MOLECULAR MARKERS OF OBESITY AND DIABETES

Christopher Dean Swagell
Bachelor of Science (Honours)
University of Queensland

CRC for Diagnostics
School of Life Sciences
Institute of Health and Biomedical Innovation
Queensland University of Technology
Brisbane, Australia

A thesis submitted for the degree of Doctor of Philosophy at the Queensland University of Technology, 2007.
I dedicate this thesis to my mentor Phil and my family Bill, Lucy and David.

We finally got there!
Declaration

The work presented in this thesis has not been previously submitted for a degree or diploma at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person(s) except where due reference is made.

Signed: ..............................................

Christopher Dean Swagell

Date: .....................
Abstract

Recently it has been shown that the consumption of a diet high in saturated fat is associated with impaired insulin sensitivity and increased incidence of type 2 diabetes. In contrast, diets that are high in monounsaturated fatty acids (MUFAs) or polyunsaturated fatty acids (PUFAs), especially very long chain n-3 fatty acids (FAs), are protective against disease. However, the molecular mechanisms by which saturated FAs induce the insulin resistance and hyperglycaemia associated with metabolic syndrome and type 2 diabetes are not clearly defined.

It is possible that saturated FAs may act through alternative mechanisms compared to MUFA and PUFA to regulate of hepatic gene expression and metabolism. It is proposed that, like MUFA and PUFA, saturated FAs regulate the transcription of target genes. To test this hypothesis, hepatic gene expression analysis was undertaken in a human hepatoma cell line, Huh-7, after exposure to the saturated FA, palmitate. These experiments showed that palmitate is an effective regulator of gene expression for a wide variety of genes. A total of 162 genes were differentially expressed in response to palmitate. These changes not only affected the expression of genes related to nutrient transport and metabolism, they also extend to other cellular functions including, cytoskeletal architecture, cell growth, protein synthesis and oxidative stress response. In addition, this thesis has shown that palmitate exposure altered the expression patterns of several genes that have previously been identified in the literature as markers of risk of disease development, including CVD, hypertension, obesity and type 2 diabetes. The altered gene expression patterns associated with an increased risk of disease include apolipoprotein-B100 (apo-B100), apo-CIII, plasminogen activator inhibitor 1, insulin-like growth factor-I and insulin-like growth factor binding protein 3. This thesis reports the first observation that palmitate directly signals in cultured human hepatocytes to regulate expression of genes involved in energy metabolism as well as other important genes.

Prolonged exposure to long-chain saturated FAs reduces glucose phosphorylation and glycogen synthesis in the liver. Decreased glucose metabolism leads to elevated rates of lipolysis, resulting in increased release of free FAs. Free FAs have a negative effect on
insulin action on the liver, which in turn results in increased gluconeogenesis and systemic dyslipidaemia. It has been postulated that disruption of glucose transport and insulin secretion by prolonged excessive FA availability might be a non-genetic factor that has contributed to the staggering rise in prevalence of type 2 diabetes. As glucokinase (GK) is a key regulatory enzyme of hepatic glucose metabolism, changes in its activity may alter flux through the glycolytic and de novo lipogenic pathways and result in hyperglycaemia and ultimately insulin resistance. This thesis investigated the effects of saturated FA on the promoter activity of the glycolytic enzyme, GK, and various transcription factors that may influence the regulation of GK gene expression. These experiments have shown that the saturated FA, palmitate, is capable of decreasing GK promoter activity. In addition, quantitative real-time PCR has shown that palmitate incubation may also regulate GK gene expression through a known FA sensitive transcription factor, sterol regulatory element binding protein-1c (SREBP-1c), which upregulates GK transcription.

To parallel the investigations into the mechanisms of FA molecular signalling, further studies of the effect of FAs on metabolic pathway flux were performed. Although certain FAs reduce SREBP-1c transcription in vitro, it is unclear whether this will result in decreased GK activity in vivo where positive effectors of SREBP-1c such as insulin are also present. Under these conditions, it is uncertain if the inhibitory effects of FAs would be overcome by insulin. The effects of a combination of FAs, insulin and glucose on glucose phosphorylation and metabolism in cultured primary rat hepatocytes at concentrations that mimic those in the portal circulation after a meal was examined. It was found that total GK activity was unaffected by an increased concentration of insulin, but palmitate and eicosapentaenoic acid significantly lowered total GK activity in the presence of insulin. Despite the fact that total GK enzyme activity was reduced in response to FA incubation, GK enzyme translocation from the inactive, nuclear bound, to active, cytoplasmic state was unaffected. Interestingly, none of the FAs tested inhibited glucose phosphorylation or the rate of glycolysis when insulin is present. These results suggest that in the presence of insulin the levels of the active, unbound cytoplasmic GK are sufficient to buffer a slight decrease in GK enzyme activity and decreased promoter activity caused by FA exposure. Although a high fat diet has been associated with impaired hepatic glucose metabolism, there is no evidence from this
thesis that FAs themselves directly modulate flux through the glycolytic pathway in isolated primary hepatocytes when insulin is also present.

Therefore, although FA affected expression of a wide range of genes, including GK, this did not affect glycolytic flux in the presence of insulin. However, it may be possible that a saturated FA-induced decrease in GK enzyme activity when combined with the onset of insulin resistance may promote the dys-regulation of glucose homeostasis and the subsequent development of hyperglycaemia, metabolic syndrome and type 2 diabetes.
Keywords
cDNA microarray analysis; Gene expression; Saturated fatty acid; Palmitate; Hepatocytes; Glucokinase; Microarray analysis; Monounsaturated fatty acid; Polyunsaturated fatty acid; Oleate; Eicosapentaenoic acid; Human hepatic cell line; Fatty acid signalling; Fatty acids; Glycolysis; Insulin; Primary rat hepatocytes
List of Publications, Manuscripts and Patents

The following collection of publications and manuscripts were prepared in conjunction with the thesis. Other non-related material that was generated over the duration of the thesis examination period is also listed.

**Thesis Associated Research Manuscripts**


**Thesis Associated Abstracts**


**Non-Associated Research Publications**


**Non-Associated Patents**


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**Abbreviations**

ABCA1 – ATP binding cassette A1  
ACC – Acetyl CoA carboxylase  
ADRP – Adipose differentiation related protein  
AGE – Advanced glycation end-products  
AMV – Avian myeloblastosis virus  
Apo – Apolipoprotein  
bHLH-LZ – Basic helix-loop-helix leucine zipper  
BSA – Bovine serum albumin  
CAD – Coronary artery disease  
cDNA – Complimentary deoxyribonucleic acid  
CDK4 – Cyclin dependent kinase 4  
CoA – Coenzyme A  
cRNA – Complimentary ribonucleic acid  
CHD – Coronary heart disease  
CRE – cAMP response element  
CVD – Cardiovascular disease  
DHA – Docosahexaenoic acid  
DMEM – Dulbecco’s modified Eagle’s medium  
dNTP – Deoxyribonucleotide triphosphate  
EDTA – Ethylenediaminetetraacetic acid  
EIF – Eukaryotic translation initiation factor  
EPA – Eicosapentaenoic acid  
FA – Fatty acid  
FAS – Fatty acid synthase  
FDFT1 – Farnesyl-diphosphate farnesyl transferase  
FDPS – Farnesyl pyrophosphate synthetase  
G-6-P – Glucose 6-phosphate  
GK – Glucokinase  
GKRP – Glucokinase regulatory protein  
GPX4 – Phospholipid hydroperoxide glutathione peroxidase  
GSH – Glutathione  
GST – Glutathione S-transferase  
H2O2 – hydrogen peroxide  
HDL – High-density lipoprotein  
HEPES – 4-2-hydroxyethyl-1-piperazineethanesulfonic acid  
HGF – Hepatic growth factor  
HNF-4α – Hepatocyte nuclear factor-4α  
Hsp90 – Heat shock protein 90  
IDL – Intermediate density lipoprotein  
IGF-1 – Insulin-like growth factor 1  
IR – Insulin receptor  
IRS – Insulin receptor substrate  
IGFBP3 – Insulin-like growth factor binding protein 3
IL-6 – Interleukin-6
JAK – Janus kinase
LBR – Lamin B receptor
LDL – Low-density lipoprotein
LIPA – Lysosomal acid lipase
LXR – Liver X receptor
MODY – Maturity onset diabetes of the young
mRNA – Messenger ribonucleic acid
MRP2 – Multi-drug resistance protein
MUFA – Monounsaturated fatty acid
NADH – nicotinamide adenine dinucleotide
NO – Nitric oxide
NFKB1 – Nuclear factor kappa B
nSREBP – Nuclear sterol regulatory element binding protein
Ob-R – Leptin receptor
P59 – Protein 59
PAI-1 – Plasminogen activator inhibitor 1
PBS – Phosphate buffered saline
PD – Pyruvate dehydrogenase
PDK1 – 3-phosphoinositide dependent kinase 1
PKB – Protein kinase B
P(3)K – phosphoinositide 3-kinase
PUFA – Polyunsaturated fatty acid
PPAR – Peroxisomal proliferator activated receptor
PPRE – Peroxisome proliferator response element
q-PCR – Quantitative real-time polymerase chain reaction
RECK – Reversion-inducing-cysteine-rich protein with Kazal motifs
ROS – Reactive oxygen species
RXR – Retinoic acid receptor
SCD – Stearyl-CoA desaturase
SDS – Sodium dodecyl sulphate
SRE – Sterol regulatory element
SREBP – Sterol regulatory element binding protein
SEPP1 – Selenoprotein P
SSC – Sodium chloride sodium citrate
STAT – Signal transducer and activator of transcription
TG – Triglyceride
TGBF2 – Transforming growth factor β2
TNF – Tumour necrosis factor
TNFR1 – Tumour necrosis factor receptor 1
VLDL – Very low-density lipoprotein
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CHAPTER ONE

INTRODUCTION
1.1 Description of Scientific Problem Investigated

Type 2 diabetes is a slow onset disease arising most often in older individuals [1], although the frequency is now increasing among children [2]. Type 2 diabetes is characterised by insulin deficiency coupled with insulin resistance that leads to a disturbance in glucose metabolism, ultimately resulting in chronic hyperglycaemia and dyslipidaemia [1; 3; 4; 5]. Approximately 200 million people worldwide are affected by type 2 diabetes and it is predicted that up to 350 million individuals will develop the disease by the year 2030 [6]. Type 2 diabetes has a massive impact on the health of Australians as the 6th leading cause of death in Australia [7] and is responsible for an enormous public health and social burden. The Australian health system expenditure on type 2 diabetes was estimated at around 800 million dollars in 2001 [8]. In addition, cardiovascular disease (CVD) is responsible for up to 80% of deaths in people suffering from type 2 diabetes [9; 10]. Other complications associated with diabetes include retinopathy, nephropathy and neuropathy.

Type 2 diabetes arises from a combination of multiple genetic and environmental factors [1; 11]. The consumption of a diet high in saturated fatty acids (FAs) has been associated with an increased incidence of type 2 diabetes as well as atherosclerosis and some forms of cancer [12]. In contrast, some diets are protective against the development of diabetes and disorders such as atherosclerosis and dyslipidaemia [13]. These include diets that are high in monounsaturated FAs (MUFAs), such as the “Mediterranean diet”, and diets high in very long chain n-3 polyunsaturated FAs (PUFAs), derived from fish oils. One explanation for the increasing incidence of type 2 diabetes may be an increased amount of saturated fat in the diet. Although it is well known that excessive weight gain and an obese state frequently precede the development of type 2 diabetes, it is not clear whether specific dietary fat intake contributes to the development of the diabetic state.

FAs have long been known to be associated with certain metabolic disorders, but it is only recently that the effects of individual FAs on gene regulation have been investigated. Since genomic profiling microarray technologies have become available studies that identify genes that are regulated by FAs are being performed [14; 15; 16; 17]. Recent evidence has identified that certain nutrients, including FAs, are important regulators of gene expression. Previously identified transcription factors include the
“FA sensors”, peroxisomal proliferator activated receptors (PPARs), and the mediator of insulin action, sterol regulatory element binding protein-1c (SREBP-1c). Therefore, the focus of this research was to investigate the effects exhibited by FAs on hepatic gene expression and metabolism.

1.2 Overall Objective of the Thesis
The aim of this project is to understand the role of fat in the development of pathological conditions such as metabolic syndrome and type 2 diabetes. In particular, this thesis focuses on the molecular mechanisms underlying the dysregulation of glucose and lipid metabolism in the liver.

This thesis may provide important information on the relationship between FAs and hepatic gene regulation that could potentially identify diagnostically useful molecular markers to detect those at risk of developing metabolic syndrome and type 2 diabetes. These markers could also be used to monitor the effectiveness of dietary modification and lifestyle changes. This thesis may provide data that could allow the identification of key targets for the development of prevention strategies and treatments for metabolic syndrome and type 2 diabetes.

Hypothesis
We propose that saturated fat alters the regulation of hepatic gene expression contributing to the development of impaired glucose metabolism, hyperglycaemia, metabolic syndrome and type 2 diabetes.

In addition, we propose that saturated fatty acids specifically alter the regulation of the key hepatic enzyme glucokinase (GK), contributing to perturbations of whole body glucose homeostasis.

The Model
In this thesis we attempted to isolate the direct effects of FAs on gene regulation in the liver from secondary whole body physiological effects of dietary fats. In order to do this we used primary hepatocytes and cultured hepatic cell lines incubated in the presence of FAs.
1.3 **Specific Aims of the Thesis**

- Characterise novel mRNA expression patterns in response to the saturated FA, palmitate, by microarray analysis in a human hepatic cell line.
- Identify potential gene targets that are regulated by FA in the liver, that may be associated with an increased risk of disease development.
- Confirm altered gene expression identified by microarray analysis by quantitative real time-PCR.
- Compare changes in the short-term gene regulation by saturated, MUFAs and PUFAs by microarray analysis in a human hepatic cell line.
- Identify regulatory elements in the GK promoter and characterise the transcription factors that interact with them.
- Identify specific FA regulation of transcription factors and other important metabolic genes involved in GK gene transcription by using quantitative real time-PCR.
- Investigate the effect of FAs on GK enzyme activity and the cellular distribution of the GK enzyme.
- Characterise FA mediated regulation of glucose phosphorylation, glycolysis and β-oxidation.

1.4 **Account of Scientific Progress Linking the Scientific Papers**

Previously, microarray studies have focused on transcriptional responses to fatty acids in cultured pancreatic β-cells [14] and clonal insulin-producing cells and *in vivo* using high fat fed mice [15; 17]. These studies have identified that FAs can directly signal and regulate gene expression in both systems. However, to date there have been no investigations into the direct effect of FA on gene expression in hepatic tissue. This issue was addressed in Chapter Three by measuring the effect of palmitate on gene expression in cultured hepatocytes.

Several MUFAs and PUFAs have been shown to be potent regulators of gene transcription in liver. For example, PUFAs act to reduce SREBP-1c mRNA levels [18] while PPARα is activated upon PUFA binding [19]. PPARα activation increases the rate of transcription of a number of target genes involved in lipid and cholesterol metabolism [20]. In addition, SREBP-1c has emerged as a major mediator of insulin
action on hepatic expression of genes involved in glycolysis and \emph{de novo} lipogenesis, such as GK \cite{21, 22}. GK is the main regulatory enzyme controlling hepatic glucose metabolism and glycolytic flux. It catalyses the phosphorylation of glucose to glucose 6-phosphate and is activated by insulin. Therefore, alterations in GK gene expression may contribute to perturbations in glucose homeostasis, resulting in hyperglycaemia. In order to identify the role that saturated fat plays in the regulation of glucose homeostasis, Chapter Four describes that analysis of GK promoter activity in cultured hepatocytes, in addition to quantitative real-time PCR analysis of transcription factors that regulate GK in response to palmitate.

Metabolic syndrome is characterised by hyperglycaemia and insulin insensitivity. One explanation for this can be illustrated by maturity-onset diabetes of the young (MODY-2), which is caused by a mutation in the GK gene that results in a mild form of type 2 diabetes \cite{23, 24}. While studies have shown that GK does not contribute to a genetic predisposition to the common form of type 2 diabetes, it may be that dietary fat is a non-genetic factor that perturbs GK expression. Reduced GK expression could decrease the flux through the glycolytic pathway, resulting in increased plasma glucose concentration and development of hyperglycaemia and type 2 diabetes. Therefore, in Chapter Five investigations into the effect of fat on GK enzyme activity, glucose phosphorylation, glycolysis and \(\beta\)-oxidation were performed in isolated primary rat hepatocytes.

\textbf{Chapter Three – Expression analysis of a human hepatic cell line in response to palmitate.}
\textit{Biochemical and Biophysical Research Communications} 2005; 328:432-441.

