NOVEL GENES IN THE LIVER OF DIABETIC *PSAMMOMYSES OBESUS*

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Abstract

Type 2 diabetes mellitus is a metabolic disease characterised by defects in insulin secretion and insulin action and disturbances in carbohydrate, fat and protein metabolism. Hepatic insulin resistance contributes to hyperglycemia and also leads to disturbances in fat metabolism in type 2 diabetes. *Psammomys obesus* is a unique polygenic animal model of type 2 diabetes and obesity, ideally suited for studies examining physiological and genetic aspects of these diseases.

To identify metabolic abnormalities potentially contributing to the obesity and diabetes phenotype in *P. obesus*, indirect calorimetry was used to characterise whole body energy expenditure and substrate utilisation. Lean-NGT, obese-IGT and obese-diabetic animals were examined in fed and fasted states and following 14 days of dietary energy restriction.

Energy expenditure and fat oxidation were elevated in the obese-IGT and obese-diabetic groups in proportion to body weight. Glucose oxidation was not different between groups. Obese-diabetic *P. obesus* displayed elevated nocturnal blood glucose levels and fat oxidation. Following 14 days of dietary energy restriction, body weight was reduced and plasma insulin and blood glucose levels were normalised in all groups. Glucose oxidation was reduced and fat oxidation was increased. After 24 hours of fasting, plasma insulin and blood glucose levels were normalised in all groups. Energy expenditure and glucose oxidation were greatly reduced and fat oxidation was increased. Following either dietary energy restriction or fasting, energy expenditure, glucose oxidation and fat oxidation were not different between groups of *P. obesus*.

Energy expenditure and whole body substrate utilisation in *P. obesus* was similar to that seen in humans. *P. obesus* responded normally to short term fasting and dietary energy restriction. Elevated nocturnal fat oxidation rates and plasma glucose levels in obese-diabetic *P. obesus* may be an important
factor in the pathogenesis of obesity and type 2 diabetes in these animals. These studies have further validated *P. obesus* as an ideal animal model of type 2 diabetes and obesity.

It was hypothesised that many genes in the liver of *P. obesus* involved in glucose and fat metabolism would be differentially expressed between lean-NGT and obese-diabetic animals. These genes may represent significant factors in the pathophysiology of type 2 diabetes. Two gene discovery experiments were conducted using suppression subtractive hybridisation (SSH) to enrich a cDNA library for differentially expressed genes.

Experiment 1 used cDNA dot blots to screen 576 clones with cDNA derived from lean-NGT and obese-diabetic animals. 6 clones were identified as overexpressed in lean-NGT animals and 6 were overexpressed in obese-diabetic animals. These 12 clones were sequenced and SYBR-Green PCR was used to confirm differential gene expression. 4 genes were overexpressed (>1.5 fold) in lean-NGT animals and 4 genes were overexpressed (>1.5 fold) in obese-diabetic animals.

To explore the physiological role of these genes, hepatic gene expression was examined in several physiological conditions. One gene, encoding thyroxine binding globulin (TBG), was confirmed as overexpressed in lean-NGT *P. obesus* with *ad libitum* access to food, relative to both obese-IGT and obese-diabetic animals. TBG expression decreased with fasting in all animals. Fasting TBG expression remained greater in lean-NGT animals than obese-IGT and obese-diabetic animals. TBG expression was not significantly affected by dietary energy restriction. TBG is involved in thyroid metabolism and is potentially involved in the regulation of energy expenditure.

Fasting increased hepatic site 1 protease (S1P) expression in lean-NGT animals but was not significantly affected in obese-IGT and obese-diabetic animals. S1P expression was not significantly affected by dietary energy restriction. S1P is involved in the proteolytic processing of steroid response
element binding proteins (SREBP). SREBPs are insulin responsive and are known to be involved in lipid metabolism.

Gene expression studies found TBG and S1P were associated with obesity and diabetes. Future research will determine whether TBG and S1P are important in the pathogenesis of these diseases.

Experiment 2 used SSH and cDNA microarray to screen 8064 clones. 223 clones were identified as overexpressed in lean-NGT *P. obesus* and 274 clones were overexpressed in obese-diabetic *P. obesus* (p<0.05). The 9 most significantly differentially expressed clones identified from the microarray screen were sequenced (p<0.01). 7 novel genes were identified as well as; sulfotransferase related protein and albumin. These 2 genes have not previously been associated with either type 2 diabetes or obesity. It is unclear why hepatic expression of these genes may differ between lean-NGT and obese-diabetic groups of *P. obesus*. Subsequent studies will explore the potential role of these novel and known genes in the pathophysiology of type 2 diabetes.
1.1 TYPE 2 DIABETES MELLITUS

1.1.1 Definition and Diagnosis
Type 2 diabetes mellitus is a metabolic disease characterised by defects in insulin secretion and insulin action, and disturbances in carbohydrate, fat and protein metabolism that result in hyperglycemia (ADA, 2000). Symptoms associated with type 2 diabetes may include polyuria, polydipsia and weight loss which may be accompanied by polyphagia (ADA, 2000). Type 2 diabetes accounts for over 85 percent of all cases of diabetes worldwide (WHO, 1994). Impaired glucose tolerance (IGT) is a metabolic stage between normal glucose homeostasis and diabetes. IGT is a major risk factor for the subsequent development of type 2 diabetes (ADA, 2000).

The World Health Organisation (WHO) define diabetes and IGT based on plasma glucose concentrations following overnight fasting and/or two hours after a 75g glucose load assessed by an oral glucose tolerance test (OGTT). The WHO criteria are outlined below (WHO, 1994).

<table>
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<tr>
<td>Venous Plasma Glucose (mmol/L)</td>
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<td>Fasting</td>
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<tr>
<td>Normal Glucose Tolerance (NGT)</td>
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Type 2 diabetes is associated with an increased risk of mortality, and is among the five leading causes of death in most developed countries (Caterson and Zimmet, 1997). Furthermore the increased morbidity accompanying type 2 diabetes is associated with complications including; hypertension and
macrovascular diseases such as ischemic heart disease, stroke, peripheral vascular damage, as well as microvascular complications including retinopathy, neuropathy and nephropathy (Klein, 1995). Within Australia the annual cost of diabetes is estimated to be A$1 billion (McCarty et al., 1996).

The metabolic syndrome, also referred to as syndrome X, represents a group of metabolic disorders that are risk factors for cardiovascular disease (Reaven, 1988; Zimmet et al., 2001). Definition of the metabolic syndrome has recently been refined as; the presence of glucose intolerance (including type 2 diabetes, IGT or insulin resistance) and at least two of the following; hypertension, obesity dyslipidemia or microalbuminuria (Alberti and Zimmet, 1998). Hyperuricaemia, hypercoagulability and hyperleptinaemia have also been proposed as components of the metabolic syndrome (Zimmet et al., 2001). Insulin resistance has been proposed as one of the key components of the metabolic syndrome.

1.1.2 Prevalence
The prevalence of type 2 diabetes is increasing rapidly worldwide, with an estimated 150 million people currently affected, and the prevalence expected to increase by 40 percent by 2010 (McCarty and Zimmet, 1994). Type 2 diabetes occurs in almost all populations, although prevalence rates vary considerably (King and Rewers, 1993). The recent AusDiab study found the prevalence of type 2 diabetes in Australian men and women to be 8 percent and 6.8 percent respectively (Dunstan et al., 2002). A similar prevalence rates is seen in the American population (Mokdad et al., 2000).

The prevalence of type 2 diabetes is substantially higher in many “traditional” populations that have adopted a “modern” lifestyle including Nauruans (41%) (Dowse et al., 1991) and Pima Indians (50%) (Knowler et al., 1990), with the latter displaying the highest known prevalence of type 2 diabetes worldwide. Estimates of the prevalence of diabetes in Australian Aborigines have ranged from 4.5 to 12.7 % (Guest and O'Dea, 1992).
The prevalence of insulin resistance in subjects with one or more components of the metabolic syndrome was examined in the Bruneck Study (Bonora et al., 1998). Insulin resistance was present in the majority of subjects with type 2 diabetes (83.9%), IGT (65.9%) and hypertension (58.0%). Insulin resistance was also common in subjects with dyslipidemia (hypercholesterolemia, low HDL cholesterol or hypertriglyceridemia). Furthermore, the prevalence of insulin resistance increased when subjects displayed more than one metabolic disturbance (Bonora et al., 1998).

A large scale prospective study in Botnia examined the risk of CVD in subjects with the metabolic syndrome as defined above (Isomaa et al., 2001). In women and men, respectively, the metabolic syndrome was seen in 10 and 15% of subjects with NGT, 42 and 64% of those with impaired fasting glucose or IGT, and 78 and 84% of those with type 2 diabetes. The risk for coronary heart disease and stroke was increased threefold and cardiovascular mortality was increased fivefold in subjects with the syndrome (Isomaa et al., 2001).

1.1.3 Aetiology

The rapid increase in the incidence of both obesity and type 2 diabetes in “traditional populations” such as the Pima Indians and Nauruans parallels the modernisation of their lifestyle. The “thrifty genotype” hypothesis, originally proposed by Neel (1962), suggests that evolution has favored individuals who could efficiently store food energy as fat during periods where food supplies were plentiful, as they were better equipped to survive the subsequent periods of famine. This “thrifty genotype” has become disadvantageous with the transition from a “traditional” to a “modern” lifestyle, which is characterised by a constant supply of food. The increased consumption of energy dense foods with a concomitant decrease in physical activity in the current environment has resulted in a rapid increase in the incidence of obesity and type 2 diabetes (Zimmet et al., 1990). Both genetic and environmental factors contribute to the development of type 2 diabetes.
1.1.3.1 Genetic Factors

Since the “thrifty genotype” hypothesis was first proposed 40 years ago, various studies have sought to determine the genetic contribution to the development of type 2 diabetes.

Population studies have examined the effect of foreign admixture on the prevalence of diabetes in “traditional” populations. Despite the same environment the prevalence of type 2 diabetes amongst full blooded Nauruans and those with Caucasian admixture were 83 and 17 percent respectively for subjects over the age of 60 (Serjeantson et al., 1983). Similar results were found with Australian Aborigines (Williams et al., 1987) and Pima Indians (Knowler et al., 1990).

Family studies have demonstrated an increased risk of developing type 2 diabetes if a first degree relative is diabetic (Keen et al., 1982; Cheta et al., 1990; Thomas et al., 1994). A study in Pima Indians demonstrated an increased risk of developing type 2 diabetes if one parent was diabetic (2.3 times) and even greater risk if both parents were diabetic (3.9 times) (Knowler et al., 1981). However these studies may overestimate the heritability of type 2 diabetes due to the difficulty in distinguishing between genetic factors and the shared family environment.

Studies examining the concordance rates of type 2 diabetes between monozygotic twins, who share 100 percent of their genes, and dizygotic twins, who share only 50 percent, provide evidence of heritability without the influence of familial environmental effects. Matsuda & Kuzuya (1994) found concordance rates in Japanese monozygotic and dizygotic twins were 87 and 43 percent respectively. A similar study in American monozygotic and dizygotic twins found concordance rates of 58 and 17 percent respectively (Newman et al., 1987). Despite the lower concordance rates in the American twins, both of these studies demonstrated higher concordance rates between monozygotic twins, strongly suggesting that genetic factors contribute to the development of type 2 diabetes. There is also evidence to suggest that insulin resistance is a heritable trait as twin studies have found insulin resistance
correlates within monozygotic twins, but not dizygotic twins (Lehtovirta et al., 2000).

Population, family and twin studies have all demonstrated the significance of genetic factors in the development of type 2 diabetes. However, type 2 diabetes is a heterogeneous disease with many subtypes and the mode of inheritance remains controversial (McCarthy and Menzel, 2001).

Type 2 diabetes occurs more often when diabetes is present maternally rather than paternally (Pettitt et al., 1988; Alcolado and Alcolado, 1991; Thomas et al., 1994). The “thrifty phenotype” hypothesis proposes that the intrauterine environment may contribute to the development of type 2 diabetes later in life (Hales and Barker, 1992). Alternatively imprinting of maternal genes may occur, or the mitochondrial genome may be important as mitochondrial genes are inherited maternally. Mutations have been described in mitochondrial DNA that cause a subtype of diabetes, which includes hearing loss (Ballinger et al., 1992; Reardon et al., 1992). However, mitochondrial mutations are not a common cause of type 2 diabetes in the general population (McCarthy and Menzel, 2001).

Maturity onset diabetes of the young (MODY) is a heterogeneous subtype of type 2 diabetes, which accounts for 2-5 percent of all cases of type 2 diabetes (Froguel, 1998). MODY is characterised by autosomal dominant inheritance of diabetes with an early age of onset and a primary defect in insulin secretion (Froguel, 1998). Five different variations of MODY have been described, and each is the result of a defect in a single gene. MODY2 results from a glucokinase mutation (Froguel et al., 1992) and MODY4 results from a mutation in the insulin promoter factor 1 (Stoffers et al., 1997). Mutations in the hepatocyte nuclear factors HNF-4α, HNF-1α and HNF-1β are responsible for MODY 1, 3 and 5 respectively (Yamagata et al., 1996a; Yamagata et al., 1996b; Horikawa et al., 1997). Recently discovered mutations in the transcription factor neurogenic differentiation 1/β-cell E-box transactivator 2 (NeuroD1/Beta2) are a rare cause of MODY (Malecki et al., 1999).
Although specific genes have been identified as contributing to MODY and mitochondrial diabetes, the mode of inheritance and the genes involved in the more common forms of type 2 diabetes remain uncertain (McCarthy et al., 1994).

1.1.3.2 Environmental Factors

The importance of environmental factors in the development of type 2 diabetes has been demonstrated in a study of Pima Indians (Ravussin et al., 1994). Arizona Pima Indians have adopted a “modern” lifestyle and have the highest known prevalence of type 2 diabetes in the world (37% in females and 54% in males). In contrast Mexican Pima Indians continue to lead a “traditional” lifestyle and have very low prevalence of diabetes (11% in females and 6% in males). This discrepancy is present despite the common genetic background of these two populations. Therefore, an individual’s genotype can determine their susceptibility to developing type 2 diabetes, but environmental factors ultimately determine whether or not they become diabetic (Ravussin et al., 1994).

The Nurses’ Health Study, a 16 year prospective study of 84,941 women found overweight or obesity was the most important predictor of type 2 diabetes development. Other important factors included; lack of exercise, a poor diet (high trans fat content and glycemic load, low in cereal fibre and low ratio of polyunsaturated to saturated fat), current smoking and abstinence from alcohol (Hu et al., 2001).

Obesity is a major risk factor for type 2 diabetes. Longitudinal studies have demonstrated an increased risk of developing type 2 diabetes in obese subjects relative to lean controls (Haffner et al., 1990; Everhart et al., 1992). Furthermore, this risk increases with the duration of obesity (Everhart et al., 1992). Central obesity is a strong risk factor for type 2 diabetes independent of total adiposity (Bergstrom et al., 1990; Haffner et al., 1990). Therefore, both the accumulation and distribution of body fat are important factors in the development of type 2 diabetes. Genetic factors are also involved in both the
accumulation and distribution of body fat (Bouchard et al., 1988; Bouchard et al., 1990; Allison et al., 1996).

A high energy intake and in particular a high fat intake have been associated with the development of type 2 diabetes in cross sectional (Tsunehara et al., 1990; Marshall et al., 1991) and longitudinal (Marshall et al., 1994) studies. Low levels of physical activity also increase the risk of subsequently developing type 2 diabetes, independent of obesity (Manson et al., 1992; Lynch et al., 1996; Hu et al., 1999). A high energy, high fat diet combined with low levels of physical activity are important environmental factors contributing to the development of type 2 diabetes. A high energy intake and low energy expenditure will also increase adiposity, further increasing the likelihood of developing diabetes.

The nutrition principles recommended by the American Diabetes Association (ADA) for treatment and prevention of type 2 diabetes include; weight loss by reducing energy intake and increasing physical activity levels, and reducing total fat and saturated fat contents to less than 30 and 10 percent of total energy intake respectively (Franz et al., 2002). Lifestyle interventions are likely to be particularly important for individuals with an increased genetic susceptibility to the development of diabetes. Strategies to promote lifestyle changes within Western populations to reduce the prevalence of type 2 diabetes have largely been ineffectual, as prevalence rates continue to increase (Zimmet et al., 2001). Gene discovery studies can identify novel factors involved in the pathogenesis of type 2 diabetes, potentially leading to new therapeutic approaches.

1.1.4 Regulation of Glucose Homeostasis

Glucose is the primary fuel for the central nervous system (CNS), therefore blood glucose levels must be carefully regulated to ensure adequate supply to this essential organ. The regulation of glucose homeostasis can be separated into two phases, that following the ingestion of an oral glucose load or mixed meal (postprandial) and that in the 6 to 12 hours allowing ingestion
Postprandial and postabsorptive glucose metabolism is summarised in Figure 1.1.

**Figure 1.1.** Glucose metabolism in the postprandial and postabsorptive phases (Dinneen *et al.*, 1992).

### 1.1.4.1 Postprandial Glucose Homeostasis

Plasma glucose concentrations increase following glucose ingestion. To utilise the available glucose and maintain plasma concentrations within the normal range three simultaneous processes occur; increased pancreatic insulin secretion, suppression of endogenous glucose production (predominantly liver), and increased glucose uptake in splanchnic tissues (liver and gut) and peripheral tissues (predominantly skeletal muscle and adipose tissue) (DeFronzo, 1992; Dinneen *et al.*, 1992).

The pancreas, skeletal muscle, adipose tissue, and liver are of primary importance in maintaining glucose homeostasis and defects in each of these tissues are potentially involved in the pathogenesis of type 2 diabetes.

#### 1.1.4.1.1 Pancreas

Insulin and glucagon are the key pancreatic hormones responsible for maintaining glucose homeostasis. The postprandial increase in plasma glucose signals the β-cells of the pancreas to secrete insulin, while suppressing glucagon secretion from the α-cells of the pancreas.
Following glucose ingestion, two distinct phases of insulin secretion occur. The first phase is characterized by a rapid rise in plasma insulin and is completed within 10 minutes. The second phase begins within 10 to 20 minutes of glucose ingestion and is of greater duration and magnitude (Pratley and Weyer, 2001). Insulin stimulates glucose uptake in peripheral tissues and suppresses endogenous glucose production (EGP), thereby reducing plasma glucose concentrations (DeFronzo, 1992; Dinneen et al., 1992).

The relationship between the plasma glucose concentration and the mean plasma insulin response to a 100g oral glucose load in normoglycemic, impaired glucose tolerant and hyperglycemic subjects resembles an inverted "U" shape, and is referred to as Starling's Curve of the pancreas (Figure 1.2) (DeFronzo, 1988). This same relationship is seen between fasting plasma glucose and insulin concentrations with the progression from NGT to diabetes (DeFronzo et al., 1989). As fasting plasma glucose concentrations increase, there is a progressive increase in insulin secretion. Overt diabetes is established as plasma glucose concentrations increase further and the compensatory increase in insulin secretion can not be maintained, leading to β-cells failure and a further increase in plasma glucose (DeFronzo, 1988).

![Figure 1.2. Starling's curve of the pancreas (DeFronzo, 1988).](image)

Several disturbances in insulin secretion occur with type 2 diabetes including delayed or blunted first phase insulin secretion and fasting hyperinsulinemia, indicative of insulin resistance. Suppressing first phase insulin secretion with somatostatin has been shown to reduce intravenous glucose tolerance (Calles-Escandon and Robbins, 1987). The first phase insulin secretion is particularly
important for suppression of EGP (Luzi and DeFronzo, 1989) and may also prime insulin sensitive tissues for glucose uptake (Castillo et al., 1994).

Several cross sectional studies have shown blunted or delayed first phase insulin secretion in subjects with type 2 diabetes (Meneilly et al., 1993), as well as those with IGT (Matsumoto et al., 1997) and the offspring of diabetic parents (Pimenta et al., 1995; Thorburn and Proietto, 1999). Several longitudinal studies have shown that a low acute insulin secretory response is a predictor of diabetes development (Skarfors et al., 1991; Lillioja et al., 1993), however, other studies have found conflicting results (Warram et al., 1990; Martin et al., 1992). A longitudinal study in Pima Indians found the acute insulin response declined 27 percent with the transition from NGT to IGT and another 51 percent with the transition from IGT to diabetes. In contrast AIR increased 30 percent in subjects that did not progress to diabetes (Weyer et al., 1999).

