NOTE TO USERS

Dissertation

THE ROLE OF CERAMIDE IN THE ANTAGONISM OF INSULIN SIGNAL TRANSDUCTION BY FREE FATTY ACIDS IN SKELETAL MUSCLE

Submitted by
José Antonio Chávez
Department of Biochemistry and Molecular Biology

In partial fulfillment of the requirements for the
Degree of Doctorate of Philosophy
Colorado State University
Fort Collins, Colorado
Fall, 2004

COLORADO STATE UNIVERSITY

July 16, 2004

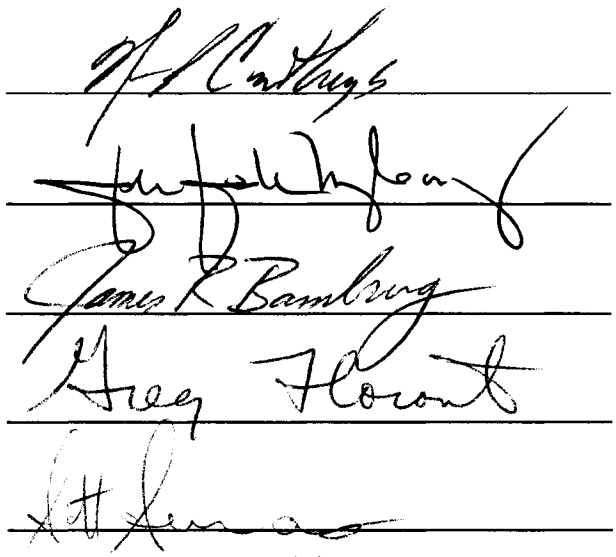
WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JOSE ANTONIO CHAVEZ ENTITLED

THE ROLE OF CERAMIDE IN THE ANTAGONISM OF INSULIN SIGNAL TRANSDUCTION BY FREE FATTY ACIDS IN SKELETAL MUSCLE

BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF

Doctorate of Philosophy

Committee on Graduate Work



Adviser

Department Head

ABSTRACTS OF DISSERTATION

The role of ceramide in the Antagonism of Insulin Signal Transduction by Free Fatty Acid in Skeletal Muscle

Obesity is highly associated with insulin resistance and is the major risk factor for Type 2 diabetes mellitus and Metabolic Syndrome X. However, the connection between expanded adipose mass and the development of insulin resistance in skeletal muscle, the tissue responsible for the majority of postprandial glucose uptake, is not clearly established. Recent studies suggest that circulating factors released by adipose tissue such as free fatty acids (FFAs) induce insulin resistance in peripheral organs by promoting the accumulation of ectopic fat-derived metabolites capable of attenuating insulin action. Specifically, numerous studies implicate the sphingolipid ceramide as a potential mediator linking lipid oversupply to the antagonism of insulin action. Ceramide is a direct product of fatty acyl-CoA that has been shown to accumulate in insulin resistance states and to inhibit early steps in insulin signaling. The studies described herein investigate the hypothesis that overaccumulation of ceramide is an important event in the development of muscle insulin resistance. We first demonstrated that induced ceramide buildup by exposing cultured C2C12 myotubes to long-chain saturated FFAs inhibits insulin stimulation of Akt/PKB, a key regulator of glucose uptake and anabolic metabolism. To further evaluate the contribution of ceramide to the saturated FFA-dependant inhibition of insulin signaling, we employed different strategies to modulate intracellular ceramide levels in C2C12 myotubes. First, inhibitors of the *de novo* ceramide synthesis prevented the

antagonistic effects of saturated FFAs on insulin signaling. Second, inhibitors of ceramide catabolism recapitulated and augmented the effects of saturated FFAs on both intracellular ceramide accumulation and the inhibition of insulin signaling. Third, overexpression of human acid ceramidase, an enzyme that catalyzes the lysosomal degradation of ceramide, protected cells from the inhibitory effects of long-chain saturated FFAs. Collectively, these studies demonstrated that ceramide is the principal intermediate linking saturated FFAs to the inhibition of insulin signaling, and implicate aberrant ceramide accumulation in the development of muscle insulin resistance.

José Antonio Chávez
Department of Biochemistry and Molecular Biology
Colorado State University
Fort Collins, CO 80523
Fall 2004

ACKNOWLEDGEMENT

I express my most sincere gratitude all the persons that have contributed to my academic success. First, I thank all teachers, instructors and professors that I have had during all my student years. From many of them, I have learned not only science, but also many other important aspects of life. Many are in Perú, in my beloved school "La Inmaculada" and in my college "Universidad Peruana Cayetano Heredia". I give a special acknowledgement to Dr. James Mattoon, who gave the first opportunity to study in the US. He was my mentor during my master studies and certainly one of the persons who directly helped me to reach to this point. I am truly thankful to him I also want to thank my PhD student advisory members: Dr. James Bamburg, Dr. Norm Curthoys, Dr. Jennifer Nyborg and Dr. Greg Florant for their counseling, expertise, and for giving me the proper directions to complete my research project.

I would like to give a special thank to my thesis advisor Dr. Scott Summers, for welcoming me in his laboratory, and for the help and assist during planning and accomplishment of the experiments, especially when things were critical. He truly was an excellent adviser, a funny boss, and better friend. Besides, I thank Scott for guiding me through the writing process, and for supporting me during all my presentations. I also thank all member of the Summers' lab for their support and specially for their friendship. Thank you Suzanne Stratford, Farida Chamberlain, Kyle Hoehn, Li-Ping Wang, Trina Knotts, William Holland, Julia Truong, and Jessica

Rubio. I could not leave behind my good friend Dr. Carlina Barillas-Mury for helping me with courses during the first years of my PhD. It was very pleasant to discuss science with her over pizza and few beers.

I thank my family for believing in me during all these time. Their encouragement and presence were significant in all aspects. I specially thank my parents for giving everything in life. They always believed that education was the best gift that anyone can receive from their parents, and I certainly agree with that. They were the most enthusiastic that I complete my studies. I dedicate this dissertation to my father, who unfortunately passed away without being able to see me becoming a doctor. I know for sure that he proud and happy for me right at this moment. Finally, I give my deepest gratefulness to Norma. She has been by my side since the beginning of my PhD, giving me love and support. Nothing could have been achieved without her. She rescued me and made me a better person.

TABLE OF CONTENT

ABBREVIATIONS

^{1}H NMR:	nuclear magnetic resonance spectroscopy
4E-BP:	4E-binding proteins
5'TOP:	5' terminal oligopyrimidine
AC:	acid Ceramidase
AS160:	Akt substrate 160
BSA:	bovine serum albumin
C2-Cer:	C2-ceramide
CAPK:	ceramide activated protein kinase
CAPP:	ceramide activated protein phosphatase
DAG:	diacylglycerol
DMEM:	Dulbecco's modified Eagle's medium
ER:	endoplasmic reticulum
FBS:	fetal bovine serum
FFA:	free fatty acid
FOXO:	forkhead box class O transcription factors
GFAT:	fructose 6-phosphate amidotranferase
GFP:	green fluorescent protein
GlcNAc:	acetyl-glucosamine
GSK3β:	glycogen synthase kinase 3-beta
HRP:	horseradish peroxidase
IMCL:	intramyocellular lipid
IR:	insulin receptor
IRS:	insulin receptor substrate
KO:	knock out
LCACoA:	long chain acyl coenzyme A
LPL:	lipoprotein lipase
MAPK:	mitogen activated protein kinase
MTOR:	mammalian target of rapamycin
NOE:	N-oleoylethanolamine

OA:	okadaic acid
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PDK1:	phosphoinositide dependent kinase-1
PDMP:	D-L-threo 1-phenyl-2-decanoylamino-3-morpholino-1-propanol
PH:	pleckstrin homology
PHAS-1:	phosphorylated heat-and acid-stable polypeptide regulated by insulin-1
PI3K:	phosphatidylinositol 3-kinase
PIP_2:	phosphatidylinositol (3,4) biphosphate
PIP_3:	phosphatidylinositol (3,4,5) biphosphate
PKB:	protein kinase B
PKC:	protein kinase C
PP1:	protein phosphatase 1
PP2A:	protein phosphatase 2A
PPAR:	peroxisome proliferators activated receptors
PTB:	phosphotyrosine binding domain
RAC:	related to A and C protein kinase
S1P:	sphingosine 1-phosphate
SK:	sphingosine kinase
SPT:	serine palmitoyltranferase
TAG:	triacylglycerol
TLC:	thin layer chromatography
TNF-α:	tumor necrosis factor-alpha
WHO:	world health organization

CHAPTER I

The Role of Lipids in the Pathogenesis of Muscle Insulin Resistance in Type 2 Diabetes and Obesity

1.1 Introduction to Diabetes Mellitus

Diabetes mellitus is a chronic disease characterized by abnormally high levels of glucose in the blood due to deficiency in insulin secretion, insulin action or both (1). The resulting hyperglycemia of diabetes is associated with long-term damage and subsequent dysfunction of various organs causing serious complications. These generally occur after several years of diabetes and affect especially the blood vessels (microangiopathy) in the eye, kidney and nerves. The frequency of arterial disease (atherosclerosis or macroangiophaty) is also markedly increased during the diabetes onset. Thus, individuals with uncontrolled diabetes are at high risk of coronary hearth disease, stroke and peripheral vascular disease.

The majority of cases of diabetes fall into two major categories of pathogenesis. Type 1 diabetes mellitus is caused by an absolute deficiency of insulin secretion. It presents mainly in childhood and early adults and accounts for about 10-20% of cases. Type 1 diabetes is thought to be caused by an autoimmune destruction of the insulin-producing β cells of the islets of Langerhans (2). In type 2 diabetes, the cause of the disease is a combination of factors including resistance of the body's cells to the action of insulin coupled with an inadequate insulin secretory response (3). Type 2 diabetes usually starts in middle age or in the elderly and is by far the most common form of this disease, representing about 80-90% of all cases (3). Genetic and environmental factors contribute to the heterogeneous etiology behind the development of this complex metabolic disease.

Type 2 diabetes has a large public health impact, and is the first metabolic disease in the world to reach epidemic proportions. According to the World Health Organization (WHO), Type 2 diabetes afflicts more than 140 million people worldwide and over the next decade, is projected to affect in excess of 212 million (4). People living in developing countries and ethnic minorities in industrial societies are afflicted with highest frequency. In the United States, 6.5% of the population has diabetes (5). This amounts to more than 16 million people of all ages, races, educational levels, weights, and geographic distributions (6). Moreover, the complications resulting from diabetes are significant causes of morbidity and mortality (7).

In developed countries, diabetes care is responsible for up to 5% of total healthcare costs. In the US alone, the total annual economic cost of diabetes in 2002 was estimated to be 132 billion dollars, both because of medical expenditures and lost productivity (8). If diabetes prevalence rates remain constant, the number of people with diabetes could increase to more than 25 million over the next decade, which would carry with it an annual cost of approximately $160 billion dollars per year (8). Such estimates do not account for the losses attributable to pain and suffering incurred by people with diabetes, as well as to families and friends of those burdened with the disease.

The impact of diabetes on healthcare emphasizes the importance of more intensive disease management, and the advent of new medical technologies to affront this problematic disease.

1.2 Overview of the Physiology of Insulin Secretion and Action

Normal subjects maintain blood glucose levels within a relatively narrow range (i.e. around 5 mM or 90 mg/dl) by balancing glucose entry into the bloodstream from the liver and from intestinal absorption after meals, and glucose uptake into the peripheral tissues (e.g. skeletal muscle, adipose tissue, etc.). The sources of hepatic glucose production are glycogenolysis (the breakdown of glycogen, the storage form of glucose) and gluconeogenesis (the synthesis of new glucose from noncarbohydrate precursors). After 8 to 12 hours without food intake, gluconeogenesis by the liver is the main source of circulating glucose. At this time, glucose is utilized mainly by the brain and red blood cells, whereas the rest of the tissues use free fatty acids (FFAs). Glucose is the main fuel for the brain, and this organ depends critically on the maintenance of normal blood glucose levels.

After digestion, glucose and other nutrients pass into the bloodstream and are taken up by peripheral tissues (primarily liver, skeletal muscle, and adipose). In the postprandial state, β cells within the islets of Langerhans in the pancreas sense circulating glucose levels and automatically secrete the appropriate amount of the hormone insulin to promote the clearance of blood glucose to normal levels. Between meals, β cells secrete low, basal amounts of insulin.

Insulin lowers circulating glucose levels by suppressing glucose output from the liver (glycogenolysis and gluconeogenesis) and by promoting glucose uptake and storage in muscle and fat, the two primary sites of insulin-dependent glucose disposal (Figure

1.1). Relatively low amounts of insulin, such as the low, basal level found between meals, are sufficient to suppress hepatic glucose output. Higher concentrations of insulin, like those found postprandially, dramatically stimulate glucose uptake. Therefore, insulin is a key regulator of glucose homeostasis. An absence of insulin results in an elevated blood glucose concentration, while an excess of the hormone results in hypoglycemia. Both of the above circumstances can be life threatening. The basic functions of insulin appear to be well conserved among animal species as disparate as nematodes, fruit flies and mammals. Upon entering the muscle cells, the enzyme hexokinase rapidly phosphorylates glucose, which is subsequently stored as glycogen due to the insulin-mediated activation of the enzyme glycogen synthase (GS), or oxidized to generate ATP synthesis in the mitochondria. The ability of insulin to stimulate glucose transport and glycogen synthesis is one of its most important physiologic actions.

Insulin also has a profound influence on fat and protein metabolism in order to meet the energy demands of the organism. Ingested fat is stored as triacylglycerides (TAG) in the central lipid droplet of adipocytes. Because of its high caloric content and efficient storage form, adipose tissue TAG represents the major source of stored fuel available for mobilization when energy requirements are increased or when glucose availability is reduced. Insulin plays a central role in the synthesis, storage, mobilization and utilization of adipose tissue TAG (9). The integrated physiologic effects of insulin on lipid metabolism facilitate TAG storage (lipogenesis), and restrain its breakdown (lipolysis) and release of FFAs from endogenous fat stores (9).

Since the breakdown of protein into their constitutive amino acids is a perpetual process, it is important for the maintenance of body protein homeostasis that protein synthesis also occurs on a continuous basis. Insulin participates actively in the control of proteolysis, protein synthesis, amino acid oxidation, and amino acid transport. Insulin's primary function is to decrease proteolysis, which results in decreased amino acid availability and to increase protein deposition, which is amplified in the presence of exogenous amino acids (9).

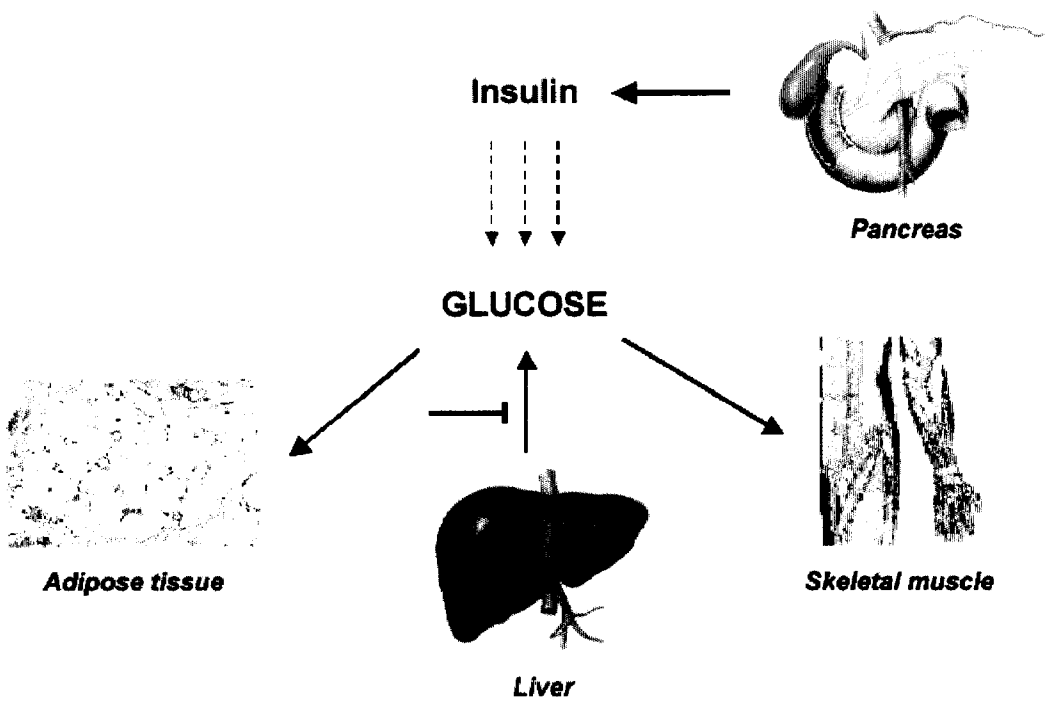


Figure 1.1 Physiologic effects of insulin. Elevated concentrations of glucose in blood stimulate release of insulin by pancreatic β cells. The effects of insulin on glucose metabolism vary depending on the target tissue. Insulin facilitates entry of glucose into skeletal muscle and adipose while simultaneously repressing glucose efflux from the liver. Diabetes mellitus results from defects in insulin action (insulin resistance) and/or insulin secretion

1.3 Signaling Transduction Pathways Mediating Insulin Action

The mechanism by which insulin stimulates the glucose uptake into muscle and fat tissue involves the redistribution of the glucose transporter isoform GLUT4 from an intracellular location to the plasma membrane, a process known as GLUT4 translocation. Insulin effects the movement of specific intracellular vesicles containing GLUT4 to the cell surface, where they fuse with the plasma membrane. This action acutely increases the number of glucose transporters available to facilitate entry of glucose into the cell interior (Figure 1.2). Targeted disruption of GLUT4 selectively in muscle (10) or adipose (11) causes overt diabetes in transgenic mice, indicating that GLUT4-mediated glucose transport is essential to the maintenance of normal glucose homeostasis. The critical role of GLUT4 translocation in regulating whole-body metabolism explains the intense effort by many laboratories to understand this process at the molecular levels and how this might be altered in diabetes.

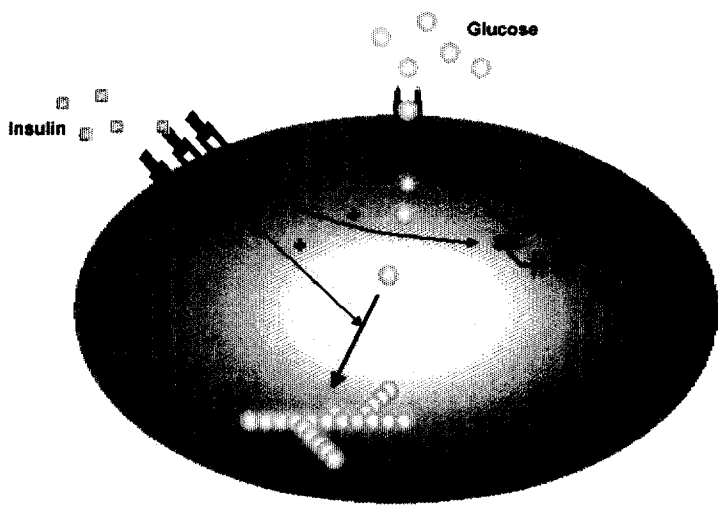


Figure 1.2. Cellular effects of insulin action. Under basal conditions, GLUT4 glucose transporters are largely localized in cytoplasmic vesicles of muscle and adipose cells. Binding of insulin to its cell surface receptors triggers a signaling pathway that promotes the fusion of those vesicles with the plasma membrane, thus increasing the number of glucose transporters exposed to the extracellular milieu. Insulin also stimulates the storage of glucose in the form of glycogen

Insulin stimulates its pleiotropic effects through the activation of a signaling cascade initiated by its binding to a cell surface receptor (Figure 1.3). Almost all mammalian tissues contain insulin receptors, although the number varies greatly, with the highest number being present in insulin-sensitive tissues such as skeletal muscle and adipose (2-3 x 10^5 receptors per cell) (12). The insulin receptor (IR) is a glycoprotein consisting of two α subunits (135 kd) and two β subunits (95 kd) covalently linked through disulfide bonds to form an $\alpha_2\beta_2$ heterotetramer. The α subunit is entirely extracellular and contains the sites for insulin binding, whereas the β subunit has a small extracellular portion, a transmembrane domain, and an intracellular insulin-regulated tyrosine protein kinase activity (12). The insulin receptor behaves as a classic allosteric enzyme in which the binding of insulin to the α subunit results in a conformational change that allows the activation of intrinsic tyrosine kinase activity of the β subunit (13, 14). Subsequent autophosphorylation of specific tyrosine amino-acids residues within the receptor's β subunit in turn results in activation of the kinase activity toward various intracellular protein substrates. Tyrosine kinase activity of IR is essential for intracellular insulin action.

The major cytoplasmic substrate for the enzyme activity of the IR is a group of docking proteins known as the insulin receptor substrates (IRS's). The best characterized of these is a 185-kd protein called insulin receptor substrate-1 (IRS1). Based on its predicted amino acid sequence, IRS1 contains 22 potential tyrosine phosphorylation sites, numerous sites for serine phosphorylation sites and a specific phosphotyrosine binding (PTB) domain (15). The PTB domain is critical for recognition and interaction of

phosphotyrosine residues in activated IR (15). Once phosphorylated, IRS1 serves as a docking protein, which recruits and activates multiple downstream targets.

One important signaling pathway emanating from IRS1 that mediates multiple insulin effects begins with the binding and activation of the enzyme phosphatidylinositol (PI) 3-kinase (PI3K) (16). PI3K is a heterodimer composed of a regulatory 85-kd subunit (usually referred as p85 subunit) and a catalytic 110-kd subunit (p110) (17). The p85 subunit contains two Src-homology 2 (SH2) domains, which direct noncovalent interactions with specific phosphotyrosine-containing peptide motif, and one SH3 domains, a region that directs protein-protein interaction to sites independent of tyrosine phosphorylation (17). Binding of the p85 subunit to tyrosine phosphorylated IRS1 enables the formation of a ternary complex and is an activation step that results in a marked increase in PI3K activity of the p110 catalytic subunit (18).

PI 3-kinase, in turn, phosphorylates specific membrane phosphoinositides such as phosphatidylinositol 4-monophosphate and phosphatidylinositol 4,5-biphosphate on the D-3 position of the inositol ring to generate phosphatidylinositol-3,4-biphosphate ($PI(3,4)P_2$) and phosphatidylinositol-3,4,5-triphosphate ($PI(3,4,5)P_3$) respectively (19). The cellular levels of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ depend on the activity of PI3K and serve as second messengers for essentially all metabolic responses to insulin.

Several studies have confirmed a role for PI3K on insulin-induced activation of glucose transport. For instance, inhibition of PI3K activity using a dominant negative

mutant (20) or pharmacological agents, such as wortmannin (21) or LY294002 (22), abolishes insulin-stimulated glucose uptake and inhibits GLUT4 vesicle translocation to the plasma membrane. On the other hand, expression of constitutively active PI3-kinase stimulates these effects (23, 24).

The increased levels of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ activate downstream events by recruitment cytosolic enzymes to the plasma membrane as a result of directly binding to their pleckstrin homology (PH) domain. PH domain is the structure most frequently encountered in proteins capable of binding specifically and with high affinity D-3 phosphoinositides. Various studies have shown that $PI(3,4)P_2$ and $PI(3,4,5)P_3$ contribute to activation of several serine/threonine kinase proteins including 3-phosphoinositide-dependant protein kinase-1 (PDK1) and Akt or protein kinase B (Akt/PKB) (25).

1.3.a *Regulation of Akt/Protein Kinase B (PKB)*

Akt/PKB was cloned in 1991 by three independent laboratories. These groups identified the enzyme as Related to A and C Protein Kinase (RAC) (26), Protein Kinase B (PKB) (27) and cAkt (cellular homologue of the Akt retrovirus oncogene) (28). However, the first name has been dropped to avoid confusion with the small GTP-binding protein Rac. Three closely related isoforms Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ) are now known, each composed of an N-terminal PH domain and a serine/threonine-rich C-terminal kinase catalytic domain (26-30). They belong to a subfamily of protein kinases that regulate a diverse array of cellular functions including

protection from apoptosis, stimulation of cellular growth and proliferation, differentiation and intermediary metabolism (31).

Numerous hormones, growth factors, cytokines, and transforming oncogenes activate Akt/PKB. Akt/PKB activation depends on the presence of $PI(3,4)P_2$ and to a lesser extent on $PI(3,4,5)P_3$ (32). Although initial studies indicated that the interaction between Akt/PKB and the phospholipids directly enables Akt/PKB activation, subsequent studies revealed that the kinase is activated instead by phosphorylation (33). Indeed, the interaction of $PI(3,4,5)P_3$ with the PH domain of Akt/PKB promotes the translocation of the enzyme to the plasma membrane where it undergoes a conformational change resulting in exposure of two regulatory phosphorylation sites: One resides in the activation loop of the kinase domain (Thr308), and the other is located ~160 residues C-terminal to this site in a non-catalytic region of Akt/PKB (Ser473) (34). Both phosphorylations are required for full activation of the enzyme. PDK1 has been characterized as the kinase that phosphorylates Akt/PKB at Thr308. The identity of the kinase that phosphorylates Akt/PKB at Ser473 (usually referred as PDK2), and the mechanism underlying regulation of this second phosphorylation, remains elusive.

Several lines of evidence suggest that Akt/PKB functions downstream of PI3K. First, insulin stimulation of Akt/PKB is inhibited by LY294002 and/or wortmannin in L6 myotubes (35, 36), human myoblasts (37), human skeletal muscle (38) and rat adipocytes (39). Second, expression of dominant negative forms of PI3K blocks Akt/PKB activation by Platelet-derived Growth Factor (PDGF) (40). Third, expression of constitutively

active PI3K (targeted to the membrane by either N-terminal myristylation or C-terminal farnesylation) results in Akt/PKB activation (41).

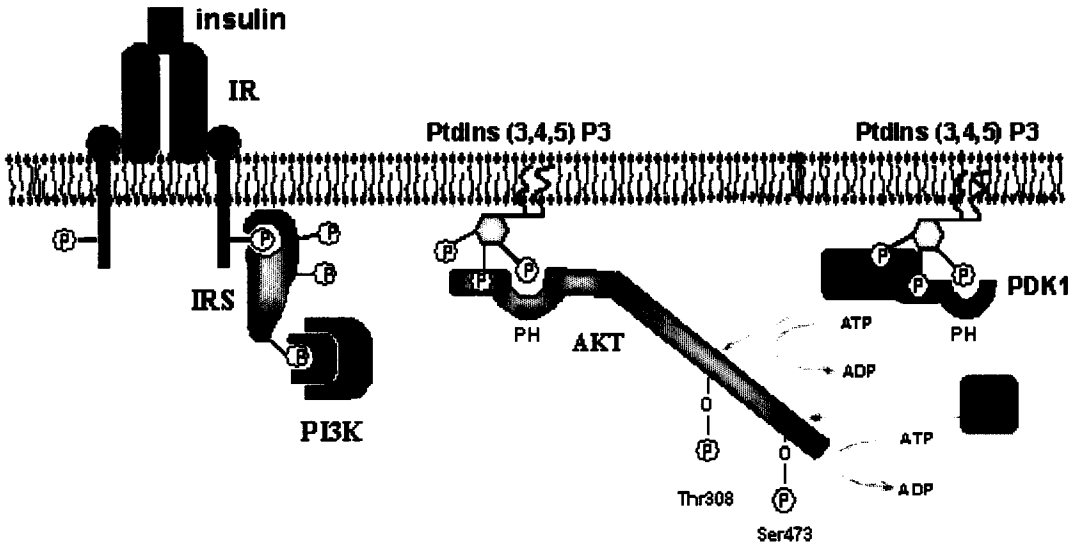


Figure 1.3. Mechanism of Akt/PKB activation. Insulin activates the receptor's intrinsic tyrosine kinase activity, promoting the tyrosine phosphorylation of the receptor itself and a family of intracellular adaptor molecules (termed insulin receptor substrate (IRS) proteins]. Phosphorylation of IRS proteins effects the recruitment of proteins that contain SH2 domains, leading to the activation of downstream effectors enzymes including phosphatidyl inositol 3-kinase (PI3K). Once activated, PI3K phosphorylates the inositol head group of membrane phosphatydilinositides, generating the phospholipids signaling molecules PIP_2 and PIP_3. Akt/PKB binds to membrane associated D3 of PIP_2 and PIP_3 via its PH domain, where it undergoes phosphorylation at Thr308 (by PDK1) and at Ser473 (by the elusive PDK2 kinase).

1.4 The Central Role of Akt/PKB in Metabolic Insulin Action

Activated Akt/PKB has been shown to mediate various metabolic effects of insulin (Figure 1.4). For instance, overexpression of either wild-type or constitutively active form of Akt/PKB in 3T3-L1 adipocytes and L6 myotubes mimics insulin-induced GLUT4 translocation, glycogen and protein synthesis (42, 43). Akt/PKB can be rendered constitutively active by either coupling it to membrane targeting sequences (i.e.

myristylation) or by exchanging both phosphorylation sites with acidic residues. Conversely, expression of a dominant negative form of Akt/PKB (kinase inactive mutant) significantly inhibits these metabolic responses of insulin in some cell lines (44-46). In addition to these experiments, further studies have investigated the regulation of specific isoforms of Akt/PKB and their involvement in insulin action. Microinjections of antibodies to the Akt2/PKBβ isoform into 3T3-L1 adipocytes partially blocked insulin stimulation of GLUT4 translocation (47). Moreover, experiments using RT-PCR and isoform specific antibodies revealed that expression of Akt2/PKBβ, but not Akt1/PKBα, increases as 3T3-L1 fibroblasts differentiate into insulin responsive, metabolic active adipocytes (48). Additional studies support the involvement of the Akt2/PKBβ isoform in glucose metabolism. Interestingly, knockout mice lacking the Akt2/PKBβ gene are impaired in the ability of insulin to lower blood glucose because of defects in the action of the hormone in fat and skeletal muscle (49). However, in striking contrast to Akt2/PKBβ null mice, Akt1/PKBα-deficient mice are normal with regard to glucose tolerance, suggesting that Akt2/PKBβ isoform is indispensable in the maintenance of normal glucose homeostasis *in vivo* (50). Thus, Akt2/PKBβ has been implicated as the central mediator of insulin-stimulated glucose transport in skeletal muscle and adipose tissue.

1.5 Downstream Targets of Akt/PKB

A variety of approaches have been applied to the identification of physiological Akt/PKB substrates leading to insulin-induced biological actions. The designation of a protein as a direct substrate of Akt/PKB requires that the candidate be directly

phosphorylated by Akt/PKB at the motif RXRXXT/S, where X is any amino acid and T/S is threonine or serine residues, respectively. Only a few well-supported Akt/PKB substrates have been identified so far (Figure 1.4). Nonetheless, among these are unquestionably crucial multifunctional effectors of insulin's action.

1.5a *Akt/PKB Substrate 160*

The insulin-regulated translocation of GLUT4 containing vesicles is a critical process necessary for the maintenance of normal glucose homeostasis. Intense efforts have been directed toward defining the signaling elements that link insulin receptor activation to the trafficking of GLUT4 vesicles in adipocytes and muscle cells.

Recently, a novel protein of 160 kd has been identified as the key connection between signaling from Akt/PKB to the trafficking of the GLUT4 vesicles. This has been designated as AS160 for Akt substrate of 160 kd (51). The most prominent feature of AS160 is that it contains five phosphorylation sites that lie in the RXRXXS/T motif preferred by Akt/PKB, and a GTPase activating protein domain (GAP domain) for the Rab family of small GTP-binding proteins. AS160 also contains two predicted phosphotyrosine binding (PTB) domains toward its amino terminus (51). Rab proteins in their active GTP-bound form are key components in the fusion of vesicles with membranes, and also in some cases participate in vesicle movement. In the absence of insulin, the Rab that presumably functions in GLUT4 translocation is maintained in its inactive GDP form by the GAP activity of AS160. Rabs have an intrinsic capacity to hydrolyze their bound GTP to GDP; however this activity is typically very low. Insulin-

stimulated Akt/PKB activity elicits phosphorylation of AS160, inactivating its GAP activity and increasing the amount of active Rab triggering GLUT4 translocation. However, the identification and characterization of the Rab that is required for GLUT4 translocation has not been accomplished yet.

