-7 hr after glucose ingestion is therefore quantitatively as important as glucose metabolism in the muscle tissue for the physiological carbohydrate homeostasis following glucose ingestion (Consoli 1992).

In contrast to the previously mentioned situation type II diabetic subjects have a blunted relative insulin mediated stimulation of muscle glucose disposal, this not an absolute decrease in uptake of glucose due to the elevated glucose and insulin levels in diabetic
subjects (DeFronzo 1985; Damsbo 1991). This insulin resistant glucose disposal in an important metabolic defect that is involved in the development and maintenance of type II diabetes (Eriksson 1989; Warram 1990; Martin 1992). In order to determine the early cellular defect that may explain the development of insulin resistance in type II diabetes, studies have examined the cellular mechanisms involved in insulin stimulated glucose uptake.

The binding of insulin to its receptor initially acts to increase the translocation of specific glucose transporters from intracellular sites to the plasma membrane and increase the activity of transporters already present in the plasma membrane (Joost 1989). It has been determined that glucose transport into cells is the rate limiting step for glucose disposal and there are several families of glucose transporters in different tissues involved with this process (Kahn 1992). Upon entry to the cell glucose is phosphorylated and is metabolised via one of two pathways. One being the oxidative pathway which is primarily the oxidation of glucose via the glycolysis (Thiebaud 1982) and the second is the non-oxidative pathway, which is largely glycogen synthesis (Thiebaud 1982).

1.4.3.4 Defects In The Regulation of Peripheral Glucose Disposal In Type II Diabetes

Investigations of the defects in the insulin mediated stimulation of glucose disposal in type II diabetes can be separated into a number of different categories, these include; alterations that occur prior to insulin binding and insulin receptors; alterations in the transmission of the signal; reduced activity of glucose transporters; reduced oxidative metabolism of glucose and reduced non-oxidative metabolism of glucose.

1.4.3.4.1 Insulin Binding & Receptors

It has been shown that insulin binding to monocytes and adipocytes from type II diabetic subjects is reduced by approximately 20 to 30% (DeFronzo 1992). This reduction in insulin binding is thought to be due to reduced numbers of insulin receptors rather than an alteration in the affinity of the receptor for insulin (DeFronzo 1992). However most studies fail to support a reduction in the number of insulin receptors or receptor affinity to insulin in liver and skeletal muscle of diabetics (Olefsky 1981; Dohm 1988). Instead the majority of studies demonstrate the reductions in insulin mediated glucose disposal in type II diabetes are due to post receptor defects (Olefsky 1981; Bogardus 1984; Haring 1993).
1.4.3.4.2 Insulin Signalling

The molecular signalling pathways that lead to the stimulation of glucose uptake in various tissues have not been completely elucidated. Until recently, it was not known whether the analogous effects of insulin and exercise on skeletal muscle glucose uptake occur via similar or different molecular signals (Goodyear 1998). For insulin action, the cascade of signalling events is initiated by insulin binding to the extracellular α-subunit of the insulin receptor, autophosphorylation of tyrosine residues in the receptor β-subunit, tyrosine phosphorylation of the insulin receptor substrates IRS-1 and IRS-2, and activation of phosphatidylinositol 3-kinase (PI 3-kinase) (reviewed in Cheatham 1995). As for exercise, several studies have clearly demonstrated that these proximal insulin-signalling steps are not components of the signalling mechanism by which exercise stimulates glucose uptake, since contractile activity does not stimulate autophosphorylation of isolated insulin receptors (Treadway 1989), receptor and IRS tyrosine phosphorylation (Goodyear 1995; Wojtaszewski 1996), or PI 3-kinase activity (Goodyear 1995; Wojtaszewski 1996). Furthermore, wortmannin, a PI 3-kinase inhibitor, does not inhibit contraction-stimulated glucose transport in vitro (Lee 1995; Lund 1995; Yeh 1995). These signalling studies demonstrate that the underlying molecular mechanisms leading to the insulin- and exercise-induced stimulation of glucose uptake in skeletal muscle are distinct (Goodyear 1998). In non-diabetic subjects the insulin receptor PI 3-kinase activity increases with increasing insulin concentrations, while in type II diabetic subjects, there is a decreased expression of the p85 regulatory subunit of PI 3-kinase independent of changes in insulin receptor number (Goodyear 1998). However, it remains unclear whether defects in this insulin signalling mechanism contribute to the insulin resistance in type II diabetes (DeFronzo 1992).

1.4.3.4.3 Glucose Transport

A number of studies have demonstrated that concentration of the glucose transporter GLUT 4 is reduced in skeletal muscle from type II diabetic subjects (Sinha 1991; Garvey 1992). In vitro incubation of isolated insulin sensitive cell lines or muscle groups with elevated glucose solutions reduces the amount of available GLUT 4 (van Putten 1985; Sasson 1986) protein and amount of GLUT 4 mRNA (Bourey 1990; Yki-Jarvinen 1992). However, studies in type II diabetic subjects have failed to show alterations in either GLUT 4 protein or mRNA (Pedersen 1990). These studies do not
exclude the possibility of a defect in the activity of the GLUT 4 transporter (Schalin-Jäntti 1992). Reductions in glucose transport in the absence of altered amounts of transport protein suggest the existence of a reduced transporter activity (Schalin-Jäntti 1992). However in offspring of two diabetic parents or first degree relatives of type II diabetic subjects (Eriksson 1989), reductions in glucose utilisation are accounted for by intracellular defects. Therefore, alterations in the expression of GLUT 4 or changes in the activity of the transporters in skeletal muscle do not adequately explain the in vivo insulin resistance in type II diabetes (Kahn 1992).

1.4.3.4.4 Oxidative & Non-oxidative Glucose Metabolism

Moderate postprandial elevations in plasma insulin levels stimulate both oxidative (glycolysis) and non-oxidative (glycogen storage) pathways of glucose metabolism to similar degrees in non-diabetic subjects (Thiebaut 1982; Fery 1993). In the postprandial state reductions in glucose uptake in type II diabetes are the result of an approximately equal impairment in the oxidative and non-oxidative pathways (Fery 1993). The impairment of glucose oxidation at physiological insulin concentrations in type II diabetic subjects has been shown to result from a reduced ability of insulin to stimulate the glycolytic rate limiting enzyme, pyruvate dehydrogenase (Mandarino 1987). However, other studies have shown that the ability of insulin to suppress fat oxidation is markedly impaired in type II diabetes and that the increased fat oxidation may be a contributing factor lowering the rate of glucose oxidation (Damsbo 1991; Del Prato 1993).

Although the impairment of glucose oxidation and non-oxidative glucose metabolism in type II diabetes are equivalent at physiological insulin levels, increased insulin levels under hyperinsulinemic clamp conditions clearly demonstrates that reduced non-oxidative glucose disposal is the major intracellular abnormality responsible for the defect in insulin action in type II diabetes (Shulman 1990; Thorburn 1990; Beck-Nielsen 1992). Furthermore, under experimental conditions of combined euglycemia and hyperinsulinemia, reduced non-oxidative glucose metabolism accounts for the majority of the reduction in whole body glucose disposal (DeFronzo 1992). However data which characterises the defects in non-oxidative glucose metabolism further into non-oxidative glycolysis (lactate production) and glycogen repletion has shown that non-oxidative glycolysis is increased in type II diabetic subjects during
hyperinsulinemia, highlighting the importance of the defect in glycogen synthesis (Del Prato 1993).

1.4.4 Summary Of The Central Role Of The Liver In Type II Diabetes

In summary, the mechanisms via which glucose homeostasis is maintained involves balance of glucose production by the liver and utilisation by peripheral tissues. Insulin controls HGO and promotes glucose utilisation by skeletal muscle. In type II diabetes, postabsorptive hepatic glucose production is increased and exhibits a positive correlation with fasting plasma glucose concentration. This increase in hepatic glucose production is the primary contributant to the fasting hyperglycemia in type II diabetes. Of the two processes by which the liver produces glucose (gluconeogenesis and glycogencolysis), gluconeogenesis appears to be increased in type II diabetes. The increase in gluconeogenesis accounts for most of the increased HGO in this condition and a positive correlation has been found in type II diabetes subjects between the rates of gluconeogenesis and fasting plasma glucose concentration. Increased production of gluconogenic precursors (lactate, alanine, glycerol) fuels the increased gluconeogenesis, but some type of intrahepatic mechanism is also present in type II diabetes that increases the hepatic conversion of these substrates into glucose. Increased hepatic fatty acid oxidation might be responsible for this increased hepatic gluconeogenic efficiency in type II diabetes. Reduced suppression of HGO after carbohydrate ingestion also plays an important role in the impairment in post prandial glucose homeostasis in type II diabetes. In type II diabetic subjects splanchnic extraction of an oral glucose load is not decreased, but HGO is suppressed less than in nondiabetic subjects after the load. Preliminary data suggest that in the postprandial state increased gluconeogenesis represents the primary mechanism responsible for the impaired suppression of HGO. Given the primary role of increased hepatic gluconeogenesis in the pathogenesis of hyperglycemia in type II diabetes and the suggested a role for fatty acids in fuelling this process. It is important to examine how fat metabolism is disturbed with type II diabetes and what role it plays in the pathogenesis of this condition.
1.5 FAT METABOLISM AND THE DEVELOPMENT OF TYPE II DIABETES

1.5.1 Defects In Lipid Metabolism In Type II Diabetes
Lipid homeostasis in mammals is regulated by intracellular lipid-related enzymes and binding proteins as well as by extracellular lipid metabolising enzymes and transport proteins. The extracellular lipid transport proteins are known as apolipoproteins (Chan 1997). These proteins are an essential structural component of the plasma lipoproteins. The plasma lipoproteins are lipid microemulsion-like particles that contain a central core of nonpolar lipids and a surface monolayer of polar lipids (primarily phospholipids) (Chan 1997). They vary greatly in size and are stabilised in the aqueous environment of the intra- and extravascular spaces by amphipathic apolipoproteins (Chan 1997).

The plasma apolipoproteins can be classified into two groups, the soluble apolipoproteins and the B apoproteins (Apo-B). The latter proteins differ from the former group of proteins in that they are water-in-soluble and are tightly associated with the lipid components of the lipoprotein particles (Chan 1997). Unlike the soluble apolipoproteins, they do not exchange between lipoproteins or other lipid surfaces (Chan 1997).

Tissues throughout the body have an enzyme, lipoprotein lipase (LPL), on their surfaces that captures circulating triglycerides from lipoproteins passing in the blood (Cryer 1987). LPL hydrolyses the triglycerides to fatty acids and monoglycerides and passes these products into the cells’ interior (Cryer 1987). Apo-B, lipoprotein lipase and other key regulators of fat metabolism are discussed in greater detail in section (1.7).

1.5.1.1 Hyperlipidemia In Type II Diabetes
The main feature of hyperlipidemia in type II diabetes is elevated plasma triglyceride contained predominantly within the very low density lipoprotein (VLDL) fraction (Howard 1987; Hagan 1989). It has been suggested that the over production of VLDL’s is due to an increased availability of substrates for triglyceride synthesis (Mancini 1980; Howard 1987). The increased production of VLDL’s is not accompanied by increased apolipoprotein concentrations, suggesting increased triglyceride within the VLDL (Howard 1987), which may adversely effect the clearance of triglyceride rich VLDL’s from the plasma (Reaven 1988). The extent of the
hyperlipidemia is related to diabetic control, degree of obesity and the presence of secondary causes of hyperlipidemia such as renal dysfunction or primary lipid disorders (Betteridge 1989).

Many hypertriglyceridemic type II diabetic patients also appear to have a defect in the clearance of triglyceride rich lipoproteins. The polypeptide which forms the structural frame work of the VLDL particle is apolipoprotein B (Apo-B) (Herbert 1983). VLDL Apo-B elevations usually accompany hypertriglyceridemia and decreased removal rates as well as increased synthesis of VLDL Apo-B have been described in small numbers of type II diabetic patients (Brown 1994). Increased body fat in such patients has been strongly correlated with the increased VLDL Apo-B production (Dunn 1984). Studies in which patients with type II diabetes have been matched with equally obese nondiabetic subjects also implicate excess body fat as a primary factor in the higher VLDL Apo-B production (Taskinen 1986).

Although many mechanisms contribute to hypertriglyceridemia in type II diabetes, insulin resistance appears to be the common basis. The insulin resistant state impairs the normal suppression of fatty acid release from adipose tissue (Frayn 1993). Consequently, the flux of free fatty acids to the liver increases and overproduction of VLDL these substrates occurs when hyperinsulinemia is present (Syvanne 1997). Insulin is also involved in another defect of hepatic VLDL metabolism. Acute hyperinsulinemia, such as after a meal, suppresses the production of large buoyant VLDL particles (VLDL1) in the liver in non-diabetic subjects but not in type II diabetic patients (Malmstrom 1996; Syvanne 1997). These VLDL1 particles have been suggested to be analogous to chylomicrons, which should be released only in the fasting state when lipids from food are not available (Syvanne 1997). Thus, one function of insulin in non-diabetic subjects is to maintain the balance between intestinally derived and liver derived triglyceride rich lipoproteins (Syvanne 1997). In type II diabetes this regulation fails, inappropriate production of VLDL by the liver occurs and the balance favours hypertriglyceridemia (Syvanne 1997).

Although reduced clearance of VLDL triglycerides has been found frequently in patients with mild to moderate hypertriglyceridemia, the reason for this abnormality is unclear. The clearance of VLDL's and chylomicrons from the plasma is mediated by lipoprotein lipase which cleaves the triglyceride into glycerol and free fatty acids (FFA) (Eckel 1989). Lipoprotein lipase measured in postheparin plasma of type II diabetic patients is
usually within the normal range of activities of non-diabetics (Pykalisto 1975; Taskinen 1982; Pfeifer 1983; Taskinen 1986). However, the distribution and regulation of LPL may differ in diabetics; studies have shown reductions in adipose tissue lipoprotein lipase (Taskinen 1986). The VLDL particles may be larger with relatively more triglyceride in many type II diabetic patients and enrichment in Apo-C has also been reported (Gabor 1980). Apo-CIII may inhibit lipoprotein lipase and may also inhibit the uptake of VLDL remnants by the liver (Shelburne 1980; Aalto-Sctala 1992). In patients that have further elevations in triglyceride levels defects in clearance appear to play a larger role. Under such conditions, not only are VLDL elevated, but chylomicrons are found in fasting plasma (Brown 1994). Chylomicron remnant clearance has been reported to be defective in diabetics with mild to moderate hypertriglyceridemia (Haffner 1984), but when triglyceride levels are extremely high, significant numbers of large chylomicrons are present in the plasma (Brown 1994). It has been suggested that these extreme triglyceride elevations may be the result of a primary but partial deficiency in lipoprotein lipase with the superimposed disturbed lipid metabolism of type II diabetes (Dunn 1984).

1.5.2 Free Fatty Acids And Type II Diabetes

A further supply of FFA involves the degradation of stored triglycerides within adipocytes by the enzyme hormone sensitive lipase, a process known as lipolysis and lipolytic rate is influenced by several factors. Epinephrine is the most important stimulator of lipolysis, insulin is the most potent inhibitor of lipolysis and adenosine is also a potent inhibitor of lipolysis (Coppack 1994). However, none of these factors are themselves responsive to changes in fatty acid availability (Wolfe 1998). In type II diabetic subjects we see an increased rate of lipolysis (Nurjhan 1992) due to an increased fat mass and resistance to the suppressive action of insulin on lipoprotein lipase activity (Yki-Jarvinen 1987; Groop 1989). The presence of increased lipolysis liberates increased levels of FFA and glycerol into the blood stream and favours FFA oxidation by insulin sensitive tissues during the postabsorptive period (Groop 1991).

The increased rate of lipolysis and elevated levels of FFA in type II diabetic subjects has marked effects on glucose uptake basely and following insulin stimulation. Studies conducted in healthy subjects have shown that acute elevation of plasma FFA by intravenous lipid/heparin infusion inhibited total body glucose uptake dose dependently after a lag period of 3-4 hours (Boden 1994). However, inhibition of carbohydrate oxidation occurred approximately 2 hr earlier than the inhibition of glucose uptake. The
latter was reversed approximately 3 hours after discontinuation of lipid/heparin infusions (Boden 1991). Thus, there appeared to be more than one mechanism by which FFA inhibited insulin stimulated glucose uptake (Boden 1994). Other studies showed that the lipid induced inhibition of insulin stimulated glucose uptake was linear (Boden 1994; Boden 1995) and occurred similarly in healthy subjects and in patients with Type II diabetes (Thiebaud 1982; Bonadonna 1989; Felley 1989; Boden 1994; Boden 1995; Roden 1996).

While the precise mechanisms operating in the previously described lipid infusion studies remains unclear a diverse range of acute and chronic effects have been demonstrated in an attempt to elucidate how FFA interact with or regulate glucose metabolism.

1.5.3 Acute Impact Of FFA On Glucose Homeostasis
1.5.3.1 The Randle Hypothesis
The original hypothesis that explained substrate competition between glucose and fatty acids was proposed 35 years ago by Randle and colleagues and was based on observations in both heart and diaphragm, in which elevated levels of fatty acid oxidation inhibited glucose oxidation (Randle 1963; Randle 1964). From these and other observations the concept of the glucosfatty acid cycle (Randle Hypothesis) was formed. The process can be explained in two stages:

1) Elevated levels of fatty acid oxidation in the mitochondria lead to increased ratios of acetyl-CoA/CoA, NADH/NAD and ATP/ADP. These increased ratios, especially acetyl-CoA/CoA, stimulate pyruvate dehydrogenase kinase which phosphorylates pyruvate dehydrogenase (PDH) to its inactive form thus inhibiting glucose oxidation (Randle 1963; Randle 1964; Taylor 1973; Jeanrenaud 1985).

2) Elevated levels of acetyl-CoA result in excessive production of citrate. Citrate has been shown to inhibit the kinase activity of the bifunctional enzyme phosphofructo-2-kinase (PFK-2)/fructo-2,6-bisphosphatase (Randle 1988) which converts fructose-6-phosphate (F-6-P) to fructose-2,6-bisphosphate (F-2,6-BisP). F-2,6-BisP has a stimulatory effect on phosphofructo-1-kinase (PFK-1) and an inhibitory effect on fructose-1,6-bisphosphatase (F-1,6-BisPase) (Iric 1983). Therefore, low levels of F-2,6-Bisp would inhibit the action of PFK-1 and allow the reverse reaction converting
fructose-1,6-bisphosphate (F-1,6-BisP) to F-6-P. This reversal may serve to stimulate the gluconeogenic pathway (Randle 1988).

This sequence of events, represented in Figure (1.1), suggests that excessive rates of free fatty acid 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 an accumulation up of glucose-6-phosphate which in turn inhibits hexokinase activity and therefore limits the rate at which glucose is taken up by the cell (Randle 1988). It has been speculated that this would lead to the inhibition of not only glucose oxidation but glycogen synthesis secondary to reduced glucose transport (DeFronzo 1988).

Since it was first proposed the Randle hypothesis has been the most popular and enduring explanation for the control of glucose and fat availability and oxidation. However there has been a number of subsequent studies that have been unable to support the hypothesis. The original glucose fatty acid cycle was based entirely on in vitro results from experiments on rat heart and diaphragm muscle metabolism (Randle 1963). Several in vitro studies have been done since that time, with conflicting results. Whereas some studies showed an inhibitory effect of fatty acids on glucose oxidation in rat skeletal muscle (Rennie 1976), others found no such effect (Berger 1976; Ruderman 1980; Oakes 1997). Maizels et al. (1977) proposed that a fatty acid effect on glucose oxidation may only occur in red muscle under some circumstances, such as when the rate of glycolysis is increased. Further more other studies (Grundleger 1982) was conducted in high fat fed rats, the high fat diet reduced insulin stimulation of active PFK-1 in skeletal muscle. However the levels of F-2,6-BisP were significantly greater. This finding was supported by a corresponding increase in insulin stimulated active PFK-2 and a reduction in active F-2,6BisPase. These results suggest that F-2,6-BisP is not a key regulatory of glycolysis in skeletal muscle (Grundleger 1982).
Figure 1.1: The Randle Cycle
1.5.3.2 The Glucose Fatty Acid Cycle Reversed

Recently it has been proposed that fatty acid oxidation is largely controlled at the site of oxidation, which is determined by the availability of glucose, rather than by its availability via lipolysis. This so called 'Glucose fatty acid cycle reversed' was proposed by Wolfe et al. (1998) and is based around studies where they tested the effect of acutely elevating glucose availability and oxidation on fatty acid oxidation in the setting of constant fatty acid concentrations (Sidossis 1996). In their investigations, normal volunteers were studied in the basal state and during a hyperinsulinemic, hyperglycemic clamp and it was found that increased availability of glucose inhibited fat oxidation, despite the constant availability of fatty acids (Sidossis 1996). The authors felt that this result was precisely contrary to that predicted by the traditional Randle cycle and led them to conclude that the availability of glucose (rather than fatty acids) is the predominant determinant of the substrate oxidation mix (Wolfe 1998).

The mechanism by which glucose inhibits fatty acid oxidation was suggested to be due to glucose or insulin restricting long chain fatty acid (LCFA) entrance into the mitochondria (Sidossis 1996). Such a mechanism was demonstrated when constant infusions of $^{1,13}$C-oleate, a LCFA, and $^{1,13}$C-octanoate, a medium chain fatty acid were given in the basal state and during a hyperglycemic and hyperinsulinemic state (Sidossis 1996). Oleate, but not octanoate, requires carnitine acyltransferase to gain access to the mitochondrial matrix (Wolfe 1998). Thus it was suggested, if glucose or insulin limit LCFA entrance into the mitochondria, then during their clamp long chain acylcarnitine formation should be decreased causing a decrease in oleate but not octanoate oxidation (Sidossis 1996). During the clamp procedure, oleate oxidation decreased whereas octanoate oxidation remained unchanged in the transition from basal to clamp conditions (Sidossis 1996). The conclusion that glucose or insulin directly limits fatty acid oxidation by restricting LCFA entrance into the mitochondria was supported by a decrease in intramuscular acyl-carnitine concentration during the clamp procedure compared to the basal state (Sidossis 1996).

1.5.3.3 Acute FFA Induced Alterations In Glucose Utilisation

An alternative to the Randle hypothesis or the reverse substrate interaction has been proposed by Boden et al. (1995). They suggested that elevated plasma levels of FFA produced a defect in two processes other than the oxidation of fat or carbohydrate including: 1) inhibition of insulin stimulated glucose transport and/or phosphorylation;
or 2) inhibition of muscle glycogen synthase activity. This group has analysed glucose fluxes through all major pathways of intracellular glucose utilisation using non-invasive techniques (Boden 1995). It was shown that lipid/heparin infusion inhibited rates of glucose uptake, glycogen synthesis and glycolysis to the same extent (Boden 1995). These results were most compatible with a FFA induced defect at the level of transport and/or phosphorylation since the primary inhibition of glycogen synthesis or glycolysis would be expected to result in disproportionately reduced rates of these pathways (Boden 1995).

The presence of a glucose transport or a phosphorylation defect has been supported by two further studies. Firstly glycogen synthase activity was demonstrated to be unaltered in muscle biopsies obtained 4 hours after lipid/heparin infusion (at a time when glucose uptake was significantly inhibited) (Boden 1994). Whereas, more recently it was found that glucose oxidation and muscle glycogen synthesis were 50%-60% lower following a lipid infusion compared with a glycerol infusion and were associated with a ~90% decrease in the increment in intramuscular glucose-6-phosphate concentration (Dresner 1999). This second study further suggest that glucose transport was the rate controlling step, due to the presence of significantly lower intracellular glucose following the lipid infusion (Dresner 1999).

The mechanisms responsible for the FFA induced defects in glucose transport and/or phosphorylation and glycogen synthesis are not known. Putative mechanisms include 1) activation of the hexosamine pathway, 2) inhibition of glucose transporter gene expression and 3) changes in cellular membrane fluidity.

1) Marshall et al. have shown in cultured rat hepatocytes that prolonged exposure to high glucose concentrations produced insulin resistance (glucose toxicity) by activating the hexosamine pathway (Traxinger 1989; Marshall 1991). This pathway accounts for only 1-3% of glucose flux under normal conditions and results in the generation of several metabolites which are important substrates for glycoprotein and phospholipid synthesis (Boden 1996). In addition fat induced insulin resistance was associated with accumulation of UDP-N-acetyl glucosamine, an end product of the hexosamine pathway and that the same degree of insulin resistance could be reproduced by increasing UDP-N-acetyl hexosamine in skeletal muscle of rats (Hawkins 1997).
2) Long and Pekala (1996) have shown that several long chain fatty acids decreased mRNA levels of the insulin responsive glucose transporter GLUT 4 in fully differentiated 3T3-L1 cells by decreasing GLUT 4 gene transcription and by destabilising the GLUT 4 message. Thus, increased FFA may induce insulin resistance by directly inhibiting GLUT 4 gene expression in muscle (Long 1996).

3) Lastly, FFA may induce changes in cell membrane fluidity. Insulin receptors are imbedded in the lipid bilayer of plasma membranes. There is some evidence to suggest that altering the fatty acid content of membranes can alter insulin receptor accessibility, insulin binding and action. For example, increasing polyunsaturated fatty acid content has been found to increase membrane fluidity, insulin binding and action whereas decreasing their content had the opposite effect (Ginsberg 1981; Grunfeld 1981; Borkman 1993; Farias 1987).

1.5.3.4 Acute Changes In Free Fatty Acids and Hepatic Insulin Resistance

The issue of whether FFA inhibit insulin suppression of hepatic glucose production (HGP), remains somewhat controversial. A number of studies have reported that acute elevation of plasma FFA increased HGP in patients with Type II diabetes and in non-diabetic controls during hyperinsulinemic clamp protocols (Ferrannini 1983; Fanelli 1993; Saloranta 1993; Boden 1994). However, acute reduction of plasma FFA with nicotinic acid or acipimox has been reported to either increase (Saloranta 1993) to decrease (Fulcher 1992; Worm 1994) or unalter HGP (Puhakainen 1993). Several plausible hypotheses exist for these divergent results (Boden 1998).

Firstly, while there is consistent in vitr producton and animal evidence that FFA promote gluconeogenesis (Williamson 1966; Williamson 1969; Jomain-Baum 1975; Gonzalez-Manchon 1989; Morand 1993). Yet it is also evident that increased gluconeogenesis may not necessarily increase HGP. A recent study in high fat fed rats found that when lipid oxidation was blocked acutely by the chemical agent etomoxir there was no significant effect on HGP in fat fed rats (Oakes 1997). This lack of effect was attributed to a compensatory increase in net hepatic glycogenolysis (Oakes 1997). Thus, there appears to be an intrahepatic mechanism which regulates HGP by decreasing glycogenolysis when gluconeogenesis is elevated and vice versa (Clore 1991; Puhakainen 1993).
Secondly, FFA are insulin secretagogues. Therefore, elevated plasma FFA levels may increase HGP by stimulating gluconeogenesis, on one hand, but on the other hand, they may raise insulin secretion which will then inhibit HGP (Boden 1998). Evidence in favour of a stimulatory effect of FFA on HGP was obtained in overnight fasted normal volunteers in whom plasma FFA were raised acutely by infusing lipid/heparin while insulin was clamped at basal concentrations (Boden 1991). Under these conditions, HGP and plasma glucose levels rose dramatically (Boden 1991). Therefore, the available human data suggest that FFA can increase HGP but the extent of the increase is controlled to some extent by the FFA mediated stimulation of insulin secretion (Boden 1998). Yet in the longer term sustained FFA may have detrimental actions on both insulin secretion and hepatic glucose homeostasis.

1.5.4 Chronic Actions Of FFA

In the previous section evidence was presented for distinct acute actions of FFA on hepatic and peripheral tissue glucose metabolism. In addition there is a considerable body of evidence examining the chronic interactions between FFA and glucose.

1.5.4.1 Fat Feeding

One frequently utilised experimental model to examine the impact of chronically elevated fatty acids is the consumption of high fat diets. In rodents ad libitum feeding of high fat diets leads to whole body and tissue specific insulin resistance (Kraegen 1986; Storlien 1986). The insulin resistance is widespread among tissues including the liver and skeletal muscle (Kraegen 1986; Storlien 1986). Similarly when insulin resistant suckling rats were weaned onto high carbohydrate diets they became insulin sensitive, but remained insulin resistant when weaned onto a high fat diet (Issad 1989). Further more, in rodents, there is a consistent theme across a range of studies indicating that manipulations that result in increased lipid availability, either circulating or stored in muscle, results in impaired insulin action (Storlien 1991). Conversely, dietary interventions such as energy restriction (Esriva 1992), or chronic pharmaceutical treatment with agents such as thiazolidinediones and etomoxir (Oakes 1994), that decrease lipid availability improve insulin action (Storlien 1997).

There are relatively few studies in humans where dietary intake has been manipulated and where insulin action has been measured by currently accepted techniques. Even among the studies where dietary lipid amount and type had been altered, there is little consensus about the outcome (Storlien 1997). Some of the positive results were in
studies with extraordinary high levels of carbohydrate (69-75% of calories) (Kolsterman 1979; Swinburn 1991), while with more realistic levels the results were generally negative (Storlien 1997). A major problem when comparing these studies is the duration of the respective study, usually weeks (Storlien 1997). However, in a study which modified the fat content of subjects diets for a year, emphasising monounsaturated, oleic and n-3 fatty acids, and the effects on insulin action were assessed by the hyperinsulinemic, euglycemic clamp technique. Overall, there was an improvement in insulin action on the fat modified diet in contrast to a general deterioration in metabolic control on the high carbohydrate control diet (Storlien 1997).

Another approach to intervention studies is to examine evidence from epidemiological studies linking dietary measures with clinical outcomes. These epidemiological studies provide a general consensus that high fat, particularly saturated fat, intake is associated with indices of insulin resistance and a greater likelihood of developing glucose intolerance and type II diabetes (Storlien 1997).

The combination of the previously described animal and human studies provides evidence that in general chronic over consumption of dietary fat has detrimental effects on insulin action and may lead to the development of type II diabetes. However it is important to note that over consumption dietary fat is not the only means via which fat stores remain chronically elevated. Underlying genetic or metabolic mechanisms, such as obesity, which maintain elevated fat levels are equally as important. The range of mechanisms via which chronic elevation of fat contributes to the development of disease shall be discussed next.

1.5.4.2 Tissue Lipid Accumulation

In a number of studies which used biopsies from human skeletal muscle have suggested a relationship between skeletal muscle lipids and insulin sensitivity (Krassak 1999). One such study in normoglycemic women found that intracellular lipid content, as assessed histologically by oil red O staining of muscle fibres, was inversely correlated with muscle insulin sensitivity (Phillips 1996). This measure of insulin sensitivity was correlated in a similar fashion with muscle triglyceride concentrations (Phillips 1996). Intracellular lipids were also related to waist-to-hip ratio and fasting plasma NEFA, but not to glucose tolerance or whole body insulin sensitivity (Phillips 1996). Another study (Pan 1997) reported an inverse correlation between muscle triglyceride and insulin sensitivity as estimated during euglycemic and hyperglycemic
clamp tests in insulin resistant and obese Pima Indians. Measures of obesity (BMI, percentage body fat, waist-to-hip ratio) were also inversely correlated with insulin stimulated whole body glucose uptake, but not with muscle triglyceride concentrations (Pan 1997). In the same study it was found that muscle triglyceride content predicts insulin sensitivity independently of all measures (Pan 1997). In a more recent study which used non-invasive nuclear magnetic resonance spectroscopy it was found that an inverse correlation existed between intramyocellular lipid content and whole body glucose uptake as well as fasting NEFA (Krissak 1999). These studies demonstrate, in muscle at least, that the accumulation of fat within cells can have detrimental effects on glucose metabolism. However it is important to note that very little of the fat accumulating in the cell remains as FFA, most of it is stored as triglyceride or phospholipid with a small labile pool turned over as cytosolic long chain acyl-CoA (LC-CoA), formed from FFA by the enzyme fatty acyl-CoA synthetase. Each one of these fat subtypes may have an action on carbohydrate metabolism and insulin action. In addition the cellular location of the various fat sub types may be of importance.

1.5.4.3 Membrane Composition

Phospholipids are structural lipids essentially confined to membranes (Storlien 1997). They form a major lipid component of the membrane bilayer: from proportionally the largest component of plasma membrane to almost all the lipid in mitochondrial membrane (Storlien 1997). Studies in rats (Storlien 1987), Caucasians (Borkman 1993) and Pima Indian (Pan 1995) populations, demonstrated a clear positive relationship between the percentage of more unsaturated lipids in the muscle structural membrane lipids and insulin action (Storlien 1997). However, the reverse was found to occur in a Swedish population where there was a negative correlation with saturated fatty acid composition of the plasma membrane and insulin action (Vessby 1994). An explanation for this difference was attributed to high levels of dietary n-3 fatty acid consumed in the Swedish population (Vessby 1994). It has been suggested that n-3 fatty acids improve insulin sensitivity or prevent insulin resistance (Storlien 1987; Storlien 1991). Alternatively the differences in the Swedish population may reflect a genetic predisposition to incorporation of specific fatty acids into membranes (Storlien 1997).

In contrast to these studies in humans, an investigation that looked at the phospholipid composition of Psammomys obesus, an animal model of type II diabetes, found no significant differences in phospholipid composition of liver and muscle (Collier 1997).
Thus it was concluded from these results that significant disturbances in glucose homeostasis and hyperinsulinemia may develop independently of tissue fatty acid composition (Collier 1997).

However, other studies have shown that when dietary sources of the long chain highly unsaturated fatty acids are incorporated into membrane structural lipids, there is an improvement in insulin action (Borkman 1993; Storlien 1993; Vessby 1994; Pan 1995). Seafood is perhaps being the best source of such fatty acids (Storlien 1993). However, the overall unsaturation index (UI) of the average westernised diet is much less (~80) than the UI of skeletal muscle phospholipids (160 to 170) (Storlien 1993). This means that the major dietary PUFAs must be both elongated and desaturated to be transformed into fatty acids that are suggested to be associated with insulin sensitivity (Storlien 1993). This is accomplished by the fatty acid desaturase and elongase enzymes. Finally investigations in infants suggest that there is a range of endogenous desaturase and elongase (enzymes which control the elongation and desaturation of fatty acids) activities across individuals and that this may play a role in early signs of glucose dysregulation (Baur 1994).

Thus it has been suggested that changing the characteristics of the membrane, by changing the phospholipid fatty acid composition, could then have potent effects on the ability of insulin to effect translocation/insertion of glucose transporters and/or their intrinsic activity when inserted into the plasma membrane (Rosholt 1994). Equally, changing the membrane lipid profile will change the ‘leakiness’ of membranes to ions and thus the energy requirements to maintain homeostasis (Else 1987; Brand 1990). These may provide mechanistic explanations for the observed correlational relationships between muscle membrane phospholipids and insulin action (Storlien 1993).

1.5.5 Lipotoxicity

Another hypothesis that has been proposed to explain how chronic elevations of fat modulate glucose metabolism and lead to the subsequent development of type II diabetes is the ‘lipotoxicity’ hypothesis (Unger 1995). The underlying concept behind this hypothesis is that chronic elevations of lipid within cells leads to alterations in the biochemical pathways primarily involved with the metabolism of glucose which ultimately results in impaired insulin action and diabetes.
1.5.5.1 Pancreatic Lipotoxicity

The concept of lipotoxicity was first postulated by Unger et al. to explain how the excessive amount of fat accumulating in diabetic the β-cell was impairing insulin secretion and contributing to the development of type II diabetes (Unger 1995). Efforts to elucidate how fatty acids influence β-cell functions have led to a series of important findings. Studies in isolated rodent islets, at concentrations of glucose that were stimulatory for insulin secretion were found to suppress the oxidation of LCFA (Berne 1975; Tamarit-Rodriguez 1984; Vara 1986). The use of agents such as 2-bromostearate and 2-bromopalmitate, which are known inhibitors of carnitine palmitoyltransferase 1 (CPT-1), the outer mitochondrial membrane enzyme that governs the flow of LCFA through the β-oxidation pathway (McGarry 1989; McGarry 1997), were shown to stimulate insulin release from perfused islets (Tamarit-Rodriguez 1984), the Syrian hamster insulinoma (HIT) cell line (Prentki 1992) and the perfused rat pancreas (Bedoya 1984). Furthermore, it was established that mitochondria isolated from rat islets expressed CPT-1 activity and as is the case for all tissues studied to date (McGarry 1997), the islet enzyme could be potentially inhibited by malonyl-CoA, the product of the acetyl-CoA carboxylase (ACC) reaction (McGarry 1997). Of particular interest, exposure of rat islets of HIT cells to glucose and a number of other secretagogues resulted in an increase of the cellular malonyl-CoA content (Corkey 1989; Prentki 1992); moreover, in HIT cells this increase was roughly in proportion to the extent of stimulation of insulin release (Corkey 1989). In addition, the insulin producing cell line derived from rat islet (INS-1) cells responded to high glucose concentrations with a major increase in transcriptional activity of the ACC gene (Brun 1993). Finally, exogenous long chain fatty acids appreciably potentiated glucose stimulated insulin secretion (GSIS) from rat islets (Tamarit-Rodriguez 1984) and HIT cells (Prentki 1992), in the later, a concomitant rise in the concentration of LC-CoA esters was observed.

The mechanisms responsible for mediating the alterations in GSIS in the presence of elevated FFA involves the multi-enzyme regulation of β-cell glucose metabolism (Liu 1998). Glucokinase determines the kinetics of glucose entry into glycolysis. Its high $K_m$ confers an $EC_{50}$ for glucose usage/insulin secretion that is in the physiological glucose range (Matschinsky 1990; Newgard 1995). β-cells also contain hexokinase, but its activity at physiologic glucose values is minimal because of allosteric inhibition.
by glucose-6-phosphate (G-6-P) (Giroix 1984). G-6-P is in equilibrium with fructose-6-phosphate (F-6-P) and therefore the steady state of G-6-P is governed by control of the outflow by phosphofructokinase (PFK) (Liu 1998). It was found that there was altered glucose sensing in rat islets cultured with FFA for 24 hr (Liu 1998). At the same time G-6-P content was lowered 60%, while the PFK \( V_{\text{max}} \) increased 50% (Liu 1998). Triacsin C, an inhibitor of fatty acyl-CoA synthetase (ACS) which produces LC-CoA, reversed these changes (Liu 1998) and shifted the secretion curve (Hosokawa 1997). Thus long-chain fatty acids act through generation of LC-CoA to increase PFK activity, which lowers the cellular level of G-6-P and deinhibits hexokinase (Liu 1998). Because the decrease in G-6-P exceeded the increase in PFK \( V_{\text{max}} \) and because increased hexokinase activity would be expected to increase G-6-P, another factor affecting PFK seemed likely (Liu 1998). This other factor was found to be the PFK inhibitor citrate (Liu 1998). This was due to citrate synthase activity (CS) being reduced in the presence of the FFA, thus a reduction in citrate levels further stimulates PFK activity and G-6-P catabolism (Liu 1998), these potential interactions are represented in Figure (1.2).