The eventual failure of the pancreas to sustain the hyperinsulinemia necessary to compensate for insulin resistance is a necessary step for the development of overt type 2 diabetes. The cause of this pancreatic failure remains to be elucidated. Hyperglycemia itself, or “glucotoxicity”, has been proposed as the cause of β-cell failure in type 2 diabetes (Garvey, 1992). “Lipotoxicity” has also been suggested, as long term exposure of pancreatic islets to excess free fatty acids inhibits glucose induced insulin secretion and biosynthesis and may also lead to apoptosis (Unger, 1995).

1.1.4.1.2 Peripheral and Hepatic Tissues
Glucose clamp studies, which maintain the plasma insulin concentration at a fixed level, have been used to examine insulin stimulated glucose disposal under various glucose concentrations. Del Prato et al (1993) performed three clamp studies in NGT and type 2 diabetic subjects. Under euglycemic conditions glucose uptake was decreased in diabetic subjects. Glycolysis, glycogen synthesis, and glucose oxidation were reduced and nonoxidative glycolysis and lipid oxidation were increased. Hyperglycemia and
hyperinsulinemia were each able to normalise whole body glucose uptake in diabetic subjects. Hyperinsulinemia also normalised glycogen synthesis and lipid oxidation but did not normalise the reduced glucose oxidation or the increased nonoxidative glycolysis. Hyperglycemia increased glycogen synthesis, but did not normalise the defects in glucose and fat oxidation or nonoxidative glycolysis. These studies highlighted multiple defects in glucose metabolism, some of which can be overcome by hyperinsulinemia and hyperglycemia.

Quantitatively, glucose clamp studies have generally found reduced glucose disposal, predominately skeletal muscle to be the most important determinate of hyperglycemia in type 2 diabetic subjects. Reduced suppression of EGP and reduced splanchnic glucose uptake only made minor contributions to hyperglycemia (DeFronzo et al., 1985; Golay et al., 1988a; Golay et al., 1988b; Del Prato et al., 1993).

Several studies have examined glucose disposal following an oral glucose load or mixed meal. Postprandial glucose appearance is elevated in type 2 diabetic subjects, relative to NGT controls (Firth et al., 1986; Ferrannini et al., 1988; McMahon et al., 1989; Mitrakou et al., 1990; Fery et al., 1993; Kelley et al., 1994). However, controversy exists over the relative contribution of reduced glucose disposal and elevated EGP to postprandial hyperglycemia.

Kelley et al (1994) examined type 2 diabetic and controls subjects following a mixed meal. Cumulative glucose appearance over the 5 hour period was elevated in the diabetic subjects and was entirely due to reduced suppression of EGP. Cumulative glucose utilisation and oxidation were not different to control subjects, nor was splanchnic glucose uptake. Similar results were seen by Fery et al (1993) following a 75g glucose load. Cumulative glucose disposal over the entire 5 hour period was not different to controls, and the increased glucose appearance was entirely due to elevated EGP. Mitrakou et al (1990) also found a reduced suppression of EGP in diabetic subjects after a 75g glucose load, with no difference in skeletal muscle glucose uptake between groups. Type 2 diabetic subjects displayed reduced splanchnic
glucose uptake in the study by (Mitrakou et al., 1990), however this was not found in other studies.

Elevated EGP was also found in type 2 diabetic subjects in a number of other studies (Firth et al., 1986; Ferrannini et al., 1988; McMahon et al., 1989; Fery et al., 1993). Ferrannini et al (1988) found that reduced postprandial glucose utilisation in diabetic subjects was quantitatively more important than elevated EGP following an oral glucose load, however the duration of this study was 3.5 hours, compared to 5 hours for the studies described above.

Taken together the results of these studies indicate both hepatic and peripheral disturbances contribute to postprandial hyperglycemia.

Quantitatively studies examining oral disposal of glucose or mixed meals attribute a greater proportion of postprandial hyperglycemia to hepatic defects than those studies utilising the glucose clamp technique. Although glucose clamp studies are often considered to be the gold standard for assessing insulin resistance, the methodological differences between these two measures of insulin stimulated glucose metabolism limit the conclusions that can be drawn regarding peripheral glucose disposal and EGP in free living subjects.

Within the liver insulin suppresses EGP by decreasing the transcription of gluconeogenic enzymes encoding phosphoenol pyruvate carboxykinase (PEPCK), fructose 1,6 bisphosphatase and glucose 6-phosphatase. Insulin also decreases the transcription of glycolytic enzymes including glucokinase and pyruvate kinase and lipogenic enzymes including fatty acid synthase and acetyl CoA carboxylase (Saltiel and Kahn, 2001). Insulin may regulate the transcription of many of these enzymes via sterol regulating element binding proteins (SREBP), which are insulin responsive transcription factors involved in fat and cholesterol metabolism (Osborne, 2000).

Insulin not only regulates glucose metabolism directly through actions on hepatic and peripheral tissues, but indirectly through actions on fat metabolism. In adipose tissue lipolysis is inhibited by insulin (Swislocki et
Adipose tissue is also insulin resistant in type 2 diabetic subjects and is associated with increased triglyceride breakdown and elevated plasma free fatty acid (FFA) concentration (Golay et al., 1987; Skowronski et al., 1991). An elevated FFA concentration could contribute to peripheral and hepatic insulin resistance.

Insulin resistance is also associated with obesity, particularly central obesity. Approximately 40 percent of overweight subjects are insulin resistant, and insulin resistance increases with weight gain and decreases with weight loss (Ferrannini et al., 1991; Ferrannini et al., 1997). Central obesity has also been associated with insulin resistance. Central adipose stores have a greater proportion of visceral adipose tissue, which is more metabolically active than subcutaneous adipose tissue (Montague and O'Rahilly, 2000). Visceral adipose tissue drains directly to the liver via the portal vein contributing to hepatic insulin resistance (Kissebah and Krakower, 1994). The liver is therefore exposed to an increased flux of FFA and precursors for glucose synthesis, promoting hepatic glucose production and VLDL secretion.

Elevated plasma FFA concentration can lead to hepatic accumulation of triglycerides and fatty acid derived metabolites, including diacylglycerol, fatty acyl CoA and ceramides, with net effect being the induction of hepatic insulin resistance (Kim et al., 2001; Kelley and Goodpaster, 2001). The increased hepatic FFA availability contributes to elevated EGP (Ferrannini et al., 1983; Rebrin et al., 1995) due to increased gluconeogenesis (Morand et al., 1993). The elevated plasma FFA concentration, along with hyperinsulinemia, also increases hepatic very low density lipoprotein (VLDL) triglyceride secretion (Malmstrom et al., 1997). Hepatic glucose and fat metabolism are therefore interdependent.

An elevation in plasma FFA concentration also inhibited insulin stimulated glucose disposal in skeletal muscle of normoglycemic and hyperglycemic subjects (Boden et al., 1994; Boden and Chen, 1995). This reduced glucose disposal was found to be due to reduced glucose uptake and subsequently
reduced glucose oxidation and glycogen synthesis (Boden et al., 1994; Boden and Chen, 1995).

In summary, reduced suppression of EGP is a major cause of postprandial hyperglycemia. Reduced peripheral glucose disposal also contributes to the elevated blood glucose levels. Increased lipolysis in adipose tissue contributes to hepatic and peripheral insulin resistance.

1.1.4.2 Postabsorptive Glucose Homeostasis
During the postabsorptive phase glucose homeostasis is dependent on the balance between whole body glucose disposal and endogenous glucose production (predominantly by the liver). In normoglycemic subjects EGP is approximately 2mg/kg/min (DeFronzo, 1988). Approximately 75-80 percent of postabsorptive glucose disposal is non-insulin dependent and predominantly occurs in the CNS and splanchnic tissues. The remaining 20-25 percent of glucose disposal is insulin dependent and occurs in peripheral tissues, predominantly skeletal muscle and also adipose tissue (DeFronzo et al., 1985; Baron et al., 1988; Ferrannini and Groop, 1989).

Glucagon stimulates gluconeogenesis and EGP as well as lipolysis. Del Prato et al (2002) has suggested type 2 diabetic subjects may display elevated plasma glucagon concentrations and may also have increased sensitivity to the hormone. These disturbances could increase EGP, contributing to hyperglycemia in type 2 diabetic subjects. However, a causative relationship between elevated plasma glucagon and hyperglycemia has not been established.

Several studies have examined whole body glucose oxidation rates in the postabsorptive phase. Bogardus et al (1984) found glucose oxidation to be reduced and fat oxidation to be increased in type 2 diabetic subjects. Similarly Franssila-Kallunki and Groop (1992) found increased fat oxidation. However, other studies have found whole body glucose oxidation and fat oxidation rates in diabetic and subjects to be similar to NGT subjects (Vaag et al., 1992; Fery et al., 1993; Kelley et al., 1994). Postabsorptive hyperglycemia in type 2
diabetes is therefore unlikely to be due to systemic reduction in glucose oxidation.

Basal metabolic rate (BMR), expressed relative to FFM, is increased type 2 diabetic subjects (Bogardus et al., 1986b; Franssila-Kallunki and Groop, 1992). Furthermore, when glycaemic control is improved either pharmacologically or with a reduced energy intake, BMR is often reduced (Ravussin et al., 1983; Bogardus et al., 1986b; Franssila-Kallunki and Groop, 1992). BMR correlates with EGP (Franssila-Kallunki and Groop, 1992), suggesting elevated gluconeogenesis, which is an energy expensive process, is likely to contribute to the elevated BMR in type 2 diabetes (Franssila-Kallunki and Groop, 1992).

Glucose uptake by the CNS is not different between diabetic and control subjects in absolute terms, which is not surprising given the importance of a constant regular supply of glucose to this organ (Grill, 1990). Fasting hyperglycemia must therefore result from reduced peripheral glucose disposal or increased EGP.

1.1.4.2.1 Peripheral Tissues
Skeletal muscle is responsible for the majority of peripheral glucose utilisation. In the postabsorptive phase plasma glucose and insulin levels are low, inhibiting glucose uptake into skeletal muscle and adipose tissue. Therefore, in postabsorptive conditions fat is preferentially oxidised, utilising plasma FFA and intramuscular triglyceride stores, with glucose oxidation predominately utilising intramuscular glycogen stores (Groop et al., 1991; Felber and Golay, 1995). Hyperglycemia in type 2 diabetes is accompanied by hyperinsulinemia, indicating type 2 diabetic subjects have a defect in insulin mediated glucose uptake in skeletal muscle.

Fery et al (1993) found postabsorptive hyperglycemia in severely diabetic subjects was due to both elevated EGP and reduced glucose disposal. Similar results were seen in other studies (Firth et al., 1986; Ferrannini et al., 1988). In contrast, studies of moderately diabetic subjects have found reduced
glucose clearance (glucose utilisation divided by plasma glucose concentration) (DeFronzo et al., 1989; Hother-Nielsen and Beck-Nielsen, 1991; Fery et al., 1993). Furthermore, these studies failed to detect any elevation in EGP suggesting peripheral, predominantly skeletal muscle, insulin resistance is the primary cause of fasting hyperglycemia in the early stages of type 2 diabetes.

DeFronzo (1992) suggested that the fasting hyperinsulinemia that accompanies moderate type 2 diabetes is inadequate to stimulate whole body glucose uptake but adequate to suppress EGP. This conclusion is supported by the relationship between EGP and fasting plasma glucose demonstrated by DeFronzo (1988). EGP was not correlated with fasting plasma glucose in normoglycemic or moderately diabetic subjects, however a significant positive correlation was found in overtly diabetic subjects. DeFronzo (1988) suggested that EGP increases significantly with the reduction in plasma insulin concentration that accompanies the development of overt diabetes.

1.1.4.2.2 Liver
Although many studies have demonstrated that increased EGP is a major cause of fasting hyperglycemia in overt diabetes (Bogardus et al., 1984; DeFronzo, 1988; Gerich, 1991; Kelley et al., 1994; Perriello et al., 1997), the relative contribution of increased EGP in the progression from normoglycemia to moderate diabetes remains controversial. Gerich (1991) demonstrated increased EGP in subjects with plasma glucose concentrations of 6-7 mmol/L. A subsequent study by Perriello et al (1997) with normoglycemic and moderately diabetic (plasma glucose <7.8 mmol/L) and overtly diabetic (≥7.8 mmol/L) subjects demonstrated increased EGP in both diabetic groups. However, normal rates of fasting EGP have been found in other studies of mildly diabetic subjects (DeFronzo, 1988; Kelley et al., 1994).

Disturbances in hepatic glucose metabolism are evident in the progression from normoglycemia to moderate fasting hyperglycemia even if absolute EGP is not increased. The liver must be insulin and glucose resistant in moderate
diabetic subjects as, this condition is characterised by elevated plasma glucose and insulin concentrations, both of which normally act to suppress EGP (DeFronzo et al., 1983). EGP is therefore increased relative to plasma glucose and insulin concentrations in the early stages of the development of type 2 diabetes, and increases further as hyperglycemia worsens.

EGP is the result of both glycogenolysis and gluconeogenesis. Defects in either of these pathways could account for the increased postprandial and postabsorptive EGP that occurs in type 2 diabetes. Glycogenesis is catalysed by glycogen synthase. Glycogenolysis is catalysed by the enzyme glycogen phosphorylase. Glycogen synthase and phosphorylase therefore jointly coordinate glycogen synthesis and degradation and contribute to the regulation of EGP. Gluconeogenesis utilises non-carbohydrate precursors including lactate from anaerobic glycolysis, alanine and glutamine from amino acid metabolism and glycerol from lipolysis, to synthesise glucose (Dinneen et al., 1992). PEPCK is the rate-limiting enzyme in gluconeogenesis. Other key enzymes in gluconeogenesis include fructose 1,6-biphosphatase and glucose 6 phosphatase.

Type 2 diabetic subjects have been shown to have increased gluconeogenesis during the postprandial (Nurjhan et al., 1986) and postabsorptive (Consoli and Nurjhan, 1990; Magnusson et al., 1992; Perriello et al., 1997) phases. Consoli & Nurjhan (Consoli and Nurjhan, 1990) demonstrated a threefold increase in gluconeogenesis in type 2 diabetic subjects compared to controls, which accounted for 80 percent of the increased EGP. Fasting hyperglycemia in these subjects was positively correlated with gluconeogenesis but not glycogenolysis. The gluconeogenic pathway is therefore primarily responsible for the elevated postabsorptive EGP, and may also predominate postprandially. Increased conversion of both lactate and alanine to glucose have been associated with the elevated gluconeogenesis in type 2 diabetic subjects (Consoli et al., 1990; Nurjhan et al., 1992). Elevated gluconeogenesis in type 2 diabetes is due to both an increased supply of gluconeogenic precursors and increased efficiency of substrate conversion in the liver (Consoli et al., 1990).
In summary, postabsorptive hyperglycemia is due to increased EGP as well as reduced peripheral glucose disposal. The former may become quantitatively more important as fasting glucose concentrations increase. Increased gluconeogenesis is likely to be the principal cause of the elevated EGP, although glycogenolysis may also contribute.

1.1.4.3 Dynamic Glucose Homeostasis
Daily maintenance of glucose homeostasis is dependent on the postabsorptive regulation of whole body glucose metabolism and the postprandial disposal of mixed meals. There is evidence to suggest diurnal variations in insulin secretion and insulin sensitivity also influence glucose homeostasis.

Boden et al (1996b) examined insulin secretion in normoglycemic subjects with plasma glucose clamped at 5, 8.8 and 12.6 mmol/L for 68 hours. Serum insulin concentration changed in a circadian (approximately 24 hour) rhythm, increasing from a nadir between midnight and 6:00AM and reaching a peak between noon and 6:00PM.

A subsequent study found this circadian rhythm in insulin secretion was absent in type 2 diabetic subjects during a hyperglycemic clamp (11.1 mmol/L) (Boden et al., 1996a). However, glucose infusion rate, reflecting insulin sensitivity, displayed circadian (approximately 24 hour) rhythm, increasing from a nadir in the morning (approximately 8:00AM) to a peak in the evening (approximately 7:00PM). The decrease in GIR was due to increased EGP rather than decreased glucose disposal. FFA and cortisol levels may have contributed to the circadian changes in EGP as these variables were correlated (Boden et al., 1996a).

Disturbances in circadian insulin secretion may be secondary to the metabolic abnormalities associated with type 2 diabetes. However, there is evidence to suggest that NGT first degree relatives of type 2 diabetic subjects have disturbances in circadian insulin secretion, suggesting these disturbances may contribute to the development of type 2 diabetes (Boden et al., 1999).
In summary, the development of hyperglycemia in type 2 diabetes is primarily due to insulin resistance, although pancreatic failure and insulin deficiency occurs as insulin sensitivity decreases and the blood glucose concentration increase further. Insulin resistance is evident in peripheral and hepatic tissues. Elevated EGP and reduced suppression of EGP are likely to contribute substantially to the postabsorptive and postprandial hyperglycemia respectively. Reduced peripheral glucose disposal also contributes to the hyperglycemia in type 2 diabetes. Insulin resistance in adipose tissue results in increased lipolysis and elevated FFA concentrations. The increased plasma FFA concentration is likely to contribute to both hepatic and peripheral insulin resistance, increasing EGP and reducing glucose disposal respectively. EGP is responsible for the diurnal variation in glucose homeostasis in type 2 diabetes.

### 1.2 GENE DISCOVERY IN TYPE 2 DIABETES

The establishment of a genetic component to the development of type 2 diabetes has stimulated research to identify these genes. Several different gene discovery strategies have been utilised, including candidate gene approaches, genome wide scans and gene expression approaches.

#### 1.2.1 Candidate Gene Approaches

Candidate gene approaches identify a gene based on a biological function, and attempt to establish a relationship between variation within the gene and a phenotype. Association studies may examine the frequency of a polymorphism within a candidate gene in diabetic and non-diabetic subjects or examine the relationship between the polymorphism and linear variables such as plasma glucose concentrations. The polymorphism examined may be directly involved in the development of the disease, or in linkage disequilibrium with a causative mutation. These studies are limited, as they require initial knowledge of the disease process.

Another candidate gene approach involves manipulating the gene of interest and examining the phenotypic changes that occur. Gene expression can be
increased using transgenic techniques or alternatively gene expression can be
reduced or abolished using antisense and gene knockout techniques. These
genetic manipulations can be specific to a tissue of interest. Although these
studies are useful, they create a non-physiological condition, limiting the
relevance to pathogenesis of the human disease.

Disturbances in hepatic glucose metabolism contribute to the development of
type 2 diabetes. Genes expressed in the liver which encode proteins involved
in insulin signaling, glucose uptake and release, glycolysis, gluconeogenesis
and glycogen synthesis and degradation are all candidates for contributing to
the development of type 2 diabetes. Several key genes are discussed below.

1.2.1.1 Insulin Receptor

Insulin receptor knockout mice develop severe diabetes with defects in the
pancreas, skeletal muscle, adipose tissue and liver, including reduced hepatic
glycogen stores (Cinti et al., 1998). Muscle specific insulin receptor knockout
mice remain glucose tolerant, suggesting other tissues, such as liver, are
important in glucose disposal in this animal (Bruning et al., 1998). In contrast
liver specific insulin receptor knockout mice are hyperinsulinemic with severe
insulin resistance and develop diabetes by the age of 2 months. However,
fasting hyperglycemia returned to normal by the age of 4 months (Michael et
al., 2000). Although the knockout of liver insulin receptors is not sufficient to
produce sustained diabetes, these studies have demonstrated the importance of
insulin receptors in the liver in maintaining glucose homeostasis.

The insulin receptor gene is located on chromosome 19p13.3-13.2. Several
linkage studies have failed to detect linkage with mutations in the insulin
receptor and type 2 diabetes (Elbein et al., 1988; O'Rahilly et al., 1988; Cox et
al., 1989; Elbein and Sorensen, 1991; Elbein et al., 1992b). Association
studies in Mexican Americans (Raboudi et al., 1989) and Caucasian, Hispanic
and Chinese Americans (McClain et al., 1988; Xiang et al., 1989) have found
an association between insulin receptor polymorphisms and type 2 diabetes.
However several studies with these and other mutations have failed to find an
association with type 2 diabetes (Takeda et al., 1986; Hitman et al., 1987;
Elbein, 1989; Morgan et al., 1990; Oelbaum et al., 1991; Sten-Linder et al., 1991). Mutations in the insulin receptor are therefore unlikely to be a common cause of type 2 diabetes.

1.2.1.2 Insulin Receptor Substrates
Insulin receptor substrate (IRS)-1 is the major substrate of the insulin receptor as well as the insulin like growth factor receptors. IRS-1 knockout mice are insulin resistant in skeletal muscle, but maintain normal glucose levels (Araki et al., 1994; Tamemoto et al., 1994). Hepatic insulin resistance is prevented by IRS-2 compensation (Patti et al., 1995). IRS-2 knockout mice develop mild to severe diabetes with reduced β-cell mass (Kubota et al., 2000). Muscle remains insulin sensitive, while the liver is insulin resistant. IRS-1 does not compensate for defective hepatic IRS-2 (Kubota et al., 2000). IRS-2 may therefore be an important component of hepatic insulin signaling.

The IRS-2 gene is located on chromosome 13q34. A common non-conservative amino acid substitution (G1057D) within IRS-2 was associated with type 2 diabetes increased in Italian subjects (Mammarella et al., 2000), however this has not been confirmed in other studies (Fritsche et al., 2001; Wang et al., 2001). Other association and linkage studies with IRS-2 and type 2 diabetes have also been negative (Kalidas et al., 1998; Almind et al., 1999; tHart et al., 2002). Mutations in IRS-2 are therefore unlikely to be a common cause of type 2 diabetes.

1.2.1.3 Glucose transporters
The glucose transporter family includes five different transporters (GLUT1-5), each with different tissue distribution and chromosomal location. GLUT2 is the liver and pancreas isoform and is located on chromosome 3q26.1-26.3 (Thorens et al., 1988). GLUT2 is involved in hepatic glucose uptake and release (Pessin and Bell, 1992).

An association study in Caucassians British subjects found a significant association between a Taq I polymorphism in GLUT2 and type 2 diabetic
subjects with a family history of diabetes (Alcolado et al., 1991). A linkage study in Pima Indians found weak linkage between GLUT2 and acute insulin response but not type 2 diabetes (Janssen et al., 1994). Several other association and linkage studies have failed to find an association between GLUT2 and type 2 diabetes (Matsutani et al., 1990; Li et al., 1991b; Patel et al., 1991; Baroni et al., 1992; Elbein et al., 1992a; Moller et al., 2001). One patient has been identified with diabetes caused by a mutation in GLUT2, although the diabetes in this patient was thought to be caused by a β-cell defect (Mueckler et al., 1994). Mutations in GLUT2 are unlikely to be a common cause of type 2 diabetes.

1.2.1.4 Glucokinase
Glucose entering the hepatocyte through GLUT2 is converted to glucose 6 phosphate by glucokinase. This reaction maintains low intracellular glucose concentrations and facilitates glucose uptake (Matschinsky, 1990). Glucokinase may also regulate glucose release (Matschinsky, 1990). A transgenic study expressing human glucokinase in the liver of mice reduced fasting glucose, insulin and lactate levels and improved glucose tolerance (Hariharan et al., 1997).

The glucokinase gene is located on chromosome 7p13. A mutation in glucokinase is responsible for MODY2 (Froguel et al., 1992), therefore many studies have examined glucokinase mutations in the common form of type 2 diabetes. A study in African Americans found the Z+4 allele was associated with an increased risk of developing type 2 diabetes and a younger onset of diabetes (Chiu et al., 1992b). The Z+2 allele increased the risk of developing diabetes in Mauritian Creoles (Chiu et al., 1992a). Other variants have been associated with insulin sensitivity (Elbein et al., 2001) and hepatic insulin sensitivity (Chiu et al., 2000b). However studies in Pima Indians, and American, British and French Caucasians did not find association with these or other mutations in the glucokinase gene (Matsutani et al., 1992; Permutt et al., 1992). Linkage studies with glucokinase have also been negative (Cook et
Glucokinase mutations are therefore unlikely to be a common cause of type 2 diabetes.

1.2.1.5 Phosphoenol Puruvate Carboxykinase
Phosphoenol pyruvate carboxykinase (PEPCK) is expressed in liver and kidneys as well as adipose tissue and the small intestine (Beale et al., 1985). PEPCK converts oxaloacetate to phosphoenol pyruvate and is the rate limiting enzyme in gluconeogenesis (Rognstad, 1979). Several studies have demonstrated increased gluconeogenesis in type 2 diabetes (Consoli and Nurjhan, 1990; Perriello et al., 1997). PEPCK is located on chromosome 20q13.31. An association study failed to find any mutations in the promoter region of PEPCK (Ludwig et al., 1996). Mutations in the PEPCK gene are yet to be associated with type 2 diabetes.

1.2.1.6 Glycogen Synthase
Glycogen synthase is the key enzyme in glycogenesis and therefore the storage of glucose in both liver and muscle. The glycogen synthase gene is located on chromosome 19. A study of Finnish subjects found an association between the Xba I polymorphism in the glycogen synthase gene and type 2 diabetes (Groop et al., 1993). However several other studies have failed to find an association between this and other polymorphisms in glycogen synthase and type 2 diabetes (Kadowaki et al., 1993; Zouali et al., 1993; Wang et al., 1998). Type 2 diabetes is unlikely to result from mutations in glycogen synthase in most cases.

1.2.1.7 Hepatocyte Nuclear Factors
Hepatocyte Nuclear Factors (HNF’s) are expressed in the liver and to a lesser extent in the pancreas, kidney and gut (Blumenfeld et al., 1991; Pontoglio et al., 1996). Mutations in the HNF isoforms 1α, 1β and 4α result in the development of MODY (Yamagata et al., 1996a; Yamagata et al., 1996b; Horikawa et al., 1997). MODY is characterised by reduced insulin secretion, rather than insulin resistance implicating the pancreas rather than the liver in this form of diabetes (Froguel, 1998). Nevertheless, HNF’s are involved in
hepatocyte differentiation and are transcription factors for many liver specific enzymes involved in glucose and fat metabolism including PEPCK (Stoffel and Duncan, 1997).

HNF-1α knockout mice develop dwarfism and diabetes due to reduced IGF-1 and insulin. Disturbances of hepatic glucose metabolism are evident with accumulation of complex carbohydrate, thought to be glycogen in hepatocytes (Lee et al., 1998). Homozygote knockouts of liver HNF-4α die during embryogenesis. Heteroygotes do not develop diabetes, although they have disturbances of lipid homeostasis with increased hepatic lipid storage and reduced circulating lipids (Hayhurst et al., 2001).

Associations studies with HNF genes have been conducted in many populations. Mutations in HNF1α have been associated with; type 2 diabetes in Finns (Rissanen et al., 2000), early onset of type 2 diabetes in American subjects (Triggs-Raine et al., 2002) and insulin sensitivity in Oji-Cree Canadians (Chiu et al., 2000a). However, several other studies with these and other mutations have failed to find any association with type 2 diabetes (Baier et al., 2000a; Rissanen et al., 2000; Elbein et al., 2001). Association studies with HNF1β in Danish Caucasians (Ek et al., 2001) and Japanese (Babaya et al., 2001) have failed to find any relationship with type 2 diabetes. A mutation in HNF4α has been associated with type 2 diabetes in Japanese subjects (Sakurai et al., 2000), however sequencing the entire coding region of HNF4α in Pima Indians failed to find any mutations associated with diabetes (Baier et al., 2000a). Further studies are necessary to determine if HNF mutations are important in the pathophysiology of type 2 diabetes.

The polymorphisms in the genes discussed above are unlikely to cause type 2 diabetes. However, many of these studies may not have used a sufficient number of subjects, limiting the statistical power to detect small effects. Alternatively genetic variations in the upstream promoter and enhancer regions of the genes may be associated with type 2 diabetes. Given type 2
diabetes is a heterogeneous disease, subphenotyping may be necessary to detect the effects of genes with small effects.

1.2.2 Identification of Novel Genes
Identification of novel genes involved in the development of type 2 diabetes requires different strategies. Gene discovery studies can be based on identifying gene mutations or differential gene expression.

1.2.2.1 Whole Genome Scans
Linkage studies identify Quantitative Trait Loci (QTL) when polymorphic markers co-segregate with a phenotype, such as type 2 diabetes, in two or more familial generations. Whole genome scans utilise anonymous markers spread evenly throughout the entire genome. Following the identification of a QTL, fine scale mapping and positional cloning strategies are used to identify the specific gene generating the linkage. Genome wide scans with positional cloning are an expensive and time consuming gene discovery approach. Furthermore these studies are not well suited to detecting genes with small phenotypic effects in complex polygenic diseases such as type 2 diabetes (Elbein et al., 1994).

Genome wide scans have identified loci associated with phenotypes related to type 2 diabetes in various populations, including Mexican Americans (Hanis et al., 1996; Duggirala et al., 1999; Mitchell et al., 2000), Finns from the isolated Botnia region (Mahtani et al., 1996; Parker et al., 2001), Pima Indians (Hanson et al., 1998), Utah Caucasians (Elbein et al., 1999), Canadian Oji-Cree (Hegele et al., 1999), Ashkenazi Jews (Permutt et al., 2001) and Chinese Han (Luo et al., 2001). The identification of the specific genes generating the linkage within these loci is continuing.

The whole genome scan in Mexican Americans identified the 2q37.3 locus, referred to as NIDDM1 (Hanis et al., 1996). NIDDM1 interacts with a gene on chromosome 15 to increase susceptibility to type 2 diabetes (Cox et al., 1999). Positional cloning has identified the calpain-10 gene within the NIDDM1 locus, and identified polymorphisms within this gene that are associated with
type 2 diabetes (Horikawa et al., 2000). Haplotypes of polymorphisms within calpain-10 contribute to the development of type 2 diabetes in Mexican Americans, and to a lesser extent in Germans and Finns from Botnia (Horikawa et al., 2000).

Calpains are calcium dependent processing proteases implicated in various processes including intracellular signaling, proliferation and differentiation (Carafoli and Molinari, 1998) and may be involved in the insulin induced downregulation of IRS-1 (Smith et al., 1996). Calpain-10 is expressed in liver as well as pancreatic islets and muscle. A calpain10 polymorphism has been associated with decreased mRNA levels in skeletal muscle and insulin resistance in nondiabetic subjects (Baier et al., 2000b). Genetic variation in calpain-10 was associated with hyperglycemia and reduced insulin secretion in a British population (Lynn et al., 2002). Exposure of mouse pancreatic islets to calpain inhibitors for 4 hours enhanced glucose induced insulin secretion (Sreenan et al., 2001). Exposure of isolated muscle strips to these inhibitors reduced insulin stimulated glucose uptake and glycogen synthesis and also reduced insulin stimulated glucose uptake in adipocytes (Sreenan et al., 2001). Continuing research is necessary to determine the precise physiological role of calpain-10 in the pathophysiology of type 2 diabetes.

1.2.2.2 Gene Expression Approaches
Another gene discovery approach is to examine the expression of a gene, rather than genetic mutations, for an association with a phenotype. Gene expression approaches rely on the tight connection between the function of a gene product and its expression profile. Generally each gene is expressed in the specific cell and under the specific conditions in which its product makes a contribution to fitness/cellular function (Brown and Botstein, 1999).

The thousands of genes that are expressed within a tissue of interest at any given time limit the efficacy of gene expression studies to identify genes associated with a disease phenotype. Oligonucleotide and cDNA microarray techniques can be used to simultaneously examine the expression of thousands of genes. Importantly, generating cDNA from RNA does not require previous
knowledge of the cDNA sequence. Furthermore, cDNA can be species and tissue specific and environmental conditions can be manipulated.

The rapid advancement of this technology means that microarray is a cost and labour effective approach for gene expression profiling and gene discovery in complex diseases such as obesity and type 2 diabetes.

**1.2.2.1 cDNA Microarray**

Microarray protocols vary between laboratories. A simplified protocol is shown schematically in Figure 1.3 and described below (Duggan et al., 1999). Templates for genes of interest are obtained from purposely, or randomly, selected clones of interest and amplified by PCR. Following purification and quality control, aliquots are printed on coated glass microscope slides using a computer controlled robotic arrayer. RNA from the sample of interest and also a reference are fluorescently labeled with Cy3- or Cy5-dUTP, respectively, during reverse transcription. The labeled cDNA are combined and purified and allowed to hybridise to the clones of interest on the array. Laser excitation of the incorporated targets yield an emission with a characteristic spectra, which is measured using a scanning confocal microscope. Monochrome images from the scanner are imported into software, merged, and a false colour image generated.

![Figure 1.3. Schematic diagram of the cDNA microarray procedure (Duggan et al., 1999).](image)
Data from an experiment is viewed as the ratio of Cy3/Cy5. A clone producing a ratio of 1 is equally expressed in the sample of interest and the reference sample. Ratios >1 and <1 represent clones overexpressed and underexpressed in the sample respectively. Gene expression information for each clone can be compiled from many RNA samples in many experimental conditions, including diabetic and nondiabetic.

The ability of cDNA microarray to efficiently screen a randomly selected library of clones and identify genes that are differentially expressed with a phenotype is limited by the abundance of undesirable clones. These include; clones only containing poly (A) mRNA tails, clones with very short cDNA inserts, clones only containing the 3’ half of the oligo(dT) primer used to synthesis cDNA, chimeric clones (different mRNAs artificially joined during ligation) (Bonaldo et al., 1996). Furthermore, highly abundant mRNA transcripts inhibit the identification of low copy number mRNAs. Three classes of mRNA transcripts have been identified based on their frequency within somatic cells; superprevalent (10-15 mRNAs that represent 10-20% of mRNA mass), intermediate (1000-2000 mRNAs, 40-45%) and complex (15,000-20,000 mRNAs, 40-45%) (Bonaldo et al., 1996). Normalisation and subtraction approaches have been developed to increase the efficiency of identifying complex mRNAs.

Normalisation reduces the frequency of all clones within a narrow range. Normalising, therefore avoids the repetitive identification of the same superprevalent cDNAs (Bonaldo et al., 1996). Subtraction approaches can subsequently remove previously identified cDNA, or alternatively enrich for yet to be identified cDNAs. Subtraction approaches, therefore increase the likelihood of identifying new cDNA’s (Bonaldo et al., 1996). Normalisation and subtraction of cDNA libraries can dramatically improve the efficiency of cDNA microarray screening.

1.2.2.2 Suppression Subtractive Hybridisation
Suppression subtractive hybridisation (SSH) compares two cDNA populations, enriching for genes that are overexpressed in one of the
populations (Lisitsyn and Wigler, 1993; Diatchenko et al., 1996). Differentially expressed genes can be cloned and identified with cDNA microarray or traditional approaches such as cDNA dot blots. Several obesity and diabetes genes have been identified using SSH, including resistin (Steppan et al., 2001) and adipogene (Larose et al., 2001).

1.3 ANIMAL MODELS OF TYPE 2 DIABETES

Although humans are the best models in which to explore the pathophysiology of type 2 diabetes, the invasive nature of many experiments has limited their usage. Animal models of type 2 diabetes have been explored to further our understanding of this disease.

1.3.1 Monogenic Animal Models

Several animal models have been identified with single gene mutations that cause both obesity and type 2 diabetes. Studies of these animals have led to significant advances in our understanding of the pathogenesis of these diseases.

1.3.1.1 Obese Mouse

An autosomal recessive mutation with full penetrance in the obese (ob/ob) mouse produces a phenotype characterised by infertility, severe obesity, hyperphagia, hyperinsulinemia and insulin resistance (Coleman, 1978). Hyperglycemia develops, although the severity depends on the background strain of the animal. When the ob mutation is expressed in the C57BL/6J mouse diabetes is mild, due to compensatory hyperinsulinemia (Coleman and Hummel, 1973). Severe diabetes occurs when the ob mutation is expressed in C57BL/KsJ mice, due to β-cell failure and insulin deficiency (Coleman and Hummel, 1967).

The ob gene encodes the adipocyte secreted protein leptin. Two mutations have been described in the ob gene resulting in either a truncated protein or complete absence of leptin (Zhang et al., 1994). Obese mice display hyperphagia and reduced energy expenditure relative to controls.
Administration of leptin reduced food intake and increased energy expenditure and increased fat oxidation resulting in substantial weight loss in ob/ob mouse (Campfield et al., 1995; Pellemounter et al., 1995; Halaas et al., 1997; Hwa et al., 1997).

1.3.1.2 Diabetes Mouse
The diabetic (db/db) mouse has an autosomal recessive mutation with full penetrance, which produces a phenotype identical to the obese mouse when these mutations are expressed on the same background strain (Coleman, 1978). The db mutation therefore produces severe diabetes when expressed in the C57BL/KsJ mouse (Coleman, 1978).

The db gene was identified as the leptin receptor (OB-R) (Tartaglia et al., 1995). The db mutation causes abnormal splicing of the leptin receptor and inhibits leptin signal transduction (Lee et al., 1996). Therefore, the phenotype of the obese and diabetic mice is due to leptin deficiency and leptin resistance respectively.

1.3.1.3 Zucker Rat
The Zucker (fa/fa) rat has an autosomal recessive mutation in the leptin receptor gene (Chua et al., 1996a). The fa mutation is different to the db mutation (Chua et al., 1996b), although it produces a similar phenotype to the obese and diabetic mice including the early development of obesity, hyperphagia, hyperinsulinemia and insulin resistance, and mild hyperglycemia (Zucker, 1965; Zucker and Antoniades, 1972).

Continuous inbreeding of fa/fa rats with the highest plasma glucose has led to the diabetic ZDF/drt-fa rat. These rats are less obese and have lower plasma insulin than the original strain and more severe hyperglycemia that occurs in male rats only (Friedman et al., 1991). The development of hyperglycemia in these rats is accompanied by reduced glucose oxidation (Etgen and Oldham, 2000).
Expressing the *fa* mutation in the Wistar Koyoto rat (WKY/NDr-t-fa) also produces a diabetic phenotype in male rats similar to the ZDF/drt-fa rat (Sugiyama *et al.*, 1989b). Female WKY/NDr-t-fa rats can become diabetic with dietary intervention (Sugiyama *et al.*, 1989a).

1.3.1.4 KK Mouse

The KK mouse develops moderate obesity, hyperinsulinemia and moderate hyperglycemia when fed a high energy diet (Ikeda, 1994). Hyperglycaemia is more prominent in males (Shafrir, 1992). The obesity and diabetes was thought to be polygenic in nature, but is now considered the result of a dominant gene with low penetrance (25%) (Butler and Gerritsen, 1970).

Crossing the KK mouse with the yellow agouti mouse (A<sup>y</sup>), a monogenic obese mouse with moderate hyperglycemia developed the KKA<sup>y</sup> mouse. The KKA<sup>y</sup> mouse has more severe hyperglycemia and diabetes than the KK mouse (Iwatsuka *et al.*, 1970).

1.3.1.5 Otsuka Long-Evans Tokushima Fatty Rat

The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is an inbred rat strain with hyperphagia, obesity, increased visceral adiposity, hyperinsulinemia, insulin resistance and hyperglycemia when compared to the Long-Evans Tokushima Otsuka (LETO) strain (Kawano *et al.*, 1992; Ishida *et al.*, 1995). The OTLEF rat develops late onset diabetes at approximately 18-25 weeks of age. Pancreatic β-cell failure and insulin deficiency occurs after the age of 40 weeks (Kawano *et al.*, 1992). Postabsorptive EGP is elevated in diabetic and pre diabetic OLETF rats (Shiba *et al.*, 1998).