A recent study by the Lienhard group strongly suggests that insulin-stimulated phosphorylation of AS160 is required for GLUT4 translocation in 3T3-L1 adipocytes (52). Specifically, overexpression of AS160 lacking several of the putative Akt/PKB phosphorylation sites in 3T3-L1 adipocytes markedly inhibited insulin-stimulated GLUT4 translocation compared with wild-type AS160. Moreover, this inhibition did not occur when the GAP activity in these mutants was inactivated by a point mutation indicating that inactivation of the Rab GAP function is required for GLUT4 translocation (52).

1.5b *Glycogen Synthase Kinase-3*

In humans, greater than 70% of postprandial glucose is ultimately stored as glycogen in peripheral tissues. As stated above, an important role of insulin is to promote glycogen accumulation. In fact, impaired glycogen metabolism is associated with diabetes and may represent a critical event in the pathophysiology of the disease. Insulin promotes glycogen synthesis both by increasing rates of glucose transport and by activating glycogen synthase (GS), which catalyzes the final step in glycogen synthesis by adding activated glucosyl groups to growing polysaccharide chains. The regulation of GS is complex and involves allosteric activators as well as regulation by

phosphorylation/dephosphorylation. In particular, the activity of GS is potently inhibited by its phosphorylation on four serine residues on the C-terminal portion. Glycogen Synthase Kinase-3b (GSK3β) is one of the kinases responsible for inactivating GS, and this enzyme is itself inactivated via phosphorylation by Akt/PKB.

GSK3 was purified as a kinase capable of phosphorylating and inactivating GS. Molecular cloning revealed two GSK3 genes that encode very similar polypeptides; GSK3α (51 kd) and GSK3β (47 kd), which are expressed ubiquitously in mammalian tissues (53). GSK3 is fully active in unstimulated cells, and undergoes a rapid inhibition in response to insulin and other growth factors. Upon insulin stimulation, Akt/PKB activity results in the phosphorylation at a single serine residue situated N-terminal to the catalytic domain (Ser21 in GSK3α and Ser9 in GSK3β) and in the subsequent inhibition of GSK3 (36). The insulin-stimulated inhibition of GSK3 diminishes GS phosphorylation and promotes its activation, contributing to the stimulation of glycogen synthesis. Like the activation of Akt/PKB, the inhibition of GSK3 by insulin is prevented by the PI3K inhibitors wortmannin and LY294002 (36).

Numerous studies suggest the significance of GSK3 in the insulin regulation of glycogen synthesis. For example, overexpression of a GSK3 S9A mutant, which is insensitive to the activity of Akt/PKB, in adipocytes results in suppression of insulin-stimulated GS activity (54). Similarly, the presence of constitutively active Akt/PKB in L6 muscle cells significantly blocks GSK3 activity and leads to GS activation in the absence of insulin (43), whereas a dominant-negative Akt/PKB results in 50% inhibition

of insulin-stimulated GS activation in these cells (44). However, the absolute contribution of GSK3 to the regulation of GS remains uncertain since several signaling molecules that are not influenced by GSK3 can modulate this process. Indeed, insulin-stimulated glycogen synthesis may involve activation of a protein phosphatase type 1 (PP1), which has been shown to dephosphorylate GS leading to its activation. PP1 is a serine/threonine-specific phosphatase implicated in the regulation of glycogen synthesis (55).

1.5c *Mammalian Target of Rapamycin*

The ability of insulin to activate protein synthesis at the translational level is one of the central anabolic responses of insulin. Insulin promotes protein synthesis in muscle and fat cells by stimulating the initiation and elongation steps in protein translation.

An important step for protein synthesis initiation consists of the preparation of the mRNA 5' untranslated segment for scanning by the 40S ribosomal complex. This process involves the binding of an mRNA 5' cap structure (7-methyl GTP) by the cap-binding protein eIF-4E. One regulatory mechanism for this process involves the control of availability of eIF-4E, which is negatively regulated through the binding of eIF-4E to a family of abundant 4E-binding proteins (4E-BPs) (the latter also known as phosphorylated heat-and acid-stable polypeptide regulated by insulin or PHAS-1) (56). The interaction of eIF-4E with 4E-BP1 is abolished by the insulin-activated multisite phosphorylation of the 4E-BP1, which releases eIF-4E and subsequently promotes its incorporation into the initiation complex (57). The protein kinase responsible for the

phosphorylation of 4E-BP1 is the mammalian Target of Rapamycin (mTOR), a direct target of activated Akt/PKB (58).

mTOR is a 289-kd serine/threonine kinase orthologue of target of rapamycin-1 (TOR1) and TOR2 in *Saccharomyces cerevisiae* (59). Direct evidence indicates that insulin-stimulated Akt/PKB kinase activity is clearly required for the effects of mTOR on 4E-BP phosphorylation. First, the insulin-stimulated phosphorylation of 4E-BP/PHAS is completely inhibited by wortmannin (57). Also, dominant-inhibitory Akt/PKB partially blocks 4E-BP phosphorylation by insulin in L6 muscle cells (44), and constitutively active, membrane targeted Akt/PKB results in robust phosphorylation of the protein (60). In addition, translation of mRNAs encoding polypeptides of the translational apparatus itself (e.g. ribosomal proteins and elongation factors) and various "growth-regulated" proteins requires the presence of continuous tract of 5 to 14 pyrimidines residues immediately at the transcriptional initiation site. Insulin selectively promotes the translation of mRNA containing this 5' terminal oligopyrimidine (5' TOP) by a wortmannin-sensitive mechanism, indicating that the PI3K pathway also controls the regulatory apparatus that gates the initiation of 5' TOP mRNAs (61). The incorporation of 5' TOP mRNAs into polysomes requires the phosphorylation and activation of the 40S ribosomal protein S6 by its immediate upstream regulator; p70 S6 kinase (62). Similarly, activation of p70 S6 kinase is achieved through an ordered series of phosphorylation of the p70 polypeptide catalyzed by mTOR. Thus, the Akt/PKB activity is crucial for the expression of 5' TOP mRNAs, a function that accounts for its role in protein synthesis.

1.5d *The Forkhead Domain Transcription Factors*

Insulin regulates the transcription of genes involved in a variety of biological phenomena such as cellular growth and differentiation. Therefore it is likely that many of the effects of Akt/PKB signaling result in the regulation of gene expression. Genetic analyses in the nematode worm *Caenorhabditis elegans* established that PI3K suppresses the function of DAF-16, a Forkhead (FOX) transcription factor that regulates organism lifespan (63). Subsequent studies revealed evolutionarily conserved orthologues of DAF-16 in higher eukaryotes. These proteins are members of a family of Forkhead box class O (FOXO) transcription factors. Members of the Forkhead superfamily are characterized by the presence of putative consensus sequences for Akt/PKB phosphorylation (RXRXXS/T) and a conserved 110-amino acid DNA binding domain, called the "forkhead" domain that binds to the major groove of DNA (64). Although the mechanism by which Akt/PKB regulate FOXO activity is not completely clear, it is believed that Akt/PKB can directly phosphorylate FOXO transcription factors, and thereby inhibit their ability to induce the expression of target genes (65). In the absence of stimulation, FOXOs are localized in the nucleus, where they activate transcription of target genes. Upon activation by insulin, Akt/PKB phosphorylates FOXOs at the specific regulatory sites, eliciting their relocalization from the nucleus to the cytoplasm and preventing them from stimulating gene transcription (65). In addition, FOXO transcription factors appear to be able to regulate transcription through DNA-independent mechanisms. FOXO transcription factors have been found to play critical roles in regulation of proliferation, apoptosis and control of oxidative stress.

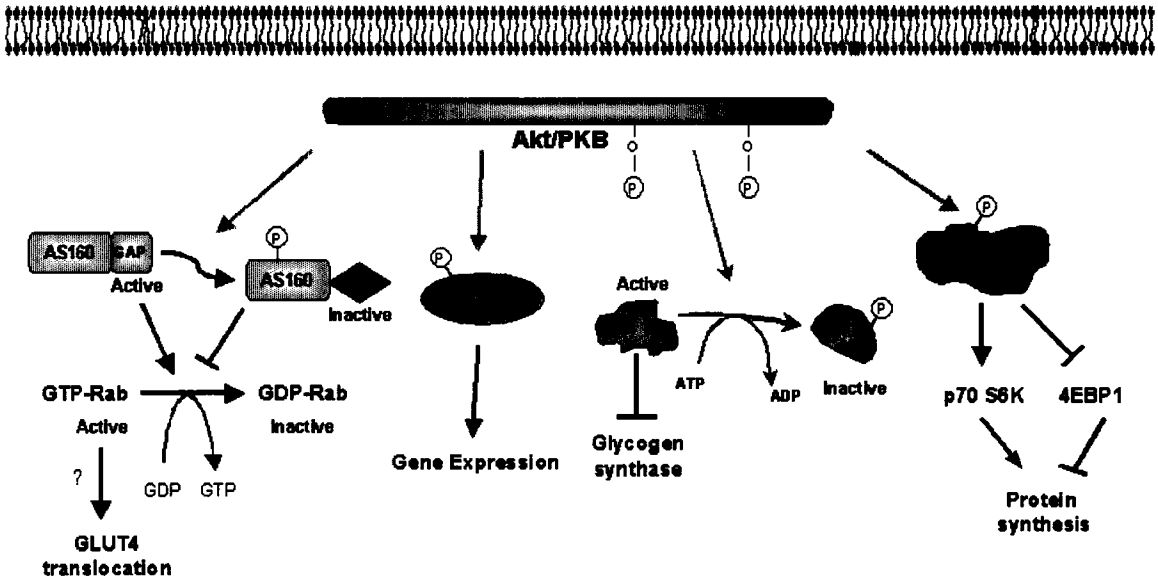


Figure 1.4. Schematic diagram depicting the central role of Akt/PKB in insulin action. Activated Akt/PKB has a number of known substrates, the phosphorylation of which account at least in part for the insulin's effects on the stimulation of glucose transport (AS160), stimulation of glycogen (GSK3) and lipid synthesis (mTOR) and changes in expression of specific genes (FOXO).

1.6 Non-PI3 Kinase Pathway

While many of insulin's action occur through activation of PI3K, other pathways are also employed. The best characterized is one shared with other growth factors leading to activation of members of the mitogen activated protein kinase (MAPK) family of serine/threonine kinases. This pathway is initiated by phosphorylation of Shc by the IR. Upon tyrosine phosphorylation by IR, Shc is able to complex with another adaptor protein, Grb2, through the SH2 domain on Grb2. Grb2 exists in a constitutive complex with the guanine nucleotide exchange factor Son of Sevenless (SOS). The Grb2/SOS complex resides in the cytoplasm but upon binding to Shc-associated IR, is recruited to the inner surface of the plasma membrane. There, SOS is brought into proximity with the

membrane localized small G-protein Ras, activating Ras to its GTP form. As a result, Ras-GTP complexes with the protein kinase Raf and leads to its activation. Activated Raf, then, phosphorylates and thereby activates the protein kinase MEK, and MEK phosphorylates and consequently activates the p42/p44 forms of MAPK (66).

The MAPK cascade represents the major mechanism mediating nuclear effects of insulin on gene expression (67). MAPK phosphorylates diverse transcription factors and so regulates the expression of specific genes (67). Even so, numerous studies indicate that this pathway has no involvement in the acute metabolic responses to insulin.

1.7 The Role of Lipids in the Development of Insulin Resistance

Insulin resistance is a state in which cells of peripheral tissues do not respond appropriately to normal dose of insulin. Insulin resistance is a major characteristic of nearly all individuals with metabolic disorders including Type 2 diabetes, obesity, hypertension, dyslipidemia, cardiovascular disease and other abnormalities, which together constitute a condition defined as Metabolic Syndrome X (68). A great proportion of individuals with one or more of the conditions of metabolic syndrome X are obese. The high prevalence of obesity in Type 2 diabetes and Metabolic Syndrome X indicates that obesity may be of pathophysiologic importance in the development of insulin resistance. However, not only the presence, but also the distribution of obesity has been shown to influence the risk of insulin resistance. For instance, central (abdominal) obesity is strongly associated with insulin resistance while fat stores in other locations is less strongly linked to these pathologies (69). One hypothesis for this

phenomenon is that adipocytes in the central depot are particularly resistant to the antilipolytic action of insulin and can release FFAs into the portal vein, exposing peripheral tissues to high FFAs concentrations (70). As it will be discussed later on, FFAs can antagonize the effects of insulin on skeletal muscle and liver. Thus, a hypothesized progression of the Type 2 diabetes is as follows. First, insulin gradually fails to stimulate glucose uptake in skeletal muscle, whereas in the liver insulin losses the ability to suppress glucose production. This, in turn, leads to an elevation in circulating glucose levels, which forces the pancreas to produce increasing amounts of insulin. As insulin resistance worsens, the pancreas is forced to produce ever-increasing amounts of insulin. If the insulin resistance becomes so severe that the pancreas is not able to produce enough insulin to meet the body's needs, then, glucose tolerance deteriorates and the patient develops diabetes mellitus (Figure 1.5).

Although the major contributor to the development of insulin resistance is obesity, other factors are implicated in the etiology of this disease. These include environmental factors, such as lifestyle, smoking, diet, and exercise level, and genetic factors. However, the extent to which genetic and environmental factors specifically determine or influence the development of insulin resistance is uncertain.

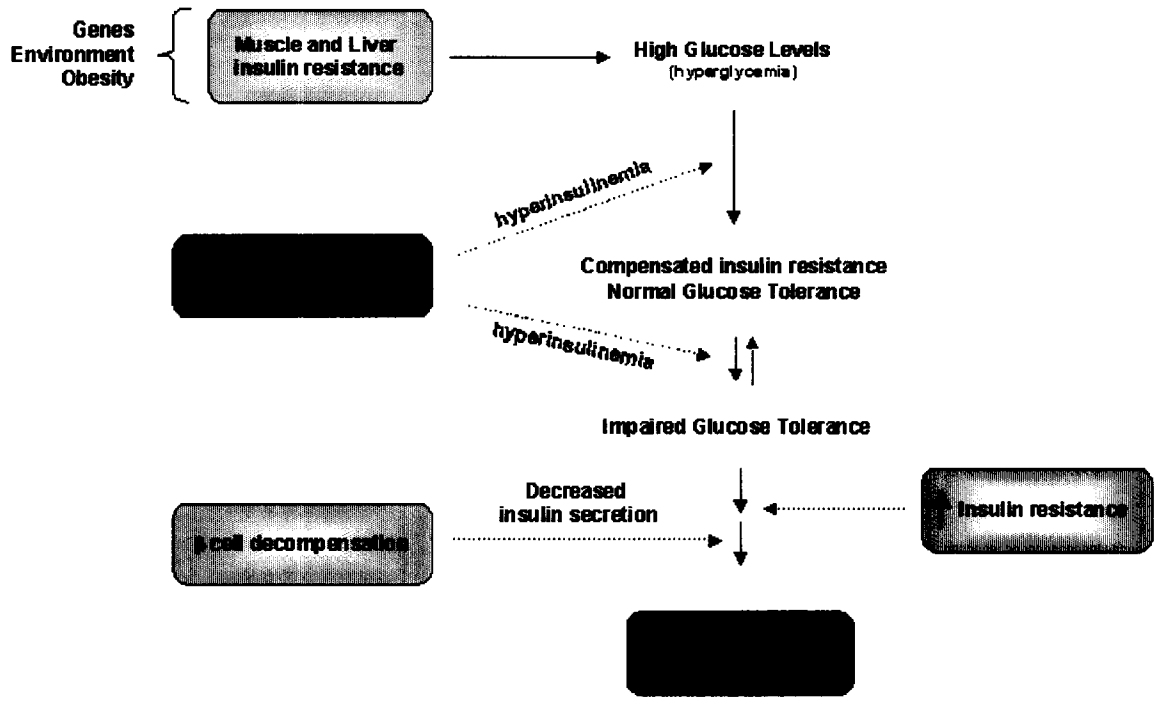


Figure 1.5. Major steps in the development of Type 2 diabetes. Insulin resistance is the first lesion, due to factors such as obesity, genetic predisposition or environmental influences. The resulting hyperglycemia is compensated by increased secretion of insulin by the β cells, sufficient to maintain normal glucose tolerance. Worsen of insulin resistance precedes the development of Impaired Glucose Tolerance. Eventually, compensation fails because islet β-cell function declines. Marked deterioration of β cells function is a late event in the process that leads to the development of Type 2 diabetes

1.8 Increased Lipid Availability in Human Insulin Resistance States

The question arises as to how obesity might contribute to the development of insulin resistance in key tissues. A likely mechanism involves the release of one or more messengers from the expanded adipose tissue that inhibits insulin action on skeletal muscle or the liver. Several candidates for such a role have been proposed including leptin (71), tumor necrosis factor-alpha (TNF-α) (72), resistin (73), adiponectin (74) and FFAs (75).

A large body of evidences indicates that in humans, FFAs are a likely link between obesity and insulin resistance. For example, it has been shown that plasma FFA concentrations are dramatically elevated in patients with severe Type 2 diabetes (76) and in the Zucker fatty (*fa/fa*) rat, which is a well-established animal model for type 2 diabetes (77). This hypothesis has been supported by experiments demonstrating that insulin resistance can be induced by directly exposing cultured cells or isolated tissues to FFAs (78, 79). Also, targeted overexpression of lipoprotein lipase (LPL), the primary enzyme mediating the liberation of FFAs from triacylglicerides (TAG) found in plasma lipoproteins, increases lipid deposition and promotes insulin resistance in either skeletal muscle or liver of mice (80). However, the most direct demonstration in humans and rodents was provided by TAG/heparin infusions, which resulted in acute FFA elevation via action of heparin to induce LPL action to liberate FFAs from the infused TAG. This acute physiological elevation of plasma FFA decreased insulin sensitivity in both muscle and liver (81-85). The FFA-induced inhibition developed only 3 to 4 hours after start of the lipid infusion, disappeared 3 to 4 hours after return of plasma FFA to normal levels, and was dose dependent (82). In addition, FFAs are also elevated when insulin resistance is induced chronically by placing rats on a high-fat diet for several weeks (86). Thus, it is believed that similar mechanisms are operative in the induction of insulin resistance by acute FFA elevation or by placing animals on a high dietary intake of lipids.

1.9 Putative Mechanism of Lipid-induced Muscle Insulin Resistance

Skeletal muscle insulin resistance is particularly relevant to Type 2 diabetes because it is the tissue responsible for the majority of whole body insulin-mediated

glucose disposal. Based on the abundant evidence that lipid oversupply induces insulin resistance states, a number of hypotheses have been suggested to define the mechanism by which increased lipid availability could lead to muscle insulin resistance.

1.9a *Glucose Fatty Acid Cycle*

The idea that an increase in plasma FFA levels causes insulin resistance was first introduced by Randle *et.al.* in the 1960s. The Randle group proposed that increased availability and oxidation of FFA reduces glucose oxidation and therefore glucose uptake (87). His group observed that increased FFA flux would lead to increased acetyl CoA in mitochondria, which in turn could inhibit pyruvate dehydrogenase activity, producing an elevation in citrate concentration. Citrate allosterically inhibits the activity of phosphofructokinase, leading to an accumulation of glucose 6-phosphate which in turn inhibits hexokinase and glucose phosphorylation, thus reducing glucose utilization, a common feature of insulin resistance and diabetes. Although the ability of elevated FFAs levels to reduce glucose oxidation may play a role in insulin resistance in some situations (82-84), the contribution of the glucose-FFA cycle to the reduction of insulin-stimulated glucose uptake is less certain. For example, although lipid infusion induces a prompt inhibition of glucose oxidation, insulin stimulation of glucose uptake remains unaffected for several hours (3-4 hours after lipid infusion). Moreover, in high-fat fed rat models of insulin resistance, the presence of a blocker of FFA oxidation had little ability to restore impaired insulin stimulation of glycogen synthesis and GS activity (88). Finally, the reduction in glycogen synthesis occurs in association with decreased glucose 6-phosphate, rather than the increased levels predicted by Randle's hypothesis. This

impairment has been attributed to a reduction in glucose transport/phosphorylation associated with decreased insulin signaling activity (89). These studies suggest that mechanism other than, or additional to, Randle cycle effects are involved in the reduction of skeletal muscle insulin resistance by increased availability of FFAs.

1.10 Intramuscular Lipid Accumulation

Another model suggests that lipid environment leading to muscle insulin resistance is not a result of a direct FFA provision from the circulation but to the accumulation of fat-derived metabolites, which can inhibit insulin action (Figure 1.6). In fact, numerous studies have demonstrated that excess of muscle lipid availability promotes an increase in TAG content. Previously, these were results of studies based on muscle biopsy (90, 91). However, it has proven problematic to separate a mixture of muscle and adipose cells from intracellular TAG molecules. An important recent improvement is the use of proton nuclear magnetic resonance (^{1}H NMR) to access intramyocellular lipids. This technique can distinguish signals from intramyocellular and extra-myocellular lipid, obviating earlier worries about possible contamination of muscle biopsies with adipose tissue. ^{1}H NMR analysis indicates an inverse correlation between muscle TAG content and insulin sensitivity in subjects with body mass indexes ranging from normal to obese (92). Furthermore, according with those studies, intramyocellular TAG content correlates more tightly with the severity of insulin resistance than most known risk factors (93). This phenomena is now recognized as one of the most consistent indicators of whole-body insulin resistance.

Intramuscular TAG accumulation is now evident in a wide array of experimental models, including insulin resistance induced acutely by lipid infusion in both humans (94) and rodents (85, 95). Genetic forms of obesity such as Zucker rats (96) as well as dietary animal models of insulin resistance (97) also exhibit increased intramyocellular TAG accumulation. Moreover, prolonged inhibition of fatty acid oxidation in muscle by pharmacological inhibition of carnitine-palmitoyl transferase 1 (CPT-1), the key enzyme for the transport of long-chain acyl-CoA compounds into the mitochondria, impairs insulin-mediated glucose disposal in rats via a mechanism related to the accumulation of TAG (97).

More support comes from human lipodystrophy syndromes, where an absence of peripheral adipose tissue, and consequent lack of a peripheral store of fatty acids, leads to insulin resistance associated with increased TAG content in muscle (98). Recent development of a genetically modified mouse model that is deficient in white adipose tissue has increased the understanding of the profound consequences of lipodystrophy (99). These "fatless" mice have severe insulin resistance and other features of human lipoatrophic diabetes including an increase in intramyocellular TAG (99). Interestingly, a dramatic reduction of insulin resistance is observed in this model when adipose tissue is restored by transplantation (100) indicating the importance of adipose tissue as a "sink" for fatty acids that prevents an overload of lipid supply to other tissues.

Although the close coupling between increased intramyocellular TAG content and the development of insulin resistance is compelling, the mechanism behind the effect is

yet to be elucidated. However it appears unlikely that intramyocellular TAG itself directly impairs insulin action. For example, TAG accumulation in muscle cells is not invariably associated with insulin resistance. Notably, the muscle of trained endurance athletes has been shown to be highly insulin-sensitive despite the presence of high levels of intramyocellular TAG (101). Moreover, short term (4-weeks) exercise training in humans improved muscle insulin sensitivity in the absence of measurable changes in muscle TAG content (102). To account to this discrepancy, it has been proposed that TAG accumulation is merely a marker for some other more harmful related FFA moiety such as long chain acyl-CoA, ceramide or diacylglycerol, whose appearance is associated to elevations in intramuscular TAG content (94, 103).

1.10a *Long-chain Acyl-CoAs.*

Long-chain acyl-CoAs (LCACoAs) are the activated form of intracellular FFA, produced by the action of acyl CoA synthase (ACS). Interestingly, there is a consistent link between decreased insulin sensitivity and LCACoAs in muscle. LCACoA content of skeletal muscle has shown to be increased in insulin-resistant animals (85, 88, 104, 105) and humans (104). In isolated soleus muscle strips, insulin-stimulated glucose uptake and glycogen synthesis was reduced by incubation with fatty acids in a manner that correlates with the accumulation of intracellular LCACoA (106). Furthermore, experimental maneuvers designed to acutely reduce the intramuscular LCACoA content of high-fat fed rats augmented insulin-stimulated glucose uptake into skeletal muscle (107).

LCACoA might relate to mechanisms by which increased fat availability in muscle leads to insulin resistance. Findings so far point to the ability of LCACoA to modulate enzyme activity. Thompson *et.al.*, (108) for example, showed that LCACoAs can inhibit hexokinase activity in homogenates of human and rat soleus muscle *in vitro*. This inhibition showed to be additive to the inhibition of hexokinase by glucose-6-phosphate (Randle's mechanism) suggesting that increase in intramuscular lipid metabolites could alter insulin-mediated glucose metabolism by decreasing the rate of glucose flux into the tissue (108). LCACoAs may also interfere with muscle glucose utilization through the direct activation of protein kinase C (PKC) isozymes, an enzyme that has been linked to insulin resistance in muscle in a wide variety of models. For instance, LCACoAs have been shown to activate PKCs in *in vitro* assays (109), and in isolated fibroblasts (110). Some PKC subtypes can phosphorylate IRS proteins on serine or threonine residues, reducing its tyrosine phosphorylation by the IR and decreasing activation of IRS-1-associated PI3K activity (111).

Furthermore, LCACoAs have also been shown to activate nuclear receptors providing the possibility that intracellular lipid availability can modulate gene expression. The most relevant of these receptors is the peroxisome proliferators activated receptors (PPARs) and in particular PPAR-γ which is important for adipose tissue proliferation and fat deposition (112). PPAR-γ is also expressed in skeletal muscle, although in lower abundance than in adipose tissue (113). However, PPAR-γ expression has been shown to be significantly increased in insulin resistance diabetic subjects (113), further suggesting its involvement in the regulation of metabolism and insulin action in muscle.

FFAs in muscle are activated to LCACoAs before transport across mitochondrial membranes by carnitine palmitoyltransferase (CPT), and then metabolized in the mitochondria by β-oxidation. In addition, LCACoAs are the substrates for the esterification of FFAs for the synthesis of TAG and other less abundant second-messengers lipids such as diacylglycerol (DAG) and ceramide. Thus, it is believed that elevated tissue lipid availability can activate lipid signaling molecules derived from LCACoAs leading to attenuation of insulin action. The most direct support of this hypothesis is provided by the use of R-etomoxir, an oxirane carboxylic acid derivative that specifically inactivates CPT-1. In both humans (114) and rodents (97), insulin sensitivity was decreased by R-etomoxir suggesting that skeletal muscle insulin resistance may be aggravated by accumulation FFA-derived metabolites that inhibit insulin signaling.

1.10b *1,2-Diacylglycerol*

1,2-Diacylglycerol (DAG) is an intermediate of both triglyceride and phospholipid metabolism. DAG can be generated by *de novo* synthesis, through the esterification of LCACoA into glycerol-3-phosphate backbones, or by the breakdown of phospholipids, particularly phosphatidylinositol-4,5-bisphosphate and phosphatidylcholine, by phospholipases C and D, respectively. DAG is an important second messenger involved in intracellular signaling and has been shown to accumulate in the insulin-resistant muscle of high-fat fed rats (115), Zucker rats (116) as well as transgenic mice with muscle-specific overexpression of lipoprotein lipase (LPL) (105). Numerous animal studies indicate that DAG may have detrimental effects on insulin

action through its ability to activate PKC isoforms [reviewed in (117)]. Increased expression and/or activation of the novel, DAG-sensitive PKC isozymes ε and/or θ have been associated with reduced insulin action in skeletal muscle of rats during lipid infusions (95, 118), as well as in rodent models of insulin resistance including obese Zucker rats (119) and high-fat fed rats (115). In a study of high-fat fed rats there was evidence of chronic activation of PKC ε and PKC θ in association with elevated DAG levels (115). Moreover, rapid reversal of insulin resistance in high-fat fed rats by acute dietary intervention (120) or by insulin sensitizers (121) is associated with a normalization of activation of these PKC isozymes.

In humans, fatty-acid-induced muscle insulin resistance was recently found to be associated with increased DAG mass and with PKC isoform (β2 and δ) activation (94). The finding generally supports the relevance of the rodent DAG/PKC/insulin resistance scenario to humans, although the relative roles of the different PKC isoforms involved is unclear. In isolated human skeletal muscle strips and adipocytes, activation of PKC by 12-deoxyphorbol 13-phenylacetate 20-acetate, a phorbol ester that mimics the effects of DAG, reduced insulin-stimulated glucose uptake whereas pharmacological inhibition of PKC activity increased insulin-stimulated glucose uptake by twofold in association with elevated insulin receptor tyrosine phosphorylation and PI 3-kinase activity (122). These data further support the notion that inappropriate activation of PKCs may interfere with insulin action by promoting serine/threonine phosphorylation of the insulin receptor and/or IRS-1, thus preventing tyrosine phosphorylation of these proteins that are necessary for the function of the insulin signaling cascade and stimulation of glucose

transport. Direct evidence for increased serine phosphorylation of IRS-1 associated with fatty-acid-induced insulin resistance was recently reported in lipid-infused rats (95). Moreover, covalent modification of IRS-1 on serine/threonine residues was shown to impair its insulin-induced tyrosine phosphorylation, the activation of PI 3-kinase, and the stimulation of glucose transport (123). Despite the abundance of evidence for its key role in the development of insulin resistance, the serine phosphorylation of IRS-1 by LCACoA-derived metabolites has remained incompletely understood.

1.10c *Ceramide*

Ceramide is a derivative of sphingomyelin, a phospholipid component of cell membranes, which can be generated by the hydrolysis of sphingomyelin through the action of sphingomyelinase or via *de novo* synthesis using LCACoAs as precursors (124). Ceramide is a bioactive lipid that plays important roles as a signaling molecule in animals. Indeed, numerous studies have shown that ceramide can alter the activity of kinases, phosphatases and transcription factors through which it is thought to play a role in the regulation of cell proliferation, differentiation and apoptosis (125). Increased cellular production of ceramide has also been implicated in the pathogenesis of insulin resistance and in the impaired utilization of glucose. Intracellular ceramide levels are elevated in skeletal muscle from obese insulin-resistance humans (126) and in peripheral tissues of several rodent models of insulin resistance including Zucker fatty rats (116) and mice overexpressing LPL (105). Moreover, exercise-induced elevation in muscle glucose uptake in Wistar rats was shown to be inversely related to the total content of ceramide in the muscles (127).

Several lines of evidence in cultured cells further implicate that ceramide may play a role in insulin resistance through its inhibition of glucose metabolic pathways. Incubation of 3T3-L1 adipocytes with a membrane-permeable C2-ceramide inhibited insulin-stimulated glucose transport by 50% by reducing GLUT4 translocation as a consequence of the direct inhibition of the phosphorylation Akt/PKB (128). Similarly, in L6 myotubes, C2-ceramide impairs the insulin-dependent activation of Akt/PKB leading to a complete loss of insulin-stimulated glucose transport and glycogen synthesis (129). Ceramide-induced inhibition of Akt/PKB occurs independently of any change in the ability of insulin to stimulate phosphorylation of IRS-1 or activate PI3-kinase and thus represents an independent pathway for the modification of insulin signaling.

Recently, it was demonstrated that incubation of C2C12 murine muscle cells with palmitate led to an increase in *de novo* intracellular ceramide, a decrease in Akt/PKB phosphorylation, and a decreased ability of insulin to stimulate glycogen synthesis (103). Taken together, this provides considerable evidence for the concept that LCACoA-induced ceramide accumulation might have important roles in the inhibition of glucose uptake in skeletal muscles. Although the identity of direct targets of ceramide that mediate its effects on insulin action remains elusive, several candidates through which ceramide could exert these effects has been proposed. These include; the ceramide-activated protein kinase (CAPK) (also known as kinase suppressor of Ras) (130), the atypical PKC isoforms ζ (PKC ζ) (131), and the ceramide-activated protein phosphatase (CAPP), which is a member of the protein phosphatase 2A (PP2A) family (132).