Due to the lack of data focusing on the direct effects of FA signalling in hepatic tissue, in Chapter Three we utilised microarray gene expression analysis to investigate the signalling of the saturated FA, palmitate, in a human hepatic cell line, Huh-7. This is the first report showing that palmitate regulates expression of numerous genes via direct molecular signalling mechanisms in cultured human hepatocyte cell line. Observations of altered hepatic regulation in response to palmitate include genes that are involved in FA transport, cholesterol catabolism, oxidative stress response, cell cycle, cell growth and proliferation. This thesis also identified several genes that are differentially regulated upon palmitate exposure that have been previously reported as markers of
increased disease risk. These genes include apolipoprotein-B100 (apo-B100) that is implicated in obesity, hypertension and diabetes [25], and apo-CIII, which is associated with high levels of apo-CIII-containing very low density lipoproteins (VLDLs) and hypertriglyceridemia [26]. Other genes associated with disease include the up-regulated plasminogen activator inhibitor-1, which is associated with renal fibrosis and diabetic nephropathy, and the down-regulated insulin-like growth factor-1 and insulin-like growth factor binding protein-3 that are characteristic of a diabetic state [27]. This in vitro molecular study supports in vivo data that saturated FA is a potential contributor to the development of diseases, such as metabolic syndrome and type 2 diabetes.

Chapter Four – Regulation of gene expression by fatty acids in human hepatic cell lines.
Submitted November 2006 - Experimental Cell Research.

In Chapter Three, palmitate was shown to be a regulator of hepatic gene expression after 48 hours exposure. Chapter Four of this thesis reports the effects of palmitate on GK promoter activity and the possible role of palmitate in regulating GK gene expression. Palmitate reduced GK promoter activity after 48 hours in the human hepatic cell line, Huh-7, transfected with GK reporter constructs. In addition, quantitative real-time PCR analysis of SREBP-1c expression, a positive regulator of GK gene transcription, revealed a reduction in mRNA levels in response to palmitate, possibly explaining the observed reduction of GK promoter activity. This thesis has generated evidence implicating a possible role of palmitate in the perturbation hepatic gene expression in Huh-7 cells. A second study was carried out to compare the effects of saturated FA, MUFA and PUFA in a second cell line, HepG2. A microarray expression analysis was performed to identify direct short-term regulation of hepatic gene expression after 1.5 hours. This comparison revealed that MUFAs and PUFAs initiate a similar pattern of hepatic gene regulation, while saturated FA failed to initiate a short-term regulatory response in hepatic cells.


As GK promoter activity and SREBP-1c mRNA expression was reduced by palmitate, Chapter Five of this thesis examines the effects of FA and insulin signalling on GK enzyme activity and glucose metabolism in cultured primary rat hepatocytes at
concentrations that mimic those after a meal. This thesis identified that the saturated FA, palmitate, and the PUFA, eicosapentaenoic acid, significantly lowered total GK enzyme activity, even in the presence of the activator insulin. In addition, the translocation of GK enzyme from the inactive, nuclear bound, to the active cytoplasmic state was unaffected by FA exposure, possibly indicating an overall reduction in the availability of cytoplasmic GK to phosphorylate glucose. However, all of the FAs tested failed to inhibit glucose phosphorylation and flux through the glycolytic in the presence of insulin. This suggests that in these experimental conditions the level of residual cytoplasmic GK is sufficient to buffer a decrease in GK enzyme activity induced by FA exposure. However, it is possible that the onset of insulin resistance or loss of insulin action is necessary before the effects of FAs on hepatic glucose metabolism are observed. This observation is consistent with the conclusion that the presence of FAs in a normal mixed meal may have little direct effect on the capacity of the liver to uptake, phosphorylate and metabolise glucose. Alternatively, FAs may affect the liver indirectly, for example by modulating the production of hormones such as insulin or glucagon by the pancreas.
References


CHAPTER TWO

LITERATURE REVIEW
2.1 Obesity

During the mid 20th century an unprecedented change in caloric availability has taken place in many Western and developing countries. One explanation is that throughout human history recurrent cycles of under-nutrition and preloading of calories for subsequent energy utilisation has been the normal pattern of human nutrition. However, in today’s society this has been replaced with continuous over-nutrition. This consequence has been greatly amplified by a permanent state of under exertion imposed by sedentary lifestyles and advances in modern technologies. It is now obvious that diseases of long-term over-nutrition have become increasingly prevalent [1]. This is due to the overwhelming incapability of compensatory mechanisms that normally buffer the metabolic change in calorie balance and short-term over-nutrition. Obesity is now so common within the world’s population that it has replaced malnutrition and infectious diseases as the most significant contributor to ill health [2]. The current epidemic of obesity poses a significant public health problem as it now afflicts more than 300 million people worldwide [3]. The global epidemic of obesity results from a combination of programmed genetic susceptibility, increased availability of high-energy foods and decreased requirement for physical activity in modern society.

Obesity can be defined as excess adiposity to an extent that is detrimental to an individual’s health. The body mass index (BMI) is commonly used as a measure of obesity with a BMI of 18.5 – 24.9 kg/m² defining the desirable range in Caucasians (Table 1). An individual’s BMI is calculated by dividing their weight in kilograms (kg) by their height in metres squared (m²). However, it must be recognised that individual BMI measurements may differ in the actual degree of adiposity and does not differentiate between muscle and adipose tissue. This means that individuals such as athletes, who have a high muscle to fat ratio, can be defined as ‘overweight’. Another measurement of obesity that can be used to assess the distribution of body fat is the waist-to-hip ratio (WHR). The WHR is the measurement of the ratio of an individual’s waist circumference to hip circumference. Abdominal fat is associated with more health problems than carrying extra weight around their hips or thighs. A WHR of 0.90 or less is considered healthy for men and a ratio of 0.80 or less is considered a sign of good health for women. A WHR of 1 or higher signals an increased risk of ill health. Other more practical methods to measure body fat include ‘skin-fold’ thickness and ‘bioelectrical impedance’ analysis. These methods can measure the percentage of body
fat by using skin fold callipers and the bioelectrical impedance of an individual’s body tissue conductivity to electricity, respectively.

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<tr>
<th>Weight Status</th>
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<tr>
<td>Underweight</td>
<td>≤ 18.5</td>
<td>Increased</td>
</tr>
<tr>
<td>Normal</td>
<td>18.5 - 24.9</td>
<td>Normal</td>
</tr>
<tr>
<td>Overweight</td>
<td>25.0 - 29.9</td>
<td>Increased</td>
</tr>
<tr>
<td>Obese</td>
<td>≥ 30.0</td>
<td>High</td>
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Table 1 – BMI Classification of Obesity [4].

Obesity is a principal independent risk factor for type 2 diabetes and an excess of body fat, especially visceral obesity [5], and the duration of obesity [6] has a range of potentially harmful consequences. Increasing weight gain and obesity leads to adverse metabolic effects on blood pressure, cholesterol and triglyceride (TG) levels, and insulin resistance. In addition, weight gain leads to insulin resistance through several mechanisms placing a greater demand on the pancreatic capacity to produce insulin, which also declines with age, leading to the development of clinical type 2 diabetes. Physical inactivity, which is both a cause and consequence of weight gain, also contributes to insulin resistance. Obesity is also associated with hypertension, dyslipidaemia (characterised by increased levels of TGs, cholesterol and low-density lipoproteins (LDLs) and decreased levels high-density lipoproteins (HDLs)), insulin resistance (characterised by increased blood insulin levels), impaired glucose tolerance and, type 2 diabetes [7; 8; 9; 10]. Furthermore, risks of coronary heart disease (CHD), cardiovascular disease (CVD), ischemic stroke and type 2 diabetes increase steadily with an increasing BMI.

Obesity can be considered to be a single disorder with multiple causes. Body weight is determined by an interaction between genetic, environmental and psychosocial factors acting through the physiological mediators of energy intake and expenditure [2]. Obesity runs in families but the influence of the genetic aetiology of obesity may be exacerbated by non-genetic factors, such as dietary intake. Apart from rare obesity-associated syndromes, the genetic influences seem to operate through susceptibility genes. Such genes may increase the risk, however alone they are not essential or sufficient to explain the development of disease. Candidate genes for obesity can be chosen for their possible involvement in body fat composition, anatomical distribution of fat, food intake and energy metabolism and expenditure. Implicit to the susceptible-
gene hypothesis is the role of environmental factors that unmask latent tendencies to develop obesity. However, predictions about possible interactions between genes and the environment are difficult because there may be a delay in an individual’s exposure to an ‘obesogenic’ environment, and/or alterations in lifestyle and the uncertainty about the precise timing of weight gain.

### 2.1.1 Mutations Associated with Obesity

Genes that predispose to obesity in humans and animals have already been identified and indicate the importance of genetic factors that are associated with the development of disease [11]. The protein produced from the leptin gene is involved in the regulation of food intake and energy expenditure and mutations in this gene result in morbidly obese humans and rats. Similarly, mutations in the leptin receptor exhibit the same phenotype, however these mutations are very rare and there is little evidence that the vast majority of overweight individuals carry mutations in leptin or its receptor. Candidate genes associated with human obesity also include receptors that are important in mechanisms of thermogenesis, such as β3-adrenergic-receptor gene and the family of uncoupling proteins [2; 12]. Other factors involved in appetite regulation include neuropeptides. These include neuropeptide Y and agouti-related protein, that stimulate appetite and decrease metabolic rate respectively, and α-melanocyte stimulating hormone (α-MSH) that inhibits appetite and increases metabolic rate. α-MSH acts through a family of melanocortin receptors, mutations in which are relatively common in severely obese individuals, representing approximately 3-5% of the obese population.

### 2.2 Metabolic Syndrome

The metabolic syndrome is associated with increased risk of type 2 diabetes and CVD and is thought to be a major component of these modern day epidemics that affects at least one in five adults. The metabolic syndrome is characterised by a cluster of metabolic abnormalities including abdominal obesity, decreased HDL cholesterol, elevated TGs, elevated blood pressure, impaired glucose regulation and hyperinsulinaemia with underlying insulin resistance (Box 1) [13]. Although the pathogenesis of the metabolic syndrome is poorly understood, it is likely that a sedentary lifestyle and dietary factors combined with genetic factors contribute to its development.
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- **Central obesity** (defined as waist circumference $\geq 94$ cm for men and $\geq 80$ cm for women) plus any two of the following four factors:
  - **raised serum triglyceride level** ($\geq 1.7$mmol/L)
  - **reduced serum HDL-cholesterol level** ($< 1.03$mmol/L in males and $< 1.29$mmol/L in females), (or specific treatment for these lipid abnormalities)
  - **raised blood pressure** (systolic blood pressure $\geq 130$mmHg or diastolic blood pressure $\geq 85$mmHg), or treatment of previously diagnosed hypertension
  - **impaired fasting glycaemia** (fasting plasma glucose $\geq 5.6$mmol/L), or previously diagnosed type 2 diabetes

*Box 1* – The 2005 International Diabetes Federation definition of the metabolic syndrome [14; 15].

The most important feature of the metabolic syndrome is the association with type 2 diabetes and atherosclerotic CVD. Prospective studies have shown that individuals with metabolic syndrome are twice as likely to die, three times more likely to suffer a myocardial infarction [16] and five times more likely to develop type 2 diabetes than those who do not [17]. In addition it has been estimated that approximately 29% of Australians aged over 25 have the metabolic syndrome [18].

### 2.3 Type 2 Diabetes

Type 2 diabetes or non-insulin dependent diabetes is a slow onset disease arising most often in older individuals [7], however the frequency of this disease is now increasing among children [19]. Type 2 diabetes is one of the most pressing health problems in the world today and accounts for up to 85-90% of all diabetic cases [20]. According to the 2004-05 National Health Survey 582,800 Australians (approximately 3% of the population) reported having type 2 diabetes and it is predicted that by the year 2010 approximately 1 million Australians will have developed it. In addition, the worldwide incidence of type 2 diabetes is projected to increase from approximately 135 million in 1995 to 299 million in 2025 [21].

Type 2 diabetes is characterised by the chronic elevation of blood glucose levels above normal range and a progressive deterioration of insulin regulation that ultimately results in the development of insulin resistance. Further characteristics of type 2 diabetes and insulin resistance include post prandial hyperglycaemia, increased hepatic glucose production and abnormalities in carbohydrate, lipid and protein metabolism [22; 23; 24; 25]. Insulin resistance is initially characterised by hyperinsulinaemia that is followed
by a decrease in efficiency of insulin signalling of glucose metabolism in liver, muscle and adipose tissue. Initially, hyperinsulinaemia compensates for this decrease in insulin signalling, therefore preserving normal glucose tolerance. However, this is followed by either increases in insulin resistance or decreases in insulin secretory response, or both, that results in the development of impaired glucose tolerance and hyperglycaemia.

Diagnosis of type 2 diabetes generally occurs after 40 years of age and many diabetics are obese. It is estimated that up to 44% of type 2 diabetics within Australia are obese [23]. In addition, type 2 diabetes is associated with a 2- to 4-fold increased risk of developing CVD [26] and is the major cause of mortality in diabetic patients, accounting for up to 80% of all deaths [27; 28; 29]. This has been attributed to the clustering of several risk factors including insulin resistance, hypertension, obesity and dyslipidaemia [30; 31]. Furthermore, poorly controlled or undiagnosed type 2 diabetes can lead to further complications including retinopathy, nephropathy, peripheral and autonomic neuropathy, stroke and CVD.

There is convincing evidence that type 2 diabetes has a genetic component as studies show a high concordance rate among monozygotic twins of approximately 41-55% [31]. Similarly, offspring of parents suffering from type 2 diabetes and longitudinal studies of families have shown that insulin resistance is a major risk factor for the development of the disease [32; 33]. Monogenic forms of diabetes have been identified such as maturity-onset diabetes of the young (MODY) and maternally inherited diabetes with deafness (MIDD) however these cases are difficult to identify and are only account for a small percentage of those suffering from diabetes. Although a genetic component has been identified for a small number of those affected from diabetes, it does not explain the majority of diabetes cases. However, recent evidence has shown that type 2 diabetes is associated with environmental factors such as lifestyle and diet. Recent studies suggest that type 2 diabetes is a polygenic disease involving numerous polymorphisms in many genes that encode proteins involved in insulin signalling, insulin secretion and intermediary metabolism in combination with the additional impact of environmental factors, such as diet and fat intake.
2.3.1 Maturity-Onset Diabetes of the Young

MODY is a genetically heterogeneous monogenic form of type 2 diabetes. It is estimated that these mutations account for 2-5% of those suffering from type 2 diabetes [34]. These monogenic forms are characterised by early age of diagnosis, usual before 25 years of age, autosomal dominant inheritance and defective glucose-stimulated insulin secretion. Clinical studies have shown that prediabetic MODY subjects have normal insulin sensitivity but suffer from abnormal glucose-stimulated insulin secretion, suggesting that pancreatic β-cell dysfunction may be the primary defect of the disorder. 

A number of monogenic forms of diabetes have been identified involving mutations in several different genes resulting in 6 subtypes of MODY. Mutations in these genes provide insight into the cascade of transcriptional factors that control the expression of pancreatic β-cell gene expression.

MODY-1, MODY-3 and MODY-5 are characterised by mutations located in the hepatocyte nuclear factor-4α (HNF-4α), HNF-1α and HNF-1β genes, respectively [35; 36; 37]. Mutations in these genes result in severe defects in glucose tolerance, glucose-stimulated insulin secretion and renal function [38]. HNF-4α is also a known regulator of glucokinase (GK) gene expression that may further contribute to the development of MODY-1. Another subtype of MODY is caused by a mutation in the GK gene (MODY-2) causing a mild form of type 2 diabetes due to a reduction in GK affinity for glucose [39]. MODY-4 is characterised by mutations in the insulin promoter factor-1 gene which is involved in early embryonic development of the pancreas [40]. Phenotypes of MODY-4 range from normal to impaired glucose tolerance to overt type 2 diabetes [41]. Finally, MODY-6 results from a mutation in the NEUROD1 gene that is involved in the development and function of pancreatic islet cells [42]. However, the identification of these rare mutations does not directly identify genetic causes of obesity and diabetes in the population as a whole. This suggests that environmental factors, such as diet, combined with a predisposed genetic background may be necessary to result in the development of type 2 diabetes.

2.4 Risk Factors Associated with Obesity, Metabolic Syndrome and Type 2 Diabetes

The first CVD associated with obesity and type 2 diabetes to be identified was coronary artery disease (CAD) [43]. A second group of disorders now threatens to replace it as a
major health problem in Western societies, the complications of obesity, which includes insulin resistance, hyperglycaemia, dyslipidaemia and hypertension [10]. A large increase in body fat is accompanied by profound changes in physiological functions. Generalised obesity results in alterations in total blood volume and cardiac function, whereas the distribution of fat around the thoracic cage and abdomen restricts respiratory expiration altering respiratory function. The intra-abdominal visceral deposition of adipose tissue, which characterises upper body obesity, is a major contributor to the development of hypertension, elevated plasma insulin concentrations and insulin resistance, dyslipidaemia and type 2 diabetes [44]. In addition, different fat deposits vary in their responsiveness to hormones that regulate lipolysis which also varies according to fat distribution [45]. Cortisol may also contribute to enhanced lipolysis by further inhibiting the anti-lipolytic effect of insulin. These factors all contribute to an exaggerated release of free fatty acids (FAs) from abdominal adipocytes into the portal system. Free FAs have a deleterious effect on insulin signalling in the liver and contribute to increased hepatic gluconeogenesis and hepatic glucose release that is observed in obesity.

Patients with type 2 diabetes are at high risk of developing a range of debilitating complications that can lead to premature disability and death. Mortality rates are two to three times higher among diabetic patients than in the rest of the population. Approximately 75% of deaths among patients with type 2 diabetes are due to CHD, with such patients experiencing a more than doubled risk of heart attack or stroke [27; 28]. In addition, microvascular complications can result in nephropathy and is a leading cause of renal disease in diabetics [46]. Furthermore, as many as 40% of non-insulin diabetics develop evidence of hepatic steatosis, or ‘fatty liver’, which is a condition that leads to hepatic fibrosis and cirrhosis in a subset of individuals [47].

### 2.4.1 Insulin Resistance

Insulin resistance is characterised by increases in blood insulin levels and an inability to suppress hepatic glucose production. Insulin stimulates the removal of blood glucose and inhibits gluconeogenesis and the release of glucose by the liver and acts to prevent the breakdown of TGs to free FAs in adipose tissue. However, insulin resistance results in impaired glucose tolerance via a number of mechanisms including resistance to peripheral glucose uptake and metabolism, impaired glycogen synthesis and increased
gluconeogenesis in the liver. This can develop as a result of a number of factors such as reduced number of insulin receptors, impaired insulin receptor binding or a disruption in post-receptor insulin-signalling transduction.

Figure 2.1 – Fatty acid intake and mechanism of insulin resistance [48].

Hepatic lipid accumulation is associated with the development of insulin resistance and results in an impaired suppression of hepatic glucose output by insulin (Figure 2.1) [49]. The accumulation of hepatic lipids appears to be most likely due to an increased delivery of TGs from adipose tissue and/or dietary FAs to the liver [50]. In obese individuals, especially those with high levels of abdominal obesity, hypertension and dyslipidaemia, the ability of insulin to exert these effects is impaired. In addition, individuals predisposed to type 2 diabetes have a compromised ability of pancreatic β-cells to secrete insulin, resulting in incomplete suppression of hepatic glucose production increasing levels of hyperglycaemia [51]. In addition, increased portal free FA flux to the liver stimulates gluconeogenesis further contributing to the development of hyperglycaemia [52].

Insulin resistance has been shown to be the best predictor of diabetes and is the central and underlying feature of type 2 diabetes [53] and is caused by a number of factors including obesity, sedentary lifestyle, pregnancy, hormone imbalance and dietary intake [22; 23]. Insulin resistance has been shown to correlate with CAD [54; 55; 56] and
hyperinsulinaemia and is a positive independent risk for CHD [57]. Insulin resistance also contributes to the development of cardiovascular risk factors, including dyslipidaemia, hypertension, type 2 diabetes and impaired fibrinolysis.

2.4.2 Hyperglycaemia
Hyperglycaemia is characterised by raised levels of circulating blood glucose levels. In adults the normal blood glucose levels range from 4-8 mmol/l. Hyperglycaemia results as a consequence of insulin resistance due to a decreased ability of tissues, such as the liver and muscle, to take up, phosphorylate and metabolise glucose. In addition, hyperglycaemia reduces nitric oxide (NO) availability, due to increases in oxidative stress levels, thereby reducing the efficacy of an endothelium-derived vasodilator system that participates in vasculature homeostasis [58]. Furthermore, hyperglycaemia increases the levels of oxidation of LDLs and glycated LDLs. Oxidised LDLs are important modulators of atherosclerosis and cardiovascular death that promote inflammation and the formation of atheromatous plaques.

Acute hyperglycaemia has been shown to impair endothelial function [59] and patients with poor long-term glycaemic control show more evidence of early atherosclerosis [60]. Poor glycaemic control can result in an increase of microvascular disease, for example, a 1% increase in glycated haemoglobin may result in a 70% increased incidence of retinopathy [61]. In addition, circulating advanced glycation end-products (AGEs) bind to plasma lipoproteins and delay their clearance, a mechanism that has been implicated in dyslipidaemia and diabetic nephropathy.

2.4.3 Oxidative Stress
Oxidative stress plays a key role in the pathogenesis of vascular complications and in type 2 diabetes. Hyperglycaemia-induced oxidative stress directly promotes endothelial dysfunction via several mechanisms including glucose autoxidation, the formation of AGEs and activation of the polyol pathway (Figure 2.2) [62]. Other circulating factors that are elevated in diabetics, include free FAs and leptin, and contribute to increase production of reactive oxygen species (ROS).
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**Figure 2.2** – Overview of reactive oxygen species sources in diabetes and their links to atherosclerosis [62]. Abbreviations: oxLDL, oxidised LDL, FFA, free fatty acids, AGEs, advanced glycation end-products, VSMC, vascular smooth muscle cells, ROS, reactive oxygen species.

Intracellular hyperglycaemia leads to increased levels of glucose autoxidation leading to the overproduction of nicotinamide adenine dinucleotide (NADH) and flavin adenosine dinucleotide. Excess NADH generates an increase in the mitochondrial proton gradient and electrons are transferred to oxygen resulting in superoxide production [63]. It is thought that mitochondrial-derived superoxide causes increased diacylglycerol synthesis and subsequent protein kinase C activation [64].

AGE formation is the result of an irreversible non-enzymatic covalent bonding of ketone or aldehyde groups of reducing sugars to free amino acid groups of proteins and other molecules [65]. In diabetic patients, AGE has been implicated in atherosclerotic lesions and tissue AGE concentrations increase with the severity of disease [66]. AGEs are proposed to contribute to atherosclerosis by modifying the extracellular matrix and circulating lipoproteins, as well as binding to the AGE receptor that is present on many vascular cells [65]. This is mediated through AGE receptor production of ROS, possibly via NAD(P)H oxidase [67], and the subsequent activation of transcription factors and expression of inflammatory mediators [68; 69].

The polyol pathway also contributes to increased ROS generation through the involvement of the enzymes aldose reductase and sorbitol dehydrogenase. The first
enzyme, aldose reductase, is responsible for the reduction of glucose to sorbitol using NADPH. Under normal conditions this conversion of glucose to sorbitol is a minor reaction, however during hyperglycaemia up to 30-35% of glucose is metabolised by this pathway [70]. The activity of this pathway results in a decreased availability of NADPH, which in turn reduces glutathione regeneration and NO synthase activity, thereby increasing oxidative stress levels [64]. The second enzyme, sorbitol dehydrogenase, oxidises sorbitol to fructose with the generation of NADH. This increased NADH production can also induce mitochondrial superoxide production via NAD(P)H oxidase activity [71].

### 2.4.4 Diabetic Dyslipidaemia

Diabetic dyslipidaemia is a cluster of potentially atherogenic plasma lipid and lipoprotein abnormalities. In type 2 diabetics, dyslipidaemia is associated with impaired endothelial function [72] and is an important and common risk factor for CVD [73]. CVD is the world’s leading cause of morbidity and mortality. Importantly, dyslipidaemia frequently precedes type 2 diabetes, indicating that a disturbance of lipid metabolism may be the primary event in the development of type 2 diabetes. The three major characteristics of dyslipidaemia include increased levels of plasma TGs, increased levels of small-dense LDL cholesterol and reduced concentrations of HDL cholesterol [74; 75].

LDLs are also associated with endothelial dysfunction [76] and are an independent risk factor for CHD [77]. LDLs are smaller and more-dense than HDLs and have an increased tendency to undergo oxidation [78], especially when glycated in the presence of hyperglycaemia and enhanced oxidative stress [79]. In addition to the reduction of HDL cholesterol levels, the HDL particles are modified and are smaller and denser [80]. This alteration in HDL particle size is due to increased lipolysis of TG rich particles [81].

### 2.4.5 Hypertension

The development of stroke and microvascular disease in diabetic patients is associated with hypertension. Hypertension can be defined as a systolic blood pressure above 140 mmHg or a diastolic blood pressure above 90 mmHg (Expert panel on detection 2002). The risk of hypertension is approximately five times higher among the obese population
and approximately two times higher in the diabetic population compared to non-obese and non-diabetic populations, respectively [82; 83; 84]. In addition, up to two-thirds of cases of hypertension are linked with excessive weight gain [85], and 85% of hypertension arises in individuals with BMI values above 25 kg/m². Hypertension is associated with the development of microvascular complications, stroke and atherosclerosis via trauma to arterial endothelium and possible contribution to plaque growth. In Australia, 69% of diabetic patients have hypertension [86], identifying this condition as a major contributor to cardiovascular mortality in type 2 diabetes [82; 83].

Hypertension has also been shown to be independently associated with insulin resistance [87], and insulin resistance may precede the development of hypertension [88]. Several mechanisms have been proposed to explain the association of insulin resistance and hypertension that include an increased sympathetic nervous system activity [89], proliferation of vascular smooth muscle cells, altered cation transport [90], increased sodium reabsorption [91] and a deficiency in the production of NO [92].

**2.4.6 Microvascular Complications**

Microvascular complications associated with type 2 diabetes include retinopathy, nephropathy and peripheral neuropathy that may lead to blindness, renal failure and limb amputation, respectively [93]. Individuals with insulin resistance including those suffering type 2 diabetes, visceral obesity and hypertension have a tendency to exhibit impaired endothelium-dependent vasodilatation. Evidence suggests that endothelial injury, activation and dysfunction are associated with early events in atherogenesis [94]. Hyperglycaemia, dyslipidaemia and hypertension can also cause damage to the endothelium resulting in an imbalance in endothelial production of vasoconstrictors when compared to vasodilators. In particular, there is a decrease in NO production which has potent antiatherogenic affects including modulation of platelet aggregation and adhesion, reduced lipid peroxidation, monocyte adhesion and vascular smooth muscle cell proliferation [95].

Diabetic retinopathy is a leading cause of blindness and visual disability causing damage to the small blood vessels in the retina, resulting in loss of vision. Retinopathy is prevalent in about 15% of patients who have had type 2 diabetes for more than 15 years. In addition, approximately 2% of diabetic sufferers become blind, while about
10% develop severe visual handicap [96]. Nephropathy develops in approximately 20% of people with type 2 diabetes. Abnormalities associated with nephropathy can be detected in most patients who have had diabetes for five to ten years and are associated with an increase in cardiovascular mortality [97; 98]. In addition, peripheral neuropathy can cause sensory loss in feet and legs resulting in the loss of protective sensation which is a leading cause for limb amputation [93].

2.5 Key Hormones Influencing Type 2 Diabetes

2.5.1 Insulin
Insulin is an anabolic hormone with powerful effects on a range of physiological processes involving mitogenic and metabolic events. The most important and examined role of insulin is its regulation of glucose homeostasis. The maintenance of normal glucose metabolism requires a tightly coordinated control of insulin secretion and action. Insulin is stored in granules in pancreatic β-cells and is secreted in response to increasing plasma glucose levels [99]. Elevated insulin levels stimulate glucose uptake in hepatic, skeletal and adipose tissue, increases in glycogen synthesis and gluconeogenesis and inhibition of glycogenolysis, ultimately reducing plasma glucose levels. Malfunction of either release of insulin by pancreatic β-cells (ie β-cell dysfunction) or insulin action (ie peripheral insulin resistance) leads to the development of type 2 diabetes.

Insulin is a member of a gene family that includes insulin-like growth factors I, II and relaxin. The insulin gene is transcribed and translated as pre-proinsulin, which is cleaved to produce insulin. Expression of the insulin gene is under the control of glucose concentrations, in which glucose binds to a glucose response element stimulating gene expression. Insulin is a major regulator of hepatic glucose metabolism and controls the expression of specific hepatic genes. For example, the glucokinase (GK) gene encoding the first enzyme involved in hepatic glycolysis is dependent on insulin for its transcription [100; 101; 102]. In addition to these well established short-term actions, insulin exerts a number of long term effects, many of which are mediated by changes in gene regulation involved in amino acid uptake, lipid metabolism, cell growth, development and survival [103].
2.5.2 Insulin Signalling

Insulin signalling and transduction involves a series of phosphorylation events that occur through the activity of certain protein kinases [104; 105]. Insulin binds to the insulin receptor (IR), which comprises of two extracellular α-subunits involved in ligand binding and two intracellular β-subunits that transduce the insulin signal to the cell. These subunits are linked by disulphide binds to form a heterodimeric complex [106]. The β-subunits have intrinsic tyrosine kinase activity that, upon insulin binding, activates intracellular signalling proteins by phosphorylation of tyrosine residues [107].

![Image of insulin signalling pathway]

**Figure 2.3 – Regulation of insulin signalling** [108]. Abbreviations: INS – insulin, IGF – insulin-like growth factor, IGF1R – insulin-like growth factor 1 receptor, PTB – phosphotyrosine-binding domain, PH – pleckstrin homology domain, TNFaR – tumour necrosis factor α receptor, IL-6R – interleukin-6 receptor, GLP-1r – glucagon-like peptide-1 receptor, pY – phosphotyrosine, pS – phosphoserine, PKC – protein kinase C, E2 – ubiquitin conjugating enzymes, Jnk – c-Jun N-terminal kinase., for other abbreviations refer to text.

Insulin binding to its receptor stimulates receptor tyrosine autophosphorylation and subsequent recruitment of the insulin-receptor substrate (IRS) **(Figure 2.3)** [109]. IRS-1 is responsible for insulin action in muscle, while IRS-2 is specific for propagating the insulin signal in the liver [110]. IR tyrosine phosphorylation facilitates the docking of IRS through binding of the phosphotyrosine-binding domain. The C terminal of the IRS
contains numerous tyrosine and serine phosphorylation sites that bind common effector enzymes including phosphoinositide 3-kinase (PI(3)K), the phosphatase SHP2 and the tyrosine kinase fyn [108]. The PI(3)K is activated during the association with IRS proteins and converts PI(4,5)P2 to PI(3,4,5)P3 that recruits protein kinase B (PKB) and 3-phosphoinositide-dependent kinase-1 (PDK1) to the plasma membrane where PDK1 activates PKB. PKB activation results in the phosphorylation of many substrates that are involved in cell survival, regulation of growth and glycogen synthesis and the control of gene expression.

Other mechanisms of insulin that effect the regulation of insulin signalling and the development of insulin resistance include inflammation and activity of protein or lipid phosphatases. Proinflammatory cytokines including interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) are secreted from leukocytes during inflammation and adipose tissue. TNF-α regulates various kinases including the activation of c-Jun kinase (Jnk) that phosphorylates serine residues of IRS-1 and IRS-2 resulting in the inhibition of insulin-stimulated tyrosine phosphorylation [111]. In addition, IL-6 increases the expression of genes that mediate ubiquitin-mediated degradation of IRS-1 and IRS-2 promoting insulin resistance. IL-6 induces the expression of SOCS proteins, SOCS1 and SOCS2 that recruit the ubiquitin based ligase, elongin B and C, into the IRS-protein complex to mediate ubiquitylation [112]. Ubiquitin-mediated degradation of IRS-1 and IRS-2 may be the mechanism of cytokine-induced insulin resistance that contributes to diabetes and β-cell failure.

### 2.5.3 Leptin

The main physiological regulator of body weight is leptin. Leptin acts as an adiposity signal in a negative feedback loop that regulates food intake and energy expenditure. Leptin is produced predominantly by white adipose tissue and plasma leptin concentrations correlate directly with the percentage of body fat [113]. The amount of adipose tissue mass, bodyweight and appetite is controlled by leptin via receptors in the hypothalamus and other tissues by decreasing food intake and increasing energy expenditure and thermogenesis [114; 115].

Insulin secretion stimulates leptin release that results in a raised expression of lipolytic genes. The role of leptin is to protect non-adipose tissues from non-oxidative
metabolism of FAs during time periods of over nutrition by increasing \( \beta \)-oxidative metabolism of surplus FAs and reducing lipogenesis [116]. In addition, leptin has been shown to repress acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS) gene expression which play a key role in lipid synthesis. At an individual’s stable weight the amount of circulating leptin promotes a state in which food intake equals energy expenditure. However, in obese humans, leptin signalling does not result in decreased food consumption. This is due to leptin resistance or insensitivity. Environmental factors such as a high fat diet have been shown to affect leptin sensitivity leading to leptin resistance although the basis for this is poorly understood.

2.5.4 Leptin Signalling

Signal transduction of leptin signalling is dependent on the phosphorylation of the leptin receptor, Ob-R. Ob-R is a single membrane spanning receptor of the cytokine-receptor superfamily [117]. Five splice variants of the Ob-R have been identified that contain truncated cytoplasmic domains designated Ob-R\(_A\), Ob-R\(_C\) to Ob-R\(_F\) [118; 119]. Ob-R\(_B\) is the long isoform that contains a 303 amino acid cytoplasmic domain that signals by activating Janus kinase -2 (JAK-2). Activation of JAK-2 subsequently phosphorylates the signal transducer and activator of transcription-3 (STAT3) [120]. Phosphorylation of STAT3 initiates dimerisation and translocation into the nucleus and the induction of gene expression [121].

In the hypothalamus, leptin binds to Ob-R\(_B\) stimulating specific signalling cascades that result in the inhibition of several orexigenic neuropeptides, while stimulating several anorexigenic peptides. The down-regulation of orexigenic peptides by leptin includes neuropeptide Y, melanin-concentrating hormone, orexins and agouti-related protein. The anorexigenic neuropeptides that are up-regulated are \( \alpha \)-melanocyte-stimulating hormone, which acts on melanocortin-4 receptor, cocaine and amphetamine-regulated transcript and corticotrophin-releasing-hormone [122].

2.6 Key Enzyme Involved in Insulin Action and Hepatic Glycolysis

2.6.1 Glucokinase

Key enzymes involved in lipid biosynthesis and glycolysis may also provide important information about the mechanisms that lead to the development of obesity and type 2
diabetes. GK is the major regulatory enzyme of hepatic glucose metabolism, controlling the supply of substrate to the glycolytic, lipogenic, glycogenic and pentose phosphate pathway. GK is the liver specific member of the hexokinase family of enzymes that catalyses the rate limiting step of glycolysis by phosphorylating glucose resulting in the subsequent synthesis of glucose 6-phosphate (G-6-P). As GK regulates hepatic glucose and lipid metabolism, changes in the regulation of GK may result in significant changes in glycolysis and lipogenesis. This suggests that an impairment of GK activity may play a major role in precipitating glucose intolerance and the development of type 2 diabetes [123].

2.6.2 Regulation of Glucokinase

The regulation of GK activity is complex. The GK gene can be transcribed from two different promoters. Extrahepatic expression is initiated from the \( \beta \)-cell specific promoter localised 20 kilobases upstream of the gene, whereas hepatic expression is controlled by the liver specific promoter adjacent to the GK gene [124; 125]. Together these isoforms of GK act to co-ordinately regulate plasma blood glucose concentrations and homeostasis [126]. In pancreatic \( \beta \)-cells, GK acts as a ‘glucose sensor’ ensuring that insulin release is appropriate to the plasma glucose concentration in which glucose phosphorylation is tightly coupled to insulin secretion (Weinhouse, 1976; Megalson and Matchinsky, 1983) (Matchinsky et al, 1998). In liver, GK is thought to be essential for the unique metabolic functions of this tissue and is subjected to both transcriptional and post-translational control mechanisms. Under basal glucose concentrations (<5.5mM) the majority of hepatic GK is bound to the GK regulatory protein (GKRP) in the nucleus (16-181), however when subjected to high glucose concentrations (10-30mM), GK is released from the GKRP and translocates into the cytoplasm where it exists in an unbound state and becomes active (14,151). GK plays an important role in the maintenance of blood glucose concentrations (Ferre et al, 1996), and impairment of GK activity may play a significant role in precipitating glucose intolerance in response to a high fat diet (Chrisholm and O’Dea, 1987).

Insulin and glucagon (via cAMP) both play a role in the regulation of expression of the GK gene [100; 102; 127; 128; 129]. Insulin increases, whereas glucagon decreases, GK gene transcription [130; 131]. After a meal, the increase in blood glucose leads to
induction of liver glycolysis, glycogenesis, and lipogenesis. In this situation, the increasing plasma insulin concentrations stimulate GK synthesis and increases glucose phosphorylation to G-6-P [132; 133]. Unlike hexokinases, GK is not inhibited by G-6-P, therefore this metabolite could be involved in the stimulation of GK gene expression in response to increases in blood glucose levels. Some studies performed in vitro on cultured hepatocytes indicate that GK activation is necessary for the induction of glycolysis and glycogen synthesis [134].

The transcriptional regulation of GK is under the control of a number of transcription factors including sterol regulatory element binding protein-1c (SREBP-1c), liver X receptor α (LXRα), HNF-4α and peroxisomal proliferator activated receptor γ (PPARγ). Insulin-induced GK gene expression is mediated through SREBP-1c, a potent activator of gene transcription [135; 136; 137]. This has been demonstrated by the identification of two functional sterol regulatory elements (SREs) in the rat GK promoter [138]. In addition, SREBP-1c basal expression levels are mediated by the induction of LXRα [139; 140; 141]. Furthermore, a functionally active HNF-4α responsive element has been identified in the rat GK promoter and has been shown to induce GK promoter activity [142]. Recently, it has been shown that PPARγ can activate GK gene transcription in response to increasing glucose concentrations [143].

In addition to changes in the rate of transcription of the GK gene, alterations in the kinetic regulation of the protein can also affect its activity. The kinetic properties of GK and the regulation of GK levels by insulin and glucagon may lead to a situation in which GK has a key role in regulating and integrating glucose metabolism in the liver. In the liver, but not the pancreas, GK activity is regulated by a 68 kDa GK regulatory protein (GKRP), which competitively inhibits GK binding to glucose [144]. In the presence of low (5 mM) concentrations of glucose, GK is bound to a GKRP in the nucleus of the cell and is inactivated [145]. When glucose concentrations rise (>20mM) or in the presence of a number of activators including sorbitol, fructose and fructose 1-phosphate, GK and the GKRP dissociate. GK then translocates into the cytoplasm, and becomes active, and consequently increases the rate of glucose phosphorylation [145]. In addition, transgenic mice with additional copies of the GK gene exhibit increased hepatic glucose metabolism and as a result, blood glucose concentrations are diminished.
Conversely, ‘knock-out’ mice lacking the hepatic, but not the pancreatic GK gene, exhibit mild hyperglycaemia and impaired glucose metabolism [146; 147]. This data further support the importance of the role GK plays in the pathogenesis of type 2 diabetes.

2.6.3 Other Enzymes Involved in Glucose Metabolism

Key enzymes involved in lipid biosynthesis, fatty acid oxidation and glycolysis may also provide important information about the mechanisms that lead to obesity and diabetes. These enzymes include acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS), involved in lipid biosynthesis and GK, the initial step of glycolytic pathway. GK is the major regulatory enzyme of hepatic glucose metabolism, controlling the supply of substrate to the glycolytic, lipogenic, glycogenic and pentose phosphate pathway. GK is a member of the hexokinase family of enzymes that catalyses the synthesis of glucose-6-phosphate (G-6-P) from glucose and ATP. GK is found predominantly in hepatocytes and pancreatic β-cells and plays an important role in the maintenance of blood glucose concentrations [126]. This suggests that an impairment of GK activity may play a major role in precipitating glucose intolerance in response to a high fat diet [123; 148].

Long-chain fatty acids and their derivatives are not only constituents of cellular structures, they also function as regulatory molecules affecting all phases of cellular activities. ACC is the rate-limiting enzyme in the biogenesis of long-chain fatty acids [149]. ACC catalyses the carboxylation of acetyl-CoA that results in the production of malonyl-CoA. Malonyl-CoA is then used for long-chain fatty acid synthesis by the multifunctional enzyme, FAS, which catalyses the synthesis of palmitic acid [150; 151]. Genes belonging to the lipogenic pathway have a more complex regulation than glycolytic genes because their expression depends on both high insulin and glucose concentrations and glucose metabolism regulated by GK [133].

2.7 Current Treatments of Type 2 Diabetes

2.7.1 Lipid-Lowering Agents

Two classes of lipid-lowering agents are currently being used as therapeutic treatments to decrease the risk of CAD, myocardial infarction and stroke in type 2 diabetics. The
first class, fibrates, exhibit lipid-modulating effects that are mediated through their capability to mimic the structure of free FAs [152]. PPAR\(\alpha\) is the molecular target of fibrates, such as gemfibrozil, which is used in the treatment of hypertriglyceridaemia [153; 154]. Fibrate binding and activation of PPAR\(\alpha\) alters the expression of genes involved in HDL-cholesterol metabolism including apo-AI and apo-AII, lipoprotein lipase, scavenger receptor B1 and ABCAI. In conditions of dyslipidaemia, fibrates promote a reduction in VLDL and LDL and increase HDL fractions attenuating an atherogenic lipid profile [155].

A second class of lipid-lowering agents, statins, have been shown to be effective in reducing cardiac death and CAD in diabetic subjects [156; 157]. Statins are 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors and act to lower cholesterol synthesis. Statins prevent atherosclerotic macrovascular complications and postpone the development of microvascular complications, such as nephropathy and retinopathy. In addition, statins may attenuate inflammation, oxidative stress, coagulation, platelet aggregation and improve insulin resistance, fibrinolysis and endothelial functions and help to prevent thrombosis [158; 159]. Increasing evidence suggests that statins are the current treatment of choice to prevent vascular complications in diabetic patients with hypercholesterolemia [160].

### 2.7.2 Hypoglycaemic Agents

Three classes of pharmacological agents have been recommended for the treatment of hyperglycaemia that include: 1) Sulfonylureas and thiazolidinediones (act to increase insulin sensitivity), 2) Biguanides (acts to suppress hepatic glucose production) and 3) \(\alpha\)-glucosidase inhibitors (alter gastrointestinal tract glucose absorption).

Sulfonylureas bind to an ATP-dependent K+ (KATP) channel on the cell membrane of pancreatic beta cells. This inhibits a tonic, hyperpolarizing outflux of potassium, which causes the electric potential over the membrane to become more positive. This depolarization opens voltage-gated Ca\(^{2+}\) channels. The rise in intracellular calcium leads to increased fusion of insulin granulae with the cell membrane, and therefore increased secretion of (pro)insulin. There is some evidence that sulfonylureas also sensitize \(\beta\)-cells to glucose, that they limit glucose production in the liver, that they decrease lipolysis (breakdown and release of fatty acids by adipose tissue) and decrease
clearance of insulin by the liver. Sulfonylureas exert their beneficial effects on glucose homeostasis by increasing insulin sensitivity and reducing blood glucose level [161; 162]. In addition, sulfonylureas affect lipid metabolism by elevating plasma HDL-cholesterol by reducing the number of small-dense LDL particles and plasma TG levels. In monotherapy, sulfonylureas induce a significant decrease of fasting blood glucose [163; 164] that is maintained over time [165].

Thiazolidinediones, also known as "glitazones", bind to PPARγ, a type of nuclear regulatory proteins involved in transcription of genes regulating glucose and lipid metabolism. These PPARs act on Peroxisome Proliferator Responsive Elements (PPRE). The PPREs influence insulin sensitive genes, which enhance production of mRNAs of insulin dependent enzymes. The final result is better use of glucose by the cells.

The biguanide, metformin, reduces intestinal glucose absorption, increases peripheral tissue uptake of circulating blood glucose and reduces hepatic glucose production. In addition, metformin reduces insulin requirements for adequate glucose metabolism, though has little effect on insulin secretion [166; 167]. Acarbose is an inhibitor of the α-glucosidase enzyme that reduces postprandial blood glucose by suppressing carbohydrate absorption from the small intestine [168].

2.7.3 Hypertensive Agents
Antihypertensive therapy in diabetic individuals includes the administration of angiotensin converting enzyme (ACE) inhibitors. ACE inhibitors offer a cardio protective effect that may be related to their beneficial effects on endothelial function. They act to prolong the action of bradykinin via the inhibition of the enzymatic breakdown leading to increased blood flow. ACE inhibitor therapy may also improve insulin resistance associated with hypertension and type 2 diabetes, this is possibly due to the role of bradykinin in insulin-stimulated glucose uptake.

2.8 Dietary Lipids and a High Fat Diet

2.8.1 Lipids
Lipids are a heterogeneous group of water insoluble and organic molecules that consist of long-chain FAs. In the body, lipids are utilised as an energy source, cellular
membranes components and precursors of molecules involved in numerous biological processes including steroid hormones, vitamins, bile acids and eicosanoids. Major forms of clinically important lipids found in human plasma include cholesterol, cholesteryl esters, TGs, phospholipids and glycolipids. TGs are stored within adipose tissue which supplies other tissues with energy in the form of free FAs. Lipids are continually synthesised and metabolised in cells such that their steady-state levels are maintained. When the metabolism of lipids is disturbed or disrupted various pathological disorders can develop including dyslipidaemia, obesity and type 2 diabetes.

2.8.2 Fatty Acid Homeostasis
In the fed state, which in humans is up to four hours after a meal, carbohydrates and lipids enter the circulation in the form of glucose and chylomicrons, respectively. The majority of dietary glucose is taken up by the liver for glycogen synthesis or metabolised via glycolysis (Figure 2.4). In the fed state, hepatic GK is induced by increasing insulin levels via insulin-induced activation of SREBP-1c. Increased GK enzyme activity promotes the metabolism of glucose via glycolysis to produce to acetyl-CoA [136; 137]. Acetyl-CoA is a precursor for lipogenesis and the production of FAs. The FAs that are produced via lipogenesis are subsequently converted to TGs and packaged into VLDLs for transport to adipose tissue for storage.

Long-chain FAs and their derivatives are not only constituents of cellular structures, they also function as regulatory molecules affecting all phases of cellular activities. ACC is the rate-limiting enzyme in the biogenesis of long-chain FAs [149]. ACC catalyses the carboxylation of acetyl CoA resulting in the generation of malonyl CoA. Malonyl CoA is then used for long-chain FA synthesis by the multifunctional enzyme, FAS, which catalysis the synthesis of palmitate [150; 151]. Genes belonging to the lipogenic pathway have a more complex regulation than glycolytic genes because their expression depends on both high insulin and glucose concentrations and glucose metabolism regulated by GK [133].
The energy requirement of non-adipose tissues is tightly coupled to FA delivery. For example, plasma free FA levels rise during exercise and fasting to meet metabolic energy needs. In metabolically active tissues, an increase in the level of FA oxidation results in a decreased level of non-oxidised FAs present in these cells. However, during chronic over-nutrition and decreased metabolic activity, FA influx into tissues may exceed FA usage. This leads to a compensatory up-regulation of FA oxidation that is required to maintain intracellular FA homeostasis. It has been suggested that a surplus of FAs may up-regulate PPAR\(\alpha\) and oxidative machinery to prevent an over-accumulation of non-oxidised lipids [170; 171; 172; 173; 174]. PPAR\(\alpha\) is a member of the family of transcription factors involved in the up-regulation of FA oxidative enzymes including, carnitine palmitoyl transferase-1 and acyl CoA oxidase [170; 171]

### 2.8.3 High Fat Diet, Hepatic Glycolysis and Gluconeogenesis

The saturated fat, palmitate, has been shown to inhibit glycolysis in liver by a mechanism also associated with glucose sparing [175]. Berry et al. [176] have shown...
Chapter 2 – Literature Review

that the inhibition of hepatic glycolysis during FA oxidation is associated with substantial enhancement of flux through the glucose/G-6-P cycle, and consequent glucose sparing. Glucose sparing is the process whereby glucose is taken up by the liver and phosphorylated to G-6-P, but is immediately recycled to glucose without undergoing further metabolism [176]. A role of glucose cycling has been implicated in the development of hyperglycaemia and is associated with type 2 diabetes [177; 178]. In hepatocytes from streptozotocin-induced insulin-dependent diabetic rats, increased rates of glucose cycling partly resulted in elevated rates of endogenous FA oxidation [179]. As endogenous FA oxidation may be increased in the liver of high fat fed rats, it is possible that glucose cycling may be enhanced, thereby contributing to the development of hyperglycaemia.

Hue et al. suggest that the catalysis of glucose phosphorylation by GK is inhibited by fatty acyl-CoA [175; 180]. The addition of FA also stimulates gluconeogenesis in liver cells that is due to an enhanced rate of FA oxidation increasing the concentration of acetyl-CoA. Excess acetyl CoA allosterically activates pyruvate carboxylase, the enzyme that catalyses the conversion of pyruvate to oxaloacetate. It has been suggested that the stimulation of gluconeogenesis by FAs is a result of the activation of this enzyme [181]. In addition, pyruvate dehydrogenase (PD) is allosterically inhibited by acetyl-CoA [182]. PD is responsible for the conversion of pyruvate to acetyl CoA. A decreased in PD activity results in an increased concentration of pyruvate, a precursor for gluconeogenesis. Therefore, this may contribute to an increase in the rate of glucose synthesis from the gluconeogenic precursors, pyruvate and lactate.

The inhibitory effects of FA oxidation on hepatic glycolysis and glucose utilisation have been associated with a decrease in the cellular concentration of fructose 1,6-bisphosphate [175; 176]. This decline has been attributed to an inhibition of 6-phosphofructo-1-kinase contributing to decreases of flux through glycolysis [183]. Similarly the effect of the GKRP is greatly reinforced by fructose 6-phosphate and antagonised by fructose 1, 6-bisphosphate. This results in the increased activity of GKRP to sequester GK inhibiting enzyme translocation to the cytoplasm. This ultimately reduces the amount of active cytoplasmic GK available to phosphorylate and metabolise glucose. A reduction in hepatic GK activity has been shown to contribute to the metabolic perturbations seen in high fat fed animals [127; 128; 145].
2.8.4 High Fat Diet and Insulin Resistance

Evidence strongly suggests that insulin resistance and type 2 diabetes arise from both the combination of multiple genetic and environmental factors [7; 184]. One factor that may contribute to the increasing incidence of type 2 diabetes is the consumption of a diet that is high in fat, particularly saturated fats [128; 185]. The accumulation of intracellular FAs, especially in hepatic tissue, appears to play an important role in the pathogenesis of insulin resistance [50]. Insulin resistance, induced as a result of a high intake of saturated fat, impairs glucose uptake and metabolism in a number of tissues.

In liver, a high fat diet causes hyperglycaemia and is associated with a loss of the insulin-induced inhibition of hepatic gluconeogenesis and insulin-induced stimulation of hepatic glycolysis [186]. In muscle, a high fat diet decreases insulin-stimulated glucose uptake and reduces insulin-stimulated oxidative and non-oxidative glucose disposal [123; 127]. In adipose tissue, a high fat diet has been associated with a decrease in insulin-stimulated glucose uptake, although this does not appear to play a major role in diet-induced hyperglycaemia [187]. This may not be a direct effect of insulin insensitivity but may be associated with an increase in the supply of FA to the liver [128; 188].

Prolonged exposure to long-chain FAs reduces glucose phosphorylation and glycogen synthesis in liver. Decreased glucose metabolism leads to elevated rates of lipolysis resulting in increased release of free FAs. Free FAs have a negative effect on the action of insulin on the liver, which in turn results in increased gluconeogenesis and systemic dyslipidaemia. This is believed to be due to increasing levels of long-chain FAs being incorporated into the plasma membrane of the cell, resulting in a decreased ability of insulin to bind its receptor in target tissues and transduce a signal [189]. These factors contribute to the prevailing systemic hyperinsulinaemia and consequently to decreased skeletal insulin sensitivity with reduced glucose uptake and decreased glucose stimulated insulin release [190; 191].

The elevation in plasma free FA concentration leads to an inappropriate maintenance of glucose production and an impairment of hepatic glucose utilisation (impaired glucose tolerance). Reduced hepatic clearance of insulin leads to increased peripheral (systemic) insulin concentrations and to a further down-regulation of insulin receptors. In the initial phases of this process, the pancreas can respond by maintaining a state of
compensatory hyperinsulinaemia preventing glucose tolerance. With increasing plasma concentrations of free FAs, the insulin resistant individual cannot continue to maintain this state of compensatory hyperinsulinaemia and hyperglycaemia prevails. Hyperinsulinaemia and insulin resistance are both significant correlates of a dyslipoproteinaemic state and contribute to the characteristic alterations of plasma lipid profile associated with obesity: elevated fasting plasma triglyceride concentration, reduced HDL-cholesterol, marginal elevations of cholesterol and LDL-cholesterol concentrations and increased number of apo-B-carrying lipoproteins [2].

Circulating free FAs are elevated in insulin-resistant states and have also been suggested to contribute to insulin resistance in muscle by reducing glucose transport or inhibiting glucose phosphorylation [192]. Elevated free FA levels have been shown to be associated with a reduction in insulin-stimulated insulin-receptor substrate-1 (IRS-1) phosphorylation and IRS-1-associated PI(3)K activity [192]. The reduced PI(3)K activity may be due to a direct effect of intramuscular free FAs on PI(3)K or alterations in upstream insulin signalling events. This reduction in kinase activity is believed to be responsible for perturbations in the translocation of the glucose transporter 4 (GLUT4) to the plasma membrane and may be responsible for a decreased level of glucose uptake. A decreased level of glucose transport and phosphorylation causes a reduction in muscle glycogen synthesis and is paralleled by a decrease in glucose oxidation. In addition, recent studies have shown that an increase in saturated FA [193; 194] and refined carbohydrate intake and decreased dietary fibre intake [195] can also result in reduced insulin sensitivity and abnormal glucose tolerance.

Saturated FAs decrease insulin sensitivity by decreasing membrane fluidity and reducing the translocation and activity of GLUT4. Increased FA uptake coupled with deficient FA oxidation lead to cellular damage and lipotoxicity as a result of increased TG accumulation. High levels of circulating FAs increase FA translocase expression and FA transport proteins within muscle tissue. This leads to lower FA oxidation due to decreased carnitine palmitoyl transferase activity as a result of increased malonyl CoA concentrations, reduced glycogen synthase activity and impairment of insulin signalling and glucose transport [48].
2.9 Transcription Factors Involved in Fatty Acid Signalling

2.9.1 Sterol Regulatory Element Binding Protein-1c
SREBPs have been established as a family of transcription factors that regulate the expression of a range of enzymes responsible for endogenous cholesterol, TG, FA and phospholipid synthesis [137; 196]. SREBPs belong to the basic helix-loop-helix-leucine zipper (bHLH-LZ) family that consist of three isoforms SREBP-1a, -1c and -2. SREBP-1a and -1c are produced from the same gene through alternative transcription start sites and SREBP-2 is transcribed from a separate gene. In liver, SREBP-1c is the predominantly expressed isoform and is a major target of insulin action inducing glucose metabolism and regulation of FA synthesis [137]. In contrast, SREBP-2 is relatively selective in transcriptionally activating cholesterol biosynthetic genes [197].

SREBPs are synthesised as membrane bound precursors localised to the endoplasmic reticulum and nuclear envelope. Activation of SREBP occurs through the sequential two-step cleavage process, catalysed by the SREBP cleavage activating protein, to release the mature NH$_2$-terminal active domain containing the bHLH-LZ domain. The bHLH-LZ domain contains a nuclear localisation signal that binds directly to importin and facilitates SREBP transport into the nucleus, where the nuclear form of SREBP is designated (nSREBP). Within the nucleus, nSREBP binds to the sterol regulatory element (SRE) which is an enhancer region present in target genes that is able to activate transcription.

Increased levels of SREBP-1c protein cleavage and subsequent activation are responsible for increased levels of glucose metabolism and lipogenesis. SREBP-1c expression is decreased during fasting but increases markedly upon carbohydrate feeding. Insulin has been shown to induce SREBP-1c transcription leading to increases of both ER membrane-bound precursors and the nuclear form of the transcription factor [198]. The effects of insulin on SREBP-1c expression are mediated by a PI(3)K dependent pathway [198; 199]. However, the downstream effectors remain unclear, however evidence suggests that both PKB/Akt [199; 200] and PKC$\lambda$ may be involved [201]. The insulin-induced activation of SREBP-1c explains the ability of insulin to enhance the conversion of glucose to FAs.
SREBP-1c transcription can also be induced by the activation of LXRα to generate FAs required for the formation of cholesterol esters to buffer free cholesterol concentrations [202]. LXRα mediates basal expression levels of SREBP-1c via retinoic acid receptor (RXR) and LXR DNA binding sites in the SREBP-1c promoter [139; 140; 141]. In addition to the regulation of SREBP-1c through LXRα activation, the LXR response elements are required for the full induction of SREBP-1c by insulin. This has lead to the hypothesis that SREBP-1c induction by insulin may be due to increased activity of LXRα though insulin-induced increases in the production of LXR ligands [203].

2.9.2 Liver X Receptor α
LXRα is a nuclear hormone receptor with high levels of hepatic expression and is activated by oxysterols and derivatives of the cholesterol synthetic pathway. The role of LXRα is to induce the expression of a number of genes involved in cholesterol efflux and clearance [204]. LXRα expression is predominately restricted to tissues involved in lipid metabolism including liver, kidney, small intestine, spleen and adipose tissue [205]. LXRs bind to their target DNA sequence as a heterodimer with RXR that can be activated by ligands for either LXR or RXR. LXRs control the expression of several genes required in cholesterol metabolism including CYP7A1, that is involved in the conversion of cholesterol to bile salts, and other genes involved in cholesterol efflux including ATP-binding cassette A1 transporter [206] and apolipoprotein E [207].

LXRs also modulate lipoprotein metabolism through the control of modifying enzymes such as lipoprotein lipase [208], cholesterol ester transfer protein [209], and phospholipid transfer protein [210]. In addition, LXRα has been shown to play a role in the up-regulation of FA and TG synthesis and secretion by directly inducing SREBP-1c gene expression, thereby regulating the expression of genes inducing FAS, stearoyl-CoA desaturase-1 (SCD-1) and ACC [211]. It has also been found that LXRα can directly stimulate FAS [212] and ACC [213] transcription through LXRα regulatory elements present in their promoters. Recent evidence indicates that LXRα may also act as an insulin mediating factor in the liver and play a key role in insulin-stimulated lipogenesis [214]. This possibility is due to the fact that SREBP-1c is a LXRα target and that LXR has been shown to induce basal SREBP-1c gene expression [139; 140;
Due to these observations, it has been postulated that maximal stimulation of lipogenic gene expression requires the activation of gene transcription from both SREBP-1c and LXRα.

2.9.3 Hepatocyte Nuclear Factor-4α
HNF-4α is a member of the nuclear receptor family of transcription factors and is highly expressed in the liver and acts as a positive transcriptional regulator of many hepatic genes [215]. HNF-4α binds DNA as a homodimer and its activity is modulated by the high affinity binding of long chain fatty acyl-CoA [216]. In addition, HNF-4α is acetylated [217], phosphorylated [218] and can bind SMADS 3 and 4, suggesting that its activity may be controlled by multiple pathways [219]. Gene knock-out studies of the HNF-4α gene disrupt a large number of genes involved in many aspects of mature hepatocyte function. This evidence suggests that HNF-4α acts as a positive transcriptional regulator of hepatic genes that involve the control of energy metabolism, xenobiotic detoxification, bile acid synthesis and serum protein production [220]. Recently, HNF-4α has been suggested to play a broader role in the hepatic and pancreatic regulation of glucose homeostasis and can transcriptionally induce hepatic GK gene expression [142]. This suggests a potential role of HNF-4α regulation in the development of type 2 diabetes.

2.9.4 Peroxisomal Proliferator Activated Receptor α
Peroxisomal proliferator-activated receptors (PPARs) are a family of nuclear hormone receptors that function as transcription factors. PPARs act through target genes to regulate diverse aspects of lipid metabolism, including FA oxidation, adipocyte development, lipoprotein metabolism and glucose homeostasis [221]. PPARs bind the promoter as a heterodimer bound to RXR and modify target gene transcription after binding to specific peroxisomal proliferator response elements (PPREs) [165]. Activation of gene transcription can take place through ligand binding of either the PPAR or RXR receptor, however binding of both ligands induces a more potent activator of transcription [222]. Target genes of PPARs belong to lipid transport and metabolism pathways and are subjected to regulation of gene transcription by FAs. PPARs act as FA sensors and transduce nutritional stimuli into changes in gene expression.
The PPARα isoform is highly expressed in tissues displaying a high catabolic rate of FAs such as liver, skeletal muscle, heart and kidney [221]. PPARα regulates a number of genes involved in β-oxidation and is also a major regulator of energy homeostasis. These genes participate in aspects of lipid catabolism such as FA uptake through membranes, FA binding in cells, FA oxidation (in microsomes, peroxisomes and mitochondria) and lipoprotein transport and assembly. Activation of PPARα increases β-oxidation stimulating energy production but also shortens long-chain fatty acids preventing lipid accumulation and toxicity. In liver, PPARα activation leads to the up-regulation of FA transport protein and long-chain acyl-CoA synthetase genes [154; 223]. In addition, PPARα increases apo-AI and apo-AII while decreasing apo-CIII levels resulting in increased HDL levels and decreased TG levels [224].

In the fasting state, the energy sources of the body shift from carbohydrate and lipid metabolism towards lipid metabolism. FAs stored during feeding are released from adipocytes and taken up by the liver and are oxidised to acetyl-CoA and subsequently ketone bodies. These processes are strongly stimulated by PPARα, expression of which is elevated during fasting [225]. In addition, FAs are ligands for PPARs, therefore it is possible that FAs released from adipose tissue can stimulate their own metabolism by PPARα activation. Experiments with PPARα null mice show that PPARα is important in the hepatic response to fasting. During fasting, these mice suffer from defects in FA oxidation and ketogenesis. This results in elevated plasma free FAs, hypoketonaemia, hypothermia and hypoglycaemia [225; 226]. The hypoglycaemia emphasises the important interplay between FA and glucose metabolism in energy homeostasis.

Interestingly, PPARs take an important part in the control of factors involved in the development of cardiovascular disease, particularly in patients with type 2 diabetes. These factors include the regulation of glucose and lipid metabolism, endothelium function, inflammation, thrombosis and fibrinolysis. Thus, the use of PPARs agonists in clinical practice has not only the potential to improve glucose and lipid metabolism but also to reduce atherosclerosis in diabetic patients.
2.9.5 Fatty Acid Gene Regulation

Dietary FAs regulate gene expression by controlling the activity or abundance of key transcription factors. Investigations into the FA regulation of hepatic gene expression have identified that SREBP-1c, LXRα, HNF-4α and PPARα are important in the control of glycolysis and lipogenesis (Figure 2.5) [227; 228]. Non-esterified FAs directly bind PPARα, HNF-4α, RXR and LXRα acting like hydrophobic hormones that control gene expression [227]. However, FAs can also regulate other transcription factors, such as SREBP-1c, through indirect mechanisms. Recently, SREBP-1c and PPARα have been established as key targets for polyunsaturated FAs (PUFAs) in the control of hepatic gene expression, in which PPARα induces FA oxidation and SREBP-1c induces FA synthesis.

Figure 2.5 – Regulation of hepatic gene expression by non-esterified fatty acids [228].

Dietary PUFAs have been well established as negative regulators of hepatic lipogenesis [229; 230]. Shimano et al. (1999) showed that suppression of lipogenic gene expression by PUFAs in the liver was primarily due to a decrease in the mature form of SREBP-1c
This suppression of hepatic gene expression is caused by three independent mechanisms. Firstly, the abundance of the mature SREBP-1c protein in the liver is decreased by dietary PUFA exposure primarily at a posttranslational level. PUFA does not accelerate the degradation of mature SREBP-1c protein, however it interferes with the proteolytic cleavage of mature SREBP-1c protein resulting in an immediate suppression of lipogenic gene transcription [232; 233; 234]. Secondly, PUFAs accelerate the decay of SREBP-1c mRNA which in turn lowers the hepatic content of SREBP-1c precursor [232]. Thirdly, the basal transcription of the SREBP-1c gene requires an endogenous sterol that activates LXR. The LXRs enhance transcription of the SREBP-1c gene by binding to a consensus recognition sequence in the enhancer region.

This anti-lipogenic action of PUFA reflects decreases in mRNA levels of hepatic enzymes including GK, ACC, FAS, SCD, ATP citrate lyase, malic enzymes, G-6-P dehydrogenase and pyruvate kinase. Similarly, SREBP-1c mRNA levels are decreased when cells are exposed to monounsaturated FAs (MUFAs) and PUFAs. SREBP-1 gene knockout studies have shown that mice had severely impaired levels hepatic mRNA transcription of FA synthetic genes, such as ACC, FAS and SCD [231]. In addition, PUFAs have been shown to down-regulate GK, ACC, FAS and SCD expression by reducing levels of mature/nuclear SREBP-1c [47]. The outcome is a lower capacity for lipogenesis, and a decrease in hepatic triglyceride output [229; 230; 232].

In rodent liver and hepatoma cells, transcription of the SREBP-1c gene is stimulated by oxysterols that bind to LXRα. LXRα regulates the expression of genes involved in hepatic bile acid synthesis [235]. Unsaturated FAs act as competitive antagonists to LXRα, which explains the ability of unsaturated FAs to lower levels of SREBP-1c mRNA [236]. This data suggests that SREBP-1c may be a master gene for the regulation of lipid and glucose metabolism in the liver and could be a gene involved in metabolic dysfunctions, such as type 2 diabetes, obesity and hepatic insulin resistance syndromes [137; 237].

The transcriptional activity of HNF-4α is activated upon saturated FA binding, while binding of PUFAs inhibits the effects on HNF-4α gene transcription [238]. HNF-4α
can regulate the expression of hepatic genes involved in lipoprotein metabolism (apo-CII, apo-CIII, apo-AII and apo-IV), iron metabolism (transferrin), carbohydrate metabolism (L-pyruvate kinase, glucose-6-phosphatase) and bile acid synthesis (CYP7A) [228]. In addition, saturated FAs and PUFAs can stimulate the induction of PPARα target genes of lipid and carbohydrate metabolism [239]. PPARα is responsible for increasing the rate of transcription of genes such as, acyl CoA synthase, acyl CoA oxidase, carnitine acyl transferase-1, FA transport protein, FA binding protein and SCD [240].

2.10 Summary

Obesity is a widespread disease of increasing prevalence, and therefore is rapidly becoming a major health issue in modern society. 60% of the Australian population is overweight and more than 5% have type 2 diabetes, representing an enormous cost to individuals with these conditions and the community at large. A number of mutations have been identified in genes that are involved in the development of obesity and diabetes. However, these mutations only account for 3-5% of the overweight population. In experimental animals, a high saturated FA intake rapidly induces hyperglycaemia, insulin resistance and impairs glucose uptake and metabolism in a number of tissues. Enzymes involved in glucose and lipid metabolism in the liver, such as GK, are important factors in the regulation of blood glucose levels in which perturbations may lead to the development of disease such as metabolic syndrome and type 2 diabetes. In contrast, a diet high in MUFAs or PUFAs may actually protect against the development of these disorders. However, the molecular mechanisms of FA regulation have not yet been fully elucidated.

We propose that consumption of a diet high in saturated fat results in alterations in the regulation of hepatic gene expression via a direct mechanism of FA signalling that contributes to the development of the metabolic syndrome and type 2 diabetes. Specifically, FA may be able to directly regulate metabolism in the liver by altering the rate of transcription of key target genes involved in glucose and lipid metabolism. As a specific example we propose that alterations in the regulation of hepatic GK enzyme activity may contribute to perturbations of whole body glucose homeostasis that result from consumption of a diet high in saturated fat.
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CHAPTER THREE

EXPRESSION ANALYSIS OF A HUMAN HEPATIC CELL LINE IN RESPONSE TO PALMITATE

Christopher D. Swagell\textsuperscript{a}, Debra C. Henly\textsuperscript{b,c}, C. Phillip Morris\textsuperscript{a}.

\textsuperscript{a} Cooperative Research Centre for Diagnostics, Queensland University of Technology, GPO Box 2434, Brisbane, Qld 4001, Australia.

\textsuperscript{b} School of Molecular and Microbial Sciences, University of Queensland, Brisbane, Qld 4067, Australia.

\textsuperscript{c} School of Health Sciences, Bond University, Gold Coast, Qld 4229, Australia.

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STATEMENT OF AUTHORSHIP

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Swagell, C.D. (candidate)
Involved in the conception and design of the project, generated all experimental data, interpreted and analysed all experimental data and wrote the manuscript.

Signed: ........................................ Date: .........................

Henly, D.C. (supervisor)
Involved in the conception and design of the project, assisted in the interpretation and analysis of all experimental data and critically reviewed the manuscript.

Signed: ........................................ Date: .........................

Morris, C.P. (supervisor)
Involved in the conception and design of the project, assisted in the interpretation and analysis of all experimental data and critically reviewed the manuscript.

Signed: ........................................ Date: .........................
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CHAPTER FOUR

REGULATION OF HUMAN HEPATOCYTE GENE EXPRESSION BY FATTY ACIDS

Christopher D. Swagell\textsuperscript{a}, Debra C. Henly\textsuperscript{b}, C. Phillip Morris\textsuperscript{a}.

\textsuperscript{a} Cooperative Research Centre for Diagnostics, Queensland University of Technology, GPO Box 2434, Brisbane, Qld 4001, Australia.

\textsuperscript{b} School of Health Sciences, Bond University, Gold Coast, Qld 4229, Australia.

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REGULATION OF HUMAN HEPATOCYTE GENE EXPRESSION BY FATTY ACIDS

Experimental Cell Research *(Submitted November 2006)*

Swagell, C.D. (candidate)
Involved in the conception and design of the project, generated all experimental data, interpreted and analysed all experimental data and wrote the manuscript.

Signed: ........................................ Date: .................................

Henly, D.C. (supervisor)
Involved in the conception and design of the project, assisted in the interpretation and analysis of all experimental data and critically reviewed the manuscript.

Signed: ........................................ Date: .................................

Morris, C.P. (supervisor)
Involved in the conception and design of the project, assisted in the interpretation and analysis of all experimental data and critically reviewed the manuscript.

Signed: ........................................ Date: .................................
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CHAPTER FOUR

APPENDIX 4.1
**Appendix 4.1**

**Table A4.1**  Genes up-regulated by FAs in HepG2 cells after 1.5 h of incubation

<table>
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<tr>
<th>Affymetrix Accession no.</th>
<th>Gene Description</th>
<th>Palmitate</th>
<th>Oleate</th>
<th>EPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>216153_x_at</td>
<td>Reversion-inducing-cysteine-rich protein with Kazal motifs</td>
<td>2.0</td>
<td>9.5</td>
<td>15.2</td>
</tr>
<tr>
<td>218792_s_at</td>
<td>B-box and SPRY domain containing</td>
<td>-</td>
<td>5.6</td>
<td>2.9</td>
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<tr>
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</tr>
<tr>
<td>200988_s_at</td>
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</tr>
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<td>3.8</td>
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<tr>
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<td>claudin 10</td>
<td>-</td>
<td>3.7</td>
<td>5.8</td>
</tr>
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<td>204404_at</td>
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<td>jerky homolog (mouse)</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>211456_x_at</td>
<td>MT-1H-like protein</td>
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<td>Zinc finger protein 419</td>
<td>-</td>
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*a Fold changes were determined by microarray hybridisation (n = 2 microarrays/treatment) of individual cell cultures exposed to 150 μM palmitate, 150 μM oleate and 50 μM EPA.

(-) mRNA expression profiles that were not significantly altered when compared to control incubations.
Table A4.2  Genes down-regulated by FAs in HepG2 cells after 1.5 h of incubation

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<tr>
<th>Affymetrix Accession no.</th>
<th>Gene Description</th>
<th>Palmitate</th>
<th>Oleate</th>
<th>EPA</th>
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<td>204176_s_at</td>
<td>Kelch-like 20 (drosophila)</td>
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<td>200598_s_at</td>
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### Chapter 4 – Regulation of Human Hepatocyte Gene Expression by Fatty Acids

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<td>karyopherin (importin) h3</td>
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<td>hyperparathyroidism 2 (with jaw tumor)</td>
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<td>procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), (protein disulfide isomerase; thyroid hormone binding protein p55)</td>
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<td>ATPase, H+ transporting, lysosomal accessory protein 2</td>
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<td>200751_at</td>
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<td>interleukin enhancer binding factor 2, 45kDa</td>
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<td>syndecan 2</td>
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<td>mannosyl (α-1,6)-glycoprotein β1-1,2-N-acetylgalactosaminyltransferase</td>
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<td>PLASMA GLUTATHIONE PEROXIDASE PRECURSOR (HUMAN)</td>
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<td>200782_at</td>
<td>annexin A5</td>
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*Fold changes were determined by microarray hybridisation (n = 2 microarrays/treatment) of individual cell cultures exposed to 150 μM palmitate, 150 μM oleate and 50 μM EPA.

(-) mRNA expression profiles that were not significantly altered when compared to control incubations.
### Table A4.3 Additional MatInspector Promoter Analysis for “Regulation of Human Hepatocyte Gene Expression by Fatty Acids”

MatInspector analysis of the hepatic glucokinase promoter showing the positive strand orientation. Numbering of the binding site position starts from the 5′ end and continues to the transcription start site. The Matrix Family Library Version 6.0 (November 2005) was used in this analysis.

<table>
<thead>
<tr>
<th>Family/matrix</th>
<th>Further Information</th>
<th>Position</th>
<th>Core sim.</th>
<th>Matrix sim.</th>
<th>Sequence (red: ci-value &gt; 60 capitals: core sequence)</th>
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<td>V$STAT/STAT.01</td>
<td>Signal transducers and activators of transcription</td>
<td>21 - 39</td>
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<td>0.924</td>
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<td>V$CEBP/CEBPB.01</td>
<td>CCAAT/enhancer binding protein beta</td>
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<td>V$RBPF/RBPJK.02</td>
<td>Mammalian transcriptional repressor RBP-Jkappa/CFB1</td>
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<td>Ikaros 2, potential regulator of lymphocyte differentiation</td>
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<td>1</td>
<td>0.955</td>
<td>tcagagGATGttgggs</td>
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<td>V$RXRF/VDR_RXR.02</td>
<td>Bipartite binding site of VDR/RXR heterodimers without a spacer between directly repeated motifs</td>
<td>207 - 231</td>
<td>0.823</td>
<td>0.757</td>
<td>aagagGATaaggtggtgccaac</td>
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<td>V$OCTB/TST1.01</td>
<td>POU-factor Tst-1/Oct-6</td>
<td>208 - 220</td>
<td>1</td>
<td>0.941</td>
<td>aagAGTAaaggg</td>
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<td>V$HEAT/HSF2.02</td>
<td>Heat shock factor 2</td>
<td>223 - 247</td>
<td>1</td>
<td>0.959</td>
<td>tcagacctctAGAAggtgccaga</td>
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<td>V$HEAT/HSF1.01</td>
<td>Heat shock factor 1</td>
<td>233 - 257</td>
<td>1</td>
<td>0.891</td>
<td>atagacagaggctAGAAtgccggg</td>
</tr>
<tr>
<td>V$GREF/GRE.01</td>
<td>Glucocorticoid receptor, C2C2 zinc finger protein binds glucocorticoid dependent to GREs</td>
<td>238 - 256</td>
<td>0.893</td>
<td>0.911</td>
<td>agagtcctcaaggtGCtgg</td>
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<td>V$STALE/MEIS1.01</td>
<td>Binding site for monomeric Meis1 homeodomain protein</td>
<td>261 - 271</td>
<td>1</td>
<td>0.96</td>
<td>tgcTGTCaaca</td>
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<td>V$MYBL/VMYB.02</td>
<td>v-Myb</td>
<td>274 - 286</td>
<td>1</td>
<td>0.996</td>
<td>cttAACGgcctct</td>
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<tr>
<td>V$OCT1/OCT1.05</td>
<td>Octamer-binding factor 1</td>
<td>287 - 301</td>
<td>0.95</td>
<td>0.915</td>
<td>tcCATCcttagttagg</td>
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<tr>
<td>V$SRFF/SLF.03</td>
<td>Serum response factor</td>
<td>289 - 307</td>
<td>1</td>
<td>0.845</td>
<td>cttcctacATTGGcttg</td>
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<td>V$XBBF/RFX1.01</td>
<td>X-box binding protein RFX1</td>
<td>308 - 326</td>
<td>1</td>
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<td>V$SF1F/SF1.01</td>
<td>SF1 steroidogenic factor 1</td>
<td>310 - 322</td>
<td>1</td>
<td>0.965</td>
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<td>Hmx2/Nkx5-2 homeodomain</td>
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<td>1</td>
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<td>V$PAX2/PAX2.01</td>
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<td>328 - 350</td>
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<td>V$FKHD/HFH2.01</td>
<td>HNF-3/Fkh Homolog 2 (FOXD3)</td>
<td>342 - 358</td>
<td>0.806</td>
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<td>V$SORY/GRY.01</td>
<td>Sex-determining region Y gene product</td>
<td>343 - 355</td>
<td>1.093</td>
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<td>V$CABL/CABL.01</td>
<td>Multifunctional c-Abi src type tyrosine kinase</td>
<td>344 - 354</td>
<td>1.097</td>
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<td>Hmx2/Nkx5-2 homeodomain transcription factor</td>
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<td>Nerve growth factor-induced protein C</td>
<td>380 - 396</td>
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<td>0.827</td>
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<td>V$EGRF/WT1.01</td>
<td>Wilms Tumor Suppressor</td>
<td>382 - 398</td>
<td>1.00</td>
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<td>V$EKLF/BKLF.01</td>
<td>Basic krueppel-like factor (KLF3)</td>
<td>382 - 398</td>
<td>0.964</td>
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<td>V$MAZF/MAZR.01</td>
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<td>382 - 394</td>
<td>1.00</td>
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<td>V$SP1F/TIEG.01</td>
<td>TGFbeta-inducible early gene (TIEG) / Early growth response gene alpha (EGRalpha)</td>
<td>385 - 399</td>
<td>0.915</td>
<td>ggtGGGGggtcag</td>
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<td>V$CSEN/DREAM.01</td>
<td>Downstream regulatory element-antagonist modulator, Ca2+-binding protein of the neuronal calcium sensors family that binds DRE (downstream regulatory element) sites as a tetramer</td>
<td>393 - 403</td>
<td>0.978</td>
<td>gtGTCAgggca</td>
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<td>V$OCTP/OCT1P.01</td>
<td>Octamer-binding factor 1, POU-specific domain</td>
<td>431 - 443</td>
<td>0.931</td>
<td>tgtATATgacag</td>
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<td>V$QCTP/OCT1P.01</td>
<td>Octamer-binding factor 1</td>
<td>433 - 447</td>
<td>0.771</td>
<td>taTATGcacaggtgc</td>
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<td>V$MYOD/TAL1_E2A.01</td>
<td>Complex of Lmo2 bound to Tal-1, E2A proteins, and GATA-1, half-site 1</td>
<td>436 - 452</td>
<td>0.98</td>
<td>atgcaCAGGtgccctt</td>
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<td>V$ZBP/ZNF202.01</td>
<td>Transcriptional repressor, binds to elements found predominantly in genes that participate in lipid metabolism</td>
<td>446 - 468</td>
<td>0.756</td>
<td>gcccttCCCCatctgccc cccacc</td>
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<td>V$NEUR/NEUROG.01</td>
<td>Neurogenin 1 and 3 (ngn1/3) binding sites</td>
<td>450 - 462</td>
<td>0.944</td>
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<td>V$ZBP/ZNF219.01</td>
<td>Kruppel-like zinc finger protein 219</td>
<td>453 - 475</td>
<td>0.947</td>
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<td>457 - 479</td>
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<td>460 - 482</td>
<td>0.998</td>
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<td>462 - 476</td>
<td>1.00</td>
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<td>Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers</td>
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<td>0.872</td>
<td>ccccccCGCCccccat gccatgg</td>
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<td>Muscle Initiator Sequence</td>
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<td>0.906</td>
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<td>V$PAX3/PAX3.01</td>
<td>Pax-3 paired domain protein, expressed in embryogenesis</td>
<td>565 - 583</td>
<td>0.804</td>
<td>aTCGTatcaccctccg cag</td>
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<td>Olfactory neuron-specific factor</td>
<td>584 - 606</td>
<td>0.824</td>
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<td>NF-kappaB (p50)</td>
<td>594 - 606</td>
<td>0.75</td>
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<td>V$STAF/STAF.01</td>
<td>Se-Cys tRNA gene transcription activating factor</td>
<td>600 - 622</td>
<td>0.792</td>
<td>atccCCCCActattcaca gtcctg</td>
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Chapter 4 – Regulation of Human Hepatocyte Gene Expression by Fatty Acids

119
| V$OCTP/OCT1P.01 | Octamer-binding factor 1, POU-specific domain | 605 - 617 | 0.789 | 0.874 | ccaCTATtcacaa |
| V$OAZF/ROAZ.01 | Rat C2H2 Zn finger protein involved in olfactory neuronal differentiation | 632 - 648 | 1 | 0.738 | tgGCAcagggcttct |
| V$AREB/AREB6.01 | AREB6 (Atp1a1 regulatory element binding factor 6) | 651 - 663 | 1 | 0.943 | tggccACCTgatttt |
| V$AREB/AREB6.04 | AREB6 (Atp1a1 regulatory element binding factor 6) | 655 - 667 | 1 | 0.991 | taccCTTTCgag |
| V$SP1F/GC.01 | GC box elements | 698 - 712 | 0.872 | 0.891 | tgaagGGGTGGgggtgg |
| V$AREB/AREB6.01 | AREB6 (Atp1a1 regulatory element binding factor 6) | 651 - 663 | 1 | 0.943 | taccCTTTCgag |
| V$AREB/AREB6.04 | AREB6 (Atp1a1 regulatory element binding factor 6) | 655 - 667 | 1 | 0.991 | taccCTTTCgag |
| V$SP1F/GC.01 | GC box elements | 698 - 712 | 0.872 | 0.891 | tgaagGGGTGGgggtgg |
| V$AREB/AREB6.01 | AREB6 (Atp1a1 regulatory element binding factor 6) | 651 - 663 | 1 | 0.943 | taccCTTTCgag |
| V$AREB/AREB6.04 | AREB6 (Atp1a1 regulatory element binding factor 6) | 655 - 667 | 1 | 0.991 | taccCTTTCgag |
| V$SP1F/GC.01 | GC box elements | 698 - 712 | 0.872 | 0.891 | tgaagGGGTGGgggtgg |
| V$AREB/AREB6.01 | AREB6 (Atp1a1 regulatory element binding factor 6) | 651 - 663 | 1 | 0.943 | taccCTTTCgag |
| V$AREB/AREB6.04 | AREB6 (Atp1a1 regulatory element binding factor 6) | 655 - 667 | 1 | 0.991 | taccCTTTCgag |
| V$SP1F/GC.01 | GC box elements | 698 - 712 | 0.872 | 0.891 | tgaagGGGTGGgggtgg |
| V$AREB/AREB6.01 | AREB6 (Atp1a1 regulatory element binding factor 6) | 651 - 663 | 1 | 0.943 | taccCTTTCgag |
| V$AREB/AREB6.04 | AREB6 (Atp1a1 regulatory element binding factor 6) | 655 - 667 | 1 | 0.991 | taccCTTTCgag |
| V$SP1F/GC.01 | GC box elements | 698 - 712 | 0.872 | 0.891 | tgaagGGGTGGgggtgg |
| V$AREB/AREB6.01 | AREB6 (Atp1a1 regulatory element binding factor 6) | 651 - 663 | 1 | 0.943 | taccCTTTCgag |
| V$AREB/AREB6.04 | AREB6 (Atp1a1 regulatory element binding factor 6) | 655 - 667 | 1 | 0.991 | taccCTTTCgag |
| V$SP1F/GC.01 | GC box elements | 698 - 712 | 0.872 | 0.891 | tgaagGGGTGGgggtgg |
| V$AREB/AREB6.01 | AREB6 (Atp1a1 regulatory element binding factor 6) | 651 - 663 | 1 | 0.943 | taccCTTTCgag |
| V$AREB/AREB6.04 | AREB6 (Atp1a1 regulatory element binding factor 6) | 655 - 667 | 1 | 0.991 | taccCTTTCgag |
| V$SP1F/GC.01 | GC box elements | 698 - 712 | 0.872 | 0.891 | tgaagGGGTGGgggtgg |
| V$AREB/AREB6.01 | AREB6 (Atp1a1 regulatory element binding factor 6) | 651 - 663 | 1 | 0.943 | taccCTTTCgag |
| V$AREB/AREB6.04 | AREB6 (Atp1a1 regulatory element binding factor 6) | 655 - 667 | 1 | 0.991 | taccCTTTCgag |
| V$SP1F/GC.01 | GC box elements | 698 - 712 | 0.872 | 0.891 | tgaagGGGTGGgggtgg |
| V$AREB/AREB6.01 | AREB6 (Atp1a1 regulatory element binding factor 6) | 651 - 663 | 1 | 0.943 | taccCTTTCgag |
| V$AREB/AREB6.04 | AREB6 (Atp1a1 regulatory element binding factor 6) | 655 - 667 | 1 | 0.991 | taccCTTTCgag |
| V$SP1F/GC.01 | GC box elements | 698 - 712 | 0.872 | 0.891 | tgaagGGGTGGgggtgg |
| V$AREB/AREB6.01 | AREB6 (Atp1a1 regulatory element binding factor 6) | 651 - 663 | 1 | 0.943 | taccCTTTCgag |
| V$AREB/AREB6.04 | AREB6 (Atp1a1 regulatory element binding factor 6) | 655 - 667 | 1 | 0.991 | taccCTTTCgag |
| V$SP1F/GC.01 | GC box elements | 698 - 712 | 0.872 | 0.891 | tgaagGGGTGGgggtgg |
| V$AREB/AREB6.01 | AREB6 (Atp1a1 regulatory element binding factor 6) | 651 - 663 | 1 | 0.943 | taccCTTTCgag |
| V$AREB/AREB6.04 | AREB6 (Atp1a1 regulatory element binding factor 6) | 655 - 667 | 1 | 0.991 | taccCTTTCgag |
| V$SP1F/GC.01 | GC box elements | 698 - 712 | 0.872 | 0.891 | tgaagGGGTGGgggtgg |
Chapter 5 – Effect of Fatty Acids, Glucose, and Insulin on Hepatic Glucose Uptake and Glycolysis

CHAPTER FIVE

EFFECT OF FATTY ACIDS, GLUCOSE, AND INSULIN ON HEPATIC GLUCOSE UPTAKE AND GLYCOLYSIS

Christopher D. Swagell a, C. Phillip Morris a, Debra C. Henly b,c.

a Cooperative Research Centre for Diagnostics, Queensland University of Technology, GPO Box 2434, Brisbane, Qld 4001, Australia.

b School of Molecular and Microbial Sciences, University of Queensland, Brisbane, Qld 4067, Australia.

c School of Health Sciences, Bond University, Gold Coast, Qld 4229, Australia.

Chapter 5 – Effect of Fatty Acids, Glucose, and Insulin on Hepatic Glucose Uptake and Glycolysis
STATEMENT OF AUTHORSHIP

EFFECT OF FATTY ACIDS, GLUCOSE, AND INSULIN ON HEPATIC GLUCOSE UPTAKE AND GLYCOLYSIS

*Swagell, C.D. (candidate)*
Involved in the conception and design of the project, generated all experimental data, interpreted and analysed all experimental data and wrote the manuscript.

Signed: [Signature]  
Date: 21-11-06

*Morris, C.P. (supervisor)*
Involved in the conception and design of the project, assisted in the interpretation and analysis of all experimental data and critically reviewed the manuscript.

Signed: [Signature]  
Date: 21-11-06

*Henly, D.C. (supervisor)*
Involved in the conception and design of the project, assisted in interpretation and analysis of all experimental data and critically reviewed the manuscript.

Signed: [Signature]  
Date: 21-11-06
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CHAPTER FIVE

APPENDIX 5.1
Appendix 5.1

Additional Detailed Methods for “EFFECT OF FATTY ACIDS, GLUCOSE, AND INSULIN ON HEPATIC GLUCOSE UPTAKE AND GLYCOLYSIS”

Glucokinase Enzyme Kinetic Measurements

Glucokinase Assay

To measure total GK activity, hepatocytes were washed with 150 mM NaCl and then lysed with 600 µL of 250 mM sucrose, 20 mM MOPS, 3 mM EDTA and 2 mg/mL digitonin, pH 7.4. After 8 minutes of incubation with this buffer, the cell lysate was removed and centrifuged at 14,000 g for 5 minutes.

To measure the activity of GK in the unbound, cytoplasmic fraction, cells were initially washed with 150 mM NaCl. To lyse the plasma membrane, cells were incubated with 600 µL of 300 mM sucrose, 2 mM DTT, 5 mM MgCl₂, 3 mM HEPES, 0.04 mg/mL digitonin, pH 7.2. The lysate containing the cytoplasmic fraction was removed from the well and centrifuged at 14,000 g for 5 minutes, the supernatant was removed and the activity of GK assayed. After removal of the cytoplasmic fraction from the well, 600 µL of 150 mM KCl, 3 mM HEPES, 2 mM DTT, 0.05 mg/mL digitonin, pH 7.2 was added to the well to release the non-cytoplasmic fraction. After scraping the well, the buffer containing the bound GK fraction was removed and centrifuged. The supernatant was assayed for GK activity.

\[
\text{Glucose} + \text{ATP} \xrightarrow{\text{GK}} \text{Glucose-6-phosphate} + \text{ADP}
\]

\[
\text{Glucose-6-phosphate} + \text{NAD}^{+} \xrightarrow{\text{G6PDH}} \text{6-Phosphogluconate} + \text{NADH} + \text{H}^{+}
\]

The NADH concentration formed according to the reaction above, measured by the change in absorbance at 340 nm, is proportional to the amount of glucokinase enzyme (GK – glucokinase, G6PDH – glucose-6-phosphate dehydrogenase).

The GK activity kinetic assay was performed in a 96 well plate that contained 100 µL of buffer (100 mM glucose, 100 mM TRIS, 100 mM KCl, 12 mM MgCl₂, 1 mM EDTA,
2.5 mM Dithiothreitol, 5 mM ATP, 0.5 mM NAD$^+$), pH 8.0 that was incubated at 37°C for 10 mins. Add 60 µL of cell lysate and 20 µL G6PDH (Leuconostoc mesenteroides, 1 U/L) to the preheated buffer in the 96 well plate. Assays were incubated at 37°C and measurements were taken at 340 nm for 20 mins at 5 sec intervals. Activity was expressed as mUnits/mg protein where 1 mUnit represents 1 nmol glucose phosphorylated per minute at 37°C.

The protein content of the cell fractions was measured by a Bradford assay using a BioRad (California, USA) protein assay kit.

**Purification and Neutralisation of Incubated Culture Medium**
Culture medium isolated from cell culture incubations were neutralised to precipitate unwanted cellular protein from supernatant in preparation for spectrophotometric analysis. At 48 h 1 mL of medium was removed from cell culture incubations and 1 mL of 1 M perchloric acid (PCA) was added to each sample and incubated on ice for 20 mins. Samples were centrifuged at 14 000g for 5 mins and supernatant removed without disturbing the pellet. The samples were neutralised with approximately 260 µL of MOPS solution (2 M KOH, 0.3 M MOPS, 100 mM KCL). The pH of the solution should fall with the range pH 5-5 – 7.0, if below pH 5.5 then add 10 µL of MOPS solution and measure pH. If the pH is over 7.0 then add 10 µL of PCA and pH again, continue until the pH falls within range. Incubate samples on ice for 30 mins and centrifuge at 14 000g for 3 mins. Remove supernatant and perform spectrophotometric assays or store at -20°C.

**Spectrophotometric Measurements**

**Lactate Assay**
The amount of lactate present in the culture medium was measured in a 96 well plate that contained 100 µL of buffer (100 mM TRIS, 100 mM KCL, 12 mM MgCl, 1 mM EDTA, 2.5 mM Dithiothreitol, 5 mM ATP and 0.5 mM NAD$^+$, pH 8.0) and was incubated at 37°C for 10 mins. Add 60 µL of neutralised culture medium and 20 µL lactate dehydrogenase (LDH) (27.3 kU/L) to the preheated buffer in the 96 well plate. Assays were incubated at 37°C and measurements were taken at 340 nm for 20 mins or until the readings stabilised.
Chapter 5 – Effect of Fatty Acids, Glucose, and Insulin on Hepatic Glucose Uptake and Glycolysis

L-Lactate + NAD$^+$ + Hydrazine Hydrate $\xrightarrow{\text{LDH}}$ Pyruvate Hydrazine + NADH + H$^+$

The NADH concentration formed according to the reaction above, measured by the change in absorbance at 340 nm, is proportional to the amount of L-lactate.

**Pyruvate Assay**

The amount of pyruvate present in the culture medium was measured in a 96 well plate that contained 100 µL of buffer (100 mM TRIS, 100 mM KCL, 12 mM MgCl, 1 mM EDTA, 2.5 mM Dithiothreitol, 5 mM ATP and 0.5 mM NADH, pH 8.0), and was incubated at 37°C for 10 mins. Add 60 µL of neutralised culture medium and 20 µL LDH (27.3 kU/L) to the preheated buffer in the 96 well plate. Assays were incubated at 37°C and measurements were taken at 340 nm for 20 mins or until the readings stabilised.

Pyruvate + NADH + H$^+$ $\xleftrightarrow{\text{LDH}}$ Lactate + NAD$^+$

The NADH concentration formed according to the reaction above, measured by the change in absorbance at 340 nm, is proportional to the amount of pyruvate.

**Acetoacetate Assay**

The amount of acetoacetate present in the culture medium was measured in a 96 well plate that contained 100 µL of buffer (100 mM TRIS, 100 mM KCL, 12 mM MgCl, 1 mM EDTA, 2.5 mM Dithiothreitol, 5 mM ATP and 0.5 mM NADH, pH 8.0), and was incubated at 37°C for 10 mins. Add 60 µL of neutralised culture medium and 20 µL 3-hydroxybuturate dehydrogenase (3-HBDH) (530 U/L) to the preheated buffer in the 96 well plate. Assays were incubated at 37°C and measurements were taken at 340 nm for 20 mins or until the readings stabilised.

Acetoacetate + NADH + H$^+$ $\xleftrightarrow{3\text{-HBDH}}$ D-3-Hydroxybuturate + NAD$^+$

The NADH concentration formed according to the reaction above, measured by the change in absorbance at 340 nm, is proportional to the amount of acetoacetate.
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3-Hydroxybuturate Assay
The amount of 3-hydroxybuturate present in the culture medium was measured in a 96 well plate that contained 100 µL of buffer (100 mM TRIS, 100 mM KCl, 12 mM MgCl₂, 1 mM EDTA, 2.5 mM Dithiothreitol, 5 mM ATP and 0.5 mM NAD⁺, pH 8.0), and was incubated at 37°C for 10 mins. Add 60 µL of neutralised culture medium and 20 µL 3-HBDH (710 U/L) to the preheated buffer in the 96 well plate. Assays were incubated at 37°C and measurements were taken at 340 nm for 20 mins or until the readings stabilised.

\[
\text{HBDH} \\
\text{D-3-Hydroxybuturate} + \text{NAD}^+ \leftrightarrow \text{Acetoacetate} + \text{NADH} + \text{H}^+
\]

The NADH concentration formed according to the reaction above, measured by the change in absorbance at 340 nm, is proportional to the amount of D-hydroxybuturate.

Isotopic Glucose Phosphorylation and Glycolysis Measurements
To measure rates of glucose phosphorylation and glycolysis, liver cells were cultured for 24 h with 2 nM or 10 nM insulin and 15 mM glucose in combination with the fatty acid to be tested. At the end of this period, the culture medium was replaced with fresh medium containing 15 mM [2-³H]glucose (0.75 μCi) to measure glucose phosphorylation or 15 mM [6-³H]glucose (0.75 μCi) (New England Nuclear, Boston USA) to measure glycolytic flux. The incubations were continued for a further 24 h and samples collected for analysis at the end of this time. Radiolabelled products of glucose metabolism (lactate, pyruvate, amino acids, and water) were separated from glucose by ion exchange chromatography. Rates of glucose phosphorylation were measured as ³H₂O released from [2-³H]glucose. Glycolysis was estimated from the recovery of isotope derived from [6-³H]glucose in H₂O, lactate, pyruvate and amino acids. In all experiments, there was at least 90% recovery of isotope.

Column Preparation:
For each separation three columns are required that gravity feed into one another. The top column contains H⁺ resin, the middle column contains acetate resin and the bottom column contains borate resin. The columns are plugged with dacron wool and wash...
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with 2 mL of water. Add 8 mL (equivalent to 2 cm) of the appropriate resin each column and elute with 5 mL of water, repeat three times. Join the columns in the appropriate order and wash twice with 5 mL of water.

Separation of Metabolites
Load 1 mL of supernatant onto the top column and wash sample onto the column twice with 0.5 mL water and discard eluate. Place a collection vessel under the bottom column and wash five times with 5 mL of water and collect the eluate. Mix the pooled eluate and take 1 mL for counting (This fraction contains $^3$H-water). Remove and separate the columns for individual elution of bound fractions.

Top Column:
Wash the column with 5 mL of water, repeat and discard the eluate. Place a collection vessel under the bottom column and elute with 5 mL of 2N NH$_4$OH, repeat four times and collect the eluate. Mix the pooled eluate and take 1 mL for counting (This fraction contains amino acids).

Middle Column:
Wash the column with 5 mL of water, repeat and discard the eluate. Place a collection vessel under the bottom column and elute with 5 mL of 1M acetic acid, repeat six times and collect the eluate. Mix the pooled eluate and take 1 mL for counting (This fraction contains the lactate). Place a new collection vessel under the column and elute with 5 mL 4N formic acid, repeat five times. Mix the pooled eluate and take 1 mL for counting (This fraction contains pyruvate).

Bottom Column:
Wash the column with 5 mL of water, repeat and discard the eluate. Place a collection vessel under the bottom column and elute with 5 mL of 1M acetic acid, repeat four times and collect the eluate. Mix the pooled eluate and take 1 mL for counting (This fraction contains glucose).

1 mL of samples eluted from the columns was mixed with 5 mL of Ready Safe scintillation cocktail mixture. Scintillation counting was performed on the LS 6500 Scintillation System (Beckman).
CHAPTER SIX

GENERAL DISCUSSION
6.1 General Discussion

This thesis was based on the hypothesis that diets containing high levels of saturated FA may alter hepatic gene regulation via direct mechanisms of FA signalling. It was further proposed that these alterations in gene expression may contribute to impaired glucose metabolism that ultimately results in the development of hyperglycaemia and insulin insensitivity that leads to the onset of metabolic syndrome and type 2 diabetes. The human hepatoma cell lines and isolated primary rat hepatocytes used in this study were chosen facilitate the characterisation of direct FA signalling from secondary, endocrine or long-term signalling events seen in whole animal studies.

This thesis aimed to define the molecular mechanisms of FA signalling that are involved in hepatic gene regulation and nutrient metabolism. The evidence obtained from this study contributes to the current literature and identifies a potential role for saturated FA in the development of metabolic syndrome and cardiovascular disease (CVD). Specifically, this thesis demonstrates that saturated FA can reduce hepatic glucokinase (GK) promoter activity and GK enzyme activity in addition to regulating transcription factors that influence GK gene transcription that may perturb glucose metabolism. In addition, this work identified for the first time that saturated FA altered the expression of genes that have previously been shown to be associated with metabolic disease states, including metabolic syndrome, CVD and type 2 diabetes.

6.2 Palmitate Differentially Regulates Hepatic Gene Expression

Metabolic syndrome and CVD are characterised by a number of risk factors that are associated with the development disease. Work reported in this thesis provides convincing evidence that the saturated FA, palmitate, contributes to the development of these risk factors. These include alterations in expression of genes involved in lipoprotein metabolism, oxidative stress response, cholesterol synthesis and fibrinolysis. Specifically, genes altered by palmitate that are associated with increased risk include, apolipoprotein-B100 (apo-B100), apo-CIII, plasminogen activator inhibitor-1 (PAI-1), insulin-like growth factor-I (IGF-I) and insulin-like growth factor binding protein 3 (IGFBP3).

Insights into the mechanisms by which a high saturated fat diet may induce macrovascular disease, such as atherosclerosis, are also provided in this thesis.
Namely:

1. an increase of apo-B100 gene expression may indicate an increased production of apo-B100-containing atherogenic lipoprotein particles.
2. the observed increase in the expression of the apo-AII gene may reduce reverse cholesterol efflux to the liver and facilitate cholesterol deposition in peripheral tissues.
3. increases in cholesterologenic gene expression may indicate an increase in cholesterol production.
4. the observed increase in the level of apo-CIII gene expression may reduce triglyceride (TG) catabolism and promote increased levels of hepatic TG secretion.
5. the induction of oxidative stress response genes may indicate an increase in oxidative stress and/or potential depletion of the antioxidant, glutathione.

6.3 Palmitate Increases the Production of Atherogenic Lipoproteins

This thesis has demonstrated that hepatic apo-B100 mRNA expression was up-regulated in response to high levels of palmitate (Figure 6.1). This data suggest that palmitate may promote a state of increased atherogenic lipoprotein particle production that has previously been shown in the literature to be associated with an increased risk of developing CHD [1]. In addition, it has been identified that increased apo-B100 levels are associated with an increased risk of obesity, hypertension and type 2 diabetes in adolescents [2] and is strongly associated with CHD in men aged 45-76 years [3]. Therefore, the evidence from this thesis may further implicate the role of palmitate as a predisposing environmental factor that contributes to the development of these metabolic disease states. Furthermore, as only one apo-B100 molecule is present on each lipoprotein particle [4], it can be used to predict the total number of circulating atherogenic plasma lipoprotein particles present in an individual. This observation provides the potential of using circulating apo-B100 levels as an indicator of a high dietary intake of saturated FA that may be used to predict the risk of metabolic disease development in healthy individuals.
Figure 6.1 – Diagrammatical representation of saturated FA regulation of gene expression. Genes in green are up-regulated and genes in red are down-regulated. Abbreviations: TG – triglyceride, C – cholesterol, NEFA – non-esterified fatty acid, apo – apolipoprotein, HL – hepatic lipase, LPL – lipoprotein lipase, ADRP – adipose differentiation-related protein, FDPS – farnesyl pyrophosphate synthetase, FDFT1 – farnesyl-diphosphate farnesyl transferase, VLDL – very low-density lipoprotein, sVLDL – small very low-density lipoprotein, LDL – low-density lipoprotein, sLDL – small low-density lipoprotein, IDL – intermediate-density lipoprotein, HDL – high-density lipoprotein, HDLR – high-density lipoprotein receptor.

This thesis has also provided evidence that palmitate exposure results in increased hepatic apo-AII and ABCA1 gene expression (Figure 6.1). Previously, it has been shown that transgenic mice over-expressing apo-AII exhibit a decreased capacity to promote in vitro cellular cholesterol efflux [5]. This suggests the possibility that palmitate may reduce the anti-atherogenic effects exhibited by HDLs and result in excess cholesterol accumulation in peripheral tissues. In addition to a possible
reduction in reverse cholesterol transport, this thesis identifies that palmitate increases the expression of cholesterologenic genes, including farnesyl pyrophosphate synthetase and farnesyl-diphosphate farnesyl transferase (Figure 6.1). This may indicate increased production of cholesterol that ultimately leads to a raised level of cholesterol-rich circulating atherogenic lipoprotein particles. This suggests that palmitate exposure may reduce HDL mediated reverse cholesterol transport from peripheral tissues to the liver, increase cholesterol synthesis and potentially promote cholesterol deposition and accumulation in vascular tissue, thereby increasing the risk of developing atherosclerosis.

6.5 Palmitate Increases Apo-CIII Gene Expression
Evidence generated from this thesis suggests that a diet high in saturated FA may contribute to increased hepatic TG secretion by raising apo-CIII levels. Plasma apo-CIII has been recognised as a useful marker that is associated with TG-rich lipoproteins and is an indicator of coronary disease risk in healthy individuals [6; 7]. Plasma apo-CIII is associated with apo-B100-containing and apo-AI-containing lipoproteins and has the ability to exchange between TG-rich lipoproteins and HDLs. In healthy individuals, the majority of apo-CIII is bound to HDL, whereas in hypertriglyceridaemic individuals, apo-CIII is mainly bound to TG-rich particles [8]. Hypertriglyceridaemia is characterised by increased levels of apo-CIII containing VLDLs which play a crucial role in the identification of atherosclerosis risk and development of CHD [9].

In addition, elevated plasma apo-CIII levels have been identified in diabetic subjects [10] and have been shown to delay the apo-E-dependent uptake and metabolism of TG-rich particles by the liver. This is due to the suppression of lipoprotein lipase and/or hepatic lipase activity and a reduced binding of TG-rich particles and their remnants to the LDL receptor [11; 12]. Recently, it has been demonstrated that overweight or insulin insensitive individuals also have increased levels of VLDL-apo-CIII and this is linked to an increased production of TG-rich VLDLs [13].

6.6 Palmitate Exposure Alters Gene Expression that Depletes Antioxidant Availability and Raises Oxidative Stress Levels
In this thesis we demonstrate that palmitate exposure alters the expression of oxidative stress response genes that could potentially result in the depletion of glutathione (GSH),
a potent antioxidant. Increased expression of selenoprotein P, phospholipid hydroperoxide glutathione peroxidase, glutathione S-transferase A2, glutathione S-transferase P and multi-drug resistance protein suggests that hepatic oxidative stress response has increased in response to palmitate. In addition, a number of genes that are reported to induce glutamate cysteine ligase expression were down-regulated, including activator-1 and nuclear factor kappa-B. Glutamate cysteine ligase is responsible for the production of GSH, and therefore decreased glutamate cysteine ligase expression may result in reduced GSH production. This data suggests that a possible depletion of GSH, combined with a decrease in GSH production, could lead to an increased level of free radical formation and oxidative stress exposure in the liver.

Increased free radical production coupled with GSH depletion may promote increased oxidation of lingering TG and cholesterol-rich LDLs in the circulation. Oxidised-LDLs exhibit an impaired ability to dock to LDL-receptors lengthening the time that oxidised-LDLs remain in the circulation. When combined with a reduced ability to facilitate reverse cholesterol efflux, this may increase cholesterol deposition within vascular tissue and contribute to the development of atherosclerosis and CVD. The regulation of genes identified in this thesis may explain the connection between a high saturated FA intake and the progression from hypertriglyceridaemia to the development of atherosclerosis and CVD.

6.7 Palmitate Decreases IGF-I and IGFBP3 Expression

The insulin-like growth factor (IGF) system plays an important role in the control of cellular growth, metabolism and survival. Biological increases in the availability of IGF activity can predispose to certain cancers, while a deficiency appears to increase the risk of diabetes and CHD [14]. Circulating IGFs are highly regulated by specific IGFBPs. Of these, IGFBP-3 accounts for more than 90% of circulating IGF activity. In addition, when IGFs are bound to IGFBPs the half-life of bound IGF is greatly enhanced.

Recently, it has been shown that individuals with obesity, dyslipidaemia, hypertension and insulin resistance have significantly lower levels of IGF-I [15]. Furthermore, circulating IGF-I levels are shown to decrease with increasing blood glucose concentrations and with the duration of diabetes [16]. IGF-I gene knockout animals display hyperinsulinaemia and skeletal muscle insulin resistance [17]. Additionally, a
polymorphism in the IGF-I gene promoter has been shown to be associated with an increased risk of type 2 diabetes and CVD [18; 19; 20]. Changes in IGF-I regulation have been linked to a reduction in insulin sensitivity, which has been shown to precede the development of type 2 diabetes [14]. In this thesis, we have shown that palmitate incubation decreased IGF-I and IGFBP3 gene expression.

6.8 Palmitate Alters Expression of Fibrinolytic Genes
A further complication of diabetes is nephropathy, which is characterised by excessive accumulation of extracellular matrix (ECM) in the kidney. The regulatory process of ECM synthesis and degradation is controlled by the plasminogen activator (PA)/plasmin/PAI system. This system involves the conversion of plasminogen to plasmin that is catalysed by PA which is under the regulation of PAI-1. Increased levels of PAI-1 reduce PA activity, which results in increased ECM deposition that is a characteristic of pathological conditions associated with renal fibrosis and excessive ECM accumulation in the kidney, such as diabetic nephropathy [21]. In addition, hydrogen peroxide has been shown to up-regulate PAI-1 mRNA and protein expression and the basal expression of PAI-1 is increased upon depletion of cellular GSH [22]. In this thesis, we observed an increase in the hepatic gene expression level of PAI-1 upon palmitate incubation. This may indicate an effect of saturated FA on hepatic ECM remodelling that promotes ECM accumulation similar to that observed in pathological conditions, such as diabetic nephropathy. If our observation of changes in expression of PAI-1 in response to saturated fat translates into increased PAI-1 levels in blood, a diet high in saturated fat may induce a prothrombotic state that could contribute to the development of atherosclerotic plaque formation.

6.9 MUFAs and PUFAs are Potent Regulators of Gene Expression
Epidemiological studies have shown an inverse correlation with intake of monounsaturated FAs (MUFAs) and polyunsaturated FAs (PUFAs) and the development of CVD such as atherosclerosis [23; 24]. Recently, it has been shown that the degree of unsaturation of dietary FAs can affect plasma lipoprotein composition as well as the expression of adhesion molecules and other pro-inflammatory factors that influence the thrombogenicity that is associated with atherosclerosis development. Evidence suggests that both MUFAs and PUFAs appear to reduce total and LDL-cholesterol compared with saturated FAs [25; 26] and protect against atherosclerosis.
[24]. In contrast, a high intake of saturated FA is strongly correlated with death from CHD [27].

Evidence from this thesis has shown that MUFAs and PUFAs may promote the expression of a panel of genes that would be protective against the development of metabolic syndrome and atherosclerotic risk if expressed in vivo. These include a reduction in the mRNA expression of squalene epoxidase by both MUFAs and PUFAs and decreased PAI-1 binding protein mRNA expression. Both of these changes would be expected to potentially reduce cholesterol synthesis and the further risk of thrombosis. This is in contrast to longer-term palmitate incubations that showed increased expression levels of the cholesterolgenic genes farnesyl pyrophosphate synthetase and farnesyl-diphosphate farnesyl transferase.

6.10 Palmitate Reduces Glucokinase Promoter and Enzyme Activity

In this thesis we proposed that alterations in the regulation of hepatic glucokinase (GK) activity contribute to perturbation of whole body glucose homeostasis as a result of the consumption of a diet high in saturated fat. Chapter four of this thesis identified that palmitate exposure can alter GK gene regulation and promoter activity that may be implicated in the dys-regulation of glucose homeostasis. Insulin increases the expression of sterol regulatory element binding protein-1c (SREBP-1c) which is known to induce hepatic GK gene expression [28]. In contrast to previous studies [29], we observed a modest reduction in SREBP-1c mRNA levels upon palmitate incubation. Furthermore, palmitate was capable of causing a significant reduction in hepatic GK promoter activity. Therefore, this study has identified that palmitate can reduce GK promoter activity through the reduction in SREBP-1c gene expression in vitro. If this translates into similar changes in vivo, saturated fat consumption would result in reduced GK enzyme production and reduced uptake and glucose metabolism in the liver.

6.11 Palmitate Failed to Reduce Glucose Phosphorylation and Glycolytic Flux in the Presence of Insulin

This study demonstrated that in the presence of insulin, palmitate exposure significantly reduced total GK enzyme activity [30]. However, the rates of glucose phosphorylation and glycolytic flux remained unchanged upon palmitate exposure. In addition,
investigations into the distribution of free GK and bound GK indicated that there was no evidence that the interaction between GK and glucokinase regulatory protein was altered in response to incubations with palmitate. Furthermore, in our experimental conditions, we identified an excess of residual unbound GK enzyme that may explain the failure of palmitate to significantly reduce the rate of glucose phosphorylation and glycolytic flux, despite showing a significant reduction in GK enzyme activity.

This observation of reduced hepatic GK promoter and enzyme activity, due to palmitate incubation, may bring to light an underlying factor of hyperglycaemia. For example, in insulin responsive individuals, insulin action may provide a sufficient level of GK gene expression that compensates for saturated FA-induced inhibition of GK expression and enzyme activity. However, in insulin-resistant individuals, saturated FA-induced inhibition may be sufficient to reduce normal glucose metabolism. Circulating free FAs are elevated in insulin-resistant states and have been suggested to contribute to insulin resistance by reducing glucose transport or inhibiting glucose phosphorylation [31]. Therefore, high levels of prolonged saturated FA exposure in combination with the onset of insulin resistance may promote the dys-regulation of glucose phosphorylation, inhibiting the flux through glycolysis leading to the development of hyperglycaemia and onset of type 2 diabetes.

6.12 Implications of this thesis
Previously, human studies have attempted to evaluate the relationship between total fat intake and obesity, metabolic syndrome and type 2 diabetes. This thesis supports the hypothesis that a consumption of a diet high in saturated fat is associated with the development of these conditions. In addition, this thesis contributes to the current literature defining the potential molecular mechanisms of saturated FA signalling in the liver that may contribute to the development of metabolic syndrome and type 2 diabetes.

The treatment of metabolic syndrome and type 2 diabetes aims to improve insulin sensitivity and to prevent the associated metabolic and cardiovascular abnormalities that are characteristic of these disease states. Since many individuals with metabolic syndrome are overweight, dietary modification should be primarily focused on weight reduction. Evidence generated for this thesis provides support that dietary modification for the treatment of metabolic syndrome should incorporate a limited intake of saturated
fat. In addition, a moderately increased consumption of MUFA and PUFA should be encouraged as they do not induce similar detrimental metabolic effects when compared to saturated FA.

This thesis also identifies genes that could be used as markers of increased risk of developing metabolic syndrome and type 2 diabetes as a result of high saturated fat consumption. These gene markers could be used as potential targets to identify those who are at risk of developing metabolic disease and could also be used to monitor the effectiveness of modified diets, such as a reduced saturated fat intake, and lifestyle changes. This thesis supports the established observation that saturated fat plays a major role in the development of metabolic syndrome, type 2 diabetes and macrovascular complications, including atherosclerosis and CVD.

6.13 Future Directions
The data that has been generated from this thesis has provided evidence that FAs can directly regulate hepatic gene expression that has the potential to translate into alterations of metabolic pathway flux, especially glucose metabolism. Therefore, the further characterisation of saturated FA signalling of GK promoter activity would target the site-directed mutagenesis of the SREBP-1c regulatory element (SRE) core nucleotide sequence. This would identify the contribution of the reduction of GK promoter activity that is due to saturated FA regulation of SREBP-1c. Further investigations into the remaining 10% reduction of promoter activity would involve the generation of smaller deletion constructs of the pGK3-GK_D vector to localise the regulatory promoter region. This would be followed by site-directed mutagenesis of potential regulatory elements to identify novel transcription factor binding sites that are involved in saturated FA regulation of SREBP-1c.

Further observations from time-course q-PCR analysis data from this thesis have also provided evidence that FAs are able to directly regulate hepatic gene expression of a number of transcription factors. This data showed that palmitate did not alter hepatic gene expression at 1.5 hours but was sufficient to directly regulate hepatic gene expression at 48 hours. These observations indicate that palmitate may require longer incubation periods to induce changes in gene regulation and suggest that the subsequent time periods used in this study were not sufficient to detect significant changes in
metabolic gene expression that could translate into perturbations of metabolic pathway flux and glucose metabolism. Therefore, time-course analysis should be performed to investigate longer time periods of 72 and 96 hours up to and including one week. To follow on from this point, the observation that EPA and oleate could significantly alter hepatic gene expression after the short-term 1.5 hour incubation suggests an increased ability to directly regulate hepatic mRNA expression levels. Therefore, a comprehensive comparison of the direct signalling effects of saturated, MUFA and PUFA at a longer incubation period should also be performed to allow a comparison of expression patterns that may be distinctly regulated by these individual FAs.

Finally, the investigations of the effects of FAs signalling and translation into metabolic effects in the absence of insulin and/or insulin-resistant conditions should be further explored. It is possible that FA induced reduction of glucose metabolism may only become apparent after the onset of insulin resistance. Therefore, measurements of the rate of glucose phosphorylation and glycolysis could be performed on primary hepatocytes isolated from insulin receptor knockout mice or in the absence of insulin. These results could be compared to insulin responsive hepatocytes in an attempt to differentiate the effect of FA signalling observed within these two independent states.

6.14 Major Findings of this Thesis

1. The saturated FA, palmitate, initiated a transcriptional response of hepatic gene expression in the Huh-7 cell line after 48 hours of incubation. A total of 162 genes displayed altered gene expression upon palmitate exposure.

2. A number of markers of risk that are associated with metabolic syndrome, CVD and type 2 diabetes were differentially regulated upon palmitate exposure. These genes include apo-B100, apo-CIII, PAI-1, IGF-I and IGFBP3.

3. An increased level of mRNA expression of genes involved in oxidative stress response was observed upon palmitate exposure.

4. Expression analysis of the hepatic GK promoter identified that palmitate incubation resulted in reduced promoter activity after 48 hours.

5. Incubation periods of 48 hours resulted in palmitate regulating the expression of hepatic transcription factors involved in the regulation of
glycolysis and FA oxidation. Palmitate incubation resulted in the reduction of SREBP-1c mRNA levels while increasing PPAR mRNA levels.

6. A short-term incubation period of 1.5 hours with palmitate failed to induce significant changes in the regulation of hepatic gene expression.

7. Short-term incubation periods of 1.5 hours with the MUFA, oleate and PUFA, EPA, initiated similar patterns of changes in hepatic gene regulation.

8. GK enzyme activity was reduced by palmitate and EPA incubations after 48 hours in primary rat hepatocyte.

9. Incubation of palmitate, oleate, linolenate, EPA and DHA failed to affect the interaction and distribution of GK and GKRP.

10. Incubation of primary rat hepatocytes with palmitate, oleate, linolenate, EPA and DHA failed to reduce glucose phosphorylation, glycolytic flux and β-oxidation in the presence of insulin.
Chapter 6 – General Discussion

Bibliography


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