Relative to control animals, OLETF rats have significantly reduced energy expenditure relative to body weight, and slightly elevated fat oxidation (Ichikawa *et al.*, 2000). Energy expenditure profiles in young (8 week old) normoglycemic OLETF and control rats demonstrate a diurnal variation with reduced expenditure in the light phase. The diurnal variation is abolished
following the development of hyperglycemia in OLETF rats (24 week old) (Ichikawa et al., 1998).

The OLETF rat has also been proposed as a polygenic animal model, however these rats were found to lack the cholecystokinin (CCK)-A receptor due a genetic abnormality (Funakoshi et al., 1994). The disrupted CCK-A gene induces hyperphagia in OLETF rats, contributing to the development of obesity and diabetes (Miyasaka et al., 1994).

1.3.2 Polygenic Animal Models
Type 2 diabetes in humans is a polygenic disease. Therefore, polygenic animal models more closely resemble the human condition and are advantageous for gene discovery purposes. Several polygenic animal models develop both obesity and type 2 diabetes.

1.3.2.1 New Zealand Obese Mouse
The New Zealand Obese (NZO) mouse is an inbred mouse strain that develops an early onset moderate obesity, increased visceral adiposity, hyperphagia, hyperleptinemia, hyperinsulinemia, and moderate hyperglycemia (Veroni et al., 1991). NZO mice have increased EGP from an early age (Veroni et al., 1991).

The NZO mouse may be a polygenic animal model although this is yet to be demonstrated. Given this strain is inbred, the metabolic abnormalities in the NZO mouse may be the result of a major gene with low penetrance similar to the KK mouse. NZO mouse are insensitive to peripheral leptin administration despite being sensitive to centrally administered leptin, suggesting a defect in leptin transport into the brain may contribute to the obesity in this animal (Halaas et al., 1995).

1.3.2.2 Macaca mulatta (Rhesus Monkey)
Obesity develops in approximately 50 percent of rhesus monkeys in captivity with ad libitum access to food, and body fat is predominantly stored centrally (Hansen and Bodkin, 1993). Food intake does not vary greatly between
animals suggesting differences in energy expenditure or substrate utilisation may contribute to the development of obesity (Hansen and Bodkin, 1993).

Many obese rhesus monkeys develop diabetes after maturity, between the ages of 15 and 21, although some monkeys remain normoglycemic (Hansen and Bodkin, 1986). Diabetes is accompanied by polydipsia, polyuria, glycosuria, polyphagia and eventual weight loss, similar to humans (Hansen and Bodkin, 1986).

A six year longitudinal study found that approximately 30 percent of obese adult rhesus monkeys developed overt type 2 diabetes, and a further 50 percent progressively developed impaired glucose tolerance, suggesting that they may eventually become diabetic (Hansen and Bodkin, 1986). The phenotypic heterogeneity in these monkeys as they develop impaired glucose tolerance resembles that seen in humans. Pre diabetic rhesus monkeys displayed elevated fasting insulin levels and increased glucose stimulated insulin secretion (Hansen and Bodkin, 1986). Increased EGP develops in parallel with increased fasting plasma glucose (Bodkin et al., 1989).

Long term (8.5 years) dietary energy restriction of rhesus monkeys to 70 percent of their normal food intake, reduced fasting insulin and glucose stimulated insulin concentrations, and increased insulin sensitivity and glucose tolerance compared to control monkeys (Gresl et al., 2001). Similarly, preventing the development of obesity in rhesus monkeys with a weight maintaining diet has been shown to improve glucose tolerance and insulin sensitivity and to prevent the development of hyperinsulinemia (Hansen and Bodkin, 1993).

The physiological and genetic similarity between rhesus monkeys and humans, and the heterogeneous development of type 2 diabetes and obesity in these animals, suggest they would be an ideal animal model for the study of these diseases. However, the use of rhesus monkeys is impractical given these are relatively large animals (up to 20kg), that typically live for 30 years and may remain normoglycemic for 20 years (Hansen and Bodkin, 1986; Hansen
and Bodkin, 1993). Furthermore, primates are emotional and intelligent animals raising serious ethical issues.

### 1.3.2.3 *Psammomys obesus* (Israeli Sand Rat)

*Psammomys obesus*, commonly known as the Israeli sand rat, is an outbred animal model of obesity and type 2 diabetes. *P. obesus* belong to the Gerbillinae family and are native to the deserts of North Africa and the Middle East (Shafrir and Gutman, 1993). In their natural environment these animals survive on a diet of low energy salt bush (*atriplex halimus*). To obtain sufficient energy these animals consume large quantities of salt bush, estimated to be up to 65 percent of their body weight (Pinshow, 1993). *P. obesus* also have a relatively low BMR, estimated to be 60 percent of that expected for rodents of similar mass (Pinshow, 1993). Neither obesity or diabetes have been reported in *P. obesus* in the wild (Shafrir and Gutman, 1993).

Early studies by (Hackel *et al.*, 1966) found that maintaining *P. obesus* on an *ad libitum* diet of laboratory chow caused some animals to becoming severely glucose intolerant and hyperinsulinemic, and some also became hypoinsulinemic with ketoacidosis and died. Conversely, some animals maintained glucose tolerance at the expense of hyperinsulinemia, while others were phenotypically unaffected. Restricting the food intake of *P. obesus* to 30 calories per day was shown to prevent glucose intolerance and diabetes (Hackel *et al.*, 1967).

The colony of *P. obesus* at the Geelong campus of Deakin University is maintained on a standard laboratory chow diet. The distribution of body weight within this colony approximates a normal distribution and resembles that seen in human populations (Figure 1.4) (Walder *et al.*, 2000). A familial effect accounts for 51 percent of the variation in body weight (Walder *et al.*, 2000).
Cross sectional studies have demonstrated an inverted “U” shaped relationship between plasma glucose and insulin concentrations (Figure 1.5) (Barnett et al., 1994a). This closely resembles Starling’s curve of the pancreas seen in humans (DeFronzo, 1988). Longitudinal studies on the development of diabetes within *P. obesus* demonstrate a similar relationship between glucose and insulin concentrations (Barnett et al., 1994a). A familial effect accounts for up to 26 percent of the variation in blood glucose and plasma insulin concentrations in these animals (Walder et al., 2000).

**Figure 1.4.** Frequency distribution of body weight in 16 week old *P. obesus* (Walder et al., 2000).

**Figure 1.5.** Plasma glucose and insulin concentrations in 19 week old *P. obesus* (Barnett et al., 1994a).

Diabetic *P. obesus* display a range of metabolic abnormalities including hyperphagia, obesity, hyperinsulinemia and elevated plasma cholesterol and triglycerides (Barnett et al., 1995). Fasting plasma glucose concentrations are
positively correlated with both hepatic glucose production and the metabolic clearance rate of glucose (Barnett et al., 1994a; Collier et al., 1997). Peripheral and hepatic insulin resistance precedes overt diabetes in P. obesus (Kalderon et al., 1986; Shafrir and Gutman, 1993). EGP has been associated with increased glycogen synthase and PEPCK activity suggesting elevated gluconeogenesis and glycogenolysis (Barnett et al., 1995). Hepatic insulin resistance coincides with elevated hepatic triglyceride content (Zoltowska et al., 2001).

P. obesus display the characteristics of a polygenic animal model of obesity and type 2 diabetes, and are therefore an ideal animal model for the study of these diseases. Furthermore the heterogeneity of phenotypic responses seen in P. obesus upon consumption of a relatively high energy diet closely resemble that seen in various traditional human populations that have adopted a modern lifestyle including Pima Indians and Nauruans (Knowler et al., 1981; Zimmet et al., 1990). These environmental changes are characterised by the consumption of energy dense foods with little physical activity and are thought to unmask a strong genetic predisposition to the development of obesity and type 2 diabetes (Zimmet et al., 1990).

1.4 SUMMARY

Type 2 diabetes mellitus is a metabolic disease characterised by defects in insulin secretion and insulin action, and disturbances in carbohydrate, fat and protein metabolism that result in hyperglycemia. The liver contributes to the development of type 2 diabetes through reduced suppression of postprandial EGP and elevated postabsorptive EGP. The aetiology of this disease includes a strong genetic component. However, specific genes involved in the pathogenesis of type 2 diabetes remain elusive. Gene discovery approaches based on gene expression can identify novel genes associated with this disease. P. obesus is a unique polygenic animal model of obesity and type 2 diabetes, displaying many metabolic abnormalities seen in humans. However, it is unclear if disturbances in whole body energy metabolism and substrate
utilisation occur in these animals. These animals are ideally suited for studies aiming to identify genes associated with type 2 diabetes.

### 1.5 AIMS

Firstly, this study aimed to characterise whole body energy metabolism and substrate utilisation in *P. obesus*, to further validate these animals as an ideal model of obesity and type 2 diabetes. Lean-NGT, obese-IGT and obese-diabetic animals were examined with indirect calorimetry in several metabolic states including; fed, fasted and dietary energy restricted.

Secondly, this study aimed to identify novel genes differentially expressed in the liver of lean-NGT and obese-diabetic *P. obesus*. Suppression subtractive hybridisation was used to enrich a cDNA library for differentially expressed genes. These genes were subsequently screened with cDNA dot blots and cDNA microarray to identify individual genes associated with type 2 diabetes.
Energy Metabolism in *Psammomys obesus*

### 3.1 ABSTRACT

*Psammomys obesus* is a polygenic animal model of obesity and type 2 diabetes. Three studies were conducted to characterise energy expenditure and substrate utilisation in lean-NGT, obese-IGT and obese-diabetic *P. obesus*.

Experiment one examined *P. obesus* with *ad libitum* access to food. Energy expenditure and fat oxidation were elevated in the obese-IGT and obese-diabetic groups in proportion to body weight. Glucose oxidation was not different between groups. Obese-diabetic *P. obesus* displayed elevated nocturnal blood glucose levels and fat oxidation.

Experiment two examined *P. obesus* following 14 days of dietary energy restriction. Body weight was reduced and plasma insulin and blood glucose levels were normalised in all groups. Glucose oxidation was reduced and fat oxidation was increased. Following dietary energy restriction, energy expenditure, glucose oxidation and fat oxidation were not different between groups of *P. obesus*.

Experiment three examined *P. obesus* after 24 hours of fasting. Plasma insulin and blood glucose levels were normalised in all groups. Energy expenditure and glucose oxidation were greatly reduced and fat oxidation was increased. Following fasting, energy expenditure, glucose oxidation and fat oxidation were not different between groups of *P. obesus*.

Energy expenditure and whole body substrate utilisation in *P. obesus* was similar to that seen in humans, and these animals responded normally to fasting and dietary energy restriction. Energy expenditure and fat oxidation were elevated in obese-IGT and obese-diabetic animals in proportion to body weight. *P. obesus* respond normally to short term fasting and dietary energy restriction.
restriction. Elevated nocturnal fat oxidation rates and plasma glucose levels in obese-diabetic *P. obesus* may be an important factor in the pathogenesis of obesity and type 2 diabetes in these animals. These studies have provided further validation of *P. obesus* as an ideal animal model of obesity and type 2 diabetes.

### 3.2 INTRODUCTION

The pathogenesis of obesity and type 2 diabetes are interrelated. Obesity is the strongest determinant of type 2 diabetes with over 85 percent of type 2 diabetic patients obese (WHO, 1994). Obesity occurs as a result of a chronic imbalance between energy intake and expenditure.

Obesity is associated with elevated energy expenditure however, relative to fat free mass energy expenditure is similar to lean controls (Bogardus *et al.*., 1986a; Nelson *et al.*, 1992). Longitudinal studies have shown that low relative energy expenditure is predictive of subsequent weight gain (Ravussin *et al.*, 1988). Furthermore, a high respiratory quotient (RQ), indicative of a low ratio of fat to glucose oxidation, has also been shown to predict weight gain in some studies (Zurlo *et al.*, 1990; Seidell *et al.*, 1992). Some studies with post-obese subjects have found reduced resting energy expenditure (Geissler *et al.*, 1987; Weigle *et al.*, 1988), and reduced fat oxidation (Larson *et al.*, 1995; Wyatt *et al.*, 1999; Weyer *et al.*, 2000a), relative to weight matched controls suggesting a predisposition to weight gain in some subjects.

Elevated basal metabolic rate (BMR) has been found in some type 2 diabetic subjects (Ravussin *et al.*, 1983; Bogardus *et al.*, 1986b; Fontvieille *et al.*, 1992; Franssila-Kallunki and Groop, 1992), possibly due to the energy cost of gluconeogenesis, which is elevated in type 2 diabetes (Franssila-Kallunki and Groop, 1992). Whole body glucose oxidation rates have been negatively correlated with plasma glucose concentrations in type 2 diabetic subjects (Bogardus *et al.*, 1984) and elevated whole body fat oxidation has also been found (Franssila-Kallunki and Groop, 1992). However, other studies have found similar glucose and fat oxidation rates in diabetic subjects (Vaag *et al.*, 1984).
1992; Fery et al., 1993; Kelley et al., 1994), suggesting hyperglycemia is unlikely to be due to reduced systemic glucose oxidation.

Reducing dietary energy intake and increasing energy expenditure are the primary weight loss recommendations for obese patients, particularly those with type 2 diabetes (Franz et al., 2002). Weight loss improves insulin sensitivity and glucose tolerance (Christiansen et al., 2000). Caloric restriction and short term fasting also improve insulin sensitivity and glucose tolerance prior to any change in body weight, due to the reduced glucose intake and decreased endogenous glucose production (EGP) (Christiansen et al., 2000).

*Psammomys obesus* is a unique polygenic animal model of obesity and type 2 diabetes. When housed in laboratory conditions with *ad libitum* access to standard rodent chow, some animals remain lean and healthy whereas others develop obesity and type 2 diabetes (Hackel et al., 1966; Barnett et al., 1994a).

The aim of the present study was to characterise whole body energy balance and substrate utilisation in lean-NGT, obese-IGT and obese-diabetic *P. obesus*, to further characterise *P. obesus* as an animal model of obesity and type 2 diabetes. Energy metabolism will be examined using indirect calorimetry in animals with *ad libitum* access to food, as well as animals following a 24 hour fast and following 14 days of dietary energy restriction.

### 3.3 RESEARCH DESIGN AND METHODS

Research design and methods are discussed in detail in Chapter 2 and described briefly below.

#### 3.3.1 Characterisation of Energy Metabolism

Characterisation of baseline energy metabolism in *P. obesus* was conducted with two separate experiments. Experiment 1 utilised an Oxymax Equal Flow Indirect Calorimeter (Columbus Instrument, Ohio, USA) to measure fat
oxidation, carbohydrate oxidation, RQ and total energy expenditure (TEE) as described previously (Weir, 1990; Frayn, 1983). Physical activity was measured while animals were in the calorimeter using the Opto-Varimax Mini-Infrared Animal Activity Monitoring System (Columbus Instruments, Ohio, USA).

At 16 weeks of age lean-NGT (n=10), obese-IGT (n=10) and obese-diabetic (n=10) *P. obesus* were separated and daily food intake was measured for 14 days. At 18 weeks of age body weight was measured and animals underwent 24 hours of indirect calorimetry. Animals had *ad libitum* access to food and water. Blood samples were collected for measurement of blood glucose with an enzymatic glucose analyser (Yellow Springs Instruments, Yellow Springs, USA) and plasma insulin (Insulin RIA, Phadeseph, Uppsala, Sweden).

Experiment 2 examined the diurnal influence on energy metabolism in another group of *P. obesus*. At 16 weeks of age lean-NGT (n=8), obese-IGT (n=8) and obese-diabetic (n=8) *P. obesus* were separated and daily food intake was measured for 14 days. At 18 weeks of age, blood samples were collected every 6 hours over a 24 hour period. Animals had *ad libitum* access to food and water. Blood glucose concentrations were measured, as were plasma insulin, cortisol (Orion Diagnostica, Finland), leptin (Linco Research Inc, St Charles, USA) and FFA (Free Fatty Acids Half-micro Test, Roche Diagnostics, Mannheim, Germany).

### 3.3.2 Dietary Energy Restriction

Energy metabolism was characterised in *P. obesus* before and after dietary energy restriction. At 16 weeks of age lean-NGT (n=13), obese-IGT (n=15) and obese-diabetic (n=12) *P. obesus* were separated and daily food intake was measured for 14 days. At 18 weeks of age body weight was measured, blood samples were collected and animals underwent 24 hours of indirect calorimetry. Animals had *ad libitum* access to food and water.

Animals were separated into control and restricted groups for a 14 day experimental period. Groups were matched for sex, age and body weight.
Control animals had *ad libitum* access to food, whereas restricted animals were given 66 percent of the average food intake of their respective group during the baseline period. Body weight and food disappearance were measured daily. At the completion of the restriction period blood samples were taken and animals were placed in the indirect calorimeter for another 24 hours.

### 3.3.3 Fasting

Energy metabolism was characterised in *P. obesus* while fasting. Lean-NGT (n=5), obese-IGT (n=5) and obese-diabetic (n=5) 18-week old *P. obesus* were placed in an indirect calorimeter for 48 hours. Animals were allowed *ad libitum* access to food for the first 24 hours and then fasted for the remaining 24 hours. Blood samples were collected at 0, 24 and 48 hours from the beginning of the calorimetry period for measurement of blood glucose and plasma insulin.

### 3.3.4 Statistical Analyses

Statistical analyses were conducted using SPSS (Version 10, SPSS inc, Chicago, USA). A Kolmogorov-Smirnov test was used to examine the distribution of data. Normally distributed data were analysed using an independent samples T test or paired samples T test. One way ANOVA was used with the LSD post hoc test when variance between groups was homogeneous, and with the Games-Howell post hoc test when variances were not homogenous. Non-normally distributed data was analysed using a Mann-Whitney test. The relationship between linear variables was assessed using Pearson and Spearman correlations for normally and non-normally distributed data respectively. Total energy expenditure was adjusted for differences in body weight using linear regression as previously described (Ravussin and Bogardus, 1989). Statistical significance was set at p<0.05.
3.4 RESULTS

3.4.1 Characterisation of Energy Metabolism
Baseline energy metabolism was examined in lean-NGT, obese-IGT and obese-diabetic *P. obesus*. Phenotypic and metabolic data for these animals is shown in Table 3.1. Relative to lean-NGT animals, obese-IGT animals were hyperinsulinemic, and obese-diabetic animals were hyperinsulinemic and hyperglycaemic. Obese-diabetic *P. obesus* consumed more food than lean-NGT animals, although this did not reach statistical significance (p=0.065).

Table 3.1 Phenotypic and metabolic characteristics of *P. obesus*. TEE = total energy expenditure, RQ = respiratory quotient, CHO = carbohydrate. * Significantly different to lean-NGT (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Lean-NGT</th>
<th>Obese-IGT</th>
<th>Obese-Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>211 ± 0.4</td>
<td>249 ± 7*</td>
<td>240 ± 5*</td>
</tr>
<tr>
<td>Plasma Insulin (mU/l)</td>
<td>57 ± 18</td>
<td>379 ± 190*</td>
<td>531 ± 101*</td>
</tr>
<tr>
<td>Blood Glucose (mmol/l)</td>
<td>3.8 ± 0.2</td>
<td>4.1 ± 0.4</td>
<td>16.0 ± 1.0*</td>
</tr>
<tr>
<td>Food Intake (g)</td>
<td>12.7 ± 0.4</td>
<td>13.0 ± 0.9</td>
<td>14.5 ± 1.0</td>
</tr>
<tr>
<td>TEE (kJ/day)</td>
<td>104 ± 6</td>
<td>139 ± 22*</td>
<td>133 ± 5*</td>
</tr>
<tr>
<td>RQ (ratio)</td>
<td>0.88 ± 0.01</td>
<td>0.86 ± 0.01</td>
<td>0.85 ± 0.01</td>
</tr>
<tr>
<td>CHO Oxidation (mg/min)</td>
<td>2.2 ± 0.2</td>
<td>2.5 ± 0.2</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>Fat Oxidation (mg/min)</td>
<td>0.4 ± 0.1</td>
<td>0.7 ± 0.1*</td>
<td>0.8 ± 0.1*</td>
</tr>
<tr>
<td>Activity (counts)</td>
<td>189 ± 19</td>
<td>245 ± 38</td>
<td>260 ± 25</td>
</tr>
</tbody>
</table>

TEE was elevated in obese-IGT and obese-diabetic *P. obesus* relative to lean-NGT animals (p<0.05, Table 3.1). TEE was positively correlated with body weight ($r^2=0.304$, p<0.001, Figure 3.1), and after correction for body weight, TEE was not different between groups. Physical activity levels were not different between groups.
Mean RQ tended to be lower in obese-diabetic *P. obesus* relative to lean-NGT animals (*p*=0.075), indicative of elevated fat oxidation relative to glucose oxidation (Table 3.1). Analysis of the 24 hour RQ profile revealed significant diurnal variation in obese-diabetic *P. obesus* with a peak at 1500 hours and a nadir at 0100 hours (Figure 3.2).