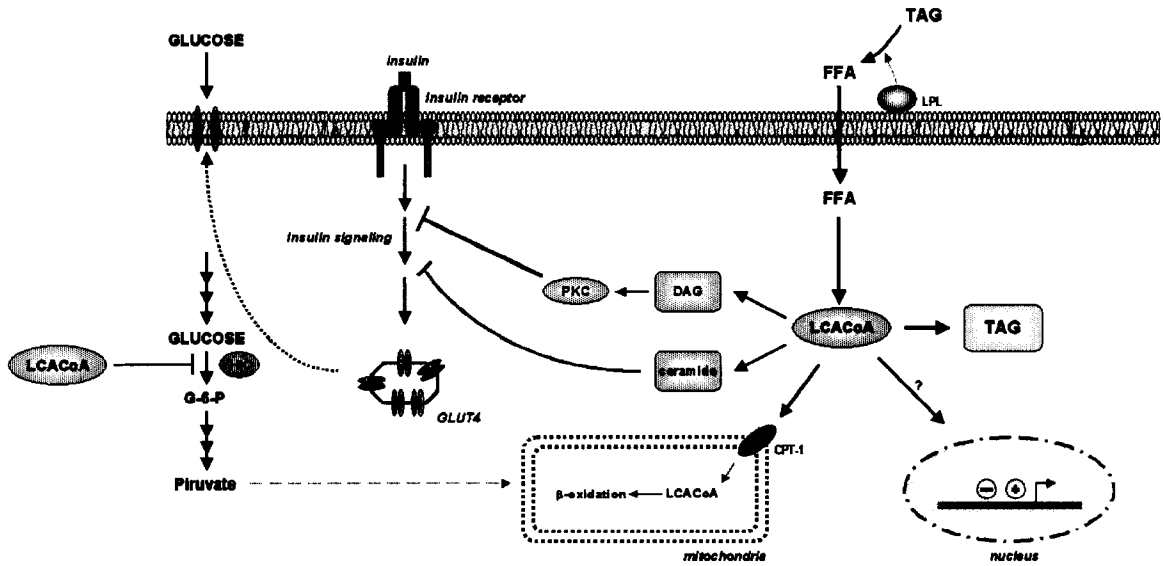


Figure 1.6. Some possible mechanism by which intramuscular lipids may interfere with muscle insulin action and glucose metabolism to produce insulin resistance. Muscle lipid accumulation may be manifested as increased levels of stored triacylglyceride (TAG or of cytosolic long-chain acyl CoA (LCACoA). LCACoA can influence glucose metabolism by altering directly key enzymes activities (e.g. Hexokinase (HK) or protein Kinase C (PKC). Mitochondrial LCACoA oxidation may influence glucose oxidation via the classic glucose-fatty (Randle) cycle. Accumulation of specific LCACoA-derived metabolites (e.g. DAG or ceramide) may alter insulin-signaling pathways through distinct effectors. LCACoA may change gene expression by acting as ligands for nuclear transcription activators (e.g. PPARγ).

1.11 The Hexosamine Pathway

Another consequence of muscle lipid oversupply is an increased carbon flux through the hexosamine pathway, which has also been associated to the development of insulin resistance. In the presence of increased fatty acid availability, a greater proportion of fructose-6-phosphate is converted to glucosamine-6-phosphate by the rate-limiting enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT). Following acetylation and uridylation, result in increased concentrations of the end-product UDP-N-acetyl-glucosamine (UDP-GlcNAc). This pathway acts as a nutrient-sensing mechanism

to decrease the rate of glucose uptake upon elevated hexose phosphates, as a result of increased lipid supply. Direct evidence for a role for hexosamine products in the generation of insulin resistance has been presented. Infusion of rats with either glucosamine (GlcN) or uridine (133), or overexpression of GFAT in transgenic mice (134), increases flux through the hexosamine pathway and induces skeletal muscle insulin resistance. Nonetheless, the role of the hexosamines in lipid-induced insulin resistance has recently been challenged after studies showing that elevations in plasma FFAs induced insulin resistance with no marked increase in skeletal muscle content of UDP-GlcNAc (135). It is believed that the product of this pathway, GlcNAc serves as a substrate for protein glycosylation so that alterations in regulated *N*- or *O*-linked glycosylation could play a role for its effects on insulin action. In particular, the widespread occurrence of cytosolic *O*-linked *N*-acetyl glucosamine on transcription factors, RNA polymerase, and nuclear pore proteins suggests a potential role for hexosamines in altering gene expression. Nevertheless, the levels at which hexosamines affect the expression of genes of the insulin signaling remains to be clarified.

1.12 Conclusion

Insulin resistance is a common feature of Type 2 Diabetes and other metabolic states including obesity and Metabolic Syndrome X. Despite myriad research studies, the molecular defects underlying the development of insulin resistance remain unknown. However, it is clear that the development of insulin resistance is associated with disturbances in lipid metabolism, which increase the concentration of circulating FFAs and promote their accumulation within peripheral tissues, particularly in muscle, the

major site of insulin-stimulated glucose disposal. Human, animal and *in vitro* studies have examined mechanism whereby lipids could lead to muscle insulin resistance. Randle glucose-fatty acid cycle is the classical mechanism by which FFAs might influence muscle glucose metabolism and insulin action. However, the clear association that exist between increased accumulation of intramyocellular TAG and other FFAs metabolites (i.e. long-chain acyl coenzyme A, DAG, ceramide), and insulin resistance has prompted investigators to hypothesize that additional effects, others than impairment of glucose oxidation, are implicated in the development of insulin resistance by FFAs. These lipid metabolites are important second messenger involved in a wide variety of signal transduction pathways that mediate cell-specific biological responses, and it is therefore likely that they impede glucose transport by activating pathways that interfere with insulin signal transduction.

1.13 Dissertation Layout

The objective of the studies presented in the following chapters is to determine the importance of ceramide on the development of insulin resistance in cultured cells.

We began our studies by identifying an insulin-responsive cell line in which exogenous FFAs blocked insulin signaling and its metabolic action. C2C12 myotubes and 3T3-L1 adipocytes, two well-established cell models of insulin signaling were used for this purpose. Chapter 2 evaluates the repercussions of excess FFAs delivery to the muscle and adipose cells. These experiments were also able to delineate the importance

of the FFA species on the accumulation of these lipid metabolites and the inhibition of insulin action on both cell lines.

To determine whether ceramide was required for the inhibition of insulin action by FFAs in C2C12 myotubes, we designed two strategies described in both Chapter 3 and 4. First, we attempted to block the inhibitory effects of saturated FFAs with pharmacological agents that prevent ceramide biosynthesis or metabolism. This approach permitted us to answer whether ceramide itself, or alternatively another ceramide metabolite, was the principal mediator of the inhibitory effects of FFAs. A second approach to delineate a role for ceramide in FFA-induced inhibition of insulin action was to overexpress in the myotubes genes involved in ceramide degradation, such as acid ceramidase. Results presented in chapter 4 not only support the central role of ceramide accumulation in the inhibition of muscle insulin sensitivity by exogenous FFAs, but also could suggest that strategies aimed at regulating aberrant ceramide deposition could be utilized for the treatment of insulin resistance *in vivo*.

The identification of ceramide as an important lipid-derived inhibitors of insulin signaling and action could ultimately lead to an increased understanding of the molecular basis for the development of insulin resistance, as well as to the identification of novel therapeutic targets.

CHAPTER 2

Characterizing the Effects of Saturated Fatty Acids on Insulin Signaling and Ceramide and Diacylglycerol Accumulation in 3T3-L1 Adipocytes and C2C12 Myotubes.

This chapter describes work published in Archives of Biochemistry and Biophysics (Chávez and Summers, 2003). All experiments presented herein were carried out by Chavez JA.

Chavez JA, Summers SA *Arch Biochem Biophys.* 2003 Nov 15;419(2):101-9

2.1 Abstract

A strong correlation between intramyocellular lipid concentrations and the severity of insulin resistance has fueled speculation that lipid oversupply to skeletal muscle, fat, or liver may desensitize these tissues to the anabolic effects of insulin. To identify free fatty acids (FFAs) capable of inhibiting insulin action, we treated 3T3-L1 adipocytes or C2C12 myotubes with either the saturated FFA palmitate (C16:0) or the monounsaturated FFA oleate (C18:1), which were shown previously to be the most prevalent FFAs in rat soleus and gastrocnemius muscles. In C2C12 myotubes, palmitate, but not oleate, inhibited insulin-stimulation of glycogen synthesis, as well as its activation of Akt/Protein Kinase B (PKB), an obligate intermediate in the regulation of anabolic metabolism. Palmitate also induced the accrual of ceramide and diacylglycerol, two lipid metabolites that have been shown to inhibit insulin signaling in cultured cells and to accumulate in insulin resistant tissues. Interestingly, in 3T3-L1 adipocytes, neither palmitate nor oleate inhibited glycogen synthesis or Akt/PKB activation, nor did they induce ceramide or DAG synthesis. Using myotubes, we also tested whether other saturated fatty acids blocked insulin signaling while promoting ceramide and DAG accumulation. The long chain fatty acids stearate (18:0), arachidate (20:0), and lignocerate (24:0) reproduced palmitate's effects on these events, while saturated fatty acids with shorter hydrocarbon chains [i.e. laurate (12:0) and myristate (14:0)] failed to induce ceramide accumulation or inhibit Akt/PKB activation. Collectively these findings implicate excess deliver of long-chain fatty acids in the development of insulin resistance resulting from lipid oversupply to skeletal muscle.

2.2 Introduction

The peptide hormone insulin stimulates the uptake and storage of glucose and other nutrients into adipose tissue and skeletal muscle, while simultaneously repressing glucose efflux from the liver. Insulin resistance occurs when a normal dose of insulin is incapable of eliciting these anabolic responses, and the condition is a major contributor to the pathogenesis of Type 2 Diabetes Mellitus (136) and Metabolic Syndrome X (68). Multiple studies reveal an association between increased lipid availability and insulin resistance, suggesting a causative role for lipid oversupply to skeletal muscle, adipose tissue, and the liver in the development of the insulin resistant condition (111). Moreover, recent studies utilizing ^{1}H NMR indicate that intramyocellular triacylglyceride (TAG) concentrations correlate more tightly with insulin resistance than any other commonly used risk factors including body mass index, percent of body-fat mass, or age (93). A hypothesis emerging from these studies is that insulin resistance results from the accumulation of one or more fat-derived metabolites that antagonize insulin signal transduction (93, 111).

Insulin accelerates glucose entry into skeletal muscle and fat by stimulating the translocation of the glucose transporter GLUT4 from intracellular vesicles to the plasma membrane. Subsequently, insulin regulates metabolic enzymes to the storage of the incoming glucose as either glycogen, triglyceride, or protein (137, 138). Insulin initiates these anabolic actions through a signaling pathway triggered by a heterotrimeric receptor with intrinsic tyrosine kinase activity (139). The activated enzyme phosphorylates intracellular substrates (IRS proteins) that recruit and activate a variety of signaling

molecules (140). Most notably, IRS proteins activate phosphatidylinositol 3-kinase (PI3K), which is an obligate intermediate in insulin's metabolic (141-143), anti-apoptotic (144, 145), and mitogenic effects (146). PI3K, by catalyzing the phosphorylation of specific phosphoinositides to generate the lipid products phosphatidylinositol-3,4-bisphosphate (PI-3,4-P_2) and phosphatidylinositol-3,4,5-trisphosphate (PIP_3), initiates intracellular signaling cascades by recruiting cytoplasmic enzymes to the plasma membrane. One particular target of PI-3,4-P_2 and PIP_3 is Akt/Protein Kinase B (PKB), a serine/threonine kinase that mediates many of PI3K's metabolic and anti-apoptotic actions (147-149).

Ceramide and diacylglycerol are fatty acyl-CoA metabolites shown to accumulate in peripheral tissues of insulin resistant rodents and to inhibit insulin signaling and action in various isolated tissues or cultured cells, including 3T3-L1 adipocytes and C2C12 myotubes (116, 122, 128, 150-154). The studies described herein investigated the efficacy of various saturated free fatty acids (FFAs) as either (a) antagonists of insulin signaling and/or (b) inducers of ceramide and diacylglycerol accumulation in these two cell types. Findings obtained revealed that long-chain FFAs [i.e. palmitate (16:0), stearate (18:0), arachidate (20:0, and lignocerate (24:0)] effectively induced the synthesis of both metabolites and inhibited the activation of Akt/PKB in C2C12 myotubes. Conversely, saturated FFAs with hydrocarbon chains shorter than palmitate [i.e. laurate (12:0) and myristate (14:0)] failed to induce ceramide or inhibit Akt/PKB in either cell type. Interestingly, palmitate, which was the most potent inhibitor of Akt/PKB, failed to induce ceramide or DAG accumulation or to inhibit insulin signaling in 3T3-L1

adipocytes. Collectively these findings identify long-chain saturated fats, through their ability to induce the synthesis of inhibitory bio-metabolites such as ceramide or DAG, as potential antagonists of insulin action in skeletal muscle.

2.3 Experimental Procedures

2.3a *Reagents*:

Fetal bovine serum was from Atlas Biologicals (Fort Collins, CO), and silica gel 60 thin layer chromatography (TLC) plates were from Merck (Darmstadt, Germany). The FFAs, ceramide standards, and FFA-free bovine serum albumin (BSA) were obtained from Sigma Chemicals (St. Louis, MO). Low glucose Dulbecco's Modified Eagles's Medium (DMEM), was from Life Technologies (Rockville, MD). Antibodies utilized included the following: rabbit polyclonal anti-phospho-Akt (Ser-473), anti-phospho-GSK3β (serine-9), and anti-mitogen activated protein kinase (MAPK) antibodies from Cell Signaling; a mouse anti-glycogen synthase kinase 3β (GSK3β) antibody from Transduction Labs (Lexington, KY); a rabbit anti-phospho-MAPK antibody from Promega (Madison, WI); and rabbit polyclonal anti-Akt and horseradish peroxidase conjugated anti-rabbit and anti-mouse antibodies from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3b *Cell Culture*:

C2C12 myoblasts were maintained at 37°C in Dulbecco's modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS). For differentiation into myotubes, the myoblasts were grown to confluency and the media replaced with DMEM

containing 10% horse serum. Myotubes were used for experiments 4 days following differentiation.

2.3 c *Immunoblot Analysis*:

Cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose and immunoblotted using methods described previously (155). Antibody binding was detected using the Enhanced Chemiluminescence Plus kit from Amersham Biosciences (Piscataway, NJ) according to manufacturers instructions.

2.3d *Glycogen Synthesis Assay:*

The incorporation of glucose into a glycogen pellet was determined as described previously (54). Lipid-pretreated myotubes in 6-well plates were incubated for 1 h in 1 ml/well serum free-DMEM containing D-[U-^{14}C]glucose (4 μCi/ml) in the absence or presence of 100 nM insulin. Cells were washed four times with 2 ml of ice-cold PBS and scraped into 250 μl/well 1 M KOH. Extracts were heated to 100 °C for 10 min. After addition of 40 μl of a saturated solution of Na_2SO_4, glycogen was precipitated by the addition of 700 μl ice-cold acetone and incubation at -70 °C for 15 min. Samples were centrifuged at 3500 RPM and supernatants aspirated. Pellets were washed by resuspension in 50 μl of water followed by addition of 500 μl of ice-cold acetone and recentrifugation. Final pellets were dissolved in 200 μl of water andcounted for radioactivity.

2.3e *2-Deoxgyglucose Uptake Assay:*

The 2-deoxyglucose assay was performed using methods previously described (128). Adipocytes (12-well culture dishes) were incubated in 0.5ml Leibovitz's L-15 media/0.2%BSA for 1.5-2 hours in air incubator. Cell were washed once with PBS and once with Kreb's-Ringer bicarbonate buffer supplemented with 0.2%BSA (KRP/0.2%BSA), and incubated for another 30 min in KRP/0.2%BSA. Insulin was added, and the cells incubated for 15 min. The assay was initiated by the addition of [^{14}C-(U)]2-deoxyglucose (0.5 μCi/sample) and glucose (5mM final concentration). The assay was terminated after 15 min by washing cells three times in ice-cold PBS. Cells were solubilized in 0.05% SDS in PBS and cell-associated radioactivity determined by scintillation counting.

2.3f *FFA Treatment*:

Free fatty acids were administered to cells by conjugating them with FFA-free bovine serum albumin (BSA). Briefly, FFAs were dissolved in ethanol and diluted 1:100 in DMEM (with or without 1% FBS) containing 2% (w/v) BSA. C2C12 myotubes or 3T3-L1 myotubes were incubated with the FFAs (0.75 mM final concentration) in 1% FBS-DMEM for 16 hours, washed with PBS, and then incubated with the FFA in serum free DMEM for 3 hours prior to experiment initiation.

2.3g *Ceramide Assay*:

Myotubes were lysed in ice-cold 1M NaCl (0.25-ml) and transferred to glass tubes. Ice cold $CHCl_3$:CH_3OH (1:2, v/v, 0.75 ml) was added and the suspension was

vortexed vigorously. After the subsequent addition of 0.25 ml of 1 M NaCl and 0.25 ml of $CHCl_3$, samples were incubated on ice for 10 minutes and phases were separated by centrifugation (2000xG for 5 min). Ceramide content in the extract was determined using a radiometric diacylglycerol assay kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

2.4 Results and Discussion

To elucidate the molecular mechanisms underlying the inhibitory effects of lipid oversupply on insulin action, we set out to identify an insulin-responsive cell line in which insulin signaling or action was sensitive to exogenous FFAs. We began by studying two well-established models of insulin signaling: C2C12 myotubes and 3T3-L1 adipocytes (Figure 2.1). Insulin dramatically stimulates early signaling events, including the phosphorylation and activation of the anabolic enzymes Akt/PKB and glycogen synthase kinase 3β (GSK3β) and the mitogenic enzyme mitogen-activated protein kinase (MAPK), in either cell type (Figure 2.2A). By contrast, however, anabolic responses to insulin, such as the stimulation of glucose uptake and glycogen synthesis, are differentially sensitive to the hormone in the two cell types. For example, although insulin dramatically stimulates rates of both glucose uptake (~3-fold) (Figure 2.2B) and glycogen synthesis (~8-fold) (Figure 2.2C) in 3T3-L1 adipocytes, its effect on either event is much smaller (~1.1 fold and 2-fold, respectively) in C2C12 myotubes. Thus, C2C12 myotubes apparently lack some of the intracellular machinery necessary to achieve maximal insulin responsiveness. The studies described herein investigated the ability of various FFAs to (a) antagonize insulin signaling and insulin-stimulated glycogen synthesis and (b) induce the accumulation of ceramide and DAG in these two cell types.

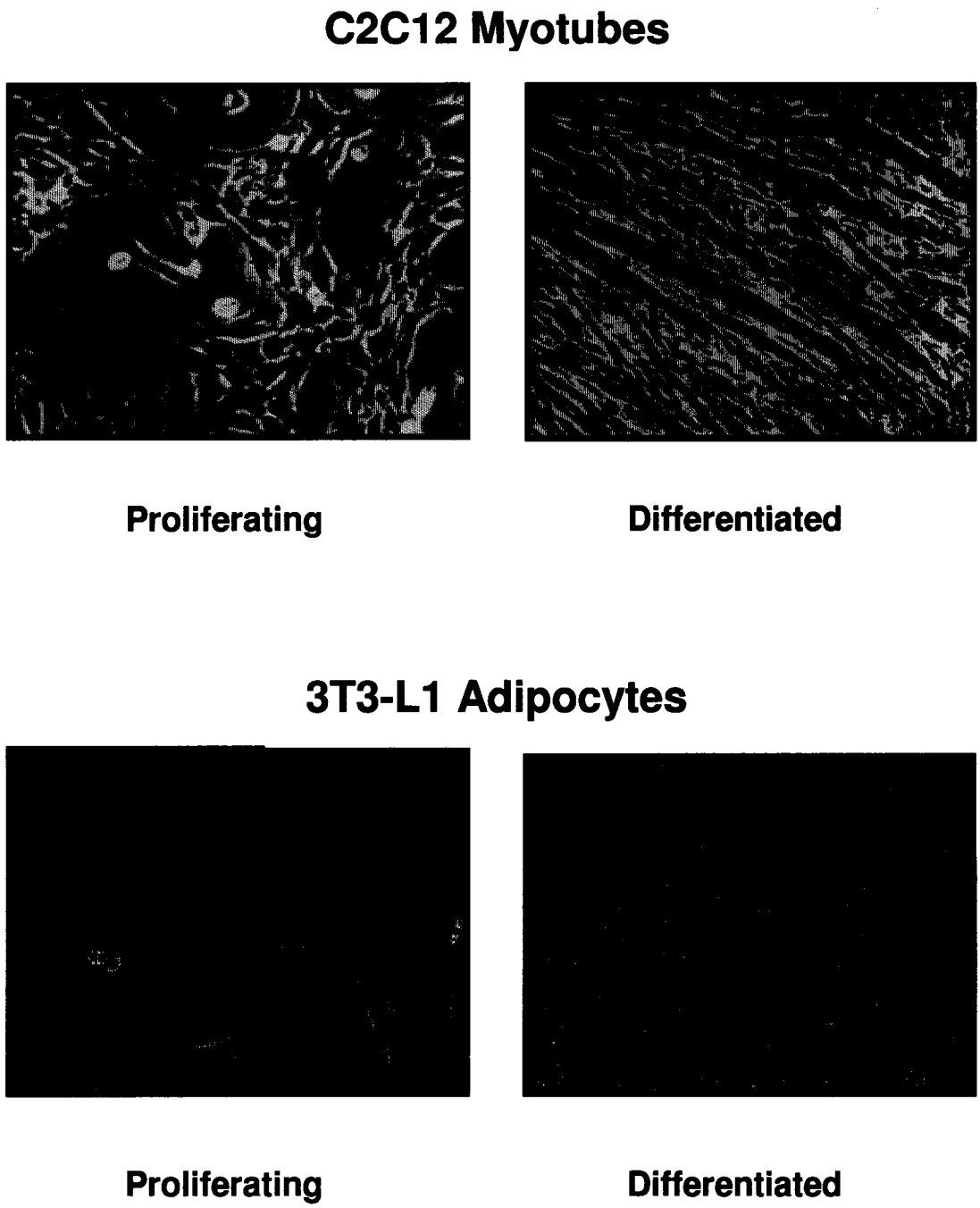


Figure 2.1. Phase micrograph depicting morphology of C2C12 and 3T3-L1 cells before and after differentiation. Cells were differentiated as described in the Methods section and photographed at 20X magnification

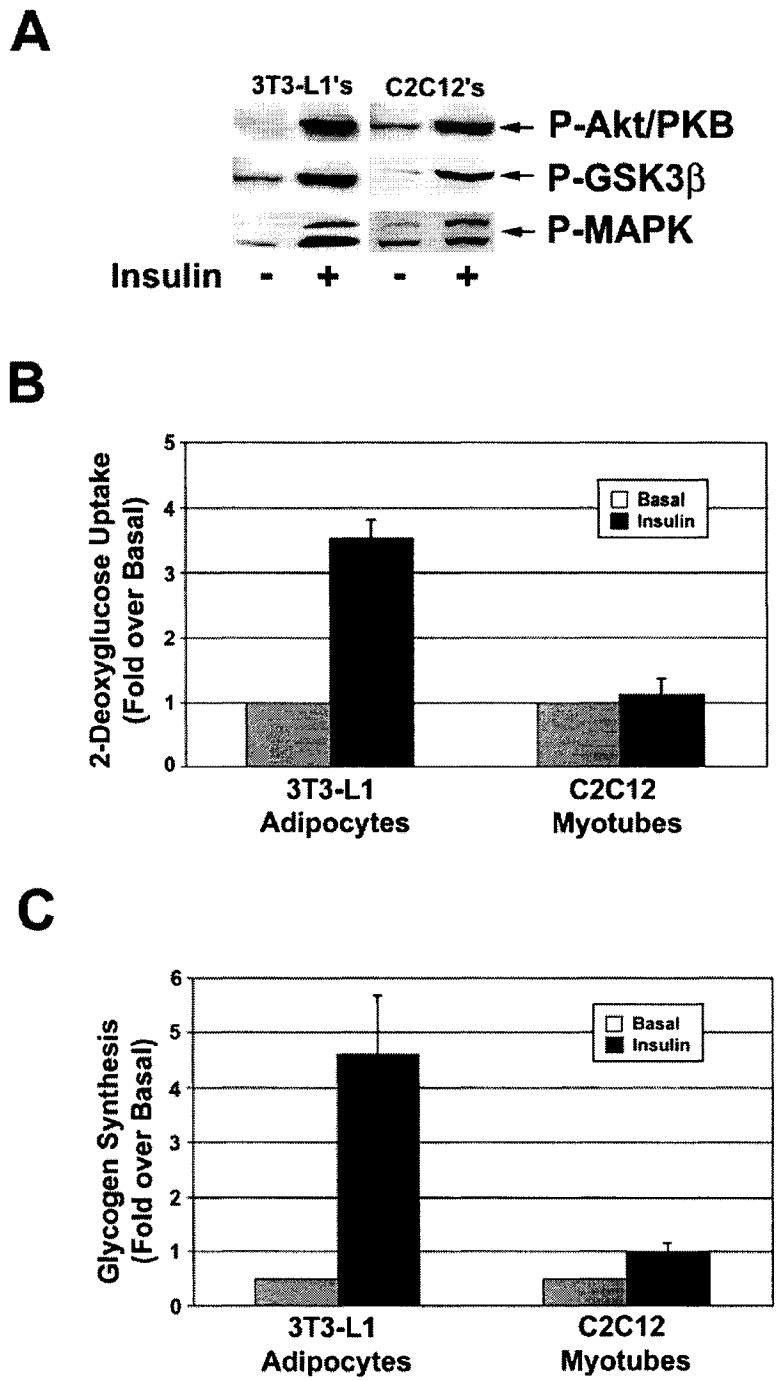


Figure 2.2: Comparing insulin signaling and action in 3T3-L1 adipocytes and C2C12 Myotubes A. Differentiated 3T3-L1 adipocytes and C2C12 myotubes were stimulated with insulin (100 nM) for 10 minutes prior to lysis. Cell lysates were then resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antibodies. Detection was by enhanced chemiluminescence. Data are representative of three independent experiments. **B.** 3T3-L1 adipocytes and C2C12 myotubes were treated with insulin as in (A) above and rates of 2-deoxyglucose were determined. Findings are reported as the mean fold-stimulation over basal rates +/- S.E.M. **C.** 3T3-L1 adipocytes and C2C12 myotubes were treated with insulin as in (A) above and rates of glucose incorporation into glycogen were determined. Findings are reported as the mean fold-stimulation over basal rates +/- S.E.M.

2.4a *Comparing the effects of palmitate and oleate on insulin signaling and anabolic metabolism in C2C12 myotubes and 3T3-L1 adipocytes*

Palmitate (C16:0) and oleate (18:1) are the most prevalent saturated and monounsaturated FFAs, respectively, in the circulation. Moreover, they comprise the largest percentage of the diglyceride fraction of rat soleus or gastrocnemius (white or red) muscle (156). The addition of either palmitate or oleate to 3T3-L1 adipocytes failed to have any effect on rates of insulin-stimulated glycogen synthesis (Figure 2.3A). Interestingly, however, palmitate, but not oleate, completely ablated the small (~2-fold) insulin-stimulation of glycogen synthesis in C2C12 myotubes (Figure 2.3B). Under these conditions, no signs of cell death were observed in either cell type. The observation that the saturated fatty acid palmitate inhibited insulin action, while the unsaturated fat did not, is consistent with numerous dietary studies in human populations indicating that saturated fats markedly decrease insulin responsiveness in peripheral tissues, while unsaturated fats have weaker or in some cases insulin-sensitizing effects [reviewed in (157)]. Moreover, these data suggest that skeletal muscle is differentially sensitive to the regulatory effects of saturated FFAs on insulin action.

A substantial body of evidence suggests that the mechanism by which lipid oversupply antagonizes insulin action involves disruption of the insulin-signaling cascade responsible for GLUT4 translocation and the regulation of anabolic metabolism [reviewed in (93)]. Similarly, the inhibitory effects of palmitate on glycogen synthesis in C2C12 myotubes appeared to result, at least partially, from its ability to block the phosphorylation and activation of Akt/PKB. For example, using a phospho-specific

antibody that recognizes an activating phosphorylation site (S473) in Akt/PKB, we found that palmitate reduced phosphorylation of this residue in C2C12 myotubes (Figure 2.4A). To confirm that the inhibition of Akt/PKB phosphorylation reduced Akt/PKB activity, we evaluated the phosphorylation state of glycogen synthase kinase 3β, a substrate of Akt/PKB that links it to the regulation of glycogen synthase (54). Again, palmitate, but not oleate, blocked insulin-stimulated phosphorylation of GSK3β in C2C12 myotubes (Figure 2.4A). Interestingly, palmitate stimulated the phosphorylation of the p44 isoform of MAPK, an enzyme linking insulin to the regulation of gene expression, in myotubes (Figure 2.4A). As predicted by the glycogen synthesis experiment (Figure 2.3), 3T3-L1 adipocytes were refractory to all of the palmitate effects on Akt, GSK3β, and MAPK (Figure 2.4B). Thus, saturated fats selectively modulated insulin-signaling pathways in skeletal muscle, but were without effect in 3T3-L1 adipocytes.

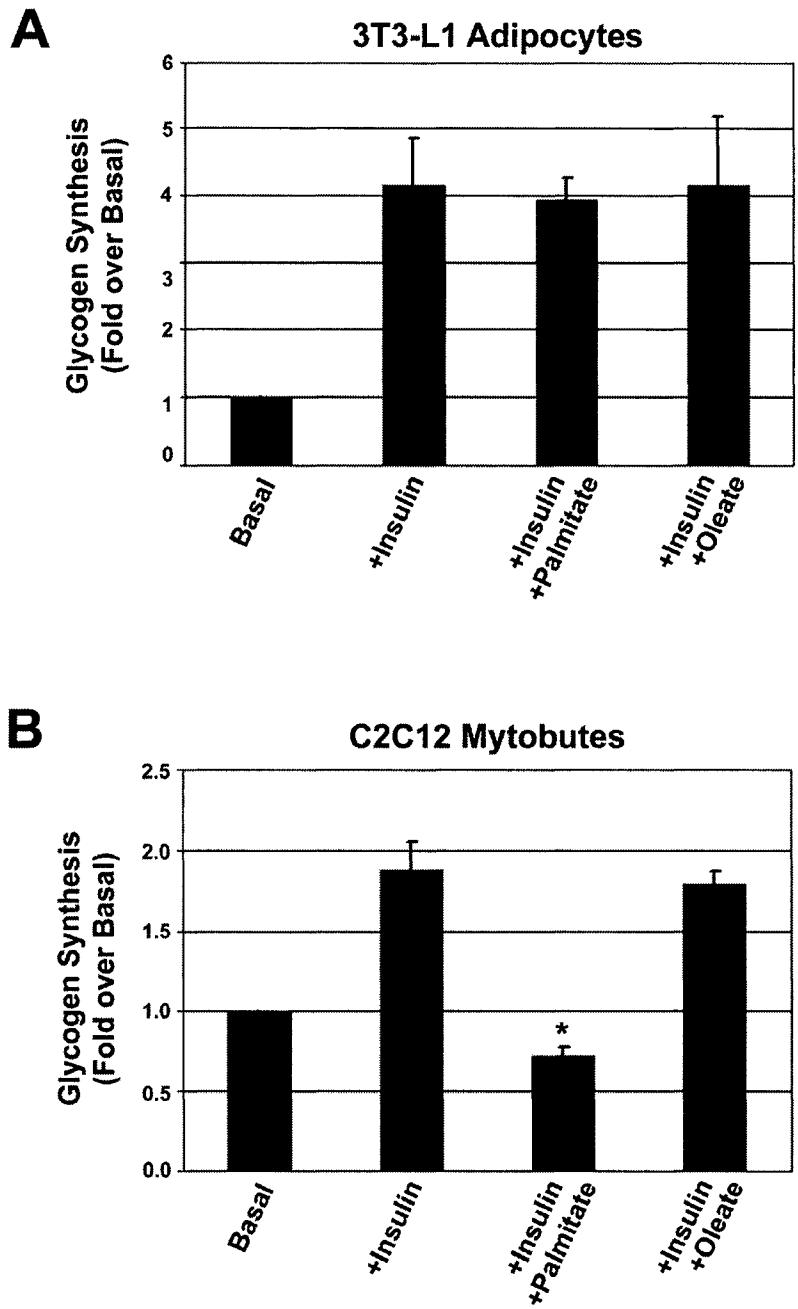


Figure 2.3: Comparing the inhibitory effect of palmitate on insulin-stimulated glycogen synthesis in 3T3-L1 adipocytes and C2C12 myotubes. 3T3-L1 adipocytes **(A)** and C2C12 myotubes **(B)** were incubated in the presence or absence of palmitate (0.75 mM, 16 hours) prior to stimulation with insulin (100 nM, 10 minutes). Glucose incorporation into glycogen was determined as described in the Materials and Methods section. Findings are reported as the mean fold-stimulation over basal rates +/- S.E.M. The asterisk denotes that the value was statistically different than the (+) insulin sample at $p<0.05$.