![Diagram](image)

**Figure 1.2:** Potential Interactions In The Pancreatic \( \beta \)-cell.
1.5.5.2 Extrapancreatic Lipotoxicity

The role of elevated cytosolic lipids, LC-CoA (formed from FFA by the enzyme fatty acyl-CoA synthetase) and malonyl-CoA (the product of the acetyl-CoA carboxylase reaction) derived from glucose, having detrimental effects in organs other than the pancreas has been well characterised. Malonyl-CoA is the product of the first step of the de-novo lipogenic pathway and is a potent inhibitor of β-oxidation via its inhibition of CPT (McGarry 1997). LC-CoA have been shown to have a number of effects including; altering the expression of genes implicated in carbohydrate and lipid metabolism, elevating steroid hormone production by the adrenal gland, elevated HGP and increased fat deposition in adipose tissue (Prentki 1996). Thus it has been suggested that the same metabolic events (elevated LC-CoA and malonyl-CoA) may explain the altered function of various organs in obesity and type II diabetes (Prentki 1996).

Consistent with this view, artificial elevation of plasma FFA levels in humans during an insulin clamp leads to impaired insulin stimulation of glucose utilisation and possible glycogen synthase activation in muscle (Bonadonna 1989; Boden 1991; Kelley 1993; Ebeling 1994). Furthermore, glucose has been shown to modulate the accumulation of malonyl-CoA, in skeletal muscle (Saha 1995). Finally, fat feeding rats produced insulin resistance in muscle associated with increases in triglyceride content (Storlien 1991) and LC-CoA levels (Chen 1992).

Increases in malonyl-CoA concentration in rodent muscle have been found in a wide variety of insulin resistant states (Saha 1994; Saha 1995). In contrast, decreased levels of malonyl-CoA have been observed in muscle after exercise (Winder 1990) and electrically induced contraction (Duan 1993), conditions associated with increased insulin sensitivity (Prentki 1996). Also improvements in insulin sensitivity in muscle of obese hyperinsulimic rodents due to treatment with a thiazolidinedione was associated with a decrease in the concentration of malonyl-CoA (Kurowski 1996).

In the liver, it has been established that FFA enhance gluconeogenesis (Williamsson 1966; Williamson 1969; Jomain-Baum 1975; Gonzalez-Manchon 1989; Morand 1993), inhibit glycolysis (Morand 1994) and alter gluconeogenic gene expression (Antras-Ferry 1994). FFA have also been implicated as possible messengers of insulin mediated inhibition of HGO (Rebrin 1995). It was originally demonstrated by McGarry et al. (McGarry 1977; McGarry 1978; Boyd 1980; McGarry 1980) that changes in
malonyl-CoA level play a key role in the disposition of FFA in the liver and in the regulation of ketogenesis and gluconeogenesis, suggesting a role for the effector signal malonyl-CoA in regulating HGP (Prentki 1996).

Thus far we have examined how carbohydrate and lipid metabolism is altered in type II diabetes. It was demonstrated that apart from the presence of dyslipidemia in type II diabetes FFA have an important role to play in the pathogenesis of this disease. Increased rates of lipolysis in type II diabetes leads to elevated circulating levels of FFA, which have detrimental effects on glucose uptake, glucose oxidation or the reverse where glucose inhibits fat oxidation. FFA also acutely effect glucose transport, phosphorylation and glycogen synthase activity. Chronic elevation of FFA leads to insulin resistance and increased intracellular fat accumulation, as well as being suggested to alter plasma membrane composition. Importantly chronic elevations of FFA and accumulation of fat with tissues have been suggested to have toxic actions in tissues such as the pancreas, where fat accumulation results in defective GSIS. Toxic actions of chronic FFA exposure have also been found in extrapancreatic tissues such as muscle, where glucose utilisation is impaired. Further more, in the liver FFA have been suggested to chronically regulate HGP. Central in the mediation of these chronic actions is the accumulation of intracellular fat and possibly fat as LC-CoA and malonyl-CoA.

1.6 REGULATION OF GENE EXPRESSION BY FATTY ACIDS

Apart from the role of fatty acids that play in membrane structure and signal transduction, it has become clear that they also have play a role in controlling metabolic processes and influence the genetic expression of many enzymes involved with lipid and carbohydrate metabolism (Sessler 1998). The most extensively studied fatty acids are the polyunsaturated fatty acids (PUFA). Flick et al. (1977) and Jeffcoat and James (1977) showed that a diet containing 60% linoleic acid fed to rats decreased the activity of liver enzymes involved in lipogenesis. Further studies have observed that the expression of lipogenic enzymes increased in rat pups during the transition from a high fat to a low fat diet post weaning (Clarke 1990). The antilipogenic action of PUFA reflects the unique ability of these fatty acids to reduce the hepatic activities of enzymes in lipogenesis and glycolysis (Clarke 1994; Jump 1994). Such suppression of enzymatic activities does not represent a fatty acid mediated impairment in enzyme
catalytic efficiency, but rather reflects a decrease in hepatic enzyme content caused by a PUFA mediated suppression of enzyme synthesis (Clarke 1993; Clarke 1994). To date, many hepatic genes have been shown to be regulated by the fat component of the diet. The levels of expression of genes encoding rodent malic enzyme, acetyl-CoA carboxylase (ACC) (Salati 1986), L-type pyruvate kinase, fatty acid synthase (FAS) (Clarke 1990), glucose transporter 4 (GLUT 4) (Tebbey 1994), S14 protein (Clarke 1990) and stearoyl-CoA desaturase (SCD1) (Ntambi 1992; Landschulz 1994) are all known to be decreased (60-90%) by dietary n-3 and n-6 PUFA (Clarke 1993; Jump 1993; Clarke 1994). A clearer understanding of how PUFA decrease enzyme synthesis came with the demonstration that PUFA decreased hepatic FAS mRNA abundance and that this was the consequence of an inhibition of gene transcription (Blake 1990; Clarke 1990; Jump 1994). Moreover, the selective control of lipogenic gene expression can be recreated in cultured hepatocytes. This eliminates the idea that PUFA exert their effects by modifying insulin or glucagon release (Clarke 1994).

1.6.1 Fatty Acid Control Of Oxidation
In addition to suppressing rates of hepatic glycolysis and lipogenesis by inhibiting the expression of genes encoding for glycolytic and lipogenic enzymes, n-6 and n-3 fatty acids are potent inhibitors of hepatic triglyceride synthesis (Strum-Odin 1987) and stimulators of fatty acid oxidation and ketogenesis (Wong 1984; Clarke 1993; Takada 1994). Pathway flux studies indicate the n-6 and n-3 species suppress the activity of diacylglycerol acyltransferase (Strum-Odin 1987), suggesting a decrease in triglyceride formation. A key factor for the enhanced hepatic oxidation of fatty acids is the induction of peroxisomal acyl-CoA oxidase (Reddy 1994; Clarke 1997). Acyl-CoA oxidase catalyses the initial rate limiting step in peroxisomal fatty acid oxidation (Reddy 1994; Clarke 1997). Importantly, diets rich in very long chain fatty acids, such as fish oil, are associated with peroxisomal proliferation and greater acyl-CoA oxidase expression (Takada 1994; Clarke 1997). Thus, the hepatic capacity for peroxisomal oxidation is increased (Clarke 1997). PUFA not only induce the expression of acyl-CoA oxidase, they have also been shown to stimulate the transcription of mitochondrial HMG-CoA synthase, a key regulatory enzyme in the synthesis of ketones (Rodriguez 1994). This induction of ketone synthesis associated with PUFA is paralleled by an increase in hepatic ketone output (Wong 1984). Clearly, PUFA function is to coordinately regulate the fate of hepatic fatty acids (Clarke 1997). Moreover, a defect in any of these adaptive responses would potentially increase hepatic triglyceride accumulation or output and modulate hepatic and peripheral fuel metabolism.
1.6.2 Fatty Acid Regulation Of Genes In Extrahepatic Tissues

Although fatty acid regulation in hepatic tissues is the most intensively studied, regulation of gene expression by PUFA in other tissue types has also been observed. Amri et al. (Amri 1991) showed that an adipocyte-specific gene was regulated by the fatty acid linolenate. More recently, Jones et al. (1996) observed a 75% decrease in adipose tissue steroyl-CoA desaturase 1 (SCD1) mRNA when lean or obese Zucker rats were fed a diet high in PUFA. Similar results have been observed in tissue culture systems. In the 3T3-L1 adipocyte cell line, arachidonic acid (AA) decreased SCD1 mRNA stability in a dose-dependent manner, as did linoleic, linolenic and eicosapentaenoic acid (EPA) (Sessler 1996). This is also the case with GLUT 4 (Tebbey 1994; Long 1996). Fatty acids also initiate differentiation of cultured cells into monocytes and granulocytes, as well as induce cell necrosis and apoptosis (Finstad 1994). Furthermore, PUFA increase the expression of Thy-1 antigen on a T lymphocyte cell line (Deglon 1995). However, in a different T lymphoma cell line, AA decreases SCD2 expression (Tebbey 1993). PUFA have also been shown to regulate the expression of the genes, L-FABP, apolipoprotein A-IV and apolipoprotein C-III (Niot 1997); the cardiac myocyte's Na+ channel gene; the pancreatic cell's ACC gene (Brun 1997) and the brain SCD2 gene (DeWille 1993).

1.6.3 Molecular Mechanisms Of Fatty Acid Gene Regulation

The molecular mechanisms of fatty acid regulation of gene expression are still poorly understood. PUFA suppression of several hepatic genes was shown initially to be due largely to a decrease in the rate of gene transcription (Ntambi 1992; Clarke 1993; Jump 1993; Landschulz 1994). More recently, studies on A-9 desaturase (SCD1), GLUT 4 in adipocytes and the A-9 desaturase 2 (SCD2) gene in lymphocytes have shown that the effect of PUFA on gene expression can affect both gene transcription and mRNA stability (Tebbey 1994; Sessler 1996). In mature adipocytes, the half-life of SCD1 mRNA is 67% lower in cells treated with AA (Sessler 1996), whereas GLUT 4 mRNA stability is reduced by 43% (Tebbey 1994). Preadipocyte fatty acid-induced expression of adipocyte fatty acid binding protein is also suspected to be regulated through message stability because transcription of the gene was not increased with fatty acid treatment (Distel 1992).

The most recent studies have focused on the mechanism by which PUFA regulate gene transcription. These studies are based on the idea that a cis-acting PUFA responsive element (PUFA-RE) are located in the promoter region of the PUFA-regulated genes.
To alter gene transcription, a transcription factor (putative PUFA-binding protein) would bind to a PUFA-RE blocking or enhancing gene transcription (Sessler 1998). There are several potential candidates for proteins that bind the PUFA-RE. It was shown that peroxisome proliferators such as WY14643, fibrates and thiazolidinediones were involved in activating peroxisomal β-oxidation, the same events activated by fatty acids (Sessler 1998). PUFA activate peroxisome proliferator-activated receptors (PPAR), and have therefore been hypothesised to be the endogenous activator of this receptor (Gottlicher 1992). Lipogenic genes, such as S14 and FAS, are repressed by both peroxisome proliferators and PUFA (Sessler 1998). On the basis of these reports, it could then be speculated that PUFA act via a peroxisome proliferator responsive element and that once activated, PPAR, along with their heterodimer partner, retinoid X receptor, were the common mediators of positive and negative effects on lipogenic and β-oxidative genes, respectively (Sessler 1998). The functions of PPARs, peroxisome proliferator responsive elements and retinoid X receptors are covered in greater detail in (section 1.8.1).

Yet there have been several additional reports showing that the PPAR are not the sole mediators in PUFA-coordinated gene regulation. Ren et al. (1996) and Baillie et al. (1996) have provided support of PPAR-independent PUFA regulation of the rat S14 gene. Furthermore, recent reports provide definitive evidence that PUFA do not repress gene expression by acting as the endogenous activators of PPAR. Ren and colleagues (1997) used a PPARα deficient rat strain to show that some PUFA effects are PPAR-independent. A diet containing fish oil high in (n-3) PUFA did not induce mRNAs of the acyl-CoA oxidase or CYP4A2 genes (Ren 1997), this was interpreted to suggest that PPARα is required for the normal PUFA induction of these genes (Ren 1997). However, the same diet repressed S14 and FAS mRNAs expression by 70%, levels of repression equal to that observed in normal rats (Ren 1997). Thus, it was felt that PPARα was not required for (n-3) PUFA to repress gene expression (Ren 1997).

Because the PPAR does not mediate PUFA repression of all the known gene transcription factors, the search for a PUFA-specific transcription factor has begun. The stearoyl-CoA desaturase 1 (SCD1) and stearoyl-CoA desaturase 2 (SCD2) genes were the first to exhibit the binding of nuclear proteins to their PUFA-responsive regions (Waters 1997). The binding of a protein to the PUFA-RE of the SCD1 and
SCD2 genes (Waters 1997) is thought to implicate a polyunsaturated fatty acid binding protein (PUFA-BP) dependent mechanism for repression of gene transcription (Waters 1997). It is hypothesised that the interaction of an effector molecule with the PUFA-BP results in repression of gene transcription (Waters 1997). Arachadonic acid (AA) was able to repress SCD1 and GLUT 4 gene expression in adipocytes by 78% (Long 1996; Sessler 1996). Whereas, Clarke et al. (Clarke 1997) showed that the AA analog 5,8,11,14-eicosatetraynoic acid (ETYA), a potent inhibitor of Δ6 desaturase, blocked the repression of hepatic FAS by (n-6) PUFA. However, ETYA could still induce acetyl-CoA oxidase. These results suggested that AA must undergo Δ6 desaturation before becoming a potent negative regulator of hepatic gene transcription (Clarke 1997). Suggesting that a PUFA metabolite may be directly involved with regulating gene transcription in different tissues (Sessler 1998).

1.6.4 Overview of Liver, Fatty Acids And Diabetes
In the previous sections it has been discussed how the liver and increased HGO is central in the development of type II diabetes. While the mechanisms behind this are still unclear it is now well established that elevated supply and tissue accumulation of fatty acids is an important factor in the dysregulation of hepatic glucose metabolism. This may occur both allosterically and at the gene transcriptional level, although the precise mechanisms and interactions have yet to be clarified. However it is increasingly clear that changes mediated at the level of gene transcription are central to the final metabolic outcome. Therefore it can be hypothesised that increased fatty acids may also have important actions on hepatic lipid metabolism, particularly at the gene level. It has been well characterised that intracellular lipids are potent regulators of lipid specific gene expression in tissues such as adipose tissue and pancreas, yet the effect of increasing lipid supply and accumulation in the liver and the actions this has on gene transcription have yet to receive systematic investigation.

It may be expected that increasing accumulation of fatty acids in the diabetic liver may activate lipid specific pathways. This activation may result in hepatic lipid oxidation remaining elevated in the face of persistent hyperglycemia, such that the continued higher rates of lipid oxidation promotes gluconeogenesis. Similarly gene changes may continue to promote lipid export contributing to the hypertriglyceridemia in the diabetic state. It is therefore necessary to examine the key regulatory steps in hepatic lipid metabolism which may be expected to be pivotal in determining the uptake, transport,
oxidation, storage or export of lipids. Given that gene expression is an important controller of fat metabolism, by measuring the activity or level of gene expression of key lipid metabolising enzymes and transport proteins we can obtain an indication of how hepatic lipid metabolism may be altered in type II diabetes and also how any interventions may impact of liver fat metabolism. In addition having an understanding of the mechanisms that may be regulating gene expression may provide insight as to how any changes are mediated.

1.7 HEPATIC LIPID METABOLISM

The key points in hepatic lipid metabolism that shall be covered in the following sections can be categorised into the following processes; uptake of lipids from circulation, cytosolic transport to either oxidation, storage or re-esterification and importantly in hepatic lipid metabolism the repackaging of fats into VLDL particles for extrahepatic export. In addition we shall discuss two key lipid responsive transcription factors which have been shown to mediate transcriptional control over the enzymes and transport proteins.

1.7.1 Liver Lipid Uptake And Lipoprotein Lipase

Fats either from dietary sources or released from storage in adipose tissue generally travel in circulation bound up in lipoprotein particles. As such when the lipid contained within these lipoproteins, the dominate form being triglyceride, is required by cells it must first be liberated from the lipoproteins which assist in the transport of the fat.

Transport of triglyceride-fatty acids from the plasma into tissues is thought to be dependent on the activity of the hydrolytic enzyme lipoprotein lipase (LPL) (Nilsson-Ehle 1981). Its hydrolytic actions enable the recognition and subsequent uptake of triglyceride rich chylomicrons and VLDL's by organs such as the liver (Chang 1996). LPL resides in liver, adipose tissue, skeletal muscle, heart, mammary tissue and brown adipose tissue (BAT) (Nomura 1984; Kuuscla 1997) and is found attached to the endothelium of capillaries which supply these organs (Cryer 1987). In humans two LPL mRNA transcripts have been identified, a 3.2- and 3.6-kb transcript are found in adipose tissue whereas muscle only expresses the larger 3.6-kb transcript (Ranganathan 1995). The activity of adipose tissue and brown adipose tissue (BAT) LPL is under adrenergic control (Kuuscla 1997), with adrenergic stimulation inhibiting or enhancing
activity respectively (Kuusela 1997). In the postprandial state when dietary carbohydrate provides energy for muscle and heart, LPL activity in adipose tissue is high whereas muscle and heart activity is low. This is due to triglycerides being channelled to storage in adipose tissue; in the fasted state, in contrast, LPL activity in adipose tissue is low while in muscle and heart it is high (Cryer 1987). It is generally agreed that insulin is a major factor responsible for the changes in LPL activity in these tissues (Nilsson-Ehle 1981).

In adult animals, LPL is made in many tissues of the body, although the adult liver contains smaller amounts of LPL compared with adipose tissue and muscle (Merkel 1998). LPL gene expression in adult liver can be induced at times of metabolic stress, in the presence of hypertyglyceridemia or as a result of cytokine action (Tracey 1992; Hardardottir 1994; Merkel 1998). Liver LPL remains vital in the metabolism of chylomicrons, as LPL must first hydrolyse chylomicrons and their remnants prior to clearance by the hepatocyte (Chang 1996). In addition LPL in conjunction with hepatic triglyceride lipase is responsible for shunting circulating triglycerides into VLDL production and β-oxidation (Merkel 1998).

The response of LPL to obesity and diabetes is varied, however the results from the various studies can be grouped into three broad categories. Firstly are the studies where LPL activity is associated with the concentration of plasma insulin. These studies have generally been conducted in adipose tissue and demonstrated with the onset of obesity plasma insulin levels were elevated as was LPL activity (Chan 1982; Murase 1985; Rebuffe-Scrive 1993). When diabetes was chemically induced, by agents such as streptozotocin or alloxan, plasma insulin levels and adipose tissue LPL activity were reduced (Nomura 1984; Wilson 1987; Saheki 1996). Therefore this first group of studies demonstrates that increases or decreases in insulin levels appear to influence the activity of LPL in adipose tissue. The second group of investigations have been carried out in organs such as the liver, heart and skeletal muscle where LPL activity appears to be independent of circulating insulin levels and still related to the presence of diabetes (Chan 1982; Saheki 1996). These studies again used alloxan to chemically induce diabetes and looked at cardiac and skeletal muscle LPL activity. Alloxan treatment decreased plasma insulin levels and adipose tissue LPL but it did not alter cardiac or skeletal muscle LPL activity (Chan 1982; Saheki 1996). This lack of change was attributed to the stable tissue mass of cardiac and skeletal muscle which did not change with the development of diabetes, unlike adipose mass which decreased in conjunction
with reduced LPL and insulin levels (Chan 1982; Saheki 1996). The third group of studies have been conducted in insulin resistant humans (Maheux 1997) and rodents (Rodrigues 1997) where post heparin released plasma LPL activity was measured. In these studies it was found that with increasing insulin resistance plasma triglyceride levels where elevated whereas LPL activity was reduced (Maheux 1997; Rodrigues 1997). It was postulated that the relationship between LPL activity and plasma triglyceride concentration is associated with the inability of insulin to stimulate LPL transcription or to increase the intracellular mRNA stability of adipose tissue LPL in insulin resistant individuals (Maheux 1997).

In summary association studies suggest the presence of diabetes alters the activity of adipose tissue LPL via changes in insulin level or insulin action. That is, an absence of insulin or the presence of insulin resistance may reduce LPL activity. In organs such as the liver LPL activity may not be under a tight control by insulin and the change in activity are due to modulations in fat supply.

1.7.2 Fat Transport Within The Liver And Fatty Acid Binding Proteins

The uptake of long chain fatty acids (LCFAs) and subsequent movement within the cell have historically been described as a passive, diffusional process (Sweetser 1987). This was considered to be the same process, whether it was uptake by the small intestine or uptake of LCFA by individual tissues such as liver or muscle (Sweetser 1987). In contrast, recent evidence suggests that cellular transport and diffusion of LCFA, rather than a passive process, is regulated by a carrier mediated process (Sweetser 1987; Veerkamp 1991), the kinetic nature of which has been described in a number of different cells (Glatz 1990).

At present, a number of cellular proteins involved in fat metabolism have been described and are collectively termed fatty acid binding proteins (FABPs) (Veerkamp 1991). Each FABP was named according to the tissue in which it was first described; L-FABP (liver), I-FABP (intestine), A-FABP (adipose tissue) and H-FABP (heart) (Veerkamp 1991). The nucleotide sequence has been established for genes encoding a number of FABPs, included in these are; rat L-FABP and I-FABP; mouse L-FABP, I-FABP, H-FABP, A-FABP and myelin-FABP; human L-FABP, A-FABP and I-FABP (Veerkamp 1995). The organisation of these gene appears to be identical with 3400-4000 nucleotides containing 4 exons and 3 introns, with exon 1 highly conserved.
between the different FABPs (Veerkamp 1995). These cytosolic FABPs are all in the 14–16 kDa size range and account for up to five percent of soluble proteins (Sweetser 1987; Glatz 1990). In addition, there is a FABP (40kDa) in the plasma membrane, referred to as plasma membrane fatty acid binding protein (PM-FABP) and is believed to span the entire phospholipid bilayer as an integral membrane protein (Stremmel 1988; Clarke 1989). The PM-FABP appears to be identical in all tissues and is not considered to be rate limiting for LCFA uptake (Stremmel 1986; Glatz 1990). It has been suggested that the cytoplasmic FABPs act as a shuttle system translocating LCFA through the aqueous phase of the cytoplasm or function in a ‘stepping stone’ fashion allowing LCFA to move from one intracellular location to another (Sweetser 1987; Clarke 1989; Veerkamp 1991).

The content of the L-FABP type is dynamically regulated in differing physiological and pharmacological conditions (Veerkamp 1995). Hormones appear to provoke marked effects on L-FABP content in rat liver (Veerkamp 1995). Female rats have a higher L-FABP content than male rats as testosterone decreases, but oestrogens increase the content (Bass 1985; Veerkamp 1995). Hypothyroidism decreases while adrenalectomy and hyperthyroidism increase L-FABP content of liver (Nakagawa 1994). Oestrogens increase the concentration of L-FABP mRNA in cultured human hepatoma cells (Veerkamp 1995). Stimulating fatty acid oxidation via high fat diets increase the FABP content of rat adipose tissue, intestine as well as content and mRNA of rat liver (St. John 1987; Bass 1988; Malewiak 1988). In a further dietary study with increasing proportion of calories derived from dietary lipids, more modest elevations in the FABP content of the liver and skeletal muscle have been reported, in parallel with the increased rates of fatty acid oxidation (Veerkamp 1991). Similarly agents such as clofibrin acid and peroxisomal proliferators increase L-FABP content in liver and intestine of rodents (Bass 1985), but have no effect on H-FABP content in heart and skeletal muscle (Paulussen 1989). These agents raise the L-FABP mRNA level by an enhanced transcription rate of the L-FABP gene (Bass 1985; Besnard 1993), indicating a common mechanism, distinct from that by which hormones regulate the level (Nielsen 1994).

Interestingly FABPs are bound by long chain fatty acids such as hexadecanedioic acid which activate PPAR (Brandes 1990). The presence of FABP in the nucleus has been observed for L-FABP in rat and bovine liver (St. John 1987; Bass 1988; Malewiak 1988) and for H-FABP in bovine heart (Dreyer 1992) and in locust flight muscle.
Studies conducted in either obese or diabetic animals generally report increased FABP activity. In obese rats higher hepatic levels of cytoplasmic FABPs have been demonstrated compared to their lean counterparts (Malewiak 1988). In addition, streptozotocin induced diabetic rats, which demonstrated pronounced elevations in the rate of fatty acid oxidation demonstrate increased H-FABP activity (Veerkamp 1996) and mRNA levels (Carey 1994) in skeletal muscles. Yet hepatic FABP levels appear to be downregulated (Veerkamp 1996). A higher molecular weight FABP in the placenta of pregnant diabetic women was found with greater fatty acid binding capacity than the FABP found in the placenta of non-diabetic women (Thomas 1986).

An interesting association between polymorphisms in the I-FABP gene and insulin sensitivity was reported (Prochazka 1993). Significant linkage of elevated post glucose challenge insulin responses with I-FABP polymorphisms has been further reported in Mexican Americans (Mitchell 1995). The polymorphism in I-FABP linked to the detrimental alterations in insulin sensitivity has been shown to result in an amino acid substitution (Ala54 to Thr54) which increases the affinity of the FABP for long chain fatty acids (Baier 1995). This was hypothesised to change the type and amount of fatty acids absorbed from the intestine, which could ultimately impact on insulin sensitivity (Baier 1995). However even though this I-FABP polymorphism results in a physiological change, subsequent studies in a number of populations have failed to link this polymorphism with type II diabetes directly. In a population of Aboriginal Canadians the Ala54Thr I-FABP polymorphism was liked with BMI, percentage body fat and fasting triglyceride levels but not with diabetes (Hegele 1996). Similarly in Japanese men no link was found with diabetes or impaired glucose tolerance (Yamada 1997) and again in three European populations no link was found with insulin resistance or type II diabetes (Pihlajamaki 1997; Vidgren 1997; Vionnet 1997). Although subsequent studies suggest that the Ala54Thr I-FABP polymorphism may not be the direct cause of insulin resistance or type II diabetes for a period of time I-FABP was an exciting candidate gene that may have contributed to the pathogenesis of these diseases.
1.7.3 Liver Fat Export And Very Low Density Lipoprotein Synthesis And Apolipoprotein-B Production

In addition to uptake and cytosolic transport of fats the liver is the major source of plasma lipoproteins, hepatic lipoprotein production is important to the understanding of the mechanisms involved in the development of hyperlipoproteinemia in type II diabetes. The most common alteration of lipoproteins in type II diabetes is an elevation in VLDL, as reflected by either increased total triglyceride or VLDL triglyceride concentrations (Howard 1987).

The biosynthesis of VLDL in the liver is a complex process requiring the coordinate synthesis and assembly of a variety of lipid and apolipoproteins components in the lumen of the endoplasmic reticulum and the Golgi apparatus of hepatocytes (Gibbons 1990; Vancé 1990). Integral in the assembly and secretion of VLDL particles is apolipoprotein B (Apo-B), a polypeptide which forms the structural frame work of the VLDL particle and without which hepatic triglyceride secretion is not possible (Herbert 1983). In addition, the Apo-B proteins differ from the other apolipoproteins in that they are water insoluble and are tightly associated with the lipid components of the lipoprotein particles (Chan 1997). Unlike the soluble apolipoproteins they do not exchange between lipoproteins or other lipid surfaces (Chan 1997).

Apo-B isolated from plasma lipoproteins exists in two distinct molecular weight forms, Apo-B100 and Apo-B48, which are products of a common gene (Hodges 1992). Both forms of Apo-B are synthesized and secreted by the liver (Hodges 1992) as well as Apo-B100 and Apo-B48 demonstrating tissue specific expression (Inui 1997). In humans ApoB100 is synthesized in the liver and Apo-B48 in the small intestine (Hodges 1992). In both rats and humans Apo-B48 mRNA is produced by a unique mechanism know as RNA editing. Apo-B mRNA editing involves a site specific C to U cytidine deamination reaction which is mediated by an enzyme complex containing a catalytic subunit, apobec-1 (Hodges 1992; Scott 1994; Davidson 1995). Since each VLDL particle is thought to contain only one molecule of Apo-B100 (Catapano 1981) measuring the mRNA level of this mRNA species provides an index of VLDL synthesis.

Few studies have examined Apo-B metabolism in type II diabetes, although the limited results provide some evidence for a clearance defect (Haffner 1984). Subjects with type II diabetes have a decrease in fractional catabolic rate for Apo-B (Haffner 1984).
Haffner et al. (1984) found slower clearance of Apo-B similar to that observed for VLDL triglyceride. Studies which suggest an over production of Apo-B in type II diabetes include investigations by Sigurdsson et al. (1976), where it was observed that type II diabetic subjects had increased production rates of Apo-B compared to normolipidemic controls. Kisselbах et al. (Kisselbах 1982) also observed that Apo-B production was increased in both normolipidemic and in hyperlipidemic mildly diabetic subjects. On the other hand two studies by Howard et al. (Taskinen 1986; Howard 1987) among obese diabetics found that individuals with type II diabetes did not have an elevation in Apo-B production compared with weight matched controls.

Similar to the human data studies in animal models of obesity and diabetes have also displayed alterations in Apo-B metabolism. In Wistar fatty rats it has been shown that animals suffering from type II diabetes have elevated levels of VLDL production and defects in VLDL catabolism from the circulation (Kazumi 1996). This situation was further exacerbated following fructose feeding, which stimulates de-novo lipogenesis, and stimulated VLDL production in previously healthy control rats (Yoshino 1989). Obese animals with lesions of the ventromedial hypothalamus have been reported to show an increased abundance of Apo-B mRNA in the liver compared with controls (Inui 1997). Given the key role the liver plays in VLDL metabolism, measuring the level of Apo-B gene expression would provide an indication of the rate VLDL synthesis and liver fat export.

1.7.4 Liver β-oxidation

Fatty acids are a major source of energy for hepatocytes. The transport of LC-CoA groups into the mitochondrial matrix to undergo β-oxidation is effected by the mitochondrial carnitine palmitoyltransferase enzyme system.

1.7.4.1 Carnitine Palmitoyltransferase

The carnitine palmitoyltransferase (CPT) system, comprising the outer mitochondrial membrane protein CPT 1 and inner mitochondrial membrane protein CPT 2 (Figure 1.3), was first conceptualised as a mechanism for mitochondrial fatty acid transport in the early 1960's by the independent studies of Fritz et al. (Fritz 1963) and Bremer et al. (Bremer 1962). Despite the elegance and unique features of their formulation little further development of the CPT system occurred until 1973 when it was found that inherited defects at the CPT locus can cause serious disease in humans (DiMauro
1973). Four years later it was elucidated, at least in the liver, CPT 1 plays a pivotal role in the regulation of hepatic fatty acid oxidation and ketogenesis by virtue of the inhibition of CPT 1 by malonyl-CoA, the product of the acetyl-CoA carboxylase reaction (McGarry 1977). Since that time it has emerged that the malonyl-CoA/CPT 1 partnership is not restricted to liver. It now seems likely that it also constitutes a key element in glucosed fatty acid cross talk in tissues such as heart (Saddik 1993) and skeletal muscle (Saha 1994; Trumble 1995) under a variety of physiological and pathological conditions. Furthermore, there is growing evidence pointing to an important role of malonyl-CoA/CPT 1 interaction in the regulation of insulin secretion from the pancreatic β-cell (Prentki 1992; Chen 1994; Newgard 1995), raising the possibility that abnormalities at this level might be a factor in the altered β-cell function in obesity and type II diabetes (McGarry 1997). In addition CPT 1 has attracted attention as a potential site for pharmacological intervention in conditions of excessive fatty acid oxidation such as poorly controlled diabetes (Anderson 1995). Finally, patients with inherited defects at the CPT locus, particularly CPT 2 (a number of them resulting in infant mortality) are now being reported with increasing frequency (Taroni 1993). These subjects have abnormal muscle fat metabolism and in severe forms fat metabolism can be altered in other tissues (Taroni 1993).

Examination of the studies which have measured CPT 1 activity animal models of diabetes has produced mixed results. However, the findings of these studies can be classified into two groups. Firstly the studies that have induced, usually severe, diabetes by a chemical agent such as streptozotocin demonstrate increased CPT activity with the onset of diabetes, in a wide range of organs (Brady 1987; Cook 1987; Grantham 1988; Wall 1989; lida 1991; lida 1993; Park 1995). The mechanism behind this induction of enzyme activity may be related to the hypoinsulinemia which accompanies chemically induced diabetes. Thus, the utilisation of glucose as a fuel source is reduced and metabolism is shifted towards oxidation of fat. The second groups of studies are those which have used animal models which spontaneously develop diabetes and in these studies fat oxidation and CPT activity is not increased when diabetes is present (Zammit 1985; Brady 1986; Lopaschuk 1987; Brady 1991).
Studies on the regulation of the CPT genes are less abundant than measure of enzyme activity and have generally been restricted to understanding how changes in nutritional and hormonal status, as well as the administration of pharmacological agents, affect expression of the CPT proteins in rat liver. Manipulations that enhance the capacity for hepatic fatty acid oxidation, such as fasting, fat feeding, induction of diabetes or treatment of rats with peroxisomal/mitochondrial proliferating agents, all cause an increase in the mRNA and activity levels of CPT 1 and CPT 2 (McGarry 1997). The effects are variable and sometimes profound. For example, whereas in 48 hr fasted or streptozotocin diabetic rats the specific activity of CPT 1 in liver mitochondria was found to increase by 2-3 fold the CPT 1 mRNA rose by 7.5 fold to 15 fold, respectively (Park 1995). Compared with hypothyroid rats, hyperthyroid animals exhibited a 40 fold elevation in liver CPT 1 mRNA abundance this was paralleled by changes in CPT 1 activity (Mynatt 1994).
1.7.4.2. Acetyl-CoA Carboxylase

The rate of hepatic $\beta$-oxidation is continually changing between meals. There is a general shift towards utilisation of fatty acids as an energy source, however in the postprandial period the rate fat oxidation is downregulated. Central in the control of this process is the enzyme acetyl-CoA carboxylase (ACC). The initial product of ACC, malonyl-CoA is both an intermediate in the de-novo synthesis of fatty acids and an inhibitor of CPT 1 (McGarry 1997). In tissues in which de novo fatty acid synthesis does not occur, such as the pancreatic $\beta$-cell (Prentki 1996) and heart (Awan 1993; Saddik 1993), malonyl-CoA still appears to function as a regulator of CPT 1 activity (Saha 1997). The importance of malonyl-CoA as a regulator of CPT warrants the investigation of ACC.

ACC catalyses the ATP dependent carboxylation of acetyl-CoA to malonyl-CoA in the rate limiting step of long chain fatty acid biosynthesis (Volpe 1973; Lane 1974; Numa 1984). In liver the predominant ACC species is a 265 kDa ACC, often refereed to as ACC$_\alpha$ (Ha 1994), whereas the dominant form of the enzyme in skeletal muscle is a 280 kDa protein referred as ACC$_\beta$ (Widmer 1996). The activity of liver ACC is modulated acutely by changes in the phosphorylation state of specific serine residues induced by 5'-AMP kinase or an adenosine 3',5'-cyclic monophosphate dependent protein kinase (Hardie 1989; Ha 1994) and chronically by changes in enzyme protein due to alterations in gene expression (Kim 1989). In general, insulin and glucose increase the activity of ACC in liver by inhibiting 5'-AMP kinase and by inducing its synthesis, whereas glucagon, catecholamines and LCFA have the opposite effects (Witters 1988; Hardie 1989; Kim 1989). Citrate is thought to be the major precursor of the cytosolic acetyl-CoA from which malonyl-CoA is synthesized and it is an allosteric activator of ACC in vitro (Kim 1989). A role of citrate in ACC regulation in vivo is unclear however (Hardie 1989; Kim 1989).

ACC is regulated by a wide range of physiological stimuli. The enzyme activity of ACC in the liver of rats was shown to decrease by 96% after a 48 hr fast (Pape 1988). However ACC activity increased approximately 60 fold after 48 and 72 hr of refeeding a fat free diet (Pape 1988). These changes in enzyme activity were attributed to changes in the amount of ACC protein. There was a 96% decrease in ACC protein after 48 hr of fasting and a greater than 80 fold increase in the amount of ACC protein occurred when
fasted animals were re-fed for 48 or 72 hr (Pape 1988). Similar changes were observed in the amount of ACC mRNA present under the conditions of fasting and re-feeding, with no detectable ACC mRNA present in the 48 hr fasted animals and increases in mRNA on re-feeding (Papc 1988).

Investigations in diabetic animals show that both hepatic and adipose tissue ACC activity is reduced with the onset of disease. When diabetes was chemically induced by streptozotocin or alloxan ACC activity was reduced by 50-60%, whereas ACC mRNA was almost eliminated after the onset of diabetes (Nakanishi 1970; Miethke 1986; Pape 1988). However when diabetic animals received insulin these effect were reversed and when control animals were treated with insulin there was an increase in ACC activity and mRNA (Pape 1988). These studies provide evidence for the role of insulin modulating ACC activity and gene expression, as previously mentioned (Pape 1988; Witters 1988; Hardie 1989; Kim 1989).

1.7.5 Uncoupled Liver Fat Oxidation And Uncoupling Proteins
In recent times the role of uncoupled respiration in the development of obesity and type II diabetes has received an increasing amount of attention. This is due to discovery of at least two new uncoupling protein isoforms which are expressed throughout various human tissues and which have been associated with obesity and type II diabetes (Bao 1998).

Uncoupling proteins (UCPs) constitute a subgroup of mitochondrial carrier proteins that are localized in the inner mitochondrial membrane (Klaus 1991). By dissipating proton gradients, they act to uncouple respiration from oxidative phosphorylation and convert fuel to heat (Skulachev 1998). So far, three homologous UCP isoforms have been identified. UCP-1 was the first UCP to be discovered and is thought to be expressed exclusively in brown adipose tissue (Skulachev 1998). It plays an important role in adaptive thermogenesis and energy expenditure in rodents (Himms-Hagen 1989), as cold exposure, increased food intake, β-adrenergic stimulation and thyroid hormones induce UCP-1 gene transcription and activity (Silva 1997).

In the past two years two additional members of the UCP family, which are expressed in adult tissues, have been identified: UCP-2 and UCP-3. UCP-2 is widely expressed in human skeletal muscle, fat, heart, placenta, lung, liver, kidney and pancreas (Gimeno 1997). Within the liver UCP-2 is highly expressed in the immunocompetent
Kupffer cells, suggesting a particular function of UCP-2 in macrophages (Larrouy 1997). A similar conclusion was reached in a study by Kopecky et al. (Kopecky 1998), where it was suggested that in the liver UCP-2 plays a role in hematopoietic development. Their study demonstrated a unique developmental pattern of UCP-2 expression in the liver of mice, where the level of UCP-2 mRNA was about 30 fold higher in foetuses compared to that in adults and started to decline immediately after birth.

In white adipose tissue of ob/ob and db/db mice, the UCP-2 transcript is induced approximately fivefold compared to lean littermate controls (Gimeno 1997), indicating that the upregulation of UCP-2 expression may be a compensatory change to counteract genetically induced obesity (Bao 1998). In type II diabetic subjects UCP-2 mRNA levels were increased 2.7 fold, an effect that could not be explained by obesity (Bao 1998). The UCP-2 gene has been mapped to human chromosome 11q13 and mouse chromosome 7 in regions that have been linked to obesity and hyperinsulinemia (Fleury 1997).

UCP-3 exhibits more limited tissue specific expression confined to skeletal and cardiac muscle and fat tissue (Boss 1997; Vidal-Puig 1997). UCP-3 is expressed as two distinct isoforms, long form of UCP-3 (UCP-3L) and short form of UCP-3 (UCP-3S) due to additions of deletions to the the final portion of the sequence encoding the COOH-terminal 37 amino acid residues (Solanes 1997). However the expression of UCP-3 is not regulated by differential effects on UCP3L and UCP3S forms of mRNA (Bao 1998). In addition like UCP-2 the expression of UCP-3 in muscle tends to increase as a function of obesity and type II diabetes (Bao 1998).

1.7.6 Liver Fatty Acid Re-esterification And Storage And Glycerol-3-Phosphate Acyltransferase

The flux of fatty acids towards either oxidation or storage is an important regulatory process within the liver. Also given that defects in either fat oxidation and storage have both been implicated in the development of type II diabetes studying these processes is important. An enzyme which has a key role in this process of directing fats into storage is the enzyme glycerol-3-phosphate acyltransferase.

Glycerol-3-phosphate acyltransferase (GPAT) catalyses the committed step of triacylglycerol and phospholipid biosynthesis by generating lysophosphatidic acid in
mammals (Bell 1980; Brindley 1985). GPAT activity is present in the microsomal membrane fraction which is the principle site of glycerol lipid synthesis and also in the outer mitochondrial membrane (Monroy 1972; Schlossman 1976; Coleman 1980). In liver, 50% of total activity is found in the mitochondrial fraction, while in most other tissues microsomal GPAT activity is about 10 times that of the mitochondria fraction (Bremer 1976; Schlossman 1976).

The partitioning of fatty acids for esterification from those for oxidation is partly carried out by GPAT and is known to be under environmental, nutritional and hormonal control. In the liver of obese Zucker rats it has been shown that the entire process of reesterification is elevated (Moir 1994; Jamdar 1995). Central in this process is an increased level of GPAT gene expression which is accompanied by an elevation of enzyme activity (Moir 1994; Jamdar 1995). It was concluded by the authors that the perturbation observed in lipid metabolism may contribute to the increased deposition of body fat in Zucker rat (Jamdar 1995). In contrast to these findings in obese animals, studies in the liver of streptozotocin diabetic rats found no changes in GPAT activity (Dang 1984). However, in the same animals microsomal and mitochondrial GPAT activity in adipose tissue was reduced (Saggerson 1987), while in BAT only mitochondrial GPAT activity was decreased (Mitchell 1991), demonstrating the possibility of different regulation in different tissues.

The effects of insulin and glucagon on transcription of the GPAT gene have been examined by administering insulin to diabetic animals and glucagon or dibutyryl cAMP to normal animals (Sul 1998). GPAT mRNA levels were low in streptozotocin diabetic mice and increased by fourfold within 1 hr after insulin injection (Shin 1991). Within 6 hr, insulin caused a maximum 19 fold stimulation, comparable to increases in GPAT mRNA caused by feeding mice a high carbohydrate, fat free diet (Lakshmanan 1972; Shin 1991). Cycloheximide abolished the insulin effect suggesting that transcriptional regulation of the GPAT genes by insulin requires ongoing protein synthesis (Paulauskis 1989; Shin 1991). Administration of glucagon or dibutyryl cAMP during refeeding of animals subjected to fasting completely blocked the elevation of the GPAT transcription rates, which suggests that glucagon, via cAMP, antagonises the insulin effect and inhibits transcription of mitochondrial GPAT genes (Paulauskis 1989).
1.7.7 Summary Of Key Hepatic Lipid Pathways

In order to more clearly understand how each of the previously described lipid metabolising enzymes or transport proteins participate in the metabolism of fat within the liver, the following figure was constructed (Figure 1.4).

Figure 1.4: Representative Liver Cell.

By measuring the activity or level of gene expression of lipid metabolising enzymes and transport proteins an indication of how hepatic lipid metabolism may be deranged in type II diabetes can be obtained. In addition, any interventions that may impact of liver fat metabolism can also be studied. Given that the activity or abundance of a number of
the previously mentioned lipid metabolising enzymes and transport proteins is influenced by the rate at which their respective mRNA is expressed. How this transcriptional control may be mediated by two key lipid responsive transcription factors shall be discussed next.

1.8 TRANSCRIPTIONAL REGULATORS

The expression of genes important in the uptake, transport, oxidation, storage and export of fatty acid in the liver are likely to be regulated by elements of a common pathway. Important in the transcriptional alteration of many genes in lipid metabolism are the peroxisome proliferator-activated receptors.

1.8.1 Peroxisome Proliferator-Activated Receptors

Peroxisome Proliferator-Activated Receptors (PPARs) are members of the superfamily of the nuclear hormone receptors that function as ligand-dependent transcription factors (Schoonjans 1996). Upon ligand activation they regulate the expression of genes containing specific response elements, called PPREs (Osumi 1991; Tugwood 1992).

Three receptor subtypes have been identified, PPARα, β (or δ) and γ (Dreyer 1992; Gottlicher 1992; Schmidt 1992; Sher 1993; Zhu 1993; Kliewer 1994; Tontonoz 1994; Amri 1995; Aperlo 1995). These receptors heterodimerize with the retinoid X receptor (RXR) and alter the transcription of target genes after binding to PPREs. The PPREs consist of a hexameric nucleotide direct repeat of the recognition motif (TGACCT) spaced by one nucleotide (Schoonjans 1996). After activation, PPARs control the expression of genes implicated in intra- and extracellular lipid metabolism, such as genes encoding enzymes involved in peroxisomal β-oxidation pathway (Osumi 1991; Tugwood 1992; Zhang 1992; Bardot 1993; Marcus 1993), cytochrome P450 (Muerhoff 1992; Palmer 1994), 3-hydroxy-3-methylglutarly-CoA synthase (Rodriguez 1994), medium chain acyl-CoA synthetase (Gulick 1994), CPT (Brandt 1998; Mascaro 1998), UCP2 (Aubert 1997), LPL (Schoonjans 1996), L-FABP (Sterchele 1994; Belury 1998) and the acyl-CoA synthetase gene (Schoonjans 1995).

The transcriptional activity of the PPAR subtypes is enhanced by a multitude of chemical compounds, including fatty acids, thiazolidinedione antidiabetic agents, prostaglandins, peroxisome proliferators and fibrate hypolipemic drugs (Schoonjans
1996). Whereas all these compounds are known to activate PPARs, only PPARγ directly binds antidiabetic thiazolidinediones (TZD) (Forman 1995; Lehmann 1995) and prostaglandin derivatives (Forman 1995; Kliwer 1995), but not the other activators. Fatty acids more selectively activate PPARβ, whereas fibrates are more selective as PPARα activators (Kliwer 1994). In addition to ligand selectivity for the PPAR subtypes, the expression patterns of the various PPAR subtypes are also distinct. PPARα is predominantly expressed in liver, heart, kidney, intestinal mucosa and BAT (Beck 1992; Braissant 1996). PPARβ is abundant and ubiquitously expressed, whereas PPARγ has a more restricted expression pattern, with two isoforms (Wahli 1995). The PPARγ2 isoform is predominantly expressed in adipose tissue (Zhu 1993; Tontonoz 1994) and has been demonstrated to play a pivotal role in adipocyte differentiation (Tontonoz 1994).

A key report that led to identification of TZDs as ligands for PPARγ came from Harris and Kletzien (Harris 1994), who showed that pioglitazone increased transcriptional activity from the aP2 enhancer and apparently did so through a differentiation-linked DNA site. When this differentiation site was cloned and identified as the PPARγ/RXR heterodimer, the question was raised as to whether TZD drugs were acting as direct agonists for PPARγ (Spiegelman 1998). These studies identified BRL49653 and pioglitazone as direct ligands of PPARγ with positive action on gene transcription (Forman 1995; Lehmann 1995). Importantly, the TZDs were also shown to be highly selective for PPARγ, as they had very minimal activity towards PPARα or PPARβ (Spiegelman 1998).

The evidence that PPARγ is the major receptor mediating the antidiabetic activity of TZDs is very strong, based on the following lines of pharmacological evidence. 1) Each of the TZD drugs binds to and activates PPARγ in the same concentration range that has antidiabetic activity (Willson 1996). 2) Among many TZDs surveyed, the rank order of potency of their antidiabetic activities closely matches the rank order of
their affinities for PPARγ (Willson 1996). 3) Potent and selective ligands for PPARγ outside of the TZD class have now been developed on the basis of their activation of PPARγ. These have antidiabetic actions in preclinical models of insulin resistance and diabetes (Spiegelman 1998). 4) Ligand stimulation of RXR, the heterodimeric partner of PPARγ, also improves insulin sensitivity in vivo (Mukherjee 1997). Taken together, these data make a compelling case that PPARγ is the major functioning receptor for the common TZD actions in diabetes (Spiegelman 1998).

In the liver, as mentioned previously, PPARα is the most prevalent species and it has been shown to play a key role in regulating hepatic fatty acid metabolism. This is demonstrated by Aoyama et al. (1998), where a PPARα null mouse was used to determine the physiological role of PPARα in liver. Several mitochondrial fatty acid metabolising enzymes including very long chain acyl-CoA dehydrogenase, long chain acyl-CoA dehydrogenase, short chain specific 3-ketoacyl-CoA thiolase and long chain acyl-CoA synthetase were expressed at lower levels in PPARα null mice (Aoyama 1998).

Similar to the PPARα null mice Zucker fatty rats also have reduced levels of hepatic lipid oxidation together with enhanced lipogenesis (Murakami 1998). Although in Zucker rats these abnormalities in fat metabolism could be overcome by the TZD derivative KRP-297, which was shown to increase mRNA levels and the activity of the enzyme acyl-CoA oxidase, known to be unregulated by PPARα (Osumi 1991; Murakami 1998). These effects was also demonstrated in primary rat hepatocytes and was suggested to be due to the direct action of the TZD on PPARα (Murakami 1998).

Although they are widespread and influence transcription of a broad range of genes PPARs are one of a number of important transcriptional regulators. A second important family are the sterol regulatory element-binding proteins.
1.8.2 Sterol Regulatory Element-Binding Proteins

Sterol regulatory element-binding proteins (SREBPs) were purified based on their ability to bind a 10-bp sequence, the sterol regulatory element-1 (SRE-1), initially identified in the promoter region of the LDL receptor gene (Brown 1997). Subsequent isolation of the human SREBP cDNAs indicated that three proteins, SREBP-1A, SREBP-1C and SREBP 2 are derived from two separate SREBP genes (Brown 1997). SREBP 1A and 1C mRNAs result from alternatively splicing exon 1 of the SREBP 1 gene (Brown 1997). Adipocyte determination and differentiation factor 1 (ADD1), the rat homologue of SREBP-1C, was cloned independently as a result of studies aimed at identifying a nuclear protein that bound the E-box motif in the promoter of the FAS gene (Tontonoz 1993). ADD1 expression was shown to be required for adipogenesis and for induction of a number of mRNAs, including FAS and GPAT, that occur during the differentiation process (Tontonoz 1993; Kim 1996; Ericsson 1997).

SREBP-1C is synthesized as a 125 kDa protein that contain two transmembrane domains that anchor the protein to the endoplasmic reticulum (Brown 1997). When cellular sterol levels are low, two distinct proteolytic events release a mature 68 kDa NH$_2$ terminal fragment of SREBP-1C, which then translocates to the nucleus, binds to the promoters of target genes and activates transcription (Brown 1997). Conversely, when levels of cellular sterols are high, proteolytic processing of SREBP-1C is diminished, nuclear levels of the mature proteins decline and transcription of target genes is low (Brown 1997).

A number of genes have been shown to be regulated at the transcription level by mature SREBPs. These included the following genes involved in cholesterol homeostasis; HMG-CoA synthase, HMG-CoA reductase, farnesyl diphosphate synthase, squalene synthase, the LDL receptor and sterol-CoA desaturase (Ericsson 1996; Brown 1997; Guan 1997). The following genes involved in fatty acid synthesis and triglyceride synthesis; FAS, ACC and GPAT (Tontonoz 1993; Kim 1996; Lopez 1996; Ericsson 1997; Magana 1997), as well as the SREBP 2 gene itself (Sato 1996). In addition it has been shown that SREBP 1 also activates PPARγ through production of an endogenous ligand (Kim 1998) and stimulates leptin gene expression (Kim 1998).

The liver of transgenic animals expressing dominant-positive SREBP-1A were enlarged up to fourfold, due to the accumulation of massive amounts of triglyceride and cholesterol ester (Shimano 1996). Despite the build up of these end products, the
mRNAs encoding multiple enzymes in the cholesterol and fatty acid biosynthetic pathways were elevated (Shimano 1996). These included the mRNAs for HMG CoA reductase, HMG CoA synthase, farnesyl diphosphate synthase, squalene synthase, ACC, FAS and sterol CoA desaturase. As a result the livers of the animals overproduced cholesterol and fatty acids (Shimano 1996). The white adipose tissue of these SREBP-1A transgenic mice was normal in young animals, but it involuted as the animals became older (Shimano 1996). In contrast animals expressing equivalent amounts of SREBP-1C had a milder phenotype (Shimano 1997). The livers of these animals was not significantly enlarged, showed only modest accumulation of triglyceride and a slight increase in the mRNAs encoding the fatty acid biosynthetic enzymes (Shimano 1996). The mRNAs encoding the cholesterol biosynthetic enzymes were not elevated. Finally SREBP 2 transgenic mice demonstrated activation of both the pathways for cholesterol and fatty acid biosynthesis, with greater activation of the cholesterol pathway (Horton 1998). Interestingly in all of these SREBP transgenic mice that had little adipose tissue but significant liver accumulation of fat displayed a diabetic phenotype (Horton 1998).

1.8.3 Overview

In summary, it appears that the liver plays a central role in the pathogenesis of type II diabetes and alterations in hepatic fat metabolism, specifically lipid accumulation, may contribute to the development of disease. However, it is not clear whether these alterations in fat metabolism may manifest as changes in the level of gene expression of key lipid metabolising enzymes and transport proteins, which are under the transcriptional regulation of lipid responsive transcription factors. In order to determine if this is the case we need means by which diabetic status and/or fat metabolism can be manipulated. This would allow the examination of whether it is alterations in hepatic fat metabolism that are contributing to the onset of disease or if the changes in fat metabolism are a secondary abnormality. However, it is not always feasible to conduct detailed or invasive studies in humans to examine molecular, biochemical or metabolic changes which occur in obesity and type II diabetes. Moreover, genetic analysis in humans is limited due to the slow evolution of disease, which limits the collection of multi-generation pedigrees (Weatherall 1992). Animal models therefore have a valuable role in investigations on the endocrine, metabolic and genetic basis of obesity and type II diabetes (Shafrir 1992).
1.9 ANIMAL MODELS OF OBESITY AND TYPE II DIABETES

A large range of animal models have been identified and used in the study of obesity and type II diabetes, most of which are rodents (Shafrir 1992). Rodents with long lasting genetic diabetes or insulin resistance have been used, such as the Zucker fa/fa rat, Wistar-Kyoto diabetic rat, ob/ob mice, the NZO mouse and others have been obtained by selective in breeding (Coleman 1978; Veroni 1991; Shafrir 1992; Zarjevski 1992). The major limitation of this group of animals is that they are genetically homogenous, which is unlike human populations (Shafrir 1992). Other rodents have been induced to develop varying degrees of glucose intolerance, but this requires the destruction of the pancreas with streptozotocin and alloxan or infiltration of the pancreas with fat caused by high fat feeding (Bringolf 1972; Collier 1985; Portha 1989; Pascoe 1990).

1.9.1 Single Gene Models Of Obesity And/Or Type II Diabetes

All of the commonly utilised strains of single-gene mutant obese rodents arose spontaneously from animals inbred for many generations. They have disease phenotypes which are either definitively affected (homozygous mutants) or healthy controls (heterozygotes and wild-type), although rodents heterozygous for several of the obesity genes display increased susceptibility to high-fat diets and other metabolic stresses.

The ob/ob (obese) mouse, which is known to be leptin deficient (Zhang 1994), exhibits a phenotype consisting of severe, early-onset obesity with insulin resistance, hyperglycemia and hyperinsulinemia which is strain- and background-dependent (Bray 1972). For example, C57BL/Js ob/ob mice develop severe diabetes and insulinopenia, while C57BL/6J ob/ob mice develop only transient insulin resistance that is compensated by β-cell hypertrophy (Friedman 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 1992). Ob/ob mice exhibit a myriad of associated metabolic and hormonal abnormalities including defective thermogenesis and impaired gonadotrophin secretion, resulting in infertility (Friedman 1992). Interestingly, ob/ob mice are insulin sensitive and have normal circulating glucose and insulin
concentrations prior to weaning, hyperinsulinemia developing when the animals are about 4 weeks old followed later by hyperglycemia (Gethuth 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 1966), including the strain-dependent severity of diabetes described above (Friedman 1992). It is now known that the db mutation results in a leptin receptor defective in signal transduction (Chen 1996; Lee 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 leptin, as predicted by the parabiosis experiments of Coleman in the early 1970s (Coleman 1973; Coleman 1978).

The Zucker fa/fa (fatty) rat (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 1996; Iida 1996; Phillips 1996; Takaya 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, hypoglucaconemia (Mathe 1995), and they exhibit over expression of lipogenic enzymes including LPL (Dugail et al. 1992), resulting in increased hepatic and adipose tissue lipogenesis (Langley 1992), and polydipsia (Bray 1972). Hyperphagia is not necessary for the development of obesity in these animals (Cleary 1987), suggesting a defect in the regulation of energy expenditure, but is marked in ad libitum fed animals compared with controls (Bray 1972).

The fat/fat mouse is characterised by slowly-developing obesity from puberty onwards which is preceded by hyperinsulinemia (Roberts 1996). The gene defect in these animals has recently been shown to be a point mutation (Ser202Pro) in the carboxypeptidase E (CPE) gene (Naggert 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 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 1996), however not all of these mice are hyperglycemic, indicating that defective insulin processing may not the only mechanism resulting in 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 1995).

The tub mutation arose spontaneously in a C57BL/6J mouse colony causing the slow development of obesity and hyperinsulinemia after weight gain, but not hyperglycemia or hypercorticoosteronemia (Coleman 1990). The tub gene was recently cloned, and the mutation in obese animals identified (Noben-Trauth 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 over expressed in the hypothalamus (Kleyn 1996; Noben-Trauth 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 (Kleyn 1996; Noben-Trauth 1996).

1.9.2 Inbred Polygenic Models Of Obesity and Type II Diabetes
Polygenic animal models of obesity and type II diabetes provide an opportunity to investigate the interactions between various components of the overall phenotype in a situation more closely resembling that found in humans. Few truly polygenic rodent models of obesity actually exist in outbred colonies, and of those, only one, Psammomys obesus, which will be discussed in more detail below, exhibits the full phenotypic range from lean to grossly obese, and glucosc 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). Type II diabetes 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 (Nagert 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 (Veroni 1991; Proietto 1993). 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 1993). 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 bred albino mice of similar length (Veroni 1991). 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 1992). These animals are innately hyperphagic and slowly accumulate abdominal visceral fat compared with controls (Ishida 1995), which is followed by hyperinsulinemia, hypertriglyceridemia and hyperglycemia in males (Sato 1995).

1.9.3 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 1955) and have defective BAT thermogenesis (Cooney 1987) and increased fatty acid synthesis compared with controls (Cooney 1989). Adrenalectomy reduced body weight and circulating insulin and glucose levels in these animals, as well as significantly improving glucose tolerance (Blair 1994). Other methods for creating hypothalamic lesions include injections of monosodium glutamate and direct electrolytic destruction (Shafritz 1990).
The application of molecular biology has had a significant impact on the generation of animal models of obesity and type II diabetes. 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 (Erickson 1996), transgenic mice over expressing one of the SREBP transcription factors as discussed earlier (Horton 1998) 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 1995).

1.9.4 Chemical Cytotoxic Agents Specific For β-cells

Several chemicals are selectively toxic to the pancreatic β-cells; the principle among them being: alloxan and streptozocin, which may be deemed as glucose with a highly reactive nitrosourea side chain (Shafrir 1990).

1.9.4.1 Alloxan

The β-cell toxicity of alloxan was discovered serendipitously while testing the nephrotoxicity of uric acid derivatives in rats and rabbits (McLetchie 1982). Alloxan is rapidly taken up by the β-cells and has a direct effect on islet membrane permeability (McLetchie 1982). Morphologic abnormalities have been described suggesting the disruption of the β-cell membrane (Orci 1976). There is evidence that alloxan acts at the site of hexose transport as it inhibits GSIS (Tomita 1974). Alloxan also interferes with the generation of glucose derived energy in the islets by inhibiting the glycolytic flux and pyruvate oxidation (Jain 1978; Borg 1979; Ishibashi 1979). Both glucose at high concentration and the nonmetabolizable 2-deoxy- and 3-O-methylglucoses, which share the entry site, block the diabetogenic action of alloxan and restore insulin production (Jain 1978; Borg 1979; Ishibashi 1979).

Administration of alloxan is most effective by intravenous injection in a dose of 40 to 50 mg/kg (Shafrir 1990). It produces irreversible functional β-cell damage within minutes and structural changes within hours in most rodents, dogs, cats, rabbits, monkeys, sheep, cattle, fish and birds (Shafrir 1990). The response to alloxan may be divided into three phases: initial hyperglycemia lasting approximately 2 hr, probably
due to liver glycogenolysis, followed by transient hypoglycemia at approximately 6 hr, due to the outpouring of insulin from the damaged cells, then a permanent hyperglycemia starting at 12 hr (Shafrir 1990).

1.9.4.2 Streptozocin
The diabetogenic actions of streptozocin (STZ) was detected by Upjohn Laboratories during testing of potential antibiotics from Streptomyces achromogenes, hence its name (Shafrir 1990). It chemical exhibits a broad spectrum antibiotic action, but also destroys the β-cells after a single injection (Shafrir 1990). It is effective in different species at dosages ranging from 50 to 200 mg/kg in rats, dogs, mice, Chinese hamsters, monkeys, miniature pigs and rabbits. It is more effective than alloxan in certain species eg, in guinea pigs and Syrian hamsters which do not develop permanent hyperglycemia after alloxan (Shafrir 1990). Animals treated with STZ, although highly insulin deficient, do not usually require insulin treatment for survival. In fact, a mild diabetic state may be induced in rats by a single low dose of approximately 35 mg/kg (Shafrir 1990).

An impressive body of knowledge on the mechanism of STZ diabetogenicity has been accumulated. Its nitrosourea moiety is responsible for β-cell toxicity, while the deoxyglucose moiety facilitates its transport across the cell membrane (Rossini 1977). The α-anomer of STZ shows higher potency, parallel to the greater effect of the α-glucose anomer on insulin secretion, suggesting the involvement of the membrane glucoreceptor in β-cell penetration (Rossini 1977).

Okamoto et al. (Okamoto 1988) have put forward the concept for a uniform mechanism of activation of alloxan, STZ and similar molecules. They consider fragmentation of β-cell DNA as the crucial event caused by accumulation of superoxide and OH radicals and/or DNA alklylation. Breaks in the DNA strands are responsible for the deterioration in insulin synthesis and secretion. This also starts immediately the repair processes, involving the activation of poly (ADP-ribose) synthase and the associated NAD utilisation. Okamoto et al maintain that the NAD depletion is so precipitous that it becomes irreversible and results in virtual cessation of NAD-dependent energy and protein metabolism and thus cell necrosis (Okamoto 1988).
1.9.5 Obesity And Type II Diabetes In Primates

The use of primates, such as Macaque (Howard 1990) or Rhesus monkeys (Hamilton 1972), which are known to be prone to obesity and type II diabetes 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 1992), and there are some difficulties involved in studying primates. 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 type II diabetes may not be evident for many years (at least 10 in most cases), greatly increasing the cost and difficulty of research (Hansen 1986). Between the ages of 10 and 25 years, a proportion of Rhesus monkeys held in captivity become obese, hyperglycemic and hyperinsulinemic (Bodkin 1993; Bodkin 1996), with hypertriglyceridemia and altered insulin receptor splicing noted in some diabetic animals (Bodkin 1993; Huang 1994; Huang 1996). These animals are likely to represent a very good animal model of human obesity and type II diabetes, but the inherent difficulties in confining such large animals, coupled with the extensive time periods involved in the development of obesity and type II diabetes make Rhcsus monkeys a difficult prospect for the study of these diseases.

Other than the Rhesus monkey there is another model of obesity and type II diabetes which exhibits a complete spectrum of phenotypic responses when held in captivity, *Psammomys obesus*, was the focus of all the studies in this thesis and will be discussed in detail below.
1.10 *Psammomys obesus*

*Psammomys obesus* (the Israeli Sand Rat or Fat Sand Rat) is a unique animal model of obesity and type II diabetes. Several aspects of metabolism in *P. obesus* closely resemble those found in human obesity and type II diabetes, and many studies performed in this animal have confirmed its status as an important animal model of obesity and type II diabetes. In particular, *P. obesus* are representative of those human populations which develop high prevalence rates of obesity and type II diabetes in response to the change from a traditional to a "Western" lifestyle, such as Pima Indians, Australian Aborigines and Pacific Islanders (Knowler 1981; Zimmet 1993).

The common name of "Sand Rat" is inaccurate as these animals belong to the subfamily Gerbillinae. The precise taxonomy of the sub-family Gerbillinae is disputed, being variously included in the family Muridae (Nowak 1991) or Cricetidae (Harrison 1991). In either case, *P. obesus* are rodents native to the Sahara-Arabian deserts from Algeria to the Sudan in North Africa and extending eastwards to Arabia (Harrison 1991). These arid environments support mainly halophytic vegetation, and *P. 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 1990). 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 1972; Degen 1988; Kam 1988). For these reasons *P. obesus* scrapes the leaves of *Atriplex halimus* with their teeth to remove electrolytes (Degen 1988; Kam 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).

*P. obesus* have a relatively low basal metabolic rate (BMR), only 57-60% of that expected for a rodent of similar size (Degen 1993; Pinshow 1993). The average daily metabolic rate of *P. 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 1963; Shkolnik 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 *P. obesus* to consume large quantities of this food source to maintain a neutral energy balance. It has been estimated that, in the field, *P. obesus* consume 50-65% of their body mass in plant material daily (Pinshow 1993), and up to 68% under laboratory conditions (Kam 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 *P. obesus* often locate their burrows at the base of these plants, they can expend minimal energy on foraging (Degen 1993).

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

No evidence of obesity, hyperglycemia or hyperinsulinemia have ever been recorded in *P. obesus* freshly trapped from the wild (Shafrir 1993). However, it is now well established that when fed *ad libitum* laboratory chow, a significant proportion of *P. obesus* become obese and develop type II diabetes. Initially, investigators at Duke University studied *P. 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 *P. obesus* colony that a number of the animals became obese and developed type II diabetes (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 *P. obesus* had a mean body weight of 141 ± 11 g, laboratory animals fed solely on fresh vegetables weighed 148 ± 9 g, while those fed laboratory chow had body weight of 251 ± 11 g (Schmidt-Nielsen 1964). *P. 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 *P. obesus* were unaffected by a diet of laboratory chow (Schmidt-Nielsen 1964; Hackel 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 *P. 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 1966; Miki 1967). The fact that
only some *P. obesus* develop obesity and/or type II diabetes 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 1994).

1.10.1 Dietary Studies In *Psammomys obesus*

Early studies showed that *P. 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, hyperglycemia, glycosuria, cataracts and reduced glucose tolerance were observed (Hackel 1965; Hackel 1966). These changes were heterogeneous with some animals progressing rapidly to marked glucose intolerance, hypoinsulinemia and death, whereas others were more resistant and remained glucose intolerant and hyperinsulinemic, while still other *P. obesus* were apparently unaffected by the laboratory chow diet (Hackel 1966).

A proportion of *P. obesus* became hyperphagic when fed a laboratory chow diet, compared to their food intake on a vegetable diet (Hackel 1966). Studies in which energy intake was restricted have shown that the development of obesity and type II diabetes can be significantly reduced in *P. 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 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 1967). In addition, a diet with a high component of wheat straw, which was relatively low caloric compared to laboratory chow, caused a reduction in body weight, body fat, blood glucose and plasma insulin concentrations and increased reproductive success in *P. obesus* (Adler 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 type II diabetes including percentage body fat, blood glucose and plasma insulin concentration (Adler 1986).

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 *P. obesus*, and reduced blood
glucose and 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 (Barnett 1994).

In summary, 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 type II diabetes in *P. obesus*.

### 1.10.2 Characterisation Of *Psammodys obesus*

The range of glycemic response to a chow diet enabled a *P. obesus* colony to be characterised by glucose and insulin levels in the fed state (Kalderon 1986). Four groups of animals were identified; group A (normoglycemic, normoinsulinemic); group B (normoglycemic, hyperinsulinemic); group C (hyperglycemic, hyperinsulinemic) and group D (hyperglycemic, normoinsulinemic) (Kalderon 1986). Longitudinal data have also been presented although glucose was the only metabolite measured (Adler 1988). Unfortunately, in these cross-sectional and longitudinal studies *P. obesus* were not matched for age (Kalderon 1986; Adler 1988). In addition, the blood samples were only taken in the fed state which suggested the possibility that the variation in glucose and insulin response may have been partly due to variation in the time period between the last meal and the collection of the sample (Barnett 1994).

In our laboratory, we have previously shown that *P. obesus* offered an ad libitum diet of standard laboratory chow displays a continuous range of phenotypic responses. A significant proportion of the animals becomes hyperphagic and obese, with increased body fat content (Barnett 1994). 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 *P. obesus* may have a genetic predisposition to over eat whenever food is available, in keeping with the thrifty gene hypothesis (Collier 1997).

Other abnormalities noted in obese *P. obesus* include hypertriglycerideremia and hypercholesterolemia relative to lean littermates and reduced spontaneous physical activity when housed in a cage with an exercise wheel (Collier 1997). Energy restriction studies in adult *P. 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 1994; Barnett 1995).

The heterogeneous response of *P. obesus* to a laboratory chow diet has led to a system of classification of the animals into four groups for experimental purposes as described in Table 1.3. The system is based on arbitrary cut off values for blood glucose (8 mmol/L) and plasma insulin (150 mU/L), which combined with body weight enables animals to be allocated to one of the four groups.

Table 1.3: Classification Of *Psammomys obesus*.

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood Glucose</th>
<th>Plasma Insulin</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>&lt;8 mmol/L</td>
<td>&lt;150 mU/L</td>
<td>lean normoglycemic, normoinsulinemic</td>
</tr>
<tr>
<td>B</td>
<td>&lt;8 mmol/L</td>
<td>≥150 mU/L</td>
<td>obese normoglycemic, hyperinsulinemic</td>
</tr>
<tr>
<td>C</td>
<td>≥8 mmol/L</td>
<td>≥150 mU/L</td>
<td>obese hyperglycemic, hyperinsulinemic</td>
</tr>
<tr>
<td>D</td>
<td>≥8 mmol/L</td>
<td>&lt;150 mU/L</td>
<td>lean hyperglycemic, hypoinsulinemic</td>
</tr>
</tbody>
</table>

(Kalderon 1986) (Barnett 1994).

Cross-sectional analyses of populations of *P. 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 and primate models of type II diabetes (DeFronzo 1988; Hamilton 1972). This is very important, as it makes *P. obesus* the only rodent model of obesity and type II diabetes known which mimics the pattern found in human populations (Barnett 1994). In addition, longitudinal studies in *P. obesus* have shown that individual animals tend to progress around the curve in a clockwise direction over time (Barnett 1994).

Studies investigating these groups of *P. obesus* have shown that the both Group B and Group C *P. obesus* were hyperphagic and obese compared with Group A animals (Barnett 1995; Collier 1997). Analysis of a large number of animals showed that the degree of hyperphagia and obesity tended to be greater in Group C than in Group B animals, however both were significantly different to Group A *P. obesus* (unpublished data). Some of the other metabolic defects observed in *P. obesus* are detailed below.
1.10.3 Metabolic Abnormalities In *Psammomys obesus*

A large number of earlier studies compared *P. obesus* with other species, most notably the albino rat. The physiological relevance of comparing these two species in many instances is inappropriate, as cross-species comparison at best can provide crude indications of differences in metabolic pathways from which candidate pathways can be used to examine more closely its role in the development of obesity and type II diabetes in *P. obesus*.

Degeneration of pancreatic β-cells was noted in early studies of *P. obesus* fed a diet of laboratory chow (Schmidt-Nielsen 1964). Subsequent studies have shown β-cell degranulation, vacuolation and glycogen nephrosis (Hackel 1965) and infiltration of fibroblasts, fibrocytes and lymphocytes (Petkov 1985).

Immunocytochemical studies suggest that the β-cells of Group C *P. obesus* are in a chronic hypersecretory state during which impaired processing of proinsulin appears to take place (Bendayan 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 *P. obesus* pancreata for insulin (Gadot 1994). These abnormalities were partially corrected by fasting, which reduces the demand on insulin secretion. The authors concluded that Group C *P. 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 1994).

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

In addition to possible defects in insulin secretion in *P. obesus*, several studies have investigated the role of the insulin receptor in this animals. Insulin receptors from hepatocytes of Group A *P. 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 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 1994).

A pronounced resistance to the actions of insulin has been documented in muscle and adipose tissue of *P. 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 1977; Kohler 1980). Hepatic insulin resistance has also been noted, as attested by an increased rate of gluconeogenesis (Shafrir 1993).

Group A *P. obesus* have a relatively high VLDL-TG uptake and LPL activity in adipose tissue compared with albino rats (Kalderon 1983; Kalderon 1986). The VLDL-TG uptake by adipose tissue of *P. obesus* was 5-fold increased, and LPL activity 10-fold that of albino rats (Kalderon 1983; Kalderon 1986). In addition, the activity of LPL in *P. obesus* heart and skeletal muscle was relatively low compared with albino rats (Chajek-Shaul 1988) and the high activity of LPL in adipose tissue was not reduced by a 20 hr fast or treatment with cholera toxin as it was in albino rats (Chajek-Shaul 1988). These findings suggest that the metabolism of *P. obesus*, as a species, is geared toward the channelling 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 *P. obesus* a decreased rate of fat store utilisation has been observed in comparison to albino rats (Frenkel 1972).
Other metabolic observations in Group A *P. 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 1986). These findings suggest that, relative to albino rats, *P. 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 *P. 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 1986).

Fatty acid synthesis in *P. obesus* occurs almost entirely in the liver in contrast to other rodents, but consistent with higher mammals (Kalderon 1983). *P. obesus* adipose tissue is rich in LPL and in the capacity to store the performed lipids, but poor in the enzymes involved in lipogenesis compared with albino rats (Kalderon 1983). Injection of labelled triglycerides in *P. 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 *P. obesus* (Gutman 1990). These factors indicate a state of readiness in *P. obesus* to store lipids whenever available.

Further studies found that hepatic pyruvate dehydrogenase, a key enzyme in glucose oxidation, had decreased activity in *P. obesus* compared with albino rats (Nakai 1997). In addition, the activity of hepatic 3-hydroxyl-CoA dehydrogenase was 4-fold higher in *P. 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 *P. obesus* (Nakai 1997). In the same study diet-induced diabetes (group C) resulted in a decreased active form of liver pyruvate dehydrogenase complex and increased activity of liver 3-hydroxyl-CoA dehydrogenase, suggesting that the diabetic conditions further suppressed glucose oxidation and promoted fatty acid oxidation (Nakai 1997).

As mentioned previously, the studies described above compared *P. obesus* with other species and do not explain why some animals become obese and/or diabetic 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 *P. obesus* (Group B) was associated with several metabolic changes including hyperphagia and obesity (Barnett 1995; Collier 1997). The obesity in these animals was due to increased adipose tissue mass in all major body fat stores (Barnett 1995). 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 1986). The lack of hyperglycemia in Group B *P. obesus* despite the reduction in peripheral glucose uptake was possibly attributable to a fall in IIHG in this group compared with Group A animals, as inferred from a reduced PEPCK activity (Kalderon 1986).

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

In Group C *P. 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, indicating a worsening of insulin resistance (Kalderon 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 1986). Despite these observed changes in adipose tissue metabolism, Group C *P. obesus* had the highest levels of adipose tissue mass, and were clearly obese relative to Group A animals (Kalderon 1986; Barnett 1994).

In contrast to the reduced VLDL-TG uptake in adipose tissue of Group C compared with Group B *P. 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 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 *P. obesus* (Kalderon 1986). The additional triglyceride synthesis by the liver would then be directed towards
muscle or retained in the liver. Because both glucose uptake and utilisation were decreased in Group C muscle (Kalderon 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 P. obesus compared with Group A, while the metabolic clearance rate of labelled glucose tended to be lower in Group C animals (Habito 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 1995). Interestingly, glucose uptake was increased in the soleus and gastrocnemius muscles of P. obesus from Group B compared with group A despite no difference in glycemia (Habito 1995).

The role of increased fatty acid oxidation in the pathogenesis of obesity and type II diabetes in P. 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 HGP, as well as glucose uptake in several tissues (Barnett 1996). In addition, etomoxir treatment reduced plasma insulin levels in hyperinsulinemic animals (Barnett 1996). These results imply that the elevated fatty acid oxidation levels found in P. obesus may contribute significantly to the development of type II diabetes in these animals.

Finally an investigation, that looked at the phospholipid composition of P. obesus liver and muscle found no significant differences between groups A, B and C (Collier 1997). A minor difference in individual fatty acids was demonstrated in group D (increased liver 20:4n-6 and increased muscle 22:5n-3); however, the unsaturation indices in the liver and muscle were not significantly different between any of the groups (Collier 1997). Thus it was concluded from these results that significant disturbances in glucose homeostasis and hyperinsulinemia may develop independently of tissue fatty acid composition (Collier 1997). Interestingly this study did show a significantly higher percentage of total lipid in groups B and C compared with group A (Collier 1997). This finding raises the possibility that an excessive accumulation of total
lipid may be leading to derangement in hepatic metabolism which result in the pathogenesis of obesity and type II diabetes in *P. obesus*.

All of these previous studies indicate that *P. obesus* develops obesity and type II diabetes in a heterogeneous fashion, similarly to susceptible human populations. The metabolic changes that accompany the development of obesity and type II diabetes in *P. obesus* make it an ideal animal model in which to study these two disease states. The following chapters of this thesis detail studies that were carried out in order to determine the role of hepatic fat metabolism in the development of obesity and type II diabetes in *P. obesus*. 
1.11 AIMS

Defects in fat metabolism are central to the aetiology and pathogenesis of obesity and type II diabetes. The liver plays a central role in these disease states via its regulation of glucose and fat metabolism. In addition, accumulation of fat within the liver has been associated with changes in key pathways of carbohydrate and fat metabolism. However a number of questions remain: 1) Is fat accumulation in the liver a primary or secondary event in the development of obesity and type II diabetes? 2) Does accumulation of liver fat result in alterations in the expression of key genes involved with fat metabolism? 3) Are key lipid-responsive transcription factors altered by liver fat accumulation in obese and/or diabetic \textit{Psammomys obesus}?

To study these questions \textit{Psammomys obesus} has been utilised, this polygenic rodent model of obesity and type II diabetes develops obesity and diabetes in a similar pattern to susceptible human populations. In addition dietary and environmental changes to \textit{Psammomys obesus} will be employed to create different states of energy balance, which shall allow the regulation of liver fat gene expression to be examined. These investigations include:

1. Fat accumulation and fatty acid binding proteins in lean, obese and diabetic \textit{Psammomys obesus}.
   The accumulation of fat in adipose tissue and liver will be measured in lean, obese and obese diabetic \textit{Psammomys obesus}. The amount of fat accumulation will be associated with changes in blood glucose, plasma insulin and cytosolic fatty acid binding protein levels.

2. Characterisation of hepatic lipid enzymes, transport protein and lipid responsive transcription factor gene expression in lean, obese and diabetic \textit{Psammomys obesus}.
   The relationship between hepatic lipid accumulation, obesity and type II diabetes and liver gene expression of key fat metabolising enzymes, transport proteins and lipid responsive transcription factors will be examined.
3. The effect of acute and chronic energy restriction on hepatic lipid metabolism in *Psammomys obesus*.

Effects of acute and chronic energy restriction will be assessed in obese and diabetic *P. obesus*. The impact of energy restriction on pre-existing levels of hepatic fat metabolising enzymes, transport proteins and lipid responsive transcription factors gene expression will be examined.

4. The effect of sucrose feeding on the development of obesity and type II diabetes in *Psammomys obesus*.

Sucrose diets provide the opportunity to examine the impact of elevating energy intake, hepatic lipogenesis and liver lipid accumulation on the expression of fat metabolising enzymes, transport proteins and lipid responsive transcription factors.

5. The effect of nicotine treatment in lean and obese *Psammomys obesus*.

Nicotine treatment shall be used as a means of increasing hepatic lipid supply due to its ability to enhance lipolysis. The impact of nicotine treatment on body weight, blood glucose, plasma insulin, liver fat accumulation and key genes involved with fat metabolism will be assessed.

6. The effect of high dose leptin administration on hepatic fat metabolism in *Psammomys obesus*.

Leptin has been shown to deplete tissue triglyceride stores. It is unclear if leptin has actions in the liver, nor is it clear if leptin impacts on liver triglyceride stores.

It is hypothesised that fat accumulation within the liver is a primary defect in the aetiology and pathogenesis of obesity and type II diabetes. Fat accumulating in the liver is the result of changes in the gene expression of key enzymes and proteins involved with fat uptake (LPL), fat transport (L-FABP), fat oxidation (CPT and UCP-2), fat re-esterification or storage (GPAT) and export of fat from the liver (Apo-B) and these changes are regulated by key lipid responsive transcription factors.
Chapter 2

MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

2.1.1 Animal Housing
Weaned *P. obesus* of mixed sex, obtained from a breeding colony maintained at Deakin University, were used for all experimental procedures. Prior to any experimental intervention the animals were maintained in a ventilated, temperature controlled environment (21±1°C), 50% humidity on a 12 hour light-dark cycle. The animals were housed in groups of 2-4, separated based on sex in standard plastic rodent cages with an absorbent layer of wood shavings on the base of the cage, which was changed at least twice a week. Standard pelleted rodent chow (Barastoc A.R.M. mix, Melbourne, Australia) and water were supplied *ad-libitum* at all times.

2.1.2 Ethics Approval
All experimental procedures were in accordance with the guidelines for the treatment of animals by the National Health and Medical Research Council and formally approved by the Deakin University Animal Ethics Committee. Approval was granted for all experimental protocols under the title of “What are the Physiological Roles of Fatty Acid Binding Proteins?” (Ethics approval numbers A4/94 and A9/96).

2.1.3 Classification Of Experimental Animals
At age 16 weeks, animals were classified into four groups based on their fed blood glucose and plasma insulin concentrations as previously described (Barnett 1994). Group A were normoglycemic-normoinsulinemic (glucose < 8.0 mmol/L, insulin < 150 µU/ml), Group B were normoglycemic-hyperinsulinemic (glucose < 8.0 mmol/L, insulin ≥ 150 µU/ml), Group C were hyperglycemic-hyperinsulinemic (glucose ≥ 8.0 mmol/L, insulin ≥ 150 µU/ml) and Group D hyperglycemic-hypoinsulinemic (glucose...
≥ 8.0 mmol/L, insulin ≤ 150 μU/ml). Although group D animals were not used prior to any experimental interventions.

2.2 IN-VIVO EXPERIMENTATION

2.2.1 Tail Vein Blood Collection

Blood was collected into sodium fluoride, sodium heparin tubes (Sigma Chemical Co. St Louis, USA) treated plastic microfuge tubes. Coating the inside of the microfuge tubes with sodium fluoride inhibited red blood cell glycolysis and heparin prevented blood coagulation. The microfuge tubes were prepared by mixing sodium fluoride (400mg) and sodium heparin (3000 IU) in 50 ml deionised water, 100 μl this solution was dispensed into each microfuge tube. The solution was dried by evaporation at 70°C for 12 hr.

In all experiments tail vein blood samples were taken from the rats by excising the tip (<3mm) from the tail. Approximately 100 μl of blood was collected from the tail vein into fluoride/heparin treated microfuge tubes and immediately stored at 4°C for blood glucose analysis.

2.3 ANALYSIS METHODS

2.3.1 Whole Blood Glucose Measurement

Blood glucose concentration was measured using a YSI glucose analyser (Sidekick 1500, Yellow Springs Instrument Co., Ohio, USA). Glucose was determined via the glucose oxidase reaction, in which glucose is converted to gluconic acid liberating hydrogen peroxide. The glucose oxidase is immobilized in a polycarbonate and cellulose membrane attached to a platinum anode and the current generated is proportional to the quantity of hydrogen peroxide produced. The glucose analyser was calibrated against a 10 mmol/L glucose standard with a 25 mmol/L linearity check as directed by the manufactures instructions. After blood glucose analysis samples were centrifuged at 20000 g for 5 min at 4°C and plasma removed for storage at -20°C until required for further analysis.
2.3.2 Plasma Triglycerides

Plasma triglycerides were analysed on an automatic analyser (Hitachi 706, Japan) using commercial colorimetric assays (Boehringer Mannheim, Mannheim, Germany). Plasma triglycerides were determined using the GPO-PAP method. Plasma triglycerides esters are cleaved by lipase (≥ 3 U/ml) to glycerol which is phosphorylated by glycerol kinase (≥ 0.2 U/ml) forming glycerol-3-phosphate. The glycerol phosphate is oxidised by glycerol phosphate oxidase (≥ 2.5 U/ml) to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide initiates the oxidation of phenol by phenol oxidase (≥ 1.1 U/ml) which binds to 4-aminophenazonc (0.77 mmol/L) forming 4-(p-benzoquinone-mono-imino) the UV absorbance of which is measured at 540 nm. A linear relationship exists for plasma triglyceride concentration and UV absorbance at 540 nm. The triglyceride concentrations are calibrated against a calibration serum for automated systems (triglyceride concentration 1.77 mmol/L, Boehringer Mannheim, Mannheim, Germany). Normal physiological (Precinorm Lipid, value 1.62 mmol/L, range 1.39-1.85 mmol/L, Boehringer Mannheim, Mannheim, Germany) and pathological (Precipath Lipid, value 4.29 mmol/L range 3.69-4.89, Boehringer Mannheim, Mannheim, Germany) external standards were randomly included in each sample tray.

2.3.3 Non-Esterified Fatty Acids

Non-esterified fatty acids (NEFA) were determined using an enzymatic colorimetric method (Wako Pure Chemicals, Osaka, Japan). The assay is based on the principle that NEFA in serum when treated with acyl-CoA synthetase in the presence of adenosine triphosphate, magnesium cations and CoA, forms the thiol ester of CoA, acyl-CoA, as well as the byproducts adenosine monophosphate and pyrophosphate. In the second portion of the procedure, the acyl-CoA is oxidised by added acyl-CoA oxidase to produce hydrogen peroxide which in the presence of added peroxidase allows the oxidative condensation of 3-methyl-N-ethyl-N-(b-hydroxyethyl)aniline (MEHA) with 4-aminoantipyrine to form a purple colored product with absorption maximum at 550 nm. Hence, the amount of NEFA in the sample can be determined from the optical density measured at 550 nm.
The assay procedure involved addition of 1ml of colour reagent A (acyl-coenzyme A synthetase 3 U, ascorbate oxidase 30 U, coenzyme A 7 mg, ATP 30 mg, 4-aminoanilpyrina 3 mg, phosphate buffer 0.05 mol/L, magnesium chloride 3 mmol/L) to 50 µl of sample serum or NEFA standard solution (oleic acid 1.0 mEq/L), all tubes were mixed and incubated at 37°C for ten minutes. After the incubation 2.0 ml of colour reagent B (acyl-coenzyme A oxidase 132 U, peroxidase 150 U, MEHA 1.2 mmol/L) was added to all reaction mixes and then allowed to incubated at 37°C for ten minutes, after which time all reactions were allowed to equilibrate to room temperature for five minutes. The reactions were then read for optical density at 550 nm versus a reagent blank, distilled water in place of standard or sample. Sample concentration was determined by constructing a standard curve using oleic acid standards (0, 0.5, 1, 1.97 mEq/l) and then comparing the absorbance of the unknown samples to the known values in the curve. this process was carried out by the spectrophotometers management system (Model DU640B UV/VIS-Spectrophotometer, Beckman Instruments Aust., Sydney, Australia).

2.3.4 Plasma Insulin

Plasma insulin concentrations were determined using a commercial radioimmunoassay (Phadeseph Insulin RIA, Pharmacia Diagnostics AB, Sweden). Unknown plasma concentrations are measured by the competitive exclusion of $^{125}$I-labelled insulin for specific anti-insulin antibodies, thus the plasma insulin concentration (μU/ml) is inversely proportional to the bound radioactivity (CPM). The concentration of insulin is determined by constructing a standard curve using human standards (0, 3, 10, 30, 100 and 240 μU/ml) and then comparing the competitive binding capacity of the unknown samples to the known values of the curve. In the assay 20 µl plasma samples diluted in 30 µl assay buffer or 50 µl of standard in duplicate were added to 50 µl $^{125}$I-labelled insulin and 50 µl human anti-insulin antibody solution (raised in guinea pig), which cross-reacts with P. obesus insulin. The solutions were then incubated for 2 hrs at room temperature after which a double antibody suspension was added (Sepharose-anti-guinea pig IgG raised in sheep) and allowed to incubate for 30 min at room temperature. The bound and free insulin was then separated by centrifugation (3000 g for 20 min) with the resultant pellet containing the insulin antibody complex and the
free insulin, the supernatant was decanted off. Pellet gamma $^{125}$I radioactivity was
determined on a gamma counter (Minaxi g Auto-Gamma 5000 series, Packard
Instrument Company, IL, USA) with a counting time of 1 min.

The reported sensitivity of the assay was <2.5 μU/ml, cross reactivity with C-peptide
<0.1%, IGF-1 and IGF-2 <0.1% with an inter assay coefficient of variation <5.0%.
The range for the external standards (Lymphochekc Immunoassay control serum,
levels 1.2 and 3 Bio-Rad, Anaheim, USA) were level 1: 15.9±0.4 μU/ml, level 2:
52.3±3.0 μU/ml and level 3: 146.9±4.0 μU/ml.

2.3.5 Plasma Leptin

Plasma leptin concentrations were determined using a commercial radioimmunoassay
(Linco Multi-Species Leptin RIA Kit, Linco Research Inc., St Louis, MO). Unknown
plasma concentrations are measured by the competitive exclusion of $^{125}$I-labelled human
leptin for specific anti-leptin antibodies, thus the plasma leptin concentration (ng/ml) is
inversely proportional to the bound radioactivity (CPM). The concentration of leptin is
determined by constructing a standard curve using human standards (0, 1, 2, 5, 10, 20,
50 ng/ml) and then comparing the competitive binding capacity of the unknown
samples to the known values of the curve. In the assay 50 μl plasma samples or 50 μl
of standard in duplicate were added to 50 μl of multi species anti-leptin antibody
solution (raised in guinea pig). The solutions were then incubated for 24 hr at 4°C after
which 50 μl $^{125}$I-labelled human leptin was added and left to incubate for 24 hr at
4°C. Following the second 24 hr incubation a double antibody suspension was added
(Pharose-anti guinea pig IgG raised in sheep) and allowed to incubate for 20 min at
room temperature. The bound and free leptin was then separated by centrifugation
(3000 g for 20 min) with the resultant pellet containing the leptin antibody complex and
the free leptin, the supernatant was decanted off. Pellet gamma $^{125}$I radioactivity was
determined on a gamma counter (Minaxi g Auto-Gamma 5000 series, Packard
Instrument Company, IL, USA) with a counting time of 1 min.
2.3.6 Total Protein Determination

Total protein concentrations in tissue extracts were determined by the Peterson’s modified micro-Lowry method (Sigma Diagnostics, St Louis, MO, USA). The method is based on an alkaline cupric tartrate reagent complexing with the peptide bonds present in the proteins and forms a purple colour when a phenol reagent is added. Absorbance is read at a wavelength between 500 nm and 800 nm.

The assay procedure involves preparing five standard solutions by diluting a stock protein standard to give five final solutions of 50, 100, 200, 300, and 400 μg/ml, along with a water blank, these were used to construct a standard curve. Isolated mitochondrial samples were diluted by 1:100 and made up to 1 ml, ensuring linearity of response. To all solutions 1 ml of Lowry reagent (Cupric tartrate) was added, mixed and allowed to stand at room temperature for 20 min. After this incubation 0.5 ml Folin & Ciocalteu’s phenol reagent was added with rapid and immediate mixing. The reaction was allowed to continue for 30 min after which time blank, standards and samples were transferred to plastic disposable cuvetes and absorbance read at 750 nm. Protein concentration was calculated from which a standard curve and analysis was performed, this was carried out by the spectrophotometer’s management system (Model DU640B UV/VIS-Spectrophotometer, Beckman Instruments Aust., Sydney, Australia).

2.3.7 Tissue Fatty Acid Binding Protein Lipid Binding Capacity

The tissue FABP lipid binding capacity assay was based on the method of Glatz and Vercamp (1983). This assay was divided into four sections, 1) FABP tissue extraction, 2) FABP chromatography, 3) Preparation of 14C palmitate and 4) FABP binding assay. All of which are diagrammatically represented in Figure (2.1)

2.3.7.1 FABP Tissue Extraction

At sacrifice the liver of experimental animals was perfused with ice cold 0.9% saline removed and patted dry, weighed, freeze clamped in liquid nitrogen and stored at -70°C until further analysis. One gram of frozen liver was weighed out and homogenised in 25% (w/v) ice cold buffer A (10 mM Na₂PO₄, 154 mM KCl pH 7.4) (Sigma Chemical Co. St Louis, USA) at 0°C using a teflon glass homogeniser which was rinsed with 1.0 ml of ice cold buffer A to remove any residue. The homogenate and collected rinsing were centrifuged at 600 g for 10 min at 4°C, the supernatant was carefully removed to avoid disturbing lipids in the top of the supernatent. The supernatent was
centrifuged again at 105000 g for 90 min at 4°C. The supernatant was removed and progressed onto the chromatography process.

Figure 2.1: FABP Bio-assay Procedure
2.3.7.2 FABP Chromatography

The first chromatography step was involved with removal of any albumin present in the sample using the prepacked Econo-Pac DEAE blue cartridge anion-exchange column (Biorad, Richmond, CA). The column packing consisted of Affi-gel® blue (Biorad, Richmond, CA), described by the manufactures as a beaded, crosslinked agarose gel with covalently attached Cibacron® Blue F3GA dye (Biorad, Richmond, CA) support for selective removal of albumin. This support has an albumin binding capacity of greater than 11mg/ml and the albumin can only be desorbed with high salt concentrations. Binding of non-albumin proteins was low, with either complete elution initially or with the inclusion of low salt concentrations.

Prior to addition of sample the Econo-Pac DEAE blue cartridge was prepared for use by washing with buffer G (1.4 M NaCl, 0.10 M acetic acid, pH 3.0, 40% v/v isopropanol) (Sigma Chemical Co. St Louis, USA) for 10 min at 1.0 ml/min the cartridge was then inverted so as the flow was directed upwards to remove any air in the cartridge and rinsed with buffer E (1.4 M NaCl, 0.020 M Tris-HCl. pH 8.0) (Sigma Chemical Co. St Louis, USA) for 10 min at 2.0 ml/min followed by buffer C (0.020 M Na₂HPO₄, pH 7.1) (Sigma Chemical Co. St Louis, USA) for 10 min at 2.0 ml/min. The cartridge was then returned to the downward position and a 2 ml filtered aliquot of FABP homogenise/sample was applied to the column at 1.0 ml/min in buffer C, the sample was allowed to load onto the cartridge for 1 min prior to eluting with buffer C for 10 min at 1.0 ml/min while collecting the albumin free effluent. After sample collection the cartridge was regenerated by washing with buffer E for 5 min at 2.0 ml/min followed by buffer H (1.5 M sodium thiocyanate in buffer C) (Sigma Chemical Co. St Louis, USA) and buffer C for 10 min at 2.0 ml/min. The cartridge was then allowed to equilibrate with buffer C for 5min at 1.0 ml/min prior to purification of the next sample.

Following the elution of the albumin free extract the second chromatography step involved delipidation of the albumin free extract to remove any endogenous lipid that may interfere with the subsequent FABP binding assay. The method used was based on the 'Lipidex 1000' methods of Glatz and Veerkamp (Glatz 1983) and involves the utilization of a 10% (w/w) substituted hydroxyalkoxypropyl derivative of Sephadex G-25 (Sigma Chemical Co. St Louis, USA) to remove unbound as well as protein bound hydrophobic molecules from aqueous solutions in a temperature dependent manner.
according to the protein-lipid interaction kinetics. Hydroxyalkoxypropyl dextran treatment at 4°C results in the separation of unbound fatty acids from protein-fatty acid complexes, whereas at 37°C all fatty acids are removed.

The delipidation chromatography process took place in a temperature controlled perspex cabinet were all chromatography columns packed with hydroxyalkoxypropyl dextran, buffers and sampled used were first equilibrated at 37°C and then maintained at this temperature during the delipidation process. This delipidation step involved loading 5 ml of filtered de-albumenised effluent onto the hydroxyalkoxypropyl dextran column. The sample was applied to the column in buffer A at 1.0 ml/min and then allowed to run to waste for 30 min with buffer A at 20 ml/h, after which the fat free extract was collected for the next 2.5 hr. Between delipidation runs the columns were regenerated by washing with 10% (v/v) methanol (Sigma Chemical Co. St Louis, USA) for 10 min at 20 ml/h, then 50% (v/v) methanol for 10 min at 20 ml/h, then 10% (v/v) methanol for 10 min at 20 ml/h, then the column was flushed with buffer A for 30 min at 20 ml/h and finally allowed to equilibrate with buffer A for 10 min at 20 ml/h at 37°C before addition of the next sample.

2.3.7.3 Preparation Of C\textsuperscript{14} Palmitate
Radioactively labeled palmitic acid was prepared from a 53 mCi/mmol stock solution of C\textsuperscript{14} palmitate (ICN Pharmaceuticals, Irvine, CA). 50 nmol of C\textsuperscript{14} palmitate was diluted and mixed in 8 ml of ethanol (Sigma Chemical Co. St Louis, USA) and 8 ml of water in a round bottom flask. The pH of the solution was then adjusted to pH 8.0 with 0.2 M KOH and the ethanol removed by rotary evaporation under low pressure at 25°C for 15 min. After evaporation 4 ml of water was added to the mix to replace the volume of water lost, the C\textsuperscript{14} palmitate was then ready for use in the FABP assay.

2.3.7.4 FABP Bio-assay
The binding capacity of the fat free L-FABP extract was compared to the equivalent binding of a range of albumin standards (0, 1, 2, 4, 8, 16, 32, 64 and 128 μg of albumin). The assay involved incubating 300 μl of lipid free sample or 300 μl of albumin standard with 200 μl of C\textsuperscript{14} labelled palmitate at 37°C for ten min. Samples and standards were then cooled on ice and 100 μl of an ice-cold hydroxyalkoxypropyl dextran suspension (100 μg/ml) was added, mixed thoroughly and left to incubate at
0°C for 10 min. Samples and standards were then centrifuged for 2 min at 200000 g at 4°C. The radioactivity of a 500 μl aliquot of the supernatant from samples and standards was determined by liquid scintillation counting (RackBeta II, LKB Wallac, Turku, Finland). The binding capacity of the samples was determined by constructing a standard curve and then comparing the binding capacity of the unknown samples to the known values of the albumin curve.

2.3.8 Tissue Triglyceride Content
Tissue triglyceride content was determined using an adaptation of the Folch lipid extraction method (Folch 1957). One gram of frozen liver sample was weighed, chopped into small pieces and the transferred into a pre-rinsed (with chloroform/methanol, 2:1 ratio) 20 ml screw top glass scintillation vial. To each sample vial 10 ml of 2:1 chloroform/methanol was added, the tubes were then shaken vigorously and placed in the refrigerator overnight. Sample tubes were removed from the refrigerator and allowed to come to room temperature. Samples were then filtered into the separating funnel, rinsed with 2x5 ml of 2:1 chloroform/methanol (ensuring all of the sample was filtered) and 4 ml of 0.9% saline was added to assist in the removal of non-lipid contaminants. The separating funnel was then capped and swirled, with pressure released intermittently. Separating funnels were then placed in the refrigerator overnight, to allow the organic and aqueous phases to separate. Sample tubes were removed from the refrigerator and warmed to room temperature. The lower organic layer was transferred into a round bottom flask (pre-rinsed with chloroform) and evaporated to dryness using a rotary evaporator at 30°C. Chloroform was added to the dried organic phase, to make up a 10 ml volumetric flask (pre-rinsed with chloroform) transferred into a 20ml screw top glass scintillation vial. A 2ml aliquot of the sample was then placed into a pre-weighed 20 ml glass scintillation vial and the solution was dried off under nitrogen. Further evaporation was achieved by placing the dried down vials were then placed in a dissector overnight, then re-weighed. The amount of fat present represented the increase in vial weight, this amount of fat was then expressed as a percentage of total liver weight.
2.4 ENZYMATIC ANALYSIS METHODS

2.4.1 Carnitine Palmitoyltransferase 1 Activity
Carnitine palmitoyltransferase 1 (CPT) activity was measured in a crude mitochondrial extract based on the method of Power et al. (1994). The method was based on measuring the rate at which the mitochondria produced H\(^3\) acylcarnitine when incubated in a medium containing H\(^3\) carnitine. The assay is divided into two sections 1) the isolation of the mitochondria and 2) measurement of CPT activity.

2.4.1.1 Isolation Of Mitochondria
Mitochondria were isolated from frozen livers based on the method of Denyer et al. (Denyer 1989), in which 1 g liver portions were weighed and homogenised with a blade homogeniser in 4 ml of extraction medium (0.25 M sucrose, 5 mM Tris-HCl and 1 mM EGTA, pH 7.4 at 4°C) (Sigma Chemical Co. St Louis, USA). The homogeniser was then rinsed with 4 ml of extraction medium, collecting the rinsings. The crude homogenate was centrifuged at 800 g for 10 min at 4°C, the supernatant was removed and stored on ice while the pellet was resuspended in 4 ml of extraction medium, homogenised and centrifuged at 800 g for 10 min at 4°C. The supernatants were combined and centrifuged at 10000 g for 10 min at 4°C, after which the crude mitochondrial pellet was resuspended in 0.5 ml of mitochondrial resuspension buffer (150 mM KCl, 5 mM Tris-HCl and 1 mM EGTA, pH 7.4 at 4°C) (Sigma Chemical Co. St Louis, USA) and stored on ice until the assay for CPT activity.

2.4.1.2 Measurement Of CPT Activity
CPT activity was determined by incubating 50 μl of mitochondrial suspension in 1 ml of assay medium (150 mM KCl, 5 mM Tris-HCl, 1 mM EGTA, 1 mM dithiothreitol, 5 mM MgCl\(_2\), 5 mM ATP, 100 μM palmitoyl-CoA, 1 μg/ml rotenone, 1 μg/ml antimycin A, and 0.13% BSA, pH 7.4) (Sigma Chemical Co. St Louis, USA) equilibrated at 37°C for 5 min. The reaction was initiated by addition of 0.005 pmol [\(^3\)H]carnitine (Amersham, Buckinghamshire, England), after 1 min and 3 min the reaction was terminated by addition of 300 μl of 0.6 M HCl and samples stored on ice. The [\(^3\)H]acyl-carnitine formed was extracted by addition of 2 ml butanol. The butanol layer was removed and washed with 2 ml of butanol saturated water. The butanol layer was again removed and the amount of [\(^3\)H]acylcarnitine formed counted by liquid scintillation (RackBeta II, LKB Wallac, Turku, Finland). Results were expressed as the amount of [\(^3\)H]acyl-carnitine formed, per mg of mitochondrial protein, per minute.
Protein concentration was determined using a total protein kit (Sigma Chemical Co. St Louis, USA) as previously described.

2.5 MOLECULAR METHODS

2.5.1 RNA Extraction
For RNA extraction, 70 mg of liver 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 20000 g for 15 min at 4°C and the aqueous (upper) phase transferred to a fresh tube. An equal volume of isopropanol containing 0.2 M NaCl was added, mixed briefly and stored at -20°C for 1 hr or overnight to precipitate the RNA. The RNA was pelleted by centrifugation at 20000 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 50 µl of RNA free water (Promega, Madison, WI).

The absorption of a 400-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 400, and the constant used for quantitation of RNA was 40, this calculation was carried out by the spectrophotometers management system.

An aliquot of the RNA containing stock was taken and diluted to 0.2 µg/ml with RNA free water, 1 µl of ribonuclease inhibitor (Recombinant RNasin®, Promega, Madison, WI) was then added to the stock and diluted RNA solution which were both stored at -70°C until needed.
2.5.2 cDNA Synthesis

After the RNA was quantitated, 1 μg of RNA was then reverse transcribed to generate cDNA using the enzyme AMV Reverse Transcriptase (Promega, Madison, WI). Briefly, the RNA was added to a mixture containing 5 mM MgCl₂, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1 mM of each dNTP, 1 U/μl Recombinant RNasin and 0.5 μg Oligo(dT)₁₅ Primer per microgram RNA (Promega, Madison, WI).

After the addition of 15 U/μg AMV Reverse Transcriptase (Promega, Madison, WI), the mixture was incubated at 42°C for 1 hr before the reaction was terminated by incubation at 99°C for 10 min. The cDNA generated was stored at -70°C.

2.5.3 Polymerase Chain Reaction (PCR)

Oligonucleotidic primers for the genes of interest were chosen from the published sequence on the Genebank database or alternatively primers were selected for the gene of interest by comparing mRNA sequences from various mammals to identify highly conserved regions. Primers were then designed using ‘Primer Express’ software (Perkin Elmer, Norwalk, USA). The primer sequences used are shown in Table 2.1.

Table 2.1: Sequences Of PCR Primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer (5'-3')</th>
<th>Antisense primer (5'-3')</th>
<th>Size of cDNA, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>GGCTACAGCTTCACCACCAC</td>
<td>GCTTGCTGTACACATCCTGC</td>
<td>480</td>
</tr>
<tr>
<td>LPL</td>
<td>CCAGCTGGACCTAACTTGGAG</td>
<td>CTCCTCAATGGGTTAATGTC</td>
<td>200</td>
</tr>
<tr>
<td>L-FABP</td>
<td>GCCCATATGAACTTCTGGCG</td>
<td>CTGGGATCTCCAATCTCTCT</td>
<td>389</td>
</tr>
<tr>
<td>Apo-B</td>
<td>AAGTAC</td>
<td>TGCTGACTCTCTCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CACGGATATGATAGTGCTCAT</td>
<td>CTGAATTCATTTCAATGGGA</td>
<td>246</td>
</tr>
<tr>
<td></td>
<td>GAOACAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPT</td>
<td>CTCCCTGAGCAGTTACCAATG</td>
<td>CTTGCGCTGGGTAAGACTAT</td>
<td>176</td>
</tr>
<tr>
<td>ACC</td>
<td>ACTCCAGGAGACAGCAGATC</td>
<td>TCTGCCAGTCCAATTCTAGC</td>
<td>535</td>
</tr>
<tr>
<td>UCP-2</td>
<td>AACAGTCTCACAACAGGCG</td>
<td>AGCATGGAAGGGGCAAGTG</td>
<td>471</td>
</tr>
<tr>
<td>GPAT</td>
<td>TGATGACGGAGGAGAGCGTC</td>
<td>AGACAGTATGTTGGCACTCTC</td>
<td>504</td>
</tr>
<tr>
<td>PPARα</td>
<td>TCAGTACATGTCCTGCTGTAG</td>
<td>GTGCAGGGCCGCCTCATAC</td>
<td>483</td>
</tr>
<tr>
<td></td>
<td>ATCT</td>
<td>TCGCGGG</td>
<td></td>
</tr>
<tr>
<td>SREBP-1C</td>
<td>ATCGCGCGGAGAGTCGCGG</td>
<td>ACTGTTTCTGTTTGATGA</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>GIAGCGTC</td>
<td>GCTGGAGCAT</td>
<td></td>
</tr>
</tbody>
</table>
PCR was performed by adding 300 ng of cDNA to a reaction mix containing 20 mM Tris-HCl (pH 8.4 at 20°C), specific MgCl₂, 50 mM KCl, 0.2 mM of each dNTP, 100 pmol each primer and 1.0 U Taq DNA Polymerase (Life Technologies, Gaithersburg, MD). The samples were amplified in the linear phase of the PCR reaction for 22-40 cycles, optimised for each gene, using the following parameters: 94°C for 30 sec, specific annealing temperature for 30 sec and 72°C for 1 min. The specific MgCl₂ concentration, annealing temperature and linear phase for each gene investigated are shown in Table 2.2. β-Actin primers were used as a control for all quantitation and were amplified in the linear phase for 22 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min. Levels of mRNA were expressed as the ratio of signal intensity for the sample genes to that of β-Actin. PCRs were performed in a Perkin Elmer 9600 thermal cycler (Perkin Elmer, Norwalk, USA) in a total volume of 20μl in 0.5ml thin walled PCR tubes (Perkin Elmer, Norwalk, USA).

Table 2.2: PCR Conditions

<table>
<thead>
<tr>
<th>Gene</th>
<th>MgCl₂ (mmol)</th>
<th>Annealing Temperature (°C)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>β Actin</td>
<td>12.5</td>
<td>58</td>
<td>22</td>
</tr>
<tr>
<td>LPL</td>
<td>20</td>
<td>55</td>
<td>40</td>
</tr>
<tr>
<td>L-FABP</td>
<td>15</td>
<td>66</td>
<td>36</td>
</tr>
<tr>
<td>Apo-B</td>
<td>10</td>
<td>55</td>
<td>37</td>
</tr>
<tr>
<td>CPT</td>
<td>20</td>
<td>55</td>
<td>40</td>
</tr>
<tr>
<td>ACC</td>
<td>10</td>
<td>55</td>
<td>32</td>
</tr>
<tr>
<td>UCP-2</td>
<td>10</td>
<td>55</td>
<td>36</td>
</tr>
<tr>
<td>GPAT</td>
<td>10</td>
<td>55</td>
<td>32</td>
</tr>
<tr>
<td>PPARα</td>
<td>10</td>
<td>55</td>
<td>36</td>
</tr>
<tr>
<td>SREBP-1C</td>
<td>15</td>
<td>55</td>
<td>38</td>
</tr>
</tbody>
</table>
2.5.4 Semi-Quantitative Reverse Transcription PCR (RT-PCR)
To enable semi-quantitation of RT-PCRs, the linear phases of all genes studied had to be determined. This was achieved by setting up 10 duplicate PCRs for the genes of interest and β-actin as described above, and consecutively removing tubes after each group of 4 cycles. After removal the tubes were immediately quenched in ice and stored at 4°C before agarose gel electrophoresis. The PCR products were electrophoresed on a 1.4% agarose gel, visualised using ethidium bromide and quantitated by computer integrated densitometry (Kodak ds 1D system, Kodak, New Haven CT) and the linear phase of each PCR determined, the linear phase for each gene are shown in Table 2.2 as the number of cycles.

2.5.5 Electrophoresis
10 µl of each PCR product was fractionated by agarose gel electrophoresis in a 1.4% gel containing 0.5 µg/ml ethidium bromide at 7 V/cm for 60 min in a DNA SubCell system (Bio-Rad, Anaheim, USA). The gels were then photographed using a computer integrated digital camera (Kodak ds 1D system, Kodak, New Haven, CT), under ultraviolet transillumination (model 4000, Stratagene, LaJolla, USA) at 302 nm.

2.5.6 DNA Sequencing
Due to the unique nature of P. obesus there has been little or no DNA sequences published, in order to determine if the PCR products formed represented the respective gene of interest all PCR products were verified by DNA sequencing. PCR products were excised from a 1% TAE agarose 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). DNA sequences were then aligned with other species using the Blast program on the Genbank database. The relative homology of the respective P. obesus genes are compared with other mammalian sequences as shown in Table 2.3.
Table 2.3: Homology Of PCR Products From *Psammomys obesus* With Human And Other Rodent Sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Rat</th>
<th>Mouse</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>74</td>
<td>67</td>
<td>70</td>
</tr>
<tr>
<td>LPL</td>
<td>87</td>
<td>87</td>
<td>83</td>
</tr>
<tr>
<td>L-FABP</td>
<td>85</td>
<td>84</td>
<td>78</td>
</tr>
<tr>
<td>Apo-B</td>
<td>89</td>
<td></td>
<td>84</td>
</tr>
<tr>
<td>CPT</td>
<td>87</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>ACC</td>
<td>93</td>
<td></td>
<td>89</td>
</tr>
<tr>
<td>UCP-2</td>
<td>73</td>
<td>73</td>
<td>68</td>
</tr>
<tr>
<td>GPAT</td>
<td>89</td>
<td>85</td>
<td>73</td>
</tr>
<tr>
<td>PPARα</td>
<td>87</td>
<td>87</td>
<td>84</td>
</tr>
<tr>
<td>SREBP-1C</td>
<td>65</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.6 STATISTICAL ANALYSIS

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 appropriate chapter. Statistical comparisons between two groups of data were determined using a paired Student's 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

DISPROPORTIONATE INCREASE OF FATTY ACID BINDING PROTEINS IN THE LIVERS OF OBESE DIABETIC PSAMMOMYS OBESUS.

3.1 SUMMARY

To investigate the role of FABPs in the regulation of fat metabolism, the relationship between FABP levels and lipid accumulation in P. obesus, a genetically heterogenous rodent model of obesity and type II diabetes was examined. Accumulation of fat within the liver was elevated in both the obese and obese diabetic animals. Interstial adipose tissue FABP levels increased in parallel with the elevated adipose tissue mass in both non-diabetic obese and diabetic obese P. obesus. In contrast, I-FABP levels were found to increase disproportionately to liver triglyceride mass only in the diabetic obese P. obesus. when compared to either the obese or lean animals. Thus, with the onset of diabetes the regulation of hepatic L-FABP is altered, suggesting that increased FABP levels may contribute to the altered hepatic metabolism present in the diabetic state.

3.2 INTRODUCTION

FABPs are a distinct group of cytosolic transport proteins which have been shown to specifically bind and transport LCFA through the aqueous environment of the cytosol (Sweetser 1987; Veerkamp 1991). Each FABP was named according to the tissue in which it was first described; L-FABP (liver), I-FABP (intestine), A-FABP (adipose tissue) and II-FABP (heart) (Veerkamp 1991). It has been suggested that the cytoplasmic FABPs act as a shuttle system translocating LCFA through the aqueous phase of the cytoplasm or function in a 'stepping stone' fashion allowing LCFA to move from one intracellular location to another (Sweetser 1987; Clarke 1989; Veerkamp 1991). Whether FABPs play a key role in regulation of fat metabolism remains unclear.
P. obesus, a native of North Africa and the Middle East are diurnally active above ground (Shafrir 1993). Their staple diet consists of stems and leaves of saltbush (Shafrir 1993). However, when fed a diet of standard laboratory chow P. obesus can develop diabetes (Shafrir 1993). Interestingly, a range of metabolic responses has been demonstrated in the transition from a diet low in energy to a diet of high-energy density, with some animals remaining lean and healthy, while others develop obesity and moderate to severe diabetes (Kalderon 1986). This range of response mirrors susceptible human populations (Shafrir 1993). Results of studies which inhibited free fatty acid oxidation in P. obesus implied that elevated fatty acid oxidation was at least partly responsible for the increased basal glucose turnover and increased HGP in diabetic P. obesus (Habito 1995; Barnett 1996). Obese diabetic P. obesus also display abnormalities in lipid metabolism including, elevated circulating cholesterol and circulating triglyceride levels (Barnett 1994).

To examine the role of FABPs in the regulation of fat metabolism we aim to investigate the relationship between FABP levels and lipid accumulation in the livers of P. obesus, a heterogeneous model of obesity and type II diabetes.

3.3 METHODS

3.3.1 Animals
Animals were housed with ad libitum food and water, 12 hr light dark cycle at 21±1°C. The high energy density of the diet, relative to the herbivorous diet that P. obesus subsists on in its native habitat, induces heterogenous weight gain and a distribution of glucose tolerances (Barnett 1994). At 12 weeks of age thirty animals were bled and the blood samples assessed for plasma glucose, insulin and triglyceride concentrations. The animals were classified into three groups: A, normoglycemic-normoinsulinemic (n=11); B, normoglycemic-hyperinsulinemic (n=11); or C, hyperglycemic-hyperinsulinemic (n=8).

3.3.2 Analytical Methods
Scrum insulin concentrations were determined by a radio-immunoassay (Phadeossep, Kabi Pharmacia Diagnostics, Sweden), using a human insulin standard. Tissues were rapidly removed and freeze clamped in liquid nitrogen for future analysis. Tissue FABP content was measured based on the radiochemical method of Glatz and Veerkamp (Glatz 1983). Tissue homogenates were prepared in Tris-HCl buffer centrifuged for 10 min at 600 g and the supernatant centrifuged
again for 90 min at 105000 g at 4°C. Albumin was removed from the cytosolic homogenate by affinity chromatography and then delipidated using a hydrophobic dextran column. The resultant FABP extract was incubated with [14C] palmitate at 37°C for 10 min and unbound [14C] removed using hydrophobic dextran and centrifugation (20000 g for 2 min at 4°C). The resulting supernatant containing the bound [14C] palmitate was assayed for radioactivity by liquid scintillation counting. Tissue lipid content was measure by chloroform methanol extraction (Folch 1957).

3.3.3 Statistical Analyses
All results are expressed as mean ± SEM. Statistical significance was evaluated using one-way ANOVA with the aid of a multiple comparison test (Tukey-HSD procedure) to establish where the differences between groups were significant. In all cases P values of 0.05 or less were considered to indicate statistical significance.

3.4 RESULTS

3.4.1 Anthropometric And Blood Variables
Animals in group A were lean, normoglycemic and normoinsulinemic (Table 3.1). Group B were obese, although maintained normoglycemia despite hyperinsulinemia and group C were both obese and diabetic.

Table 3.1: Comparison between body weight, glucose, insulin and fat accumulation for group A, B and C P. obesus.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight (g)</th>
<th>Glucose (mmol/L)</th>
<th>Insulin (mU/L)</th>
<th>Scapular fat (g)</th>
<th>Liver Lipid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>189.6±10.9</td>
<td>3.5±0.15</td>
<td>52.8±7.1</td>
<td>1.93±0.45</td>
<td>4.2±0.34</td>
</tr>
<tr>
<td>B</td>
<td>225.9±15.8*</td>
<td>4.2±0.24</td>
<td>311.2±42.7*</td>
<td>4.16±0.73*</td>
<td>6.5±0.78*</td>
</tr>
<tr>
<td>C</td>
<td>235.1±9.5*</td>
<td>10.6±0.58*</td>
<td>479.7±118.5*</td>
<td>3.93±0.56*</td>
<td>5.8±0.56*</td>
</tr>
</tbody>
</table>

* Indicates significantly different from group A.
3.4.2 Adipose Tissue

Interscapular fat mass was used as an indicator of whole body adipose tissue lipid accumulation. Group B and C animals demonstrated a significant increase in adipose tissue mass when compared with lean group A animals (Table 3.1). Total FABP present (Figure 3.1) in the interscapular adipose tissue of group B (26.8±8.3 mg) and group C animals (28.2±4.2 mg) was significantly greater than group A (7.5±0.9 mg, P<0.05). When interscapular fat FABP content was expressed as the ratio of FABP to interscapular fat mass (Figure 3.2) it was found that all groups had a similar ratio.

![Graph showing FABP in adipose and liver tissue](image)

Figure 3.1: Total amount of cytosolic FABP in adipose and liver tissue in group A, B and C P. obesus. * Indicates a significant difference from group A.

3.4.3 Liver

Hepatic lipid accumulation was measured in all animals (Table 3.1). Both obese diabetic group C animals (5.8±0.56%) and obese non-diabetic group B animals (6.5±0.78%) had a significantly increased hepatic lipid content compared with lean group A animals (4.2±0.34%, p<0.05). Total FABP present (Figure 3.1) in the liver of group C animals (251.4±55 mg) was significantly greater when compared to group A (101.3±18.2 mg). Liver FABP levels did not differ in group B (151.8±43.3 mg) when compared to the control group. When liver FABP content was expressed as the ratio of the FABP levels to fat accumulation in the liver (Figure 3.2), group A (0.37±0.07) and group B (0.31±0.05) had similar ratios. However, group C animals (0.71±0.11) demonstrated an approximate doubling of the ratio of FABP to fat accumulation in the liver compared to the other study groups.
Figure 3.2. Ratio of cytosolic FABP to adipose tissue mass or FABP to liver triglyceride content in group A, B and C P. obesus. * Indicates a significant difference from group A.

3.5 DISCUSSION

Adipose tissue FABP concentrations and liver fat accumulation were significantly increased in both obese and obese diabetic P. obesus. However the ratio of FABP to interscapular fat mass was unchanged indicating that adipose FABP levels reflected the level of fat storage. In contrast, obese diabetic animals demonstrated a significant increase in hepatic cytosolic FABPs and exhibited a disproportionate elevation in the ratio of FABP to fat in the same tissue. This suggests that there is a dysregulation of FABP levels in obesity and type II diabetes.

The glucose and insulin concentrations in the fed state of the animals analysed in the current study were comparable with previous studies in P. obesus (Barnett 1994) demonstrating a range of values from normal through to the elevated concentrations seen in the diabetic state. This range of responses mirrors those seen in susceptible human populations (Shafir 1993). The increased body weight observed in both group B and C was consistent with our previous studies in P. obesus (Barnett 1994).

The accumulation of interscapular fat in all three groups was used as an indicator of obesity in these animals. This clearly demonstrated that the increased body weight present in both groups B and C could be largely attributed to an increase in adipose tissue mass. The increase in total adipose FABP in the interscapular fat
and the elevated FABP to fat mass ratio in the same tissue suggests that in adipose tissue FABP levels are linked with adipose tissue mass. As previously reported (Veerkamp 1991) and confirmed in this study adipose tissue cytosolic FABP levels are linked with fat storage.

With increasing body weight and onset of diabetes elevated levels of fat accumulated in livers of *P. obesus*. However the total amount of FABP present and the ratio of FABP to hepatic lipid accumulation in the liver was markedly and disproportionately elevated in the obese diabetic group C animals. Thus the changes in hepatic FABP bio-activity are not linearly correlated with hepatic lipid accumulation. This would suggest that the increased FABP levels in the liver of obese and diabetic *P. obesus* may act to promote excessive liver fat oxidation. Elevated fat oxidation increases hepatic glucose production with suppressed glucose oxidation and elevated rates of gluconeogenesis. Evidence for this hypothesis is found in previous studies in our laboratory where it was demonstrated that obese diabetic *P. obesus* exhibits elevated hepatic glucose production which was reduced following free fatty acid inhibition (Habito 1995; Barnett 1996).

In conclusion, obese and obese diabetic *P. obesus* have significantly elevated body weight, adipose tissue mass and hepatic lipid accumulation. Adipose tissue FABPs are elevated in obesity and reflect the adipose tissue mass. In contrast liver cytosolic FABPs exhibited a disproportionate elevation in the ratio of FABP to fat in obese diabetic *P. obesus*. In further studies we aim to investigate if these abnormalities in fat metabolism, hepatic lipid accumulation and hepatic FABP changes, are more wide spread and manifest as alterations in key lipid metabolising enzymes and transport proteins in the livers of obese diabetic *P. obesus*. 
Chapter 4

HYPERGLYCEMIA ALTERS HEPATIC LIPID OXIDATIVE GENE EXPRESSION IN OBESE PSAMMOMYS OBEUS.

4.1 SUMMARY

Intracellular lipid may be an important regulator of gene expression. In this study we examined the relationship between hepatic lipid accumulation, obesity and type II diabetes with the liver gene expression of key fat metabolising enzymes and transport proteins. These investigations were conducted in the polygenic rodent model P. obesus. The development of obesity and hyperinsulinemia in P. obesus was associated with excessive liver lipid accumulation and an increase in the mRNA levels of the key re-esterification enzyme GPAT. Similarly, obese P. obesus which were diabetic (hyperglycemia and hyperinsulinemia) also displayed both elevated liver lipid accumulation and increased GPAT gene expression. However, diabetic P. obesus also had markedly increased expression of the lipid responsive transcription factors PPARα and SREBP-1C, as well as CPT, L-FABP and ACC mRNAs. The changes in gene expression appeared to be more closely linked with the development of diabetes and not the accumulation of fat within the liver. These findings indicate that intracellular lipid accumulation in diabetic P. obesus is not the major determinant of hepatic lipid-specific gene expression. This study suggests that hyperglycemia may be important in regulating hepatic lipid-specific genes.
4.2 INTRODUCTION

Type II diabetes is a heterogeneous disorder resulting from complex interactions between genetic and environmental factors. Key metabolic defects in type II diabetes include fasting hyperglycemia and excessive plasma glucose levels following either a meal or glucose ingestion (DeFronzo 1989; Consoli 1992). This hyperglycemia may in turn act to impair both insulin action and pancreatic beta-cell function (Yki-Jarvinen 1994). A number of studies have shown that hyperglycemia correlates closely with the maintenance of excessive liver glucose production, due primarily to increased gluconeogenesis (DeFronzo 1985; Groop 1989). In the genetically heterogeneous rodent model of obesity and type II diabetes, *P. obesus*, those animals which spontaneously develop diabetes elevated liver glucose production has been identified as a principle factor in the aetiology and maintenance of hyperglycemia (Habito 1995). The rate of liver glucose production has been shown to correlate closely to circulatory plasma non-esterified fatty acids (NEFA) concentrations (Boden 1996). However, rather than promoting hepatic fatty acid oxidation (Diraison 1998) and therefore hepatic gluconeogenesis, via classical allosteric gluconeogenic enzyme activation (Boden 1996), fatty acids in diabetic states appears to be preferentially re-esterified and stored as intracellular triglycerides (Diraison 1998; Kraegen 1991; Oakes 1994). The increased intracellular lipids may act indirectly to enhance gluconeogenesis by modulating the transcription rates of genes coding for gluconeogenic and glycolytic enzymes (Antras-Ferry 1994; Liimatta 1994; Massillon 1997). Studies have further shown that fatty acids are important regulators of genes coding for lipid metabolising enzymes (Clarke 1996; Pretki 1996) however, it remains to be determined whether increasing intracellular lipids modulates these genes.

Previously, obese diabetic *P. obesus* have been shown to display abnormalities in hepatic lipid metabolism including increased liver fat deposition, together with elevated circulating NEFA and triglycerides levels (Barnett 1994; Lewandowski 1997). Thus the propensity for excessive lipid storage may represent a key metabolic defect contributing to the development of type II diabetes in *P. obesus*. This study aims to 1) determine if the accumulation of fat within the liver is causal in the development of obesity and type II diabetes and 2) if any changes in fat metabolism are mediated through lipid responsive transcription factors and genes important in lipid metabolism in the livers of a cross-sectional sample of *P. obesus*. 
4.3 METHODS

4.3.1 Animals

A colony of mixed sex *P. obesus* was maintained on a 12 hr light 12 hr dark cycle at 21±1°C and fed *ad libitum* standard rat chow (Barastoc, Pakenham, Australia). Consistent with previous reports from our laboratory (Barnett 1994), the animals displayed wide spontaneous variation in weight gain, due to increased adiposity accompanied by varying degrees of hyperinsulinemia and hyperglycemia (Collier 1997). *P. obesus* (n=48) at 16 to 20 weeks of age were allocated into three groups based on body weight, blood glucose and plasma insulin as described previously (Barnett 1994). Group A were lean, normoglycemic, normoinsulinemic; Group B were obese normoglycemic, hyperinsulinemic; Group C were obese, hyperglycemic, hyperinsulinemic (Barnett 1994). All animals were sacrificed by anaesthetic overdose, tissues removed, freeze clamped and maintained in liquid nitrogen until analysis. Body fatness was estimated by combining the weight of the same selected fat stores (peri-renal, mesenteric, hind limb intra-muscular and suprascapular) in each animal and expressing this as a percentage of body weight.

4.3.2 Biochemical Analyses

Blood was collected from the tail vein in heparinized microtubes and blood glucose immediately determined using an automated enzymatic analyser (2300 Stat Plus, Yellow Springs Instruments, Ohio). Plasma insulin levels were determined by radioimmunoassay using a human primary antibody (Phadese ph, Kabi Pharmacia Diagnostics, Sweden). Plasma leptin levels were measured by radioimmunoassay using a multi-species primary antibody (Linco, St Charles, MO). Plasma triglyceride levels were measured colorimetrically (Bohringer-Mannheim, Mannheim, Germany) as were non-esterified free fatty acids (NEFA) (WAKO Pure Chemical Co., Japan).

4.3.3 Hepatic Carnitine Palmitoyltransferase Activity And Triglyceride Accumulation

A measure of β-oxidation, carnitine palmitoyltransferase (CPT) activity was determined based on the method of Power and colleagues (Power 1994). Mitochondria were isolated from frozen livers and CPT activity was determined by incubating 50 μl of mitochondrial suspension in 1ml of assay medium (150 mM KCl, 5 mM Tris-HCl, 1 mM EGTA, 1 mM dithiothreitol, 5 mM MgCl₂, 5 mM ATP, 100 μM palmitoyl-CoA, 1
µg/ml rotenone, 1 µg/ml antimycin A, and 0.13% BSA (pH 7.4) equilibrated at 37°C for 5 min. The reaction was initiated by addition of 0.005pmol [3H]carnitine and terminated by addition of 300 µl of 0.6 M HCl at either 1 or 3 min. [3H]acylcarnitine formed was extracted by addition of 2ml butanol, followed by a butanol saturated water wash. [3H]acylcarnitine was counted by liquid scintillation (RackBeta II, LKB Wallac, Finland). Results were expressed as [3H]acylcarnitine formed per mg of mitochondrial protein, per minute. Protein concentration was determined using a total protein kit (Sigma, St. Louis, MO). Total hepatic lipid accumulation was measured by chloroform methanol extraction (Folch 1957).

4.3.4 Liver Fatty Acid Binding Protein Bio-activity
Liver cytosolic fatty acid binding protein bio-activity measurement was based on the radiochemical method of Glatz and Veenkamp (Glatz 1983). Tissue homogenates were prepared in Tris-HCl buffer centrifuged for 10 min at 600 g and the supernatant centrifuged again for 90 min at 105000 g at 4°C. Albumin was removed from the cytosolic homogenate by affinity chromatography and then delipidated using a hydrophobic dextran column. The resultant L-FABP extract was incubated with [14C] palmitate at 37°C for 10 min and unbound [14C] removed using hydrophobic dextran and centrifugation 20 000 g for 2 min at 4°C). The resulting supernatant containing the bound [14C] palmitate was assayed for radioactivity by liquid scintillation counting.

4.3.5 Extraction Of Total RNA And Reverse Transcription (RT)-PCR Quantitation
Total RNA was extracted from 30 mg frozen liver portions by the RNAzol B method (Bresatec, Adelaide, Australia). First strand cDNA was generated from 1 µg of RNA in a 30 µl volume using the oligo(dT) primer in the first strand cDNA synthesis kit (Promega, Madison, WI). One microliter of the reverse transcription reaction mix was amplified with primers specific for LPL, L-FABP, Apo-B, CPT, ACC, UCP-2, GPAT, PPARα and SREBP-1C in a total volume of 20 µl. All sequences were from the Genbank, accession numbers and primer sequences are shown in Table 2.1 in the general material and methods section. The samples were amplified in the linear phase for 22–40 cycles, optimised for each gene, using the following parameters: 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. β-Actin primers were used as a control for all
quantitation and were amplified in the linear phase for 22 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min. Levels of mRNA were expressed as the ratio of signal intensity for the sample genes to that of β-Actin. The PCR products were electrophoresed on a 1.4% agarose gel, visualised using ethidium bromide and quantitated by computer integrated densitometry (Kodak ds 1D system, Kodak, New Haven, CT).

4.3.6 Statistical Analyses
All results are expressed as mean ± SEM. Statistical significance was evaluated using one-way ANOVA with the aid of a multiple comparison test (Tukey–HSD procedure) to establish where the differences between groups were significant. In all cases P values of 0.05 or less were considered to indicate statistical significance.

4.4 RESULTS

4.4.1 Blood Metabolites, Hormones And Anthropometric Variables
_P. obesus_ were separated into three groups on the basis of plasma glucose and insulin concentrations, as shown in Table 4.1. Group B and C _P. obesus_ had significantly elevated fed insulin concentrations, while group C alone had elevated fed blood glucose concentrations (P<0.05). Consistent with earlier studies group C _P. obesus_ were also hypertriglyceridemic in comparison with group A and B (P<0.05), however no differences were found in circulating NEFA levels among groups (Table 4.1). Plasma leptin concentrations were elevated in group B _P. obesus_, compared with group A (P<0.05) (Table 4.1). _P. obesus_ in groups B and C had significantly elevated body mass, estimated body fat and increased liver lipid accumulation compared to group A (P<0.05) (Table 4.2).
Table 4.1: Blood metabolites and hormones in lean, obese and obese diabetic *P. obesus*.

<table>
<thead>
<tr>
<th>Group</th>
<th>Glucose (mmol/L)</th>
<th>Insulin (mU/L)</th>
<th>NEFA (mEq/L)</th>
<th>TG (mmol/L)</th>
<th>Leptin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4.5±0.1</td>
<td>55±9</td>
<td>0.50±0.05</td>
<td>0.78±0.13</td>
<td>21±5</td>
</tr>
<tr>
<td>B</td>
<td>4.8±0.6</td>
<td>294±50*</td>
<td>0.30±0.04</td>
<td>0.99±0.11</td>
<td>39±5*</td>
</tr>
<tr>
<td>C</td>
<td>13.7±1.1**</td>
<td>567±94*</td>
<td>0.45±0.06</td>
<td>2.0±0.4*</td>
<td>26±2</td>
</tr>
</tbody>
</table>

Values are the mean ± S.E.M. * Significantly different from group A, P<0.05. ** Significantly different from group A&B, P<0.05.

Table 4.2: Anthropometric variables, liver lipid accumulation, L-FABP bio-activity and hepatic CPT activity in *P. obesus*.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Mass (g)</th>
<th>Body Fat (%)</th>
<th>Liver Lipid (%)</th>
<th>Bio-activity (mg/g)</th>
<th>CPT Activity (nmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>161±7.1</td>
<td>2.3±0.3</td>
<td>3.8±0.3</td>
<td>19.5±2.2</td>
<td>1.6±0.1</td>
</tr>
<tr>
<td>B</td>
<td>227±9.1*</td>
<td>3.9±0.3*</td>
<td>5.2±0.4*</td>
<td>21.0±3.4</td>
<td>1.9±0.3</td>
</tr>
<tr>
<td>C</td>
<td>224±7.2*</td>
<td>5.7±0.2*</td>
<td>5.0±0.3*</td>
<td>30.1±5.9*</td>
<td>1.6±0.1</td>
</tr>
</tbody>
</table>

All values are the mean ± S.E.M. * Significantly different from group A, P<0.05.
4.4.2 CPT Activity And Gene Expression

CPT activity did not differ significantly among groups (Table 4.2), although CPT gene expression was significantly elevated in group C compared to group B (P<0.05) (Figure 4.1). Similarly, ACC gene expression was increased only in the group C P. obesus (P<0.05) (Figure 4.1).

![Bar chart showing mRNA levels relative to β-Actin of CPT, UCP-2, ACC and GPAT in P. obesus. * Significantly different from group A P<0.05. † Significantly different from group B P<0.05.]

Figure 4.1: mRNA levels relative to β-Actin of CPT, UCP-2, ACC and GPAT in P. obesus. * Significantly different from group A P<0.05. † Significantly different from group B P<0.05.

4.4.3 L-FABP Activity And Gene Expression

There was a significant increase in L-FABP bioactivity from group A to group C P. obesus (P<0.05) (Table 4.2). Although L-FABP gene expression was significantly elevated in group C animals compared to groups A and B (group A 0.3±0.1 relative units, group B 0.3±0.1 relative units and group C 1.0±0.2 relative units, P<0.05, Figure 4.2).
4.4.4 Gene Expression Of Lipid Specific Genes; LPL, ACC, GPAT And Apo-B

The expression of the mRNA for the key enzyme for the esterification of free fatty acids to triglyceride, GPAT, was elevated in group B and C P. obesus when compared to the lean group A P. obesus (P< 0.05) (Figure 4.1). In contrast the gene expression of the lipolytic protein, LPL was reduced to 20% of the relative gene expression of group A animals in the group C P. obesus (P< 0.05) (Figure 2). Apo-B gene expression was not significantly different among groups (Figure 4.2), but did show a non-significant trend to be increased in group C. The relative expression of the UCP-2 gene was reduced by 75% in group C P. obesus (P< 0.05) (Figure 4.1).

![Graph showing mRNA levels relative to β-Actin of LPL, L-FABP and Apo-B in P obesus.](image)

* Significantly different from group A, P<0.05. † Significantly different from group B, P<0.05.
4.4.5 Gene Expression Of Lipid Responsive Transcription

Factors PPARα And SREBP-1C

Measurement of transcription factor mRNA demonstrated PPARα gene expression was 4.5 fold higher in the hyperglycemic group C animals compared with both group A and B P. obesus. The same trend was observed in the expression level of SREBP-1C with a 1.9 fold increase in group C compared with groups A and B (Figure 4.3).

![Bar graph showing mRNA levels relative to β-Actin of PPARα and SREBP-1C in ad-libitum fed P. obesus. * Significantly different from group A, P<0.05. † Significantly different from group B, P<0.05.](image)

Figure 4.3: mRNA levels relative to β-Actin of PPARα and SREBP-1C in ad-libitum fed P. obesus. * Significantly different from group A, P<0.05. † Significantly different from group B, P<0.05.
4.5 DISCUSSION

Our results demonstrated that hepatic lipid content is increased in *P. obesus* following the onset of obesity. The development of diabetes in the obese *P. obesus* population resulted in no further alteration of liver lipid accumulation. The development of obesity in *P. obesus*, without diabetes, was also accompanied by the development of hyperinsulinemia and increased GPAT mRNA levels, without significant alterations in the expression of any of the remaining genes analysed. However, the obese diabetic animals, in addition to having hyperinsulinemia and hyperglycemia, displayed significant changes in the gene expression of all of the key lipid responsive transcription factors and lipid metabolising enzymes analysed, with the exception of Apo-B.

The current study, together with previous work from our laboratory has identified that elevated liver lipid accumulation accompanies the onset obesity (Lewandowski 1997). Results of this study demonstrated a two fold elevation in the genc expression of GPAT, a key fatty acid esterification enzyme, in these obese animals (groups B and C). A similar elevation of GPAT mRNA and enzyme activity has been observed previously in obese Zucker rats (Jamdar 1995) and was suggested that these increases may stimulate hepatic phospholipid or triglyceride synthesis (Moir 1994). We may speculate in the current study that increased GPAT mRNA provides a mechanism by which fatty acids are preferentially trafficked to re-esterification and intracellular storage.

Of the animals which develop obesity and hyperinsulinemia (group B) approximately one half of these animals subsequently develop hyperglycemia (group C) (Barnett 1994). In the present study hepatic lipid accumulation was not altered in the obese animals with the development of diabetes. This finding combined with the data from the gene expression measurements suggests that the marked alterations in mRNA levels of the key lipid metabolising enzymes and lipid responsive transcription factors analysed are not solely the consequence of increased hepatic lipid accumulation. It is unclear from the results of this study what stimuli enhance the expression of lipid oxidative or transport genes, however the mRNA levels of two key lipid responsive transcription factors, PPARα and SREBP-1C were markedly increased in the obese diabetic *P. obesus*. It is well established that PPARα is an important transcriptional regulator of the LPL, L-FABP, and CPT genes (Veerkamp 1995; Staels 1997; Mascaro 1998) with
transgenic PPARα null mice demonstrating reduced hepatic expression of many lipid-oxidative genes (Aoyama 1998). Whereas SREBP-1C has been shown to transcriptionally regulate the expression of key genes responsible for cholesterol and fatty acid synthesis (Brown 1997). Included in the genes regulated by SREBP-1C activity are GPAT and ACC (Ericsson 1997; Lopez 1996). Given the regulatory role the transcription factors play in the expression of many lipid-specific genes it was not unexpected that mRNA levels of genes important in the oxidation of lipids, together with genes necessary for the uptake, transport and hepatic export of lipids were also increased.

Consistent with previous studies in obese diabetic rodents and diabetic humans (Zammit 1985; Brady 1986; Diraison 1998), hepatic fatty acid oxidation appeared unaltered in obese or diabetic P. obesus, as measured by CPT enzyme activity, the key regulator of long-chain fatty acid flux into the mitochondrial matrix. In contrast, CPT gene expression displayed a greater than two fold increase in the diabetic P. obesus. Disassociation of CPT gene expression and enzyme activity has been observed previously in diabetic rats (McGarry 1997), in which a fifteen fold increase in mRNA levels corresponded to only a three fold increase in enzyme activity. This may suggest that post-transcriptional events or protein turnover regulate protein concentrations and activity in the hyperglycemic state (McGarry 1997).

Malonyl-CoA is an important allosteric inhibitor of CPT enzyme activity (McGarry 1978), it is synthesised from cytosolic citrate under the enzymatic control of ACC. Citrate, and thus malonyl-CoA, are postulated to respond to changes in intracellular glucose flux, thus inhibiting lipid oxidation (Saha 1997). Our current results demonstrated a three fold increase in ACC mRNA levels in diabetic P. obesus, which together with the significant fasting hyperglycemia of these animals may suggest greater in-vivo inhibition of CPT activity. Also this result is consistent with previous studies by our research group that has demonstrated increased de novo lipogenesis in the liver of diabetic P. obesus (Lcwandowski 1997).

Potentially important in fatty acid oxidation and whole body thermogenesis is the recently identified uncoupling protein 2 (Skulachev 1998). Significant levels of UCP-2 gene expression were measured in this study, however previous work has demonstrated very low hepatocyte UCP-2 gene expression, with the majority of the expression in the liver localised to the Kupffer cells (Larrouy 1997). In the current
study UCP-2 expression was suppressed with the development of hyperglycemia in the obese *P. obesus*, suggesting modulated Kupffer cell uncoupled respiration, the significance of which is not known.

Lipoprotein lipase, necessary for the hydrolysis and uptake of plasma triglycerides (Nestel 1962), was shown to be suppressed with the onset of diabetes in obese *P. obesus*. This finding supports previous studies in *P. obesus* in which liver LPL enzyme activity was negatively correlated with both plasma insulin and glucose concentrations (Chajek-Shaul 1988). In contrast other rodent models of obesity and diabetes, including Wistar fatty and obese-diabetic ventromedial hypothalamic lesioned rats, have shown either no alterations or enhanced hepatic LPL activity (Inoue 1982). A physiological consequence of the down regulation of LPL with the onset of diabetes in *P. obesus* is reduced VLDL clearance (Kalderon 1986). Assuming this was the case it would add further weight to the finding of Kalderon et al who demonstrated reduced VLDL clearance in diabetic *P. obesus* accompanied by reduced adipose tissue LPL activity (Kalderon 1986).

Liver fatty acid binding protein is a cytosolic protein belonging to a family which has strong binding affinity for long chain fatty acids (Vcorkamp 1995). The regulation of L-FABP is poorly understood, although gene expression has been shown *in-vitro* to be up-regulated in the presence of elevated fatty acids (Meunier-Durmont 1996). In the current study, using a bio-assay for L-FABP activity, a non-significant tendency for increased activity in the obese diabetic animals was observed. Importantly these results also demonstrate that L-FABP gene expression was not increased in the presence of elevated hepatocyte lipid accumulation, requiring the onset of hyperglycemia prior to increased L-FABP expression.

Plasma apolipoprotein B-100 (Apo-B) concentrations have been utilised to provide an index of VLDL synthesis, as this protein is expressed uniquely by the hepatocyte, with only one Apo-B molecule incorporated per VLDL particle (Chan 1997). Apo-B gene expression tended to be increased with the onset of hyperglycemia implying that VLDL synthesis may be increased. This is consistent with a number of previous studies in Wistar fatty rats and *db/db* mice and that demonstrated increased VLDL production with the onset of diabetes (Kazumi 1996; Li 1997).
The consistent finding of the current study was altered transcription factor and lipid-specific gene expression in those obese *P. obesus* that developed significant hyperglycemia. These animals had comparable levels of hepatocyte lipid accumulation to the obese normoglycemic group. These data argue against intracellular lipid accumulation alone being the major determinant of lipid-specific gene expression.

Previous studies in pancreatic β-cells of diabetic rodents have demonstrated that hyperglycemia accompanied by intracellular lipid accumulation causes enhanced expression of the lipogenic enzymes ACC, fatty acid synthase and malic enzyme (Roche 1998). It is unresolved in the present study whether a similar situation occurs in the liver, that is alterations in gene expression are due directly to elevated glucose or secondary to a combination of elevated glucose and fat accumulation. Glucose has been shown to elevate the expression of several genes in cultured hepatocytes and adipocytes, including fatty acid synthase and ACC (Foufelle 1992; Mourrières 1997). An alternative scenario, is that hyperglycemia may alter arachidonic acid and prostanoid production (Tesfamariam 1990; Xia 1995), which may in turn activate PPARα expression, because it is well established that PPARα is activated by a range of fatty acids and members of the prostaglandin J family (Forman 1995; Clarke 1996).

The data from this study suggest that intrahepatic lipid accumulation is not associated with the changes in gene expression observed in this study and it appears that the onset of diabetes is more closely linked to the gene changes. This has major implications for the underlying hypothesis that was being tested in this thesis, that is the accumulation of fat within the liver is a primary defect in the development of diabetes and it in turn results in alterations in hepatic lipid gene expression. It is apparent that fat accumulation is secondary in the development of diabetes and the lipid responsive transcription factors and key fat metabolising enzymes studied are more closely linked with the onset of diabetes (hyperglycemia) and not liver fat content. Thus, in future studies an attempt to modulate the diabetic status of *P. obesus* shall be made in order to determine if the alterations in gene expression associated with diabetes are removed or promoted.

In conclusion, the development of obesity and hyperglycemia in *P. obesus* is associated with altered expression of key genes that code for regulators of hepatic lipid uptake, storage, oxidation and export. The alterations in gene expression are suggestive of a greater partitioning of fatty acids to storage, rather than oxidation or export in these obese diabetic *P. obesus*. Yet, hyperinsulinemic *P. obesus* that maintained
normoglycemia, displayed comparable body fatness and liver triglyceride accumulation, although rates of gene expression were not markedly altered from that of the lean normoglycemic *P. obesus*. The differences in gene expression between groups, despite similar tissue lipid accumulation argues against fatty acids acting as the principle regulators of gene expression. Although the mechanisms responsible for the altered gene expression are yet to be elucidated, the data suggests that hyperglycemia could be important permissive factor, which may act at the level of gene expression. Future investigations need to be conducted in diabetic animals where blood glucose levels are manipulated. This would allow a closer investigation of the link between hyperglycemia and alterations in hepatic lipid gene expression.
Chapter 5

EFFECT OF ACUTE AND CHRONIC ENERGY RESTRICTION ON HEPATIC LIPID METABOLISM IN *PSAMMOMYS OBESUS*

5.1 Summary

We have previously shown that hyperglycemia may be important in regulating lipid specific genes in the liver. In this study we examined what effect correcting hyperglycemia acutely (24 hr fast) and chronically (2 weeks energy restriction) had on alterations in gene expression of key fat metabolising enzymes and transport proteins in diabetic *P. obesus*. Animals were classified into three groups, Group A lean normoinsulinemic-normoglycemic, Group B obese hyperinsulinemic-normoglycemic, Group C obese hyperglycemic-hyperinsulinemic. Acutely normalising glucose and insulin levels following a 24 hr fast in group C corrected the elevated gene expression of the transcription factors PPARα and SREBP-1C, but did not change the previously altered levels of CPT, GPAT, LPL or L-FABP mRNAs. Chronic energy restriction in diabetic animals reduced body fat and L-FABP mRNA, as well as reducing PPARα and SREBP-1C mRNA. However, previously altered levels of CPT, GPAT and LPL mRNAs did not change following chronic energy restriction. From these investigations it appears that correcting the hyperglycemia present in the obese diabetic *P. obesus* can remove some but not all of the pre-existing alterations in gene expression, suggesting potentially different and complex regulation of gene expression.
5.2 INTRODUCTION

Diabetes is a disease of both carbohydrate and lipid metabolism (Groop 1989). It is well recognised that elevated levels of circulating glucose has a toxic effect and can lead to changes at the gene transcription level (Yki-Jarvinen 1990; Kahn 1997). This was evident in the data from the previous chapters where chronic elevations of glucose were found to be associated with alterations in the gene expression of fat metabolising enzymes, lipid transport proteins and transcription factors, independent of the level of intracellular fat. This finding was in contrast to data of other investigators in which it was demonstrated that accumulation of cytosolic fat which was primary determinant of alterations in gene expression in the liver, skeletal muscle and pancreas (Kraegen 1991; Unger 1995). Given the central role of the liver in whole body glucose and lipid metabolism (Consoli 1992), the manner via which hyperglycemia impacts on the gene expression of fat metabolising enzymes, lipid transport proteins and transcription factors needs further investigation.

*Psammonmys obesus* (Israeli sand rat), is a unique animal model of obesity and type II diabetes, which exhibits a complete spectrum of body weight, blood glucose and plasma insulin concentrations when fed an *ad libitum* diet of normal laboratory chow (Barnett 1994). This heterogeneous response makes *P. obesus* more analogous to the pattern of human obesity and type II diabetes than other homogenous single gene models of obesity and diabetes. Previous studies by our group have shown that acute energy restriction, in the form of a 24 hr fast, was able to normalise glucose and insulin levels in obese diabetic *P. obesus* but did not alter lipid stores (Walder 1998). Whereas chronic energy restriction in *P. obesus* that had already developed obesity and diabetes caused a reduction in blood glucose, plasma insulin, body weight and lipid store levels (Barnett 1994).

In this study we used acute and chronic energy restriction to investigate the effect of normalising glucose and insulin levels and determined what impact acute reductions in glucose and chronic reductions in glucose and lipid stores would have on the expression of key lipid metabolising enzymes, in the livers of a cross-sectional sample of *P. obesus*. 

5.3 METHODS

5.3.1 Animals
A colony of mixed sex *P. obesus* were maintained on a 12 hr light 12 hr dark cycle at 21±1°C and fed *ad libitum* standard rat chow (Barastoc, Pakenham, Australia). Consistent with previous reports from our laboratory (Barnett 1994), the animals displayed wide spontaneous variation in weight gain, due to increased adiposity accompanied by varying degrees of hyperinsulinemia and hyperglycemia (Collier 1997). At 12 weeks of age animals were selected and allocated into three groups based on body weight, blood glucose and plasma insulin as described previously (Barnett 1994). Group A were lean, normoglycemic, normoinsulinemic (n=38); Group B were obese normoglycemic, hyperinsulinemic (n=32); Group C were obese, hyperglycemic, hyperinsulinemic (n=26). Within each category (A to C) the animals were further subdivided into three randomly allocated groups. One group was maintained on *ad libitum* food, the second group was fasted for 24 hr and the third group was restricted to 11 g of food per day for two weeks. The 11 g of food per day represented a 16% reduction in food intake for group A, compared to control A animals; a 29% reduction in food intake for the group B compared to control B animals and a 46% reduction in food intake for the group C animals compared to control A animals. This level of energy restriction was selected based on previous investigations in our laboratory, where this level of energy restriction corrected hyperglycemia and hyperinsulinemia (Barnett 1994). At the end of the 24 hr fast or energy restriction period all animals were sacrificed by anaesthetic overdose between 9am and 12noon, tissues removed and freeze-clamped and maintained in liquid nitrogen until analysis. Body fatness was estimated by combining the weight of the same selected fat stores (peri-renal, mesenteric, hind limb intra-muscular and suprascapular) in each animal and expressing this as a percentage of body weight.

5.3.2 Biochemical Analyses
Blood was collected from the tail vein in heparinized microtubes and blood glucose immediately determined using an automated enzymatic analyser (2300 Stat Plus, Yellow Springs Instruments, Ohio). Plasma insulin levels were determined by radioimmunoassay using a human primary antibody (Phadeseq, Kabi Pharmacia Diagnostics, Sweden). Plasma leptin levels were measured by radioimmunoassay using a multi-species primary antibody (Linco, St Charles, MO). Plasma triglycerides were
measured colorimetrically (Bohringer-Mannheim, Mannheim, Germany) as were non-
esterified free fatty acids (NEFA) (WAKO Pure Chemical Co., Japan).

5.3.3 Hepatic Carnitine Palmitoyltransferase Activity And
Triglyceride Accumulation

A measure of β-oxidation, CPT activity was determined based on the method of Power
and colleagues (Power 1994). Mitochondria were isolated from frozen livers and CPT
activity was determined by incubating 50 µl of mitochondrial suspension in 1 ml of
assay medium (150 mM KCl, 5 mM Tris-HCl, 1 mM EGTA, 1 mM dithiothreitol, 5
mM MgCl₂, 5 mM ATP, 100 µM palmitoyl-CoA, 1 µg/ml rotenone, 1 µg/ml antimycin
A, and 0.13% BSA (pH 7.4) equilibrated at 37°C for 5 min. The reaction was initiated
by addition of 0.005 pmol [³H]carnitine and terminated by addition of 300 µl of 0.6 M
HCl at either 1 or 3 min. [³H]acylcarnitine formed was extracted by addition of 2 ml
butanol, followed by a butanol saturated water wash. [³H]acylcarnitine was counted by
liquid scintillation (RackBeta II, LKB Wallac, Finland). Results were expressed as
[³H]acylcarnitine formed per mg of mitochondrial protein, per minute. Protein
concentration was determined using a total protein kit (Sigma, St. Louis, MO). Total
hepatic lipid accumulation was measured by chloroform methanol extraction (Folch
1957).

5.3.4 Liver Fatty Acid Binding Protein Bio-activity
Liver cytosolic fatty acid binding protein bio-activity measurement was based on the
radiochemical method of Glatz and Veerkamp (1983). Tissue homogenates were
prepared in Tris-HCl buffer centrifuged for 10 min at 600 g and the supernatant
centrifuged again for 90 min at 105000 g at 4°C. Albumin was removed from the
cytosolic homogenate by affinity chromatography and then delipidated using a
hydrophobic dextran column. The resultant L-FABP extract was incubated with [¹⁴C]
palmitate at 37°C for 10 min and unbound [¹⁴C] was removed using hydrophobic
dextran and centrifugation (20 000 g for 2 min at 4°C). The resulting supernatant
containing the bound [¹⁴C] palmitate was assayed for radioactivity by liquid scintillation
counting.
5.3.5 Extraction Of Total RNA And Reverse Transcription
(RT)-PCR Quantitation
Total RNA was extracted from 30 mg frozen liver portions by the RNAzol B method (Bresatec, Adelaide, Australia). First strand cDNA was generated from 1 μg of RNA in a 30 μl volume using the oligo(dT) primer in the first strand cDNA synthesis kit (Promega, Madison, WI). One microliter of the reverse transcription reaction mix was amplified with primers specific for LPL, L-FABP, Apo-B, CPT, ACC, UCP-2, GPAT, PPARα and SREBP-1C in a total volume of 20 μl. All sequences were from the Genbank, primer sequences are shown in Table 2.1 in the general material and methods section. The samples were amplified in the linear phase for 22-40 cycles, optimised for each gene, using the following parameters: 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. β-Actin primers were used as a control for all quantitation and were amplified in the linear phase for 22 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min. Levels of mRNA were expressed as the ratio of signal intensity for the sample genes to that of β-Actin. The PCR products were electrophoresed on a 1.4% agarose gel, visualised using ethidium bromide and quantitated by computer integrated densitometry (Kodak ds 1D system, Kodak, New Haven, CT).

5.3.6 Statistical Analyses
All results are expressed as mean ± SEM. Changes in the level of gene expression are represented as a percentage of the group A control (100%). Fasting and chronic energy restricted groups are represented in separate figures relative to their respective control group. Statistical significance was evaluated using one-way ANOVA with the aid of a multiple comparison test (Tukey-HSD procedure) to establish where the differences between groups were significant. In all cases P values of 0.05 or less were considered to indicate statistical significance.
5.4 RESULTS

5.4.1 Metabolic Comparisons Between Lean, Obese And Obese Diabetic Animals

Prior to the 24 hr fast or chronic energy restriction *P. obesus* classified as group B and C had elevated body mass and liver lipid accumulation (P<0.05, Table 5.1), plasma insulin and leptin levels (P<0.05, Table 5.2) were elevated compared to group A. In addition group C animals were also hyperglycemic (P<0.05, Table 5.2), hypertriglyceridemic (P<0.05, Table 5.3) and had elevated L-FABP bio-activity (P<0.05, Table 5.4). There were no differences in plasma NEFA (Table 5.3) or CPT enzyme activity (Table 5.4) between any of the groups prior to fasting or chronic energy restriction.

Table 5.1: Effect of 24 hr fast and chronic energy restriction on body mass and liver fat accumulation

<table>
<thead>
<tr>
<th></th>
<th>Body Mass (g)</th>
<th>Liver Lipid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Δ Body mass</td>
</tr>
<tr>
<td>24 hr</td>
<td>24 hr Energy</td>
<td>Control</td>
</tr>
<tr>
<td>A</td>
<td>171±5.5</td>
<td>-5.4±1.7†</td>
</tr>
<tr>
<td>B</td>
<td>221±5.4*</td>
<td>-1.8±1.9</td>
</tr>
<tr>
<td>C</td>
<td>233±6.8*</td>
<td>1.1±1.2</td>
</tr>
</tbody>
</table>

All values are expressed as mean±SEM. * Indicates a significant difference from group A. † Indicates a significant effect of a 24 hr fast. § Indicates a significant effect of chronic energy restriction.
Table 5.2: Effect of 24 hr fast and chronic energy restriction on glucose, insulin and leptin.

<table>
<thead>
<tr>
<th></th>
<th>Blood Glucose (mmol/L)</th>
<th>Plasma Insulin (mU/L)</th>
<th>Plasma Leptin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Δ Glucose</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>24 hr</td>
<td>Energy</td>
<td>24 hr</td>
</tr>
<tr>
<td>Fast</td>
<td></td>
<td></td>
<td>Fast</td>
</tr>
<tr>
<td>Restriction</td>
<td></td>
<td></td>
<td>Restriction</td>
</tr>
<tr>
<td>A</td>
<td>4.5±0.2</td>
<td>-0.8±0.3</td>
<td>61±9</td>
</tr>
<tr>
<td>B</td>
<td>5.0±0.5</td>
<td>-1.5±0.5</td>
<td>364±61*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.6±0.2</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>12.6±0.9**</td>
<td>-7.7±0.7†</td>
<td>433±58*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-6.7±0.5§</td>
<td></td>
</tr>
</tbody>
</table>

All values are expressed as mean±SEM. * Indicates a significant difference from group A. ** Indicates a significant difference from group A&B. † Indicates a significant effect of a 24 hr fast. § indicates a significant effect of energy restriction.
Table 5.3: Effect of 24 hr fast and chronic energy restriction on triglycerides and NEFA.

<table>
<thead>
<tr>
<th></th>
<th>Plasma Triglycerides (mmol/L)</th>
<th>Plasma NEFA (mEq/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Δ Triglycerides</td>
</tr>
<tr>
<td></td>
<td>24 hr</td>
<td>Fast</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.9±0.1</td>
<td>-0.1±0.1</td>
</tr>
<tr>
<td>B</td>
<td>1.3±0.3</td>
<td>0.3±0.2</td>
</tr>
<tr>
<td>C</td>
<td>2.0±0.5*</td>
<td>1.6±0.7</td>
</tr>
</tbody>
</table>

All values are expressed as mean±SEM. * Indicates a significant difference from group A. † Indicates a significant effect of a 24 hr fast. § Indicates a significant effect of chronic energy restriction.

### 5.4.2 mRNA Comparisons Between Lean, Obese And Obese Diabetic Animals

Accompanying the metabolic changes prior to acute or chronic energy restriction in both groups B and C was an increased level of GPAT mRNA (P<0.05, Figures 5.1 and 5.2) while group C alone displayed increased mRNA levels of L-FABP, CPT, ACC, PPARα and SREBP-1C, whereas LPL was decreased in group C (P<0.05, Figures 5.1 to 5.6). Apo-B mRNA did not differ between any of the groups prior to fasting or chronic energy restriction (Figures 5.3 and 5.4).

### 5.4.3 Impact Of 24 hr Fasting And Chronic Energy Restriction On Anthropometric And Blood Variables

Twenty four hour fasting significantly reduced body mass in group A animals (P<0.05) which was not apparent in group B or C. This period of acute fasting normalised plasma insulin levels in groups B and C (P<0.05) as well as restoring normoglycemia in group C (P<0.05). Circulating levels of NEFA were increased in all groups following fasting (P<0.05). Plasma leptin in group B alone was elevated after a 24 hr fast (P<0.05). A 24 hr fast did not alter circulating triglyceride levels or liver lipid accumulation in any of the groups.
Unlike fasting energy restriction caused a reduction in body mass and normalised plasma insulin in groups B and C (P<0.05). Blood glucose levels were ameliorated in group C (P<0.05). Circulating NEFAs were elevated following energy restriction in the B and C animals (P<0.05) while leptin levels were normalised in both B and C (P<0.05). Similar to the acutely fasted animals chronic energy restriction did not alter circulating triglyceride levels or liver lipid accumulation in any of the groups.

5.4.4 Effect Of 24 hr Fasting And Chronic Energy Restriction On CPT Activity And Gene Expression

Twenty four hour fasting increased CPT activity approximately 2 fold in all groups (P<0.05), with the greatest increase in group A animals, however CPT activity did not differ between any of the groups. In contrast chronic energy restriction resulted in a halving of CPT activity in groups A and B, yet caused an increase in group C (P<0.05). Acute fasting did not alter CPT mRNA levels in any P. obesus. Chronic energy restriction increased CPT expression only in group A P. obesus (P<0.05).

Table 5.4: Effect of 24 hr fast or chronic energy restriction on CPT and L-FABP activity.

<table>
<thead>
<tr>
<th></th>
<th>CPT Activity</th>
<th>L-FABP Bio-Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol/mg mito. protein/min)</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 hr Fast</td>
</tr>
<tr>
<td>A</td>
<td>1.4±0.1</td>
<td>3.8±0.2†</td>
</tr>
<tr>
<td>B</td>
<td>1.8±0.3</td>
<td>3.4±0.3†</td>
</tr>
<tr>
<td>C</td>
<td>1.6±0.1</td>
<td>2.7±0.4†</td>
</tr>
</tbody>
</table>

All values are expressed as mean±SEM. Values for L-FABP activity are represented as the activity of the control group and the percentage change from the control group for the respective experimental group. ** Indicates a significant difference from group A and group B. † Indicates a significant effect of a 24 hr fast. § Indicates a significant effect of chronic energy restriction.
Figure 5.1: Change in mRNA level relative to control for CPT, ACC and GPAT in 24 hr fasted *P. obesus*. * Indicates a significant difference from group A. † Indicates a significant effect of 24 hr fasting.

Figure 5.2: Change in mRNA level relative to control for CPT, ACC and GPAT in chronically energy restricted *P. obesus*. * Indicates a significant difference from group A. § Indicates a significant effect of chronic energy restriction.
5.4.5 Effect Of 24 hr Fasting And Chronic Energy Restriction On L-FABP Activity And Gene Expression

Prior to energy restriction L-FABP bio-activity and gene expression was elevated in group C. Acute fasting normalised L-FABP bio-activity in group C (P<0.05). Chronic energy restriction had no significant effect on activity. The level of L-FABP expression was elevated in group B following a 24 hr fast such that both groups B and C had significantly elevated levels of mRNA compared to group A (P<0.05) post fast. Energy restriction resulted in a lowered L-FABP expression in group C such that all groups were the same (P<0.05).

![Graph showing % Change in mRNA level relative to control for LPL, L-FABP and Apo-B in 24hr fasted P. obesus. * Indicates a significant difference from group A. † Indicates a significant effect of 24 hr fasting.]

5.4.6 Effect Of 24 hr Fasting And Chronic Energy Restriction On The Gene Expression Of LPL, ACC, GPAT And Apo-B

To determine the impact of fasting and chronic energy restriction on a number of lipid metabolising pathways the expression of mRNA sequences encoding enzymes involved in hepatic long-chain fatty acid metabolism were measured. The gene expression of the lipolytic protein, LPL was increased in all groups following a 24 hr fast. ACC gene expression was increased in group B and decreased in group C (P<0.05) following a 24 hr fast which removed any differences that existed between these groups prior to fasting. A 24 hr fast caused a reduction in the expression of Apo-B in group A, but had no effect on GPAT mRNA levels in any of the groups.
Figure 5.4: Change in mRNA level relative to control for LPL, L-FABP and Apo-B in chronically energy restricted *P. obesus*. * Indicates a significant difference from group A. § Indicates a significant effect of chronic energy restriction.

Energy restriction did not alter LPL or GPAT mRNA levels in all groups. However, as Apo-B expression was reduced in group A. A uniform increase in ACC expression in all groups was observed following chronic energy restriction, however the significant elevation in group C relative to group A still persisted.

5.4.7 Effect Of 24 hr Fasting And Chronic Energy Restriction On The Gene Expression Of Lipid Responsive Transcription Factors PPARα And SREBP-1C

Acute fasting resulted in an increase of PPARα mRNA in group A while at the same time decreasing levels in group C, such that the previous elevation in group C was removed (P<0.05). A similar effect was observed in group C animals after energy restriction, a reduction in PPARα expression, which again normalised mRNA levels. The level of SREBP-1C expression was reduced following fasting in group A and C (P<0.05), whereas energy restriction also reduced SREBP-1C mRNA levels in group C, in both cases this corrected the previously elevated levels in group C (P<0.05).
Figure 5.5: Change in mRNA level relative to control for PPARα and SREBP-1C in 24 hr fasted *P. obesus*. * Indicates a significant difference from group A. † Indicates a significant effect of 24 hr fasting.

Figure 5.6: Change in mRNA level relative to control for PPARα and SREBP-1C in chronically energy restricted *P. obesus*. * Indicates a significant difference from group A. § Indicates a significant effect of chronic energy restriction.
5.5 DISCUSSION

In this study we have demonstrated that acute energy restriction (24hr fast) reduced insulin and glucose levels in obese and diabetic animals without altering fat stores. Concurrent with these changes was a normalisation of liver ACC, PPARα and SREBP-1C gene expression in the diabetic animals. Chronic energy restriction also lowered insulin and glucose levels but in addition reduced body weight and total body fat in the obese and diabetic animals. These changes were accompanied by normalisation of L-FABP, PPARα and SREBP-1C gene expression in liver.

This study confirms data from the previous chapter that the development of obesity in *P. obesus* is associated with altered GPAT gene expression. Whereas, the onset of hyperglycemia in *P. obesus* is associated with altered expression of genes that code for CPT, ACC, GPAT, LPL and L-FABP. In addition the gene expression of the lipid responsive transcription factors PPARα and SREBP-1C was increased with hyperglycemia. Hyperinsulinemic *P. obesus* that maintained normoglycemia, displayed comparable body fatness and liver triglyceride yet rates of gene expression, were markedly different from those of the diabetic *P. obesus*.

The slight reduction in body weight and constant glucose, insulin, leptin and plasma triglycerides following a 24 hr fast in the lean group A animals was consistent with previous studies in *P. obesus* and other animal models that were subject to similar periods of acute food deprivation (Barnett 1994; Dubuc 1988; Walder 1998). The increased NEFA levels and constant L-FABP activity is consistent with earlier studies in the liver of Albino rats fasted for 24-48 hr (Bass 1985; Sterchele 1994). Elevated CPT activity with unchanged mRNA in group A following the 24 hr fast is again consistent with earlier studies in Wistar rats (Brady 1987). Similar to the metabolic data the levels of hepatic lipid metabolising enzyme mRNA measured show the same response to acute and chronic energy restriction as a number of previous studies in Albino or Wistar rats. These previous studies have found constant levels of ACC activity, GPAT activity and L-FABP gene expression in fasted albino rats (Bates 1979; Munday 1991; Sterchele 1994), similarly increased LPL mRNA (Peinado-Onsurbe 1992) and decreased Apo-B mRNA (Baum 1990) have been found. Again consistent with previous fasting studies the increase in PPARα (Sterchele 1996) and decreased
SREBP-1C (Horton 1998) mRNA shows a normal response to fasting in group A *P. obesus*.

The effect of chronic energy restriction in group A *P. obesus* again resulted in no significant alterations in metabolic parameters and surprisingly limited effects on mRNA species investigated. This may be largely due to 16% reduction in food intake in group A animals was not severe enough to have pronounced physiological effects, as previously shown in *P. obesus* (Barnett 1994).

In the obese group B animals 24 hr fasting resulted in a normalisation of plasma insulin, increased leptin, increased NEFA and CPT activity all of which are consistent with previous studies in *P. obesus* or other rodent models (Bass 1985; Brady 1987; Walder 1998). This acute loss of hyperinsulinemia was accompanied by a 60% increase in the level of ACC mRNA, which suggests an increase in hepatic *de-novo* lipogenesis (McGarry 1980). A similar elevation in hepatic lipogenesis has previously been found in rats that have undergone bouts of fasting followed by re-feeding (Kochan 1997) and is though to provide a survival advantage, enabling more efficient storage of fuel for times of extended famine. Given that this increase in ACC mRNA was also accompanied by a 200% increase in L-FABP mRNA, a cytosolic transport protein that binds long chain fatty acid with high affinity (Veerkamp 1991), it can be speculated that there is increased hepatic lipogenesis and cytoplasmic transport of fatty acids to storage.

Chronic energy restriction in the group B animals had a similar metabolic effects to fasting for 24 hr with a normalisation of plasma insulin and increased NEFA levels. However, there were two differences; firstly leptin levels were reduced and secondly there was a reduction in body fatness, both of which are consistent with previous studies albino rats (Jones 1993; Cha 1998). Unlike the fasted group B, animals chronic energy restriction had no significant effect on the expression of any the fat metabolising enzymes or proteins measured as well as having no impact on the lipid responsive transcription factors measured. This lack of response may be explained in relation to glucose concentrations. We have previously suggested that changes in glucose concentration are responsible for alterations in mRNA level and glucose concentration in group B *P. obesus* did not change following chronic energy restriction. It follows if glucose is modulating gene expression and glucose did not change, then we would not
expect to see any alterations in the level of mRNA species that may be under the influence of glucose.

In group C animals acute fasting normalised glucose, insulin, increased NEFA and CPT activity, but did not alter the amount of body fat nor liver triglyceride stores, all of which are consistent with previous studies in *P. obesus* or other rodents (Bass 1985; Brady 1987; Walder 1998). Unlike the response observed in groups A and B animals acute energy restriction profoundly decreased L-FABP activity to levels comparable with control animals. This effect may be unique to diabetic animals as previous fasting studies in non-diabetic rodents show that fasting has no effect on L-FABP activity (Bass 1985).

It was suggested in the previous chapter that the hyperglycemia present in the group C animals is important in controlling the altered gene expression present in these obese diabetic animals. These current results add weight to this proposal, given that the 24 hr fast was able to reduce glucose levels but did not change the level of body fat or hepatic lipid accumulation, yet we demonstrated pronounced reductions in ACC, PPARα and SREBP-1C expression. This may suggest that the changes in gene expression are occurring secondary to acute changes in glucose levels.

Chronic energy restriction in the group C animals had a comparable effect on blood glucose and anthropometric parameters 24 hr fasting. However, body fat stores were reduced, yet surprisingly liver lipid accumulation remained unaltered. This constant level of hepatic lipid even after a period of 54% energy restriction in the group C animals may indicate a persistent dysregulation in hepatic lipid storage. A speculative mechanism which hepatic lipid levels are maintained in group C is via a maintenance of de-novo lipogenesis, as suggested by increased levels of ACC mRNA after chronic energy restriction, thus the increased ACC gene expression serves to maintain hepatic lipid levels. This fat sparing mechanism within the liver may originally have provided a survival advantage to *P. obesus* in its energy sparse native environment.

Similar to the effect of acute fasting, chronic energy restriction reduced the gene expression levels of PPARα, SREBP-1C and L-FABP. These consistent changes in lipid gene expression that occur with alterations in glucose concentration add further weight to the proposition that glucose, at least in part, is modulating the level of
expression of these lipid genes. Previous evidence for this link between PPARα and
SREBP-1C expression and glucose levels has been demonstrated. Horton et al. (1998)
re-fed fasted rats with a high carbohydrate low fat meal which was shown to rapidly
increase SREBP-1C expression (Horton 1998). Thus, the expression of SREBP-1C
appears to be associated with changes in blood glucose level both acutely and
chronically.

There are exceptions to the described changes of gene expression, mRNA levels of
CPT, GPAT and LPL did not change following chronic energy restriction, suggesting
their altered expression in the diabetic state is not under the exclusive control of blood
glucose. It might well be that the level of mRNA is altered in the presence of other
hormonal, developmental, environmental or genetic factors not measured in this study
can impact on the expression of these genes, although it is still apparent from this and
the previous study that hyperglycemia can at least in part alter the level of CPT, GPAT
and LPL gene expression.

In conclusion lean healthy P. obesus respond to acute and chronic energy restriction in
a similar fashion to other lean non-diabetic rodent species, this occurred at a metabolic
and gene level. In the obese hyperinsulinemic P. obesus acute and chronic energy
restriction normalises plasma insulin levels but did not have major effects on the level
of gene expression of lipid metabolising enzymes, transport proteins or lipid responsive
transcription factors. Acute and chronic energy restriction in obese diabetic P. obesus
resulted in a number of unique responses. Acutely normalised glucose and insulin
levels without altering fat stores and was able to correct the pre-existing alterations in
the transcription factors PPARα and SREBP-1C. Whereas chronic energy restriction
had the same effects in obese diabetic P. obesus but with the additional reduction in
body fat and L-FABP mRNA. It appears that correcting the hyperglycemia present in
the obese diabetic P. obesus can normalise some but not all of the alterations in gene
expression, suggesting potentially different and complex regulation of gene expression.
Further investigations promoting the development of hyperglycaemia in previously
non-diabetic P. obesus are warranted to further elucidate the dysregulation of gene
expression in these animals. Such interventions may include stimulating de-novo
lipogenesis via over feeding with sucrose or fructose diets, alternatively animals may be
fat fed.
Chapter 6

SUCROSE FEEDING IN PSAMMOMYS OBESUS PROMOTES THE DEVELOPMENT OF TYPE II DIABETES WITHOUT ALTERING HEPATIC LIPID GENE EXPRESSION.

6.1 SUMMARY

We have demonstrated in the two previous chapters that obese diabetic *P. obesus* have altered mRNA levels of hepatic lipid metabolising enzymes and lipid responsive transcription factors. The aims of this study were; to determine whether sucrose feeding in *P. obesus* promotes the development of obesity and diabetes and with the development of hyperglycemia were there alterations in mRNA level of hepatic lipid metabolising enzymes and lipid responsive transcription factors. These investigations were conducted in the polygenic rodent model *P. obesus*. Animals were classified into three groups, Group A (lean normoinsulinemic-normoglycemic), Group B (obese hyperinsulinemic-normoglycemic), Group C (obese hyperglycemic-hyperinsulinemic). Animals from each of these were then randomly assigned to receive *ad-libitum* food and water or *ad-libitum* food supplemented with a 5% sucrose solution substituted for drinking water. Sucrose increased body weight gain and body fatness in lean *P. obesus*. However, other metabolic parameters or the level of expression of hepatic lipid enzymes and transcription factors did not differ between groups. Obese group B *P. obesus* developed diabetes following sucrose feeding, yet mRNA levels of key lipid metabolising enzymes differed substantially from those of *P. obesus* with pre-existing diabetes. This suggests that alterations in lipid gene expression observed in the control obese diabetic animals may be due to a pre-existing genetic susceptibility or secondary to other metabolic aberrations. In the obese diabetic group C *P. obesus* sucrose feeding exacerbated the pre-existing diabetes in this group of animals and modulated the transcription of several genes analysed, including PPARα, CPT, LPL and L-FABP.
6.2 INTRODUCTION

*P. obesus* is a unique polygenic animal model of obesity and diabetes (Shafrir 1993). When fed a diet of standard laboratory chow, a proportion of animals remains lean while others develop varying degrees of obesity and type II diabetes comparable to the metabolic profile observed in susceptible human populations (Barnett 1994). In addition, obese diabetic *P. obesus* have been shown to display abnormalities in hepatic lipid metabolism including increased hepatic lipogenesis (Kalderon 1983), elevated liver fat deposition, together with elevated circulating NEFA and triglycerides levels as well as decreased insulin sensitivity (Barnett 1994; Lewandowski 1997). In the two preceding chapters it has been demonstrated that obese and obese diabetic *P. obesus* also display alterations in hepatic lipid gene expression. Obese *P. obesus* had increased mRNA levels of the re-esterification enzyme GPAT. Obese diabetic *P. obesus* in addition to the increased GPAT mRNA level, demonstrated increased expression of liver CPT, ACC and L-FABP together with decreased LPL gene expression. Furthermore, obese diabetic *P. obesus* had increased mRNA levels of the lipid responsive transcription factors PPARα and SREBP-1C. This suggests that these changes in gene expression occur secondary to metabolic abnormalities developing. In the previous chapter (Chapter 5) it was demonstrated that some of these alterations in hepatic lipid gene expression could be corrected by normalising glucose levels with 24 hr fasting or chronic energy restriction.

In has been previously reported that sucrose feeding in rats promoted weight gain (Pagliassotti 1996), impaired glucose tolerance (Storlien 1988), promoted hepatic insulin resistance (Storlien 1988), elevated serum lipid levels (Kazumi 1989), increased hepatic lipid content (Basilico 1983) and increased hepatic lipogenesis (Cohen 1972). In previous studies in *P. obesus* elevated lipogenesis was a characteristic of the diabetic state alone, therefore sucrose feeding, in addition to promoting enhanced energy intake, may promote the development of diabetes in obese insulin resistant *P. obesus*. Thus the aims of this study were firstly to investigate the impact of sucrose feeding on body weight, blood glucose, plasma insulin and liver lipid accumulation in lean, obese and diabetic *P. obesus*. Secondly to examine whether a dietary modification that promotes the development of hyperglycemia and hyperinsulinemia was able to modulate hepatic lipid specific gene expression in previously normal or obese animals.
6.3 METHODS

6.3.1 Animals
A colony of mixed sex \textit{P. obesus} were maintained on a 12 hr light 12 hr dark cycle at 21±1°C and fed \textit{ad libitum} standard rat chow (Barastoc, Pakenham, Australia). Consistent with previous reports from our laboratory (Barnett 1994), the animals displayed wide spontaneous variation in weight gain, due to increased adiposity which was accompanied by varying degrees of hyperinsulinemia and hyperglycemia (Collier 1997). At 16 weeks of age \textit{P. obesus} were selected and allocated into three groups based on body weight, blood glucose and plasma insulin as described previously (Barnett 1994). Group A were lean, normoglycemic, normoinsulinemic (n=17); Group B were obese normoglycemic, hyperinsulinemic (n=17); Group C were obese, hyperglycemic, hyperinsulinemic (n=16). Within each category (A to C) the animals randomly subdivided to receive \textit{ad libitum} food and water or \textit{ad libitum} food plus a 5% sucrose solution instead of water for three weeks. This level of sucrose supplementation resulted in a 15% increase in total energy intake for all groups receiving sucrose.

At the end of the three week feeding period all animals were sacrificed by anaesthetic overdose between 9am and 12noon, tissues removed and freeze clamped and maintained in liquid nitrogen until analysis. Body fatness was estimated by combining the weight of the same selected fat stores (perirenal, mesenteric, hind limb intra-muscular and suprascapular) in each animal and expressing this as a percentage of body weight.

6.3.2 Biochemical Analyses
Blood was collected from the tail vein in heparinized microtubes and blood glucose immediately determined using an automated enzymatic analyser (2300 Stat Plus, Yellow Springs Instruments, Ohio). Plasma insulin levels were determined by radioimmunoassay using a human primary antibody (Phadeseq, Kabi Pharmacia Diagnostics, Sweden). Plasma leptin levels were measured by radioimmunoassay using a multi-species primary antibody (Linco, St Charles, MO). Plasma triglycerides were measured colorimetrically (Bohringer-Mannheim, Mannheim, Germany) as were non-esterified free fatty acids (NEFA) (WAKO Pure Chemical Co., Japan).
6.3.3 Hepatic Carnitine Palmitoyltransferase Activity And Triglyceride Accumulation

A measure of β-oxidation, carnitine palmitoyltransferase activity (CPT) was determined based on the method of Power and colleagues (Power 1994). Mitochondria were isolated from frozen livers and CPT activity was determined by incubating 50 μl of mitochondrial suspension in 1 ml of assay medium (150 mM KCl, 5 mM Tris-HCl, 1 mM EGTA, 1 mM dithiothreitol, 5 mM MgCl2, 5 mM ATP, 100 μM palmitoyl-CoA, 1 μg/ml rotenone, 1 μg/ml antimycin A, and 0.13% BSA (pH 7.4) equilibrated at 37°C for 5 min. The reaction was initiated by addition of 0.005 pmol [3H]carnitine and terminated by addition of 300 μl of 0.6 M HCl at either 1 or 3 min. [3H]acylcarnitine formed was extracted by addition of 2 ml butanol, followed by a butanol saturated water wash. [3H]acylcarnitine was counted by liquid scintillation (RackBeta II, LKB Wallac, Finland). Results were expressed as [3H]acylcarnitine formed per mg of mitochondrial protein, per minute. Protein concentration was determined using a total protein kit (Sigma, St. Louis, MO). Total hepatic lipid accumulation was measured by chloroform methanol extraction (Folch 1957).

6.3.4 Liver Fatty Acid Binding Protein Bio-activity

Liver cytosolic fatty acid binding protein bio-activity measurement was based on the radiochemical method of Glatz and Veerkamp (Glatz 1983). Tissue homogenates were prepared in Tris-HCl buffer centrifuged for 10 min at 600 g and the supernatant centrifuged again for 90 min at 105000 g at 4°C. Albumin was removed from the cytosolic homogenate by affinity chromatography and then delipidated using a hydrophobic dextran column. The resultant L-FABP extract was incubated with [14C] palmitate at 37°C for 10 min and unbound [14C] was removed using hydrophobic dextran and centrifugation (20000 g for 2 min at 4°C). The resulting supernatant containing the bound [14C] palmitate was assayed for radioactivity by liquid scintillation counting.
6.3.5 Extraction Of Total RNA And Reverse Transcription (RT)-PCR Quantitation

Total RNA was extracted from 30 mg frozen liver portions by the RNAzol B method (Bresatec, Adelaide, Australia). First strand cDNA was generated from 1 μg of RNA in a 30 μl volume using the oligo(dT) primer in the first strand cDNA synthesis kit (Promega, Madison, WI). One microliter of the reverse transcription reaction mix was amplified with primers specific for LPL, L-FABP, Apo-B, CPT, ACC, UCP-2, GPAT, PPARα and SREBP 1C in a total volume of 20 μl. All sequences were from the Genbank, primer sequences are shown in Table 2.1 in the general materials and methods section. The samples were amplified in the linear phase for 22-40 cycles, optimised for each gene, using the following parameters: 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. β-Actin primers were used as a control for all quantitation and were amplified in the linear phase for 22 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min. Levels of mRNA were expressed as the ratio of signal intensity for the sample genes to that of β-Actin. The PCR products were electrophoresed on a 1.4% agarose gel, visualised using ethidium bromide and quantitated by computer integrated densitometry (Kodak ds 1D system, Kodak, New Haven, CT).

6.3.6 Statistical Analyses

All results are expressed as mean ± SEM. Statistical significance was evaluated using one-way ANOVA with the aid of a multiple comparison test (Tukey-HSD procedure) to establish where the differences between groups were significant. In all cases P values of 0.05 or less were considered to indicate statistical significance.
6.4 RESULTS

6.4.1 Metabolic And mRNA Comparisons Between Lean, Obese And Obese Diabetic Animals

Prior to dietary interventions P. obesus classified as groups B and C had elevated body mass, estimated body fat and liver lipid accumulation (P<0.05, Table 6.1). Plasma insulin was elevated in both groups B and C compared to group A (P<0.01, Table 6.2). In addition to these differences group C animals alone were also hyperglycemic (P<0.01, Table 6.2), hypertriglyceridemic (P<0.05, Table 6.3) and had elevated L-FABP bio-activity (P<0.05, Table 6.4). There were no differences in plasma leptin (Table 6.2), NEFA (Table 6.3) or hepatic CPT enzyme activity (Table 6.4) among the groups prior to sucrose feeding.

Table 6.1: The effect of sucrose feeding on body weight, body fat stores and liver lipid accumulation.

<table>
<thead>
<tr>
<th></th>
<th>Body Weight (g)</th>
<th>Estimated Body Fat (%)</th>
<th>Liver Fat Accumulation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Sucrose</td>
<td>Gain</td>
</tr>
<tr>
<td>A</td>
<td>161±12</td>
<td>196±10†</td>
<td>27±5†</td>
</tr>
<tr>
<td>B</td>
<td>202±12*</td>
<td>227±11*†</td>
<td>26±2†</td>
</tr>
<tr>
<td>C</td>
<td>232±5**</td>
<td>235±6*</td>
<td>4±6</td>
</tr>
</tbody>
</table>

All values are mean±SEM. * Indicates a significantly different from group A. ** Indicates a significantly different from group A&B. † Indicates a significant effect of sucrose feeding.

Accompanying the metabolic changes was alterations in hepatic gene expression prior to sucrose feeding. Both groups B and C had an increased level of GPAT mRNA (P<0.05, Figure 6.1) while only group C displayed increased mRNA levels of L-FABP, CPT, ACC, PPARα and SREBP-1C and decreased LPL gene expression (P<0.05, Figures 6.1 to 6.3). Apo-B mRNA did not differ among the groups prior to sucrose feeding (Figure 6.2).
Table 6.2: The effect of sucrose feeding on blood glucose, plasma insulin and plasma leptin.

<table>
<thead>
<tr>
<th>Glucose (mmol/L)</th>
<th>Insulin (µU/ml)</th>
<th>Leptin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Sucrose</td>
</tr>
<tr>
<td>A</td>
<td>4.5±0.3</td>
<td>5.0±0.3</td>
</tr>
<tr>
<td>B</td>
<td>4.2±0.4</td>
<td>10.8±2.3*†</td>
</tr>
<tr>
<td>C</td>
<td>13.9±1.4**</td>
<td>18.9±1.3***†</td>
</tr>
</tbody>
</table>

All values are mean±SEM. * Indicates a significantly different from group A. ** Indicates a significantly different from group A&B. † Indicates a significant effect of sucrose feeding.

6.4.2 Impact Of Sucrose Feeding On Anthropometric And Blood Parameters

Sucrose feeding caused a significant elevation in body weight and body fat in group A and B. P. obesus (P<0.05) which was not apparent in group C. Sucrose treatment increased hepatic lipid accumulation only in group C (P<0.01), increased blood glucose levels in groups B and C (P<0.05), such that group B became hyperglycemic and the pre-existing hyperglycemia in group C was further exacerbated (P<0.05). Plasma insulin levels were significantly increased in group B alone following sucrose feeding (P<0.04). Circulating NEFAs were increased in all groups following sucrose feeding (P<0.05), whereas plasma leptin and triglyceride levels were not altered by sucrose feeding.

Table 6.3: The effect of sucrose feeding on circulating triglycerides and non-esterified free fatty acids.

<table>
<thead>
<tr>
<th>Triglycerides (mmol/L)</th>
<th>NEFA (mEq/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>A</td>
<td>0.8±0.07</td>
</tr>
<tr>
<td>B</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>C</td>
<td>2.8±0.7*</td>
</tr>
</tbody>
</table>

All values are mean±SEM. * Indicates a significantly different from group A. † Indicates a significant effect of sucrose feeding.
6.4.3 Effect Of Sucrose Feeding On Oxidative Enzyme Activity And Gene Expression

Sucrose supplementation tended to lower CPT activity in group A and B, while in group C activity tended to be increased (P<0.01). In contrast, sucrose feeding in group C resulted in a 2.5 times reduction in CPT mRNA (P<0.01), abolishing the differences between groups. UCP-2 gene expression was significantly reduced in group B following sucrose feeding (P<0.05), however this did not result in any difference among groups.

Table 6.4: The effect of sucrose feeding on carnitine palmitoyl transferase activity and liver fatty acid binding protein bio-activity.

<table>
<thead>
<tr>
<th></th>
<th>CPT Activity (nmol/mg mito protein/min)</th>
<th>L-FABP Bio-activity (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Sucrose</td>
</tr>
<tr>
<td>A</td>
<td>1.6±0.2</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>B</td>
<td>1.5±0.2</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td>C</td>
<td>1.9±0.6</td>
<td>2.6±0.5**</td>
</tr>
</tbody>
</table>

All values are mean±SEM. ** Indicates a significantly different from groups A&B. † Indicates a significant effect of sucrose feeding.
Figure 6.1: The effect of sucrose feeding mRNA levels of enzymes associated with lipid oxidation or synthesis. All values are mean±SEM. * Indicates a significantly different from group A. ** Indicates a significantly different from group A&B. † Indicates a significant effect of sucrose feeding.

6.4.4 Effect Of Sucrose Feeding On L-FABP Activity And Gene Expression
Sucrose feeding resulted in a 1.5 times reduction in L-FABP bio-activity (P<0.03) and halving in L-FABP gene expression in group C (P<0.01). In both cases the pre-existing elevations of L-FABP activity and mRNA in group C was removed the such that all groups were the same.
Figure 6.2: The effect of sucrose feeding mRNA levels of proteins associated with lipid transport. All values are mean±SEM. * Indicates a significantly different from group A. ** Indicates a significantly different from groups A&B.

6.4.5 Effect Of Sucrose Feeding On The Gene Expression Of LPL, ACC, GPAT And Apo-B

To determine the impact of sucrose feeding on a number of lipid metabolising pathways the expression of mRNA sequences encoding enzymes involved in hepatic long-chain fatty acid metabolism were measured. The gene expression of the lipolytic protein, LPL was not altered by sucrose feeding and the pre-existing decreased level of mRNA in group C persisted. Similarly ACC gene expression was not effected by sucrose feeding and the pre-existing elevation in group C remained. Sucrose feeding caused a non-significant increase in Apo-B expression in group C. This increase coupled with slight reductions in groups A and B resulted in elevated levels of Apo-B mRNA in group C compared to the other two groups (P<0.03). Following sucrose feeding GPAT mRNA was reduced in group B (P<0.05), this normalised the level of gene expression such that groups A and B were the same, whereas elevated levels of GPAT mRNA persisted in group C (P<0.03).
6.4.6 Effect Of Sucrose Feeding On The Gene Expression Of
Lipid Responsive Transcription Factors PPARα And
SREBP-1C

Sucrose feeding resulted in a decrease in PPARα mRNA in group C, such that the pre-existing elevation in group C was removed (P<0.05). The level of SREBP-1C expression was reduced in all groups following sucrose feeding (P<0.05), however the pre-existing elevated levels in group C remained in comparison to sucrose fed groups A and B P. obesus (P<0.05).

Figure 6.3: The effect of sucrose feeding mRNA levels of lipid responsive transcription factors. All values are mean±SEM. * Indicates a significantly different from group A. ** Indicates a significantly different from group A&B. † Indicates a significant effect of sucrose feeding.
6.5 DISCUSSION

This study demonstrated that sucrose supplementation of the drinking water in the lean *P. obesus* resulted in an increase in body weight gain and body fatness over a three-week period. In addition obese group B animals developed diabetes following sucrose feeding, characterised by elevations in body weight, body fat, blood glucose and plasma insulin. However despite the group B animals developing significant hyperglycemia no modulations of the mRNA levels of lipid metabolizing enzymes, transport proteins or lipid responsive transcription factors were measured. These findings demonstrate that after the onset of sucrose induced diabetes in group B, animals still had a pre-diabetic gene profile. This suggests that alterations in lipid gene expression observed in the control obese diabetic animals may be due to a pre-existing genetic susceptibility or secondary to other metabolic aberrations. In the obese diabetic group C animals sucrose feeding exacerbated the pre-existing diabetes in this group of animals and reduced the level of expression of the transcription factor PPARα and lipid metabolising enzymes CPT, LPL and L-FABP which are under PPARα’s control.

*P. obesus* are a desert gerbil species which typically consume an energetically sparse diet (Shafir 1993). Unlike other rodent species the optimal concentration of sucrose, at which fluid intake was not compromised was 5% rather than the 60-70% that has been used previously (Cohen 1972; Pagliaassotti 1996). Following the three week period of sucrose supplementation the group A animals gained a significant amount of body weight and body fat compared to their control counterparts. Sucrose or fructose, feeding has consistently been shown to elevate body weight gain and body fatness (Kanarek 1982; Rattigan 1984; Kazumi 1997). Sucrose supplementation is able to create a state of positive energy balance (Beck 1996). In the current study, this was again observed with sucrose treated animals and resulted in a net increase in energy intake. The weight gain in the group A animals following sucrose feeding highlights the susceptibility of *P. obesus* to excess weight gain in all *P. obesus* even those that remain lean on standard laboratory chow.

Unlike the lean animals group B *P. obesus* had pre-existing obesity and elevations in body fat and plasma insulin levels compared to lean controls. Hepatic lipid metabolism in obese *P. obesus* is characterised by increased lipid storage and elevated GPAT mRNA levels. However sucrose feeding, particularly as a result of increased fructose
levels and elevated *de novo* lipogenesis, led to a further enhancement of body weight gain and body fat storage. In addition, these obese group B animals developed diabetes and further exacerbated their pre-existing hyperinsulinemia. This increase in plasma insulin may indicate a further impairment in insulin action, similar to that which has been shown previously in Wistar rats fed a diet rich in sucrose (Storlien 1988). At the gene level we observed a reduction in the level GPAT mRNA to that of group A animals, which suggests a reduced rate of fatty acid re-esterification (Bell 1980). This may provide a protective mechanism that prevents excessive amounts of lipid accumulating in the liver, instead fat deposition is increased in adipose tissue. Group B animals also had a decrease in the level of UCP-2 mRNA, however the physiological relevance of this change is unclear given that the new level of expression is no different from groups A and C. Also given that UCP-2 expression has been found to be localised in the Kupffer cells of the liver (Larroury 1997) and our mixed liver cell preparation most likely contained immuno-competent Kupffer cells the sucrose mediated reduction of UCP-2 mRNA observed in this study may suggest a fluctuation in immune function (Larroury 1997) not fat metabolism.

Despite significant alterations in carbohydrate intake and body composition there was little modification to mRNA levels of the measured lipid metabolising enzymes either in group A or B animals following sucrose feeding. Specifically in group B animals sucrose induced hyperglycemia and increased the severity of the hyperinsulinemia. However despite these metabolic changes these animals did not exhibit alterations in the mRNA level that accompany the chronic diabetes found in group C prior to sucrose feeding. This lack of response is interesting given that we demonstrated altered hepatic lipid gene expression, in previous chapters, in the presence of chronic hyperglycemia in *P. obesus* and glucose and insulin are both known to regulate gene expression in a number of tissues (Kahn 1997; Mourrieras 1997). A possible explanation for these differences may be the genes measured in this study are not necessary for the development of diabetes. If this is the case it may represent unique differences in the regulation of the genes for PPARα, SREBP-1C, CPT, ACC, LPL and L-FABP in genetically pre-disposed obese diabetic *P. obesus*. In addition, these genes in obese diabetic animals may be modulated by factors other than glucose and insulin which do not change following sucrose feeding. These factors may include sex steroids, insulin like growth factors or leptin receptors that were not measured in the current study.
In the obese diabetic group C animals sucrose feeding exacerbated the pre-existing diabetes. Accompany the changes in blood glucose level was increased accumulation of hepatic lipid and increased CPT activity, these elevations are similar to that shown in more severe ketotic forms of diabetes where the major fuel source for cellular respiration is fat instead of glucose (Park 1995). Accompanying the increase in hepatic lipid accumulation was an increase in Apo-B expression, suggesting increased VLDL synthesis (Chan 1997). Such an increase in VLDL production is common in other rodents that have been sucrose fed (Sparks 1986; Abraham 1994) and is thought to be a result of increased hepatic de-novo lipogenesis. It is unclear from the current data why Apo-B gene expression was increased only in the group C P. obesus. It can be speculated that group C P. obesus which demonstrated increased VLDL secretion are doing so a result of the severe diabetes they are experiencing or it may be an attempt to compensate for the elevation in hepatic lipid content.

Interestingly we observed a decrease in the expression of the transcription factor PPARα in group C animals following sucrose feeding and accompanying this reduction was a decrease in mRNA levels of CPT, LPL and L-FABP. It is known that each of these proteins is under the transcriptional control of PPARα (Veerkamp 1995; Stuehls 1997; Mascaro 1998) and PPARα expression in turn is regulated by fatty acids (Clarke 1996) which are known to suppress the expression of a number of hepatic lipid genes. Thus it may be possible the increase in hepatic lipid level following sucrose feeding in the group C animals is mediating a reduction in PPARα expression which in-turn reduced mRNA levels of the PPARα responsive genes including, CPT, LPL and L-FABP.

The first committed step of de-novo lipogenesis is under the control of the enzyme ACC (McGarry 1997). In the current study despite increased hepatic lipid levels in group C P. obesus and an increase in body fatness in groups A and B P. obesus the level of ACC gene expression in all of these groups did not change between control and sucrose fed groups. This is in contrast to earlier sucrose feeding studies where high dose sucrose feeding caused increases in total hepatic triglyceride formation (Holt 1979) and ACC enzyme activity (Cohen 1972). One possible explanation for these differences may be due to the dose of sucrose used, in this study a 5% sucrose solution
was used compared with 60-70% supplementation used in earlier studies (Cohen 1972; Holt 1979). Thus sucrose supplementation in the current study may be causing slight shift towards positive energy balance as opposed to stimulating hepatic de novo lipogenesis. Another consideration is the overall decrease in SREBP-1C gene expression across all groups following sucrose feeding. SREBP-1C is known to influence the rate of ACC gene expression (Lopez 1996), thus a uniform suppression of SREBP-1C gene expression may compensate for any stimulatory effect that sucrose feeding may have on ACC gene expression.

In conclusion sucrose feeding in lean P. obesus resulted in an increase in body weight and body fatness but did not change any other metabolic parameters or mRNA levels of any genes measured. Obese P. obesus developed diabetes following sucrose feeding, characterised by elevations in body weight, body fat, blood glucose and plasma insulin. However even though these animals developed diabetes they did not have the same mRNA profile for lipid metabolising enzymes as animals that had pre-existing diabetes. This lack of response suggests that diabetes can develop independently from changes in hepatic fat metabolism. In the obese diabetic P. obesus sucrose feeding exacerbated the pre-existing diabetes in this group of animals and reduced the level of expression of PPARα and lipid metabolising enzymes CPT, LPL and L-FABP which are under PPARα’s control.

In this and previous chapters we have not been able to demonstrate a primary role for liver lipid accumulation contributing to the development of diabetes. Furthermore modulating the diabetic state has only selective effects on liver lipid gene expression. However the question remains, does increasing the supply of lipids act to modulate these genes, this scenario shall be investigated next in P. obesus treated with nicotine, a stimulator of lipolysis.
Chapter 7

NICOTINE TREATMENT IN *PSAMMOMOMYS OBESUS* PROMOTES WEIGHT LOSS AND A SHIFT TOWARDS INCREASED HEPATIC LIPID OXIDATION.

7.1 SUMMARY

In this study we investigated the effects of nicotine treatment on hepatic lipid metabolism in lean and obese *P. obesus*, a polygenic rodent model of obesity. Nicotine was administered at a dose of 9 mg kg\(^{-1}\) day\(^{-1}\) between the scapulae via miniosmotic pumps. Nicotine treatment lowered body weight in the lean and obese *P. obesus*. Accompanying the reduction in body weight in both groups was an increase in hepatic CPT gene expression and enzyme activity. Accompanying these oxidative changes was an increase in LPL mRNA in both lean and obese groups as well as a uniform decrease in L-FABP and Apo-B gene expression. However there was no significant change in the mRNA levels of the transcription factors PPAR\(\alpha\) and SREBP-1C which are thought to be important in the regulation of genes for key hepatic lipid metabolising enzymes and proteins studied. This study demonstrated that nicotine treatment induced a shift in the metabolic flux of fat towards oxidation and away from storage in the liver.
7.2 INTRODUCTION

The negative relationship between cigarette smoking and body weight has been confirmed in large population data (NHANES II) (Klesges 1993). It is thought that these effects of smoking are nicotine mediated, as Hajek et al. have demonstrated a significantly lower body weight in users of nicotine chewing gum than non-users (Hajek 1988). Furthermore epidemiological data from populations in Nauru, Mauritius and Western Samoa have shown a significant association between smoking and leptin levels (Hodge 1997). Yoshida et al. (1991) demonstrated that nicotine treatment in mice decrease body weight and food intake together with increasing noradrenaline turnover, brown adipose tissue thermogenesis and resting metabolic rate. Investigations into nicotine's impact on fat metabolism has shown that nicotine treatment in albino rats increased heart LPL activity, mass and mRNA level as well as increasing basal lipolysis (Sztalryd 1996). In addition, it has been shown that male cigarette smokers have an overall increased rate of fat metabolism compared to non-smokers (Jensen 1995). Mechanisms that have been suggested to explain the underlying nicotine induced alterations in fat metabolism have focused around nicotine's actions in the nervous system. Chowdhury et al. suggested that nicotine caused reductions in body weight, food intake and insulin as well as increased growth hormone levels by stimulating central cholinergic and alpha-adrenoreceptor pathways (Chowdhury 1990). The stimulatory effects of nicotine on the sympathetic nervous system was suggested to account for the reduced body weight and food intake in mice (Yoshida 1991). Finally nicotine treatment is known to increase dopamine and serotonin levels both of which are known to have role in appetite regulation (Takada 1990; Summers 1995).

*P. obesus* is a unique polygenic animal model of obesity and diabetes (Shafrir 1993). When fed a diet of standard laboratory chow, a proportion of animals remains lean while others develop varying degrees of obesity comparable to the metabolic profile observed in susceptible human populations (Barnett 1994). In addition, obese *P. obesus* have been shown to display abnormalities in hepatic lipid metabolism including liver fat deposition together with decreased insulin sensitivity (Barnett 1994; Lewandowski 1997). In the previous chapters we have shown alterations in hepatic lipid gene expression in *P. obesus*, specifically mRNA levels of the re-esterification enzyme GPAT were elevated in obese *P. obesus*. Thus the aims of this study were firstly to investigate the impact of nicotine treatment on body weight, blood glucose, plasma insulin and liver lipid accumulation in lean and obese *P. obesus*. Secondly to
examine whether this pharmacological intervention, known to increase fat supply by stimulating lipolysis, is able to modulate hepatic lipid metabolism and gene expression in previously normal or obese *P. obesus*.

### 7.3 METHODS

#### 7.3.1 Animals

A colony of mixed sex *P. obesus* were maintained on a 12 hr light 12 hr dark cycle at 21±1°C and fed ad libitum standard rat chow (Barastoc, Pakenham, Australia). Consistent with previous reports from our laboratory (Barnett 1994), the animals displayed wide spontaneous variation in weight gain, due to increased adiposity which was accompanied by varying degrees of hyperinsulinemia and hyperglycemia (Collier 1997). At 16 weeks of age *P. obesus* were selected and allocated into two groups based on body weight, blood glucose and plasma insulin as described previously (Barnett 1994). The lean group were normoglycemic, normoinsulinemic (n=14); Whereas the obese group were normoglycemic, hyperinsulinemic (n=14). Within each category (lean and obese) the animals were randomly subdivided to received nicotine sulphate (9 mg kg⁻¹ day⁻¹) or saline in the sham controls for nine days. The nicotine or saline was administered subcutaneously between the scapulae with Alzet mini-osmotic pumps (model 2002), which were implanted under light anaesthesia (sodium pentobarbital, 60mg/kg). Animals were allowed to recover for 48 hr after which they were monitored daily for weight and food intake.

At the end of the nine day treatment period all animals were sacrificed by anesthetic overdose between 9am and 12noon, tissues removed and freeze clamped and maintained in liquid nitrogen until analysis. Body fatness was estimated by combining the weight of the same selected fat stores (perirenal, mesenteric, hind limb intramuscular and suprascapular) in each animal and expressing this as a percentage of body weight.

#### 7.3.2 Biochemical Analyses

Blood was collected from the tail vein in heparinized microtubes and blood glucose immediately determined using an automated enzymatic analyser (2300 Stat Plus, Yellow Springs Instruments, Ohio). Plasma insulin levels were determined by radioimmunoassay using a human primary antibody (Phadeseph, Kabi Pharmacia
Diagnostics, Sweden). Plasma leptin levels were measured by radioimmunoassay using a multi-species primary antibody (Linco, St Charles, MO). Plasma triglycerides were measured colorimetrically (Bohringer-Mannheim, Mannheim, Germany) as were non-esterified free fatty acids (NEFA) (WAKO Pure Chemical Co., Japan).

7.3.3 Hepatic Carnitine Palmitoyltransferase Activity And Triglyceride Accumulation

A measure of β-oxidation, carnitine palmitoyltransferase activity was determined based on the method of Power and colleagues (Power 1994). Mitochondria were isolated from frozen livers and CPT activity was determined by incubating 50 μl of mitochondrial suspension in 1 ml of assay medium (150 mM KCl, 5 mM Tris-HCl, 1 mM EGTA, 1 mM dithiothreitol, 5 mM MgCl₂, 5 mM ATP, 100 μM palmitoyl-CoA, 1 μg/ml rotenone, 1 μg/ml antimycin A, and 0.13% BSA (pH 7.4) equilibrated at 37°C for 5 min. The reaction was initiated by addition of 0.005 pmol [³H]carnitine and terminated by addition of 300 μl of 0.6 M HCl at either 1 or 3 min. [³H]acylcarnitine formed was extracted by addition of 2 ml butanol, followed by a butanol saturated water wash. [³H]acylcarnitine was counted by liquid scintillation (RackBeta II, LKB Wallac, Finland). Results were expressed as [³H]acylcarnitine formed per mg of mitochondrial protein, per minute. Protein concentration was determined using a total protein kit (Sigma, St. Louis, MO). Total hepatic lipid accumulation was measured by chloroform methanol extraction (Folch 1957).

7.3.4 Liver Fatty Acid Binding Protein Bio-activity

Liver cytosolic fatty acid binding protein bio-activity measurement was based on the radiochemical method of Glatz and Veerkamp (1983). Tissue homogenates were prepared in Tris-HCl buffer centrifuged for 10 min at 600 g and the supernatant centrifuged again for 90 min at 105000 g at 4°C. Albumin was removed from the cytosolic homogenate by affinity chromatography and then dephosphorylated using a hydrophobic dextran column. The resultant L-FABP extract was incubated with [¹⁴C] palmitate at 37°C for 10 min and unbound [¹⁴C] was removed using hydrophobic dextran and centrifugation (20000 g for 2 min at 4°C). The resulting supernatant containing the bound [¹⁴C] palmitate was assayed for radioactivity by liquid scintillation counting.
7.3.5 Extraction Of Total RNA And Reverse Transcription (RT)-PCR Quantitation

Total RNA was extracted from 30 mg frozen liver portions by the RNAzol B method (Bresatec, Adelaide, Australia). First strand cDNA was generated from 1 μg of RNA in a 30 μl volume using the oligo(dT) primer in the first strand cDNA synthesis kit (Promega, Madison, WI). One microliter of the reverse transcription reaction mix was amplified with primers specific for LPL, L-FABP, Apo-B, CPT, ACC, UCP-2, GPAT, PPARα and SREBP-1C in a total volume of 20 μl. All sequences were from the Genbank, primer sequences are shown in Table 2.1 in the general material and methods section. The samples were amplified in the linear phase for 22-30 cycles, optimised for each gene, using the following parameters: 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. β-Actin primers were used as a control for all quantitation and were amplified in the linear phase for 22 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min. Levels of mRNA were expressed as the ratio of signal intensity for the sample genes to that of β-Actin. The PCR products were electrophoresed on a 1.4% agarose gel, visualised using ethidium bromide and quantitated by computer integrated densitometry (Kodak ds 1D system, Kodak, New Haven, CT).

7.3.6 Statistical Analyses

All results are expressed as mean ± SEM. Statistical significance was evaluated using one-way ANOVA with the aid of a multiple comparison test (Tukey-HSD procedure) to establish where the differences between groups were significant. In all cases P values of 0.05 or less were considered to indicate statistical significance.
7.4 RESULTS

7.4.1 Metabolic and mRNA Comparisons Between Lean and Obese Animals

*P. obesus* that were classified as obese, at 16 weeks of age, had elevated body mass, liver triglyceride accumulation and a tendency for increased body fatness (P<0.05, Table 7.1). Plasma insulin and leptin levels (P<0.05, Table 7.2) were also elevated compared to their lean counterparts. Hepatic CPT enzyme activity and L-FABP activity did not differ between the obese and lean animals (Table 7.3).

Obese animals demonstrated an increased level of hepatic GPAT and UCP-2 expression compared with lean animals (P<0.05, Figure 7.1). Hepatic expression levels of the other mRNA species analysed including; LPL, L-FABP, Apo-B, CPT, ACC, PPARα and SREBP 1C did not differ significantly between lean and obese *P. obesus*. (Figures 7.1 to 7.3).

Table 7.1: A comparison between body mass, food intake, estimated body fat and liver fat in lean and obese *P. obesus* and the effect of nicotine treatment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Change In Body Mass (g)</th>
<th>Food Intake (g)</th>
<th>Estimated Body Fat (%)</th>
<th>Liver Fat (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>4.5±2.0</td>
<td>12.1±0.6</td>
<td>2.7±0.5</td>
<td>3.8±0.4</td>
</tr>
<tr>
<td>Lean Nicotine</td>
<td>-24.0±7.0†</td>
<td>16.3±1.4†</td>
<td>2.2±0.4</td>
<td>4.0±0.2</td>
</tr>
<tr>
<td>Obese</td>
<td>4.7±2.5</td>
<td>13.5±1.0</td>
<td>3.3±0.5</td>
<td>4.9±0.4*</td>
</tr>
<tr>
<td>Obese Nicotine</td>
<td>-19.0±5.0†</td>
<td>15.0±1.2</td>
<td>4.4±0.4*</td>
<td>5.8±0.8*</td>
</tr>
</tbody>
</table>

Lean and obese animals were treated for nine days with nicotine (9 mg kg⁻¹ day⁻¹) or saline as described in the methods. Values shown are the mean ± S.E.M. * Indicates significant difference between lean and obese groups (P<0.05). † Indicates significant effect of nicotine treatment (P<0.05).
7.4.2 Impact Of Nicotine Treatment On Anthropometric And Blood Variables

Nicotine treatment for nine days in lean and obese P. obesus did not alter circulating glucose or triglyceride levels. However nicotine treatment did cause a significant reduction in body mass in both lean and obese P. obesus (P<0.05). Following nicotine treatment obese P. obesus had significantly more body fat than lean animals (P<0.05), yet surprisingly liver lipid accumulation remained unaltered. Nicotine treatment in the lean group led to increased in food intake (P<0.05, Table 7.1), despite decreased body weight. In the obese P. obesus nicotine treatment resulted in a normalisation of insulin levels (P<0.05). In addition there was a significant increase in plasma leptin levels in obese P. obesus following nicotine treatment (P<0.05).

Table 7.2: A comparison between circulating glucose, insulin, leptin, NEFA and triglycerides in lean and obese P. obesus and the effect of nicotine treatment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Glucose (mmol/L)</th>
<th>Insulin (mU/L)</th>
<th>Leptin (ng/ml)</th>
<th>NEFA (mEq/L)</th>
<th>TG (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>4.3±0.3</td>
<td>60±13</td>
<td>26±2.4</td>
<td>0.17±0.03</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>Lean Nicotine</td>
<td>4.8±0.2</td>
<td>49±14</td>
<td>20±2.5</td>
<td>0.21±0.05</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>Obese</td>
<td>4.8±0.5</td>
<td>240±55*</td>
<td>37±2.1*</td>
<td>0.26±0.04*</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>Obese Nicotine</td>
<td>4.4±0.4</td>
<td>102±19†</td>
<td>47±5.7*†</td>
<td>0.3±0.04</td>
<td>0.9±0.1</td>
</tr>
</tbody>
</table>

Lean and obese animals were treated for nine days with nicotine (9 mg kg⁻¹ day⁻¹) or saline as described in the methods. Values shown are the mean ± S.E.M. * Indicates significant difference between lean and obese groups (P<0.05). † Indicates significant effect of nicotine treatment (P<0.05).
7.4.3 mRNA Levels Of Enzymes Involved With In Free Fatty Acid Uptake And Esterification

In lean and obese *P. obesus* nicotine treatment resulted in increased LPL gene expression (P<0.05). This effect was pronounced in the lean animals, such that the level of LPL mRNA in the lean group following nicotine treatment was significantly greater than that of the obese group (P<0.05). In contrast, the mRNA and activity levels of L-FABP were reduced in both lean and obese *P. obesus* after nicotine treatment (P<0.05). An additional effect of nicotine treatment in the obese group was a reduced level of GPAT gene expression (P<0.05), such that the pre-existing elevation present in the obese animals was removed.

Table 7.3: A comparison between CPT activity and L-FABP activity in lean and obese *P. obesus* and the effect of nicotine treatment.

<table>
<thead>
<tr>
<th>Group</th>
<th>CPT Activity (nmol/mg protein/min)</th>
<th>L-FABP Activity (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>0.8±0.1†</td>
<td>1.3±0.3</td>
</tr>
<tr>
<td>Lean Nicotine</td>
<td>1.7±0.2†</td>
<td>0.7±0.1†</td>
</tr>
<tr>
<td>Obese</td>
<td>1.2±0.2</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>Obese Nicotine</td>
<td>2.0±0.4†</td>
<td>0.2±0.1*†</td>
</tr>
</tbody>
</table>

Lean and obese animals were treated for nine days with nicotine (9 mg kg⁻¹ day⁻¹) or saline as described in the methods. Values shown are the mean ± S.E.M. * Indicates significant difference between lean and obese groups (P<0.05). † Indicates significant effect of nicotine treatment (P<0.05).
7.4.4 Activity And mRNA Levels Of Enzymes Involved In Free Fatty Acid Oxidation

In the lean and obese animals nicotine treatment resulted in an increase in CPT mRNA and enzyme activity (P<0.05). However nicotine treatment had no effect on the level of ACC gene expression in lean or obese *P. obesus*. In the lean group there was a significant increase in UCP-2 gene expression (P<0.05) such that the levels in both lean and obese groups were comparable after nicotine treatment.

![Graph showing mRNA levels of enzymes](image)

Figure 7.1: The effect of nicotine treatment on mRNA levels of enzymes associated with lipid oxidation or synthesis. All values are mean±SEM. * Indicates a significant difference between lean and obese groups. † Indicates a significant effect of nicotine treatment.
7.4.5 mRNA Levels Of Enzymes Involved With VLDL Synthesis

Nicotine treatment caused a reduction in the level of Apo-B mRNA in both lean and obese P. obesus (P<0.05), suggesting a reduction in the rate of VLDL synthesis.

Figure 7.2: The effect of nicotine treatment on mRNA levels of proteins associated with lipid transport. All values are mean±SEM. * Indicates a significant difference between lean and obese groups. † Indicates a significant effect of nicotine treatment.
7.4.6 mRNA Levels Of The Transcription Factors PPARα And SREBP-1C

The gene expression of the two lipid responsive transcription factors PPARα and SREBP 1C was not significantly altered by nicotine treatment in lean or obese P. obesus.

![Graph showing mRNA levels of PPARα and SREBP-1C in Control and Nicotine conditions](image)

Figure 7.3: The effect of nicotine treatment on mRNA levels of lipid responsive transcription factors. All values are mean±SEM.

7.5 DISCUSSION

This study demonstrated that nicotine treatment lowered body weight in the lean and obese P. obesus. Along with the reduction in body weight in both groups was an increase in hepatic CPT gene expression and enzyme activity as well as increased UCP-2 gene expression in lean animals. Accompanying these oxidative changes was an increase in LPL mRNA in both lean and obese groups as well as a uniform decrease in L-FABP and Apo-B gene expression. However there was no significant change in the mRNA level of the transcription factors PPARα and SREBP-1C.

The weight reducing action of nicotine observed in both groups of P. obesus in this study was consistent with numerous other investigations. Nicotine delivered in
drinking water (Chowdhury 1990), injections (Saah 1994) or via Alzet pumps (Li 1994) at various doses and over different time courses has consistently been shown to lower the body weight of rodents. In the current study the reduced body weight was not accompanied by a loss of body fat stores, in contrast to previous studies demonstrating lower body fat following nicotine treatment at similar doses (Szalay 1996). Potentially the loss of body mass in *P. obesus* may be accounted for by a loss of lean body mass or increased diuresis. However, an increase in urine out-flow is unlikely as previous studies in rats and goats has shown that nicotine induces antidiuresis (Vandeputte-Van Messom 1981; Patel 1995)

The constant NEFA and plasma triglyceride levels in both groups following nicotine treatment in in contrast to previous studies in albino rats where subcutaneous injection of nicotine twice a day for three weeks resulted in an increase in circulating triglycerides, cholesterol, LDLs and VLDLs (Latha 1993). Similarly continuous infusion of nicotine via Alzet pumps increased basal lipolysis (Szalay 1996). Although the current results did not show the same changes as these previous studies it still cannot be excluded that nicotine did not increase lipolysis. The reason for this is nicotine may still have an increase in flux of blood lipids even though absolute levels did not change and the increased LPL mRNA levels in both groups following nicotine treatment may also indicate that we have an increase in lipid flux.

The normalisation of plasma insulin levels in the obese group following nicotine treatment is consistent with a number of previous studies (Chowdhury 1990; Saah 1994). The increase in circulating leptin in the obese group may have little metabolic action, given that these animals had pre-existing hyperleptinemia. Any further increases in leptin concentration may only exacerbate the leptin resistance present in obese *P. obesus* (Walder 1998).

The first major effect observed in this study was that nicotine treatment enhanced hepatic lipid oxidation. This occurred on two levels, firstly there was an increase in hepatic β-oxidation (CPT) in both groups and secondly an increase in uncoupled oxidation (UCP-2) in the lean animals. The uniform increase in CPT gene expression and enzyme activity in both lean and obese *P. obesus* following nicotine treatment is similar to data in humans (Jensen 1995). These previous investigations were carried out in male cigarette smokers who demonstrated increased fat oxidation compared with non-smokers. In addition it was found that the rate of fat oxidation decreased in
smoking subjects upon cessation of smoking (Jensen 1995). Although these results in humans did not specify the sites where fat oxidation was increased, previous studies in rats have suggested that nicotine treatment diverts fat away from storage in adipose tissue towards utilization in muscle or liver (Sztalryd 1996). Furthermore the constant ACC mRNA level, which produces an allosteric regulator of CPT activity (McGarry 1997), suggests no compensatory regulation of CPT activity occurred following nicotine treatment, which may suggest fat oxidation is favored.

The increased gene expression of UCP-2 in the lean animals is similar to previous studies which have shown that nicotine treatment increases brown adipose tissue (BAT) thermogenesis (Yoshida 1991). The key protein involved with thermogenesis in BAT is UCP-1 (Skulachev 1998). UCPs constitute a subgroup of mitochondrial carrier proteins which by dissipating proton gradients, act to uncouple respiration from oxidative phosphorylation and convert fuel to heat (Bao 1998). Thus in the liver of nicotine treated P. obesus the increased expression of UCP-2 suggests an increase in uncoupled oxidation similar to that observed in BAT.

The second major effect observed following nicotine treatment was the down regulation of the metabolic pathways involved with hepatic fat storage within the liver and export of fat from the liver. This finding is similar to previous studies, where it was suggested that nicotine treatment directed fat away from storage in adipose tissue towards utilization in muscle and liver (Sztalryd 1996). Our results reflect this in the normalisation of GPAT gene expression in the obese group following nicotine treatment. GPAT catalyses the committed step of triacylglycerol and phospholipid biosynthesis, and a reduced mRNA level suggests a reduced rate of fatty acid re-esterification in the animals (Bell 1980). Similarly the uniform reduction in Apo-B gene expression suggest that the rate of VLDL synthesis is reduced following nicotine treatment (Chan 1997), this result is in contrast to an earlier lower dose (1.2 mg kg\(^{-1}\) day\(^{-1}\)) nicotine treatment study, which demonstrated increased circulating VLDL levels post treatment (Latha 1993). However the same study also demonstrated decreased uptake of lipoproteins after nicotine treatment, potentially suggesting that VLDL particles remained in the circulation longer, which may account for the measured elevated levels (Latha 1993). Furthermore the uniform reduction in L-FABP mRNA and activity following nicotine treatment may suggest reduced transport of fat through the cytosol (Veerkamp 1995) prior to incorporation into VLDL particles.
It can be speculated from the increased gene expression of LPL following nicotine treatment that hepatic fat metabolism is shifted towards oxidation and away from storage. A metabolic shift such as this is supported by the following observations: 1) LPL gene expression is increased in both groups following nicotine treatment, suggesting an increased rate of fat uptake by the liver (Nilsson-Ehle 1981). 2) Hepatic lipid content was not changed in either group by nicotine treatment, suggesting that fat taken up by the liver is not stored within the liver. 3) The decreased Apo-B mRNA levels discussed previously suggested that hepatic fat export was decreased. Thus, combining these findings it may be suggested that the eventual fate of the fat taken up by the liver is oxidation.

We have shown previously in earlier chapters that the transcription factors PPARα and SREBP-1C may be important in mediating the gene expression of key lipid metabolizing enzymes and proteins in the liver. PPARα is an important transcriptional regulator of the LPL, L-FABP, and CPT genes (Veeckamp 1995; Staels 1997; Mascaro 1998). Whereas SREBP-1C has been shown to transcriptionally regulate the expression of key genes responsible for cholesterol and fatty acid synthesis (Brown 1997). Included in the genes regulated by SREBP-1C activity are GPAT and ACC (Ericsson 1997; Lopez 1996). In the current study the level of gene expression of PPARα and SREBP-1C was not significantly altered by nicotine treatment. Thus it is unlikely that these two transcription factors can account for the changes observed in hepatic fat metabolism observed in this study.

In summary, nicotine treatment lowered body weight in the lean and obese P. obesus. Accompanying the reduction in body weight in both groups was an increase in hepatic CPT gene expression and enzyme activity. In the lean group there was a significant increase in UCP-2 gene expression. Accompanying these oxidative changes was an increase in LPL mRNA in both groups as well as a uniform decrease in L-FABP and Apo-B gene expression. However there was no significant change in the mRNA level of the transcription factors which regulated the expression of these genes of key hepatic lipid metabolising enzymes and proteins studied. Overall in the liver nicotine treatment caused a shift in the metabolic flux of fat towards oxidation and away from storage.

This study demonstrated that nicotine treatment did not alter the level of liver fat, yet a change in the expression profile of hepatic lipid genes was still observed. These
findings again demonstrate that the level of hepatic fat accumulation is not modulating the level of lipid gene expression. To determine if pharmacological lipid depletion is able to modulate the expression of these genes of hepatic lipid metabolising enzymes and proteins, we shall attempt to deplete tissues of triglyceride stores with leptin treatment in the next study.
Chapter 8

IMPACT OF LEPTIN TREATMENT ON LIVER LIPID METABOLISM IN PSAMMOMYS OBESUS.

8.1 SUMMARY

In this study we investigated the effects of leptin administration on hepatic lipid metabolism in lean and obese P. obesus, a polygenetic rodent model of obesity. Leptin was administered at a dose of 45 mg/kg/day by intraperitoneal injection. In lean P. obesus leptin treatment resulted in a reduction in food intake, body mass and body fatness. No changes in food intake and body weight were observed in obese leptin treated P. obesus. Lean leptin responsive P. obesus demonstrated a leptin specific elevation in hepatic CPT enzyme activity. Leptin treatment, independent of reductions in food intake, maintained LPL and suppressed both CPT and UCP-2 gene expression levels in the liver. These results demonstrate that leptin treatment may modulate liver lipid metabolism, however this action is not due to substantial alterations in the expression of genes encoding lipid metabolising enzymes.

8.2 INTRODUCTION

The morbid obesity of ob/ob mice is due to a single point mutation in the ob gene resulting in a truncated non-secreted form of the leptin protein (Zhang 1994; Tritos 1997). Leptin administration in ob/ob mice markedly suppresses food intake, lowers body weight and adiposity, while simultaneously increasing energy expenditure (Halaas 1995; Pellemounter 1995). Leptin treatment in insulin-resistant rodents, in addition to lowering body weight, is accompanied by improvements in circulating glucose, insulin and lipids (Pellemounter 1995; Chen 1996). Subsequent studies demonstrated that leptin infusion lowered HGP during a hyperinsulinemic clamp (Rossetti 1997). While intravenous or intra-cerebroventricular (ICV) leptin treatment
increase glucose turnover and uptake in the liver (Kamohara 1997). However, leptin treatment has also been shown to impact on lipid metabolism. Supraphysiological leptin concentrations depletes liver, skeletal muscle and pancreatic intracellular triglyceride stores (Shimabukuro 1997). The depletion of intracellular triglycerides may be due to increased pancreatic and skeletal muscle lipid oxidation, which has been demonstrated in-vitro following leptin treatment (Muoio 1997; Zhou 1997). This is supported by leptin increasing the mRNA levels of enzymes involved with lipolysis coupled with increased uncoupling proteins 2 and 3 expression measured in the pancreas, adipose tissue and muscle (Zhou 1997; Cusin 1998).

The actions of leptin treatment on liver lipid metabolism have yet to be examined in detail. As we have described previously the liver is a key site for lipid metabolism, including β-oxidation of fatty acids (McGarry 1980), glycerol lipid formation (Bell 1980) and VLDL synthesis (Chan 1997). These pathways are dependent upon lipid specific transport mechanisms, including L-FABP (Veerkamp 1991) and LPL (Nestel 1962). In addition it has become apparent from the studies in the previous chapters that gene expression of these key lipid metabolising enzymes and transport proteins is modulated independent of hepatic lipid accumulation.

Previous studies by our group have shown that circulating leptin is synthesised normally in P. obesus, with gene expression and circulating protein concentrations positively correlating to body weight and insulin levels (Walder 1997). In this study we examined the impact of supraphysiological administration of leptin on liver lipid metabolism in P. obesus. We aimed to determine if the triglyceride depleting actions of leptin was via modifications in the gene expression of hepatic lipid genes.
8.3 METHODS

8.3.1 Animals
A colony of mixed sex *P. obesus* were maintained on a 12 hr light 12hr dark cycle at 21±1°C and fed *ad libitum* standard rat chow (Barastoc, Pakenham, Australia). Consistent with previous reports from our laboratory (Barnett 1994), the animals displayed wide spontaneous variation in weight gain, due to increased adiposity accompanied by varying degrees on insulin resistance (Collier 1997). At 16 weeks of age 37 animals were selected and allocated into three groups based on body weight, lean (n=16), lean pair fed (n=8) and obese (n=13).

8.3.2 Leptin Administration
After the baseline period, animals were given intraperitoneal injections, three times per day (at 0800, 1600 and 2400 hr) of 15 mg per kg body weight, of recombinant murine leptin for 7 days (Amgen Inc., Thousand Oaks, California) (n=7 lean, n=7 obese). This dosage of leptin resulted in a total of 45 mg/kg/day. Equivalent volume of saline in animals randomised to a control group (n=8 lean, n=6 obese) was administered at in a comparable manner. Body weight and food intake was measured daily throughout the study. In addition, blood was collected from tail vein samples (100 μl) on days 2, 4 and 7 at 1200 hr (the midpoint between the morning and afternoon injections) for biochemical analyses. Each day the pair fed animals (n=8) were given the same amount of chow that was consumed by age-, gender- and body weight-matched lean animals receiving leptin.

At the completion of seven days treatment the animals were sacrificed by anaesthetic overdose, tissues removed and freeze clamped in liquid nitrogen. Body fatness was estimated by combining the weight of the same selected fat stores (perirenal, mesenteric, hind limb inter-muscular and suprascapular) in each animal and expressing this as a percentage of body weight.

8.3.3 Biochemical Analyses
Blood was collected from the tail vein in heparinized microtubes and blood glucose immediately determined by a enzymatic glucose analyser (2300 Stat Plus, Yellow Springs Instruments, Ohio). Plasma insulin levels were determined by radioimmunoassay using an human primary antibody (Phadceph, Kabi Pharmacia Diagnostics, Sweden). Plasma leptin levels were measured in collaboration with
Amgen Inc. (Thousand Oaks, CA) 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 20pg/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 levels were measured colorimetrically (Bohringer-Mannheim, Mannheim, Germany) as were non-esterified free fatty acids (NEFA) (WAKO Pure Chemical Co., Osaka).

8.3.4 Hepatic CPT Activity And Triglyceride Accumulation

Carnitine palmitoyltransferase, a measure of β-oxidation, was determined based on the method of Power and colleagues (Power 1994). Mitochondria were isolated from frozen livers as follows; liver portions were weighed and homogenised in 4 vol. of extraction medium (0.25 M sucrose, 5 mM Tris-HCl and 1 mM EGTA, pH 7.4). The crude homogenate was centrifuged twice at 800 g for 10 min at 4°C, retaining the supernatant on both occasions. The supernatants were combined and centrifuged at 10000 g for 10 min at 4°C, after which the crude mitochondrial pellet was resuspended in 0.5 ml of resuspension buffer (150 mM KCl, 5 mM Tris-HCl and 1 mM EGTA, pH 7.4). CPT activity was determined by incubating 50 μl of mitochondrial suspension in 1 ml of assay medium (150 mM KCl, 5 mM Tris-HCl, 1 mM EGTA, 1 mM dithiothreitol, 5 mM MgCl₂, 5 mM ATP, 100 μM palmitoyl-CoA, 1 μg/ml rotenone, 1 μg/ml antimycin A, and 0.13% BSA, pH 7.4) equilibrated at 37°C for 5 min. The reaction was initiated by addition of 0.005 pmol [³H]carnitine, after 1 min and 3 min the reaction was terminated by addition of 300 μl of 0.6 M HCl. The [³H]acyl-carnitine formed was extracted by addition of 2 ml butanol. The butanol layer was removed and washed with 2 ml of butanol saturated water. The butanol layer was again removed and the amount of [³H]acylcarnitine formed counted by liquid scintillation (RackBeta II, LKB Wallac, Turku, Finland). Results were expressed as the amount of [³H]acylcarnitine formed per mg of mitochondrial protein per minute. Protein concentration was determined using a total protein kit (Sigma, St. Louis, MO). Total hepatic lipid accumulation was measured by chloroform methanol extraction (Folch 1957).
8.3.5 Extraction Of Total RNA And Reverse Transcription (RT)-PCR Quantitation

Total RNA was extracted from 30 mg frozen liver portions by the RNAzol B method (Bresatec, Adelaide, Australia). First strand cDNA was generated from 1 μg of RNA in a 30 μl volume using the oligo(dT) primer in the first strand cDNA synthesis kit (Promega, Madison, WI). One microliter of the reverse transcription reaction mix was amplified with primers specific for LPL, Apo-B, L-FABP, CPT, ACC, GPAT and UCP-2 in a total volume of 20 μl. All sequences were from the Genbank, primer sequences are shown in Table 2.1 in the general material and methods section. The samples were amplified in the linear phase for 22-40 cycles, optimised for each gene, using the following parameters: 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. β-Actin primers were used as a control for all quantitation and were amplified in the linear phase for 22 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min. Levels of mRNA were expressed as the ratio of signal intensity for the sample genes to that of β-Actin. The PCR products were electrophoresed on a 1.4% agarose gel, visualised using ethidium bromide and quantitated by computer integrated densitometry (Kodak ds 1D system, Kodak, New Haven, CT).

8.3.6 Statistical Analyses

All results are expressed as mean ± SEM. In all cases except for change in body weight statistical significance was evaluated using one-way ANOVA with the aid of a multiple comparison test (Tukey-HSD procedure) to establish where the differences between groups were significant. Repeated measures analysis was used to determine statistical differences in body weight change. In all cases P values of 0.05 or less were considered to indicate statistical significance.
8.4 RESULTS

8.4.1 Metabolic And mRNA Comparisons Between Lean And Obese Animals

*P. obesus* that were classified as obese, at 16 weeks of age, had elevated body mass, estimated body fat and liver triglyceride accumulation (P<0.05, Table 8.1). Plasma insulin and leptin levels (P<0.05, Table 8.2) were also elevated compared to their lean counterparts. Hepatic CPT enzyme activity did not differ between the obese (2.0±0.4 nmol/mg mitochondrial protein/min) and lean animals (1.8±0.2 nmol/mg mitochondrial protein/min)(Figure 8.1).

Obese animals demonstrated an increased level of hepatic GPAT expression compared with lean animals (P<0.05, Table 8.3). Hepatic expression levels of the other mRNA species analysed including LPL, L-FABP, Apo-B, CPT, ACC and UCP-2 did not differ significantly between lean and obese *P. obesus*. (Table 8.3).

Table 8.1: A comparison between body mass, food intake, estimated body fat and liver fat in lean and obese *P. obesus* and the effect of leptin treatment or pair feeding

<table>
<thead>
<tr>
<th>Group</th>
<th>Change In Body Mass (%)</th>
<th>Food Intake (g)</th>
<th>Estimated Body Fat (%)</th>
<th>Liver Fat (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>1.7±0.05</td>
<td>12.5±0.7</td>
<td>2.7±0.5</td>
<td>4.3±0.4</td>
</tr>
<tr>
<td>Lean Leptin</td>
<td>-4.2±3.2†</td>
<td>9.7±0.9†</td>
<td>1.3±0.4†</td>
<td>3.7±0.2</td>
</tr>
<tr>
<td>Pair Fed</td>
<td>-3.8±1.5§</td>
<td>9.7±0.6§</td>
<td>1.5±0.2</td>
<td>3.5±0.3</td>
</tr>
<tr>
<td>Obese</td>
<td>-1.1±0.6</td>
<td>14.2±0.6</td>
<td>4.8±0.5*</td>
<td>5.9±0.4*</td>
</tr>
<tr>
<td>Obese Leptin</td>
<td>-1.8±0.7</td>
<td>15.0±1.1</td>
<td>4.6±0.5*</td>
<td>6.1±0.8*</td>
</tr>
</tbody>
</table>

Lean and obese animals were treated for 7 days with leptin (45 mg/kg) or pair fed as described in the methods. Values shown are the mean ± S.E.M. * Indicates significant difference between lean and obese groups (P<0.05). † Indicates significant effect of leptin treatment (P<0.05). § Indicates significant effect of pair feeding (P<0.05).
Table 8.2: A comparison between blood glucose, plasma insulin, non-esterified free fatty acids plasma triglycerides and leptin in lean and obese *P. obesus* and the effect of leptin treatment or pair feeding.

<table>
<thead>
<tr>
<th>Group</th>
<th>Glucose Pre (mmol/L)</th>
<th>Glucose Post (mmol/L)</th>
<th>Insulin Pre (μU/ml)</th>
<th>Insulin Post (μU/ml)</th>
<th>NEFA Pre (mEq/L)</th>
<th>NEFA Post (mEq/L)</th>
<th>TG Pre (mmol/L)</th>
<th>TG Post (mmol/L)</th>
<th>Leptin Pre (ng/ml)</th>
<th>Leptin Post (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>4.6±0.2</td>
<td>4.1±0.5</td>
<td>57±10</td>
<td>51±10</td>
<td>0.4±0.04</td>
<td>0.7±0.1</td>
<td>0.8±0.3</td>
<td>0.9±0.2</td>
<td>27±8</td>
<td>38±7</td>
</tr>
<tr>
<td>Lean Leptin Pair Fed</td>
<td>4.5±0.4</td>
<td>4.4±0.5</td>
<td>23±7</td>
<td>18±5</td>
<td>0.4±0.1</td>
<td>0.5±0.1</td>
<td>0.8±0.1</td>
<td>0.9±0.1</td>
<td>24±6</td>
<td>2048±552†</td>
</tr>
<tr>
<td>Obese</td>
<td>5.3±0.6</td>
<td>5.3±0.8</td>
<td>360±65*</td>
<td>309±61*</td>
<td>0.4±0.1</td>
<td>0.5±0.1</td>
<td>0.8±0.1</td>
<td>0.6±0.3</td>
<td>25±6</td>
<td>32±7</td>
</tr>
<tr>
<td>Obese Leptin</td>
<td>4.1±0.4</td>
<td>4.3±0.3</td>
<td>398±74*</td>
<td>434±123*</td>
<td>0.4±0.1</td>
<td>0.4±0.1</td>
<td>1.2±0.2</td>
<td>1.1±0.1</td>
<td>55±9*</td>
<td>1921±334†</td>
</tr>
</tbody>
</table>

Lean and obese animals were treated for 7 days with leptin (45 mg/kg) or pair fed as described in the methods. Values shown are the mean ± S.E.M for pre-treatment (Pre) and post-treatment (Post). * Indicates significant difference between lean and obese groups (P<0.05).
† Indicates significant effect of leptin treatment (P<0.05).
8.4.2 Impact Of Leptin Treatment Or Pair Feeding On Anthropometric And Blood Variables

Leptin treatment in the obese *P. obesus* did not alter body mass, estimated body fat, liver triglyceride accumulations, food intake and the plasma glucose, insulin, NEFA and triglyceride concentrations. This is despite plasma leptin concentrations increasing 30-fold with leptin treatment (57±14 ng/ml to 1921±334 ng/ml with leptin treatment, P<0.01) (Table 8.2).

In lean *P. obesus* leptin treatment lowered food intake and significantly reduced body mass compared to untreated lean *P. obesus* (P<0.05) (Table 8.1). Estimated body fat was reduced by 50% in lean treated animals compared with their lean control counterparts (P<0.05). Pair fed animals matched for food intake with the lean leptin treated animals showed a similar loss of body mass and body fat. Circulating NEFA levels were unaltered by leptin treatment or pair feeding.

8.4.3 mRNA Levels Of Enzymes Involved With In Free Fatty Acid Uptake And Esterification

In obese *P. obesus* leptin treatment did not significantly alter the hepatic gene expression for LPL, L-FABP and GPAT compared to saline treated control obese *P. obesus*. Similarly, in the lean leptin treated animals LPL, L-FABP and GPAT gene expression remained unaltered when compared to saline treated control animals. However, pair fed *P. obesus* demonstrated reduced LPL mRNA levels (P<0.05).

8.4.4 mRNA Levels Of Enzymes Involved In Free Fatty Acid Oxidation

In the obese animals leptin treatment did not alter the gene expression of CPT, ACC or UCP-2. Similarly in lean animals, neither leptin treatment nor pair feeding had any effect on the liver expression of CPT. The level of ACC mRNA was reduced following leptin treatment in lean animals and with pair feeding. However leptin treatment caused a specific decrease in UCP-2 expression in lean animals (P<0.05) as this reduction was not found following pair feeding.
Table 8.3: A comparison of mRNA expression for key lipid metabolising enzymes and transport proteins in lean and obese *P. obesus* and the effect of leptin treatment or pair feeding.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Lean</th>
<th>Lean Leptin</th>
<th>Pair Fed</th>
<th>Obese</th>
<th>Obese Leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPL</td>
<td>7.0±0.6</td>
<td>6.0±1.0</td>
<td>5.0±0.8§</td>
<td>7.0±2.0</td>
<td>5.0±1.0</td>
</tr>
<tr>
<td>L-FABP</td>
<td>3.0±0.3</td>
<td>3.0±0.4</td>
<td>2.0±0.2</td>
<td>5.0±1.0</td>
<td>3.0±0.3</td>
</tr>
<tr>
<td>Apo-B</td>
<td>7.0±1.0</td>
<td>6.0±0.7</td>
<td>6.0±0.6</td>
<td>5.0±1.0</td>
<td>5.0±2.0</td>
</tr>
<tr>
<td>CPT</td>
<td>4.0±0.8</td>
<td>4.0±0.4</td>
<td>8.0±2.0</td>
<td>5.0±1.0</td>
<td>8.0±2.0</td>
</tr>
<tr>
<td>ACC</td>
<td>7.0±0.5</td>
<td>5.0±0.3 †</td>
<td>4.0±0.9§</td>
<td>9.0±3.0</td>
<td>6.0±0.7</td>
</tr>
<tr>
<td>UCP-2</td>
<td>4.0±0.7</td>
<td>2.0±0.4 †</td>
<td>4.0±1.0</td>
<td>4.0±0.1</td>
<td>5.0±1.0</td>
</tr>
<tr>
<td>GPAT</td>
<td>6.0±0.8</td>
<td>5.0±0.6</td>
<td>6.0±0.6</td>
<td>7.0±0.4*</td>
<td>8.0±1.0</td>
</tr>
</tbody>
</table>

Lean and obese animals were treated for 7 days with leptin (45 mg/kg) or pair fed as described in the methods. Values shown are the mean ± S.E.M expressed as arbitrary units representing gene expression relative to β-Actin multiplied by a factor of ten.

* Indicates significant difference between lean and obese groups (P<0.05). † Indicates significant effect of leptin treatment (P<0.05). § Indicates significant effect of pair feeding (P<0.05).

8.4.5 Effect Of Leptin Treatment On Hepatic CPT Enzyme Activity

No change in hepatic CPT activity with leptin treatment was measured in the obese *P. obesus*. In lean animals leptin treatment significantly increased hepatic CPT activity compared with lean controls, whereas pair feeding had the opposite effect and reduced CPT activity (P<0.05)(Figure 8.1).

8.4.6 mRNA Levels Of Enzymes Involved With VLDL Synthesis

Leptin treatment or pair feeding had no significant effect in lean or obese animals with unaltered in Apo-B expression in all groups.
Figure 8.1: CPT activity in lean and obese *P. obesus* after leptin administration, saline control or pair feeding. Lean and obese animals were treated for 7 days with leptin (45 mg/kg) or pair fed as described in the methods. Values shown are the mean ± S.E.M. † Indicates significant effect of leptin treatment (P<0.05). § Indicates significant effect of pair feeding (P<0.05).

8.5 DISCUSSION

The aim of this study was to determine whether leptin treatment was able to modulate liver fat oxidation or gene expression of key regulatory enzymes of lipid metabolism in lean and obese *P. obesus*. Leptin treatment lowered food intake and body weight in the lean, but not the obese *P. obesus*. Reduced food intake, in lean *P. obesus* either following leptin treatment or in matched pair-fed controls suppressed hepatic ACC gene expression. Yet leptin treatment alone in lean *P. obesus* significantly increased hepatic mitochondrial β-oxidation and lowered UCP-2 mRNA, when compared to ad-libitum and pair-fed lean *P. obesus*.

Previously our group has reported the effectiveness of leptin treatment (45 mg/kg) in reducing body weight and body fatness only in lean *P. obesus* (Walder 1998). Although the chosen leptin treatment dose resulted in supraphysiological circulating leptin concentrations, which remained significantly elevated for the entire eight hours
between intraperitoneal administrations, body weight remained unaltered in the obese animals. The failure of significant peripheral concentrations to inhibit food intake, confirms the presence of considerable leptin resistance in obese *P. obesus* (Walder 1998), in a manner comparable to that reported previously in obese NZO mice (Halaas 1997).

Accompanying the changes in body composition observed in lean leptin treated animals liver β-oxidative capacity, as measured by CPT enzyme activity, was increased. Similar effects of leptin treatment have been shown in pancreatic islets (Zhou 1997) and skeletal muscle (Muoio 1997) providing evidence across a number of tissues that leptin may be an important regulator of lipid oxidation. Despite the increased CPT activity following leptin treatment there was no corresponding change in CPT mRNA levels. This may indicate that leptin regulates CPT enzyme activity independently of gene expression. Important in the *in-vivo* regulation of CPT is the cytosolic concentration of the allosteric inhibitor, malonyl-CoA, which is synthesised from citrate by ACC (McGarry 1980). ACC mRNA was comparably reduced in both lean leptin treated and pair-fed *P. obesus*. Yet the observation of elevated CPT activity only in the leptin treated group indicates that this action of leptin may be mediated by mechanisms other than the regulation of ACC gene expression. Interestingly, pair-fed *P. obesus* demonstrated reduced hepatic LPL gene expression, while leptin treatment resulted in the maintenance of LPL gene expression. Increased LPL gene synthesis may have increased triglyceride hydrolysis and hepatic fatty acid uptake (Nestel 1962), thereby maintaining CPT activity following leptin treatment. Clearly, further studies examining liver lipid flux and oxidation in leptin treated *P. obesus* is required to examine these speculations further.

Previous observations of decreased tissue triglyceride stores and increased fatty acid partitioning to oxidation following leptin treatment (Muoio 1997; Zhou 1997), could not be demonstrated in the liver, at the level of gene expression, in the current study. Leptin did not alter the gene expression of L-FABP, which is believed to be important in the cytosolic transport of fatty acids (Veerkamp 1991). Similarly, there was no alteration the mRNA levels of GPAT, which catalyses the initial intracellular re-esterification of fatty acids (Bell 1980). This contrasts studies in pancreatic islet cells and hyperleptinemic rats infused with AdCMV-leptin and normal rat islets cultured with 20 ng/ml of leptin for 48 hr (Zhou 1997) where GPAT mRNA levels are reduced in both cases. Reduced GPAT activity is supported by the demonstration of reduced re-
esterification in rodent perfused skeletal muscle exposed to leptin (Muollo 1997). Furthermore, gene expression of Apo-B, which has been utilised as an index of VLDL secretion (Chan 1997) was unaltered by leptin treatment.

Leptin was also demonstrated to specifically reduced UCP-2 gene expression, an important regulator of mammalian thermogenesis (Skulachev 1998). These current results are in contrast to previous studies in pancreatic islets and adipose tissue (Zhou 1997; Cusin 1998), where UCP-2 mRNA was increased following leptin treatment. Importantly UCP-2 expression has been found to be localised in the Kupffer cells of the liver, with very low gene expression reported in hepatocytes (Larroquey 1997). The mixed liver cell preparation used in the present study is likely to contain significant quantities of Kupffer cells, suggesting that leptin may be influencing uncoupled respiration primarily in these immune cells and thus potentially influencing immune function (Flier 1998).

In summary, leptin treatment specifically increased hepatic CPT activity and decreased UCP-2 gene expression independent of the changes in body composition in lean, leptin sensitive P. obesus. Leptin treatment did not alter food intake, body weight and liver lipid metabolism in obese P. obesus. These findings establish that leptin treatment is unlikely to significantly impact on the gene expression of key lipid metabolism enzymes, expressed primarily in hepatocytes. This would suggest that the impact of leptin treatment on lipid oxidation is tissue-specific, with the actions of leptin on liver lipid metabolism unlikely to significantly contribute to the enhancement of whole-body lipid oxidation.

This current study again demonstrates that hepatic lipid levels do not play a role in modulating liver fat gene expression. The reason behind this conclusion is the handful of changes that did occur in the liver with leptin treatment were not associated with changes in hepatic fat content.
CONCLUSIONS AND FUTURE DIRECTIONS

9.1 CONCLUSIONS

Obesity and diabetes are polygenic disorders characterised by complex metabolic and hormonal abnormalities. The sequence of disturbances which initiate insulin resistance and ultimately diabetes and beta-cell failure remains unclear. There is now significant evidence that fat accumulation and elevated FFA flux in diabetic states. The reciprocal regulation of fatty acid and glucosic oxidation, such that increased FFA flux may impair glucose oxidation, storage and transport is now well demonstrated in human and rodent studies. More recently, evidence has emerged that fat accumulation within tissues, rather than representing the passive storage, is an important regulator of substrate flux, oxidation and importantly gene expression.

It is generally accepted that increased HGO is central to the development of type II diabetes. While the mechanisms underlying this are still unclear it is now well established that elevated supply and tissue accumulation of fatty acids is an important factor in the dysregulation of hepatic glucose metabolism. This occurs both allosterically and at the gene transcriptional level, although the precise mechanisms and interactions have yet to be clarified. However it is increasingly clear that changes mediated at the level of gene transcription are central to the final metabolic outcome.

In an attempt to clarify this situation the major focus of these studies has been to determine if excessive accumulation of fat within the liver of *P. obesus* is a primary defect in the aetiology and pathogenesis of type II diabetes.

The initial study demonstrated that obese and diabetic *P. obesus* had significantly elevated body weight, adipose tissue mass and hepatic lipid accumulation. Adipose tissue FABPs were elevated in obesity and reflected the adipose tissue mass. In contrast liver cytosolic FABPs exhibited a disproportionate elevation in the ratio of L-FABP to fat in diabetic *P. obesus*. These L-FABP changes were speculated to indicate alterations in hepatic lipid metabolism, such as elevated fat oxidation which in turn may lead to
increased hepatic glucose production due to a suppressed rate of glucose oxidation and elevated rate of gluconeogenesis.

Further investigations demonstrated the development of diabetes in *P. obesus* was associated with altered expression of key genes that code for regulators of hepatic lipid uptake, storage, oxidation, export and the transcription factors important in regulating their expression. Included in these diabetic alterations was a decrease in LPL mRNA together with increases in L-FABP, CPT, ACC, GPAT, PPARα and SREBP-1C mRNA. These alterations in gene expression were suggestive of a greater partitioning of fatty acids to storage, rather than oxidation or export in diabetic *P. obesus*. Yet, hyperinsulinemic *P. obesus* that maintained normoglycemia, displayed comparable body fatness and liver triglyceride accumulation to the diabetic animals. However, rates of gene expression in the obese non-diabetic animals was not markedly altered from that of the lean normoglycemic *P. obesus*. The differences in gene expression between groups, despite similar tissue lipid accumulation argues against total accumulation of fatty acids acting as the principle regulators of gene expression. Although the mechanisms responsible for the altered gene expression are yet to be elucidated, the data suggests that hyperglycemia could be important permissive factor, acting at the level of gene expression. Importantly the results of these investigation effectively disprove the hypothesis that accumulation of fat within the liver represents a primary defect in the development of obesity and type II diabetes in *P. obesus*. However it was interesting to find that alterations in the expression of genes measured coincided with the development of diabetes. This in turn raised the possibility that manipulation of the diabetic state may also alter the expression of these hepatic lipid genes.

In the following study the diabetic state of *P. obesus* was manipulated via the use of energy restriction, previously shown to ameliorate elevated plasma insulin and glucose levels. In obese hyperinsulinemic *P. obesus* acute and chronic energy restriction normalised plasma insulin levels but did alter glucose concentrations or the level of hepatic lipid. In addition acute and chronic energy restriction both did not have a major impact on the gene expression of lipid metabolising enzymes, transport proteins or lipid responsive transcription factors. Acute and chronic energy restriction in diabetic *P. obesus* yielded unique responses. Acutely normalised glucose and insulin levels, without altering fat stores, corrected the pre-existing alterations in the transcription factors PPARα and SREBP-1C. Whereas chronic energy restriction had the same
effects as acute energy restriction in diabetic *P. obesus*, but with the additional reduction in body fat and L-FABP mRNA. These results suggested that correcting the hyperglycemia present in the obese diabetic *P. obesus* could remove the pre-existing alterations in L-FABP, PPARα and SREBP-1C gene expression but this effect did not extend to normalising CPT or GPAT mRNA level. These differential effects on gene expression suggest that additional regulatory mechanisms may be involved, these may include regulation of gene expression by transcription factors not measured in this study, such as CCAAT/enhancer-binding proteins or hepatic nuclear factors. Alternatively the lack of changes in CPT and GPAT mRNA levels may be related to the tightly regulated hepatic lipid concentration, which did not change with acute or chronic energy restriction.

Given that correction of diabetes with energy restriction was able to modulate the expression of genes and transcription factors associated with hepatic fat metabolism, the next study aimed to examine if overfeeding would promote the development of diabetes and in turn alter gene expression. The method of overfeeding employed was to supplement a sucrose solution for drinking water. Sucrose feeding in lean *P. obesus* increased body weight and body fatness, but did not change any other metabolic parameters or significantly alter the level of expression of hepatic lipid enzymes or lipid responsive transcription factors. Obese *P. obesus* tended to developcd diabetes following sucrose feeding. However these dietary-induced diabetic *P. obesus* did not have the same mRNA profile for lipid metabolising enzymes as animals with pre-existing spontaneous diabetes. This different response suggests that the genes measured in this study are not necessary for the development of diabetes. If this is the case it may represent unique differences in the regulation of the genes for PPARα, SREBP-1C, CPT, ACC, LPL and L-FABP in genetically pre-disposed obese diabetic *P. obesus*. In the diabetic *P. obesus* sucrose feeding exacerbated the pre-existing diabetes in this group of animals and reduced the level of expression of the transcription factor PPARα and lipid metabolising enzymes CPT, LPL and L-FABP which are under the transcriptional control of PPARα. These sucrose fed group C animals were the only group that demonstrated any alteration in the level of hepatic lipid accumulation thus far.
The results presented thus far suggest that normalisation of blood glucose levels in diabetic \textit{P. obesus} modulates the expression of L-FABP, PPAR\(\alpha\) and SREBP-1C, but induction of diabetes in obese animals did not alter the gene expression of the hepatic lipid metabolising enzymes or transcription factors measured. However in both of these previous studies although diabetic status was modulated hepatic lipid content remained constant, with the exception of group C sucrose fed animals which had additional changes in mRNA levels. In attempt to modulate the level of hepatic lipid accumulation and increase fat supply, independent of nutrient intake, lean and obese \textit{P. obesus} were treated with nicotine a stimulator of lipolysis. This treatment lowered body weight in the lean and obese \textit{P. obesus}. Accompanying the reduction in body weight in both groups was an increase in hepatic \(\beta\)-oxidation and in the lean group there was a significant increase in uncoupled respiration. Accompanying these oxidative changes was a increase in fat uptake in both groups as well as a uniform decrease cytosolic fat transport and VLDL synthesis. However there was no significant change in the mRNA level of the transcription factors PPAR\(\alpha\) and SREBP-1C which are thought to regulate the expression of genes for key hepatic lipid metabolising enzymes and proteins studied. Overall in the liver nicotine treatment caused a shift in the metabolic flux of fat towards oxidation and away from storage and this change was not mediated via the transcription factors PPAR\(\alpha\) and SREBP-1C.

In a further attempt to modulate hepatic lipid accumulation lean and obese animals were treated with leptin. Leptin treatment in rodents has been shown to lower body weight, decrease blood lipids and deplete liver, skeletal muscle and pancreatic intracellular triglyceride stores. Supraphysiological doses of leptin had no effect in obese and small effects in lean \textit{P. obesus}. In the liver leptin treatment resulted in a specific increase in hepatic \(\beta\)-oxidation and decreased UCP-2 gene expression independent of the changes in body composition in lean, leptin sensitive \textit{P. obesus}. Leptin treatment did not alter food intake, body weight and liver lipid metabolism in obese \textit{P. obesus}. These findings established that leptin treatment is unlikely to significantly impact on the gene expression of key lipid metabolizing enzymes, expressed primarily in hepatocytes. These results indicate that the impact of leptin treatment on lipid oxidation is tissue-specific, with the actions of leptin on liver lipid metabolism unlikely to significantly contribute to the enhancement of whole-body lipid oxidation.
In summary it was demonstrated that obese diabetic *P. obesus* have altered levels of LPL, L-FABP, CPT, ACC, GPAT, PPARα and SREBP-1C mRNAs independent of hepatic lipid concentrations. Acute and chronic reductions in blood glucose following energy restriction reduces the level of L-FABP, PPARα and SREBP-1C gene expression again independent of hepatic lipid accumulation. Induction of diabetes via sucrose over feeding resulted in the development of diabetes in obese *P. obesus*, however there was no change in the mRNA concentration of lipid metabolising enzymes and transcription factors that were known to be elevated in animals with chronic pre-existing diabetes. Increasing fat supply to the liver with nicotine treatment resulted in a shift of fat metabolism towards oxidation and away from storage. These changes appeared to be mediated by increased CPT, UCP-2 and LPL gene expression in conjunction with decreased L-FABP and Apo-B mRNA levels. Leptin treatment was only effective in lean *P. obesus* where it increased hepatic β-oxidation independent of changes in body composition.

Addressing the specific hypotheses: 1) Fat accumulation within the liver is not a primary defect in the aetiology and pathogenesis of obesity and type II diabetes. 2) Fat accumulating in the liver is not the result of changes in the gene expression of key enzymes and proteins involved with fat uptake (LPL), fat transport (L-FABP), fat oxidation (CPT and UCP-2), fat synthesis or storage (ACC and GPAT) and export of fat from the liver (Apo-B). 3) The key lipid responsive transcription factors PPARα and SREBP-1C are not altered by liver fat accumulation. However alterations in the levels of PPARα and SREBP-1C mRNAs are associated with the spontaneous development of diabetes in genetically predisposed *P. obesus*. In addition reductions in the level of gene expression of PPARα and SREBP-1C is associated with acute and chronic reductions of hyperglycemia, yet induction of diabetes via over feeding did not alter the level of their respective mRNAs.
9.2 FUTURE DIRECTIONS

It has become apparent from our investigations to date that fat accumulation within the liver is not a primary defect in the aetiology and pathogenesis of obesity and type II diabetes. It was also demonstrated that the accumulation of fat within the liver did not result from changes in the gene expression of key enzymes and proteins involved with fat uptake (LPL), fat transport (L-FABP), fat oxidation (CPT and UCP-2), fat re-esterification or storage (GPAT) and export of fat from the liver (Apo-B). This suggests that the increased liver fat levels in the obese and diabetic P. obesus are simply the result of excessive lipid uptake, synthesis and storage that occur after the onset of disease.

9.2.1 What Is The Role Of Liver Fat In The Development Of Diabetes Now?

While the studies within this thesis have demonstrated that hepatic lipid accumulation has little or no role in the development of diabetes in P. obesus, the data demonstrated that in the sucrose feeding study the group C animals had increased levels of hepatic fat accumulation after sucrose treatment and tis was associated with additional changes in hepatic lipid gene expression. This result suggests that excessive fat accumulation may still be having selected effects on lipid gene expression in the liver. Thus a study which may directly induce excessive lipid accumulation in all groups of P. obesus may further clarify the impact of fat accumulation on gene expression in the liver.

A convenient means via which to over stimulate hepatic lipid accumulation in P. obesus would be to increase the fat content of the animals diet. Previous studies in rats have shown that high fat diets induce profound and wide spread tissue insulin resistance (Oakes 1997). This is manifest as a reduced insulin stimulated oxidative and non-oxidative glucose disposal, particularly in skeletal muscle, the major site of insulin stimulated glucose disposal (Oakes 1997). In liver, fat feeding impairs insulin’s potency to suppress HGP (Oakes 1997). The high fat fed rat model of insulin resistance has been used extensively to elucidate physiological mechanisms of nonpharmacological (Kraegen 1989; Storlien 1991) and pharmacological (Storlien 1993; Oakes 1994) strategies for reversing or preventing insulin resistance. In addition, a fat feeding study would promote the accumulation of fat within the liver (Nomura 1987; Roth 1996) without stimulating lipogenesis and represent the situation in human subjects who consume diets high in fat (Howard 1997; Storlien 1997).
An alternative to promoting hepatic lipid accumulation via fat feeding would be to use a transgenic animal model that is known to have elevated lipid accumulation. One such model is a transgenic mouse that over expresses a truncated nuclear form of SREBP proteins (Shimomura 1998). These transgenic mice have a more than 20 fold elevation in the rate of hepatic fatty acid synthesis, which is thought to be due to increased ACC and FAS gene expression (Shimomura 1998), accompanied by increased levels of mRNAs for ATP citrate lyase, malic enzyme, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. All of which resulted in these transgenic animals having a coordinated activation of the entire unsaturated fatty acid biosynthetic process (Shimomura 1998). Therefore a transgenic model which over expressed the SREBP-1C isoform may be expected to have increased hepatic lipid deposition as a result of alterations in the mRNA levels of ACC, FAS, GPAT and Apo-B all of which are thought to be transcriptionally regulated by SREBP-1C. Thus it may be speculated that the increased deposition of fat within the liver following fat feeding or as a result of a genetic manipulation would modulate lipid gene expression so as to increase fat oxidation which may promote the development of diabetes.

9.2.2 The Link Between PPARα And SREBP-1C And Hyperglycemia

The results from the current investigations demonstrated that increased PPARα and SREBP-1C gene expression was associated with hyperglycemia, furthermore it was shown that normalising blood glucose levels also resulted in a reduction in PPARα and SREBP-1C gene expression. This association between PPARα, SREBP-1C and diabetes raises a number of questions which may be answered in future studies. Firstly, the association between PPARα and SREBP-1C mRNA levels and hyperglycemia implics that an unknown metabolic coupler may link glucose metabolism with lipid metabolism and gene transcription. Following on from this, if a metabolic coupler can be found, is it together with PPARα and SREBP-1C involved with the transcriptional control of other genes not measured that may be important in the development of diabetes.
9.2.2.1 Carbohydrate Regulation Of Gene Expression

In relation to the metabolic coupler that links glucose metabolism and gene transcription, studies to date have not yet revealed the nature of this compound. However, several candidates have been suggested and are worthy of consideration. The earliest work on this question came from Mariash & Oppenheimer (1983), who studied the synthesis of malic enzyme in hepatocytes. The critical step in signal generation was localised to a mitochondrial step at or downstream from pyruvate oxidation (Mariash 1983). A mitochondrial origin for the mediator was supported by the observation that dichloroacetic acid, a stimulator of pyruvate dehydrogenase, could also induce malic enzyme synthesis (Mariash 1983). This response did not require insulin and occurred at levels of glucose normally unable to stimulate malic enzyme synthesis (Mariash 1983). Thus, pyruvate oxidation was considered essential for generation of the metabolic signal (Mariash 1983).

A second possible metabolic coupler is glucose-6-phosphate. Fougelle et al. (1992) observed that 2-deoxyglucose stimulated expression of FAS and ACC genes in cultured adipose tissue and suggested that the generation of glucose-6-phosphate was proposed as the critical step in the process. However, it proved difficult to reconcile this hypothesis with the effects of 2-deoxyglucose in hepatocytes (Towle 1997). Numerous studies have reported no effect of this glucose analog on production of the lipogenic enzymes (Towle 1997). It could be argued that the presence of glucose-6-phosphatase in the hepatocyte might prevent the accumulation of 2-deoxyglucose-6-phosphate necessary for stimulation (Towle 1997). In an effort to overcome this, Rencurel et al. (Rencurel 1996) used high (30 mM) concentrations of 2-deoxyglucose but found no stimulation of GLUT2 mRNA. Thus, glucose-6-phosphate is not a likely candidate for the metabolic coupler in hepatocytes, but it may be important in the adipocyte (Towle 1997).

Doiron et al. (1996) recently suggested that the signal for generation of the metabolic coupler arises not from glycolysis but from the alternative pathway for glucose oxidation, the pentose phosphate pathway. This suggestion was based on the observation that xylitol at a concentration of 0.5 mM stimulated L-PK promoter activity to the same extent as 20 mM glucose in mhAT3F cells (Doiron 1996). Since xylitol is converted to xylulose-5-phosphate and enters the pentose phosphate pathway, xylulose-5-phosphate was proposed as the key intracellular signalling molecule (Doiron 1996). Intriguingly, Nishimura et al. (Nishimura 1994; Nishimura 1995) recently
identified xylulose-5-phosphate as a mediator for early effects of glucose on the activity of fructose-6-phosphate kinase/fructose-2,6-bisphosphatase. This metabolite accumulated in the liver within 2 min of glucose perfusion, slightly preceding increases in fructose-2,6-bisphosphate activity (Nishimura 1994; Nishimura 1995). Xylulose-5 phosphate activated a form of protein phosphatase 2A that dephosphorylated the bifunctional enzyme to activate its kinase activity and led to generation of fructose-2,6-bisphosphate (Nishimura 1994; Nishimura 1995). It is conceivable that such a phosphatase might also act on a transcription factor that interacts with the lipogenic enzyme genes to activate their expression. One such transcription factor may be SREBP-1C, given the association we have demonstrated between this transcription factor and hyperglycemia.

9.2.2.2 Other Genes Regulated By PPARα And SREBP-1C

It is already known that both PPARα and SREBP-1C regulate the transcription of a number of other genes important in hepatic metabolism which were not measured in the present investigations. These include the expression of genes implicated in intra- and extracellular lipid metabolism, such as genes encoding enzymes involved with peroxisomal β-oxidation pathway (Osumi 1991; Tugwood 1992; Zhang 1992; Bardot 1993; Marcus 1993), cytochrome P450 (Muehrhoff 1992; Palmer 1994), 3-hydroxy-3-methylglutarly-CoA synthase (Rodriguez 1994), medium chain acyl-CoA synthetase (Gulick 1994), and the acyl-CoA synthetase gene (Schoonjans 1995). An alteration in the transcriptional regulation of any one of the gene may directly or indirectly be associated with the development of diabetes.

Taking both the previous sections into consideration, together with the studies that form this thesis, it is apparent that PPARα and SREBP-1C are under a complex system of control. Furthermore this complex regulation extends to the target genes of PPARα and SREBP-1C which are also regulated by other transcription factors not measured in the current studies, all of which is part of the complex which forms the animal as a whole. Therefore future studies may simply expand on the current line of investigation and measure transcription factors and genes not covered by the present studies. However, even though such a line of research may uncover as yet undiscovered relationships between diabetes, transcription factors and lipid metabolism, it would be very time consuming and still may not answer the questions raised in this thesis. An alternative
approach would be to continue measuring mRNA levels for key points of lipid metabolism in a primary cell culture system, using hepatocytes from lean, obese and diabetic P. obesus. In this system it may be possible to further elucidate how carbohydrate metabolites such as xylose-5-phosphate influence PPARα and SREBP-1C gene expression and how this regulates lipid gene expression. In addition within such a system new methodologies such as antisense technology which can be used to selectively inhibit PPARα and SREBP-1C gene expression (Mandrup 1998). Thus enabling the genes which are under the control of PPARα and SREBP-1C to be studied separate from the influence these two transcription factors have over them. Finally the use of a microarray system may allow a large number of genes to be investigated at one time (Schena 1995). Which will allow the effects of any experimental intervention to be studied in a broader context, as the level of expression of many genes can be studied at once.
REFERENCES


References


References


References


References


Brandes R, RM Kaikaus, N Lysenko, RK Ockner and NM Bass (1990). Induction of fatty acid binding protein by peroxisome proliferators in primary hepatocyte cultures


References


References


glucose tolerance. A 20-year follow-up of the Finnish and Dutch cohorts of the Seven Countries Study. Diabetes Care 18(8): 1104-12.


References


References


Power GW, P Yaqoob, DJ Harvey, EA Newsholme and PC Calder (1994). The effect of dietary lipid manipulation on hepatic mitochondrial phospholipid fatty acid


Puhakainen I and H Yki-Jarvinen (1993). Inhibition of lipolysis decreases lipid oxidation and gluconeogenesis from lactate but not fasting hyperglycemia or total hepatic glucose production in NIDDM. Diabetes 42: 1694-1699.


References


References


References


References


Thomas C, C Lowy and J Evans (1986). The occurrence of different forms of fatty acid binding protein in diabetic and normal placentae of both humans and rabbits may explain altered lipid transfer in diabetic pregnancy. Diabetologia 29: 600A-601A.


Thorburn AW, B Gumbiner, F Bulacan, P Wallace and RR Henry (1990). Intracellular glucose oxidation and glycogen synthase activity are reduced in non-insulin-
dependent (Type II) diabeted independent of impaired glucose uptake. J Clin Invest 85: 522-529.


Vionnet N, EH Hani, S Lessege, A Philippi, J Hager, M Varret, M Stoffel, Y Tanizawa, KC Chiu, B Glaser, MA Permutt, P Passa, F Demenais and P Froguel