The mean fat oxidation rate was significantly elevated in obese-IGT and obese-diabetic *P. obesus* relative to lean-NGT animals (*p*<0.05, Table 3.1). Fat oxidation was positively correlated with body weight (*r*²=0.187, *p*<0.05, Figure 3.3) and relative to body weight, fat oxidation rates were not different between groups. Analysis of the 24 hour fat oxidation profile revealed significant diurnal variation in obese-diabetic *P. obesus* with a peak at 0100 hours and a nadir at 1500 hours (Figure 3.4a). Plasma FFA levels in obese-diabetic animals also exhibited a peak at 0600 hours (p<0.05, Figure 3.4b). Lean-NGT and obese-IGT *P. obesus* did not exhibit any diurnal variation in fat oxidation and plasma FFA levels.
Figure 3.2 24 hour respiratory quotient profile in *P. obesus*. Data expressed as mean ±SEM. * Significantly different to obese-IGT and obese-diabetic (p<0.05). # Significantly different to lean-NGT and obese-IGT (p<0.05).

Figure 3.3 Linear correlation between fat oxidation and bodyweight in *P. obesus* ($r^2=0.187$, p<0.05).
Figure 3.4 24 hour fat oxidation (3.4a) and plasma free fatty acid (3.4b) profiles in *P. obesus*. Data expressed as mean ± SEM. * Significantly different to obese-IGT and obese-diabetic (P<0.05). # Significantly different to obese-IGT (P<0.05). ^ Significantly different to lean-NGT (P<0.05).
The mean carbohydrate oxidation rates were not different between groups (Table 3.1). Analysis of the 24 hour glucose oxidation profile revealed a significant reduction in obese-diabetic animals between 2300-0700 hours, with a nadir at 0100 hours and a peak at 1300 hours (Figure 3.5a). Blood glucose levels were significantly diurnally elevated at 2400 hours in obese-diabetic P. obesus (p<0.05, Figure 3.5b), and tended to be elevated in lean-NGT and obese-diabetic animals.

**Figure 3.5** 24 hour glucose oxidation (3.5a) and blood glucose (3.5b) profiles in P. obesus. Data expressed as mean ±SEM. * Significantly different lean-NGT (P<0.05). # Significantly different to obese-IGT (P<0.05).
The 24 hour profile for plasma leptin concentrations in lean animals displayed a nocturnal increase with a peak between 1800-2400 hours and a nadir at 0600 hours (Figure 3.6). Obese-IGT and obese-diabetic groups did not display this diurnal variation in leptin concentrations. No diurnal variations were observed in activity levels, food intake or plasma insulin and cortisol concentrations in *P. obesus*.

![Plasma Leptin (ng/ml)](image)

**Figure 3.6** 24 hour plasma leptin profiles in *P. obesus*. Data expressed as mean ±SEM. * Significantly different to obese-IGT and obese-diabetic (P<0.05).

### 3.4.2 Dietary Energy Restriction

The effect of dietary energy restriction on energy metabolism was examined in lean-NGT, obese-IGT and obese-diabetic *P. obesus*. Two cohorts of animals were used. One group was placed on a diet restricting energy intake (restricted) and the other group was allowed *ad libitum* access to food (control). Phenotypic data for the restricted and control animals measured prior to restriction (age 18 weeks) and following restriction (age 20 weeks) are shown in Table 3.2. Phenotypic data for the control animals, measured at 18 weeks and 20 weeks are shown in Table 3.3. Body weight was significantly reduced in all restricted groups (p<0.05). Restriction significantly reduced
blood glucose and plasma insulin concentrations (p<0.05) in all groups. Following energy restriction, blood glucose and plasma insulin concentrations were not significantly different between lean-NGT, obese-IGT and obese-diabetic *P. obesus*. Amongst control animals body weight, blood glucose and plasma insulin concentrations did not significantly change over the experimental period.

**Table 3.2** Phenotypic and metabolic data for restricted *P. obesus* before and after the dietary energy restriction period. TEE = total energy expenditure, RQ = respiratory quotient, CHO = carbohydrate. * Significantly different to lean-NGT (p<0.05). # Significantly different to pre restriction (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Lean-NGT</th>
<th>Obese-IGT</th>
<th>Obese-Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>229 ± 7</td>
<td>212 ± 6#</td>
<td>234 ± 5</td>
</tr>
<tr>
<td>Plasma Insulin (mU/l)</td>
<td>67 ± 13#</td>
<td>60 ± 15</td>
<td>439 ± 109*</td>
</tr>
<tr>
<td>Blood Glucose (mmol/l)</td>
<td>3.7 ± 0.2</td>
<td>4.3 ± 0.3</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>TEE (kJ/day)</td>
<td>120 ± 7</td>
<td>115 ± 7</td>
<td>127 ± 18</td>
</tr>
</tbody>
</table>
| RQ (ratio)           | 0.87 ± 0.01| 0.82 ± 0.01#| 0.86 ± 0.02| 0.82 ± 0.01#| 0.82 ± 0.01|#| 0.81 ± 0.01#
| CHO Oxidation (mg/min)| 2.3 ± 0.2| 1.6 ± 0.2#| 2.3 ± 0.3     | 1.7 ± 0.2#| 2.4 ± 0.4| 1.6 ± 0.1#|
| Fat Oxidation (mg/min)| 0.67 ± 0.11| 0.86 ± 0.08#| 0.61 ± 0.2| 0.86 ± 0.06#| 0.81 ± 0.12*| 1.0 ± 0.1#|
| Activity (counts)    | 193 ± 32 | 277 ± 76  | 249 ± 32      | 289 ± 46  | 214 ± 26 | 246 ± 95  |

Indirect calorimetry and physical activity data, measured prior to and following dietary energy restriction, for the restricted and control groups are shown in Tables 3.2 and 3.3 respectively. Following energy restriction, TEE was reduced in all groups although this did not reach statistical significance. The change in body weight was positively correlated with the change in TEE ($r^2=0.150$, p<0.05, data not shown), and TEE remained proportional to body weight in all three groups (data not shown). Activity levels were not significantly affected by energy restriction. Amongst control animals TEE did not significantly change over the 2 week period.
Table 3.3 Phenotypic and metabolic data for control *P. obesus* before and after the dietary energy restriction period. TEE = total energy expenditure, RQ = respiratory quotient, CHO = carbohydrate. * Significantly different to lean-NGT (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Lean-NGT</th>
<th>Obese-IGT</th>
<th>Obese-Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>228 ± 8</td>
<td>232 ± 8</td>
<td>234 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma Insulin (mU/l)</td>
<td>81 ± 14</td>
<td>79 ± 19</td>
<td>313 ± 25*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood Glucose (mmol/l)</td>
<td>3.9 ± 0.2</td>
<td>4.6 ± 0.4</td>
<td>4.4 ± 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEE (kJ/day)</td>
<td>109 ± 12</td>
<td>109 ± 9</td>
<td>115 ± 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RQ (ratio)</td>
<td>0.86 ± 0.01</td>
<td>0.85 ± 0.01</td>
<td>0.87 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHO Oxidation (mg/min)</td>
<td>2.1 ± 0.1</td>
<td>1.9 ± 0.2</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Fat Oxidation (mg/min)| 0.53 ± 0.11| 0.54 ± 0.01| 0.59 ± 0.08 | 0.57 ± 0.01*
|                     |          |           |              | 0.95 ± 0.12* | 0.90 ± 0.10*|
| Activity (counts)   | 178 ± 40 | 180 ± 26  | 180 ± 27     | 215 ± 22 |
|                     |          |           |              | 211 ± 29 | 237 ± 18 |

RQ was significantly reduced with dietary energy restriction in lean-NGT, obese-IGT and obese-diabetic *P. obesus* (p<0.05). Glucose oxidation was significantly reduced in all restricted groups (p<0.05), and fat oxidation was increased in all groups, although this did not reach statistical significance in obese-diabetic animals (Table 3.2). Following energy restriction RQ, glucose oxidation and fat oxidation were not significantly different between the three groups. Amongst control animals RQ, fat oxidation and glucose oxidation rates did not significantly change over the 2 week period.

### 3.4.3 Fasting

The effect of 24 hour fasting on metabolism was also examined in *P. obesus*. Phenotypic data from lean-NGT, obese-IGT and obese-diabetic animals, prior to and following fasting is shown in Table 3.4. Fasting significantly reduced plasma insulin concentrations in obese-IGT and obese-diabetic *P. obesus* (p<0.05). Fasting significantly reduced blood glucose concentrations in
obese-diabetic *P. obesus* (p<0.05). Following fasting, blood glucose and plasma insulin concentrations were not significantly different between groups.

**Table 3.4** Phenotypic and metabolic data for *P. obesus* before and after the fasting period. TEE = total energy expenditure, RQ = respiratory quotient, CHO = carbohydrate. * Significantly different to lean-NGT (p<0.05). # Significantly different to fed (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Lean-NGT</th>
<th>Obese-IGT</th>
<th>Obese-Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fed</strong></td>
<td>Fast</td>
<td>Fed</td>
<td>Fast</td>
</tr>
<tr>
<td><strong>Body Weight</strong> (g)</td>
<td>229 ± 15</td>
<td>225 ± 16</td>
<td>249 ± 5</td>
</tr>
<tr>
<td><strong>Plasma Insulin</strong> (mU/l)</td>
<td>64 ± 25</td>
<td>47 ± 15</td>
<td>284 ± 77*</td>
</tr>
<tr>
<td><strong>Blood Glucose</strong> (mmol/l)</td>
<td>3.9 ± 0.5</td>
<td>3.9 ± 0.3</td>
<td>3.8 ± 0.4</td>
</tr>
<tr>
<td><strong>TEE</strong> (kJ/day)</td>
<td>114 ± 6</td>
<td>97 ± 2  #</td>
<td>127 ± 6</td>
</tr>
<tr>
<td><strong>RQ</strong> (ratio)</td>
<td>0.86 ± 0.01</td>
<td>0.75 ± 0.02#</td>
<td>0.84 ± 0.01#</td>
</tr>
<tr>
<td><strong>CHO Oxidation</strong> (mg/min)</td>
<td>2.0 ± 0.1</td>
<td>0.70 ± 0.1#</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td><strong>Fat Oxidation</strong> (mg/min)</td>
<td>0.70 ± 0.1</td>
<td>0.93 ± 0.01#</td>
<td>0.85 ± 0.02#</td>
</tr>
<tr>
<td><strong>Activity</strong> (counts)</td>
<td>221 ± 52</td>
<td>202 ± 24</td>
<td>212 ± 17</td>
</tr>
</tbody>
</table>

Indirect calorimetry and physical activity data for the 24 hour baseline period and the 24 hours while the animals were fasting are shown in Table 3.4. Mean fasting TEE was significantly lower than baseline in lean-NGT, obese-IGT and obese-diabetic *P. obesus* (p<0.05). Mean fasting TEE was not significantly different between groups. Activity levels were not significantly affected by fasting.

RQ was significantly reduced in lean-NGT, obese-IGT and obese-diabetic *P. obesus* (p<0.05). Glucose oxidation was significantly reduced in all groups (p<0.05), and fat oxidation was increased, although this did not reach statistical significance in the obese-diabetic animals (Table 3.4). Following fasting, RQ, glucose oxidation and fat oxidation were not significantly different between groups.
3.5 DISCUSSION

3.5.1 Energy Intake and Expenditure in *Psammomys obesus*

Obesity in *P. obesus* is accompanied by an elevation in energy expenditure, independently of the presence of diabetes. However, energy expenditure remains proportional to body weight in lean-NGT, obese-IGT and obese-diabetic animals, suggesting obesity is unlikely to be the result of a gross disturbance in energy expenditure. Food intake was not significantly different between lean and obese groups, although there was a tendency for increased intake in obese-diabetic animals. Studies in humans have shown energy expenditure, relative to FFM, is not different between lean and obese subjects supporting the findings of this study (Bogardus et al., 1986a; Nelson et al., 1992).

Dietary energy restriction reduced body weight in all animals. Energy expenditure was subsequently reduced in proportion to the reduction in body weight, and relative to body weight, energy expenditure was not different between groups. Energy expenditure was dramatically reduced with fasting and this response was not different between groups. Taken together these results suggest that obese *P. obesus* do not manifest a gross inability to regulate energy expenditure in response to dietary manipulations. A significant increase in energy intake accompanying obesity in *P. obesus* was expected, however estimating food intake from food disappearance data may not have been accurate enough to detect these differences. A study with inbred diabetes prone and diabetes resistant lines of *P. obesus* found the energy cost of weight gain was lower in the diabetes prone line suggesting differences in either tissue content or energy expenditure (Kalman et al., 1993). Longitudinal energy metabolism studies in pre-obese *P. obesus* are necessary to determine the primary cause of obesity in our outbred colony.

The reduction in energy expenditure accompanying dietary energy restriction is a survival mechanism that compensates for periods of famine (Weigle et al., 1988). The magnitude of the reduction in energy expenditure in *P. obesus* was less than that seen in other rodents (Hill et al., 1985). Baseline energy
expenditure in *P. obesus* is only 60 percent of that seen in rodents of similar mass (Pinshow, 1993), suggesting these animals have little room left for further energy conservation.

In the present study individual components of energy expenditure (BMR, thermogenesis and physical activity) were not measured directly. However, physical activity, measured as the number of infrared beams broken, was not different following dietary restriction. Studies in rhesus monkeys (Kemnitz et al., 1993) and humans (Weyer et al., 2000b) have found reduced physical activity following dietary energy restriction. Although speculative, the maintenance of physical activity levels in *P. obesus* may be an adaptive response to search for food. The most likely cause of the decreased energy expenditure accompanying dietary energy restriction is a reduction in BMR due to the decrease in body weight, however a reduction in thermogenesis is also possible.

### 3.5.2 Substrate Utilisation in *Psammomys obesus*

Whole body glucose oxidation rates were not different between lean-NGT, obese-IGT and obese-diabetic *P. obesus*. Furthermore, whole body glucose oxidation rates following dietary energy restriction and while fasting were not different between groups. Hyperglycemia is therefore unlikely to be due a defect in whole body glucose oxidation. Similarly, studies in humans have shown fasting whole body glucose oxidation rates were not different between type 2 diabetic subjects and controls (Vaag *et al.*, 1992; Fery *et al.*, 1993; Kelley *et al.*, 1994).

Fat oxidation was elevated in obese-IGT and obese-diabetic *P. obesus* and was positively correlated with body weight. Several studies in humans have found increased fat oxidation in obese subjects (Felber *et al.*, 1987; Niskanen *et al.*, 1997). Glucose and protein oxidation rates are tightly regulated to maintain net glucose and protein balance respectively (Schutz *et al.*, 1989; Jebb *et al.*, 1996). In contrast fat oxidation is largely independent of fat intake and is quantitatively dependent on the difference between total energy requirements and energy derived from glucose and protein oxidation (Schutz *et al.*, 1989).
The development of obesity, and the accompanying elevation in fat oxidation, may be sufficient to attain fat balance as body weight stabilises (Schutz et al., 1989; Flatt, 1993).

Short term fasting dramatically altered whole body energy metabolism in *P. obesus*. Carbohydrate oxidation was substantially reduced and fat oxidation was increased in all animals. The net result was a reduction in energy expenditure, independent of body weight. Blood glucose levels and whole body glucose oxidation rates were not different between diabetic and nondiabetic animals, suggesting 24 hours of fasting is sufficient to match EGP to whole body glucose disposal in diabetic animals.

Dietary energy restriction reduced body weight and normalised blood glucose and insulin levels in *P. obesus*. Whole body glucose oxidation was reduced and fat oxidation was increased. Glucose and fat oxidation rate were not different between groups. Fat oxidation rates were not significantly increased in obese-diabetic animals. This is most likely due to already elevated baseline fat oxidation rates in these animals. Several studies in humans have demonstrated fat oxidation rates in post obese subjects are lower than in never obese controls (Astrup et al., 1994; Larson et al., 1995; Wyatt et al., 1999; Filozof et al., 2000; Weyer et al., 2000a). However, several studies with mice or rats have found fat oxidation was increased (Boschmann et al., 1994) or unchanged (Chen and Heiman, 2000; Rohner-Jeanrenaud et al., 2002) with weight loss following dietary energy restriction. The discrepancy between human subjects and either rodents or gerbils may represent different physiological responses to weight loss between species. Alternatively different experimental design with respect to magnitude and rate of weight loss may also contribute to variability between studies, especially if some subjects remain in negative energy balance while others have maintained a stable body weight.

Dietary energy restriction and fasting initially reduces blood glucose levels prior to the reduction in body weight, due to the reduction in glucose intake and decreased EGP (Christiansen et al., 2000). Subsequent improvements in
the blood glucose and lipid profiles are likely to be mediated through reduced EGP, although peripheral insulin sensitivity and glucose effectiveness are also improved (Christiansen et al., 2000).

Insulin resistance increases with weight gain and decreases with weight loss (Ferrannini et al., 1991; Ferrannini et al., 1997). Therefore, visceral adiposity, which drains directly to the liver via the portal vein may potentially mediate the relationship between insulin resistance and body weight, as the liver is exposed to an increased flux of FFA and precursors for glucose synthesis, promoting hepatic glucose production and very low density lipoprotein (VLDL) secretion, and contributing to hepatic insulin resistance (Kissebah and Krakower, 1994). Surgical removal of visceral fat has been shown to increase hepatic insulin sensitivity in rats (Barzilai et al., 1999). Abdominal fat content was independently associated with blood glucose and plasma insulin concentrations in a large cross sectional study of P. obesus (Walder et al., 2000), suggesting visceral adiposity is an important determinant of glucose metabolism in these animals.

3.5.3 Diurnal Substrate Utilisation in Psammomys obesus

Unlike other animal models of obesity and diabetes, P. obesus do not display circadian variation in food intake or physical activity levels. In contrast rats consume more food and are most active during the dark phase (Mistlberger et al., 1998). Rats also display circadian variations in blood glucose concentrations, which are controlled by the suprachiasmatic nucleus (la Fleur et al., 2001). P. obesus may be a better model to explore the circadian variations in glucose homeostasis without the confounding effects of activity and food intake.

Obese-diabetic P. obesus exhibited elevated fat oxidation rates and plasma glucose levels during the dark phase. Increased activity levels and plasma FFA concentration can each increase fat oxidation (Jequier, 1998), however neither of these factors were associated with the elevated fat oxidation in P. obesus. Boden et al (1996a) found nocturnal insulin sensitivity decreased and EGP increased in type 2 diabetic subjects. Fasting EGP is elevated in obese-
diabetic *P. obesus* (Barnett *et al.*, 1995), however it is unclear if increased EGP is responsible for the nocturnal elevation in blood glucose levels as EGP was not measured in the present study. Plasma insulin and cortisol levels did not display any diurnal variations and are therefore unlikely to be responsible for the nocturnal increase in plasma glucose and fat oxidation rates. Further studies are necessary to determine the physiological significance of these metabolic disturbances.

Lean-NGT *P. obesus* display a nocturnal elevation in plasma leptin concentration. Similarly, studies in lean human subjects have found nocturnal increases (Sinha *et al.*, 1996). Basal plasma leptin concentrations in obese-IGT and obese-diabetic *P. obesus* are elevated and these animals are extremely resistant to leptin administration, relative to lean-NGT animals (Walder *et al.*, 1997). The absence of diurnal variations in obese-IGT and obese-diabetic *P. obesus* further supports the possibility of dysregulated leptin signalling in these animals.

### 3.5.4 Summary

Whole body energy expenditure and substrate utilisation in *P. obesus* were similar to that seen in humans, providing further validation of *P. obesus* as an ideal animal model of obesity and type 2 diabetes. Energy expenditure and fat oxidation were elevated in obese-IGT and obese-diabetic animals. After adjusting for body weight, neither energy expenditure nor fat oxidation were different between groups. Short term fasting and dietary energy restriction normalised blood glucose and plasma insulin levels in obese-IGT and obese-diabetic animals. Dietary energy restriction also reduced body weight in all animals. Energy expenditure, glucose oxidation and fat oxidation were not different between lean-NGT, obese-IGT and obese-diabetic animals after normalising blood glucose and plasma insulin concentrations.

Obese-diabetic *P. obesus* exhibited elevated nocturnal fat oxidation rates and plasma glucose levels, independent of plasma insulin and cortisol levels. Further studies are necessary to elucidate the importance of these nocturnal fluctuations in glucose and fat metabolism in the pathogenesis of obesity and
diabetes in these animals. Lean-NGT *P. obesus* display a nocturnal elevation in plasma leptin concentration, which is absent in obese-IGT and obese-diabetic animals, supporting the dysregulation of leptin signalling in obese *P. obesus*. 
Gene Discovery with SSH and cDNA Dot Blots

4.1 ABSTRACT

This study aimed to identify novel genes differentially expressed in the liver of lean-NGT and obese-diabetic *Psammomys obesus*. Suppression subtractive hybridisation (SSH) was used to enrich cDNA library for differentially expressed genes. cDNA dot blots were used to screen 576 clones with cDNA derived from lean-NGT and obese-diabetic animals. 6 clones were identified as overexpressed in lean-NGT animals and 6 were overexpressed in obese-diabetic animals. These 12 clones were sequenced and SYBR-Green PCR was used to confirm differential gene expression. 4 genes were overexpressed (>1.5 fold) in lean-NGT animals and 4 genes were overexpressed (>1.5 fold) in obese-diabetic animals.

To explore the physiological role of these genes, hepatic gene expression was examined in several physiological conditions. Another, larger cohort of *P. obesus* with *ad libitum* access to food was used. In addition, a cohort of *P. obesus* were fasted for 24 hours and another cohort were restricted to 67% of their usual food intake for 14 days, prior to being sacrificed.

One gene, encoding thyroxine binding globulin (TBG), was confirmed as overexpressed in lean-NGT *P. obesus* with *ad libitum* access to food, relative to both obese-IGT and obese-diabetic animals. TBG expression decreased with fasting in all animals. Fasting TBG expression remained greater in lean-NGT animals than obese-IGT and obese-diabetic animals. TBG expression was not significantly affected by dietary energy restriction. TBG is involved in thyroid metabolism and is potentially involved in the regulation of energy expenditure.

Fasting increased hepatic site 1 protease (S1P) expression in lean-NGT animals but was not significantly affected in obese-IGT and obese-diabetic animals. S1P expression was not significantly affected by dietary energy restrictions.
restriction. S1P is involved in the proteolytic processing of steroid response element binding proteins (SREBP). SREBPs are insulin responsive and are known to be involved in lipid metabolism.

Gene expression studies found TBG and S1P were associated with obesity and diabetes. Future research will determine whether TBG and S1P are important in the pathogenesis of these diseases.

4.2 INTRODUCTION

Type 2 diabetes mellitus is a polygenic disease characterised by defects in insulin secretion and insulin action, and disturbances in carbohydrate, fat and protein metabolism which are at least partly due to hepatic defects (ADA, 2000; Gerich, 1991; Perriello et al., 1997). \textit{P. obesus} is a unique animal model of obesity and type 2 diabetes, ideally suited for studies aiming to identify novel genes involved in the pathophysiology of type 2 diabetes and obesity.

Novel genes associated with diabetes can be identified using gene expression based approaches without prior knowledge of the disease process. The primary limitation of this strategy is the large number of genes that are expressed within a tissue of interest at any given time (Bonaldo et al., 1996). Subtractive hybridisation based techniques can be used to screen this large number of genes to identify those of interest based on different levels of expression in diseased and control samples (Lisitsyn and Wigler, 1993).

Suppression subtractive hybridisation (SSH) is novel a polymerase chain reaction (PCR) based subtractive hybridisation technique (Diatchenko et al., 1996). Target cDNA fragments are enriched by favourable hybridisation kinetics and PCR amplification (Hubank and Schatz, 1999). The enriched cDNA library can be cloned and putatively differentially expressed genes can be screened with cDNA dot blots.
The current study was undertaken to identify genes differentially expressed in the liver of lean-NGT and obese-diabetic *Psammomys obesus*, utilising SSH and cDNA dot blots. It was hypothesised that these genes could represent significant factors in the pathophysiology of type 2 diabetes.

### 4.3 RESEARCH DESIGN AND METHODS

Research design and methods are discussed in detail in Chapter 2 and described briefly below.

**4.3.1 Suppression Subtractive Hybridisation**

SSH studies used 18-week old lean-NGT (n=3) and obese-diabetic (n=3) *Psammomys obesus*. These were designated as SSH animals and had *ad libitum* access to food. Body weight, blood glucose (Yellow Springs Instruments, Yellow Springs, USA) and plasma insulin were measured (Insulin RIA, Phadeseph, Uppsala, Sweden). Two SSH experiments were conducted using the PCR-Select cDNA subtraction kit (Clontech, Palo Alto, U.S.A.). Experiment 1 was designed to enrich a cDNA pool for genes overexpressed in the liver of lean-NGT *Psammomys obesus*. Conversely, experiment 2 was designed to enrich cDNA for genes overexpressed in obese-diabetic *Psammomys obesus*.

**4.3.2 Cloning and cDNA Reamplification**

The enriched cDNA from the SSH experiments 1 and 2 were cloned using the T/A Cloning Kit Invitrogen, Carlsbad, USA). The PCR products were ligated into a pCR2.1 plasmid vector and chemically transformed into TOP10 *E. coli* cells. White colonies, representing successfully transfected clones, were selected and grown overnight. These clones were amplified by PCR and used to prepare cDNA dot blots.

**4.3.3 cDNA Dot Blots Screening**

Two identical nylon membranes (Bright-Star Plus, Ambion, Austin, USA) were prepared for cDNA dot blots, using The PCR Select Differential Screening Kit (Clontech, 1999). The PCR products representing the positive
clones were transferred onto nylon membranes using a dot blot apparatus (Biorad, Anaheim, USA) and cross-linked using a UV Stratalinker at 120mJ (Stratagene, Austin, USA).

The enriched cDNA from experiment 1 and 2 were used to prepare tester and driver probes respectively. Probes were each allowed to hybridise to the nylon membrane. Membranes were exposed to a phosphorous plate and examined with a phosphorimager (Molecular Dynamics, Sunnyvale, USA). A clone was identified as overexpressed in lean-NGT *P. obesus* when a stronger signal was detected from the tester probe than the driver probe.

### 4.3.4 Sequencing and Bioinformatics

Putatively overexpressed clones were reamplified from bacteria with PCR, visualised on an agarose gel with electrophoresis and excised. cDNA was purified using the UltraClæan GelSpin DNA Purification Kit (Mo Bio Laboratories, Solana Beach, USA). cDNA was sequenced by DyeDeoxy Terminator (Applied Biosystems, Foster City, USA) using an automated DNA sequencer (Applied Biosystems, Foster City, USA).

Sequences were examined for homology with known genes or expressed sequence tags (ESTs), using blast searches of databases at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

### 4.3.5 Gene Expression Studies

To validate the differential expression identified with cDNA dot blot screening and to explore the physiological role of the genes identified, three sets of gene expression studies were conducted. Firstly, to confirm the cDNA dot blot results, gene expression was examined in the lean-NGT and obese-diabetic SSH animals. Secondly, to explore any potential role in energy metabolism, gene expression was examined in fed and fasted *P. obesus*. Lean-NGT, obese-IGT and obese-diabetic animals were included. Thirdly, to further explore any metabolic role, gene expression was examined in fed and dietary energy restricted *P. obesus*. Lean-NGT, obese-IGT and obese-diabetic animals were included.
Gene expression studies utilised SYBR Green PCR used the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, USA). Primer were designed with Primer Express software (Version 1.5, Applied Biosystems, Foster City, USA). Gene expression was normalised to cyclophilin, an endogenous control. Dissociation curve analysis (Applied Biosystems, Foster City, USA) was used with the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, USA) to determine if a single PCR product was generated with the SYBR Green PCR.

4.3.5.1 Fasting
18-week old lean-NGT (n=15), obese-IGT (n=16) and obese-diabetic (n=13) *P. obesus* were separated into fed and fasted groups. Groups were matched for sex and body weight. Fed animals had *ad libitum* access to food, whereas food was withdrawn from the fasted animals for 24 hours. Body weight, blood glucose and plasma insulin were measured at the beginning and end of the fasting period.

4.3.5.2 Dietary Energy Restriction
At 18 weeks of age lean-NGT (n=13), obese-IGT (n=15) and obese-diabetic (n=12) *P. obesus* underwent 24 hours of indirect calorimetry with an Oxymax Equal Flow Indirect Calorimeter (Columbus Instrument, Ohio, USA) to measure fat oxidation, carbohydrate oxidation, respiratory quotient (RQ) and total energy expenditure (TEE). Physical activity was measured while animals were in the calorimeter using the Opto-Varimax Mini-Infrared Animal Activity Monitoring System (Columbus Instruments, Ohio, USA).

Animals had *ad libitum* access to food and water. Animals were separated into fed and restricted groups for a 14 day experimental period. Groups were matched for sex and body weight. Fed animals had *ad libitum* access to food, whereas restricted animals were given 66 percent of the average food intake of their respective group during the baseline period. Body weight and food disappearance were measured daily. At the completion of the restriction period blood samples were taken and animals were placed in the indirect
calorimeter for another 24 hours. Blood glucose and plasma insulin were measured.

4.3.6 Statistical Analysis
Statistical analysis was conducted using SPSS (Version 10, SPSS inc, Chicago, USA). A Kolmogorov-Smirnov test was used to examine the distribution of data. Normally distributed data in two groups was assessed using an independent samples T-test. Data in three or more groups was assessed using one way ANOVA, with the LSD post hoc test when variance between groups was homogeneous, and with the Games-Howell post hoc test when variances were not homogenous. Non-normally distributed data was analysed using a Mann-Whitney test. The relationship between linear variables was assessed using Pearson and Spearman correlations for normally and non-normally distributed data respectively. Statistical significance was set at p<0.05.

4.4 RESULTS

4.4.1 Suppression Subtractive Hybridisation
Lean-NGT and obese-diabetic *P. obesus* were selected for gene discovery studies, allowing for the identification of genes differentially expressed with diabetes as well as obesity. *P. obesus* were male and 18 weeks of age. Phenotypic data is shown in Table 4.1.

Experiment 1 enriched a cDNA pool for genes overexpressed in lean-NGT *P. obesus*. β actin was visible on an agarose gel after 15 cycles of PCR with non enriched cDNA (Figure 4.1a). In contrast, β actin was visible after 25 cycles with enriched cDNA. This 10 cycle difference corresponds to an approximate 1000 fold enrichment of overexpressed genes. Experiment 2 enriched cDNA for genes overexpressed in obese-diabetic *P. obesus*. β actin was visible 10 cycles later in enriched cDNA than non enriched, corresponding to a 1000 fold enrichment (Figure 4.1b). As further confirmation of enrichment, G3PDH expression in experiments 1 and 2 were reduced approximately 1000 fold (data not shown).
**Table 4.1** Phenotypic data for the *P. obesus* used in SSH experiments. Data expressed as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Lean-NGT</th>
<th>Obese-Diabetic</th>
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<tbody>
<tr>
<td>n</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>210.7 ± 7.8</td>
<td>245.3 ± 2.9</td>
</tr>
<tr>
<td>Blood Glucose (mmol/l)</td>
<td>3.9 ± 0.4</td>
<td>9.7 ± 0.3</td>
</tr>
<tr>
<td>Plasma Insulin (µU/ml)</td>
<td>64 ± 17</td>
<td>481 ± 200</td>
</tr>
</tbody>
</table>

**Figure 4.1.** β-actin PCR products from SSH experiments 1 (4.1a) and 2 (4.1b) visualised on a 1% agarose gel. PCR products from enriched and non-enriched cDNA were amplified for 15, 20, 25, 30 and 35 cycles.

**4.4.2 cDNA Dot Blots Screening**

Putatively overexpressed clones from lean-NGT (n=288) and obese-diabetic *P. obesus* (n=288) were screened with cDNA dot blots. Blots were hybridised with cDNA from experiment 1 (lean-NGT enriched) and experiment 2 (obese-diabetic enriched). 6 clones were identified as overexpressed in lean-NGT *P. obesus* and 6 clones overexpressed in obese-diabetic *P. obesus*. A representative pair of cDNA dot blots are shown in Figure 4.2. These 12 differentially expressed genes were sequenced. Blast searches of the NCBI databases (http://www.ncbi.nlm.nih.gov), 9 clones showed homology with
known genes (Table 4.2). The remaining 3 clones did not show significant homology with known genes or (EST’s) on NCBI databases.

Figure 4.2. cDNA dot blots probed with $^{32}$P labelled tester (4.2a) and driver (4.2b) enriched cDNA. Open squares indicate clones displaying evidence of overexpression.

4.4.3 Gene Expression Studies
4.4.3.1 SSH Animals
First stage confirmation of these putatively differentially expressed genes involved measuring gene expression in the liver of the animals used to generate the enriched cDNA (Table 4.1). 4 genes showed 1.5 fold or more overexpression in lean-NGT animals (Table 4.2) and 4 genes showed 1.5 fold or more overexpression in obese-diabetic animals (Table 4.3).

Table 4.2. Clones overexpressed in lean-NGT P. obesus. Homologies with known genes and ESTs on NCBI databases. Overexpression presented as the ratio of expression of lean-NGT to obese-diabetic animals.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Gene Homology</th>
<th>Genebank Accession no.</th>
<th>Overexpression</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGT 12</td>
<td>Cytochrome P450, Subunit IIa</td>
<td>NM000766</td>
<td>5.1</td>
</tr>
<tr>
<td>AGT 16</td>
<td>Novel</td>
<td>N/A</td>
<td>1.2</td>
</tr>
<tr>
<td>AGT 18</td>
<td>Zinc Finger Protein 274</td>
<td>BC009763</td>
<td>0.7</td>
</tr>
<tr>
<td>AGT 24</td>
<td>C Reactive Protein</td>
<td>X17496</td>
<td>4.5</td>
</tr>
<tr>
<td>AGT 32</td>
<td>Thyroxine Binding Globulin</td>
<td>M63991</td>
<td>7.7</td>
</tr>
<tr>
<td>AGT 33</td>
<td>Cytochrome P450, Subunit IIIa</td>
<td>AB039380</td>
<td>7.0</td>
</tr>
</tbody>
</table>
**Table 4.3.** Clones overexpressed in obese-diabetic *P. obesus*. Homologies with known genes and ESTs on NCBI databases. Overexpression of obese-diabetic genes is presented as the ratio of expression of obese-diabetic to lean-NGT animals.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Gene Homology</th>
<th>Genebank Accession no.</th>
<th>Overexpression</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGT 7</td>
<td>Site 1 Protease</td>
<td>NM053569</td>
<td>1.3</td>
</tr>
<tr>
<td>AGT 8</td>
<td>Novel</td>
<td>N/A</td>
<td>1.5</td>
</tr>
<tr>
<td>AGT 22</td>
<td>Novel</td>
<td>N/A</td>
<td>1.5</td>
</tr>
<tr>
<td>AGT 27</td>
<td>MS4A4</td>
<td>AB013102</td>
<td>1.5</td>
</tr>
<tr>
<td>AGT 30</td>
<td>HSPC055</td>
<td>AF161540</td>
<td>1.7</td>
</tr>
<tr>
<td>AGT 31</td>
<td>Huntington-Interacting Protein</td>
<td>AJ238403</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**4.4.3.2 Fed and Fasted Animals**

Second stage confirmation involved determining gene expression in a different and larger subset of animals. Lean-NGT obese-IGT and obese-diabetic *P. obesus* were used. To further elucidate any potential role in energy metabolism another set of lean-NGT obese-IGT and obese-diabetic *P. obesus* sacrificed after 24 hours of fasting were also included. Phenotypic data for these animals is shown in Table 4.4. Previous studies with *P. obesus* have shown 24 hour fasting is associated with reduced total energy expenditure and carbohydrate oxidation, and increased fat oxidation (Chapter 3.4). Fasting also normalises blood glucose and plasma insulin levels in obese-IGT and obese-diabetic groups (Chapter 3.4).

**Table 4.4.** Phenotypic data for fed and fasted *P. obesus*. Data expressed as mean ± SEM. * Indicates a significant difference compared to lean-NGT animals (p<0.05). # Indicates a significant difference compared to fed animals (p<0.05).
Using SYBR Green PCR in the larger group of animals, only AGT 32 displayed significantly greater gene expression in lean-NGT *P. obesus* relative to obese-diabetic *P. obesus*. This gene encoded Thyroxine Binding Globulin (TBG) (Figure 4.3). The 350 nucleotides of *P. obesus* TBG is 91 percent homologous to rat TBG (Genebank accession no. M63991) and 78 percent homologous to human TBG (Genebank accession no. M14019).

Hepatic TBG gene expression in *P. obesus* is shown in Figure 4.4. Expression was significantly greater in lean-NGT animals than obese-IGT (p=0.001) and obese-diabetic animals (p=0.003). TBG expression was reduced with fasting in all groups (p=0.039). Furthermore, amongst fasted *P. obesus* TBG expression was greater in lean animals than obese-IGT (p=0.014) and obese-diabetic (p=0.018) groups. TBG expression was negatively correlated with body weight (r=0.40, p=0.007, Figure 4.5a) and plasma insulin concentration in these animals (r=0.30, p=0.049, Figure 4.5b). SYBR Green PCR in the larger group of animals did not confirm any of the genes identified as overexpressed in obese-diabetic *P. obesus*.

AGT 7 gene expression was less than 1.5 fold different between lean-NGT and obese-diabetic *P. obesus* and was therefore not confirmed during the first stage of confirmation. However, the Blast search of the NCBI database found AGT 7 encoded site 1 protease (S1P) (Figure 4.6). The 480 nucleotides of *P. obesus* is 93 percent homologous to rat S1P (Genebank accession no. NM053569) and 91 percent homologous to human S1P (Genebank accession no. NM003791). S1P is a substilisin-related protease that cleaves sterol
regulatory element-binding proteins (SREBPs). SREBPs are transcription-regulating proteins, involved in feedback systems that regulate multiple enzymes in the cholesterol and fatty acid biosynthesis pathways (Brown and Goldstein, 1999; Osborne, 2000). Given the known role of S1P in metabolism, S1P gene expression was examined for further association with diabetes or obesity in fed and fasted *P. obesus*.

**Figure 4.4.** TBG gene expression in fed and fasted *P. obesus*. Data expressed as mean ± SEM. * Indicates a significant difference compared to lean-NGT animals (p<0.05). # Indicates a significant difference compared to fed animals (p<0.05).