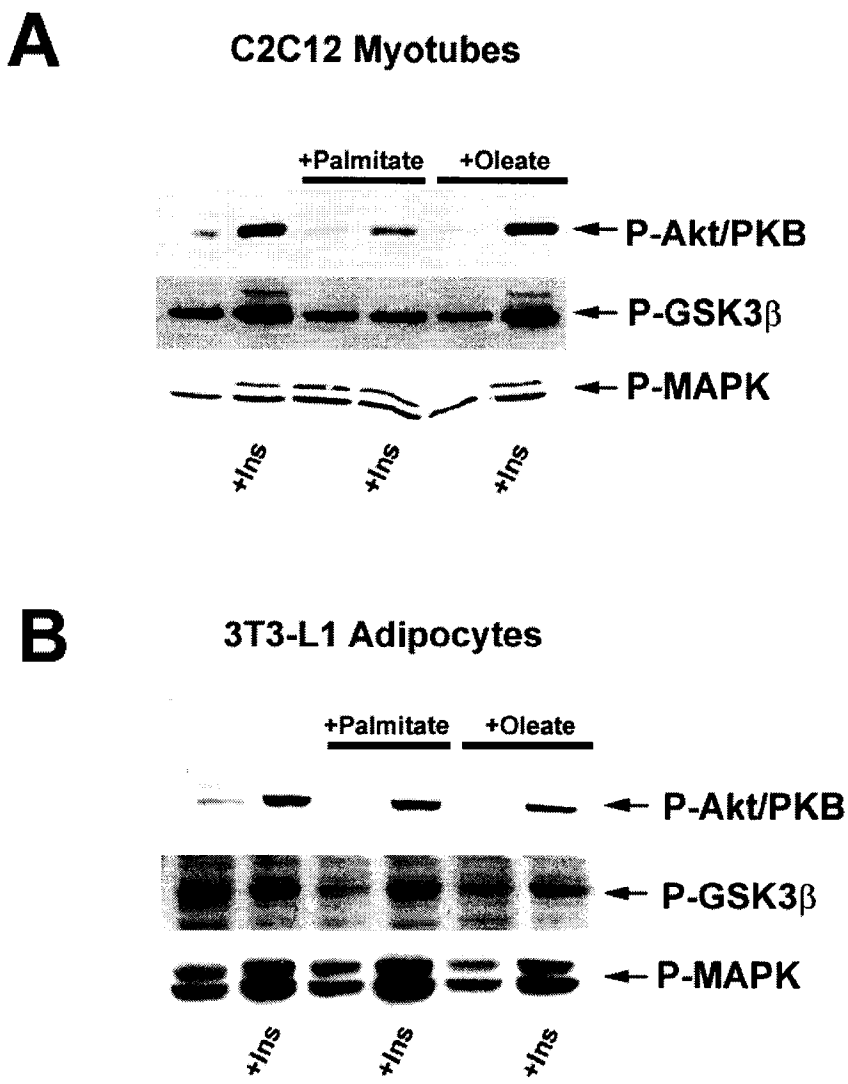


Figure 2.4: Comparing the effect of palmitate on Akt/PKB and MAPK phosphorylation in 3T3-L1 adipocytes and C2C12 Myotubes. 3T3-L1 adipocytes **(A)** and C2C12 myotubes **(B)** were treated with or without palmitate (0.75 mM, 16 hours) prior to stimulation with insulin (100 nM, 10 minutes). Cells were then lysed, resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antibodies. Equal loading and expression was confirmed by immunoblotting with antibodies recognizing total Akt/PKB, GSK3β, or MAPK (data not shown). Detection was by enhanced chemiluminescence. Data are representative of three independent experiments.

2.4b *Evaluating the effects of palmitate and oleate on ceramide/DAG accumulation in 3T3-L1 adipocytes and C2C12 myotubes*

Many investigators have hypothesized that derivatives of fatty acyl-CoA are the likely mediators of lipid-induced insulin resistance (93). Since 3T3-L1 adipocytes predominantly store fatty acids in the form of triglycerides (158), we speculated that their resistance to the inhibitory effects of palmitate may result from their ability to effectively convert fatty acyl-CoAs into triglyceride, thus preventing their conversion into other inhibitory metabolites. Ceramide and DAG are two particular fatty acyl-CoA metabolites that have been shown to accumulate in insulin resistant tissues (116) and to block insulin signaling and action in cultured cells (122, 128, 150-154). Specifically, endogenous ceramide and short-chain ceramide analogs block the activation of Akt/PKB by preventing its recruitment to PIP_3 enriched microdomains in the plasma membrane (128, 150), while phorbol esters, which mimic the effects of DAG, inhibit insulin signaling to IRS-1 (122, 151-153) and Akt (154). Moreover, both reagents have been shown to block insulin-stimulated glucose uptake and/or glycogen synthesis in 3T3-L1 adipocytes or C2C12 myotubes (103, 128). Interestingly, ceramide, in addition to inhibiting Akt/PKB activation, has also been shown to stimulate MAPK phosphorylation (137), and thus could mediate the effects of palmitate on both of these signaling intermediates.

To determine whether palmitate or oleate induced ceramide or DAG accumulation in either cell type, we employed the ceramide:DAG kinase assay. Briefly, DAG kinase can phosphorylate both DAG and ceramide to produce phosphatidic acid and ceramide 1-phosphate, respectively, which can be resolved from each other by thin layer

chromatography (TLC). When the reaction is allowed to proceed in the presence of [^{32}P]ATP, the phosphorylated products can be detected using a Storm phosphorimager (159). Figures 2.5A and 2.5B demonstrate the use of this system to quantify DAG and ceramide standards. As shown in figures 2C and 2D, palmitate induced the accumulation of both DAG and ceramide in C2C12 myotubes, while it failed to stimulate production of either in 3T3-L1 adipocytes. By contrast, oleate, which was unable to inhibit insulin signaling in either cell type, failed to stimulate ceramide or DAG accumulation under any circumstances. As shown in Figure 2.6A, palmitate induced ceramide and DAG synthesis in myotubes at concentrations as low as 250 μM, which is comparable to circulating FFA levels found in both normal and pathological states (160, 161). The effects of palmitate on both DAG and ceramide synthesis correlated tightly with its effects on Akt/PKB phosphorylation (Figure 2.6B).

Many investigators have hypothesized that certain tissues are particularly sensitive to lipid oversupply. The data presented thus far are consistent with this hypothesis, as FFAs selectively modulated insulin action in skeletal muscle, but were without effect in adipocytes. Moreover, the findings presented suggest that ability of 3T3-L1 adipocytes to maintain normal insulin sensitivity in the presence of a large lipid load may result from their ability to absorb the lipids without converting them into inhibitory bio-metabolites, such as ceramide and DAG.

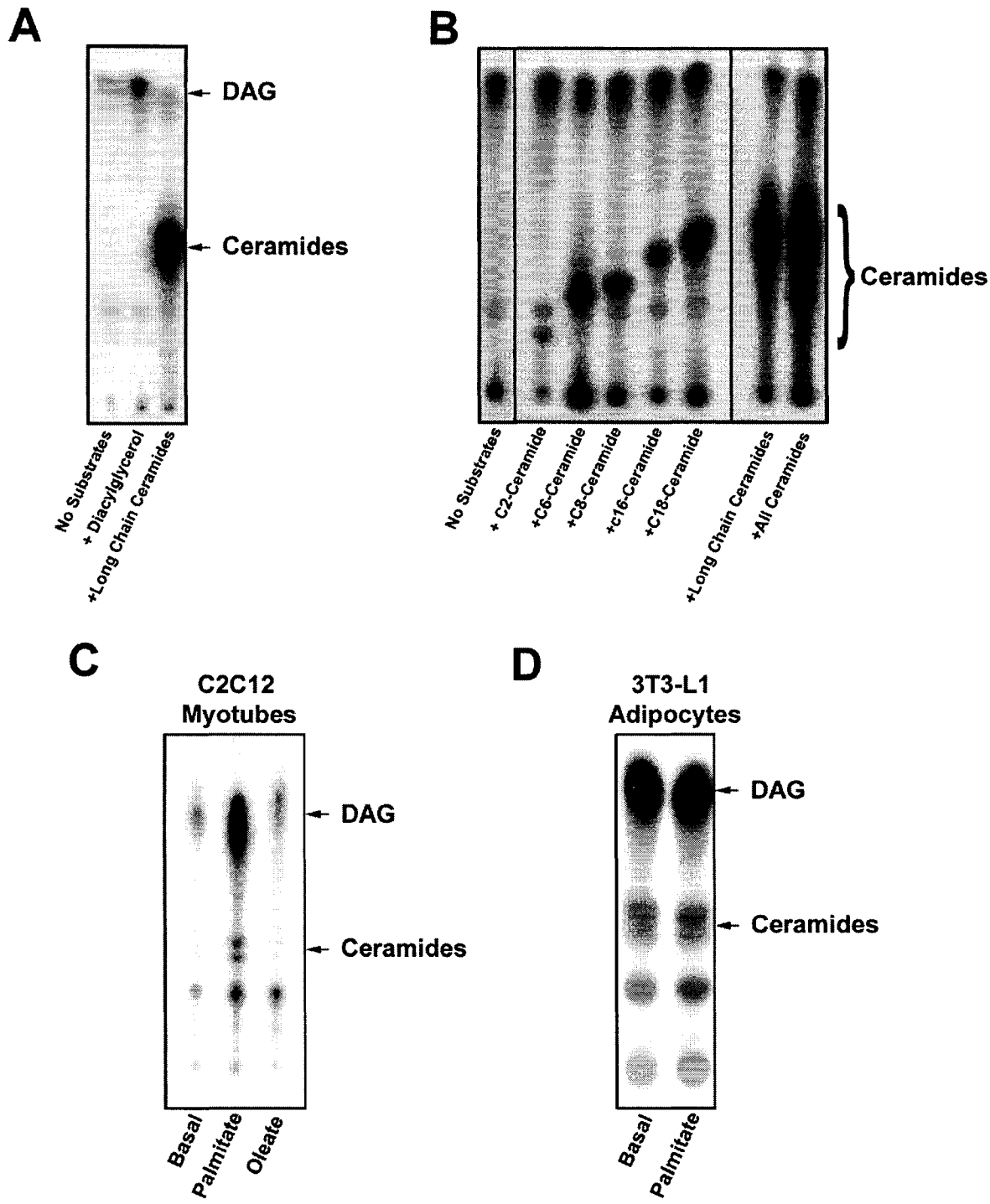


Figure 2.5: Comparing the effects of palmitate on ceramide and DAG accumulation in 3T3-L1 adipocytes and C2C12 myotubes. Ceramide or DAG standards or lipid extracts from C2C12 myotubes or 3T3-L1 adipocytes were incubated with DAG-kinase and [^{32}P]ATP as described in the Methods section. Lipids were then re-extracted, resolved by thin layer chromatography, and detected using a storm phosphorimager. Figure **A** demonstrates the use of this assay to measure DAG and long-chain ceramide species (C18 and C24:1) while Figure **B** demonstrates the ability of this system to resolve ceramide species of varying chain length (C2, C6, C8, C16, and C18 ceramide as indicated). Figures **C** and **D** are autoradiographs depicting the use of this assay to quantify ceramide and DAG levels in lipid extracts obtained from C2C12 myotubes (C) or 3T3-L1 adipocytes (D) treated with or without palmitate or oleate (0.75 mM, 16 hours). Data are representative of at least 3 different experiments.

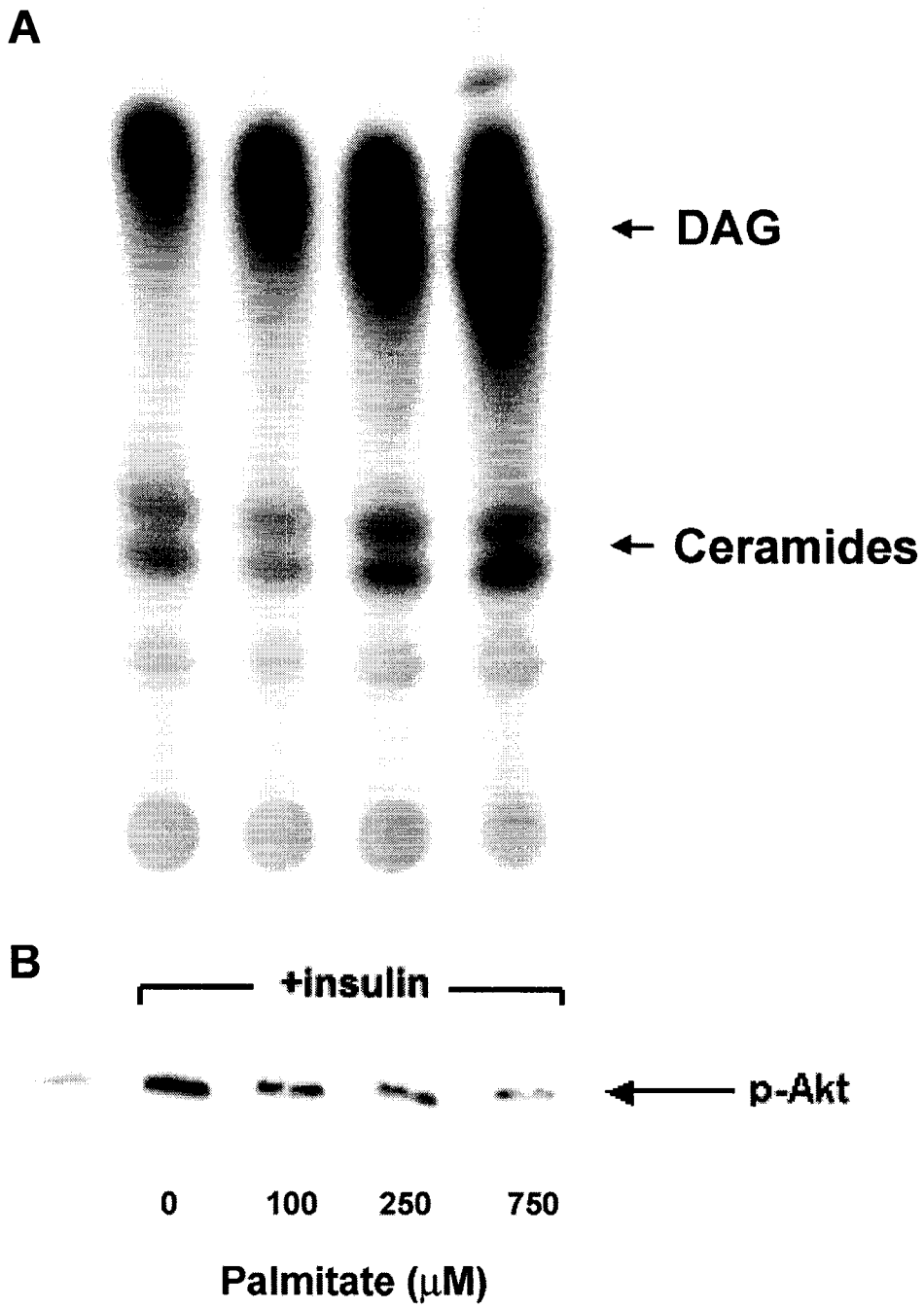


Figure 2.6: Dose response effect of palmitate treatment on C2C12 myotubes. C2C12 myotubes were treated with or without increasing concentrations of palmitate (16:0) (0 → 0.75 mM) for 16 hours prior to lipid extraction or insulin stimulation (100 nM, 10 min). **A** Ceramide and DAG levels were quantified using the DAG-kinase assay as described in the Materials and Methods section. The figure depicts a representative autoradiograph. **B** Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antibodies. Detection was by enhanced chemiluminescence. Data are representative of three independent experiments

2.4c *Evaluating the effects of other saturated fats on insulin signaling and ceramide and DAG accumulation*

We next sought to determine whether other saturated FFAs also had the capacity to inhibit insulin signaling and/or induce ceramide or DAG accumulation in C2C12 myotubes. Stearate (18:0), arachidate (20:0), and lignocerate (24:0) all inhibited insulin-stimulated phosphorylation of Akt/PKB and GSK3β, though none were as potent as palmitate (Figure 2.7). Interestingly, FFAs with shorter hydrocarbon chains [laurate (12:0) and myristate (14:0)] had no effect on insulin-stimulated Akt/PKB or GSK3β phosphorylation. These results indicated that FFAs with hydrocarbon chains as long or longer than palmitate blocked insulin signaling, while those with hydrocarbon chains shorter than palmitate had no effect.

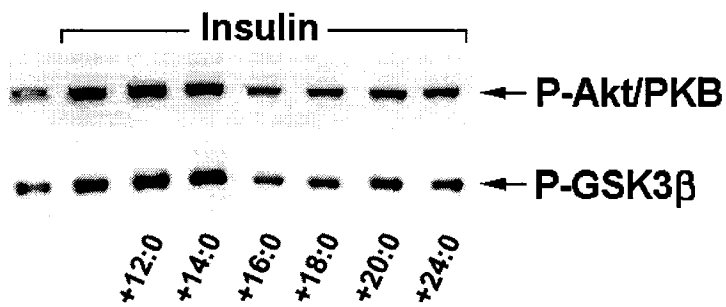


Figure 2.7: Evaluating the effect of other saturated fatty acids on Akt/PKB and GSK3β phosphorylation in C2C12 myotubes. C2C12 myotubes were treated with or without 0.75 mM laurate (12:0), myristate (14:0), palmitate (16:0), stearate (18:0), arachidate (20:0), and lignocerate (24:0) for 16 hours prior to stimulation with insulin (100 nM, 10 minutes). Cells were then lysed, resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antibodies. Equal loading and expression was confirmed by immunoblotting with antibodies recognizing total Akt/PKB or GSK3β (data not shown). Detection was by enhanced chemiluminescence. Data are representative of three independent experiments.

The long-chain saturated FFAs also significantly induced the synthesis of ceramide, while shorter-chain saturated FFAs had no effect (Figure 2.8B). However, the mechanism by which long-chain saturated FFAs stimulate ceramide synthesis remains unclear. Generally, the rate-limiting reaction in ceramide biosynthesis is the condensation of serine with palmitoyl-CoA, a reaction catalyzed by serine palmitoyltransferase (SPT) [reviewed in (162)]. The product, 3-ketosphinganine, then gets reduced, acylated, and oxidized in three subsequent reactions to produce ceramide. Palmitate increases ceramide levels by simply providing precursor palmitoyl-CoA for ceramide synthesis [reviewed in (162)] and, in at least one cell type, inducing SPT expression (163). We surmise that under the cellular conditions used herein, longer chain saturated FFAs either 'protect' the endogenous palmitoyl-CoA pool, perhaps by inhibiting its conversion to stearoyl-CoA and other long-chain fatty acids, or alternatively are converted to palmitoyl-CoA through the process of beta-oxidation. We feel it is unlikely that these FFAs regulate ceramide biosynthesis by contributing to the acylation reaction that is catalyzed by ceramide synthase, since the TLC system described should resolve ceramide species with markedly different chain lengths in that position (Figures 2.5 and 2.8).

Fatty acyl-CoA's can also induce DAG synthesis by condensing with glycerol 3-phosphate to form phosphatidic acid, which can subsequently be dephosphorylated to form DAG. All of the FFAs, with the exception of laurate, induced DAG synthesis (Figure 2.8C). Surprisingly, myristate did not inhibit Akt/PKB or GSK3β, though it did stimulate DAG accumulation to levels comparable to those achieved with an inhibitory

lipid, stearate. This observation led us to surmise that DAG derived from saturated fats was not sufficient for the inhibition of insulin action. To confirm this, we compared the effect of myristate and stearate on the regulation of insulin-stimulated glycogen synthesis. As predicted by the signaling studies, stearate inhibited insulin-stimulation of glucose incorporation into glycogen in C2C12 myotubes, while myristate had no effect (Figure 2.9). These data confirm that DAG derived from myristate did not inhibit insulin action. In general, DAG derived from saturated fats has been shown to be a poor activator of protein kinase C (164), and thus may contribute little to the inhibitory effects of saturated fatty acids on insulin signaling and anabolic metabolism.

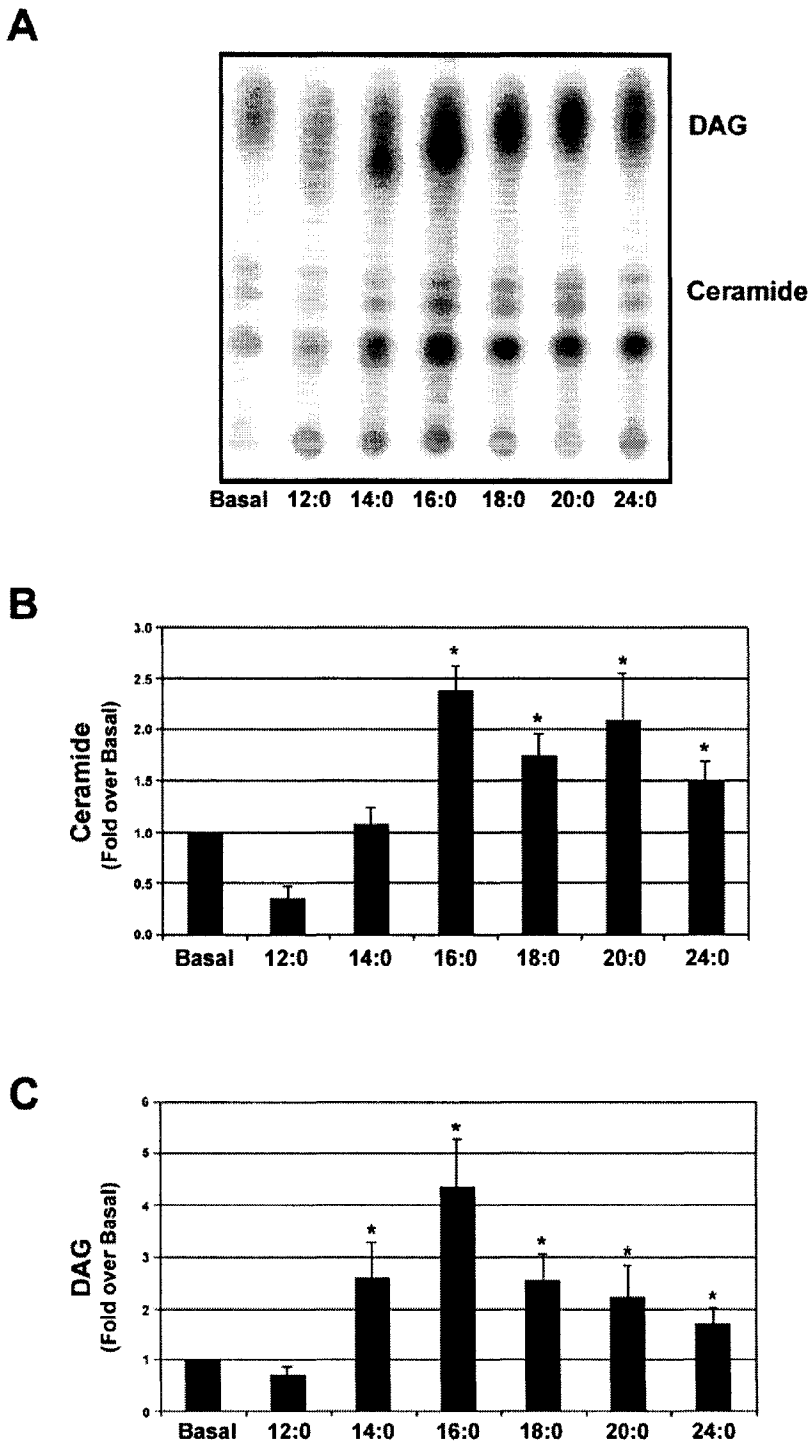


Figure 2.8: Evaluating the effect of saturated fatty acids on ceramide and DAG accumulation in C2C12 myotubes. C2C12 myotubes were treated with or without 0.75 mM laurate (12:0), myristate (14:0), palmitate (16:0), stearate (18:0), arachidate (20:0), and lignocerate (24:0) for 16 hours prior to lipid extraction. Ceramide and DAG levels were quantified using the DAG-kinase assay as described in the Materials and Methods section. Figure A depicts a representative autoradiograph. Figures B and C are quantifications of mean ceramide and DAG levels, respectively, +/- S.E.M. Asterisks denote that the values were significantly different than basal levels at $p<0.05$. Ceramide and DAG levels measured in untreated C2C12 myotubes were typically in the range of 400 pmol/mg protein and 1 μmol/mg protein, respectively.

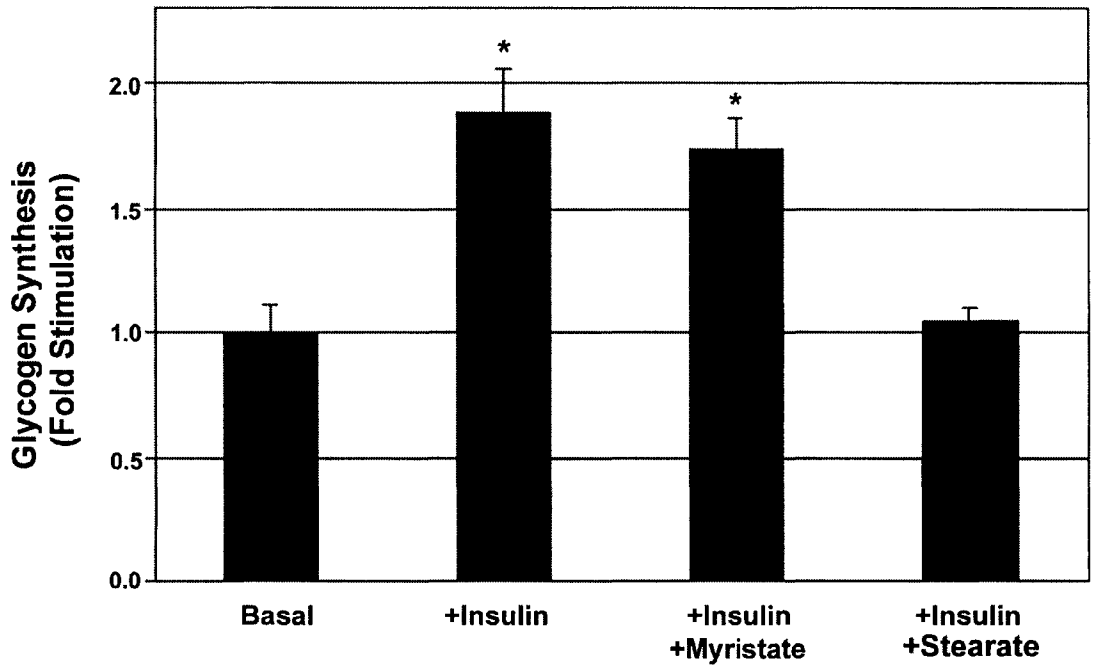


Figure 2.9: Comparing the effect of palmitate and myristate on glycogen synthesis in C2C12 myotubes. C2C12 myotubes were treated with stearate or myristate as in Figure 6 prior to stimulation with insulin (100 nM, 10 min.). Rates of glucose incorporation into glycogen were determined as described in the Materials and Methods section. Findings are reported as the mean fold-stimulation over basal rates +/- S.E.M. Asterisks denote that samples were statistically different than basal levels at $p<0.05$.

2.5 Conclusions

Greater than 80% of type 2 diabetics are obese, but the link between increased adiposity and the development of the disease is unknown. Particularly enigmatic is the observation that the majority of glucose disposal occurs in skeletal muscle, not adipose tissue, thus necessitating a link between increased adiposity and the desensitization of skeletal muscle. An idea gaining credibility is that insulin resistance results not from increased fat stores in adipose tissue, but rather results from the ectopic deposition of lipid in tissues not suited for fat storage, such as skeletal muscle and liver [reviewed in (93)]. Proponents of this hypothesis point to the usefulness of a class of antidiabetic drugs, a family of PPARγ agonists termed thiazolidinediones. These drugs have been shown to decrease plasma concentrations of triglyceride, very low-density lipoproteins, and FFAs, while simultaneously promoting the differentiation of preadipocytes into mature adipocytes capable of storing triglyceride. Thus, an emerging hypothesis is that the insulin sensitizing properties of these compounds derive from their capacity to promote storage of excess lipids in newly formed adipocytes (93).

The studies described herein support the hypothesis that aberrant fat deposition in skeletal muscle could contribute to the development of insulin resistance. Specifically, although the saturated FFA palmitate had no deleterious effects on insulin signaling or action in cultured 3T3-L1 adipocytes, it was a potent inhibitor of these events in C2C12 myotubes. Several long-chain saturated FFAs proved capable of mimicking these palmitate effects. The effects of these antagonistic FFAs could be explained by their capacity to induce the accumulation of inhibitory biometabolites, particularly ceramide,

in skeletal muscle, but not fat. Collectively these findings suggest that therapeutic strategies aimed at preventing the conversion of FFAs to these or other inhibitory metabolites may improve insulin sensitivity in skeletal muscle.

2.6 Acknowledgements

The authors thank Yusuf Hannun (Medical University of South Carolina) and Al Merrill (Georgia Institute of Technology) for helpful discussions, and Suzanne Stratford and Trina Knotts (Colorado State University) for critically reviewing the manuscript prior to submission.

CHAPTER 3

A Role for Ceramide, but not Diacylglycerol, in the Antagonism of Insulin Signal Transduction by Saturated Fatty Acids

This chapter describes work published in the Journal of Biological Chemistry (Chávez *et.al.*, 2003). All experiments presented herein were carried out by Chavez JA.

Chavez JA, Knotts TA, Wang LP, Li G, Dobrowsky RT, Florant GL, Summers SA. *J Biol Chem.* 2003 Mar 21;278(12):10297-303

3.1 Summary

Multiple studies suggest that lipid oversupply to skeletal muscle contributes to the development of insulin resistance, perhaps by promoting the accumulation of lipid metabolites capable of inhibiting signal transduction. Herein we demonstrate that exposing muscle cells to particular saturated free fatty acids (FFAs), but not mono-unsaturated FFAs, inhibits insulin stimulation of Akt/Protein Kinase B (PKB), a serine/threonine kinase that is a central mediator of insulin-stimulated anabolic metabolism. These saturated FFAs concomitantly induced the accumulation of ceramide and diacylglycerol (DAG), two products of fatty acyl-CoA that have been shown to accumulate in insulin resistant tissues and to inhibit early steps in insulin signaling. Preventing *de novo* ceramide synthesis negated the antagonistic effect of saturated FFAs towards Akt/PKB. Moreover, inducing ceramide buildup recapitulated and augmented the inhibitory effect of saturated FFAs. By contrast, DAG proved dispensable for these FFA effects. Collectively these results identify ceramide as a necessary and sufficient intermediate linking saturated fats to the inhibition of insulin signaling.

3.2 Introduction

The peptide hormone insulin stimulates the uptake and storage of glucose in skeletal muscle and adipose tissue while simultaneously inhibiting its efflux from the liver. In certain pathological conditions, including Type 2 Diabetes Mellitus (136) and Metabolic Syndrome X (68), these tissues become resistant to insulin such that a maximal dose of the hormone is unable to elicit these anabolic responses. Numerous studies suggest that the oversupply of lipid to peripheral tissues might contribute to the development of this insulin resistance. First, insulin resistant subjects frequently display signs of abnormal lipid metabolism including obesity (75), increased circulating free fatty acid (FFA) concentrations (160, 165), and elevated intramyocellular lipid (IMCL) levels (93). In fact, the size of the IMCL depots correlates more tightly with the severity of insulin resistance than most known risk factors (93). Second, experimentally exposing peripheral tissues to lipids decreases their sensitivity to insulin. For example, (a) incubating isolated muscle strips or cultured muscle cells with FFAs (78, 103, 106, 166, 167), (b) infusing lipid emulsions into rodents or humans (94, 168-170), or (c) expressing lipoprotein lipase in skeletal muscle of transgenic mice (105, 171) promotes IMCL accumulation and compromises insulin-stimulated glucose uptake. These observations have prompted investigators to hypothesize that increased availability of lipids to peripheral tissues causes insulin resistance, perhaps by promoting the accumulation of one or more fat-derived metabolites capable of inhibiting insulin action (93, 111).

The insulin receptor is a heterotetrameric tyrosine kinase receptor that mediates all of insulin's anabolic effects (139). The activated receptor phosphorylates intracellular

docking molecules (termed insulin receptor substrates, or IRS proteins) that recruit and stimulate multiple different effector enzymes (172). Phosphatidylinositol 3-kinase (PI3K) is a target of IRS proteins that is an obligate intermediate in the metabolic, anti-apoptotic, and mitogenic effects of insulin (173). PI3K phosphorylates specific phosphoinositides to generate phosphatidylinositol-3,4-bisphosphate (PI-3,4-P_2) and phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P_3), which subsequently recruit cytosolic serine/threonine kinases phosphoinositide dependent kinase-1 (PDK1) and Akt/Protein Kinase B (PKB) to the plasma membrane. The association between these phosphoinositides and the Akt/PKB pleckstrin homology domain (PH domain) promotes Akt/PKB activation by facilitating its phosphorylation on two regulatory residues (i.e. S473 and T308) (34). Studies in cultured cells involving the overexpression of constitutively active (42) or dominant negative (45, 174) forms of Akt/PKB, coupled with experiments involving the microinjection of inhibitory anti-Akt/PKB antibodies (47), indicate the enzyme's involvement in the regulation of anabolic metabolism. Moreover, knockout mice lacking the Akt2/PKBβ isoform develop a diabetes-like syndrome characterized by insulin resistance in both skeletal muscle and liver (49).

The molecular mechanisms linking FFAs to the inhibition of insulin action remain unclear. In 1963, Randle and coworkers proposed the existence of a glucose-fatty acid by which glucose and lipids could serve as competitive substrates for oxidation in muscle (87). More recent studies in either cultured cells (78, 103, 106) or rodent models of obesity and/or insulin resistance (95, 175), however, indicate that fatty acids also disrupt one or more early steps in insulin signal transduction. As shown herein, the saturated fats

palmitate and stearate, but not their mono-unsaturated counterparts oleate and palmitoleate, blocked insulin activation of Akt/PKB while concomitantly promoting the accumulation of ceramide and diacylglycerol in C2C12 myotubes. These lipid metabolites have both been shown to accumulate in tissues from insulin resistant rodents (116) and to inhibit insulin signal transduction in cultured cells (128, 129, 176-179). Specifically, studies with short-chain ceramide analogs reveal that ceramide prevents insulin-activation of Akt/PKB (128, 129, 176), while investigations with phorbol esters, which mimic the effects of DAG, indicate that DAG blocks upstream signaling events by promoting the serine phosphorylation of IRS-1 (122, 151-154, 180). Using various methods for manipulating either the synthesis or breakdown of ceramide we found that endogenously produced ceramide was both sufficient and necessary for the inhibition of insulin signaling by palmitate. Moreover, we determined that DAG was dispensable for the inhibitory palmitate effects. These findings implicate ceramide as a potentially important intermediate linking saturated fats to the development of insulin resistance.

3.3 Experimental Procedures

3.3a *Reagents*:

C2-ceramide and D-L-threo 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) were obtained from Calbiochem (La Jolla, CA), okadaic acid was from Gibco-BRL (Gaithersburg, MD), fetal bovine serum was from Atlas Biologicals (Fort Collins, CO), and silica gel 60 thin layer chromatography (TLC) plates were from Merck (Darmstadt, Germany). The following additional reagents were obtained from Sigma Chemicals (St. Louis, MO): palmitate, stearate, oleate, palmitoleate, Dulbecco's Modified Eagles's Medium (DMEM), fatty acid free bovine serum albumin (BSA), C-6 ceramide, C-16 ceramide, C-18 ceramide, fumonisin B1, myriocin, cycloserine, and N-oleoylethanolamine (NOE). Antibodies utilized included the following: rabbit polyclonal anti-phospho-Akt (Ser-473) and anti-phospho-GSK3β (serine-9) antibodies from Cell Signaling; a mouse anti-glycogen synthase kinase 3β (GSK3β) antibody from Transduction Labs (Lexington, KY); a rabbit anti-phospho MAPK antibody from Promega (Madison, WI); and rabbit polyclonal anti-Akt and horseradish peroxidase conjugated anti-rabbit and anti-mouse antibodies from Santa Cruz Biotechnology (Santa Cruz, CA).

3.3b *Cell culture*:

C2C12 myoblasts were maintained at 37°C in Dulbecco's modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS). For differentiation into myotubes, the myoblasts were grown to confluency and the media replaced with DMEM

containing 10% horse serum. Myotubes were used for experiments 4 days following differentiation.

3.3c *FFA Treatment*:

Free fatty acids were administered to cells by conjugating them with FFA-free bovine serum albumin (BSA). Briefly, FFAs were dissolved in ethanol and diluted 1:100 in 1% FBS-DMEM containing 2% (w/v) BSA. Two hours prior to performing the experiments, myotubes were placed in serum free-DMEM containing 2% BSA in either the presence or absence of FFAs.

3.3d *Immunoblot Analysis*:

Cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose and immunoblotted using methods described previously (155). Detection was done using the Enhanced Chemiluminescence Plus kit from Amersham Biosciences (Piscataway, NJ) according to manufacturers instructions.

3.3e *Phosphatidylinositol 3-Kinase Activity.*

PI3-kinase activity was assessed using the methodology of Summers *et al* (54).

3.3f *Ceramide Assay*:

Myotubes were lysed in ice-cold 1M NaCl (0.25-ml) and transferred to glass tubes. Ice cold $CHCl_3$:CH_3OH (1:2, v/v, 0.75 ml) was added and the suspension was vortexed vigorously. After the subsequent addition of 0.25 ml of 1 M NaCl and 0.25 ml

of $CHCl_3$, phases were separated by centrifugation at 3000 RPM (2000xG) for 5 min. Ceramide content in the extract was determined using a radiometric diacylglycerol assay kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

3.3g *Adenovirus Amplification, Titering, and Infection*:

Small-t cDNA served as a template for PCR amplification. The upstream primer (5'-AGATCTATGGATAAAGTTTTAAACAG-3') incorporated a *Bgl* II site and an ATG start codon and the downstream primer (5'-CTCGAGTTAGAGCTTTAAA TCTCT-3') was engineered with a *Xho* I site. The amplified products were cloned directly into TOPO2.1 (Invitrogen) and sequenced in both directions to confirm that no errors were introduced by PCR. The small-t TOPO2.1 cDNA was digested with *Bgl* II and *Xho* I and the approximate 0.5 kb small-t fragment was gel purified and subcloned into the pAdTrack-CMV shuttle vector (181). Recombinant adenoviruses were generated by homologous recombination after electroporation of small-t- with pAdEASY-1 into $RecA^+$ bacteria (BJ5183). Bacterial clones containing recombinant adenoviral DNA were verified by restriction digestion with *Pac* I, and recombinant viruses were generated by transfection of HEK 293 cells. Virus was amplified by four rounds of infection and purified from 20 x 15 cm plates of HEK 293 cells using two rounds of centrifugation through CsCl gradients. The residual CsCl was removed by dialysis against 10% glycerin in PBS in a Slide-a-Lyzer cassette. Virus was tittered using the methods of Minamide *et al.* (181), and myotubes were infected at a multiplicity of infection of 100. Experiments were performed 48 hours after infection.

3.4 Results

To identify specific FFAs capable of antagonizing insulin signal transduction, we evaluated the effect of specific saturated and monounsaturated FFAs on insulin-stimulated Akt/PKB phosphorylation and activation in C2C12 myotubes. Palmitate (C16:0) and stearate (C18:0), which comprise greater than 90% of the saturated FFAs in human serum (182), each markedly inhibited insulin-stimulation of Akt/PKB phosphorylation (Figure 3.1A). By contrast, neither oleate (C18:1), which makes up 80% of the circulating monounsaturated pool (182), nor palmitoleate (C16:1), had any effect (Figure 3.1A). Palmitate and stearate also inhibited insulin-stimulated phosphorylation of glycogen synthase kinase 3β (GSK3β) (Figure 3.1A), a substrate of Akt/PKB with numerous functions including the regulation of glycogen synthase activity (54). Palmitate inhibited Akt/PKB phosphorylation within 2-4 hours (Figure 3.1B) at a FFA concentration as low as 0.25 mM (Figure 3.1C). This concentration is comparable to that found physiologically, and is similar to that used in prior studies evaluating FFA effects in both immortalized muscle cells and isolated skeletal muscle strips. Neither palmitate nor stearate inhibited insulin-activation of PI3-kinase (Figure 3.2).

We next determined whether the inhibitory FFAs also induced the accumulation of DAG and/or ceramide. Briefly, DAG kinase can phosphorylate both DAG and ceramide to produce phosphatidic acid and ceramide 1-phosphate, respectively, which can be resolved by thin layer chromatography (TLC). When the reaction is allowed to proceed in the presence of [^{32}P]ATP, the phosphorylated products can be detected using a Storm phosphorimager (159). As shown in figure 3.3, palmitate and stearate induced

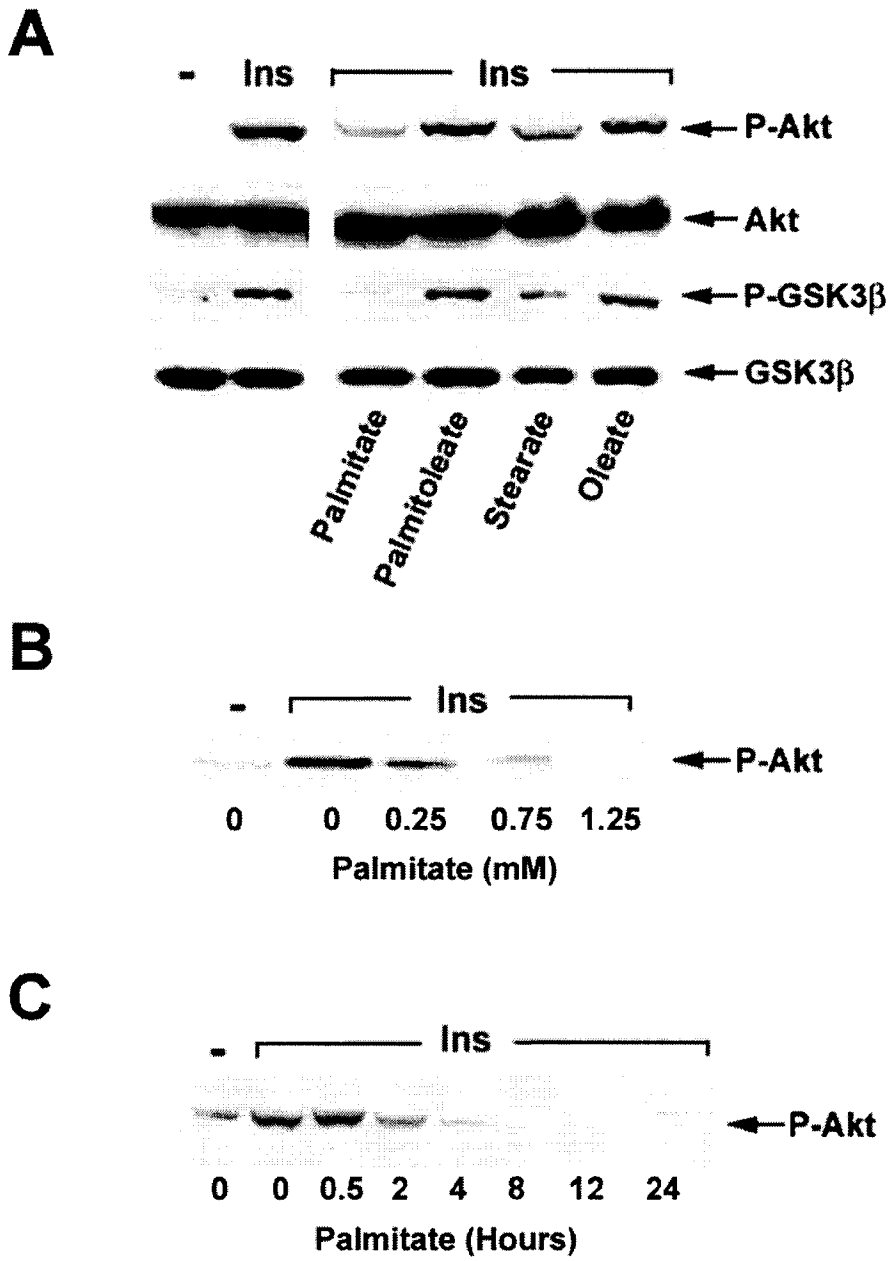


Figure 3.1: Palmitate and stearate inhibit Akt/PKB phosphorylation and activation. **A.** C2C12 myotubes were incubated in the presence or absence of the indicated FFAs (16 hours, 0.75 mM) prior to stimulation with insulin (100 nM, 10 minutes). Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antibodies. Detection was by enhanced chemiluminescence. Data are representative of three independent experiments. **B.** C2C12 myotubes were incubated with palmitate (16 hours) at the concentration indicated prior to analysis as in **(A)** above. Data are representative of three independent experiments. **C.** C2C12 myotubes were incubated with palmitate (0.75 mM) for the time indicated prior to analysis as in **(A)** above. Data are representative of three independent experiments.

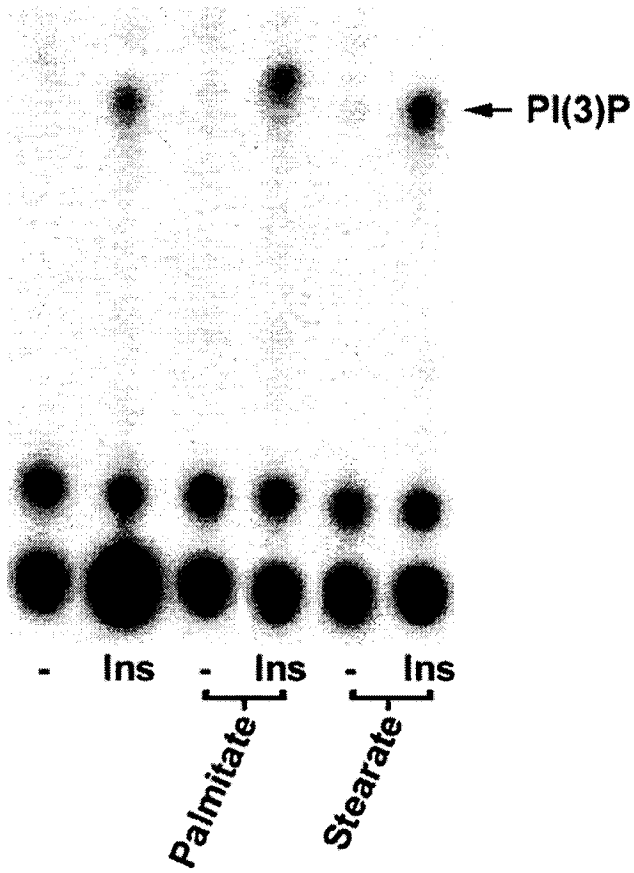


Figure 3.2: Palmitate and stearate do not inhibit insulin-stimulated PI3-kinase activity. C2C12 myotubes were incubated in the presence or absence of the indicated fatty acids (16 hours, 0.75 mM) prior to stimulation with insulin (100 nM, 10 minutes). IRS-1 was immunoprecipitated from cell lysates and incubated with [^{32}P]ATP and phosphoinositide. Lipids were extracted and resolved by thin layer chromatography. Phosphoinositide 3-phosphate [PI(3)P] produced by the phosphorylation of phosphoinositide by PI3-kinase was visualized using a Storm phosphorimager. Data are representative of three independent experiments.

ceramide accumulation 3-fold over basal levels (Figure 3.3A), whereas they induced DAG accumulation ~6-fold and 3.5-fold, respectively (Figure 3.3B). By contrast, neither oleate nor palmitate has any effect on either ceramide or DAG accumulation. Interestingly, another saturated FFA, myristate (14:0), induced DAG synthesis while having no effect on ceramide accumulation or Akt/PKB or GSK3β phosphorylation (Chapter 2).

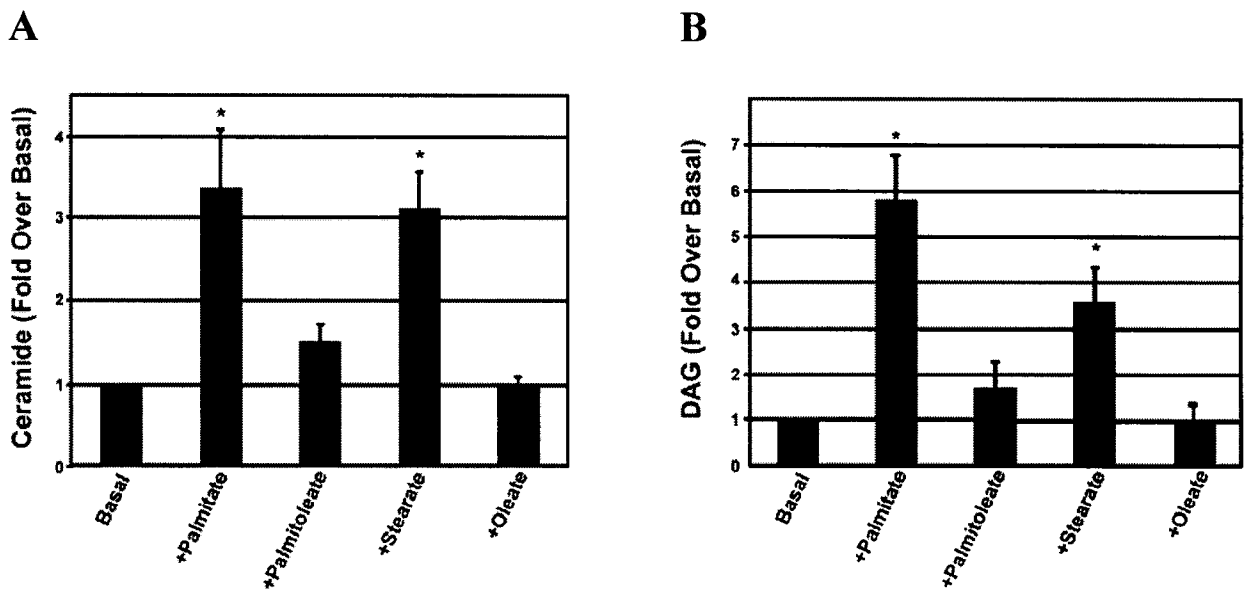


Figure 3.3: Palmitate and stearate stimulate both ceramide and diacylglycerol accumulation. Lipid extracts from C2C12 myotubes were incubated with DAG-kinase and [^{32}P]ATP as described in the Methods section. Lipids were then re-extracted, resolved by thin layer chromatography, and detected using a Storm phosphor-Imager. A and B demonstrate the levels of ceramide and DAG, respectively, in cells treated with or without the indicated fatty acids (16h,0.75 mM). Ceramide and DAG levels are presented as the mean fold increase (over basal) +/- the S.E.M. Asterisks denote that the values obtained were significantly different than basal levels ($p \leq 0.05$).

As described earlier, ceramide blocks insulin signaling by preventing the activation of Akt/PKB (103, 128), whereas DAG inhibits 'upstream' signaling events [i.e. insulin-stimulation of IRS-1 associated PI3-kinase (122, 151-154, 180)]. Since palmitate also inhibited Akt/PKB but not PI3-kinase (Figure 3.2), we hypothesized that ceramide, and not DAG, was the principal effector linking saturated FFAs to the inhibition of Akt/PKB. To test this hypothesis, we determined whether inhibitors of *de novo* ceramide synthesis could prevent palmitate's inhibition of insulin signaling. Briefly, ceramide biosynthesis requires the coordinate action of two enzymes, *i.e.* serine palmitoyl transferase and ceramide synthase [for review, see Ref (162)] (Figure 3.4). Serine palmitoyl transferase catalyzes the initial step, which involves the condensation of serine

with palmitoyl-CoA to produce 3-ketosphinganine, an sphingolipid that is subsequently reduced to form the sphingoid base sphinganine. Ceramide synthase catalyzes sphinganine acylation, producing dihydroceramide, which is then converted to ceramide by the induction of a *trans*-4,5 double bond in the sphinganine moiety.

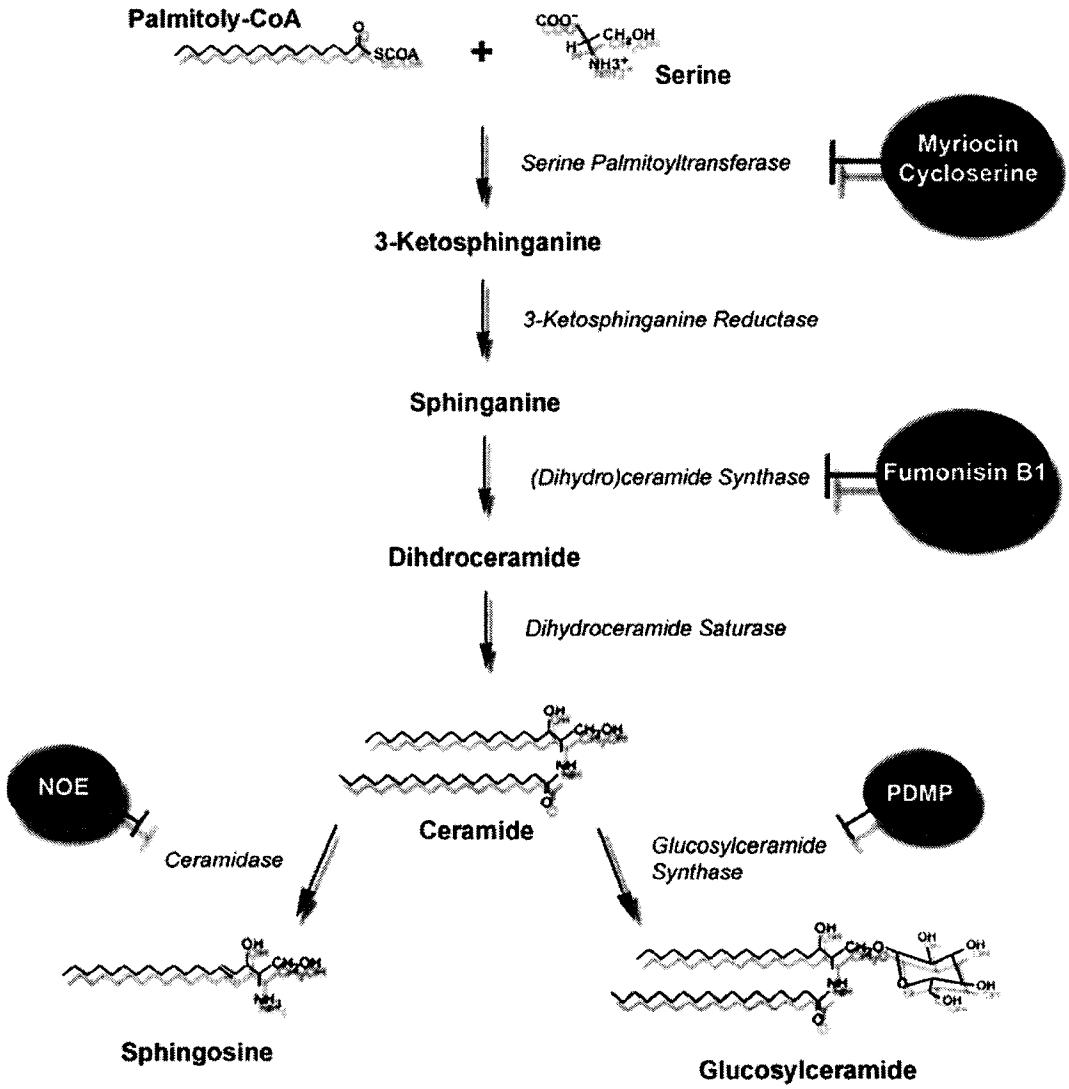


Figure 3.4: Inhibitors of ceramide synthesis or metabolism: Schematic diagram depicting the primary steps in ceramide biosynthesis.

As shown in figure 3.5A, pretreating C2C12 myotubes with myriocin, a fungal toxin that inhibits serine palmitoyltransferase, completely prevented the palmitate-induced increase in ceramide levels, but had no effect on DAG accumulation (Figure 3.5B). As predicted, myriocin completely negated the inhibitory effect of palmitate on both Akt/PKB and GSK3β phosphorylation (Figure 3.6A). Similarly, cycloserine, another specific inhibitor of serine palmitoyltransferase, also reversed the palmitate effect on Akt/PKB phosphorylation (Figure 3.6B). Fumonisin B1, a fungal toxin that inhibits a second enzyme in the biosynthetic pathway (i.e. ceramide synthase, Figure 3.4), also prevented the palmitate effect on ceramide (Figure 3.5A), but not DAG (Figure 3.5B), accumulation. Like myriocin and cycloserine, fumonisin B1 protected both Akt/PKB and GSK3β from the inhibitory effects of palmitate (Figure 3.6C). Myriocin, cycloserine, or fumonisin B1 did not affect the expression or the basal or insulin-stimulated phosphorylation of either Akt/PKB or GSK3β (data not shown). To confirm that myriocin and fumonisin B1 were acting upstream of ceramide, we evaluated whether a short-chain ceramide analog (C2-ceramide) would inhibit Akt/PKB in the presence of these toxins. According to our hypothesis, the addition of C2-ceramide should bypass the site of myriocin or fumonisin B1 action. As shown in figure 3.7, the inhibition of Akt/PKB by C2-ceramide was insensitive to both myriocin and fumonisin B1.

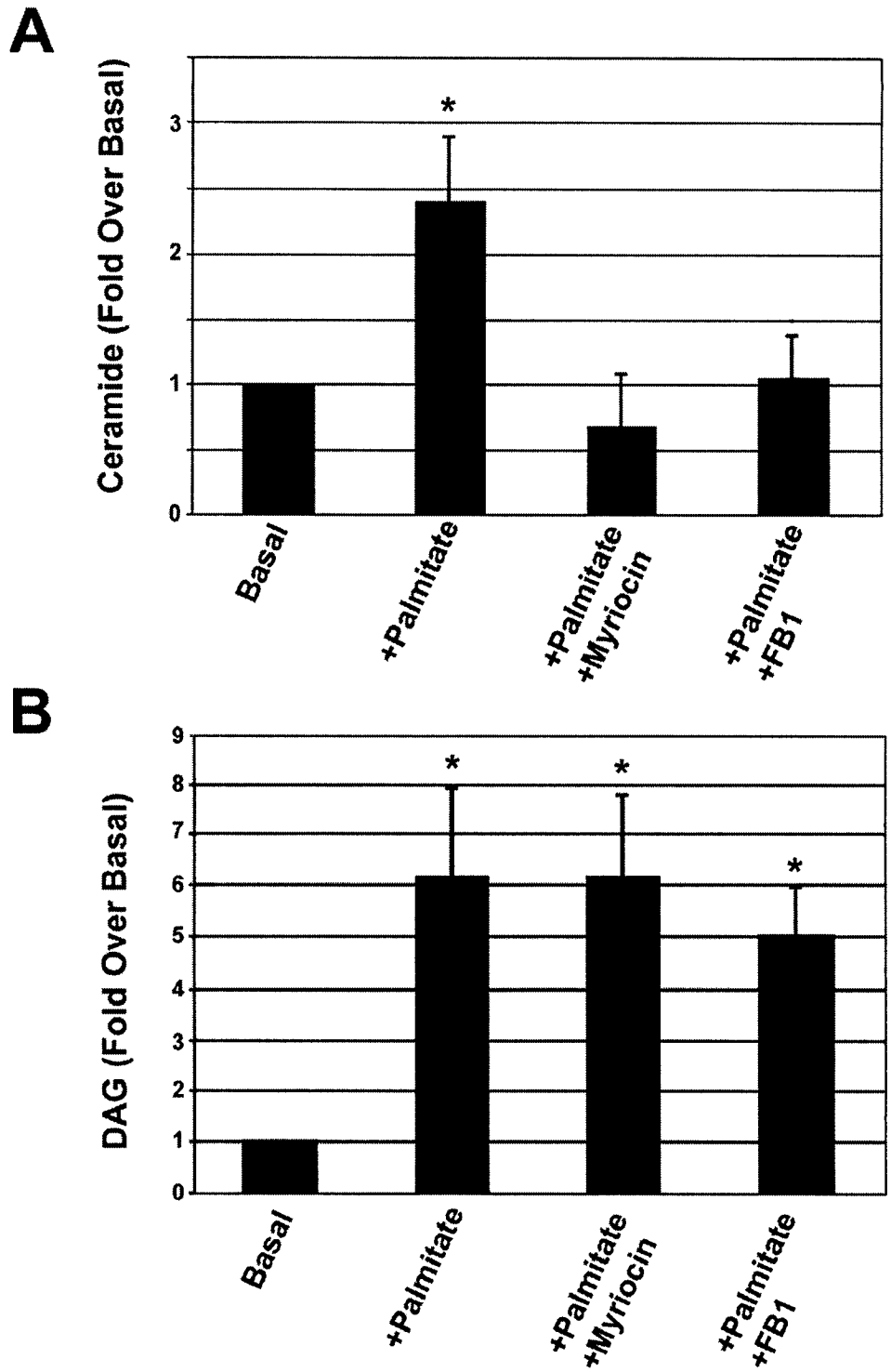


Figure 3.5: Myriocin and fumonisin B1 prevent palmitate's stimulation of ceramide synthesis. C2C12 myotubes were incubated in the presence or absence of palmitate (0.75 mM), myriocin (Myr, 10 μM), or fumonisin B_1 (FB1, 50 μM) for 16 hours prior to lipid extraction. **A.** Ceramide and **B.** DAG levels were quantified as in figure 3. Ceramide and DAG levels are presented as the mean fold increase (over basal) +/- the S.E.M. Asterisks denote that the value was significantly different than basal levels ($p \leq 0.05$).

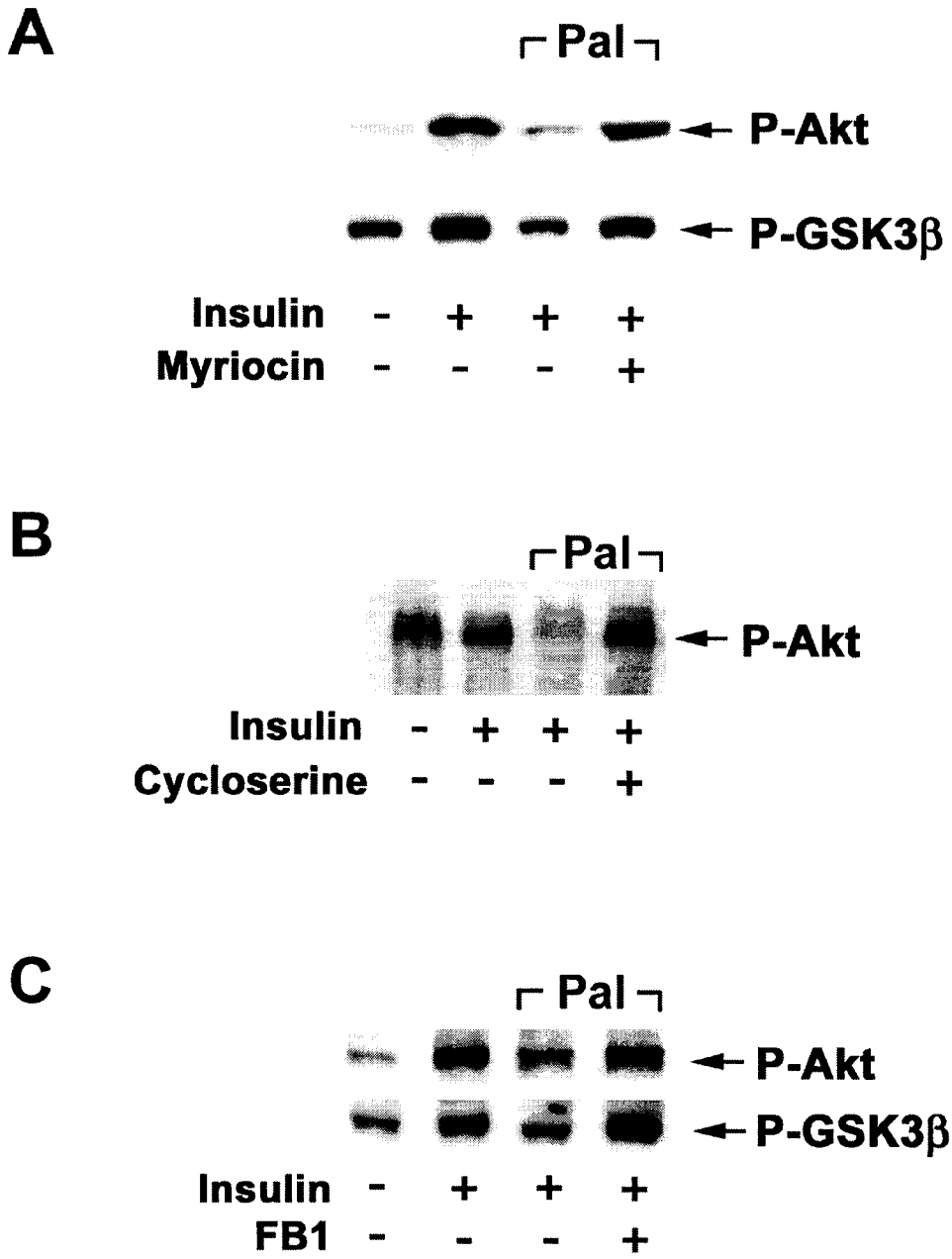


Figure 3.6: Myriocin, cycloserine, and fumonisin B1 prevent the inhibition of insulin signaling by palmitate. C2C12 myotubes were incubated in the presence or absence of palmitate (8 hours, 0.75 mM) prior to stimulation with insulin (100 nM, 10 minutes). Selected samples were treated with or without the serine palmitoyltransferase inhibitors myriocin (10 μM) or cycloserine (1mM) or the ceramide synthase inhibitor fumonisin B_1 (50 μM) just prior to adding palmitate. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antibodies. Detection was by enhanced chemiluminescence. Data are representative of at least four independent experiments.

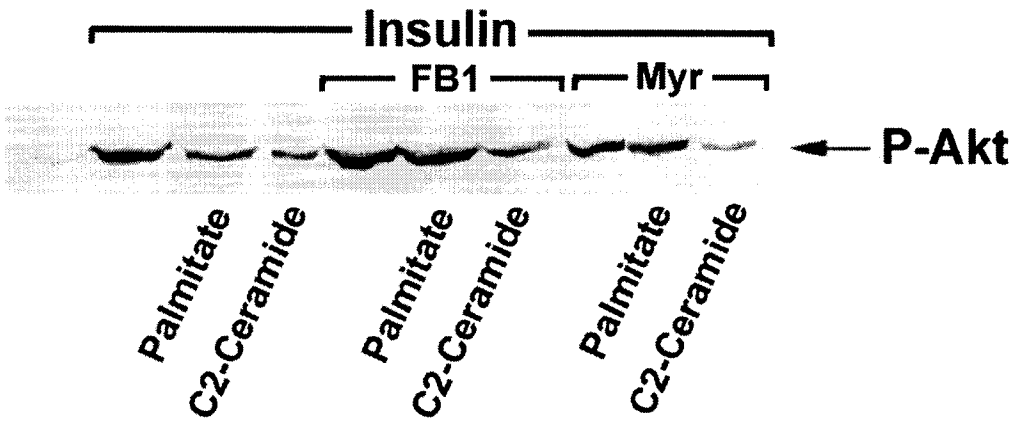


Figure 3.7: Fumonisin B1 and myriocin do not prevent the inhibition of insulin signaling by C2-ceramide. C2C12 myotubes were treated with palmitate (Pal), fumonisin B_1 (FB1), or myriocin (Myr) as in figure six, above. Some samples were also treated with C2-ceramide (Cer, 100 μM) thirty minutes prior to lysis. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antibodies. Detection was by enhanced chemiluminescence. Data are representative of three independent experiments.

Most of the work revealing that ceramide inhibits Akt/PKB relies on the use of short-chain ceramide analogs, which are questionable in terms of their ability to mimic endogenous ceramide (183). Moreover, both C2-ceramide and endogenously produced ceramide can be rapidly deacylated and glucosylated to give rise to a broad array of sphingolipid-derived molecules [reviewed in (162)]. Thus, two questions remained: first, could endogenous ceramide mimic the effects of both C2-ceramide and palmitate; second, is ceramide itself, or alternatively another ceramide metabolite, the principal mediator of the inhibitory effects of palmitate. To address this issue, we treated cells with inhibitors of ceramide glucosylation [i.e. the glucosylceramide synthase inhibitor D-L-threo 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP)] or deacylation [i.e. the ceramidase inhibitor N-oleoylethanolamine (NOE)]. These compounds were

shown previously to increase endogenous ceramide levels by blocking its normal route(s) of metabolism (184). Treating C2C12 myotubes with either compound increased cellular ceramide to levels comparable to those achieved with palmitate alone (Figure 3.8A), and both drugs markedly inhibited insulin-stimulated Akt/PKB phosphorylation (Figure 3.8B). Neither drug affected the expression of Akt or GSK3β (data not shown). Thus, increasing endogenous ceramide levels by an alternative mechanism recapitulated the inhibitory effects of palmitate on Akt/PKB phosphorylation. We next attempted to determine whether these drugs, by blocking ceramide glucosylation or deacylation, could actually augment the palmitate effect on either the accumulation of long-chain ceramides or the antagonism of insulin signaling. As predicted, the inclusion of either PDMP or NOE along with palmitate potentiated the effects of either reagent individually on both ceramide accumulation (Figure 3.8A) and the inhibition of Akt/PKB phosphorylation (Figure 3.8C and 3.8D).

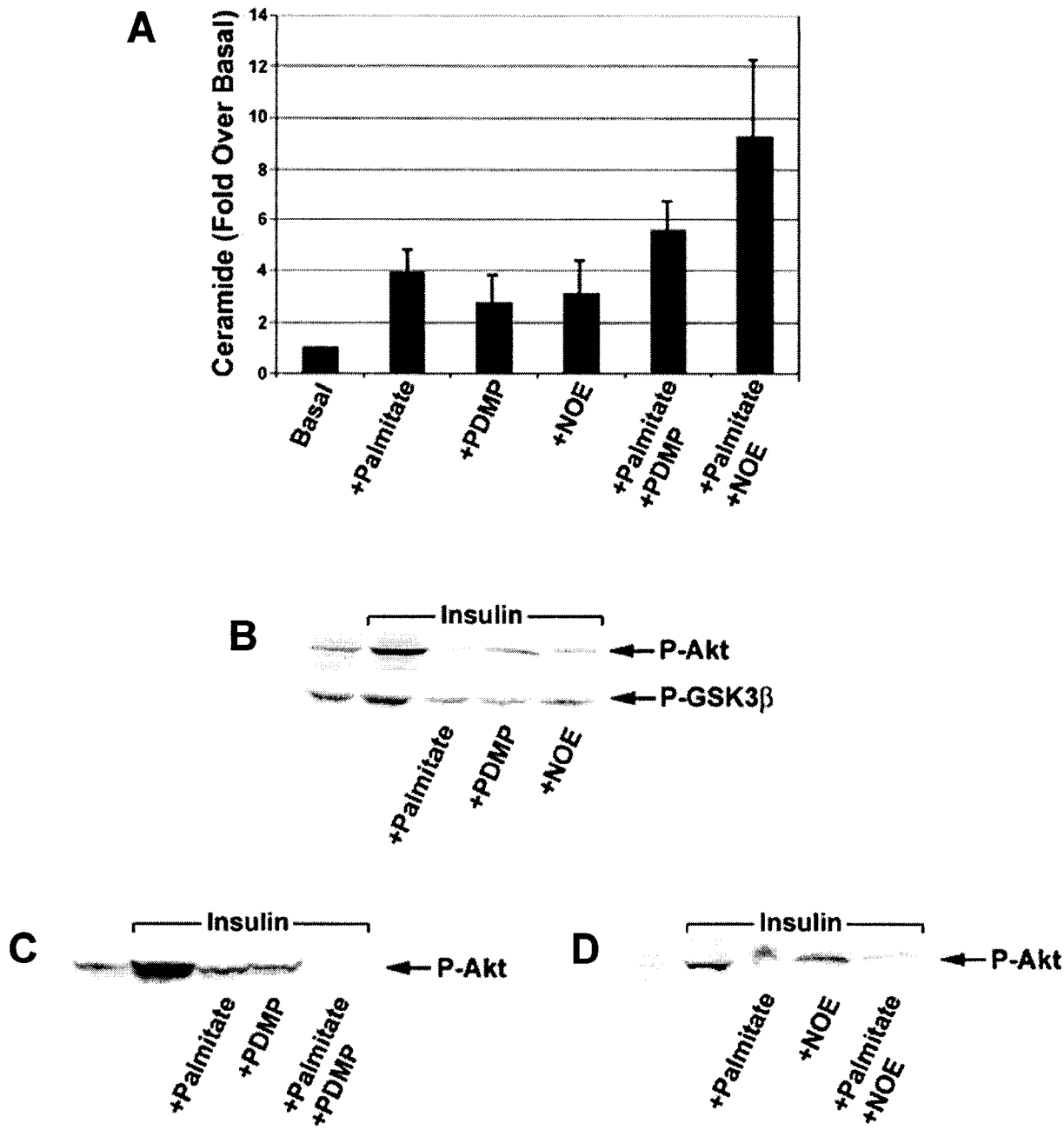


Figure 3.8: Inhibitors of ceramide glucosylation or deacylation recapitulate and augment the palmitate effect on insulin signaling. C2C12 myotubes were incubated in the presence or absence of C2-ceramide (C2) (50 μM), the glucosylceramide synthase inhibitor D-L-threo 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP, 50 μM), and/or the ceramidase inhibitor N-oleoylethanolamine (NOE, 250 μM) for 16 hours prior to stimulation with insulin (100 nM, 10 minutes). **A.** Ceramide levels were quantified as in figures 3 and 5. Ceramide levels are presented as the mean fold increase (over basal) +/- the S.E.M. **B-D.** Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antibodies. Data are representative of three independent experiments.

Ceramide has been shown to prevent Akt/PKB activation by at least two independent mechanisms. In certain cell types, okadaic acid, an inhibitor of protein phosphatase 2A (PP2A), negates the antagonistic effects of ceramide on Akt/PKB. This finding suggests that ceramide blocks insulin action by accelerating the rate of Akt/PKB dephosphorylation (132, 176, 185). In other cell types, however, okadaic acid has no effect, and ceramide instead blocks the insulin-stimulated translocation of Akt/PKB to the plasma membrane (128, 129). To test whether the effects of palmitate required PP2A in C2C12 myotubes, we pretreated cells with okadaic acid prior to treating with insulin. In the presence of okadaic acid, palmitate did not prevent insulin-stimulated Akt/PKB phosphorylation (Figure 3.9A). However, because okadaic acid stimulated Akt/PKB phosphorylation in the absence of insulin we could not distinguish between (a) OA reversing the effect of palmitate or (b) OA's stimulation of Akt/PKB being insensitive to ceramide. To more definitively determine whether palmitate was working through PP2A, we used recombinant adenovirus to overexpress the SV40 small T antigen. This protein was shown previously to inhibit PP2A activity by displacing one of the enzyme's regulatory subunits (186). SV40 small T expression completely prevented the effects of both palmitate and C2-ceramide on Akt/PKB phosphorylation (Figure 3.9B), confirming the likely involvement of PP2A in the inhibitory effects of saturated FFAs in C2C12 myotubes.

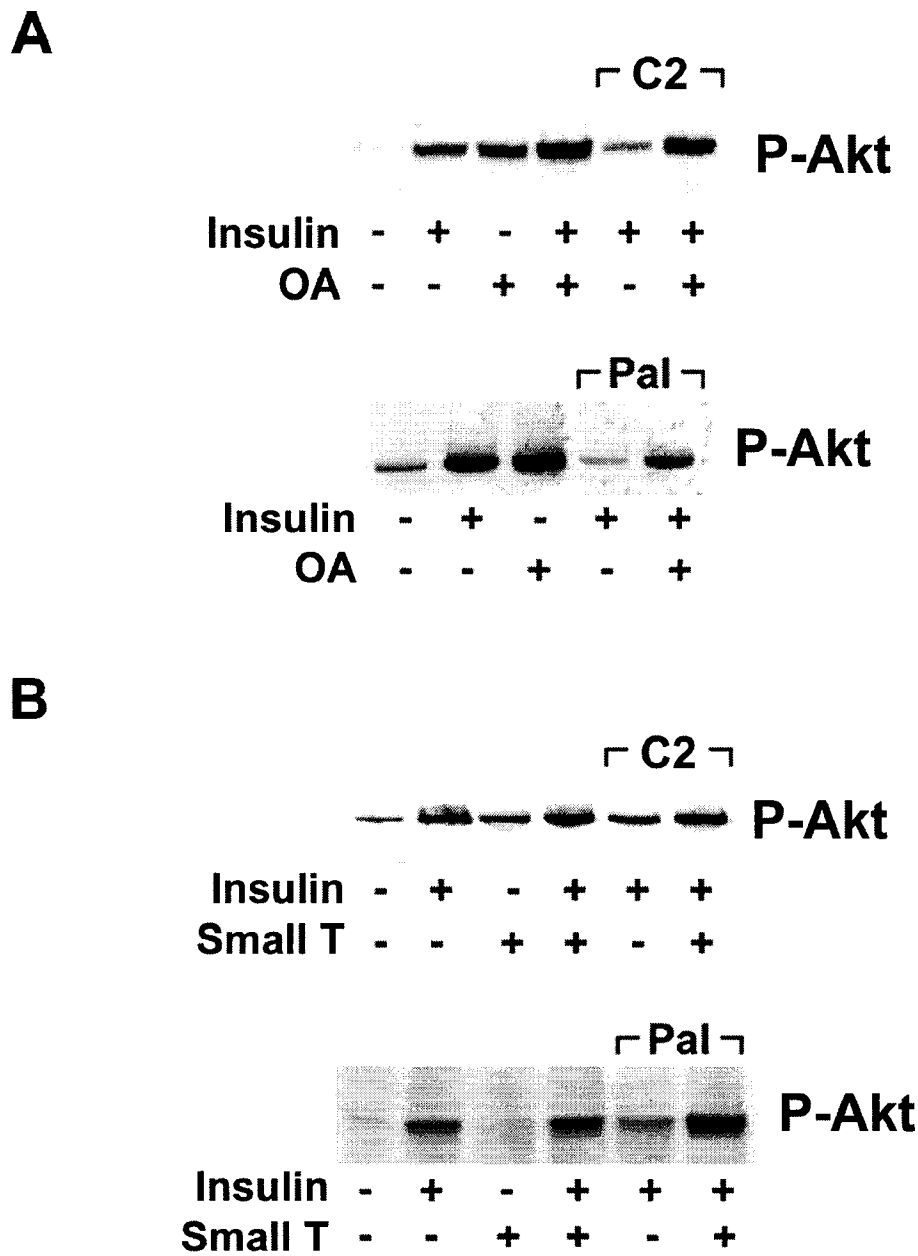


Figure 3.9: Palmitate inhibits insulin signaling via a PP2A-dependent mechanism. A. C2C12 myotubes were treated without or with palmitate (Pal, 0.75 mM, 8 hours) or C2-ceramide (Cer, 100 μM, 30 minutes). Okadaic acid was included in the indicated wells for 20 minutes prior to lysis, with in the indicated samples for the final 10 minutes. Cells were lysed, resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-phoshpho-Akt antibodies. **B.** C2C12 myotubes were incubated with adenovirus encoding either GFP (-) or GFP in combination with the SV40 small T antigen (+). Cells were then treated with palmitate (Pal, 0.75 mM, 8 hours) or C2-ceramide (Cer, 100 μM, 30 minutes) before being lysed and immunoblotted as in (A) above.

3.5 Discussion

The strong correlation between intramyocellular triglyceride levels and the severity of insulin resistance suggests that lipid oversupply to peripheral tissues could cause or exacerbate the condition. Many researchers have hypothesized that one or more derivatives of fatty acyl-CoA are likely to link inappropriate fat deposition in skeletal muscle to the inhibition of insulin signaling (93). Possible products of long chain acyl-CoAs capable of inhibiting insulin signaling include ceramide and diacylglycerol (DAG)[reviewed in (111)], but a definitive role for neither has been established. The data presented herein indicate that endogenously produced ceramide is both capable of inhibiting Akt/PKB and, more importantly, is necessary for the inhibitory effects of saturated FFAs. First, palmitate, stearate, and C2-ceramide blocked insulin signaling at the same step [i.e. by blocking activation of Akt/PKB, but not stimulation of PI3-kinase (Figures 1 and 2)] (103). Second, inhibiting *de novo* ceramide synthesis completely prevented palmitate's induction of ceramide synthesis and its antagonism of Akt/PKB and GSK3β phosphorylation (Figures 3.5-3.7). Third, preventing ceramide metabolism and/or degradation recapitulated the effects of palmitate on both intracellular ceramide accumulation and the inhibition of insulin signaling (Figure 3.8). Fourth, blocking ceramide metabolism while concomitantly adding palmitate augmented its effect on both ceramide accumulation and the inhibition of Akt/PKB phosphorylation (Figure 3.8). And fifth, expressing the SV40 small T antigen, an inhibitor of PP2A, prevented the effects of both C2-ceramide and palmitate on Akt/PKB phosphorylation (Figure 3.9). Collectively these data implicate ceramide in the insulin resistance resulting from the oversupply of saturated FFAs to skeletal muscle.

To manipulate intracellular ceramide levels in these experiments, we relied on the use of a large number of enzyme inhibitors to block ceramide synthesis or degradation. In order to minimize the possibility that our observations were the result of non-specific pharmacological effects, we employed inhibitors capable of blocking separate enzymes in the various pathways. For example, the fungal toxins myriocin and fumonisin inhibit separate enzymes that are required for *de novo* ceramide synthesis (i.e. SPT and ceramide synthase, respectively (162), and both protected C2C12 myotubes from the inhibitory effects of palmitate. Cycloserine, a serine palmitoyl transferase inhibitor that is structurally dissimilar to myriocin, also prevented the antagonistic effects of palmitate. None of these compounds affected Akt/PKB expression or activation independently (data not shown), and none were capable of blocking the antagonistic effects of exogenously added C2-ceramide, which presumably bypasses the site of action of these inhibitors (Figure 3.7). To induce ceramide accumulation we also used unique compounds with separate intracellular targets. Both PDMP and NOE, by inhibiting glucosylceramide synthase and ceramidase respectively, were able to induce ceramide accumulation and to block Akt/PKB activation (Figure 3.8). Thus, by using a broad array of inhibitors, our data conclusively indicate that ceramide is both a necessary and sufficient intermediate linking palmitate to the antagonism of insulin signaling.

Although ceramide proved to be required for the effects of palmitate, DAG derived from saturated FFAs was incapable of inhibiting insulin signaling to Akt/PKB. Specifically, in figures 3.5 and 3.6, we demonstrate conditions where insulin signaling was inhibited despite the fact that DAG accumulation was not. Moreover, we

demonstrated that attenuating the induction of DAG synthesis did not prevent the inhibitory effects of FFAs on insulin signaling (data not shown). The hypothesized involvement of DAG in insulin resistance derives from multiple prior studies both in cultured cells and rodents. For example, treating various cell lines with phorbol esters, which mimic the effects of DAG, inhibits insulin signaling to IRS-1(122, 151-153, 180) and Akt (154). Moreover, several PKC isoforms including PKCα (187, 188), β1 and β2 (189), δ (151), γ (187) and θ (151) are downstream effectors of DAG that purportedly antagonize insulin signaling by phosphorylating IRS-1 on inhibitory serine residues. Observations that DAG levels are often elevated in rodent models of insulin resistance [reviewed in (190)] have fueled speculation that the lipid may serve as a critical antagonist of insulin signaling in diseased tissues. The discrepancy between these studies and those mentioned above may be reconciled by recent observations that the acyl-chain composition in DAG may participate in its ability to activate PKC isoforms. Specifically, DAG derived from saturated fatty acids is generally a poor activator of PKC, while that produced from polyunsaturated fatty acyl CoAs is a much stronger stimulus (164). Thus, DAG may participate in the regulation of insulin signaling by polyunsaturated FFAs, while ceramide is the principle metabolite linking saturated FFAs to insulin signaling.

The mechanism by which ceramide inhibits insulin action is unclear. Ceramide has been proposed to activate PP2A, the phosphatase responsible for dephosporylating Akt/PKB in adipocytes (191). Moreover, in PC12 cells (132), brown adipocytes (176), and a human glioblastoma cell line U87MG (192), the PP2A inhibitor okadaic acid

prevents the effects of short-chain ceramide analogs on Akt/PKB phosphorylation. While these studies suggest the existence of a mechanism by which ceramide increases intracellular PP2A activity, several other reports have shown that the effects of ceramide are okadaic acid insensitive. Specifically, studies in hybrid motor neurons (193), 3T3-L1 pre-adipocytes and adipocytes (128), A7r5 vascular smooth muscle cells (194), and L6 muscle cells (129) all demonstrate a profound inhibition of Akt/PKB by C2-ceramide that is insensitive to okadaic acid. In most of these cell types, ceramide was shown to block the insulin-stimulated recruitment of Akt/PKB to the plasma membrane. Prior studies by Cazzoli *et al.* (195) indicated that the inhibitory effects of palmitate in C2C12 myotubes were also dependent upon okadaic acid. However, we found that okadaic acid in fact stimulated Akt/PKB phosphorylation in the absence of insulin, something shown for other cell types (191) (Figure 3.9A). This observation limited the drug's usefulness as a means for dissecting the pathways linking palmitate to the inhibition of Akt/PKB since one could not distinguish whether okadaic acid prevented or simply bypassed the site of ceramide action. Expressing the SV40 small T antigen, an inhibitor of PP2A, confirmed the likely involvement of PP2A in the inhibitory pathway linking both palmitate and ceramide to the regulation of Akt/PKB phosphorylation in C2C12 myotubes (Figure 3.9B).

3.6 Conclusions

Numerous studies implicate saturated fats, particularly palmitate, in the development of insulin resistance. First, palmitate is one of the most abundant FFAs found in skeletal muscle, as well as being one of the most prevalent acyl-chains found in

the diglyceride fraction of lipid extracts (156). Second, an inverse relationship exists between the consumption of palmitate and insulin sensitivity (196, 197). And third, insulin resistant muscles demonstrate accelerated rates of palmitate uptake (198). A similar cadre of results suggests that the overaccumulation of ceramide could participate in the development of insulin resistance. First, various rodent models of insulin resistance, including Zucker *fa/fa* rats (116) and mice overexpressing lipoprotein lipase (105), demonstrate elevated intramuscular ceramide levels. Second, exercise training Wistar rats improves insulin sensitivity while markedly lowering intramuscular ceramide levels (199). And third, short chain-ceramide analogs inhibit insulin-stimulated Akt/PKB activation and/or glucose uptake in 3T3-L1 adipocytes (128, 177), brown adipocytes (176), C2C12 and L6 myotubes (103, 129), and isolated rat skeletal muscle (137). The experiments described herein clearly indicate that endogenously produced ceramide is not only capable of antagonizing insulin action, but more importantly, is required for the inhibitory effects of long-chain saturated fatty acids on insulin signaling. Although aberrant ceramide accumulation is unlikely to account entirely for the diverse array of defects found in insulin resistant tissues, the findings presented herein implicate ceramide as a potentially important mediator of the deleterious effects of long-chain saturated fats. Studies attempting to block ceramide synthesis in various animal models of insulin resistance will be necessary to determine the extent to which this lipid metabolite contributes to the pathologies associated with the condition.

3.7 Acknowledgements

The authors thank Norman Curthoys, Jennifer Nyborg, and Suzanne Stratford (Colorado State University) for providing intellectual input into the study design and for critically reviewing the manuscript prior to submission.

CHAPTER 4

Ceramidase Overexpression Prevents the Inhibitory Effects of Saturated Fatty Acids on Insulin Signaling

This chapter is currently being modified for submission to the *Journal Of Biological Chemistry.* All experiments presented herein were carried out by Chavez JA.

The authors for this paper upon acceptance would be:

Jose Antonio Chavez, Julia Bär-Heine*, Konrad Sandhoff* and Scott A. Summers

Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO 80523-1870 and *Kekule-Institut fur Organische Chemie und Biochemie, Universitat Bonn, Gerhard-Domagk-Strassel, D-53121 Bonn, Germany.

4.1 Summary

Obesity is a predisposing factor for the development of insulin resistance and Type 2 diabetes mellitus. Although the connection between increased adiposity and the development of insulin resistance is unknown, numerous studies suggest that fat-derived circulating factors, such as free fatty acids (FFAs), induce muscle insulin resistance by inhibiting insulin signaling. Long-chain saturated FFAs stimulate the *de novo* synthesis and accumulation of ceramide, a cellular metabolite shown previously to inhibit insulin action. Herein we demonstrate that inducing ceramide buildup by exposing muscle cells to long-chain saturated FFAs inhibits insulin stimulation of Akt/Protein Kinase B (PKB), a central regulator of glucose uptake and anabolic metabolism. To further evaluate the contribution of ceramide to FFA-dependent inhibition of insulin action, we generated C2C12 myotubes that constitutively overexpress Acid Ceramidase (AC), an enzyme that catalyzes the lysosomal degradation of ceramide. AC overexpression prevented the inhibitory effects of long-chain saturated FFAs on insulin signaling while blocking their stimulation of endogenous ceramide accumulation. These results support the existence of a central role for aberrant ceramide accumulation in the inhibition of muscle insulin sensitivity by exogenous FFAs.

4.2 Introduction

Insulin resistance is a condition characterized by decreased sensitivity of peripheral tissues to the action of insulin. As a result, tissues cannot absorb and store glucose appropriately, leading to a compensatory increase in insulin secretion. This metabolic dysfunction leads to a cluster of abnormalities with serious clinical consequences, including Type 2 Diabetes (136) and Metabolic Syndrome X (68).

Insulin resistance is often associated with obesity, but the relationship between increased fat stores and the development of insulin resistance is not completely clear. Since skeletal muscle, and not adipose tissue, is responsible for the majority of insulin-mediated glucose disposal, many researchers have hypothesized that adipose tissue secretes a variety of circulating factors that trigger insulin resistance in other organs (200, 201). Expressly, increased release of Free Fatty Acids (FFAs) by adipose tissue has been postulated to play crucial roles in obesity-induced insulin resistance (75). Numerous arguments that support this hypothesis have been made. For example, direct exposure of cultured cells to FFAs induces a state of insulin resistance (78, 79). Also, targeted overexpression of lipoprotein lipase (LPL) in skeletal muscle and liver of mice increases lipid deposition and promotes insulin resistance (105, 171). Moreover, infusion of lipids into humans or animal models has been shown to decrease insulin sensitivity in muscle and liver (81-85). Although the molecular mechanisms by which increased availability of FFAs to peripheral tissues confers insulin resistance are not completely understood, numerous researches speculate that FFAs promote the accumulation of fat-derived metabolites capable of attenuating insulin signaling (93, 111). For instance, increased

FFA uptake from the circulation or diminished FFA oxidation in mitochondria promotes the accumulation of triacylglycerol (TAG) and other lipid metabolites within skeletal muscle while simultaneously blocking glucose utilization [Reviewed in (202)]. Studies utilizing ^{1}H NMR to measure intramyocellular lipid concentrations support this concept, as these studies indicate that intramyocellular TAG levels correlate more tightly with the severity of insulin resistance than other known risk factors [Reviewed in (202)]. In addition to promoting TAG accumulation, FFAs also stimulate the accumulation of other less abundant metabolites, such as diacylglycerol (DAG) and ceramide. Both derivatives of fatty acyl Co-A have been implicated as primary mediators of the antagonistic effects of FFAs in skeletal muscle (95, 103, 167, 203, 204).

Ceramide is a bioactive sphingolipid shown previously to block insulin action (128). Moreover, numerous studies implicate the sphingolipid in the development of fat-induced insulin resistance. First, intracellular levels of ceramide are increased in skeletal muscle from obese insulin-resistance humans (126) and in peripheral tissues of human (205) or rodent (116) models of insulin resistance induced acutely by lipid infusion. In addition, short-chain ceramide analogs or agents that induce ceramide accumulation have been shown to inhibit insulin signaling and action in various cultured cells or isolated tissues, recapitulating the antagonistic effects of FFAs on insulin-stimulated glucose metabolism (103, 206, 207).

Ceramide can be generated via *de novo* synthesis from serine and palmitoyl-CoA in the endoplasmic reticulum (ER). As we have shown previously, long-chain saturated

FFAs stimulate *de novo* synthesis in C2C12 myotubes (203). Once generated, ceramide can accumulate or can be converted into a broad array of ceramide-derived molecules. For instance, ceramide can be deacylated by ceramidases to form sphingosine, glycosylated by glucosylceramide synthase to give rise to a host of glucosylceramide derivatives, or phosphorylated by ceramide kinase to generate ceramide 1-phosphate.

Acid Ceramidase (AC) catalyzes the lysosomal hydrolysis of ceramide to sphingosine and free fatty acid (208). Studies have revealed that lysosomal degradation of ceramide by AC plays a central role in the maintenance of cellular ceramide levels (209). However, the relevance of AC activity is best described in patients suffering from Farber's disease, an inherited deficiency of AC activity that causes a fatal, massive accumulation of ceramide in lysosomes of various tissues (210).

To delineate a role for ceramide in FFAs-induced insulin resistance, we overexpressed human AC in C2C12 myotubes, a well-established cell model for the study of insulin signaling. Overexpression of AC prevented the stimulatory effects of saturated FFAs on intracellular ceramide accumulation, while simultaneously blocking their inhibitory effects on insulin signaling. These findings further support the role of ceramide in the induction of muscle insulin resistance caused by long-chain saturated FFAs and may suggest potential molecular targets for pharmaceutical intervention in the treatment of insulin resistance and Type 2 diabetes.

4.3 Experimental Procedures

4.3a *Reagents*:

Fetal bovine serum was from Atlas Biologicals (Fort Collins, CO), and silica gel 60 thin-layer chromatography (TLC) plates were from Merck (Darmstadt, Germany). The following additional reagents were obtained from Sigma Chemicals (St. Louis, MO): palmitate, stearate, arachidate, lignocerate, Dulbecco's Modified Eagles's Medium (DMEM), fatty acid free bovine serum albumin (BSA) and N-oleoylethanolamine (NOE). Antibodies utilized included the following: rabbit polyclonal anti-phospho-Akt/PKB (Ser-473) and anti-phospho-GSK3β (serine-9) antibodies from Cell Signaling (Beverly, MA); a rabbit polyclonal anti-Akt/PKB and horseradish peroxidase conjugated anti-rabbit and anti-mouse antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-acid ceramidase monoclonal antibody from BD Biosciences Pharmingen (San Diego, CA).

4.3b *Cell culture*:

C2C12 myoblasts were maintained at 37°C in Dulbecco's modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS). For differentiation into myotubes, the myoblasts were grown to confluence and the medium replaced with DMEM containing 10% horse serum. Myotubes were used for experiments 2-3 days following differentiation.

4.3c *FFA Treatment*:

Free fatty acids were administered to cells by conjugating them with FFA-free bovine serum albumin (BSA). Briefly, FFAs were dissolved in ethanol (75 mM) and diluted 1:25 in 1% FBS-DMEM containing 2% (w/v) BSA to obtain a FFA concentration of 3mM. After brief sonication and 10 min incubation at 55°C, samples were diluted to desired concentration in 1% FBS-DMEM-2% BSA, cooled at room temperature, filter sterilized, and administrated to myotubes for 14 hours. Two hours prior to performing the experiments, myotubes were placed in serum free-DMEM containing 2% BSA in either the presence or absence of FFAs.

4.3d *Immunoblot Analysis*:

Cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose and immunoblotted using methods described previously (155). Detection was performed using the Enhanced Chemiluminescence Plus kit from Amersham Biosciences (Piscataway, NJ) according to manufacturers instructions. For Acid Ceramidase expression, 4-20% LongLife precast gel from Gradipore Ltd. (Frenchs Forest, Australia) were used for protein separation.

4.3e *Ceramide Assay*:

Total lipids from myotubes were extracted by the method of Schmitz-Peiffer *et.al*, (103) and modified as described previously (203). Ceramide content in the extracts were determined using a radiometric diacylglycerol assay kit (ceramide:DAG assay) (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

Lipid samples, then, were separated by TLC using chloroform: acetone: methanol: acetic acid: water (20:8:4:4:2, by volume), as the developing solvent, and the radioactive products were detected in a phosphorimager. The regions migrating with standard ceramides were analyzed using ImageQuant® 5.1 software (Molecular Dynamics Inc.).

4.3f *Generation of C2C12 Myotubes Overexpressing Human Acid Ceramidase*:

A myc-tagged AC construct was generated by polymerase chain reaction (PCR) using human acid ceramidase cDNA (211) as template, the forward primer 5'- CTC GAG GCC GCC ACC ATG GAG CAG AAG CTG ATT TCC GAG GAG GAC CTG CCG GGC CGG AGT TGC GTC G – 3' and the reverse primer 5'- GGA TCC TCA CCA ACC TAT ACA AGG GTC – 3'. The resulting product included a *Xho*I restriction site, the start codon of the coding region followed by a c-myc (myc) tag, the 1218 bp of the coding region, and a *BamH*I restriction site at the C-terminal. The PCR fragment was ligated into pGEM-T easy vector from Promega (Madison, WI). Nucleotides 1-1218 of myc-AC were then isolated by digesting pGEM-T-myc-AC with *Not*I, and the fragment containing the PCR product was shuttled into the pLNCX1 retroviral vector using *Not*I, thus obtaining the full length myc-tagged construct (pLNCX1-myc-AC). The constructs were restriction digested to confirm proper orientation of insert and subsequently sequenced to verify that no errors were introduced by PCR. Recombinant retrovirus encoding myc-AC was generated using methods described previously (48). Briefly, the pLNCX1-AC constructs were co-transfected with plasmids encoding the *gag/pol* and *vsv* genes into 293T cells. Supernatant containing the recombinant retroviruses were collected and used to infect C2C12 myoblasts. Since pLNCX1 contains a neomycin

(G418) resistance gene, transfected populations were selected and maintained in medium supplemeted with G418 (600 μg/ml active concentration).

4.3g *Preparation of Recombinant Sphingosine Kinase:*

Human sphingosine kinase (SK) cDNA was obtained from Sarah Spiegel (Virginia Commonwealth University, Richmond, VA) and subcloned into the mammalian retroviral expression vector pLNCX1 (Clontech Laboratories, Palo Alto, CA) using *Hind*III and *Bgl*II. 3T3-L1 fibroblasts were stably transfected with the above constructs by retrovirus-mediated gene transfer as previously described (48). Recombinant SK was harvested from total cell fraction according to Olivera *et.al.*, (212). Aliquots (20 μg) of cell lysates were used for each sphingosine assay

4.3h *Sphingosine Assay.*

Four 10-cm plates containing C2C12 myotubes were incubated in serum free-DMEM containing 2% BSA in either the presence or absence of 0.75 mM palmitate for 12 hours. Cell were washed twice in ice-cold PBS and sphingosine levels were assessed using the methodology described previously (213).

4.3i *Statistical Analysis:*

Quantifiable results are presented as means +/- SEM. Differences between means were evaluated by analysis of variance and considered significant at $p<0.05$.

4.4 Results

In prior studies, we demonstrated that ceramide was necessary and sufficient for the antagonism of insulin signaling resulting from the incubation of C2C12 myotubes with long-chain saturated FFAs (206). Interestingly, N-oleoylethanolamine (NOE), a potent inhibitor of acid ceramidase (AC), recapitulated and augmented the effects of exogenous palmitate on insulin signaling (206), suggesting that ceramide was a principle metabolite linking FFAs to the antagonism of insulin signaling. Reasoning that enhanced AC expression might promote a faster removal of intracellular ceramide generated by treating with exogenous FFAs, we used recombinant retrovirus to generate stable C2C12 myotubes overexpressing human AC. Briefly, we infected C2C12 myotubes with either an empty pLNCX1 retrovirus or one encoding AC. The pLNCX1 virus carries the Neo resistance gene, and selecting the cells in medium containing G418 produced stable lines. Using a monoclonal, anti-human AC antibody we detected significant expression of AC in C2C12s infected with the recombinant virus compared with myotubes infected with the empty retroviral vector (Figure 4.1A).

Treatment with palmitate (16 hours) inhibited insulin-stimulated Akt/PKB phosphorylation, at a concentration of as low as 0.1 mM, in cells expressing an empty vector (Figure 4.1B). Palmitate also inhibited insulin-stimulated phosphorylation of glycogen synthase kinase-3 beta (GSK-3β), one of the physiological targets of Akt/PKB, implicated in glycogen synthesis (214). Akt/PKB and GSK3β levels were unchanged by any of the treatment conditions (data not shown). As predicted, AC overexpression

protected myotubes from the inhibitory effects of palmitate on insulin-stimulated phosphorylation of Akt/PKB and GSK3β phosphorylation (Figure 4.1B).

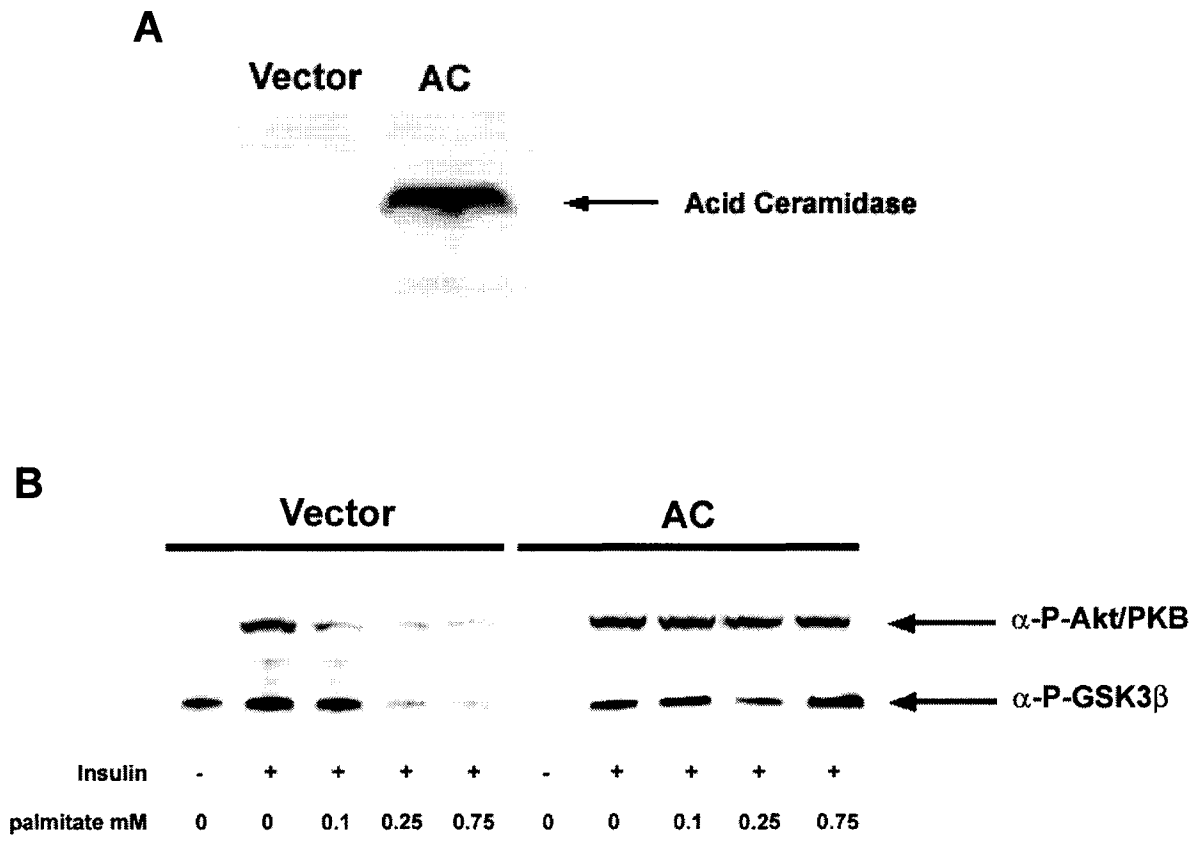


Figure 4.1: Overexpression of Acid Ceramidase in C2C12 myotubes prevents the inhibition of insulin signaling by palmitate. A. Stable C2C12 myotubes expressing an empty vector or acid ceramidase were lysed, resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an anti-Acid Ceramidase antibody. **B.** C2C12 myotubes transfected with an empty vector or acid ceramidase were incubated with palmitate (16 hours) at the concentration indicated prior to stimulation with insulin (100 nM, 10 minutes). Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antibodies. Detection was by enhanced chemiluminescence. Equal levels of expression were confirmed by immunoblotting with antibodies recognizing total Akt/PKB and GSK3β (data not shown). Data are representative of three independent experiments.

To determine whether the protection observed in AC-overexpressing C2C12 was attributable to decreased ceramide accumulation, we quantified ceramide levels in control C2C12 myotubes and those overexpressing AC using a DAG/ceramide assay. Palmitate stimulated the accumulation of ceramide in cells transfected with an empty vector (Figure 4.2A). By contrast, AC overexpression largely attenuated the increase in ceramide accumulation induced by palmitate (Figure 4.2A). As shown in figure 4.2B, AC overexpression did not have any affect on DAG accumulation caused by palmitate treatment. The effect of palmitate on ceramide synthesis correlated tightly with its effect on insulin-stimulated Akt/PKB phosphorylation and activation. Thus, enhancing turnover of newly generated ceramide in AC-overexpressing cells protected cells against the antagonistic effects of palmitate on insulin signaling.

Because sphingosine is the product of the AC reaction, it was of interest to determine whether overexpression of AC altered sphingosine levels. Briefly, sphingosine levels were determined in total cell fractions by measuring the *in vitro* incorporation of radiolabelled phosphate from ^{32}P-γ-ATP into endogenous sphingosine to form sphingosine 1-phosphate (S1P) in the presence of recombinant sphingosine kinase. Lipids were then resolved by TLC and detected using a phosphoimager. When empty vector transfectants were incubated with palmitate there was a significant 2.5-fold change in sphingosine levels. However, treatment of AC-overexpressing cells with palmitate resulted in a more than seven-fold increase in sphingosine (Figure 4.3). These data clearly indicate that the overexpressed AC was indeed active *in vivo*, and effectively metabolized ceramide to sphingosine, and thus reduced the accumulation of long-chain

ceramides that induces the inhibition of insulin signaling.

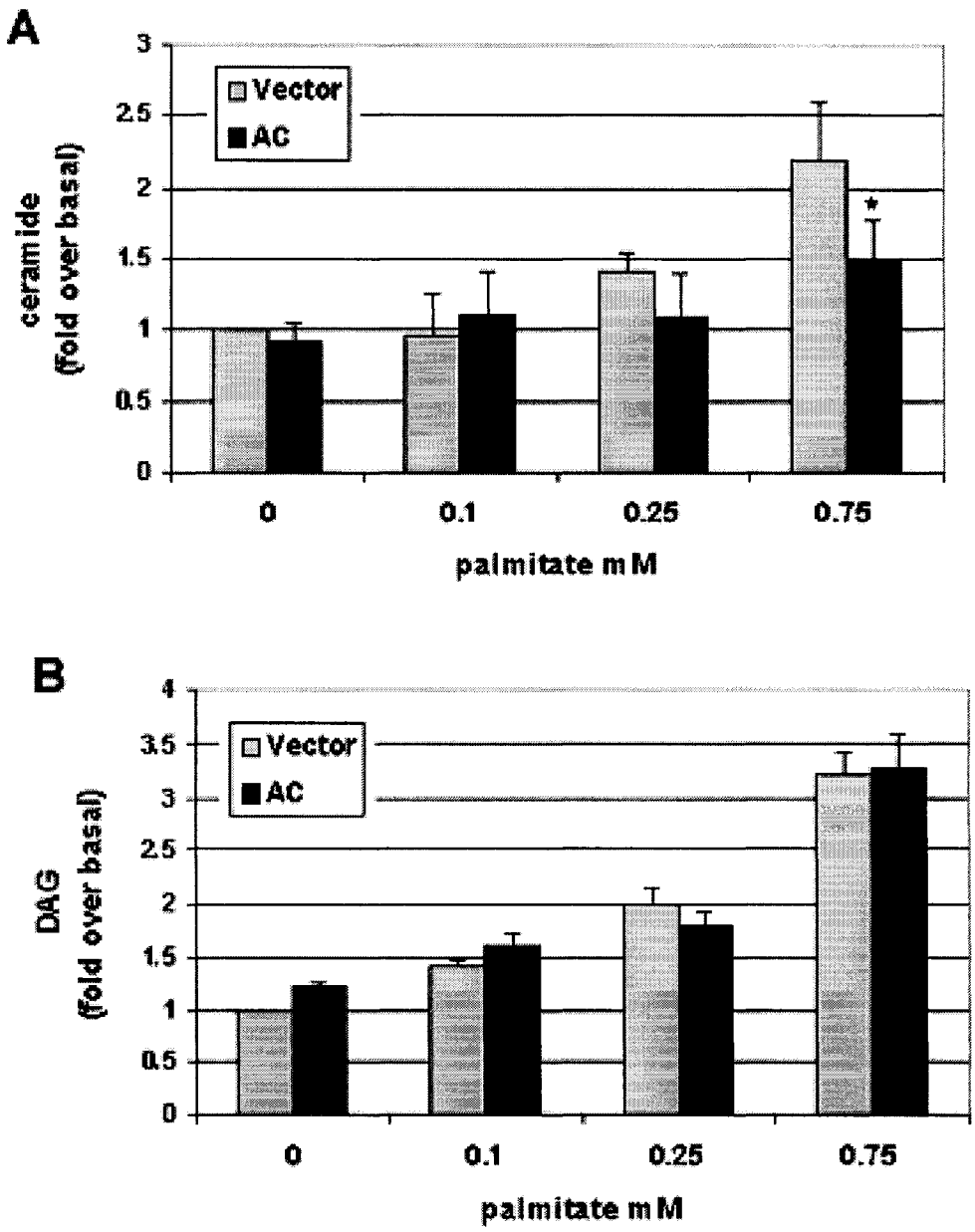


Figure 4.2: Overexpression of Acid Ceramidase prevent palmitate's stimulation of ceramide, but not DAG synthesis. Stable C2C12 myotubes expressing an empty vector or acid ceramidase were incubated in the presence or absence of the indicated palmitate concentrations (0-0.75 mM) for 16 hours prior to lipid extraction. Ceramide standards (data not shown) or lipid extracts from transfected C2C12 myotubes were incubated with DAG-kinase and [^{32}P]-γ-ATP as described in the methods section. Lipids were then re-extracted, resolved by thin layer chromatography, and detected using a storm phosphorimager. Ceramide **(A)** and DAG **(B)** levels were quantified and data presented as the mean fold increase (over basal) +/- the S.E.M. Asterisks denote that the value was significantly different than levels obtained in vector cells ($p \leq 0.05$).

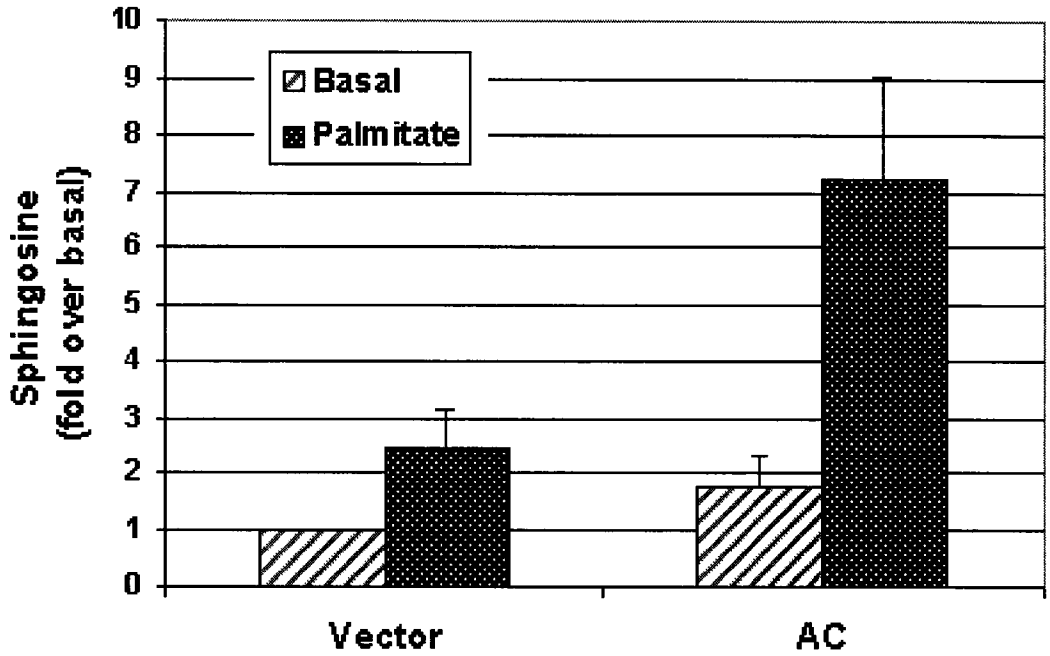


Figure 4.3: Overexpression of Acid Ceramidase stimulates the synthesis of sphingosine. Stable C2C12 myotubes expressing an empty vector or acid ceramidase were incubated in the presence or absence of palmitate (0.75 mM) for 12 hours prior to lipid extraction. Sphingosine levels were measured as described under Materials and Methods and compared to standards reactions (data not shown). Data are presented as the mean fold increase (over basal) +/- the S.E.M. Asterisks denote that the value was significantly different than basal levels ($p \leq 0.05$).

We next sought to determine whether overexpression of AC in C2C12 myotubes also had the capacity to protect cells from other long-chain saturated FFAs which have been shown previously to inhibit insulin signaling while inducing ceramide accumulation (203). Stearate (18:0), arachidate (20:0), and lignocerate (24:0) all inhibited insulin-stimulated phosphorylation of Akt/PKB and GSK3β in control cells, though at different intensities (Figure 4.4). Once again, AC overexpression negated the inhibitory effects of these FFAs (Figure 4.4). These data further identify ceramide as a critical regulator of the antagonistic effects of long-chain saturated FFAs on insulin signaling.

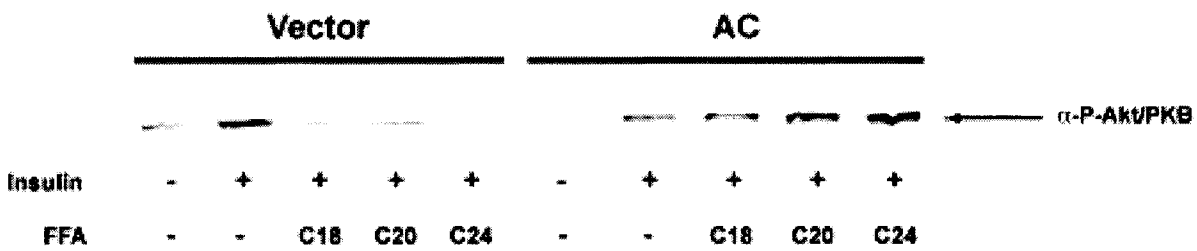


Figure 4.4: Overexpression of Acid Ceramidase prevents the inhibition of Akt/PKB and GSK3β phosphorylation by other saturated fatty acids in C2C12 myotubes. C2C12 myotubes transfected with an empty vector or with vector containing the acid ceramidase gene were treated with or without 0.75 mM palmitate (16:0), stearate (18:0), arachidate (20:0), and lignocerate (24:0) for 16 hours prior to stimulation with insulin (100 nM, 10 minutes). Cells were then lysed, resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antibodies. Equal loading and expression was confirmed by immunoblotting with antibodies recognizing total Akt/PKB or GSK3β (data not shown). Detection was by enhanced chemiluminescence. Data are representative of three independent experiments.

To confirm the mechanism by which AC overexpression protected cells from the antagonistic effects of saturated FFAs, we treated myotubes with NOE, an inhibitor of AC activity. As shown in figure 4.5, NOE clearly inhibited the insulin-stimulated phosphorylation of Akt/PKB in both controls and AC overexpressing myotubes. As expected, the inclusion of NOE along with palmitate potentiated the effects of the FFAs on Akt/PKB phosphorylation in both cell lines (Figure 4.5). However, palmitate alone did not inhibit the insulin-stimulated Akt/PKB phosphorylation in cells transfected with AC owing to the high expression of AC in this cell line. Collectively, these data confirm that the enzymatic activity of AC mediates its protective effects on insulin signaling.

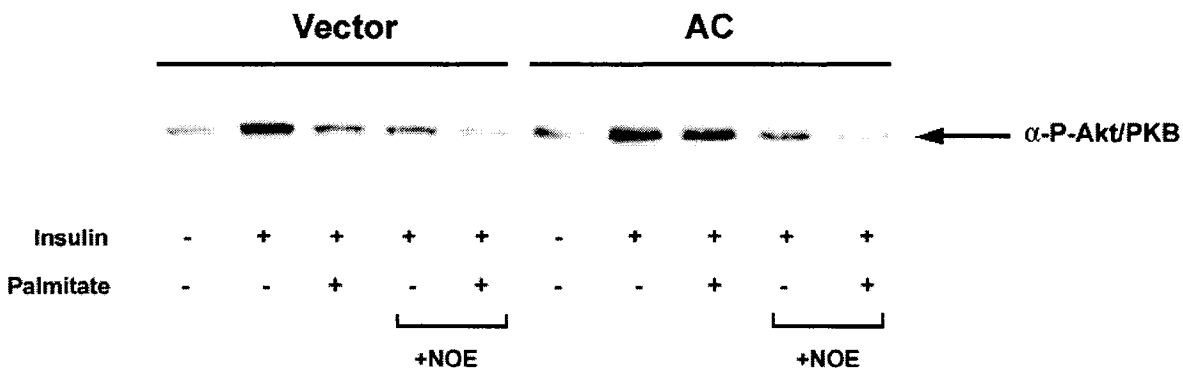


Figure 4.5: C2C12 myotubes overexpressing Acid Ceramidase are sensitive to inhibitors of ceramide deacylation. NOE recapitulates the palmitate effect on insulin signaling observed in WT C2C12. C2C12 myotubes overexpressing acid ceramidase were incubated in the presence or absence of palmitate (0.75 mM) and/or the ceramidase inhibitor N-oleoylethanolamine (NOE, 500 μM) for 16 hours prior to stimulation with insulin (100 nM, 10 minutes). Proteins in the cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antibodies. Data are representative of three independent experiments.

4.5 Discussion

The connection between obesity and metabolic diseases, such as Type 2 diabetes and Metabolic Syndrome X, is well established. According to the American Diabetes Association, nearly 90% of all people newly diagnosed with Type 2 diabetes are overweight or obese. Elevated levels of circulating FFAs have been shown to contribute to the complications of obesity, particularly insulin resistance, by promoting an excessive deposition of fat in various insulin-responsive tissues, other than adipose (93). Within peripheral tissues, FFAs are altered in the cytoplasm to form acyl-CoAs and incorporated into different pools of FFA-metabolites (*i.e.* TAG, DAG and ceramides) with potentially deleterious roles on the insulin signaling pathways and glucose metabolism. The strong correlation between intramyocellular-lipid (IMCL) levels with the severity of insulin resistance is consistent with this hypothesis (202).

The identification of novel metabolites capable of inhibiting insulin actions remains a considerable focus of attention. Numerous findings have suggested a role for sphingolipid pathways in the regulation of insulin signal transduction. Sphingolipids, particularly ceramides, have been shown to inhibit insulin signaling in cell culture and animal models of insulin resistance. Furthermore, agents that interfere with sphingolipid pathways have been recently demonstrated to modulate insulin signaling. In C2C12 myotubes for example, inhibitors of serine-palmitoyltransferase or ceramide synthase, two enzymes required for *de novo* ceramide synthesis, prevented the antagonistic effects of ceramide accumulation on insulin signaling induced by the saturated FFA palmitate (206). In addition, inhibiting ceramide metabolism or degradation with pharmacological

agents recapitulated and augmented the effects of palmitate on both intracellular ceramide accumulation and the inhibition of insulin signaling (206). These observations have given us a substantial body of evidence to support the hypothesis that increases in endogenous ceramide levels represents a potentially important mechanism underlying the development of insulin resistance. In this regard, ceramidases suggested an excellent target for the regulation of ceramide levels and its cellular responses toward insulin action.

Since ceramide accumulation has also been considered as a hallmark of apoptosis, cell cycle arrest, and growth suppression, overexpression of ceramidases has been utilized as an important tool for determining the role of ceramide in these biological events. For example, overexpression of acid ceramidase protects against apoptosis induced by TNF-α (215), an important finding given that a number of prostate cancer cell lines are know to overexpress acid ceramidase (216). Herein, we aimed to attenuate intracellular ceramide accumulation and counteract its inhibitory effect on insulin signaling by saturated FFAs.

AC overexpression blocked the antagonistic effects of palmitate on Akt/PKB phosphorylation, while also blocking the phosphorylation of a target of Akt/PKB, GSK3β. Interestingly, these effects were accompanied by a significant reduction of ceramide accumulation resulting from exposure to palmitate. Hence, the high AC expression observed in transfected myotubes indicated that the protective effect observed in our experiments resulted from a rapid removal of intracellular ceramide generated by

de novo synthesis. As mentioned above, AC overexpression did not affect the induction of DAG. Thus, despite an increase in DAG levels, cells were still protected from the antagonistic effect of palmitate. This observation further support the concept that ceramide, but not DAG, is indispensable for the inhibition of insulin signaling caused by the long chain-saturated FFA palmitate.

Palmitate induces ceramide accumulation primarily by fueling the cell with palmitoyl CoA, a substrate in the rate-limiting reaction governing ceramide synthesis. Interestingly, AC overexpression also negated the inhibitory effects of longer-chain saturated FFAs. In contrast, AC overexpression in C2C12 myotubes failed to protect cells from the short chain ceramide C2-Cer (data not shown). The susceptibility of AC overexpressing myotubes to the C2-Cer was probably due to the fact that this short ceramide analog is not a precursor of endogenous ceramide (data not shown) and hence a poor substrate for AC. It should be noted that in C2C12 myotubes, not only palmitate, but also other long-chain saturated FFAs (e.g. stearate (18:0), arachidate (20:0), and lignocerate (24:0)) efficiently induced the accumulation of similar endogenous ceramide species (most likely C16-ceramide) and inhibited insulin-stimulated Akt/PKB activation (203). Therefore, it seems quite possible that all the long-chain saturated FFAs are converted into a common ceramide product, which is catabolized efficiently by AC.

Sphingosine, the immediate product of AC activity, has been shown to have potent bioregulatory effects on a variety of cellular processes involving signal transduction mechanisms (217). Specifically, sphingosine inhibition of glucose transport

has been previously reported (218-220). These observations suggested that the sphingolipid could be the mediator that links FFAs to the inhibition of Akt/PKB. However, according to our results, endogenous sphingosine accumulation produced by AC overexpression (which diverts palmitate from accumulating as ceramide), appeared to have no effect on the inhibition of insulin signaling. These data coupled with those observed after the inclusion of NOE confirms the hypothesis that ceramide itself, but not sphingosine, is the primary metabolite responsible for the inhibitory effects of palmitate. NOE was capable to suppress insulin-stimulated Akt/PKB phosphorylation in both control and AC lines suggesting that the protection observed in the latter was merely a result of AC overexpression and the subsequent clearance of endogenous ceramide.

One question originating from these findings refers to the mechanism by which AC, an enzyme active only in acidic compartments, was capable of protecting cells from the inhibitory effects of ceramide produced through a *de novo* pathway. Although it is not clear which subcellular compartment of ceramide is responsible for the inhibition of Akt/PKB, the enzymes that govern the *de novo* pathways leading to ceramide production reside on the cytosolic side of the endoplasmic reticulum (ER) (221). One possibility is that the AC could be localizing to other compartments due to a profound overexpression, and that the enzyme could retain some enzymatic activity despite being in a suboptimal (i.e. non-acidic) environment. Another possible explanation would be that the degradation of ceramide in the lysosomal compartment that results from the AC overexpression accelerates rates of ceramide flux into the lysosomes.

Because the anti AC antibody used in this study was specific for the human isoform, we could not determine endogenous expression of AC in C2C12 myotubes. However, northern blot analysis of various murine tissues showed undetectable AC mRNA levels in skeletal muscle compared to other tissues (222). Possibly, due to the lack of AC activity in skeletal muscle, this tissue may be particularly susceptible to aberrantly accumulate ceramide when exposed to excess FFAs. Consistent with this speculation is that mammals, including humans, are not suitably adapted to process abundant dietary energy sources and avert their causative role in developing muscle insulin resistance. An answer for this conjecture may be found using *in vivo* models of Farber's Disease, an autosomal recessive disorder caused by lysosomal AC deficiency. Patients suffering from this rare disease accumulate ceramide in the lysosomal compartments of cells in nearly all tissues. However, due to their shortened life span, there is no evidence as to whether or not patients develop insulin resistance. Recent studies by Li *et.al.* showed that AC $^{-/+}$ KO mice manifest a progressive lipid storage disease which correlated with elevated ceramide levels and a reduction of AC activity (223). Yet no reports with regard to insulin resistance have been provided from phenotypic studies of these mice. Measurements of insulin-stimulated glucose disposal in AC $^{-/+}$ KO mice would be necessary in order to establish a central role of AC in the development of insulin resistance *in vivo*.

4.6 Conclusions

Ceramide has been implicated as a mediator of insulin resistance induced by the increased availability of FFAs from adipose tissues during obesity onset (126). Here, we

further evaluated the functional significance of saturated FFA-induced ceramide accumulation in the development of muscle insulin resistance by constitutively overexpressing AC in C2C12 myotubes. Overexpression of AC protected cells from the inhibitory effects of long-chain saturated FFAs on insulin signaling as a result of a rapid lysosomal ceramide degradation. These results presented clearly confirm the critical role of AC in the regulation of ceramide accumulation and its deleterious effects on the insulin-signaling pathway. Moreover, this study suggests that preventing the aberrant muscle accumulation of ceramide by promoting its metabolism into sphingosine and sphingosine-derivatives might restore normal insulin sensitivity and glucose metabolism in animal models of insulin resistance. The information obtained could point to potential targets for pharmacological manipulation for the prevention and treatment of Type 2 Diabetes.

Conclusions and Future Perspectives

The increased secretion of fatty acids by adipose tissue has been proposed to link obesity to the development of insulin resistance. Specifically, many researchers have speculated that increased availability of lipids to peripheral tissues causes insulin resistance by promoting the accumulation of fat-derived metabolites capable of inhibiting insulin action. Numerous studies support the hypothesis that the aberrant deposition of the sphingolipid ceramide in peripheral tissues contributes to the development of insulin resistance. Using C2C12 myotubes, we have investigated the role of ceramide as a potential mediator linking FFAs to the inhibition of insulin signaling. Herein we have shown the following:

As described in chapter 2, we first demonstrated that long chain FFAs, but not shorter chain saturated FFAs or unsaturated ones, block insulin signaling in C2C12 myotubes while simultaneously promoting ceramide accumulation. As described in chapters 3 and 4, we subsequently determined that ceramide was a required intermediate linking saturated FFAs to the inhibition of Akt/PKB.

First, we demonstrated that pharmacological agents that prevent ceramide biosynthesis blocked the inhibitory effects of saturated FFAs. As shown in chapter 3, the inhibitors of ceramide synthesis completely prevented the inhibition of insulin signaling by the saturated FFA palmitate in C2C12 myotubes. More interesting, these agents completely blocked palmitate's induction of ceramide, but not DAG (another known important antagonist of insulin signaling implicated the development of insulin

resistance), indicating that ceramide, or a ceramide derivative, mediates the inhibitory effects of palmitate in C2C12 myotubes. In fact, ceramide can be further metabolized by a number of intracellular enzymes to generate other molecular derivatives, many of which, have biological effects on their own. To address the question whether ceramide itself, or alternatively another ceramide metabolite was the principal mediator of the inhibitory effects of palmitate, we evaluated the effects of specific inhibitors of enzymes that mediate ceramide catabolism. Interestingly, the use of these drugs recapitulated the effects of saturated FFAs on both ceramide accumulation and the inhibition of insulin signaling indicating that ceramide is both a sufficient and necessary intermediate linking saturated fatty acids to the inhibition of insulin signaling.

A second approach to delineate a role for ceramide in saturated FFA-induced insulin resistance was to overexpress in skeletal muscle cells genes involved in ceramide degradation, such as acid ceramidase. Using this strategy, we showed in Chapter 4, that we can block the inhibitory effects of saturated FFAs on insulin signaling in C2C12 myotubes by overexpressing human acid ceramidase. These results support the central role of ceramide accumulation in the inhibition of muscle insulin sensitivity by exogenous FFAs and suggest that approaches designed to control aberrant ceramide deposition could be utilized for the treatment of insulin resistance *in vivo*.

Understanding the relevance of these findings, which were obtained in cultured cells, to the development of insulin resistance in humans will ultimately require testing the role of ceramide in animal models of the condition. Based on our findings, we

hypothesize that strategies aimed to manipulate routes of ceramide synthesis or degradation could have marked effects on insulin-stimulated glucose utilization. For example, treating animals with inhibitors of *de novo* ceramide synthesis should block the progression of insulin resistance induced by lipid oversupply. Moreover, targeted overexpression of acid ceramidase in skeletal muscle of transgenic mice might protect animals from becoming insulin resistant when placed on a high fat diet. Data obtained from these experiments would reveal whether modulating ceramide levels quantitatively improved insulin sensitivity in rodents, and would reveal whether ceramides could be a pharmaceutical target for the treatment of insulin resistance.

References

1. (2003) Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 26 Suppl 1, S5-20

2. Eisenbarth, G. S., Jackson, R. A., and Pugliese, A. (1992) Insulin autoimmunity: the rate limiting factor in pre-type I diabetes. *J Autoimmun* 5 Suppl A, 241-246

3. Genuth, S., Alberti, K. G., Bennett, P., Buse, J., Defronzo, R., Kahn, R., Kitzmiller, J., Knowler, W. C., Lebovitz, H., Lernmark, A., Nathan, D., Palmer, J., Rizza, R., Saudek, C., Shaw, J., Steffes, M., Stern, M., Tuomilehto, J., and Zimmet, P. (2003) Follow-up report on the diagnosis of diabetes mellitus. *Diabetes Care* 26, 3160-3167

4. De Courten M, B. P., Tuomilehto J, Zimmet P. (1997) *Epidemiology of NIDDM in non-europids* Vol. Chichester; Wiley,, Chichester; Wiley

5. Mokdad, A. H., Ford, E. S., Bowman, B. A., Nelson, D. E., Engelgau, M. M., Vinicor, F., and Marks, J. S. (2000) Diabetes trends in the U.S.: 1990-1998. *Diabetes Care* 23, 1278-1283

6. Mokdad, A. H., Ford, E. S., Bowman, B. A., Dietz, W. H., Vinicor, F., Bales, V. S., and Marks, J. S. (2003) Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. *Jama* 289, 76-79

7. Harris, M. I. (1998) Diabetes in America: epidemiology and scope of the problem. *Diabetes Care* 21 Suppl 3, C11-14

8. Hogan, P., Dall, T., and Nikolov, P. (2003) Economic costs of diabetes in the US in 2002. *Diabetes Care* 26, 917-932

9. Flakoll, P. J. C., M.G and Cherrington, A.D. (2000) Physiology of Insulin Action. In *Diabetes Mellitus a Fundamental and Clinical Text* (Derek LeRoith, S. I. T. a. J. M. O., ed) pp. 148-161, Lippincott Williams and Wilkins, Philadelphia

10. Zisman, A., Peroni, O. D., Abel, E. D., Michael, M. D., Mauvais-Jarvis, F., Lowell, B. B., Wojtaszewski, J. F., Hirshman, M. F., Virkamaki, A., Goodyear, L. J., Kahn, C. R., and Kahn, B. B. (2000) Targeted disruption of the glucose

transporter 4 selectively in muscle causes insulin resistance and glucose intolerance. *Nat Med* 6, 924-928

11. Abel, E. D., Peroni, O., Kim, J. K., Kim, Y. B., Boss, O., Hadro, E., Minnemann, T., Shulman, G. I., and Kahn, B. B. (2001) Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. *Nature* 409, 729-733

12. Kahn, C. R., and White, M. F. (1988) The insulin receptor and the molecular mechanism of insulin action. *J Clin Invest* 82, 1151-1156

13. Czech, M. P. (1985) The nature and regulation of the insulin receptor: structure and function. *Annu Rev Physiol* 47, 357-381

14. Shoelson, S. E., White, M. F., and Kahn, C. R. (1988) Tryptic activation of the insulin receptor. Proteolytic truncation of the alpha-subunit releases the beta-subunit from inhibitory control. *J Biol Chem* 263, 4852-4860

15. White, M. F., and Kahn, C. R. (1994) The insulin signaling system. *J Biol Chem* 269, 1-4

16. Backer, J. M., Myers, M. G., Jr., Shoelson, S. E., Chin, D. J., Sun, X. J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E. Y., Schlessinger, J., and et al. (1992) Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. *Embo J* 11, 3469-3479

17. Fry, M. J. (1994) Structure, regulation and function of phosphoinositide 3-kinases. *Biochim Biophys Acta* 1226, 237-268

18. Yonezawa, K., Ueda, H., Hara, K., Nishida, K., Ando, A., Chavanieu, A., Matsuba, H., Shii, K., Yokono, K., Fukui, Y., and et al. (1992) Insulin-dependent formation of a complex containing an 85-kDa subunit of phosphatidylinositol 3-kinase and tyrosine-phosphorylated insulin receptor substrate 1. *J Biol Chem* 267, 25958-25965

19. Cantrell, D. A. (2001) Phosphoinositide 3-kinase signalling pathways. *J Cell Sci* 114, 1439-1445

20. Hara, K., Yonezawa, K., Sakaue, H., Ando, A., Kotani, K., Kitamura, T., Kitamura, Y., Ueda, H., Stephens, L., Jackson, T. R., and et al. (1994) 1-

Phosphatidylinositol 3-kinase activity is required for insulin-stimulated glucose transport but not for RAS activation in CHO cells. *Proc Natl Acad Sci U S A* 91, 7415-7419

21. Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O., and Ui, M. (1994) Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. Studies with a selective inhibitor wortmannin. *J Biol Chem* 269, 3568-3573

22. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Kahn, C. R. (1994) Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. *Mol Cell Biol* 14, 4902-4911

23. Katagiri, H., Asano, T., Ishihara, H., Inukai, K., Shibasaki, Y., Kikuchi, M., Yazaki, Y., and Oka, Y. (1996) Overexpression of catalytic subunit p110alpha of phosphatidylinositol 3-kinase increases glucose transport activity with translocation of glucose transporters in 3T3-L1 adipocytes. *J Biol Chem* 271, 16987-16990

24. Martin, S. S., Haruta, T., Morris, A. J., Klippel, A., Williams, L. T., and Olefsky, J. M. (1996) Activated phosphatidylinositol 3-kinase is sufficient to mediate actin rearrangement and GLUT4 translocation in 3T3-L1 adipocytes. *J Biol Chem* 271, 17605-17608

25. Fruman, D. A., Rameh, L. E., and Cantley, L. C. (1999) Phosphoinositide binding domains: embracing 3-phosphate. *Cell* 97, 817-820

26. Jones, P. F., Jakubowicz, T., Pitossi, F. J., Maurer, F., and Hemmings, B. A. (1991) Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. *Proc Natl Acad Sci U S A* 88, 4171-4175

27. Coffer, P. J., and Woodgett, J. R. (1991) Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. *Eur J Biochem* 201, 475-481

28. Bellacosa, A., Testa, J. R., Staal, S. P., and Tsichlis, P. N. (1991) A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science* 254, 274-277

29. Jones, P. F., Jakubowicz, T., and Hemmings, B. A. (1991) Molecular cloning of a second form of rac protein kinase. *Cell Regul* 2, 1001-1009

30. Konishi, H., Kuroda, S., Tanaka, M., Matsuzaki, H., Ono, Y., Kameyama, K., Haga, T., and Kikkawa, U. (1995) Molecular cloning and characterization of a new member of the RAC protein kinase family: association of the pleckstrin homology domain of three types of RAC protein kinase with protein kinase C subspecies and beta gamma subunits of G proteins. *Biochem Biophys Res Commun* 216, 526-534

31. Lawlor, M. A., and Alessi, D. R. (2001) PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J Cell Sci* 114, 2903-2910

32. Aman, M. J., Lamkin, T. D., Okada, H., Kurosaki, T., and Ravichandran, K. S. (1998) The inositol phosphatase SHIP inhibits Akt/PKB activation in B cells. *J Biol Chem* 273, 33922-33928

33. Kohn, A. D., Takeuchi, F., and Roth, R. A. (1996) Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation. *J Biol Chem* 271, 21920-21926

34. Alessi, D. R., and Cohen, P. (1998) Mechanism of activation and function of protein kinase B. *Curr Opin Genet Dev* 8, 55-62

35. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. *Embo J* 15, 6541-6551

36. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378, 785-789

37. Hurel, S. J., Rochford, J. J., Borthwick, A. C., Wells, A. M., Vandenheede, J. R., Turnbull, D. M., and Yeaman, S. J. (1996) Insulin action in cultured human myoblasts: contribution of different signalling pathways to regulation of glycogen synthesis. *Biochem J* 320 (Pt 3), 871-877

38. Shepherd, P. R., Nave, B. T., Rincon, J., Haigh, R. J., Foulstone, E., Proud, C., Zierath, J. R., Siddle, K., and Wallberg-Henriksson, H. (1997) Involvement of phosphoinositide 3-kinase in insulin stimulation of MAP-kinase and

phosphorylation of protein kinase-B in human skeletal muscle: implications for glucose metabolism. *Diabetologia* 40, 1172-1177

39. Moule, S. K., Welsh, G. I., Edgell, N. J., Foulstone, E. J., Proud, C. G., and Denton, R. M. (1997) Regulation of protein kinase B and glycogen synthase kinase-3 by insulin and beta-adrenergic agonists in rat epididymal fat cells. Activation of protein kinase B by wortmannin-sensitive and -insensitive mechanisms. *J Biol Chem* 272, 7713-7719

40. Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* 81, 727-736

41. Klippel, A., Reinhard, C., Kavanaugh, W. M., Apell, G., Escobedo, M. A., and Williams, L. T. (1996) Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal-transducing kinase pathways. *Mol Cell Biol* 16, 4117-4127

42. Kohn, A. D., Summers, S. A., Birnbaum, M. J., and Roth, R. A. (1996) Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J Biol Chem* 271, 31372-31378

43. Ueki, K., Yamamoto-Honda, R., Kaburagi, Y., Yamauchi, T., Tobe, K., Burgering, B. M., Coffer, P. J., Komuro, I., Akanuma, Y., Yazaki, Y., and Kadowaki, T. (1998) Potential role of protein kinase B in insulin-induced glucose transport, glycogen synthesis, and protein synthesis. *J Biol Chem* 273, 5315-5322

44. Takata, M., Ogawa, W., Kitamura, T., Hino, Y., Kuroda, S., Kotani, K., Klip, A., Gingras, A. C., Sonenberg, N., and Kasuga, M. (1999) Requirement for Akt (protein kinase B) in insulin-induced activation of glycogen synthase and phosphorylation of 4E-BP1 (PHAS-1). *J Biol Chem* 274, 20611-20618

45. Wang, Q., Somwar, R., Bilan, P. J., Liu, Z., Jin, J., Woodgett, J. R., and Klip, A. (1999) Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. *Mol Cell Biol* 19, 4008-4018

46. Cong, L. N., Chen, H., Li, Y., Zhou, L., McGibbon, M. A., Taylor, S. I., and Quon, M. J. (1997) Physiological role of Akt in insulin-stimulated translocation of GLUT4 in transfected rat adipose cells. *Mol Endocrinol* 11, 1881-1890

47. Hill, M. M., Clark, S. F., Tucker, D. F., Birnbaum, M. J., James, D. E., and Macaulay, S. L. (1999) A role for protein kinase Bbeta/Akt2 in insulin-stimulated GLUT4 translocation in adipocytes. *Mol Cell Biol* 19, 7771-7781

48. Summers, S. A., Whiteman, E. L., Cho, H., Lipfert, L., and Birnbaum, M. J. (1999) Differentiation-dependent suppression of platelet-derived growth factor signaling in cultured adipocytes. *J Biol Chem* 274, 23858-23867

49. Cho, H., Mu, J., Kim, J. K., Thorvaldsen, J. L., Chu, Q., Crenshaw, E. B., 3rd, Kaestner, K. H., Bartolomei, M. S., Shulman, G. I., and Birnbaum, M. J. (2001) Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science* 292, 1728-1731

50. Cho, H., Thorvaldsen, J. L., Chu, Q., Feng, F., and Birnbaum, M. J. (2001) Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *J Biol Chem* 276, 38349-38352

51. Kane, S., Sano, H., Liu, S. C., Asara, J. M., Lane, W. S., Garner, C. C., and Lienhard, G. E. (2002) A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. *J Biol Chem* 277, 22115-22118

52. Sano, H., Kane, S., Sano, E., Miinea, C. P., Asara, J. M., Lane, W. S., Garner, C. W., and Lienhard, G. E. (2003) Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *J Biol Chem* 278, 14599-14602

53. Woodgett, J. R. (1990) Molecular cloning and expression of glycogen synthase kinase-3/factor A. *Embo J* 9, 2431-2438

54. Summers, S. A., Kao, A. W., Kohn, A. D., Backus, G. S., Roth, R. A., Pessin, J. E., and Birnbaum, M. J. (1999) The role of glycogen synthase kinase 3beta in insulin-stimulated glucose metabolism. *J Biol Chem* 274, 17934-17940

55. Dent, P., Lavoinne, A., Nakielny, S., Caudwell, F. B., Watt, P., and Cohen, P. (1990) The molecular mechanism by which insulin stimulates glycogen synthesis in mammalian skeletal muscle. *Nature* 348, 302-308

56. Lawrence, J. C., Jr., and Abraham, R. T. (1997) PHAS/4E-BPs as regulators of mRNA translation and cell proliferation. *Trends Biochem Sci* 22, 345-349

57. Mendez, R., Myers, M. G., Jr., White, M. F., and Rhoads, R. E. (1996) Stimulation of protein synthesis, eukaryotic translation initiation factor 4E phosphorylation, and PHAS-I phosphorylation by insulin requires insulin receptor substrate 1 and phosphatidylinositol 3-kinase. *Mol Cell Biol* 16, 2857-2864

58. Nave, B. T., Ouwens, M., Withers, D. J., Alessi, D. R., and Shepherd, P. R. (1999) Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem J* 344 Pt 2, 427-431

59. Sabatini, D. M., Erdjument-Bromage, H., Lui, M., Tempst, P., and Snyder, S. H. (1994) RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell* 78, 35-43

60. Kohn, A. D., Barthel, A., Kovacina, K. S., Boge, A., Wallach, B., Summers, S. A., Birnbaum, M. J., Scott, P. H., Lawrence, J. C., Jr., and Roth, R. A. (1998) Construction and characterization of a conditionally active version of the serine/threonine kinase Akt. *J Biol Chem* 273, 11937-11943

61. Dufner, A., Andjelkovic, M., Burgering, B. M., Hemmings, B. A., and Thomas, G. (1999) Protein kinase B localization and activation differentially affect S6 kinase 1 activity and eukaryotic translation initiation factor 4E-binding protein 1 phosphorylation. *Mol Cell Biol* 19, 4525-4534

62. Ferrari, S., and Thomas, G. (1994) S6 phosphorylation and the p70s6k/p85s6k. *Crit Rev Biochem Mol Biol* 29, 385-413

63. Lin, K., Dorman, J. B., Rodan, A., and Kenyon, C. (1997) daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. *Science* 278, 1319-1322

64. Kaufmann, E., and Knochel, W. (1996) Five years on the wings of fork head. *Mech Dev* 57, 3-20

65. Birkenkamp, K. U., and Coffer, P. J. (2003) Regulation of cell survival and proliferation by the FOXO (Forkhead box, class O) subfamily of Forkhead transcription factors. *Biochem Soc Trans* 31, 292-297

66. Bevan, P. (2001) Insulin signalling. *J Cell Sci* 114, 1429-1430

67. Kim, S. J., and Kahn, C. R. (1997) Insulin regulation of mitogen-activated protein kinase kinase (MEK), mitogen-activated protein kinase and casein kinase in the cell nucleus: a possible role in the regulation of gene expression. *Biochem J* 323 (Pt 3), 621-627

68. Hansen, B. C. (1999) The metabolic syndrome X. *Ann N Y Acad Sci* 892, 1-24

69. Ohlson, L. O., Larsson, B., Svardsudd, K., Welin, L., Eriksson, H., Wilhelmsen, L., Bjorntorp, P., and Tibblin, G. (1985) The influence of body fat distribution on the incidence of diabetes mellitus. 13.5 years of follow-up of the participants in the study of men born in 1913. *Diabetes* 34, 1055-1058

70. Martin, M. L., and Jensen, M. D. (1991) Effects of body fat distribution on regional lipolysis in obesity. *J Clin Invest* 88, 609-613

71. Cohen, B., Novick, D., and Rubinstein, M. (1996) Modulation of insulin activities by leptin. *Science* 274, 1185-1188

72. Hotamisligil, G. S., and Spiegelman, B. M. (1994) Tumor necrosis factor alpha: a key component of the obesity-diabetes link. *Diabetes* 43, 1271-1278

73. Steppan, C. M., Bailey, S. T., Bhat, S., Brown, E. J., Banerjee, R. R., Wright, C. M., Patel, H. R., Ahima, R. S., and Lazar, M. A. (2001) The hormone resistin links obesity to diabetes. *Nature* 409, 307-312

74. Arita, Y., Kihara, S., Ouchi, N., Takahashi, M., Maeda, K., Miyagawa, J., Hotta, K., Shimomura, I., Nakamura, T., Miyaoka, K., Kuriyama, H., Nishida, M., Yamashita, S., Okubo, K., Matsubara, K., Muraguchi, M., Ohmoto, Y., Funahashi, T., and Matsuzawa, Y. (1999) Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 257, 79-83

75. Boden, G. (1997) Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. *Diabetes* 46, 3-10

76. Reaven, G. M. (1988) Banting lecture 1988. Role of insulin resistance in human disease. *Diabetes* 37, 1595-1607

77. Liu, R. H., Mizuta, M., Kurose, T., and Matsukura, S. (2002) Early events involved in the development of insulin resistance in Zucker fatty rat. *Int J Obes Relat Metab Disord* 26, 318-326

78. Storz, P., Doppler, H., Wernig, A., Pfizenmaier, K., and Muller, G. (1999) Cross-talk mechanisms in the development of insulin resistance of skeletal muscle cells palmitate rather than tumour necrosis factor inhibits insulin-dependent protein kinase B (PKB)/Akt stimulation and glucose uptake. *Eur J Biochem* 266, 17-25

79. Van Epps-Fung, M., Williford, J., Wells, A., and Hardy, R. W. (1997) Fatty acid-induced insulin resistance in adipocytes. *Endocrinology* 138, 4338-4345

80. Pulawa, L. K., and Eckel, R. H. (2002) Overexpression of muscle lipoprotein lipase and insulin sensitivity. *Curr Opin Clin Nutr Metab Care* 5, 569-574

81. Ferrannini, E., Barrett, E. J., Bevilacqua, S., and DeFronzo, R. A. (1983) Effect of fatty acids on glucose production and utilization in man. *J Clin Invest* 72, 1737-1747

82. Boden, G., Jadali, F., White, J., Liang, Y., Mozzoli, M., Chen, X., Coleman, E., and Smith, C. (1991) Effects of fat on insulin-stimulated carbohydrate metabolism in normal men. *J Clin Invest* 88, 960-966

83. Kelley, D. E., Mokan, M., Simoneau, J. A., and Mandarino, L. J. (1993) Interaction between glucose and free fatty acid metabolism in human skeletal muscle. *J Clin Invest* 92, 91-98

84. Roden, M., Price, T. B., Perseghin, G., Petersen, K. F., Rothman, D. L., Cline, G. W., and Shulman, G. I. (1996) Mechanism of free fatty acid-induced insulin resistance in humans. *J Clin Invest* 97, 2859-2865

85. Chalkley, S. M., Hettiarachchi, M., Chisholm, D. J., and Kraegen, E. W. (1998) Five-hour fatty acid elevation increases muscle lipids and impairs glycogen synthesis in the rat. *Metabolism* 47, 1121-1126

86. Kraegen, E. W., Clark, P. W., Jenkins, A. B., Daley, E. A., Chisholm, D. J., and Storlien, L. H. (1991) Development of muscle insulin resistance after liver insulin resistance in high-fat-fed rats. *Diabetes* 40, 1397-1403

87. Randle, P. J., Garland, P. B., Newsholme, E. A., and Hales, C. N. (1965) The glucose fatty acid cycle in obesity and maturity onset diabetes mellitus. *Ann N Y Acad Sci* 131, 324-333

88. Oakes, N. D., Cooney, G. J., Camilleri, S., Chisholm, D. J., and Kraegen, E. W. (1997) Mechanisms of liver and muscle insulin resistance induced by chronic high-fat feeding. *Diabetes* 46, 1768-1774

89. Roden, M., Krssak, M., Stingl, H., Gruber, S., Hofer, A., Furnsinn, C., Moser, E., and Waldhausl, W. (1999) Rapid impairment of skeletal muscle glucose transport/phosphorylation by free fatty acids in humans. *Diabetes* 48, 358-364

90. Phillips, D. I., Caddy, S., Ilic, V., Fielding, B. A., Frayn, K. N., Borthwick, A. C., and Taylor, R. (1996) Intramuscular triglyceride and muscle insulin sensitivity: evidence for a relationship in nondiabetic subjects. *Metabolism* 45, 947-950

91. Pan, D. A., Lillioja, S., Kriketos, A. D., Milner, M. R., Baur, L. A., Bogardus, C., Jenkins, A. B., and Storlien, L. H. (1997) Skeletal muscle triglyceride levels are inversely related to insulin action. *Diabetes* 46, 983-988

92. Virkamaki, A., Korsheninnikova, E., Seppala-Lindroos, A., Vehkavaara, S., Goto, T., Halavaara, J., Hakkinen, A. M., and Yki-Jarvinen, H. (2001) Intramyocellular lipid is associated with resistance to in vivo insulin actions on glucose uptake, antilipolysis, and early insulin signaling pathways in human skeletal muscle. *Diabetes* 50, 2337-2343

93. McGarry, J. D. (2002) Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. *Diabetes* 51, 7-18

94. Itani, S. I., Ruderman, N. B., Schmieder, F., and Boden, G. (2002) Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha. *Diabetes* 51, 2005-2011

95. Yu, C., Chen, Y., Cline, G. W., Zhang, D., Zong, H., Wang, Y., Bergeron, R., Kim, J. K., Cushman, S. W., Cooney, G. J., Atcheson, B., White, M. F., Kraegen, E. W., and Shulman, G. I. (2002) Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J Biol Chem* 277, 50230-50236

96. Kuhlmann, J., Neumann-Haefelin, C., Belz, U., Kalisch, J., Juretschke, H. P., Stein, M., Kleinschmidt, E., Kramer, W., and Herling, A. W. (2003) Intramyocellular lipid and insulin resistance: a longitudinal in vivo 1H-spectroscopic study in Zucker diabetic fatty rats. *Diabetes* 52, 138-144

97. Dobbins, R. L., Szczepaniak, L. S., Bentley, B., Esser, V., Myhill, J., and McGarry, J. D. (2001) Prolonged inhibition of muscle carnitine palmitoyltransferase-1 promotes intramyocellular lipid accumulation and insulin resistance in rats. *Diabetes* 50, 123-130

98. Gan, S. K., Samaras, K., Thompson, C. H., Kraegen, E. W., Carr, A., Cooper, D. A., and Chisholm, D. J. (2002) Altered myocellular and abdominal fat partitioning predict disturbance in insulin action in HIV protease inhibitor-related lipodystrophy. *Diabetes* 51, 3163-3169

99. Moitra, J., Mason, M. M., Olive, M., Krylov, D., Gavrilova, O., Marcus-Samuels, B., Feigenbaum, L., Lee, E., Aoyama, T., Eckhaus, M., Reitman, M. L., and Vinson, C. (1998) Life without white fat: a transgenic mouse. *Genes Dev* 12, 3168-3181

100. Gavrilova, O., Marcus-Samuels, B., Graham, D., Kim, J. K., Shulman, G. I., Castle, A. L., Vinson, C., Eckhaus, M., and Reitman, M. L. (2000) Surgical implantation of adipose tissue reverses diabetes in lipoatrophic mice. *J Clin Invest* 105, 271-278

101. Goodpaster, B. H., He, J., Watkins, S., and Kelley, D. E. (2001) Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes. *J Clin Endocrinol Metab* 86, 5755-5761

102. Helge, J. W., and Dela, F. (2003) Effect of training on muscle triacylglycerol and structural lipids: a relation to insulin sensitivity? *Diabetes* 52, 1881-1887

103. Schmitz-Peiffer, C., Craig, D. L., and Biden, T. J. (1999) Ceramide generation is sufficient to account for the inhibition of the insulin-stimulated PKB pathway in C2C12 skeletal muscle cells pretreated with palmitate. *J Biol Chem* 274, 24202-24210

104. Ellis, B. A., Poynten, A., Lowy, A. J., Furler, S. M., Chisholm, D. J., Kraegen, E. W., and Cooney, G. J. (2000) Long-chain acyl-CoA esters as indicators of lipid metabolism and insulin sensitivity in rat and human muscle. *Am J Physiol Endocrinol Metab* 279, E554-560

105. Kim, J. K., Fillmore, J. J., Chen, Y., Yu, C., Moore, I. K., Pypaert, M., Lutz, E. P., Kako, Y., Velez-Carrasco, W., Goldberg, I. J., Breslow, J. L., and Shulman, G. I. (2001) Tissue-specific overexpression of lipoprotein lipase causes tissue-specific insulin resistance. *Proc Natl Acad Sci U S A* 98, 7522-7527

106. Thompson, A. L., Lim-Fraser, M. Y., Kraegen, E. W., and Cooney, G. J. (2000) Effects of individual fatty acids on glucose uptake and glycogen synthesis in soleus muscle in vitro. *Am J Physiol Endocrinol Metab* 279, E577-584

107. Oakes, N. D., Bell, K. S., Furler, S. M., Camilleri, S., Saha, A. K., Ruderman, N. B., Chisholm, D. J., and Kraegen, E. W. (1997) Diet-induced muscle insulin resistance in rats is ameliorated by acute dietary lipid withdrawal or a single bout of exercise: parallel relationship between insulin stimulation of glucose uptake and suppression of long-chain fatty acyl-CoA. *Diabetes* 46, 2022-2028

108. Thompson, A. L., and Cooney, G. J. (2000) Acyl-CoA inhibition of hexokinase in rat and human skeletal muscle is a potential mechanism of lipid-induced insulin resistance. *Diabetes* 49, 1761-1765

109. Orellana, A., Hidalgo, P. C., Morales, M. N., Mezzano, D., and Bronfman, M. (1990) Palmitoyl-CoA and the acyl-CoA thioester of the carcinogenic peroxisome-proliferator ciprofibrate potentiate diacylglycerol-activated protein kinase C by decreasing the phosphatidylserine requirement of the enzyme. *Eur J Biochem* 190, 57-61

110. Nesher, M., and Boneh, A. (1994) Effect of fatty acids and their acyl-CoA esters on protein kinase C activity in fibroblasts: possible implications in fatty acid oxidation defects. *Biochim Biophys Acta* 1221, 66-72

111. Schmitz-Peiffer, C. (2000) Signalling aspects of insulin resistance in skeletal muscle: mechanisms induced by lipid oversupply. *Cell Signal* 12, 583-594

112. Spiegelman, B. M. (1998) PPAR-gamma: adipogenic regulator and thiazolidinedione receptor. *Diabetes* 47, 507-514

113. Loviscach, M., Rehman, N., Carter, L., Mudaliar, S., Mohadeen, P., Ciaraldi, T. P., Veerkamp, J. H., and Henry, R. R. (2000) Distribution of peroxisome proliferator-activated receptors (PPARs) in human skeletal muscle and adipose tissue: relation to insulin action. *Diabetologia* 43, 304-311

114. Hubinger, A., Knode, O., Susanto, F., Reinauer, H., and Gries, F. A. (1997) Effects of the carnitine-acyltransferase inhibitor etomoxir on insulin sensitivity, energy expenditure and substrate oxidation in NIDDM. *Horm Metab Res* 29, 436-439

115. Schmitz-Peiffer, C., Browne, C. L., Oakes, N. D., Watkinson, A., Chisholm, D. J., Kraegen, E. W., and Biden, T. J. (1997) Alterations in the expression and cellular localization of protein kinase C isozymes epsilon and theta are associated with insulin resistance in skeletal muscle of the high-fat-fed rat. *Diabetes* 46, 169-178

116. Turinsky, J., O'Sullivan, D. M., and Bayly, B. P. (1990) 1,2-Diacylglycerol and ceramide levels in insulin-resistant tissues of the rat in vivo. *J Biol Chem* 265, 16880-16885

117. Koya, D., and King, G. L. (1998) Protein kinase C activation and the development of diabetic complications. *Diabetes* 47, 859-866

118. Griffin, M. E., Marcucci, M. J., Cline, G. W., Bell, K., Barucci, N., Lee, D., Goodyear, L. J., Kraegen, E. W., White, M. F., and Shulman, G. I. (1999) Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. *Diabetes* 48, 1270-1274

119. Qu, X., Seale, J. P., and Donnelly, R. (1999) Tissue and isoform-selective activation of protein kinase C in insulin-resistant obese Zucker rats - effects of feeding. *J Endocrinol* 162, 207-214

120. Bell, K. S., Schmitz-Peiffer, C., Lim-Fraser, M., Biden, T. J., Cooney, G. J., and Kraegen, E. W. (2000) Acute reversal of lipid-induced muscle insulin resistance is associated with rapid alteration in PKC-theta localization. *Am J Physiol Endocrinol Metab* 279, E1196-1201

121. Schmitz-Peiffer, C., Oakes, N. D., Browne, C. L., Kraegen, E. W., and Biden, T. J. (1997) Reversal of chronic alterations of skeletal muscle protein kinase C from fat-fed rats by BRL-49653. *Am J Physiol* 273, E915-921

122. Cortright, R. N., Azevedo, J. L., Jr., Zhou, