ROLE OF STEAROYL-CoA DESATURASE1 IN FATTY ACID-INDUCED INSULIN RESISTANCE

A Thesis Presented in Total Fulfilment of the Degree of Master of Applied Science

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AUGUST, 2006
PUBLICATIONS

Papers arising from the studies presented in this thesis.


DECLARATION

I, the candidate, Srijan Kumar Pinnamaneni, certify that:

a) Except where acknowledgement has been made, the work submitted is of the candidate alone.

b) The work has not been submitted previously, in whole or in part, to qualify for any other academic award.

c) The content of the thesis is the result of the work which has been carried out since the official commencement date of the approved research program.

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ACKNOWLEDGEMENTS

In this masters project I received help from different people and all of them deserve their share of gratitude from my side.

First and foremost I would like to thank my supervisor, Dr. Matthew Watt, for his guidance and encouragement in every aspect of this project. Along with the science, he taught me so many other things which are necessary for my scientific career and life. Without him, I would never have finished my project.

I would like to express my gratitude to Prof. Mark Febbraio, my second supervisor for providing support, financial aid and opportunity to work in his laboratory.

Thanks to Dr. Jong Sam Lee for providing muscle samples for my first study, Dr. Allan Tall, for the plasmid pcDNA3.1/Hygro-mSCD1, an essential component for the overexpression studies and Dr. Ntambi and his group for giving the necessary information during these studies.

I would like to acknowledge the help of Robert Southgate for plasmid preparation and his timely help during this project and Dr. Andrew Carey for teaching me parts of PCR technique.

Thanks to Nadine Watson for her patience to teach me cell culture methods and her timely observation in the laboratory.

Thanks to Mark’s post doctoral researchers Beata Skiba, Nadine Watson and Graeme Lancaster for their help in the laboratory at various steps of this project.

Special thanks to my fellow students Hayley Nicholls, Sarah Turpin, Anna Holmes, Ming Hang Stanley, Michelle Hage, Jason Chung, Joo Lee Cham and Murali for creating healthy environment for working and learning.
Very special thanks to Hema Raina for her help at various steps of my masters project.

I am grateful to my parents and especially my brother Krishna for their love and support.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-DG</td>
<td>2-deoxy glucose</td>
</tr>
<tr>
<td>αMEM</td>
<td>alpha modified eagle’s medium</td>
</tr>
<tr>
<td>ACS</td>
<td>acyl-CoA synthase</td>
</tr>
<tr>
<td>Akt</td>
<td>acute transforming retrovirus thymoma</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary deoxy ribonucleic acid</td>
</tr>
<tr>
<td>C_T</td>
<td>critical threshold</td>
</tr>
<tr>
<td>CON</td>
<td>control</td>
</tr>
<tr>
<td>CoA</td>
<td>Co-enzyme A</td>
</tr>
<tr>
<td>CPT</td>
<td>carnitine palmitoyl transferase</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DGAT</td>
<td>diacylglycerol acyl transferase</td>
</tr>
<tr>
<td>DM</td>
<td>dry matter</td>
</tr>
<tr>
<td>DMEM</td>
<td>dulbecco’s modified eagle media</td>
</tr>
<tr>
<td>FAT/CD36</td>
<td>fatty acid translocase</td>
</tr>
<tr>
<td>FATP</td>
<td>fatty acid transport protein</td>
</tr>
<tr>
<td>FABP&lt;sub&gt;c&lt;/sub&gt;</td>
<td>cytoplasmic fatty acid transport protein</td>
</tr>
<tr>
<td>FABP&lt;sub&gt;pm&lt;/sub&gt;</td>
<td>plasma membrane bound fatty acid binding protein</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>GLUT</td>
<td>glucose transporter protein</td>
</tr>
<tr>
<td>GPAT</td>
<td>glycerol-3-phosphate acyltransferase</td>
</tr>
<tr>
<td>HSL</td>
<td>hormone sensitive lipase</td>
</tr>
<tr>
<td>HOMA</td>
<td>homeostatic model assessment</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>IMTG</td>
<td>intramyocellular triglyceride</td>
</tr>
<tr>
<td>IKKβ</td>
<td>inhibitor of kappa B kinase β</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N terminal kinase</td>
</tr>
<tr>
<td>LCFA</td>
<td>long chain fatty acids</td>
</tr>
<tr>
<td>LCFA-CoA</td>
<td>long chain fatty acyl-CoA</td>
</tr>
<tr>
<td>LPAAT</td>
<td>lysophosphatidic acid acyl transferase</td>
</tr>
<tr>
<td>LXR</td>
<td>liver X receptor</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa beta</td>
</tr>
<tr>
<td>OGTT</td>
<td>oral glucose tolerance test</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acids</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferation activated receptor</td>
</tr>
<tr>
<td>PPH-1</td>
<td>phosphatidate phosphohydrolase</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>real time polymerase chain reaction</td>
</tr>
<tr>
<td>SAT</td>
<td>saturated fatty acids</td>
</tr>
<tr>
<td>SCD1</td>
<td>stearoyl CoA desaturase 1</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering ribonucleic acid</td>
</tr>
<tr>
<td>SREBP</td>
<td>sterol regulatory element binding protein</td>
</tr>
<tr>
<td>TAG</td>
<td>triacylglycerol</td>
</tr>
<tr>
<td>TBST</td>
<td>tris buffered saline tween-20</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
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ABSTRACT

Recent investigations suggest that reducing stearoyl CoA desaturase (SCD) 1 expression confers protection against obesity and insulin resistance, whereas others show that increasing SCD1 expression protects cells from lipotoxicity. The overall aim of this thesis was to establish the role of SCD1 expression in fatty acid metabolism and insulin stimulated glucose disposal in skeletal muscle.

In vitro and in vivo studies were conducted to investigate the relationship between fatty acid subtype, SCD1 expression and fuel metabolism. The role of fatty acid subtype on fatty acid metabolite accumulation and insulin resistance was initially examined in rats. Rats were provided with a low fat diet or a high fat diet consisting of predominantly saturated (SAT) or polyunsaturated fatty acids (PUFA). Rats fed a SAT diet were insulin resistant and had increased skeletal muscle diacylglycerol content whereas rats fed a PUFA diet retained insulin sensitivity and accumulated triacylglycerol rather than diacylglycerol. Interestingly, SCD1 mRNA and protein content were elevated in SAT rats compared with PUFA fed and control fed rats, indicating a possible involvement of SCD1 in the aetiology of insulin resistance. Subsequently, SCD1 expression was examined in the skeletal muscle of various rodent models of genetic and diet-induced obesity. SCD1 content was consistently upregulated in the skeletal muscle of obese rodents.

To determine whether SCD1 contributes to or protects from fatty-acid induced insulin resistance, SCD1 levels were transiently altered in L6 skeletal muscle myotubes. Short interfering (si) RNA was used to decrease SCD1 content and a pcDNA3.1/Hygro-mSCD1 vector was introduced to increase SCD1 content. Reducing SCD1 protein
resulted in marked esterification of exogenous fatty acids into diacylglycerol and ceramide. Insulin-stimulated Akt (acute transforming retrovirus thymoma) phosphorylation and 2-deoxyglucose uptake were reduced with SCD1 siRNA. Exposure of L6 myotubes to palmitate abolished insulin-stimulated glucose uptake in both control and SCD1 siRNA myotubes. Transient overexpression of SCD1 resulted in triacylglycerol esterification but attenuated ceramide and diacylglycerol accumulation and protected myotubes from fatty acid-induced insulin resistance. Further, these changes were associated with reduced phosphorylation of e-Jun Amino-Terminal Kinase (JNK) and the inhibitor of IκB kinase (IKK), both of which impair insulin signalling. These studies indicated that SCD1 protects from cellular toxicity in L6 myotubes by preventing excessive accumulation of bioactive lipid metabolites. Collectively, these experiments indicate that increasing SCD1 expression may be a protective mechanism designed to prevent insulin resistance in obese phenotypes.
CHAPTER 1

LITERATURE REVIEW
1.1 Skeletal muscle fatty acid metabolism

1.1.1 Introduction

Skeletal muscles utilise carbohydrates, fatty acids, ketone bodies and amino acids as the metabolic substrates to meet the body’s energy requirements. In non-contracting muscle cells glucose and fatty acids are the chief oxidative substrates, contributing ~10% and 90% respectively, (Dagenais et al., 1976) of the total adenosine triphosphate (ATP) turnover. Long chain fatty acids (LCFA) are the primary lipid energy source for skeletal muscle and are stored as triacylglycerol (TAG) within lipid droplets that are located in adipose tissue and, to a lesser extent, within skeletal muscle (Terjung & Kaciuba-Uscilko, 1986; Gorski, 1992). The LCFAs are released from TAGs after hydrolysis by numerous lipases including hormone sensitive lipase (HSL) and adipose triglyceride lipase (Holm et al., 2000; Zimmermann et al., 2004), complexed to albumin and enter the circulation for eventual uptake by peripheral tissues such as skeletal muscle. LCFAs are also available to the skeletal muscle as very low density lipoproteins and as TAGs in the core of circulating lipoproteins or chylomicrons; however, the contribution of these LCFAs to ATP production is considered negligible (Sorrentino et al., 1988).

1.1.2 Long chain fatty acid uptake in skeletal muscle

De novo fatty acid synthesis is limited in muscle fibers (Saggerson et al., 1992; Gaster et al., 2005) and intramyocellular triacylglycerol (IMTG) is stored in relatively small quantities (~20 mmol/kg dm) (Watt et al., 2003). Therefore, the bulk of fatty acids required for energy production are derived from extracellular sources (Saggerson et al., 1992). Fatty acids are released from TAG stores in the adipose tissue by the action of HSL and adipose triglyceride lipase (Holm et al., 2000; Zimmermann et al., 2004).
After fatty acids are released from adipose tissue, they form a complex with albumin because fatty acids are insoluble in blood.

Thus, >99% of the fatty acids carried in plasma are bound to albumin (Arici et al., 2002). The fatty acid-albumin complex is transported in the circulation for uptake by peripheral tissues, such as skeletal muscle. Previously, it was assumed that fatty acid uptake in muscle tissue occurred via passive diffusion; however, recent investigations confirmed that fatty acid transport is a highly regulated process involving numerous regulatable proteins (Bonen et al., 2003).

### Passive diffusion

The plasma fatty acid concentration in blood to cytoplasm differs between 200 and 600 µmol/L and thereby creates a concentration gradient, which acts as driving force behind skeletal muscle fatty acid extraction (Van der Vusse & Roemen, 1995). The sarcolemma is composed of two phospholipid membranes. Absorption of fatty acids to the outer layer of the plasma membrane creates a higher concentration of free fatty acid, which facilitates fatty acid flux to the inner membrane. The difference in the ionization constants ($pK_a$) results in protonation (ionization) of fatty acid inside the plasma membrane (Hamilton et al., 1994). It is believed that the transfer of fatty acids occurs via a flip-flop mechanism in which ionized fatty acids of one leaflet are exchanged with unionized fatty acids of another leaflet (Gutnecht, 1988; Schmider et al., 2000) indicating the fatty acid affinity towards ionization constant. Once they come in the proximity of inner sarcolemmal membrane the concentration of cytoplasmic fatty acid binding proteins (FABP<sub>c</sub>) facilitate the fatty acid to undergo desorption to cross the membrane and bind to it to reach intracellular sites for utilisation (Hamilton, 1998).
Although passive diffusion contributes to sarcolemmal uptake of fatty acids, it is now clear that protein-mediated uptake is also involved in this process.

**Protein-mediated uptake**

Protein mediated transport involves various membrane associated proteins including the 40 kDa plasma membrane bound fatty acid binding protein (FABP$_{pm}$), the 53 kDa native, non-glycosylated fatty acid translocase (FAT/CD36, the rat analogue of human CD36) and the 63 kDa fatty acid transport protein (FATP) 1-5. Studies in cell culture demonstrate that each of these proteins can independently increase fatty acid transport when overexpressed (Bonen *et al.*, 2002).

In mammals, the bulk of fatty acids dissociate from albumin upon entry to the interstitial space and bind with FABP$_{pm}$, which facilitates transport of fatty acid across the plasma membrane (Van der Vusse *et al.*, 1998). FAT/CD36 is present at the plasma membrane and also appears to be translocated from an intracellular location to the plasma membrane in response to insulin and contractions (Bonen *et al.*, 2003). FAT/CD36 transports fatty acids across the lipid bilayer of the plasma membrane (Abumrad *et al.*, 1993; Koonen *et al.*, 2005) and it appears as though FAT/CD36 and FABP$_{pm}$ may act in concert to facilitate this process; however, the molecular mechanisms are unresolved (Bonen *et al.*, 2003). There is also evidence that FAT/CD36 is located in subsarcolemmal and intermyofibril fractions of highly purified mitochondria, and functional assessment suggests that FAT/CD36 may translocate to the mitochondria and facilitate fatty acid oxidation (Campbell *et al.*, 2004). The FABP$_c$ (Bass, 1988; Glatz *et al.*, 1988; Veerkamp & van Moerkerk, 1993), binds to the fatty acid and activate it to form fatty acyl-CoA in presence of acyl-CoA synthase (ACS). This process facilitates the binding of fatty acyl-CoA to acyl-CoA binding protein,
which can transport the fatty acyl-CoA to various cellular locations before dissociation and entry of fatty acids into metabolic pathways including oxidation and esterification. FATP is an integrated bifunctional membrane protein, which is involved with fatty acid transport and possesses catalytic characteristics to convert the fatty acids into fatty acyl-CoA (Coe et al., 1999; Herrmann et al., 2001). FATP co-expression along with FAT/CD36 suggests its involvement with FAT/CD36 could contribute fatty acid uptake in muscles (Brinkmann et al., 2002; Gimeno et al., 2003). The activation of fatty acids into acyl-CoA by the intrinsic ACS of FATP is still under investigation (Hall et al., 2003).
Figure 1.1 Schematic representation of fatty acid transport in skeletal muscle across endothelium and plasma membrane. Fatty acid (FA), plasma membrane fatty acid binding protein (FABP<sub>PM</sub>), fatty acid translocase (FAT/CD36), fatty acid transport protein (FATP), acyl-CoA synthase (ACS), cytosolic fatty acid binding protein (FABP<sub>C</sub>), acyl CoA binding protein (ACBP), fatty acyl-CoA (FACoA).
1.1.3 Fatty acid oxidation

Upon entry to the cytoplasm, LCFAs are directed to oxidation for the production of ATP, or to storage as intracellular signalling molecules, phospholipids and triglycerides. The LCFAs entering the cytoplasm are coupled with coenzyme A in the presence of ACS to form fatty acyl-CoA. ACS appears to play an important role in regulating fatty acids into oxidative and synthetic pathways as suggested by its cellular location on the outer mitochondrial surface and plasma membrane, and proximity to proteins involved with esterification (Iijima et al., 1996).

Fatty acyl-CoAs are oxidized to acetyl-CoA in the mitochondria. Although short-chain fatty acids (C4-C10) can diffuse through the mitochondrial membrane, long LCFA-CoAs require a carrier to cross the mitochondrial membranes. To overcome the mitochondrial membrane barrier, the long chain fatty acyl-CoA esters are adapted to a carnitine-dependent shuttle mechanism (Bremer, 1983). Carnitine palmitoyl transferase (CPT)1 is an enzyme which is present in outer mitochondrial membrane and is considered a rate-limiting step to LCFA-CoA flux into the mitochondria (McGarry & Brown, 1997). CPT1 is situated on the inner surface of the outer mitochondrial membrane and converts acyl-CoA (long chain fatty acid) to acylcarnitine. Acylcarnitine is then transported across the inner mitochondrial membrane by carnitine-acylcarnitine translocase in exchange for carnitine. After reaching the mitochondrial matrix, acylcarnitine is exchanged for free carnitine in the presence of CPT2 to release free fatty acyl-CoA into the mitochondrial matrix. Along with the CPT system, a recent investigation has suggested that FAT/CD36 can transport fatty acids across the mitochondrial membranes (Campbell et al., 2004) and thereby act as an alternate pathway to transfer LCFAs into the mitochondria.
Inside, the mitochondria fatty acyl-CoA undergoes β-oxidation, which consists of a series of dehydrogenation, hydration, oxidation, and thiolysis reactions to ultimately form acetyl-CoA. The dehydrogenation reaction catalyses the conversion of fatty acyl-CoA to enoyl-CoA by acyl-CoA dehydrogenase. Enoyl-CoA is then hydrolysed by enoyl-CoA hydratase to 3-L-hydroxyacyl-CoA, which undergoes an oxidation reaction catalysed by 3-L-hydroxyacyl-CoA dehydrogenase to form β-ketoacyl-CoA. In the final step β-ketoacyl-CoA thiolase catalyzes the β-ketoacyl-CoA to form acetyl-CoA. The acetyl-CoA can then enter the Krebs cycle to produce ATP.

### 1.1.4 Fatty acid esterification

TAGs are the main storage forms of energy in muscle cells. Excess fatty acids are stored as TAGs in the form of lipid droplets in cells. Fatty acyl-CoAs in the cytoplasm serve as the major precursor to TAG synthesis, which results from a series of reactions described as the Kennedy pathway. The two major enzymes involved in this process include glycerol-3-phosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT). Excess fatty acyl-CoAs are esterified to glycerol-3-phosphate by GPAT to form lysophosphatidate. GPAT activity is increased by insulin (Saggerson et al., 1992), thyroid hormone (Baht & Saggerson, 1988) and transcriptionally activated by sterol regulatory elementary binding proteins-1 (SREBP-1) (Brown & Goldstein, 1997). Conversely, GPAT activity is inhibited by glucagon via increasing intracellular cAMP levels (Sul et al., 2000).

Lysophosphatidate is catalysed by lysophosphatidic acid acyl transferase (LPAAT) to form phosphatidate, which acts as precursor in the synthesis of TAG along with phospholipids. It is hydrolysed by phosphatidate phosphohydrolase (PPH-1) to form diacylglycerol (DAG). PPH-1 is also upregulated by insulin (Saggerson et al., 1992)
and thyroid hormone (Baht & Saggerson, 1988), while glucagon counter-regulates these other hormonal actions. DGAT then acylates DAG to form TAG and is considered a key regulator of TAG biosynthesis. Like other enzymes in the Kennedy pathway, DGAT is hormonally regulated, thyroid hormone and insulin increase DGAT activity and glucagon antagonises this effect (Coleman et al., 2000).
Figure 1.2 Schematic representation of TAG esterification. Lysophosphatidic acid acyl transferase (LPAAT), phosphatidate phosphohydrolase (PPH-1), glycerol-3-phosphate acyltransferase (GPAT), diacylglycerol acyltransferase (DGAT), acyl CoA synthase (ACS), diacylglycerol (DAG), triacylglycerol (TAG).
1.1.5 Triacylglycerol hydrolysis

TAG is a complex energy fuel stored primarily in adipose tissue, and to a lesser extent in skeletal muscle. In adipose tissue, TAGs are hydrolysed by numerous lipases including adipose triglyceride lipase, HSL and monoglyceride lipase (Holm et al., 2000; Zimmermann et al., 2004). In skeletal muscle, TAGs and DAGs are known to be hydrolysed by HSL, and studies using neutralising antibodies suggest the existence of other neutral lipases that are important for basal hydrolysis (Langfort et al., 2000; Watt & Spriet, 2004; Watt et al., 2004). HSL is controlled by β-adrenergic and contraction related mechanisms by phosphorylation at various serine residues (Langfort et al., 2000; Watt et al., 2005; Watt et al., 2006b). The fatty acids produced in this reaction are utilised for cell functions, including oxidation and re-esterification, and the liberated glycerol is transferred to liver for glucose synthesis or subjected to TAG resynthesis.
Figure 1.3 Schematic representation free fatty acid release during triacylglycerol hydrolysis. Adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), monoglyceride lipase (MGL), triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MG) and fatty acid (FA).
1.2. Fatty acid metabolites and insulin resistance

1.2.1 Introduction

Skeletal muscle is quantitatively the most important tissue for insulin and non-insulin stimulated glucose uptake, contributing between 75-95% of total uptake (Baron et al., 1988a). Insulin facilitates glucose uptake and other metabolic activities in tissues and the reduced ability of these activities lead to insulin resistance. Insulin resistance is a condition in which the cells of the body become resistant to the effects of insulin, that is, the normal response to a given amount of insulin is reduced. As a result, higher levels of insulin are needed in order for insulin to induce its effects. Various factors are attributed to the aetiology of insulin resistance. The close association of lipid oversupply and insulin resistance is well established in various tissues and have been reviewed previously (Griffin et al., 1999; Boden et al., 2001; McGarry, 2002). In cases where free fatty acid availability is increased, fatty acid metabolites including DAGs, ceramides and LCFA-CoA are proposed to reduce insulin sensitivity by interfering with various aspects of the insulin signalling cascade. Insulin resistance is the common preceeding feature of type 2 diabetes.

1.2.2 Insulin signal transduction

Insulin induces the transport of glucose via activation of glucose transporter proteins (GLUT). They are transmembrane proteins categorised as GLUT1 to GLUT 12 (Linden et al., 2006) and differ from each other in terms of tissue distribution, biochemical properties, regulatory mechanisms and physiological actions (Bell et al., 1990). Skeletal muscle is considered as the major site for blood glucose regulation by insulin. Among different glucose transporter isoforms, GLUT1 and GLUT4 are the isoforms
seen in skeletal muscle but GLUT4 is expressed in major portion and plays a significant role in insulin signalling. It commences with binding of insulin to its receptor. The insulin receptor consists of two extracellular alpha (α) and transmembrane beta (β) subunits (Muller-Wieland et al., 1993) with intrinsic tyrosine kinase activity. Insulin’s binding to the α subunit induces autophosphorylation of the β-subunit on tyrosine residues resulting in the activation of the receptor. The insulin receptor substrate, a member of protein family that appears in six different isoforms (Huang et al., 2005), then associates with the insulin receptor. Insulin receptor substrate 1 (IRS-1) is believed to be involved considerably in the regulation of glucose homeostasis in skeletal muscle and IRS-2 as an alternative (White & Kahn, 1994; Cai et al., 2003; Huang et al., 2005). This event induces the signal downstream via activation of phosphotidylinositol-3-kinase that binds to IRS-1 through its p85 subunit. This event phosphorylates the secondary messengers, phosphatidylinositol 3, 4-biphosphate and phosphatidylinositol 3, 4, 5-triphosphate (PIP₃) then activates phosphoinositol-dependent kinase-1 (PDK-1), which subsequently phosphorylates protein kinase B (PKB, also known as Akt) (Burgering & Coffer, 1995) and the atypical protein kinase C (aPKC) isoformes (aPKC ζ, ι) (Good et al. 1998). Akt along with atypical PKCs, zeta (ζ) and lambda (ι), are involved in the translocation of GLUT4 to the plasma membrane (Schmitz-Peiffer, 2000). Limited evidence suggests that other PKC isoformes contribute to GLUT4 translocation (Braiman et al., 1999; Cooper et al., 1999). Akt is also involved in glycogen synthesis by inactivating and phosphorylating glycogen synthase kinase-3, which inhibits the phosphorylation of glycogen synthase, thereby enhancing glycogen synthesis from intracellular glucose.
Figure 1.4 Schematic representation of intracellular proteins involved with binding to insulin receptors during GLUT4 translocation across the cell membrane. Phosphotidylinositol-3-kinase (PI3-K), phosphatidylinositol (3,4,5), triphosphate (PIP 3,4,5), protein kinase B (PKB or Akt), phosphatidylinositol (4,5)biphosphate (PIP 4,5), atypical protein kinase C (a PKC), phosphoinositide-dependent protein kinase-1 (PDK-1), Insulin receptor substrate1 (IRS-1).
1.2.3 Fatty acid metabolism in obesity and insulin resistance

Fatty acid metabolism is regulated by genetic, nutritional and hormonal factors (Lewis et al., 2002). Fatty acid metabolism in skeletal muscle is an essential factor that contributes to insulin resistance and obesity. Disturbances in skeletal muscle fatty acid metabolism include defects in skeletal muscle fatty acid transport (Kelley et al., 1999; Bonen et al., 2004) oxidation (Kelley et al., 1999) and increased TAG storage (Bonen et al., 2004). Generally in muscle, the delivery of free fatty acids is balanced by esterification into TAGs and other intracellular lipid pools and oxidation. One disruption in obese and diabetic conditions is an increase in circulating plasma fatty acids, which most likely reflects impaired lipolytic pathways in adipose tissue (Large & Arner, 1998). It appears that the insulin resistance commonly observed in these individuals diminishes the inhibitory effects of insulin on HSL, which in turn leads to increased lipolysis and plasma free fatty acid levels (Gelding et al., 1995; Eriksson et al., 1999). Skeletal muscle fatty acid transport is another other factor involved in the dysregulation of fatty acid metabolism. LCFA protein transporters are now considered important in explaining the aberrations of fatty acid transport in obese and insulin resistance conditions. It appears that FABP<sub>pm</sub> function is not defective in obesity, whereas FAT/CD36 is associated with an increased transport rate and fatty acid esterification in obese subjects compared to controls (Bonen et al., 2004). They also revealed that the expression of fatty acid transporters is unchanged; however, there is more FAT/CD36 at the plasma membrane consistent with increased fatty acid uptake (Bonen et al., 2004). Furthermore, the increased LCFA transport rate with diminished fatty acid oxidation causes TAG accumulation in skeletal muscle (Bonen et al., 2004).

Obesity is also characterised by a decreased capacity for mitochondrial fatty acid oxidation. Lower carnitine palmitoyl transferase (CPT) activity and oxidative enzyme
activity is reported in obesity versus lean control subjects (Simoneau et al., 1999) which was consistent with reduced tracer determined rates of fatty acid oxidation (Kelley et al., 1999). In a recent study by Kim and co-authors, defective CPT activity contributed to reduced oxidation in obese skeletal muscle (Kim et al., 2000). Collectively, these abnormalities in fatty acid metabolism leads to excessive storage of intramyocellular triglyceride (IMTG) accumulation, and other fatty acid metabolites, which leads to insulin resistance (Phillips et al., 1996; Pan et al., 1997; Watt et al., 2006a).

1.2.4 Mechanisms of fatty acid induced insulin resistance

It is well established that the accumulation of intracellular lipids is associated with insulin resistance (McGarry, 2002). Although it was long thought that TAG caused insulin resistance (Storlien et al., 1991; Pan et al., 1997; Krssak et al., 1999), this seems unlikely because TAG is sometimes elevated in instances where skeletal muscle is actually insulin sensitive (Bruce et al., 2003). It is now apparent that the generation of lipid intermediates such as LCFA-CoA, DAGs and ceramides are more likely to mediate these defects in insulin signalling. Indeed, these metabolites are often elevated in the skeletal muscle of obese and individuals with type 2 diabetes.

**Long chain fatty acyl- CoA**

LCFA-CoAs are a major link between increased lipid availability and impaired lipid metabolism. As discussed, these are the activated forms of intracellular free fatty acids that are converted in the presence of ACS into LCFA-CoA. The increased LCFA-CoA pool may interfere with insulin signalling via activating PKC theta(θ) activity (Orellana et al., 1990; Kasahara & Kikkawa, 1995), which is proposed to inhibit insulin signal transduction via serine phosphorylation at 1101. This limits tyrosine phosphorylation and subsequent downstream signalling. Alternatively, LCFA-CoA can be esterified into
Diacylglycerol

DAGs are elevated in the skeletal muscle of obese, insulin resistant phenotypes (Schmitz-Peiffer et al., 1997). Elevated DAG levels are thought to interfere with insulin signalling by activation of PKC (θ, δ, β2) isoforms (Schmitz-Peiffer, 2002). PKC β1 and β2 inhibit the tyrosine kinase activity of the insulin receptor (Bosshmaier et al., 1997) and other studies in skeletal muscle showed that the activated PKC, in presence of DAGs, induces phosphorylation on serine and threonine residues of the insulin receptor, which results in diminished tyrosine kinase activity and insulin resistance (Itani et al., 2000). The role of PKC in lipid induced insulin resistance is further confirmed by reduced insulin stimulated phosphatidylinositol-3-kinase activity (Griffin et al., 1999). The decrease in phosphatidylinositol-3-kinase activity was hypothesised to be mediated by phosphorylation on IRS-1 Ser 307, and subsequent decreases in IRS-1 tyrosine kinase activity (Yu et al., 2002). However, PKC theta (θ) is not likely to directly phosphorylate IRS-1 at Ser307 because it is not a proline-directed kinase. Instead, it is possible that PKCs may activate JNK and IKKβ, known kinases for Ser 307. This would be consistent with studies demonstrating that fat-induced insulin resistance is blocked in JNK1 and IKKβ knock-out mice (Yuan et al., 2001; Hirosumi et al., 2002). Others have demonstrated that PKC θ phosphorylates IRS-1 at
serine 1101 blocking IRS-1 tyrosine phosphorylation and downstream activation of the Akt pathway (Yu et al., 2002; Li et al., 2004).

**Ceramide**

Ceramides are generated either by *de novo* synthesis from serine and palmitoyl-CoA (Merrill & Jones, 1990) or hydrolysis of sphingomyelin in the presence of sphingomyelinase. Ceramides are elevated in the skeletal muscle of insulin resistant rodents (Turinsky et al., 1990; Lessard et al., 2004) and humans (Adams et al., 2004; Straczkowski et al., 2004). Ceramides interfere with insulin signalling at the level of Akt. Ceramides dephosphorylate Akt at the catalytically important Ser 473 site via activation of ceramide activated protein phosphatase, which is a member of protein phosphatase family (Begum et al., 1996). Ceramide also exerts a negative effect on Akt through the activation of the PKC ζ (Andjelkovic et al., 1996), which is thought to prevent translocation of Akt to the plasma membrane secondary to Akt Ser 32 phosphorylation (Powell et al., 2004). Others studies have suggested that ceramides can interfere with signalling by inhibiting tyrosine phosphorylation of IRS-1 (Summers & Nelson, 2005), although further work is required to confirm these events. Irrespective, fatty acid exposure results in ceramide accumulation, which inhibits insulin stimulated glucose uptake, GLUT4 translocation (Summers et al., 1998) and glycogen synthesis in muscle cells (Hajduch et al., 2001), thus demonstrating a causative role in insulin resistance.

**Serine/Threonine kinases are involved with fat-induced insulin resistance**

c-Jun Amino-Terminal Kinase (JNK) is activated in presence of free fatty acids and inflammatory cytokines and are elevated in obesity and type 2 diabetes (Hotamisligil et al., 1996; Griffin et al., 1999). JNK phosphorylates IRS-1 at Ser 307 (Aguirre et al.,...
2000) and the absence of JNK significantly improves insulin sensitivity and enhances insulin receptor signalling capacity in obese rodent models (Hirosumi et al., 2002). IκB Kinase β (IKKβ) is a serine kinase identified as a proximal element of the pro-inflammatory IKK/IκB/NFκB pathway. Studies in obese and insulin resistant mice demonstrate increased liver IKKβ activity (Cai et al., 2005), which is likely to result in phosphorylation on IRS-1 Ser307. Studies in mice with targeted disruption of liver IKKβ show that IKKβ acts as negative regulator in insulin signalling by reducing the association of IRS-1 with p85 subunit of phosphoinositol-3-kinase and Akt activation (Arkan et al., 2005; Cai et al., 2005). Others showed that inhibition of IKK activity by administration of salicylates prevents acute fat-induced insulin resistance in rodents and improves insulin sensitivity in type 2 diabetes patients (Kim et al., 2001b; Hundal et al., 2002). More recently, studies in L6 myotubes extend on these observations and demonstrate that NFκB activation, which is downstream of IKK, is an important mechanism of lipid-induced insulin resistance (Sinha et al., 2004). Thus, activation of the NFκB pathway by fatty acids can induce insulin resistance.

1.2.5 Acute fatty acid treatment and insulin resistance

In the past few years, much research has focused on establishing the interaction between lipid oversupply and insulin resistance. Lipid oversupply is defined as elevated plasma free fatty acids, which is often observed in obese and insulin resistant patients (Boden & Chen, 1995). Randle and co workers predicted a long time ago that elevated glucose stimulates insulin secretion and consequently controls the availability of FFA release from tissue depots to avoid the competition for substrate utilization in muscle. When plasma FFA levels are elevated, fatty acid oxidation is also increased resulting in reduced glucose uptake and oxidation. Thus, excess fatty acids may induce insulin resistance by its inhibitory action on pyruvate dehydrogenase, which further inhibits a
series of enzymes involved in glucose uptake and ultimately leading to feedback inhibition and reduced glucose uptake (Randle et al., 1963; Randle et al., 1964). Recent investigations have addressed the issue of lipid oversupply by acutely increasing plasma free fatty acids (i.e: to 1.5 to 2 mM) by infusing a TAG emulsion with heparin for several hours. Using this technique Roden et al., (Roden et al., 1996; Roden et al., 1999) demonstrated that lipid oversupply reduced insulin mediated glucose transport /phosphorylation in skeletal muscle of healthy humans. It is apparent that the increased plasma fatty acid concentration observed using this technique is associated with the accumulation of several fatty acyl-CoA metabolites including ceramide (Watt et al., 2005; Watt et al., 2006b), LCFA-CoA and DAGs (Yu et al., 2002). This is associated with IRS-1 serine 307 phosphorylation and reduced glucose transport. Furthermore, the defective insulin action could not be reversed despite the fact that cells were exposed to pharmacological insulin concentrations, thus demonstrating that lipid oversupply is a powerful mediator of insulin resistance (Yu et al., 2002).
Figure 1.5 Schematic representation of possible mechanisms of intramuscular lipid metabolite interference of insulin signalling. Fatty acid (FA), fatty acyl-CoA (FA-CoA), diacylglycerol (DAG), triacylglycerol (TAG), carnitine palmitoyl transferase (CPT), insulin receptor substrate (IRS-1), phosphatidylinositol-3-kinase (PI-3-K), phosphatidylinositol-dependent kinase (PDK), protein kinase B (akt/PKB), novel protein kinase C (nPKC). LCFA are incorporated into ceramide and DAG, which directly inhibit insulin signal transduction at Akt and IRS-1 respectively.
1.2.6 High fat diet induced insulin resistance

There is now excellent evidence to show that chronic high fat feeding induces insulin resistance. Various genetic and nutritional factors are believed to contribute to insulin resistance. Among the nutritional factors, high fat diet and its widespread availability plays the major role in inducing insulin resistance. Various studies have suggested an inverse relationship between fat content and insulin sensitivity in various models (Krissak et al., 1999; Boden et al., 2001; Kim et al., 2001a). Rodents are used as the common model of research to investigate the relationship between high fat feeding and insulin resistance. Most diet studies have found that high fat feeding results in defective insulin signalling (Hansen et al., 1998; Anai et al., 1999; Kim et al., 2004a; Herr et al., 2005; Gao et al., 2006). In insulin signal transduction, IRS1 and IRS2 mediate PI3-Kinase activation which plays an important role in GLUT4 translocation and glycogen synthesis. A study conducted by Anai and his group demonstrated that high fat feeding in rodents impairs PI 3-Kinase activation in muscle and adipose tissue (Anai et al., 1999). According to this study, high fat diets result in decreased phosphorylation of IRS1 and IRS2, which further decreased IRS association with PI 3-Kinase (Anai et al., 1999). These findings are further confirmed by another study as high fat diet impairs stimulation of glucose transport in muscle (Hansen et al., 1998). It was explained in this study that a high fat diet leads to decreased insulin-stimulated tyrosine phosphorylation in muscle and defective insulin receptor signalling with impaired GLUT4 translocation, which ultimately results in impaired glucose transport. Another important aspect in insulin resistance is activation of PKC isoforms in response to fatty acid derivatives and DAG. This activation results in inhibition of insulin signalling by inducing serine phosphorylation of IRS1, which inhibits tyrosine phosphorylation of IRS1 and downregulates the activation of PI3-Kinase (Kim et al., 2001b; Gao et al., 2006). These above studies indicate that fatty acid metabolites may interfere with
insulin signalling to induce insulin resistance. In a recent study the relationship between intramuscular fat content and insulin resistance was investigated (Kim et al., 2004b). According to this study, consumption of a high fat diet induces the expression of FATP, which leads to elevated fatty acid uptake into cells for accumulation of fatty acid metabolites and induce insulin resistance.

It also appears that the type of dietary fatty acid is equally important in this process. A classic study by Storlein and colleagues showed that rats fed saturated and unsaturated diets become profoundly insulin resistant, whereas substituting a small fraction of the diet with fish oil largely prevents this effect (Storlien et al., 1991). The variable effects of high fat diets has gained recent attention because the consumption of different fat content is thought to produce different fatty acid metabolites, some of which are linked to insulin resistance. A recent study in skeletal muscle cells showed that accumulation of different fatty acids may alter insulin function in skeletal muscle in vitro (Thompson & Cooney, 2000). According to these authors, skeletal muscle cells treated with similar concentration of palmitate, oleate, linoleate and $\alpha$-linolenate showed varied effect on glucose transport and phosphorylation. These fatty acids contributed to the accumulation of LCFA-CoA and other fatty acid metabolites, which interact with glucose transport and phosphorylation and decrease the ability of insulin to induce glycogen synthesis in vitro. Other studies in cell culture revealed that exposure to saturated fat resulted in the accumulation of DAGs and TAGs, whereas unsaturated fat accumulates as intracellular free fatty acids (Gaster et al., 2005). Others have reported that a SAT diet or treating cells with saturated fats is involved in the accumulation of fatty acid metabolites including DAGs and ceramides, and interferes in insulin stimulated glucose uptake and thereby causing insulin resistance (Maron et al., 1991; Parker et al., 1993; Montell et al., 2001). In contrast, PUFA results in the accumulation
of IMTGs and free fatty acids and serve a protective function from fatty acid induced lipotoxicity by promoting TAG accumulation (Montell et al., 2001; Chavez & Summers, 2003; Gaster et al., 2005). However, a study in rats showed that a diet high in polyunsaturated fat reduced TAG levels and improved insulin sensitivity (Jucker et al., 1999). These past investigations strongly suggest that a SAT diet is worse than a PUFA diet in causing insulin resistance; however the exact mechanisms behind this are not clear.

1.3 Stearoyl-CoA Desaturase (SCD) 1

1.3.1 Regulation of SCD1

SCD1 is a 37 kDa protein with a half life of 3-4 hrs (Heinemann & Ozols, 1998) and is expressed as different isoforms depending upon the mammalian species. SCD1 is expressed in the liver, adipose tissue (Ntambi et al., 1988), skeletal muscle, brain, kidney, spleen, heart, lung, skin (Kaestner et al., 1989), sebocytes and hair follicles (Zheng et al., 1999). SCD1 expression is low in skeletal muscle compared to adipose tissue and remains consistent with its low lipogenic activity. SCD1 expression is regulated by various dietary, hormonal and environmental factors. Dietary factors including a high carbohydrate diet (Ntambi et al., 1988; Ntambi, 1992), high plasma glucose (Waters & Ntambi, 1994), fructose (Jones et al., 1998) and cholesterol (Kim et al., 2002), and hormonal factors such as insulin (Waters & Ntambi, 1994), growth hormone (Beswick & Kennelly, 2000) and androsterone (Imai et al., 2001) induce SCD1 expression. Recently, the anti-diabetic drugs PPARα (Peroxisome proliferators activated receptors α) and LXR (Liver X receptors) agonists have been shown to increase SCD1 expression (Miller & Ntambi, 1996). In contrast, it appears that PUFAs (Waters & Ntambi, 1994, 1996), conjugated linoleic acid (Lee et al., 1998), thyroid
hormone (Waters et al., 1997), leptin (Cohen et al., 2002), glucagon (Lefevre et al., 1999), tumor necrosis factor-α (Weiner et al., 1991) and sulphur substituted fatty acids (Hovik et al., 1997) inhibit SCD1 gene expression. It is now clear that these dietary and hormonal factors influence SCD1 transcription via induction of a sterol regulatory element binding protein (SREBP) dependent mechanism (Kersten, 2001; Shimano, 2001). It is also believed that SCD1 is not controlled via post-transformational modification, although further studies are required to confirm this.

1.3.2 Role of sterol regulatory element binding protein (SREBP) 1c in SCD1 expression

SREBP plays an important role in regulating the expression of SCD1. SREBPs act as lipid sensors that undergo a proteolytic process that serves as a transcription factor for fatty acid synthetic genes including SCD1 (Heinemann & Ozols, 1998). SREBPs belong to the group of transcription factors that are attached to endoplasmic reticulum (ER) as inactive precursors. Each precursor is constructed with about 1000 amino acids residues capable of three different domain settings to serve as transcription factors. Three SREBP isoforms have been identified in the mammalian genome and include SREBP-1a, SREBP-1c and SREBP-2. SREBP-1c is a specific transcription factor of SCD1 and many studies in the liver have shown that SREBP-1c plays a critical role in SCD1 transcription (Shimomura et al., 1998; Foretz et al., 1999). Indeed, SREBP-1c deletion resulted in diminished expression of the whole class of lipogenic genes, including SCD1 (Shimano et al., 1997) whereas LXR ablation resulted in reduced expression of SREBP-1, SCD1 and fatty acid synthase suggesting its prominent role in lipogenesis (Joseph et al., 2002).
1.3.3 Functional role of SCD1 in cell function

SCD1 is an endoplasmic reticulum bound lipogenic enzyme that catalyses the conversion of saturated fats (palmitoyl and stearoyl-CoA) to monounsaturated fats (palmitoleoyl and oleoyl-CoA) (Ntambi & Miyazaki, 2004). Almost all the work investigating SCD1 function has been conducted in liver and adipose tissue. The monounsaturated fats acts as substrates for the synthesis of triglycerides, wax esters, cholesteryl esters and membrane phospholipids. The ratio of saturated to monounsaturated fats contributes to the membrane phospholipid composition and aberrations in the ratio lead to various disease states including diabetes, obesity, cardiovascular disease, neurological disease, skin disorders and cancer. Recent studies have provided much evidence regarding the physiological role of SCD1 by examining the SCD1 mutation (Zheng et al., 1999) and targeted disrupted models (SCD1\textsuperscript{-/-}) in mice (Miyazaki et al., 2001). In these models, mutations revealed that SCD1 is essential in \textit{de novo} synthesis of different lipids irrespective of the presence of other lipogenic enzymes such as DGAT and ACAT. SCD1 activity is found to be elevated in adipose tissues of obese rodents (Cohen et al., 2002); especially in leptin deficient obese mouse models (Cohen et al., 2002). Thus the SCD1 expression has physiological significance in normal and disease conditions.
Figure 1.6 Schematic representation of biochemical pathway of fatty acid metabolism involving stearoyl CoA desaturase 1. Fatty acid synthase (FAS), stearoyl CoA desaturase1 (SCD1), diacylglycerol (DAG).
1.3.4 Role of skeletal muscle SCD1 in metabolism

SCD1 activity in skeletal muscle is known to be positively correlated with body weight of human subjects (Jones et al., 1996). A recent study by Hulver et al. (Hulver et al., 2005) also demonstrated that SCD1 activity is upregulated in obese subjects, and also reported increased production of MUFAs and reduced fatty acid oxidation in primary myotubes, indicating that SCD1 expression in obese conditions contributes to abnormal lipid metabolism. Similarly, in mice, SCD1 deficiency in skeletal muscle reduced ceramide synthesis via reduced activation of serine palmitoyl transferase and decreasing its substrate by CPT1-induced increase of β-oxidation (Dobrzyn et al., 2005).

Another approach used to investigate SCD1 function is via the use of SCD1 specific antisense oligonucleotide inhibitors (Jiang et al., 2005a). Antisense oligonucleotide (ASO) treatment resulted in diminished de novo fatty acid synthesis, lipogenic gene expression and increased expression of genes involved with energy expenditure in adipose tissue. Whether these treatments have effects in skeletal muscle were not reported. Other factors to influence skeletal muscle SCD1 expression include exercise, which increased skeletal muscle SCD1 expression (Ikeda et al., 2002). The role of dietary fatty acids on SCD1 expression and activity in skeletal muscle is unknown. Most of the studies investigating the functional importance of SCD1 have utilised a recently developed murine knockout (-/-) model. In a series of investigations, Ntambi and co-workers assessed different lipogenic and fatty acid β-oxidation gene expression in SCD1 (-/-) and SCD1 (+/-) models. SCD1-/- models showed that lipogenic genes including SREBP-1, GPAT, and FAS are downregulated whereas, lipid oxidation genes including PPARα, ACO, CPT-1, VLCAD are upregulated (Ntambi et al., 2002a) in liver.
1.3.5 Role of SCD1 in insulin resistance

The weight of experimental evidence suggests that SCD1 contributes to the pathogenesis of obesity and insulin resistance. SCD1 content is elevated in the livers of insulin resistant \textit{ob/ob} and lipoatrophic mice (Cohen \textit{et al.}, 2002; Asilmaz \textit{et al.}, 2004) and in animals rendered insulin resistant by high-fat feeding (Biddinger \textit{et al.}, 2005). Moreover, SCD1 null (ab\textsuperscript{j}/ab\textsuperscript{j}, SCD1\textsuperscript{-/-}) mice (Cohen \textit{et al.}, 2002; Ntambi \textit{et al.}, 2002a) and mice treated with SCD1 specific antisense oligonucleotide inhibitors (Jiang \textit{et al.}, 2005a) exhibit a leaner phenotype and resistance to diet-induced obesity. SCD1 deficiency was associated with improved insulin signalling by activating insulin receptor tyrosine phosphorylation and down regulating the activity of protein-tyrosine phosphatase-1B, which is known to attenuate insulin signalling (Rahman \textit{et al.}, 2003). However, the interpretation of SCD1 function in SCD1 knockout mice is confused by the findings of increased energy expenditure (Ntambi \textit{et al.}, 2002a; Jiang \textit{et al.}, 2005a) and basal thermogenesis (Lee \textit{et al.}, 2004b), and adaptive alterations in metabolic genes. In a recent study (Voss \textit{et al.}, 2005), overexpression of SCD1 in myotubes resulted in reduced insulin stimulated glucose uptake; however, upon closer inspection it appears as though the authors conclusions were incorrect. In actually, untreated cells became less responsive to insulin after palmitate exposure whereas SCD1 overexpressing cells showed no such decrease. This suggests that SCD1 overexpression was in fact protective. Thus, the role of SCD1 in skeletal muscle fatty acid metabolism and insulin resistance are poorly described and provides the impetus for the studies completed in this thesis.
1.4 Aims of thesis

The primary aims of the thesis were:

(1) To examine the effects of different high fat diets on insulin sensitivity, skeletal muscle lipid metabolite accumulation, insulin resistance and SCD1 expression.

(2) To determine the role of SCD1 on substrate metabolism in fatty acid-induced insulin resistance through transient knockdown and overexpression in L6 myotubes.
CHAPTER 2
GENERAL METHODS
2.1 Animal experiments

Twenty four male Sprague-Dawley rats (initial body mass 95-110 g) were obtained from Samtako (Bio Korea, Kyoung-Ki, Korea) and housed four per cage in an environmentally controlled laboratory (temperature 22±1°C, and relative humidity, 55±2%) with a 12-hour light-dark cycle (light, 0700 to 1900). In the week before any experimental intervention, animals were given ad libitum access to standard rodent chow (Samtako, Bio Korea, Kyoung-Ki, Korea). Food intake was not determined in this study because the PUFA diet was too ‘liquid’ to obtain accurate measures of the food remaining in cages. The Animal Ethics Committee of Korea National Sport University approved all procedures performed in this study.

After one week, rats were divided into one of three experimental diet groups. Control rats (CON; n=8) were fed standard rat chow (61.8% carbohydrate, 15.7% fat, 22.5% protein expressed as % of total caloric intake). The other groups consumed high fat diets (24.3% carbohydrate; 52.8% fat; 22.9% protein) that consisted primarily of saturated fatty acids (SAT; n=8) derived from lard and coconut oil or polyunsaturated fatty acids (PUFA; n=8) derived from safflower oil (ICN Biomedicals, CA, USA). The PUFA diet consisted of linoleic acid (18:2; n-6). Animals, regardless of different dietary conditions, were provided with food and water ad libitum throughout the experimental period. Animals were maintained on their diets for eight weeks prior to testing.
2.1.1 Table of diet composition and energy contents of the diets

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<th>Ingredient</th>
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</tr>
<tr>
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<tr>
<td>Starch (from rice) [CHO]</td>
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</tr>
<tr>
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<tr>
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<tr>
<td>Olive Oil [FAT; MUFA]</td>
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<td>Safflower Oil [FAT; PFA]</td>
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Kcal/100g (% Kcal)

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<th>SAT      (100.0)</th>
<th>PUFA     (100.0)</th>
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<tr>
<td>PROTEIN</td>
<td>76.0 (22.5)</td>
<td>117.2 (22.9)</td>
<td>117.2 (22.9)</td>
</tr>
</tbody>
</table>

Values expressed as g/100g of diet. Numbers in parenthesis represent the percent of total energy content within the respective diet.
2.1.2 Oral glucose tolerance test

Following the six week experimental period, rats were overnight fasted, weighed and underwent an oral glucose tolerance test (OGTT). Glucose (20% w/v solution) was administered by oral gavage at a dose of 2.0 g/kg body mass, and blood was obtained from a tail vein prior to glucose administration and at 15, 30, 60 and 120 min thereafter. At least 2 days after OGTT and after an overnight fast (10 h), rats were anaesthetised by intraperitoneal injection of sodium pentobarbital (60 mg/kg body mass) and the vastus lateralis muscle was rapidly dissected out, immediately frozen in liquid nitrogen and stored at -80°C until analyses. A blood sample was drawn from the femoral artery and rats were sacrificed by heart removal.

2.2 Cell culture

L6 cells were cultured following aseptic techniques in 75cm² flasks containing growth media, which consisted of αMEM supplemented with 10% FBS, 1% penicillin/streptomycin (v:v). The media was replaced daily with pre-warmed media (37°C) until the cells were 60–70% confluent. Cells were washed twice with pre-warmed PBS and treated with 4 ml of 0.25% trypsin for 4 min with shaking each minute. Once the cells were completely detached the total number of cells were counted then seeded into 6 well plates at 7000 cells per cm². Cells were grown to 60% confluency before transfection.

2.2.1 Short interfering RNA (siRNA)

RNA interference (RNAi) is a process in which the introduction of double-stranded RNA (dsRNA) into the target cell degrades the complementary mRNA and suppresses the expression of the target protein (Caplen, 2004). The RNAi synthesis is a multistep
process. In this process, the RNAase III family member (e.g., Dicer in Drosophila) recognizes dsRNA and cleaved it into 21-23 nucleotides of siRNA (Agrawal et al., 2003). These siRNAs are integrated into the RNA Induced Silencing Complex (RISC). This RISC complex is aimed to the target complementary RNA species which is cleaved and results in reduced protein expression.

After reaching 60% confluence, L6 myoblasts were washed with warm PBS and trypsinised with 300µl of 0.25% trypsin for 4 min at 37°C with shaking each minute. Cells were allowed to reattach with the addition of 2 ml growth media. After 16-20 h cells were washed with warmed PBS and 1ml DMEM (without serum and antibiotics) was added to each well.

Two siRNA sequences of rat SCD1 were tested for their capacity to decrease SCD1 protein levels in L6 myotubes. Gene silencing was achieved with equal efficacy with the following pairs of 21 bp oligonucleotide sequences (accession number NM139192, siRNA ID 58064 and siRNA ID 57973, Ambion, Austin, Texas, USA). The oligonucleotide pairs were annealed and reconstituted in RNAse-free water. A negative control siRNA was also purchased (catalogue number 4613) to account for off-target effects. For transfection, the cell culture media was changed to DMEM and 50 pmol of the respective oligonucleotide sequence was added with Lipofectamine 2000 (Invitrogen) to give a final concentration of 30 nM. The transfection media was removed after 16-20h, cells were washed with PBS and differentiated as described.

2.2.2 Transient transfection procedures

Hygro-mSCD1/pcDNA3.1 (was a kind gift from Dr.Allan Tall, Division of Molecular Medicine, Department of Medicine, Columbia University, New York, USA) spotted
onto filter paper was placed in a sterile eppendorf tube with 500 µl of sterile water. It was heated for 10 min at 30°C and was freezeed until required. The DNA plasmid was diluted 1:100 and quantified on a spectrophotometer at 260 nm. The yield was calculated as absorbance at 260 nm X 40 X dilution factor / 1000. The plasmid was introduced into DH5 α (50 µl aliquots) competent cells (E.coli that takes up plasmid) by an electroporator (Gene pulser II, Biorad, Australia) with added SOC media without antibiotic. After the electroporation process this plasmid mixture was spread on a prepared agar plate and incubated overnight at 37°C. Depending upon the quantity of plasmid required, 2 or 3 colonies of plasmid from the agar plate were taken and seeded in the Luria broth (LB) and mixed on an orbital shaker at 37°C for 20 hours at 150 rpm. The LB media containing the bacteria was purified and the plasmid was obtained using a commercially available kit (Promega Wizard Midi prep Kit). The DNA yield was spectrophotometrically determined and averaged 50 μg/μl.

Cells were transfected using a mixture of DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate) and DOSPER (1,3-Di-Oleoyloxy-2(6-Carboxy-spermyl)-propylamide) in filter-sterilised HEPES buffered saline (42mM HEPES, 274 mM NaCl, 1.4 mM Na₂HPO₄, 11 mM dextrose, pH 7.1). The mixture was added to the cells in 1ml α-MEM supplemented with 10% fetal bovine serum. The transfection media was removed after 16-20 h, cells were washed with pre-warmed PBS and differentiated as described above.

2.2.3 Determination of DAG and TAG incorporation in L6 myotubes

L6 myoblasts were maintained at 37°C in 5% CO₂/95% O₂ humidified air in α modified essential medium +10% fetal bovine serum (FBS). Differentiation was induced by switching to medium containing 2% FBS when the myoblasts were ~70% confluent.
Experimental treatments were started after 2 days, by which time nearly all of the myoblasts had fused to form myotubes. Cells were incubated with 0.5 mM/L palmitate (16:0) or 0.5 mM/L linoleate (18:2) conjugated to 2% bovine serum albumin (BSA), or 2% BSA alone (CON) for 5 h. The media was supplemented with 0.25 mM/L palmitate and $^{14}$C-palmitate (0.5 µCi/ml) and cells were incubated for a further 2 h. Incorporation of the radiolabelled fatty acid was determined by lysing cells in methanol and extracting lipids in chloroform: methanol (2:1). The organic phase containing TAG and DAG was removed and dried with nitrogen gas. The lipids were reconstituted, loaded onto silica gel plates and the lipid fractions were separated by thin layer chromatography. The bands corresponding to TAG and DAG were visualised under long-wave UV after spraying with chlorofluorescein dye (0.02% w/v in ethanol), and bands were scraped for liquid scintillation counting (Tri-Carb 2500TR, Packard, Canberra, Australia). In separate experiments, TAG, DAG and ceramide content were determined in cells (described below) after 5 h fatty acid treatment. For determination of 2-deoxy-D-$[^{14}$C$]$ glucose uptake, cells were grown as described and incubated in 0.5 mM fatty acids or BSA for 5 h. Cells were washed with warm phosphate buffered saline (PBS) and incubated in MEM without (basal) or with 100 nM/L insulin for 30 min. The media was removed and 2-deoxy-D-[$^{14}$C$]$ glucose uptake (0.5 µCi/ml, 10 µM/L cold 2-DG) was added. The assay was stopped after 20 min by the addition of ice-cold PBS and cells were lysed in 0.3 M NaOH. Radioactivity was determined by liquid scintillation counting.
2.3 Analytical procedures

2.3.1 Measurement of skeletal muscle lipid metabolites

DAGs and ceramide were extracted and quantified according to the methods of Preiss (Preiss et al., 1986). Ceramide and DAG levels from untreated and palmitate treated L6 myotubes of skeletal muscle were determined using the DAG kinase method. This involved the extraction and separation of the lipids, the phosphorylation of ceramide and DAG by DAG kinase with radioactively labelled ATP (\(^{32}\text{P}\)), detection using radiography and quantification of the products by scintillation counting. The radioactive label incorporated into the products of the DAG kinase reaction is therefore proportional to the amount of ceramide and DAG in the treated myotubes. The reactions are as follows:

\[
\text{Ceramide} + \text{AT}^{32}\text{P} \quad \xleftrightarrow{\text{DAG Kinase}} \quad \text{Ceramide-1-}^{32}\text{P}
\]

\[
\text{DAG} + \text{AT}^{32}\text{P} \quad \xleftrightarrow{\text{DAG Kinase}} \quad \text{Phosphatidic Acid}
\]

After palmitate treatment of the myotubes, the media was removed and the myotubes were washed twice with warmed phosphate buffered saline (PBS). These myotubes were then lysed with ice-cold 1M NaCl and transferred to clean glass kimble tubes where 750 µl of 1:2 chloroform: methanol (v:v) was added and tubes were mixed. Then 250 µl of 1M NaCl and 250 µl chloroform was added and samples were again mixed. The samples were then incubated on ice for 30 min and centrifuged 400 x g for 20 min (Beckman Coulter Allegra 6R Centrifuge). The supernatant was removed and 400 µl of the lower phase was transferred to new kimble tubes. The lower phase was dried under nitrogen before the addition of 400 µl of 2:1 chloroform: methanol (v:v) and
vigorous mixing. Samples were transferred to new glass kimble tubes and 20 µl cardioplin/octylglucoside mix (5 mM cardiolipin, 7.5% octylglucoside (v:v), 1 mM DETAPAC, (pH 7.0) was added. The samples were mixed and incubated at room temperature (RT) for 15 min. To commence the reaction, 100 µl of reaction mixture (2X Reaction buffer: 200 mM Imidazole HCl, 200 mM NaCl, 50 mM MgCl₂, 4 mM EGTA), DAG kinase (Sigma-Aldrich), 20 mM ATP, 20 mM DTT, 0.5 µCi/µL dH₂O + ³²P-ATP)) was added to the samples and incubated for 2h at RT. The reaction was stopped with the addition of 4 ml of 2:1 chloroform: methanol (v:v). The phases were separated with 1% HClO₄ (v:v), mixed and centrifuged at 400 x g for 2 min. The upper phase was discarded and 2.5 ml of the organic phase was transferred to a new kimble tube and dried under nitrogen. The lipid was reconstituted in 65 µl of 2:1 chloroform: methanol (v:v) and 30 µl of sample was spotted onto heat activated thin layer chromatography (TLC) silica plates. The TLC plate was developed in solvent system containing chloroform: acetone: methanol: acetic acid: water, 100:40:20:20:10. The TLC plate was dried and placed in a cassette with film on top and exposed overnight. The film was developed the next morning and DAG and ceramide bands detected. The corresponding bands on the TLC plate were scraped and added to 5 ml of scintillation fluid (StarScint, Perkin Elmer Analytical Sciences). Radioactivity was determined on the 2500 TR Packard Liquid Scintillation Analyser.

2.3.2 Determination of triacylglycerol fatty acid composition

Freeze-dried muscle (~100 mg) was extracted with chloroform-methanol (2:1 by volume) containing 10 mg/L of butylated hydroxytoluene. After storage of the samples overnight at 4°C, each sample was filtered through Whatman paper, rinsed with an additional volume of extracting solvent and partitioned against 0.9% NaCl. This was
evaporated under nitrogen gas and the lipids were reconstituted in chloroform. Samples were spotted onto TLC plates and developed in a solvent consisting of petroleum ether: diethyl ether: acetic acid (85:15:3 v:v:v) and the TAG fraction was identified against a standard (Nu-Chek, 185A) and scraped into a vial. Methyl esters of fatty acids of the TAG extract were prepared by saponification using 5% H$_2$SO$_4$ in methanol. The fatty acid methyl esters were separated by capillary gas liquid chromatography using a 50m x 0.32 mm (I.D.) fused silica column bonded phase column (BPX70, SGE, Melbourne, Australia) with helium as carrier gas at a flow rate of 43cm/sec. The column oven was maintained at 125°C for 3 min and increased at a rate of 8°C/min to 220°C and this temperature was maintained for the duration of the run. Fatty acids were identified by comparison with standard mixtures of fatty acid methyl esters and the results are expressed as percent of the total TAG fraction.

2.3.3 SCD1 Activity

An index of SCD1 activity was calculated as the relative amount of product (16:1) to the corresponding substrate (16:0).

Saturation index / SCD1 activity = \[16:1(\text{n7})/16:0\] x 100.

Palmitic acid (16:0), Palmitoleic acid (16:1).

2.3.4 Blood metabolite and hormone analysis

Whole blood was obtained from the tail vein and used for blood glucose (Glucocard II, KDK, Japan) and lactate determination (Yellow Springs Instruments 1500, Yellow Springs, OH, USA) prior to anaesthesia. Whole blood was transferred to an EDTA-administered tube, centrifuged (5,000 rpm for 15 min) and the plasma collected. Free fatty acid concentration was determined by an enzymatic colorimetric method (NEFA C...
test kit, Wako, Japan) and insulin concentration was measured by enzymatic immunoassay ELISA kit (Mercodia AB, Uppsala, Sweden).

2.3.5 Fatty acid metabolism

A palmitate solution consisting of (α minimum essential medium (αMEM), 2% fatty acid free bovine serum albumin (BSA) (w: v) and 1 mM palmitate in 100% ethanol was prepared. The palmitate solution was incubated on a roller for 1 h and diluted to the desired concentration with αMEM. Where required, 14C-palmitate (Amersham biosciences, Piscataway, NJ, USA) was added to the palmitate media at a final concentration of 0.5mM. 1ml of palmitate solution is added to each well and incubated for 2 hours. After that the media is collected to determine fatty acid oxidation by the addition of 1ml of the collected media was added to a vial containing 1ml of 1M H2SO4 and a microcentrifuge tube with 1ml of 1M benzethonium hydroxide. The liberated 14CO2 was trapped in the microcentrifuge containing benzethonium hydroxide, and it was fixed in a scintillation vial and the radioactivity was counted to calculate the rate of fatty acid oxidation. Palmitate uptake was calculated as the sum of oxidation and esterification.

2.3.6 2-Deoxyglucose uptake

L6 myoblasts were maintained at 37°C in 5% CO2/95% O2 humidified air in α modified essential medium (αMEM), +10% fetal bovine serum (FBS). Differentiation was induced by switching to medium containing 2% FBS when the myoblasts were ~70% confluent. Experimental treatments were started after 2 days, by which time nearly all of the myoblasts had fused to form myotubes. Cells were incubated with 0.5 mM/L palmitate (16:0) or 0.5 mM/L linoleate (18:2) conjugated to 2% BSA, or 2% BSA alone
(CON) for 5 h and TAG, DAG and ceramide content were subsequently determined (described above). For determination of 2-deoxy-D-[\textsuperscript{14}C] glucose uptake, cells were grown as described and incubated in 0.5 mM fatty acids or BSA for 5 h. Cells were washed with warm PBS and incubated in \( \alpha \) MEM without (basal) or with 100 nM/L insulin for 30 min. The media was removed and 2-deoxy-D-[\textsuperscript{14}C] glucose (0.5 \( \mu \text{Ci/} \text{ml, } 10 \mu \text{M/L cold 2-DG} \) was added. The assay was stopped after 20 min by the addition of ice cold PBS and cells were lysed in 0.3 M NaOH. Radioactivity was determined by liquid scintillation counting.

2.3.7 Evaluation of mRNA content by real time-polymerase chain reaction (RT-PCR)

RT-PCR was employed to determine the mRNA expression of the genes of interest. Here, the relative abundance of the mRNA of interest in inversely related to the cycle number (the point we see an exponential rise in PCR product). The point at which the exponential increase in gene product occurs is called the critical threshold (\( C_T \)). We then quantified the expression of the gene of interest by employing the comparative delta \( C_T \) method. All genes of interest were expressed relative to the house-keeper gene, 18S.

**Probe and primer design**

The sequences of the primers and probe were as follows: forward primer, 5’-CAC GCC GAC CCT CAC AA-3’; reverse primer, 5’-TCT TTG ACA GCC GGG TGT TT-3’; Taqman probe, 5’-TTC TTC TCT CAC GTG GGT TGG CTG CTT-3’. Ribosomal 18S was used as a constitutive housekeeping control (Applied Biosystems, Foster City CA, USA). Before using PCR technique on SCD1 gene we performed a series of experiments to optimise the PCR conditions.
**Primer optimisation**

We conducted experiments to determine the optimal primer conditions for SCD1. Maximum fluorescence and lowest cycle number were considered as optimal primer concentration. To determine the optimal primer concentration 25µl loading volume of Taqman\textsuperscript{R} Universal PCR master mix (1X), 2ng of cDNA, 200nM of SCD1 Taqman\textsuperscript{R} probe, sterile water and SCD1 forward and reverse primers was used in each PCR reaction. The forward and reverse primers at concentrations of 50nM, 300nM, 900nM were added in different ratios in each PCR reaction.

Blank samples with no forward and reverse primers were also used as controls. Each reaction was carried out in duplicate and amplified using multiplex comparative critical threshold method (Bio-Rad, i Cycler, IQTM, Hercules, CA) with conditions set at 50\textdegree C for 2min, 95\textdegree C for 10min, followed by 40 cycles of 95\textdegree C for 15 sec and 60\textdegree C for 1min. In this experiment 300nM forward and 300nM reverse primer concentrations were considered as optimal as they resulted in the highest fluorescence at the lowest cycle.

**Probe optimisation**

To determine the optimum concentration for Taqman\textsuperscript{R} probe, experiments were conducted at different conditions. Here also in primer concentration, determination of optimum concentration was with maximum fluorescence at lowest cycle number. Each PCR reaction was carried out at a loading volume 25µl including Taqman\textsuperscript{R} Universal PCR master mix (1x), 2ng cDNA, 300nM reverse primer, 300nM forward primer, sterile water and SCD1 Taqman\textsuperscript{R} probe. In this experiment the probe was added at different concentrations ranging from 50 to 200 nM at 25 nM intervals. Each reaction was carried out in duplicate and amplified using multiplex comparative critical threshold method (Bio-Rad, i Cycler IQTM, Hercules, CA) with conditions set at 50\textdegree C.
for 2min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. In this experiment, 100 nM probe concentration was considered as optimal as that resulted in the highest fluorescence at the lowest cycle.

**cDNA optimisation**
To determine the optimum concentration for cDNA for the PCR reaction, experiments were conducted under various conditions. Here primer concentration determination was by maximum fluorescence at lowest cycle number. Each PCR reaction was carried out at a loading volume 25 µl including Taqman® Universal PCR master mix (1x), 300 nM reverse primer, 300 nM forward primer, sterile water and 100 nM SCD1 Taqman® probe. In this experiment cDNA was added at different concentrations ranging from 0 to 5 µl (2 ng cDNA = 1µg/1µl) at 0.5 µl intervals. Each reaction was carried out in duplicate and amplified using the multiplex comparative critical threshold method (Bio-Rad i Cycler IQTM, Hercules, CA) with conditions set at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. In this experiment, 1 µl cDNA concentration was considered as optimal as that resulted in the highest fluorescence at lowest cycle.

**Ribosomal 18S optimisation**
To optimise the concentration of ribosomal 18S gene experiments were conducted with a loading volume of 25µl including Taqman® Universal PCR master mix (1x), 300 nM reverse primer, 300 nM forward primer, sterile water and 100 nM SCD1 Taqman® probe, 200 ng cDNA with ribosomal 18S added at different concentrations ranging from 50 to 200 nM at 25 nM intervals including one blank without ribosomal 18S. Each reaction was carried out in duplicate and amplified using multiplex comparative critical threshold method (Bio-Rad i Cycler IQTM, Hercules, CA) with conditions set at 50°C
for 2min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1min. In this experiment 1.25 µl of ribosomal 18S (10ng) was considered as optimal.

**Comparison of SCD1 efficiency and 18S amplification**

In multiplex PCR, more than one primer pair was added in each PCR reaction using multiplex comparative C<sub>T</sub> method. This method was useful in determining reference gene ribosomal 18S and SCD1 gene in a single well. In this multiplex, the presence of 18S was detected by its ability to emit fluorescence at different wavelength. To conduct the comparative C<sub>T</sub> PCR, an experiment to determine the efficiency of amplification of 18S and SCD1 gene was conducted. To achieve this effect singleplex PCR where 18S and SCD1 genes were amplified in separate wells with different cDNA concentrations were performed to determine whether the rate of amplification was equal. This experiment resulted in the same efficiencies of 18S and SCD1 amplification over different concentrations. In this reaction 18S primers were controlled to follow the conditions of multiplex PCR. Experiments were then conducted to determine the effect of C<sub>T</sub> values on optimal primer singleplex versus primer limited multiplex 18S. To perform this experiment in singleplex, the primer concentrations were optimised for both 18S and SCD1. In multiplex PCR optimised SCD1 with limited 18S primer concentrations was used. This experiment was conducted at different cDNA concentrations along with control reactions without cDNA. Each reaction was performed in duplicate. The C<sub>T</sub> values obtained here were compared and observed as approximately similar. This demonstrated that multiplex PCR C<sub>T</sub> values have no effect in comparison with SCD1 singleplex PCR.
**RNA extraction**

L6 myotubes were treated with Trizol (Life Technologies, Sydney, Australia) for RNA isolation. For muscle samples 1 ml of reagent was used to lyse the cells. The cells were scraped with Trizol and the solution transferred to an eppendorf tube and 200μl chloroform was added followed by centrifugation at 17000 x g for 15 min to separate the phases. The upper aqueous phase was transferred to a fresh eppendorf tube, 200μl isopropanol was added and the solution was mixed then centrifuged at 12000 x g for 20 min. The supernatant was removed and the pellet was washed with 75% ethanol. The RNA pellet was dried and partially dissolved in 10μl (1 X RNAse free water).

**RNA quantification**

RNA was quantified by spectrophotometry (Hitachi U 2000 spectrophotometer, Australia). 1 μl of RNA was diluted in 100 μl of dH2O and the absorbance was determined at 260nm. The absorbance of 1 unit at 260 nm is equal to 40 μg of RNA/ml. RNA concentration was calculated accordingly.

**Reverse transcription**

RNA samples were reverse transcribed using Taqman Reverse Transcription Reagents (Applied Biosystems, Melbourne, Australia). 1 X TaqMan RT Buffer, DEPC Water, 5.5 mM MgCl, 500 μM dNTP (2.5 mM each), 2.5 μM random hexamer, 0.4 U/μl RNase Single inhibitor, 1.25 U/μl Reverse transcriptase, 0.01 μg/μl of diluted sample (for respective reaction).

**Taqman real time-polymerase chain reaction**

The cDNA samples were loaded in duplicate in a 96 well microtitre plate in a total volume of 25 μl. Each reaction contained 10ng cDNA, 12.5 μl iQ super mix
(BioRad)(which contains 100 mM KCl, 40 mM Tris-Hcl pH 8.4, 0.4 mM dNTP, 50 U/µl iTaq DNA polymerase, 6 mM MgCl₂, 300 nM TaqMan primer, 100 nM probe, 0.6µl 18s rRNA control primer). Probes were designed with the FAM reporter dye on the 5’ end and VIC dye to the 3’ end. 18S rRNA was used in the same reaction as a constitutively expressed house-keeping gene. For all PCR experiments, negative controls are all constituents without cDNA. PCR amplification was performed using an iCycler iQ Real Time Detection System version 3.021 (BioRad, Australia). The delta (Δ) C_T method was used to calculate the expression of each sample of the target gene. For each sample, a threshold cycle (C_T) was obtained for the target gene and the control gene (18S r RNA). The C_T for 18s r RNA was subtracted from the C_T of the target gene to obtain C_T(Δ C_T) for each sample. The difference in the Δ C_T values is calculated for each gene of interest between the control and the target gene (Skiba et al., 2005).

2.3.8 Determination of protein content by western blotting

Protein extraction

L6 myotubes or skeletal muscle samples were lysed in ice cold lysis buffer, respectively. Lysis buffer consisted of 20 mM HEPES (pH 7.4), 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol (DTT), 1 mM Na₃VO₄, 1% TritonX-100, 10% glycerol, 5 µl/ml protease inhibitor (Sigma) and 5 µl/ml phosphatase inhibitor (Sigma). For skeletal muscle, tissue was freeze dried and homogenized for 10 sec on ice using a Polytron homogeniser (Biolab Group, Australia) at full speed. For cell culture, cells were placed on ice and washed with warmed PBS. Lysis buffer was added and the adherent cells were rapidly removed with a plastic plate scraper. For both tissue and culture, the cell solution was transferred into an eppendorf tube and centrifuged at
16,000 x g for 20 min. The supernatant was transferred to an eppendorf tube and rapidly frozen in liquid nitrogen for later analysis.

**Protein determination**

The supernatant containing cellular protein was diluted 1:50 with water and the protein content was determined using the bicinchoninic acid (BCA) method using BSA as a standard (Sigma). The samples and standards were determined in duplicate in a 96 well plate using a commercially available kit (MicroBCA™ Protein Assay Reagent Kit, Quantum Scientific, Australia). The plate was incubated for 30 min at 55°C and absorbance was determined at 560 nm on a spectrophotometer (Victor³™ 1420, Multilabel Counter, Wallac). The protein content in each well was calculated from the linear regression obtained from the standards absorbance reading.

**Solubilisation of proteins**

For solubilising the proteins Lamelli’s buffer was prepared. The 4 X Lamelli’s buffer consisted of 40% glycerol (v:v), 8.2% SDS (Sodium Dodecyl Sulphate)(w:v), 0.5 M Tris-Hcl(v:v), 1% Bromophenol blue(v:v), adjusted to pH 6.8. Immediately prior to use, 31mg DTT was added to 500µl of Lamelli’s buffer. Lamelli’s buffer and an appropriate volume of water was added to lysates, mixed and heated at 95°C for 5 min. Samples were spun at 5,000 x g for 1 min prior to loading.

**Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)**

Polyacrylamide gels were made in a mini multicasting chamber (Biorad, Melbourne, Australia). Running gels (6-12%) were poured and stacking gel was added. The cassette was placed in an electrophoresis tank filled with running buffer (25 mM Tris (pH 8.8), 3 mM SDS, 192 mM glycine). A 6µg molecular weight protein standard
(Precision plus Protein Standards, Biorad) and solubilized protein were loaded into individual wells. The gel was run at 120 V until proteins had passed through the stacking gel and was increased to 150 V thereafter.

**Transfer and antibody treatment**

The proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, Sydney, Australia) and soaked in cold transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, v/v) for 5 min before protein transfer. The transfer was conducted at a constant current of 0.06 A per gel for 90 minutes. After the transfer, the membrane was washed with Tris buffered saline with Tween (TBST; 20 mM Tris, 140 mM NaCl, 0.05% Tween 20, pH 7.6). The membrane was blocked with 5% skim milk powder (w/v) in TBST on a rocker at room temperature for 1 h. After blocking, the membrane was washed with TBST and incubated with primary antibody (1:1000 monoclonal goat SCD1 antibody, SantaCruz Biotechnology), 2.5% skim milk, TBST, 0.1% NaN₃ overnight on a rocker at 4 °C. The membrane was washed three times with TBST and was incubated with secondary antibody (1:2000 antigoat antibody, 2.5% skim milk, w:v, and TBST) for 1 h. The membrane was washed 5 x 5 min with TBST. The immunoreactive bands were detected with 2 ml of chemiluminescence reagent (ECL™ Western Blotting Detection Reagents, Amersham Biosciences, England) and exposed using the Chemidoc EQ system. Proteins were quantified using Quantity One software version 9 (Biorad Laboratories, Hercules, CA, USA).

### 2.4 Statistics

Data are expressed as the mean ± SEM. Statistical analysis were performed by a one-way or two-way analysis of variance with a Student-Newman-Keuls post hoc test. Statistical significance was set at P < 0.05.
CHAPTER 3

SATURATED, BUT NOT POLYUNSATURATED FATTY ACIDS, PROMOTE SCD1 EXPRESSION, INDUCE LIPID METABOLITE ACCUMULATION AND CAUSE INSULIN RESISTANCE
3.1 Introduction

The excessive accumulation of TAG in non-adipose tissue is associated with the development of insulin resistance and type 2 diabetes. Skeletal muscle is a major site for insulin-stimulated glucose disposal (Baron et al., 1988a) and the accumulation of TAG within lipid droplets in skeletal muscle is positively correlated to the severity of insulin resistance (Pan et al., 1997; Forouhi et al., 1999; Krssak et al., 1999). However, insulin sensitivity and IMTG increase after endurance exercise training (Goodpaster et al., 2001a; Helge et al., 2001; Bruce et al., 2004) and TAG storage protects chinese hamster ovary cells from lipotoxicity, indicating that IMTG per se does not cause insulin resistance but may be a proxy for other lipid metabolites that directly interfere with insulin signalling. Indeed, skeletal muscle insulin resistance is mediated by intramyocellular accumulation of fatty acyl-CoA metabolites, such as DAGs and ceramides, which are elevated in insulin resistant states (Qu et al., 1999b, a; Yu et al., 2002; Adams et al., 2004; Lessard et al., 2004) and directly interfere with insulin signal transduction (Kanety et al., 1996; Schmitz-Peiffer et al., 1999; Yu et al., 2002; Chavez et al., 2003b; Stratford et al., 2004).

Whilst increased availability of fatty acids has been linked to skeletal muscle insulin resistance (Bachmann et al., 2001; Boden et al., 2001), it appears that the type of fatty acid is also critical. Epidemiological evidence suggests that the consumption of a Western diet that is high in saturated fats closely correlates with the development of insulin resistance in humans (Maron et al., 1991; Parker et al., 1993). Direct examination in muscle cells in vitro indicates that saturated fatty acids cause insulin resistance whereas unsaturated fatty acids exert a protective effect, or even improve insulin sensitivity (Montell et al., 2001; Chavez et al., 2003b).
Saturated fats are less readily oxidised (Leyton et al., 1987; Gaster et al., 2005), and accumulate as DAG and ceramide in vitro, whereas mono- and polyunsaturated fats accumulate as IMTG or free fatty acids (Schmitz-Peiffer et al., 1999; Montell et al., 2001; Gaster et al., 2005), thus providing a link between fatty acid subtype and insulin resistance. Numerous dietary studies in rodents and humans indicate that saturated fat significantly worsens insulin resistance, while monounsaturated and polyunsaturated fatty acids have a less pronounced effect, or even improve insulin sensitivity (Riccardi et al., 2004). The biochemical and molecular processes linking saturated fats to insulin resistance remain unresolved, but may relate to altered membrane phospholipid fatty acid composition and membrane fluidity and stability (Storlien et al., 1991), changes in lipogenic gene transcription (Clarke, 2004), the type of fatty acids within TAG (Matsui et al., 1997; Andersson et al., 2002) and direct interference with insulin signalling (Kanety et al., 1996; Schmitz-Peiffer et al., 1999; Yu et al., 2002; Chavez et al., 2003b; Stratford et al., 2004). Lipid metabolites that interfere with insulin signal transduction accumulate in tissues of insulin resistant animals (Turinsky et al., 1990; Schmitz-Peiffer et al., 1997; Qu et al., 1999a; Yu et al., 2002; Lessard et al., 2004) however, the effects of markedly altering the types of dietary fatty acids on the accumulation of these lipid metabolites in whole animals are unknown.

The primary aim of this study was to examine the role of diets rich in either saturated or polyunsaturated fatty acids on skeletal muscle fatty acid metabolite accumulation and whole body insulin sensitivity. It was hypothesised that a diet high in saturated fatty acids would increase the contents of skeletal muscle DAG and ceramide and induce insulin resistance, whereas a diet high in polyunsaturated fatty acids would not affect DAG and ceramide contents or impact upon insulin sensitivity. To confirm the biochemical responses to fatty acids, independent of potential changes in metabolic or
hormonal fluxes, we also incubated rat L6 myotubes, an *in vitro* skeletal muscle model, with saturated and unsaturated fatty acids and hypothesised that the *in vivo* effects would persist *in vitro*.

SCD 1 is an endoplasmic reticulum bound enzyme that converts saturated fatty acids (primarily 16:0; 18:0) to monounsaturated fatty acids. Global SCD1 deficiency produces a lean, obesity-resistant, insulin sensitive phenotype (Ntambi *et al.*, 2002a); however, preventing the ability to desaturate fatty acids should lead to insulin resistance because saturated fats accumulate as DAG and ceramide. While polyunsaturated fatty acids reduce and saturated fatty acids increase SCD1 expression in liver (Ntambi, 1999); the role of dietary fatty acids on SCD1 expression and activity in skeletal muscle is unknown, despite the importance of skeletal muscle in fuel metabolism and insulin resistance. Accordingly, the second aim of this study was to investigate the role of dietary fatty acids on skeletal muscle SCD1 gene and protein expression *in vivo*.

**3.2 Experimental design**

The animal experiments in this study were conducted by Dr. Jong Sam Lee (Eulji University, South Korea) and were approved by the Animal Ethics Committee of Korea National Sports University. Male Sprague-Dawley rats (Samtako, Biokorea, Kyoungki, South Korea) were obtained and maintained as described (p.35) and grouped as control, SAT and PUFA diet treated groups. After 8 weeks of diet treatment, animals were fasted overnight and an OGTT was conducted. Two days later, rats were overnight fasted and anaesthetised. Muscle and fasting blood samples were obtained. The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated from blood samples to calculate the fasting blood glucose and insulin levels (fasting insulin (pM/l) x fasting glucose (mM/l)/135). The muscle samples were immediately
frozen in liquid nitrogen. Muscle TAG, DAG and ceramide content were assessed, as were the TAG fatty acid subtypes using gas chromatography. SCD1 mRNA, protein and activity were also assessed. In separate experiments, the effects of fatty acids were assessed in L6 myotubes. Myotube TAG, DAG and ceramide accumulation and insulin stimulated glucose disposal were determined (for procedures refer to general methods).

3.3 Results

Animal characteristics and glucose tolerance

Animal weights were not different between groups after 8 weeks diet (CON, 391 ± 9g; SAT, 409 ± 7g; PUFA 380 ± 4 g). Both high fat diets increased plasma free fatty acids by ~30% but were without effect on fasting blood glucose (Table 3.1). Fasting plasma insulin was increased in animals fed SAT compared with CON fed animals, and reduced in animals fed PUFA (Table 3.1). The HOMA-IR index was increased in SAT and decreased in PUFA compared with CON (Figure 3.1A). These data indicate that SAT rats were insulin resistant and that insulin sensitivity was improved in PUFA rats. The glucose area under the curve was not different between groups (Figure 3.1B); however, plasma insulin levels were elevated in SAT compared with CON, indicating insulin resistance in these animals (Figure 3.1C). Conversely, plasma insulin levels were decreased in PUFA compared with CON indicating improved insulin sensitivity (Figure 3.1C).
Table 3.1 Body mass and fasting plasma metabolites in rats fed a chow diet (CON) or high-fat diet consisting of either saturated (SAT) or polyunsaturated (PUFA) fatty acids.

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>SAT</th>
<th>PUFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass (g)</td>
<td>391 ± 9</td>
<td>409 ± 7</td>
<td>380 ± 4</td>
</tr>
<tr>
<td>FFA (µM/L)</td>
<td>354 ± 29</td>
<td>460 ± 23*</td>
<td>448 ± 25*</td>
</tr>
<tr>
<td>Glucose (mM/L)</td>
<td>3.99 ± 0.16</td>
<td>4.61 ± 0.15</td>
<td>4.26 ± 0.10</td>
</tr>
<tr>
<td>Insulin (pM/L)</td>
<td>27 ± 5</td>
<td>76 ± 24*</td>
<td>13 ± 3*†</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=8. * different from CON, † different from SAT, P<0.05.
Figure 3.1 Effect of dietary fatty acid subtype on insulin sensitivity in rats.

(A) The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated from fasting blood glucose and insulin levels. HOMA-IR = (fasting glucose x fasting insulin)/22.5, where glucose is expressed as mM/L and insulin µU/L. In separate experiments, overnight fasted rats were provided with an oral glucose load and blood samples were taken at regular intervals over 120 min. (B) Glucose area under the curve and (C) plasma insulin were determined from tail bleeds. Values are means ± SEM, n=8. * different from CON, † different from PUFA, P<0.05.
**Skeletal muscle lipid content is influenced by dietary content**

Lipids were assessed in skeletal muscle after an 8 h fast. Skeletal muscle TAG was increased in SAT and PUFA compared with CON, and PUFA TAG content tended (P=0.08) to be greater than SAT (Figure 3.2A). Skeletal muscle DAG content was increased in SAT and PUFA compared with CON (Figure 3.2B). DAG content was greater in SAT vs PUFA. Ceramide content in rodent muscles was variable and was not affected by dietary content (Figure 3.2C).

**Effect of dietary fatty acid composition on skeletal muscle triacylglycerol fatty acids**

Dietary fatty acid composition exerted a profound effect on the fatty acid profile in skeletal muscle TAG. SAT increased the proportion of saturated fatty acids and decreased unsaturated fatty acids within TAG compared with CON (Figure 3.3A). The elevation in saturated fats with SAT could be attributed to increases in 12:0, 14:0 and 18:0 (Figure 3.3B). Interestingly, 18:1 was markedly elevated and 18:2 decreased in SAT versus CON. PUFA increased the percentage of unsaturated fats in TAG, which was largely due to increased 18:2. PUFA also decreased the proportion of all saturated fatty acid in TAG compared with CON and SAT (Figure 3.3B). The proportion of saturated fatty acids was 32, 42 and 17% for CON, SAT and PUFA, respectively.

**SCD1 is increased by a saturated fatty acid diet**

SCD1 mRNA was unaffected by PUFA and tended (P=0.11) to increase with SAT compared to CON (Figure 3.4A). We examined SCD1 protein expression by Western blot and report increased SCD1 in SAT compared with CON and PUFA (Figure 3.4C). An index of SCD1 enzyme activity, which was calculated as the relative amount of product (16:1) to the corresponding substrate (16:0), was increased (P<0.05) in SAT compared with CON and was reduced (P<0.05) in PUFA (Figure 3.4D). The difference
in SCD1 activity depending upon various diets indicated that SCD1 expression increases with SAT compared to other diets.
Figure 3.2 Effect of dietary fatty acid subtype on muscle lipid metabolites. Overnight fasted rats were sacrificed and muscle triglyceride (A), diacylglycerol (B) and ceramide (C) were determined. Values are means ± SEM, n=8. * vs CON, † vs SAT (P<0.05).
**Figure 3.3** Effect of dietary fatty acid subtype on skeletal muscle triacylglycerol fatty acids. Overnight fasted rats were sacrificed, muscle triglyceride were extracted and analysed for fatty acid composition by gas chromatography. (A) Percent saturated and unsaturated fatty acids within triacylglycerols (TAGs), (B) fatty acid profile within triacylglycerol (TAG). Values are means ± SEM, n=8. * vs CON (P<0.05).
Figure 3.4 Effect of dietary fatty acid subtype on SCD1 expression. Muscle from overnight fasted rats was rapidly dissected and examined for SCD1 mRNA (A) and SCD1 protein (C). A representative immunoblot is shown in (B). SCD1 activity index (D). Values are means ± SEM, n=8. * vs CON, † vs PUFA (P<0.05).
**Effect of palmitate and linoleate on fatty acid metabolite content and glucose uptake in L6 myotubes**

To enable biochemical studies to be conducted in the absence of possible confounding factors, such as alterations in circulating metabolites and hormones, we performed experiments in L6 myotubes. While the metabolic action of these cells to insulin is blunted and direct comparisons with *in vivo* studies should be interpreted with caution, L6 myotubes nevertheless possess the proteins required for fatty acid and glucose uptake and metabolism. Glucose uptake was determined in L6 myotubes after 5 h treatment with 0.5 mM palmitate (saturated) or linoleate (polyunsaturated) fatty acids. Palmitate decreased insulin stimulated glucose uptake, whereas linoleate pre-treatment did not affect glucose uptake (Figure 3.5A). The addition of palmitate to culture media resulted in significant DAG (5-fold) and ceramide (2-fold) accumulation (Figure 3.5 C-D). TAG content determined by fluorometric analysis after 5 h incubation revealed a small increase with palmitate (Figure 3.5B). TAG was increased by linoleate treatment (Figure 3.5B), whereas DAG and ceramide content were unchanged (Figure 3.5E-F). These data indicate that palmitate-induced ceramide and DAG accumulation are associated with insulin resistance in skeletal muscle cell culture (Schmitz-Peiffer *et al.*, 1999; Montell *et al.*, 2001; Chavez *et al.*, 2003a). They also indicate that linoleate promotes synthesis of TAG and does not affect insulin stimulated glucose uptake.
**Figure 3.5** Lipid storage and glucose uptake in L6 myotubes after 5 h treatment with palmitate or linoleate. (A) 2-deoxyglucose uptake with or without insulin. (B) $^{14}$C-palmitate incorporation into triacylglycerol (TAG) and (C) diacylglycerol (DAG). (D) L6 myotube triacylglycerol. (E) diacylglycerol and (F) ceramide content. Values represent 2 independent experiments, n=4 per experiment. * versus CON, † versus PUFA (P<0.05).
3.4 Discussion

The present study demonstrates that a diet high in SAT induces muscle DAG accumulation and saturation of the TAG pool, and is associated with insulin resistance. Conversely, animals fed a diet high in PUFA retained insulin sensitivity despite small increases in muscle DAG, which may result from enhanced TAG storage. Thus, fatty acid oversupply per se is not the major determinant of fat-induced insulin resistance, but rather dietary fat composition (Storlien et al., 1991; Storlien et al., 2001).

An important caveat of the present study relates to the interpretation of insulin resistance. The OGTT and HOMA assessments do not allow for assessment of tissue specific effects on insulin-stimulated glucose uptake. While skeletal muscle accounts for >80% of insulin mediated glucose disposal (Baron et al., 1988a), and is likely to represent events occurring at the whole body level, it is not certain that the observed whole body changes are due to altered skeletal muscle function. In this regard, it appears that glucose intolerance may only become apparent when insulin resistance occurs in numerous tissues (Yu et al., 2002). Thus, the present data relating to insulin resistance should be interpreted with these considerations in mind.

DAGs and ceramides antagonise insulin signalling in vitro and this is the first study to directly examine the effects of dietary fatty acid composition on the accumulation of these lipid metabolites in vivo. DAGs are increased in fatty acid-induced insulin resistance (Yu et al., 2002) and are proposed to interfere with insulin signalling via novel PKC activation (Yu et al., 2002). In this study, DAG was markedly elevated in SAT and coincided with insulin resistance, observations that are consistent with the current and previous studies in muscle cell culture that ascribe a direct role for saturated, but not unsaturated fatty acids, in this process (Cazzolli et al., 2001; Montell
et al., 2001; Chavez et al., 2003b; Gaster et al., 2005). However, the in vivo finding that DAGs were moderately elevated in PUFA rats, where insulin action was improved, indicates that total DAG accumulation may not be essential for insulin resistance and that other factors such as a critical threshold of intracellular DAG, cellular localization of DAG or the molecular DAG species may be important mediators of insulin resistance. Collectively, these data indicate that an abundance of dietary SAT induces insulin resistance, possibly via a marked increase in DAG content, whereas smaller increases in DAG were not associated with insulin resistance in animals fed an isocaloric diet rich in polyunsaturated fatty acids.

Ceramide is a second messenger in the sphingomyelin signalling pathway and is produced via the hydrolysis of sphingomyelin and from de novo synthesis (palmitoyl-CoA and serine). Ceramides antagonise insulin signalling (Turinsky et al., 1990; Schmitz-Peiffer et al., 1999; Simoneau et al., 1999; Chavez et al., 2003b) and ceramide accumulation correlates with the development of skeletal muscle insulin resistance in vivo (Adams et al., 2004; Straczkowski et al., 2004). In contrast with the original hypothesis, dietary fatty acid composition did not affect muscle ceramide content. This was unexpected because long chain saturated fatty acids are thought to exclusively act as the substrate for ceramide de novo synthesis (Summers & Nelson, 2005); however, a recent study that infused a lipid emulsion primarily composed of linoleate (18:2) induced ceramide generation and insulin resistance in humans (Straczkowski et al., 2004). A dissociation between ceramide content and insulin sensitivity has been observed previously (Itani et al., 2002; Yu et al., 2002; Lessard et al., 2004) and, consistent with the present findings, indicates that ceramides may not induce insulin resistance in vivo.
IMTG content positively correlates with insulin resistance (Pan et al., 1997; Forouhi et al., 1999; Krssak et al., 1999), which has led to the assumption that excess IMTG storage is undesirable. However in some circumstances, such as after endurance exercise training, increased TAG is associated with enhanced insulin sensitivity (Goodpaster et al., 2001a; Helge et al., 2001; Bruce et al., 2004; Straczkowski et al., 2004). In the present study, IMTG was increased in rats fed a SAT, and to a greater extent, in rats on the PUFA diet. These data are consistent with the studies conducted in L6 myotubes demonstrating increases in IMTG with linoleate compared with palmitate treatment. Unsaturated fats promote TAG accumulation in various cell types (e.g., cardiac, pancreatic, Chinese hamster ovary cells) see (Listenberger et al., 2003) and this and previous experiments in skeletal muscle culture (Montell et al., 2001) have demonstrated increased TAG storage after incubation with unsaturated fats. Thus, unsaturated fatty acids may protect cells from “lipotoxicity” by promoting uptake of fatty acids into an inert triglyceride lipid pool, rather than bioactive lipids such as DAGs and ceramides (Listenberger et al., 2003). The observations in PUFA rats of elevated muscle TAG and reduced DAG content lower basal plasma insulin and enhanced insulin sensitivity compared with SAT supports this possibility. The reasons for IMTG accumulation was not addressed in this study but may relate to the preference of DGAT (converts DAG to TAG) for unsaturated rather than saturated fatty acids (Coleman & Bell, 1976; Sauro & Strickland, 1990).

The fatty acid profile of different body tissues at least partially reflects the fatty acid composition of the diet. The fatty acid profile of TAG was compared between groups in this study. Eight weeks SAT diet increased the degree of saturation within skeletal muscle TAG, which was due to increases in short (12:0, 14:0) and long (18:0) chain fatty acids. It is not known whether an increase in TAG saturation contributes to insulin...
resistance. It is possible that the excess stearate (18:0) within this pool is hydrolysed and provides a substrate for DAG and ceramide production, thereby affecting insulin signalling. In contrast with SAT, PUFA increased the degree of unsaturation within IMTG, which was primarily due to increased linoleate (18:2). Increased unsaturation of the muscle membrane fatty acids is associated with improved insulin sensitivity (Storlien et al., 1991) and the lipid-lowering agent, bezafibrate, reduces the saturation in muscle TAGs and improves insulin sensitivity (Matsui et al., 1997). The greater degree of desaturation in TAG in this study is associated with improved insulin sensitivity, and conversely, an increase in saturation correlates with insulin resistance.

SCD1 is an endoplasmic reticulum bound enzyme that converts SATs to monounsaturated fatty acids. The current understanding of the biological role of SCD1 derives from studies in liver and others conducted in SCD1 null (ab1/ab1, SCD1<sup>-/-</sup>) (Cohen et al., 2002) mice (Cohen et al., 2002; Ntambi et al., 2002a) and mice treated with SCD1-specific antisense oligonucleotide inhibitors (Jiang et al., 2005a). Although global knockout studies demonstrate SCD1 deficiency to induce a leaner phenotype and resistance to diet-induced obesity (Cohen et al., 2002; Ntambi et al., 2002a), the interpretation of SCD1 function is confused by the finding of increased energy expenditure (Cohen et al., 2002; Ntambi et al., 2002a) and basal thermogenesis (Lee et al., 2004b) in these animals. Moreover, there is very limited data describing SCD1 regulation in skeletal muscle. In the present study, skeletal muscle SCD1 gene and protein expression was examined in response to various diets. SCD1 was not different between chow-fed and polyunsaturated fat-fed rats, which is not consistent with the findings in liver that polyunsaturated fatty acids oppose the induction of SREBP-1c, and inhibit SCD1 gene expression (Waters & Ntambi, 1996; Clarke, 2004). In contrast, SCD1 gene and protein expression were elevated in insulin resistant SAT rats. These
results show an increase in DAG with a concomitant increase in SCD1, suggesting a possible causative role of SCD1 in DAG accumulation and insulin resistance. However, SCD1 desaturases fatty acids and those fatty acids are preferentially oxidised or stored as TAGs. It is possible that the SCD1 increase occurs in an attempt to reduce the increase in saturated fat and DAGs in skeletal muscle. However, the findings of increased SCD1 and insulin resistance in SAT and decreased SCD1 and insulin sensitisation in PUFA could be interpreted to mean that SCD1 causes insulin resistance. Studies that directly test this hypothesis warrant further investigation.

Chronic high fat feeding induces insulin resistance in rodents, however, the role of specific dietary fats is not well characterised. Previous reports indicate that insulin resistance (Storlien et al., 1991; Jucker et al., 1999) or glucose intolerance (Marotta et al., 2004) occurs in rats fed a diet high in n-6 PUFAs, whereas rats fed diets high in n-3 and with low n-6: n-3 ratios maintain normal insulin sensitivity. In this study, an ~20% improvement in HOMA-IR in rats fed a PUFA diet consisting mainly of n-6 fatty acids (n-6:n-3 PUFA of >75:1), an effect observed previously in type 2 diabetes patients (Heine et al., 1989). The discrepancies between this and previous rodent studies (Storlien et al., 1991; Jucker et al., 1999; Marotta et al., 2004), whilst difficult to pinpoint, may be due to the duration of treatment (8 weeks in the present study vs 3-4 weeks). With respect to the time course effects, PUFAs upregulate genes involved in fat oxidation and decrease genes involved in lipid synthesis / storage (Sampath & Ntambi, 2005). Although the transcriptional response is rapid, the time required for a phenotype change may take weeks to manifest and thus explain the observed differences between studies. A more likely explanation is that the chow-fed animals were mildly insulin resistant. These animals gained weight at a similar rate to the PUFAs suggesting increased food intake. It is also possible that the small amount of saturated fats in the...
chow diet (~10% of total calories) resulted in slight insulin resistance in the controls. These possibilities remain to be determined. Another possibility is that the animals in the PUFA group may not have accumulated as much body fat as the SAT group due to the liquid nature of the diet. Unfortunately, measures of adiposity and food intake were not assessed in this study. Despite these differences, it appears that an n-6 PUFA diet is more desirable than a SAT diet, but that n-3 PUFA substitution for n-6 is advantageous for insulin action. Thus, whilst a diet supplemented with n-3 PUFAs enhances insulin action (Preiss et al., 1986; Storlien et al., 1991; Storlien et al., 2001), our data suggest that a diet rich in n-6 PUFAs is not detrimental to insulin action as previously described and may even result in modest improvements in insulin action.

In conclusion, the results of the present study demonstrate that a diet high in saturated fatty acids induces insulin resistance whereas polyunsaturated fatty acids modestly enhance insulin sensitivity. The insulin resistance in the SAT rich diet was associated with elevated DAG content and increased saturation of muscle TAG. These effects were partially ameliorated with polyunsaturated fatty acids, possibly via increased incorporation into TAG.
CHAPTER 4

STEAROYL-CoA DESATURASE1 PROTECTS AGAINST FATTY ACID-INDUCED SKELETAL MUSCLE INSULIN RESISTANCE IN L6 MYOTUBES
4.1 Introduction

As mentioned previously, skeletal muscle is an important tissue for insulin-mediated glucose uptake and lipid accumulation in skeletal muscle is associated with defective insulin signalling, reduced glucose uptake, resulting in insulin resistance and type 2 diabetes (McGarry, 2002; Unger, 2002; Eckel et al., 2005). Indeed, in the previous study it was shown that saturated fatty acids induce insulin resistance in rats and L6 myotubes (Figure 3.1).

SCD1 is expressed in various tissues such as adipose tissue, liver and skeletal muscle is considered as critical protein in the regulation of insulin action in skeletal muscle. The amount and distribution of saturated and unsaturated FAs effects many cellular processes including phospholipid composition and membrane fluidity, generation of second messengers in signalling cascades, metabolic fuel storage in the form of TAGs, cellular differentiation and apoptosis (Listenberger et al., 2003; Busch et al., 2005; Scaglia & Igal, 2005). With reference to insulin resistance, recent studies have indicated that exposure of skeletal muscle to excess saturated FA results in the generation of the fatty acyl-CoA metabolites, DAG and ceramide, that directly interfere with insulin signal transduction (Schmitz-Peiffer et al., 1999; Montell et al., 2001; Yu et al., 2002; Chavez et al., 2003a; Chavez & Summers, 2003). Thus, inhibiting SCD1 function would be expected to result in the accumulation of fatty acid metabolites that are deleterious to insulin signalling and accordingly, the development of FA-induced insulin resistance.

Paradoxically, the weight of experimental evidence suggests that SCD1 contributes to the pathogenesis of obesity and insulin resistance. SCD1 content is elevated in the
livers of insulin resistant ob/ob and lipoatrophic mice (Cohen et al., 2002; Asilmaz et al., 2004) and in animals rendered insulin resistant by high-fat feeding (Biddinger et al., 2005). Moreover, SCD1 null (ab/ab, SCD1−/−) mice (Cohen et al., 2002; Ntambi et al., 2002b) and mice treated with SCD1-specific antisense oligonucleotide inhibitors (Jiang et al., 2005b) exhibit a leaner phenotype and resistance to diet-induced obesity (Cohen et al., 2002; Ntambi et al., 2002b). However, the interpretation of SCD1 function in these studies is confused by the findings of increased energy expenditure (Ntambi et al., 2002b; Jiang et al., 2005b) and basal thermogenesis (Lee et al., 2004a), and adaptive alterations in metabolic genes.

Skeletal muscle is an important tissue for fat metabolism (van Hall et al., 2002) and insulin-stimulated glucose disposal (Baron et al., 1988b), and is accordingly a pivotal tissue for energy homeostasis and insulin resistance. Currently, the biological role of skeletal muscle SCD1 in these processes remains poorly understood. A recent study indicated that SCD1 was elevated in skeletal muscle of obese individuals and contributed to a decrease in fatty acid oxidation and increased TAG esterification (Hulver et al., 2005). It is unlikely that SCD1 contributes to skeletal muscle insulin resistance via this pathway since TAG turnover is relatively slow (Sacchetti et al., 2002) and increased IMTG actually correlates with enhanced insulin sensitivity (Goodpaster et al., 2001b). Accordingly, we directly examined the role of SCD1 on fatty acid metabolism and insulin resistance in L6 myotubes, an in vitro skeletal muscle model. With the use of transient knockdown and overexpression models, we demonstrate that SCD1 is required for normal cell function and that increases in SCD1 content protects muscle cells from FA-induced insulin resistance.
4.2 Experimental design

For animal experiments eight-week old male C57Bl/6J mice (n=12) and four-week old male Wistar rats (n=12) were purchased from Monash Animal Sciences. Male db/db mice were a kind gift from Dr. Greg Tesch (Monash University, Victoria, Australia). Mice were fed either chow (4% calories from fat) or high fat diets (53% fat) and were provided with food and water ad libitum for ten weeks. Overnight fasted animals were anaesthetised by intraperitoneal injection of sodium pentobarbital (60 mg/kg body mass) and the gastronemius muscle was rapidly dissected out, immediately frozen in liquid nitrogen and stored at -80°C until analyses. Animals were sacrificed by lethal injection of sodium pentobarbital. All experimental procedures were approved by the RMIT Animal Ethics Committee.

L6 myoblasts were used in all experiments involving SCD1 knockdown and overexpression. Cell culture techniques were used as described in the general methods (p.37). L6 myoblasts were grown to 50-60% confluence then treated with siRNA or cDNA for 16-20 hrs (p.37-39). After the differentiation, myotubes were subjected to different experiments. SCD1 mRNA was evaluated by using RT-PCR and protein content by western blot. Fatty acid oxidation, palmitate uptake, lipid metabolite accumulation and glucose uptake experiments were conducted separately for knockdown and overexpression models.

4.3 Results

SCD1 expression is upregulated in the skeletal muscle of obese rodents

Previous studies in liver indicate that SCD1 expression is increased in obese and/or insulin resistant animals (Biddinger et al., 2005). The effects of high-fat feeding were
first determined on SCD1 expression in skeletal muscle. SCD1 protein expression was markedly elevated in skeletal muscle of mice (C57Bl/6J) and rats (Wistar) made obese by high fat diets (53% fat) provided with water (Figure 4.1A). Densitometry revealed that SCD1 expression was elevated by ~35% after high fat feeding (Figure 4.1B). We next examined the expression of SCD1 in the skeletal muscle of another rodent model of obesity/insulin resistance, the db/db mouse. SCD1 expression was low in muscle extracts of db/+ mice, whereas db/db mice displayed a marked increase in SCD1 expression (Figure 4.1 A-B). These results demonstrate that skeletal muscle SCD1 protein content is increased in obese, insulin resistant animals.
**Figure 4.1** In vivo expression of SCD1 in skeletal muscle of lean and obese rodents.

(A) Lysates were prepared from skeletal muscles of rodents fed a chow or high fat diet (HFD) for 10 weeks or from lean (+/-) or obese (+/+ db/db) mice. 60 µg of protein was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. (B) Quantification of western blots (n=4). * different from lean.
SCD1 knockdown affects fatty acid metabolism in L6 myotubes

SCD1 protein content in control siRNA treated cells was not different from native L6 myotubes, whereas SCD1 siRNA reduced SCD1 protein by 40% (Figure 4.2A). There were no differences between native L6 and control siRNA treated cells for SCD1 protein or any metabolic parameter reported herein (therefore these data are not presented). Palmitate uptake was higher (1.2-fold) in SCD1 siRNA than control cells (Figure 4.2C). Fatty acid oxidation was measured as the sum of $^3$H released into the incubating media and the accumulation of ASM (acid soluble media or partially oxidised fatty acids). Fatty acid oxidation was not different between treatments (Figure 4.2D), Fatty acid incorporation into TAG was not different between treatments (Figure 4.2E), whereas SCD1 knockdown resulted in a 2-fold increase in palmitate incorporation into DAG (Figure 4.2 F).

The cellular content of DAG and ceramide, fatty acid metabolites known to directly interfere with insulin signal transduction (Schmitz-Peiffer et al., 1999; Montell et al., 2001; Chavez et al., 2003b) was examined. When cells were grown in FA-free media, SCD1 siRNA resulted in a 2- and 3-fold increase in cellular ceramide and DAG, respectively, compared with control siRNA (Figure 4.3A-B). When cells were exposed to exogenous palmitate, ceramide and DAG content were increased above basal levels in both control and SCD1 siRNA cells. Ceramide content remained higher in SCD1 siRNA (Figure 4.3A), whereas DAG content was not different between treatments (Figure 4.3B). Taken together, these data demonstrate that reduced SCD1 protein in muscle cells results in increased sarcolemmal uptake of fatty acids that are more readily incorporated into lipid metabolites that are known to negatively impact on insulin signalling.
Figure 4.2 SCD1 siRNA moderately reduces fatty acid oxidation and increases the incorporation of fatty acids into diacylglycerol (DAG). (A) SCD1 protein (mean ± SEM, n=6). * Different from L6 and Con siRNA, P<0.05. (B) Representative immunoblot. (C-F) L6 myotubes were transfected with control siRNA or SCD1 siRNA, differentiated for 3 days and assessed for fatty acid metabolism using 0.5 mM \(^{3}\)H-palmitate conjugated to 2% fatty acid-free bovine serum albumin. Results are expressed as nM/h total palmitate uptake (C), palmitate oxidation (D), palmitate incorporation into triacylglycerol (E) or incorporation into diacylglycerol (F) and represent mean SEM (n=6 individual wells). * P<0.05 as assessed by unpaired t-test.
**Figure 4.3** SCD1 siRNA increased ceramide and diacylglycerol content in L6 myotubes. Ceramide (A) and diacylglycerol (B) content were assessed in myotube lysates after 5 h incubation with BSA alone or 0.5 mM palmitate conjugated to 2% fatty acid-free BSA. Results are expressed as fold change relative to Con siRNA Vehicle and represent mean ± SEM (n=10 individual wells). * P<0.05 vs Vehicle, † P<0.05 vs Vehicle within the same group.
**SCD1 knockdown induces insulin-resistance in L6 myotubes**

The effects of SCD1 siRNA on glucose uptake was assessed in the presence and absence of palmitate and insulin. Basal 2DG uptake was not different between control and SCD1 siRNA treated cells (Figure 4.4A). Insulin-stimulated 2DG uptake was increased by ~70% above basal in control siRNA L6 myotubes, which is consistent with previous values reported for this cell line (Sinha *et al.*, 2004). SCD1 siRNA abrogated the insulin-stimulated 2DG uptake observed in control cells, which coincided with ceramide and DAG accumulation (Figure 4.4A). Treating L6 myotubes with 0.5 mM palmitate for 5 h blunted insulin-stimulated 2DG uptake in control cells and 2DG uptake remained at basal rates in SCD1 siRNA cells. Akt Ser473 phosphorylation, a critical downstream event in insulin signal transduction was increased by insulin and was partially blunted by palmitate treatment in both groups (Data not shown). Akt Ser473 phosphorylation was higher in control vs. SCD1 siRNA in response to insulin (Figure 4.4B). Insulin-stimulated Akt Thr308 phosphorylation was blunted 32% and 54% by palmitate in control and SCD1 siRNA myotubes, respectively. Collectively, these data demonstrate that SCD1 is essential for the maintenance of normal insulin-stimulated glucose transport in L6 myotubes.
Figure 4.4 Glucose uptake and insulin signalling are attenuated by SCD1 siRNA.

L6 myotubes were transfected with control siRNA or SCD1 siRNA and differentiated for 3 days. (A) 2-deoxyglucose uptake was assessed in myotubes following 5 h treatment with 2% fatty acid free BSA or 0.5 mM palmitate conjugated to 2% fatty acid-free BSA, with or without 100 nM insulin. (B) In similar experiments, myotubes were treated as described above and were lysed. Lysates were probed for phospho-Akt Ser 473 protein content. * P<0.05 vs Vehicle within the same group, † P<0.05 vs Con siRNA Palmitate.
SCD1 overexpression attenuates lipid metabolite accumulation in L6 myotubes

The initial experiments demonstrated that SCD1 protected L6 myotubes from insulin resistance by preventing the accumulation of lipid metabolites; however, the addition of palmitate resulted in insulin resistance. To investigate whether SCD1 protects myotubes from palmitate-induced insulin resistance, L6 myoblasts were transiently transfected with pcDNA3.1/Hygro-mSCD1 (L6 SCD1) or control (L6) then rapidly differentiated into myotubes. SCD1 protein content in fully differentiated myotubes was increased 1.35-fold (Figure 4.5B). Interestingly, the fold increase observed in L6 myotubes was similar to the increase seen in obese, insulin resistant rodents compared with lean littermates (Figure 4.1). SCD1 overexpression did not affect palmitate uptake (Figure 4.5C) and resulted in a small (12 ± 1%), yet significant decrease in oxidation (Figure 4.5D). SCD1 overexpression reduced the incorporation of palmitate into DAG by 90% whereas label recovery in TAG was 40% greater (Figure 4.5E-F). There was no difference in ceramide and DAG contents with SCD1 overexpression when cells were incubated in FA-free media (Figure 4.6A-B). Consistent with the \(^3\)H-palmitate incorporation experiments, ceramide and DAG content were elevated 4-fold in control cells, whereas SCD1 overexpression attenuated this increase by ~50% (4.6A-B). Thus, despite small decreases in fatty acid oxidation, SCD1 overexpression results in partitioning of fatty acids into TAG and limits the flux of palmitate into ceramides and DAG.
Figure 4.5 Transient SCD1 overexpression in L6 myotubes attenuates diacylglycerol esterification but results in triacylglycerol esterification. L6 myoblasts were transfected with SCD1 or an empty vector for 20 h and cells were differentiated for 3 days. (A) SCD1 mRNA assessed after 24 h by RT-PCR. (B) SCD1 protein. * P<0.05 vs L6 as determined by unpaired t-test. (C-F) Fatty acid metabolism was assessed using 0.5 mM $^3$H-palmitate conjugated to 2% fatty acid-free BSA. Results are expressed as nM/h total palmitate uptake (C), palmitate oxidation (D), palmitate incorporation into TAG (E), or incorporation into DAG (F) and represent mean SEM (n=10 individual wells). * P<0.05 as assessed by unpaired t-test.
Figure 4.6 Effect of transient SCD1 overexpression on lipid metabolite content.

Ceramide (A) and diacylglycerol (B) content were assessed in myotube lysates after 5 h incubation with BSA alone or 0.5 mM palmitate conjugated to 2% fatty acid-free BSA. Results are expressed as fold change relative to L6 Vehicle and represent mean SEM (n=6 individual wells). * P<0.05 vs Vehicle, † P<0.05 vs L6.
**SCD1 overexpression protects L6 myotubes from FA-induced insulin resistance**

2-DG uptake in L6 SCD1 myotubes was increased both basally (20%) and following insulin stimulation (14%) compared with native L6 myotubes (Figure 4.7A). The net increase in 2-DG uptake in response to insulin was not different between cells. There were no differences in the phosphorylation of Akt (Figure 4.7B). To examine whether SCD1 protects myotubes from insulin resistance in the presence of excess saturated fatty acids, cells were incubated for 5 h with 0.5 mM palmitate. While ~75% of the insulin-stimulated glucose uptake was lost in L6 myotubes pre-treated with palmitate, only 35% of the insulin-stimulated glucose uptake was lost in L6 SCD1. Palmitate decreased insulin-stimulated Akt Ser 473 phosphorylation to a greater extent in L6 (35%) compared with L6 SCD1 cells (20%) (Figure 4.7B). Insulin-stimulated Akt Thr308 phosphorylation was decreased by 51% in L6 and 35% L6 SCD1 myotubes. There was no difference in total Akt protein (data not shown). Taken together, these results indicate that SCD1 partially protects skeletal muscle cells from fat-induced insulin resistance by blunting the suppressive effects of palmitate on the phosphorylation of Akt.
Figure 4.7 Glucose uptake and insulin signalling with SCD1 overexpression.

(A) 2-deoxyglucose uptake was assessed in myotubes following 5 h treatment with 2% fatty acid free BSA or 0.5 mM palmitate conjugated to 2% fatty acid-free BSA, with or without 100 nM insulin. (B) In similar experiments, myotubes were treated as described above and were lysed. Cell lysates were probed for phospho-Akt Ser 473 protein content. * P<0.05 vs Vehicle within the same group, † P<0.05 vs L6.
Serine kinase activation is influenced by SCD1 expression

The production of fatty acid metabolites within insulin responsive tissues is known to activate a host of serine kinases including IkBα, NFκB and JNK, which have been shown to impair insulin signal transduction at IRS-1 Ser 307 and glucose disposal (Yu et al., 2002; Gao et al., 2003). The effect of SCD1 protein content on the phosphorylation of JNK was determined. For all protein determinations, total protein contents were not different between conditions. In siRNA experiments, basal JNK phosphorylation was unaffected by SCD1 siRNA, whereas palmitate pre-treatment increased JNK phosphorylation to a greater degree in SCD1 siRNA compared with control siRNA L6 myotubes (Figure 4.8A). In overexpression experiments, JNK phosphorylation was not different in the basal state between L6 and SCD1 L6 myotubes (Figure 4.8C). Palmitate preincubation increased JNK phosphorylation in L6 myotubes, whereas no increase occurred in L6 SCD1 cells. The activation of the proinflammatory IkBα and NFκB was determined by measuring Ser 32 phosphorylation of IkBα, which indicates targeting of IkBα for degradation and nuclear translocation of NFκB. SCD1 siRNA resulted in increased phosphorylation of IkBα, both basally and with palmitate pre-treatment (Figure 4.8B), whereas overexpression of SCD1 protected L6 myotubes from palmitate-induced IkBα phosphorylation (Figure 4.8D).
Figure 4.8 Serine kinase activation with SCD1 knockdown and overexpression.

(A-B) L6 myotubes were transfected with control siRNA or SCD1 siRNA, then differentiated for 3 days. (C-D) L6 myoblasts were transfected with mock or SCD1 plasmids and differentiated for 3 days. Cells were exposed to control (2% BSA) media or 0.5 mM palmitate conjugated to 2% fatty acid-free BSA for 5 h. Myotubes were lysed in ice-cold buffer and subjected to SDS-PAGE and analysis of JNK phosphorylation (A and C) and IkBα phosphorylation (B and D) using polyclonal antibodies. * P<0.05 vs Vehicle within the same, † P<0.05 vs L6 SCD1 or SCD1 siRNA Palmitate where applicable.
4.4 Discussion

The above data demonstrate that SCD1 expression is elevated in the skeletal muscle of obese and diabetic rodents. SCD1 inhibition in L6 myotubes increased the accumulation of lipid metabolites, resulted in serum kinase activation and insulin resistance. In case of SCD1 overexpression, TAG esterification was increased whereas DAG and ceramide accumulation and serum kinase activation were reduced. These events were associated with protection from fatty acid-induced insulin resistance. Collectively, these data indicate that SCD1 protects skeletal muscle from lipotoxicity in the face of a high fat challenge.

Studies of mice with global genetic ablation of SCD1 (Cohen et al., 2002; Ntambi et al., 2002a) as well as mice treated with SCD1 antisense oligonucleotides inhibitors (Jiang et al., 2005a) have implicated SCD1 as a cause of obesity and insulin resistance. SCD1-/-mice are resistant to diet induced weight gain (Cohen et al., 2002; Ntambi et al., 2002a), have improved insulin signalling (Cohen et al., 2002; Rahman et al., 2003; Rahman et al., 2005) and increased fatty acid oxidation (Dobrzyn et al., 2005) and reduced fatty acid synthesis (Jiang et al., 2005a). But along with these positive effects knockdown models also developing undesirable genetic changes to compensate for the loss of SCD1.

The aim of these studies originated from conflicting observations. First, the primary role of SCD1 is to convert saturated fatty acids (primarily 16:0, 18:0) to monounsaturated fatty acids (16:1, 18:1) (Ntambi & Miyazaki, 2004). Second, incubating cells with saturated fatty acids leads to insulin resistance, secondary to increases in ceramide and DAG content (Schmitz-Peiffer et al., 1999; Montell et al., 2001; Chavez et al., 2003b). Third, global SCD1 ablation reduces skeletal muscle
ceramide accumulation (Dobrzyn et al., 2005) and improves insulin signalling and glucose tolerance (Rahman et al., 2003). To investigate the role of SCD1 in fat metabolism and the interaction with insulin action, experiments were conducted in L6 skeletal muscle myotubes with reduced (~60% of control), normal (100%) and elevated (135%) SCD1 protein content. Altering SCD1 protein content in L6 myotubes induced modest changes in fatty acid uptake and oxidation, changes that are unlikely to underpin the observed changes in lipid metabolites discussed herein. Ceramide and DAG were markedly elevated in SCD1 deficient cells, even when excess palmitate was not present in the culture media, suggesting a role of SCD1 in resulting lipid metabolism. Moreover, increasing SCD1 content largely prevented the palmitate-induced increase in ceramide and DAG.

Although the finding of the role of SCD1 is consistent with other studies in different cell types (Hulver et al., 2005) recently suggested that elevated expression of SCD1 in skeletal muscle contributes to abnormal lipid metabolism and the aetiology of obesity. While our data largely support these observations (Figure 4.5E), TAG accumulation per se is unlikely to cause insulin resistance (Kelley et al., 2002) or lipotoxicity (Busch et al., 2005), and in fact may provide a protective role by providing a sink for the deposition of fatty acyl-CoA metabolites.

In the present study, insulin-stimulated 2-deoxyglucose uptake was completely blunted in SCD1 siRNA cells, which occurred concomitantly with elevated ceramide and DAG levels. Moreover, these experiments demonstrate that basal levels of SCD1 cannot prevent palmitate-induced insulin resistance in muscle cells. Moreover, these experiments demonstrate that basal levels of SCD1 cannot prevent palmitate-induced insulin resistance in muscle cells. In contrast, overexpressing SCD1 prevented most of
the palmitate-induced reduction in insulin-stimulated glucose uptake, most likely via inhibition of ceramide accumulation. Ceramides negatively regulate insulin action downstream of PI 3-kinase by PP2A and PKCζ mediated dephosphorylation of Akt and preventing Akt plasma membrane recruitment (Powell et al., 2003; Powell et al., 2004; Stratford et al., 2004). The finding of reduced Akt Ser-473 and Thr-308 phosphorylation indicates that ceramide accumulation contributes to the palmitate suppression of glucose uptake in L6 myotubes (data not shown). JNK and the inhibitor of IκB kinase (IKK) pathways are also activated by fatty acids and directly interfere with insulin signal transduction (Yuan et al., 2001; Yu et al., 2002). Further studies examining IRS-1 serine and tyrosine phosphorylation are warranted.

In summary, the present findings provide a novel perspective on the tissue specific role of SCD1. Reducing SCD1 expression increases ceramide and DAG accumulation in L6 muscle myotubes exacerbates pro-inflammatory signalling and promotes insulin resistance. In contrast, overexpression of SCD1 provides a protective effect against FA-induced insulin resistance by limiting lipid metabolite accumulation and serine kinase activation. Thus, SCD1 may be elevated in skeletal muscles of obese animals to prevent the accumulation of fatty acyl-CoA metabolites that directly contribute to insulin resistance.
CHAPTER 5

GENERAL DISCUSSION
The objectives of this thesis were (1) to examine the effects of fatty acid subtype on fatty acid-induced insulin resistance and skeletal muscle SCD1 content and activity, and (2) to determine the role of SCD1 on substrate metabolism and fatty acid-induced insulin resistance in L6 myotubes *in vitro*.

The initial rodent study (Chapter 3) was conducted to examine the effects of dietary fatty acid subtype on skeletal muscle fatty acid metabolite accumulation and insulin resistance *in vivo*. This was based on several observations. Firstly, studies in rodents and humans demonstrate that diets high in saturated fatty acids induce insulin resistance, whereas diets high in unsaturated fatty acids generally have a less pronounced, or no effect on insulin-stimulated glucose disposal (Montell *et al.*, 2001; Riccardi *et al.*, 2004). Secondly, DAG and ceramide accumulate in response to palmitate, but not oleate, exposure in skeletal muscle myotubes (Chavez *et al.*, 2003b) and these fatty acid metabolites are known to interfere with insulin signalling. Thus, it was hypothesised that dietary saturated fatty acid induce insulin resistance *in vivo* due to the accumulation of ceramides and DAG in skeletal muscle. Results from this study showed that diets high in saturated fatty acids induce insulin resistance, which is associated with increased skeletal muscle DAG content. In contrast, a diet high in polyunsaturated fatty acids actually improved insulin sensitivity and resulted in marked increases in TAG with a modest increase in DAG. The ceramide content in both groups of rats fed high fat diets was not affected by composition, which contrasts previous studies indicating that ceramides cause insulin resistance (Turinsky *et al.*, 1990; Adams *et al.*, 2004; Gorska *et al.*, 2004; Straczkowski *et al.*, 2004). Collectively, these data indicated that the dietary fat subtype plays an important role in inducing insulin resistance, rather than fatty acid oversupply *per se*. 
An interesting observation from the studies in insulin resistant rodents was the marked increase in SCD1 mRNA, protein and activity with SAT (Figure 3.4A and B). These data are consistent with previous studies in rodents that implicate SCD1 is the aetiology of insulin resistance / type 2 diabetes (Cohen et al., 2002; Ntambi et al., 2002a). However, an alternative interpretation is that SCD1 is increased in these models as a consequence of increases in fatty acid fluxes / insulin resistance to prevent further deleterious events / lipotoxicity. Indeed, studies in other cell lines including chinese hamster ovary cells (Listenberger et al., 2001), showed that SCD1 affords protection from palmitate induced lipotoxicity. Whilst previous studies in mice with global SCD1 deletion indicate that SCD1 deficiency results in a leaner, more insulin sensitive phenotype; these studies are complicated by the findings of concomitant increases in basal energy expenditure and thermogenesis (Lee et al., 2004b).

Accordingly, an experiment was designed to directly examine the role of SCD1 on skeletal muscle fatty acid metabolism and insulin sensitivity. A transient in vitro design (ie: L6 myotubes) was implemented to allow for the determination of a tissue specific response and circumvent potential changes associated with genetic/stable SCD1 ablation (eg: increases in fatty acid oxidation proteins). This study does not, however, allow for valid conclusions to be made regarding the in vivo environment. The hypothesis of this study was that SCD1 deficiency would result in the accumulation of fatty acid metabolites within skeletal muscle, which would cause insulin resistance, whereas overexpression would protect from fatty acid induced insulin resistance by preventing the accumulation of fatty acid metabolites.

Altering SCD1 content in vitro in skeletal muscle myotubes had only minor effects on fatty acid oxidation and fatty acid uptake. Rather, decreasing SCD1 in vitro resulted in
increased esterification of palmitate into DAG and ceramide whereas increasing SCD1 expression prevented increases in these lipid metabolites and instead resulted in TAG accumulation. This is consistent with the role of SCD1 in desaturating fatty acids and the preference of DGAT, the terminal enzyme in TAG esterification, for unsaturated fatty acids. It was also shown that SCD1 in vitro is essential for the maintenance of insulin signalling in L6 myotubes, even in the absence of an exogenous fatty acid load. Moreover, native L6 myotubes were unable to cope with a palmitate load and became insulin resistant, whereas SCD1 in vitro overexpression partially protects cells from palmitate-induced insulin resistance. These findings are consistent with a protective role of SCD1 against lipotoxicity in several other cell types (Listenberger et al., 2003; Busch et al., 2005; Scaglia & Igal, 2005) and contradicts SCD1 knockout model studies (Cohen et al., 2002; Ntambi et al., 2002b; Jiang et al., 2005a). Some authors (Hulver et al., 2005) have suggested that elevated skeletal muscle SCD1 expression contributes to abnormal lipid metabolism and the aetiology of obesity. In this previous study conducted in primary human myotubes (Hulver et al., 2005), SCD1 overexpression decreased fatty acid oxidation and increased TAG synthesis. While our data largely support these observations, TAG accumulation per se is unlikely to cause insulin resistance (Kelley et al., 2002) or lipotoxicity (Listenberger et al., 2003; Busch et al., 2005; Scaglia & Igal, 2005), and in fact may provide a protective role by providing a reservoir for the deposition of other fatty acyl-CoA metabolites. Importantly, our data extend on these previous observations and indicate that TAG accumulation in SCD1 overexpressing cells protects cells from DAG and ceramide accumulation. Thus, the induction of SCD1 in skeletal muscle of obese, insulin resistant phenotypes may be a consequence rather than a cause of insulin resistance and may be initiated to prevent the progression of insulin resistance.
In chapter 3, insulin-stimulated 2-deoxyglucose uptake was abrogated in SCD1 siRNA cells and endogenous levels of SCD1 did not prevent palmitate-induced insulin resistance. In contrast, overexpressing SCD1 prevented most of the palmitate-induced reductions in insulin-stimulated glucose uptake, possibly via inhibition of ceramide accumulation. Ceramides negatively regulate insulin action downstream of phosphatidylinositol 3-kinase by protein phosphatase 2A and protein kinase Cζ mediated dephosphorylation of Akt and preventing Akt plasma membrane recruitment (Powell et al., 2003; Powell et al., 2004; Stratford et al., 2004). The finding of reduced Akt phosphorylation and activity indicates that ceramide accumulation contributes to the palmitate mediated suppression of glucose uptake in L6 myotubes. Thus, elevated ceramide synthesis and suppressed Akt action appear to contribute to the palmitate-induced insulin resistance. However, all these studies supports the hypothesis that SCD1 protects muscle cells from FA-induced insulin resistance by reducing ceramide and DAG accumulation. This interpretation supports the model of cellular lipid metabolism where TAG accumulation protects against lipotoxicity.

In conclusion, this thesis has demonstrated that SCD1 is elevated in the skeletal muscle of obese, insulin resistant rodents. While previous studies in global SCD1 knockout mice indicate that SCD1 is causative for insulin resistance, the results presented in this thesis indicate that reducing SCD1 causes insulin resistance and SCD1 overexpression protects skeletal muscle myotubes from fatty acid-induced insulin resistance. Thus, future studies should examine the role of SCD1 independent of the secondary effects associated with global SCD1 deletion. One such approach would be to examine skeletal muscle insulin sensitivity in mice with targeted disruption and / or overexpression of SCD1 in skeletal muscle.
CHAPTER 6
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Changes of Plasma Free Fatty Acids on Intramyocellular Fat Content and Insulin

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