**Figure 4.5.** Linear correlation between TBG gene expression and body weight (4.5a) and plasma insulin (4.5b) in *P. obesus*.
Hepatic S1P gene expression is fed and fasted *P. obesus* is shown in Figure 4.7. S1P expression was not different between lean-NGT, obese-IGT and obese-diabetic *P. obesus* in the fed state. Gene expression was elevated in fasted compared to fed *P. obesus* when groups were combined (p=0.003).

```
GAGGGATGNGGCCATGTGGCCTTTCGTATCTTTAAGTGACTGGTACAAC
ACTTCTGTTATGAGAAAGTGAAATTTATGATGAAAAACAAAGGCAGTGTT
GGATGCCAGATACTGGAGGAGCCCAACATCCAGCTCTGAATGAGCTGCTGTC
TGATGAAACATGGGTTGACGTGACGCTTATGAAGGGGAAATTTTGCTCTG
GCAAACCATGACATGTATTATGCGTCGGGGTGACATCCAGTTGTTACAG
AAGATGTTGTGATGCACACAGACCTTCAAGGATCAAGGATGGAGGTCTT
AAAAAAGGAGACAGCCAGTTGATGAAATTTGCTTTCCATTTGCGGCTCTAG
ATCCAGCTGAGGTTGAGACTGCTATGTGCTGTATGGAGACTTCACTGCT
TGATGACAGTCACAGACAGAAGGACTGGTTTGGCTTCTGGATGCCTCCT
TNAGTACCTCGG
```

**Figure 4.6.** S1P nucleotide sequence in *P. obesus*.

![Figure 4.7. S1P gene expression in fed and fasted *P. obesus*. Data expressed as mean ± SEM. * Indicates a significant difference compared to lean-NGT fasted animals (p<0.05). # Indicates a significant difference compared to fed animals (p<0.05).](image)

**4.4.3.3 Fed and Dietary Energy Restricted Animals**
To further elucidate the potential role of TBG in metabolism, gene expression was examined in dietary energy restricted *P. obesus*. Phenotypic data for fed and energy restricted and control animals are shown in Table 4.5. Previous studies have shown 14 days of dietary energy restriction reduced body weight in all groups and normalised blood glucose and insulin levels in obese-IGT and obese-diabetic groups (Chapter 3.4). Total energy expenditure decreased in proportion to body weight, glucose oxidation was decreased and fat oxidation increased (Chapter 3.4).

**Table 4.5.** Phenotypic and metabolic data for fed and energy restricted *P. obesus*. Data expressed as mean ± SEM. * Indicates a significant difference compared to lean-NGT animals (p<0.05). # Indicates a significant difference compared to fed animals (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Lean-NGT</th>
<th></th>
<th>Obese-IGT</th>
<th></th>
<th>Obese-Diabetic</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fed</td>
<td>Rest</td>
<td>Fed</td>
<td>Rest</td>
<td>Fed</td>
<td>Rest</td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>231.7±9.4</td>
<td>215.0±7.5</td>
<td>242.0±5.6</td>
<td>223.8±8.7</td>
<td>251.8±8.8</td>
<td>236.7±4.6*</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>3.3±0.3</td>
<td>2.1±0.4#</td>
<td>3.7±0.3</td>
<td>2.5±0.3#</td>
<td>4.5±0.2*</td>
<td>3.7±0.3*</td>
</tr>
<tr>
<td>Blood Glucose (mmol/l)</td>
<td>4.0±0.2</td>
<td>4.3±0.4</td>
<td>4.4±0.5</td>
<td>3.6±0.4</td>
<td>12.4±2.7*</td>
<td>4.6±0.5#</td>
</tr>
<tr>
<td>Plasma Insulin (µU/ml)</td>
<td>91±18</td>
<td>72±17</td>
<td>303±67</td>
<td>37±12#</td>
<td>275±60</td>
<td>58±20</td>
</tr>
<tr>
<td>TEE (kJ/day)</td>
<td>109±9</td>
<td>115±7</td>
<td>1.21±7</td>
<td>115±5</td>
<td>136±7*</td>
<td>127±7</td>
</tr>
<tr>
<td>Fat Oxidation (mg/min)</td>
<td>0.54±0.01</td>
<td>0.86±0.08</td>
<td>0.57±0.01</td>
<td>0.86±0.06#</td>
<td>0.90±0.1</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>CHO Oxidation (mg/min)</td>
<td>1.9±0.2</td>
<td>1.6±0.2</td>
<td>1.9±0.2</td>
<td>1.7±0.2</td>
<td>1.9±0.2</td>
<td>1.6±0.1</td>
</tr>
</tbody>
</table>

Gene expression studies in these animals was not normalised to a housekeeping gene, as cyclophilin expression in lean-NGT animals was different between fed and dietary energy restricted groups (p<0.05). Gene expression data was therefore normalised to an arbitrary value. The TBG expression profile previously seen in fed; lean-NGT, obese-IGT and obese-diabetic animals was reproduced in these fed animals (Figure 4.8). TBG expression was in lean-NGT animals was significantly greater than obese-diabetic animals (p=0.004) and tended to be greater than obese-IGT animals.
(p=0.066). TBG expression was not significantly affected by dietary energy restriction in *P. obesus*. Furthermore, TBG expression was not correlated with adiposity, blood glucose or insulin levels, energy expenditure, or fat and glucose oxidation rates.

Figure 4.8. TBG gene expression in fed and dietary energy restricted *P. obesus*. Data expressed as mean ± SEM.

To further elucidate the potential role of S1P in metabolism, gene expression was examined in fed and dietary energy restricted *P. obesus*. S1P gene expression is shown in Figure 4.9. S1P expression was not significantly different between lean-NGT, obese-IGT and obese-diabetic *P. obesus*, and did not change with dietary energy restriction. S1P expression did not correlate with adiposity, blood glucose or insulin levels, energy expenditure, or fat and glucose oxidation rates.
Figure 4.9. S1P gene expression in fed and dietary energy restricted *P. obesus*. Data expressed as mean ± SEM.

### 4.5 DISCUSSION

SSH and cDNA dot blot screening identified 12 genes potentially differentially expressed in lean-NGT and obese-diabetic *P. obesus*. SYBR Green PCR confirmed 8 genes were at least 1.5 fold differentially expressed. The inability to confirm all but one of the 8 genes in a different subset of animals does not indicate a failure of either the SSH or cDNA dot blot techniques, as a 1000 fold enrichment of overexpressed genes was achieved. Furthermore, 67% of the genes identified exceeded the 1.5 fold overexpression reported to be the sensitivity level of the SSH technique (Clontech, 2000). Each of these genes may potentially contribute to the diabetes phenotype of these animals, however they are unlikely to be common causes in *P. obesus* given they were only differentially expressed in a small subset of animals.

Experimental groups were sex and age matched, and there was no indication that the animals selected were not representative of the lean-NGT obese-IGT and obese-diabetic groups they were classified into. However, *P. obesus* do not fall into discrete categories of diabetic and non-diabetic. Blood glucose and insulin levels are continuous variables, and the relationship between them resembles an inverted U shape (Barnett *et al.*, 1994a), similar to that seen in humans (DeFronzo, 1988). The polygenic and heterogenous nature of type 2 diabetes in these animals makes it unlikely that two samples of diabetic animals will exhibit identical genotypes. Increasing the sample size in future SSH studies will increase the likelihood of reproducing results in different samples of animals. Genes that are differentially expressed in the majority of lean-NGT and obese-diabetic *P. obesus* are more likely to represent significant factors in the pathophysiology of type 2 diabetes.

The magnitude of differential expression of many metabolic genes may be less than 1.5 fold and therefore fall below the sensitivity level of the SSH technique (Clontech, 2000). The magnitude of differential expression between
groups does not necessarily relate to the magnitude of the physiological effect. For some tightly controlled metabolic genes a difference in gene expression of less than 1.5 fold may result in a significant physiological effect, whereas as a 2-3 fold difference in other genes may be physiologically ineffectual (Strausberg and Riggins, 2001). The SSH technique is a screening procedure designed to increase the likelihood of identifying genes differentially expressed more than 1.5 fold, the physiological effect of the differential gene expression does not influence the procedure.

TBG was identified as differentially expressed between lean-NGT and obese-diabetic *P. obesus* following the screening of 576 clones with cDNA dot blots. It is difficult to speculate on how many genes may be significantly differentially expressed between these two animal groups and how many genes need to be screened to identify them. Furthermore, the unknown level of redundancy in the enriched library makes such estimations even more difficult. It is possible that identification of 10 or more differentially expressed genes may require screening 6000 or more clones. Screening such a large number of clones with cDNA dot blots is impractical. cDNA microarray technology is an alternative approach that allows up to 20,000 clones to be screened simultaneously (Wildsmith and Elcock, 2001). Utilising SSH and cDNA microarray should facilitate the identification of many more differentially expressed genes.

### 4.5.1 Thyroxine Binding Globulin

Hepatic TBG expression was dramatically reduced in obese-diabetic *P. obesus* relative to lean-NGT *P. obesus*. Furthermore, TBG expression decreased with fasting. These results suggest TBG metabolism may be involved in energy metabolism. TBG is a plasma protein that binds the thyroid hormones (TH) thyroxine (T4) and triiodothyronine (T3) (Bartalena and Robbins, 1992). TH administration was one of the first pharmacological obesity treatments as administration of TH dramatically increases energy expenditure (Krotkiewski, 2000).
TH influence many metabolic pathways, including carbohydrate, fat and protein metabolism. TH increase endogenous glucose production, through increased gluconeogenesis, while also increasing glucose utilisation (Dimitriadis and Raptis, 2001). TH also stimulates triglyceride release from adipose tissue, increasing plasma free fatty acid concentrations as well as increasing lipid utilisation (Dimitriadis and Raptis, 2001). The paradoxical increase in glucose and fat production and utilisation may represent futile substrate cycling, increasing energy expenditure.

The calorigenic action of TH is divided into short term effects, lasting from 6 to 48 hours, and long term effects, lasting from 30 hours up to 60 days (Krotkiewski, 2000). Short term effects involve diiodothyronine (T2), an active metabolite of T3, directly interacting with mitochondrion enzymes including subunit Va of cytochrome c oxidase, resulting in enhanced respiration and heat dissipation (Krotkiewski, 2000). Long term effects involve interaction between T3 and the nuclear TH receptors (TRs). TRs bind to regulatory regions of genes containing thyroid response elements. Many genes are activated including fatty acid synthase, hepatic lipoprotein lipase, glucose 6 pyruvate dehydrogenase, malate dehydrogenase, Ca++ ATPase and Na+K+ ATPase and the uncoupling proteins (UCPs). Activation of UCPs have been proposed as a primary mechanism of TH mediated elevation of energy expenditure (Krotkiewski, 2000).

TBG is produced in the liver, and along with albumin and TBG-pre albumin binds plasma TH. Approximately 70 percent of plasma T4 and T3 are bound to TBG, with less than 0.03 percent of T4 and 0.3 percent of T3 are free in the circulation and physiologically active (Bartalena and Robbins, 1992). Elevated TBG levels are associated with a reduction in free TH levels. This situation is rapidly corrected with an increase in thyroid stimulating hormone (TSH) release and a subsequent stimulation of TH synthesis and release. The net result is an increase in bound TH and normal levels of free TH (Bartalena and Robbins, 1992).
Genetic mutations in TBG can cause complete TBG deficiency, which is associated with very low total T4 and T3 levels and elevated plasma free T3 and T4 concentrations (Mori et al., 1990; Li et al., 1991a; Yamamori et al., 1991; Ueta et al., 1997; Carvalho et al., 1998a; Carvalho et al., 1998b). Mutations can also cause an excess of TBG, increasing total plasma TH levels (Griffiths et al., 1985; Mori et al., 1995). It is unclear if TBG mutations influence body weight, energy expenditure or substrate utilisation.

The current study found TBG expression decreased with fasting in *P. obesus*. Fasting has previously been associated with reduced levels of T3, TBG, blunted response to TSH and TRH and decreased tissue sensitivity to T3 (Krotkiewski, 2000). Down regulation of the thyroid system is consistent with the reduced energy expenditure accompanying fasting in *P. obesus* (Chapter 3.4) which represents a compensatory mechanism to conserve energy.

TBG expression was reduced in obese *P. obesus*, suggesting a reduction in TBG activity may contribute to the pathophysiology of obesity, potentially mediated by TH. Although speculative, a potential reduction in energy expenditure may be small, or localised to a subset of tissues, and below the sensitivity level of the whole body indirect calorimetry system, as obese *P. obesus* do not manifest a gross defect in energy expenditure (Chapter 3.4). Disproportionately low energy expenditure, mediated by TBG, in pre-obese *P. obesus* could lead to obesity. The compensatory increased energy expenditure associated with the increased body weight could be sufficient to overcome the low TH mediated energy expenditure. Alternatively, TBG may contribute to the development of obesity independently of its role in binding plasma T4 and T3. Further research is necessary to elucidate any potential role of TBG in metabolism.

4.5.2 Site 1 Protease

Hepatic S1P expression increased with fasting in lean-NGT, but not obese-IGT and obese-diabetic *P. obesus*, suggesting that S1P may potentially be involved in energy metabolism. S1P is a protease that cleaves sterol regulatory element-binding proteins (SREBPs). SREBPs are transcription-
regulating proteins, involved in feedback systems that regulate multiple enzymes in the fatty acid biosynthesis pathways including; acetyl CoA carboxylase, fatty acid synthase, steroyl, CoA desaturase-1 (Brown and Goldstein, 1999; Osborne, 2000). SREBPs also activate transcription of genes involved in cholesterol biosynthesis including: HMG CoA synthase, HMG CoA reductase, farnesyl diphosphate synthase, squalene synthase, and genes for the low density lipoprotein receptor (Brown and Goldstein, 1999; Osborne, 2000).

The SREBP isoforms 1a and 1c are produced from a single gene, with isoform 2 produced from a different gene (Hua et al., 1995; Shimano et al., 1997a; Shimomura et al., 1999). SREBPs are embedded in the endoplasmic reticulum. Activation of SREBPs is initiated by SREBP cleavage activating protein (SCAP), which transports the SREBPs to the golgi, where they undergo proteolytic cleavage. Cleavage occurs initially with S1P, and then with site 2 protease (S2P), releasing the transcriptionally active NH(2)-terminal fragments which translocate to the nucleus (Brown and Goldstein, 1999).

The physiological role of the SREBP system has been studied extensively with gene knockout and transgenic overexpression studies in mice. Hepatic TG and cholesterol synthesis and content are increased when the hepatic expression of SREBP isoforms 1a (Shimano et al., 1997a), 1c (Shimano et al., 1997a) or 2 (Horton et al., 1998b) are increased and also when SCAP expression is increased (Korn et al., 1998). Whole body knockout of SREBP 1a and 1c (Shimano et al., 1997b) and hepatic knockout of 1c (Liang et al., 2002) increased hepatic cholesterol synthesis and content, largely due to overexpression of SREBP 2, which is preferentially involved in cholesterol metabolism. Hepatic knockout of SCAP (Matsuda et al., 2001) or S1P (Yang et al., 2001) decreases activity of all the SREBPs, decreasing hepatic synthesis and storage of TG and cholesterol. Paradoxically plasma TG concentrations decrease with all the genetic manipulations described above. Plasma cholesterol levels decrease in all studies with the exception of the transgenic overexpression of either SREBP 1c or 2, which have no effect. Taken
together these studies suggest the most favourable lipid metabolism profile occurs when the activity of all SREBPs is decreased at the level of SCAP or S1P.

24 hour fasting increases SREBP activity and hepatic TG and cholesterol synthesis (Horton et al., 1998a) and is accompanied by increased fat oxidation in *P. obesus* (Chapter 3.4). The increased hepatic S1P expression with fasting in lean-NGT *P. obesus* is consistent with increased SREBP activity. However, obese-IGT and obese-diabetic *P. obesus* do not significantly increase S1P expression, despite increased in fat oxidation. Although speculative, a blunted SREBP response to fasting may be a compensatory response to hypertriglyceridemia and hypercholesterolemia in these animals.

Hepatic S1P expression was not different between lean-NGT, obese-IGT and obese-diabetic *P. obesus*, despite the presence of hyperinsulinemia, a strong stimulant of SREBP activity (Osborne, 2000). Furthermore, hyperinsulinemic, *P. obesus* have hypertriglyceridemia and hypercholesterolemia, consistent with elevated SREBP activity. The role of S1P and SREBPs in lipid metabolism in *P. obesus* is uncertain and further studies are necessary to elucidate these roles.

4.5.3 Summary

SSH enriched a cDNA library 1000 fold for genes differentially expressed in the liver of lean-NGT and obese-diabetic *P. obesus*. 8 of the 12 genes identified form cDNA dot blots screening were differentially expressed by 1.5 fold or more. TBG was confirmed as differentially expressed in a different subset of *P. obesus*. Identification of a larger number of genes differentially expressed genes will require the screening of several thousand clones. Gene expression studies with SYBR Green PCR found TBG and S1P were associated with obesity and diabetes. Future research will determine whether TBG and S1P are important in the pathogenesis of these diseases.
Gene Discovery with SSH and cDNA Microarray

5.1 ABSTRACT

This study aimed to identify novel genes differentially expressed in the liver of lean-NGT and obese-diabetic Psammomys obesus. Suppression subtractive hybridisation (SSH) was used to enrich a hepatic cDNA library for differentially expressed genes. cDNA microarray was used to screen 8064 clones with cDNA derived from lean-NGT and obese-diabetic animals. 223 clones were identified as overexpressed in lean-NGT P. obesus and 274 clones were overexpressed in obese-diabetic P. obesus.

The 9 most significantly differentially expressed clones identified from the microarray screen were sequenced. 7 novel genes were identified as well as; sulfotransferase related protein and albumin. These 2 genes have not previously been associated with either type 2 diabetes or obesity. It is unclear why hepatic expression of these genes may differ between lean-NGT and obese-diabetic groups of P. obesus.

Future studies will utilise SYBR Green PCR to confirm differential expression of these genes, and measure hepatic expression levels in fed, fasted and dietary energy restricted P. obesus similar to experiments in Chapter 4. Subsequent studies will explore the potential role of these novel and known genes in the pathophysiology of type 2 diabetes.

5.2 INTRODUCTION

The previous gene discovery experiment (Chapter 4) utilised suppression subtractive hybridisation (SSH) to enrich liver cDNA for genes differentially expressed between lean-NGT and obese-diabetic Psammomys obesus. The enriched library was subsequently screened with cDNA dot blots. Although this approach successfully screened several hundred genes, several thousand genes may need to be screened to identify important metabolic genes
associated with type 2 diabetes. Such large scale screening requires a different approach.

Microarray technology is advantageous compared to dot blots in several aspects. Firstly, microscope slides can be exposed to two cDNA samples labelled with different fluorescent dyes simultaneously, allowing competitive hybridisations between the sample of interest and a control (Duggan et al., 1999). Secondly, microscope slides provide an impermeable surface, maximizing hybridisation rates and signal intensity (Southern et al., 1999). Thirdly, up to 20,000 clones can be screened simultaneously with cDNA microarray (Wildsmith and Elcock, 2001).

The current study was undertaken to identify genes differentially expressed in the liver of lean-NGT and obese-diabetic *Psammomys obesus*, utilising SSH and cDNA microarray.

### 5.3 RESEARCH DESIGN AND METHODS

Research design and methods are discussed in detail in Chapter 2 and described briefly below.

#### 5.3.1 Suppression Subtractive Hybridisation

SSH studies used 18-week old lean-NGT (n=4) and obese-diabetic (n=4) *Psammomys obesus*. These were designated as SSH animals and had *ad libitum* access to food until they were killed. Body weight, blood glucose (Yellow Springs Instruments, Yellow Springs, USA) and plasma insulin were measured (Insulin RIA, Phadeseph, Uppsala, Sweden).

Two SSH experiments were conducted using the PCR-Select cDNA subtraction kit (Clontech, Palo Alto, U.S.A.). Experiment 1 was designed to enrich a cDNA pool for genes overexpressed in the liver of lean-NGT *Psammomys obesus*. Conversely, experiment 2 was designed to enrich cDNA for genes overexpressed in obese-diabetic *Psammomys obesus*. 
5.3.2 Cloning
The enriched cDNA from SSH experiments 1 and 2 were cloned using a T/A Cloning Kit (Invitrogen, Carlsbad, U.S.A.). The PCR products were ligated into a pCR2.1 plasmid vector and chemically transformed into TOP10 *E. coli* cells (Invitrogen, Carlsbad, U.S.A.). White colonies, representing successfully transfected clones, were selected and grown overnight.

5.3.3 cDNA Microarray Screening

5.3.3.1 PCR Amplification and Purification of Clones
Clones from experiments 1 and 2 were PCR amplified in 384 well plates (Applied Biosystems, Foster City, USA) using 5’ amino modified primers which were complementary to the bacterial vector pCR2.1 (Geneworks, Adelaide, Australia). PCR products were purified using an ArrayIt, vacuum manifold system (TeleChem International, Sunnyvale, USA).

5.3.3.2 Printing Microarray Slides
cDNA was resuspended in 20µl of ArrayIt spotting solution (TeleChem International, Sunnyvale, USA), and 5µl of resuspended cDNA was transferred to 384 well Uniplates (Whatman Inc, Clifton, USA). cDNA was printed onto 40 microarray slides using an SDD C2 robotic arrayer (Engineering Services Inc, Toronto, Canada).

5.3.3.3 Microarray Hybridisation Experiments
Two groups of *P. obesus* were used for the microarray hybridisation experiments, including lean-NGT (n=10) and obese-diabetic (n=10). The lean-NGT and obese-diabetic groups each included 4 SSH animals.

Hybridisation experiments involved indirect labeling of target cDNA and reference cDNA with the fluorescent dyes Cy5 and Cy3 respectively (Amersham Pharmacia Biotech, Uppsala, Sweden). The labeled cDNA was added to the microarray slides and a cover slip (Hybri-Slips, Sigma, St Louis, USA) was carefully placed on top. The slides were placed in ArrayIt Hybridisation Cassettes (TeleChem, International Inc, Sunnyvale, USA).
Hybridisation chambers were incubated at 65°C for 20 hours. Unincorporated cDNA was removed from the slides with a wash procedure.

5.3.3.4 Microarray Data Analysis
Microarray slides were scanned using a confocal microscope (ScanArray Lite, BioDiscovery Inc, Los Angeles, USA) with Gene Pix Pro software (Version 3.0, Axon Instruments Inc, Union City, USA). Median Cy5 and Cy3 signal intensities for each cDNA spot were imported from Gene Pix and data transformation was conducted using Genesight (Version 3, BioDiscovery Inc, Los Angeles, USA). The ratio of Cy5 (sample cDNA) to Cy3 (reference cDNA) was calculated, providing a measure of the relative expression of each clone on the microarray slide in the cDNA sample hybridised to it. This data was normally distributed, and gene expression analysis between two groups was assessed using an independent samples T test.

5.3.4 Sequencing and Bioinformatics
Putatively overexpressed clones were reamplified from bacteria with PCR, visualised on an agarose gel with electrophoresis and excised. cDNA was purified using the UltraClean GelSpin DNA Purification Kit (Mo Bio Laboratories, Solana Beach, USA). cDNA was sequenced by DyeDeoxy Terminator (Applied Biosystems, Foster City, USA) using an automated DNA sequencer (Applied Biosystems, Foster City, USA).

Sequences were examined for homology with known genes or expressed sequence tags (ESTs), using blast searches of databases at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

5.3.5 Statistical Analysis
Statistical analysis was conducted using SPSS (Version 10, SPSS inc, Chicago, USA). A Kolmogorov-Smirnov test was used to examine the distribution of data. Normally distributed data in two groups was assessed using an independent samples T-test. Non-normally distributed data was analysed using a Mann-Whitney test. Statistical significance was set at p<0.05.
5.4 RESULTS

5.4.1 Suppression Subtractive Hybridisation

Lean-NGT and obese-diabetic *P. obesus* were selected for gene discovery studies, allowing for the identification of genes differentially expressed with diabetes as well as with obesity. All animals were male and 18 weeks of age (Table 5.1). These animals were not used in any of the experiments in Chapter 4.

Experiment 1 enriched a cDNA pool for genes overexpressed in lean-NGT *Psammomys obesus*. Figure 5.1a shows that β actin was visible on an agarose gel after 15 cycles in non-enriched cDNA and after 25 cycles in enriched cDNA. Overexpressed genes were enriched over 1000 fold. Experiment 2 enriched cDNA over 1000 fold for genes overexpressed in obese-diabetic *Psammomys obesus*, as β actin was visible 10 cycles later in enriched cDNA (Figure 5.1b). Expression of G3PDH in experiments 1 and 2 was also reduced by approximately 10 cycles (data not shown), confirming the 1000 fold enrichment of overexpressed genes in both SSH experiments.

<table>
<thead>
<tr>
<th></th>
<th>Lean-NGT</th>
<th>Obese-Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>215 ± 9.1</td>
<td>254 ± 4.9*</td>
</tr>
<tr>
<td>Blood Glucose (mmol/l)</td>
<td>4.0 ± 0.2</td>
<td>13.4 ± 1.4*</td>
</tr>
<tr>
<td>Plasma Insulin (µU/ml)</td>
<td>80 ± 11</td>
<td>429 ± 66*</td>
</tr>
</tbody>
</table>

Table 5.1 Phenotypic data for the *P. obesus* used in SSH experiments. Data expressed as mean ± SEM. * Indicates a significant difference compared to lean-NGT *P. obesus* (p<0.01).
Figure 5.1 β actin PCR products from SSH experiments 1 (5.1a) and 2 (5.1b) visualised on a 1% agarose gel. PCR products from enriched and non-enriched cDNA were amplified for 15, 20, 25, 30 and 35 cycles.

5.4.2 cDNA Microarray Screening

Putatively overexpressed clones from lean-NGT *P. obesus* (n=4224) and obese-diabetic *P. obesus* (n=3840) were screened with cDNA microarray. cDNA from lean-NGT and obese-diabetic animals were used for microarray experiments. Phenotypic data for these animals are shown in Table 5.2. The lean-NGT and obese-diabetic groups each included four animals from Table 5.1 and six other animals. These combinations were used to avoid selecting genes differentially overexpressed in animals used in the SSH experiment only, as occurred with the study in Chapter 4.

Table 5.2 Phenotypic data for the *P. obesus* used in microarray screening. Data expressed as mean ± SEM. * Indicates a significant difference compared to lean-NGT *P. obesus* (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Lean-NGT</th>
<th>Obese-Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fed</td>
<td>Restricted</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>215 ± 9.1</td>
<td>215.0 ± 7.5</td>
</tr>
<tr>
<td>Blood Glucose (mmol/l)</td>
<td>4.0 ± 0.2</td>
<td>4.3 ± 0.4</td>
</tr>
<tr>
<td>Plasma Insulin (µU/ml)</td>
<td>80 ± 11</td>
<td>72 ± 17</td>
</tr>
</tbody>
</table>

The expression of each clone on the microarray slide was measured in the cDNA of interest (labelled with Cy5) and in reference cDNA (labelled with
GENE DISCOVERY WITH SSH AND CDNA MICROARRAY

Cy3). A strong linear relationship was seen between these measures. A representative comparison is shown in Figure 5.2. Gene expression in the cDNA of interest was normalised to the expression in the reference cDNA.

![Gene expression measured with microarray in Cy5 labelled sample cDNA and Cy3 labelled reference cDNA.](image)

**Figure 5.2** Gene expression measured with microarray in Cy5 labelled sample cDNA and Cy3 labelled reference cDNA.

cDNA microarray analysis revealed 223 clones derived from lean-NGT *P. obesus* displayed significantly greater expression in lean-NGT, than obese-diabetic *P. obesus* (p<0.05). 5 of these clones were highly significantly differentially expressed (p<0.01). 274 clones derived from obese-diabetic *P. obesus* displayed significantly greater expression in obese-diabetic *P. obesus* (p<0.05). 4 of these clones were highly significantly differentially expressed (p<0.01). These 9 clones with the most significant difference in gene expression were sequenced (Tables 5.3 and 5.4 respectively). AGT A5G15 revealed 87 percent homology to *Mus musculus* Sulfotransferase Related Protein (Genebank no. AF0260752). AGT C12A15 revealed 86 percent homology to *Mus musculus* Albumin (Genebank no. AJ011413). 7 clones did
not show significant evidence of homology with known genes or ESTs on the NCBI database (http://www.ncbi.nlm.nih.gov).

Table 5.3 Clones overexpressed in lean-NGT *P. obesus*. Homologies with known genes and ESTs on NCBI databases, and statistical significance of the differential gene expression are shown.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Gene Homology</th>
<th>Genebank Accession no.</th>
<th>Overexpression</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGT A1O14</td>
<td>Novel</td>
<td>N/A</td>
<td>1.41</td>
<td>0.005</td>
</tr>
<tr>
<td>AGT A9C14</td>
<td>Novel</td>
<td>N/A</td>
<td>1.25</td>
<td>0.006</td>
</tr>
<tr>
<td>AGT A9C22</td>
<td>Novel</td>
<td>N/A</td>
<td>1.25</td>
<td>0.007</td>
</tr>
<tr>
<td>AGT A10G23</td>
<td>Novel</td>
<td>N/A</td>
<td>1.47</td>
<td>0.008</td>
</tr>
<tr>
<td>AGT A5G15</td>
<td>Sulfotransferase Related Protein</td>
<td>AF026075</td>
<td>1.32</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Table 5.4 Clones overexpressed in obese-diabetic *P. obesus*. Homologies with known genes and ESTs on NCBI databases, and statistical significance of the differential gene expression are shown.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Gene Homology</th>
<th>Genebank Accession no.</th>
<th>Overexpression</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGT C11L19</td>
<td>Novel</td>
<td>N/A</td>
<td>1.38</td>
<td>0.002</td>
</tr>
<tr>
<td>AGT C12A15</td>
<td>Albumin</td>
<td>AJ011413</td>
<td>1.77</td>
<td>0.006</td>
</tr>
<tr>
<td>AGT C6P3</td>
<td>Novel</td>
<td>N/A</td>
<td>1.41</td>
<td>0.008</td>
</tr>
<tr>
<td>AGT C8D19</td>
<td>Novel</td>
<td>N/A</td>
<td>1.28</td>
<td>0.009</td>
</tr>
</tbody>
</table>

5.5 DISCUSSION

SSH successfully enriched a cDNA library approximately 1000 fold for genes differentially expressed between lean-NGT and obese-diabetic *P. obesus*. 8064 putatively differentially expressed clones were selected for screening with cDNA microarray.

SSH and cDNA microarray identified 223 clones overexpressed in lean-NGT *P. obesus* and 274 clones overexpressed in obese-diabetic *P. obesus*. 9 of the differentially expressed clones identified from the microarray screen were sequenced. 7 novel genes were identified as well as; sulfotransferase related protein and albumin. Sulfotransferase catalyzes the sulfate conjugation of monoamines and phenolic and cathecol drugs (Cooper *et al.*, 2001). Albumin is the main plasma protein and has a good binding capacity for many minerals,
fatty acids, hormones and drugs and is involved in the regulation of the osmotic pressure of blood (Peters, 1995). These 2 genes have not previously been associated with either type 2 diabetes or obesity. It is unclear why hepatic expression of these genes may differ between lean-NGT and obese-diabetic groups of *P. obesus*.

Future studies will utilise SYBR Green PCR to confirm differential expression of these genes, similar to the confirmation experiments in Chapter 4. Hepatic gene expression will be measured in the animals used in the microarray screening as well as another independent group of lean-NGT, obese-IGT and obese-diabetic *P. obesus*. Subsequent studies to further elucidate any potential role in type 2 diabetes should measure hepatic expression in fed, fasted and dietary energy restricted *P. obesus*.

Completion of confirmation experiments with SYBR Green PCR will allow conclusions to be drawn regarding how closely cDNA microarray correlates with expression measured with SYBR Green PCR will allow conclusions to be drawn regarding the magnitude of differential expression and the level of statistical confidence necessary to confirm genes identified from microarray as differentially expressed in the livers of these animals.

The majority of previous studies utilising SSH have been of a relatively small scale and have relied on dot blots, northern blots, reverse northern blots, cDNA microarray or a combination of these approaches to screen putatively differentially expressed genes (von Stein *et al*., 1997; Kuang *et al*., 1998; Welford *et al*., 1998; Yang *et al*., 1999; Corominola *et al*., 2001; Larose *et al*., 2001; Li *et al*., 2001; Gardmo *et al*., 2002; Li *et al*., 2002). Although the accuracy, reproducibility and cost effectiveness of these approaches vary, all can be used effectively when only a few hundred clones are to be screened. However, the practicality of dot blot and northern blot based approaches can become prohibitive when several thousand clones are to be screened. The studies by von Stein *et al* (1997) and Corominola *et al* (2001) used reverse northerns to screen 5000 and 13,800 clones respectively, in what must have been time consuming and labour intensive experiments. The rapid
advancement of cDNA microarray technology has ensured this technique is an accurate, reproducible and cost effective approach for large scale gene expression screening.

Microarray technology is advantageous compared to dot blots and northern blots in several aspects. Firstly, microscope slides can be exposed to two cDNA samples labelled with different fluorescent dyes simultaneously, allowing competitive hybridisations between the sample of interest and a control (Duggan et al., 1999). Secondly, microscope slides provide an impermeable surface, maximizing hybridisation rates and signal intensity (Southern et al., 1999). Thirdly, up to 20,000 clones can be screened simultaneously with cDNA microarray (Wildsmith and Elcock, 2001). Therefore the current study was able to measure the expression of 8064 genes in 20 individual animals.

Given SSH was used to enrich the cDNA library for overexpressed genes, we expected many of the 8064 genes to be highly differentially expressed between lean-NGT and obese-diabetic groups. The study by Yang et al (1999) utilising SSH and microarray to screen 332 clones putatively differentially expressed between two breast cancer cell lines, found 21 genes were differentially expressed by more than two fold. The microarray screening in the current study utilised two groups of *P. obesus* (n=20), a heterogenous animal model of a complex polygenic disease, possibly explaining why the nine highly significantly differentially expressed genes identified in the current study were overexpressed by less than two fold.

Furthermore, the magnitude of differential expression between groups does not necessarily relate to the magnitude of the physiological effect. For some tightly controlled metabolic genes a difference in gene expression of less than 1.5 fold may result in a significant physiological effect, whereas as a 2-3 fold difference in other genes may be physiologically ineffectual (Strausberg and Riggins, 2001). Statistical confidence in differential expression in a number of animals may be a more important factor in identifying differentially expressed genes.
cDNA microarray technology can be used as an independent gene discovery approach to screen a cDNA library, without the use of SSH. However, incorporating the SSH technique to enrich the cDNA for differential expressed genes reduced the total number of clones to be screened and improved the efficiency and practicality of the technique. A large number of clones still need to be screened due to the level of redundancy. As only 12 clones were sequenced an accurate estimation of redundancy could not be determined, although it is likely to be high given redundancy can be generated in both the PCR steps of the SSH procedure as well as during clone selection.

5.5.1 Summary
In summary SSH enriched a cDNA library for genes differentially expressed between lean-NGT and obese-diabetic *P. obesus*. cDNA microarray screened 8064 clones, identifying 497 differentially expressed clones. 9 clones were sequenced revealing sulfotransferase related protein, albumin and 7 novel genes. Future studies will utilise SYBR Green PCR to confirm the differential expression of these genes. Subsequent studies will explore the potential role of these genes in the pathophysiology of type 2 diabetes.
Conclusions and Future Directions

The physiological data presented in this thesis have further validated *Psammomys obesus* as an ideal animal model of obesity and type 2 diabetes. Similar to humans, obesity was accompanied by increased energy expenditure and fat oxidation, and type 2 diabetes was not associated with a defect in whole body glucose oxidation. Furthermore, *P. obesus* responded to dietary energy restriction and short term fasting response with energy conservation and glucose sparing. Interestingly, diurnal elevations in blood glucose levels and fat oxidation were identified in obese-diabetic animals. The physiological significance of these diurnal disturbances in glucose and fat metabolism and their potential role in the pathogenesis of obesity and type 2 diabetes should be investigated further.

Longitudinal studies with healthy young *P. obesus* are necessary to identify metabolic disturbances causing obesity and diabetes in these animals. Indirect calorimetry should be used to determine whether disproportionately low energy expenditure is present in the pre-obese state. Furthermore, these studies should explore any potential disturbances in substrate oxidation that may contribute to the pathophysiology of obesity and type 2 diabetes in *P. obesus*.

Previous studies have established the polygenic and heterogeneous nature of obesity and type 2 diabetes in *P. obesus*. This study has provided additional evidence of the similarity between human subjects and *P. obesus* with respect to the pathophysiology of obesity and type 2 diabetes. These animals are therefore ideally suited for studies designed to identify genes involved in the pathophysiology of these diseases.

This thesis aimed to identify hepatic genes differentially expressed with the presence of obesity and type 2 diabetes in *P. obesus*. Suppression subtractive hybridisation (SSH) was successfully used to enrich a cDNA library for differentially expressed genes. cDNA dot blots were used to successfully
SSH and cDNA microarray screening identified thyroxine binding globulin (TBG) and site 1 protease (S1P) as genes associated with obesity and diabetes in *P. obesus*. TBG may be involved in the development of obesity through its role in thyroid hormone metabolism. Future studies should examine the relationship between energy metabolism and the concentration of free and bound thyroid hormones in the circulation of *P. obesus*. Furthermore, the effect of thyroid hormone administration should be examined utilising indirect calorimetry. To establish a causal role for TBG, gene knockout or antisense experiments could be conducted to decrease TBG activity, and transgenic or adenoviral studies could increase TBG activity in *P. obesus*. Modulation of TBG may represent a novel therapeutic approach for the treatment of obesity.

S1P may be involved in the development of obesity and diabetes through its role in processing sterol regulating element binding proteins (SREBP), as SREBP1s are insulin responsive transcription factors involved in lipid metabolism. Future studies should examine the relationship between plasma and hepatic lipid levels with the hepatic expression and activity levels of SREBP isoforms (1a, 1c, 2) as well as SREBP regulators (S1P, site 2 protease and SREBP cleavage activating protein). To establish a causal role for S1P, studies should be conducted to modulate the activity of this enzyme. Gene knockout or antisense experiments could be conducted to decrease S1P activity, and transgenic or adenoviral studies could increase S1P activity in *P. obesus*. Modulating S1P mediated SREBP activity may potentially represent a new therapeutic approach to improve blood lipid and glucose profiles.

SSH and cDNA microarray screening identified 223 clones overexpressed in lean-NGT *P. obesus* and 274 clones overexpressed in obese-diabetic *P. obesus*. Sequencing the 9 clones with the most highly significant differential expression identified 7 novel genes as well as sulfotransferase related protein and albumin. These 2 genes have not previously been associated with either
type 2 diabetes or obesity. It is unclear why hepatic expression of these genes may differ between lean-NGT and obese-diabetic groups of *P. obesus*.

Time constraints prevented the studies necessary to confirm the differential gene expression of these 9 genes. SYBR Green PCR should be used to firstly confirm the gene expression in the 20 animals used in the microarray screen followed by another group of lean-NGT, obese-IGT and obese-diabetic *P. obesus*. Subsequent studies to explore any potential role of these genes in type 2 diabetes and metabolism should measure hepatic gene expression in fed, fasted and dietary energy restricted *P. obesus*. Genes identified from these gene expression studies as potentially involved in the pathophysiology of type 2 diabetes could be examined further using *in vitro* or *in vivo* studies to modulate gene activity with either gene knockout, antisense, transgenic or adenoviral studies, to establish any casual role in the pathophysiology of type 2 diabetes.

The two SSH experiments conducted have demonstrated the effectiveness of the technique to enrich a cDNA library for differentially expressed genes. Dot blots and microarrays were both used effectively to screen cDNA libraries. Microarray technology was advantageous to dot blots in two main areas; firstly expression levels were measured in thousands of genes in a single experiment, and secondly experiments were conducted with 20 individual liver cDNA samples, thereby avoiding the need to pool samples together. Although cDNA microarray technology could be used as an independent gene discovery approach to screen a cDNA library, incorporating the SSH technique enriched the cDNA for differential expressed genes and reduced the total number of clones to be screened. Therefore the combination of SSH and cDNA microarray techniques maximises the efficiency and practicality of the individual techniques and can be a powerful gene discovery strategy.

Future gene discovery studies should continue to focus on the liver, given the role of the liver in both glucose and fat metabolism and the established role in the pathophysiology of type 2 diabetes. However, other tissues with metabolic defects contributing to diabetes including skeletal muscle, pancreas and
adipose tissue, particularly visceral adipose tissue should also be examined. Identification of genetic defects contributing to the pathogenesis of type 2 diabetes will hopefully lead to new and improved therapeutic approaches.
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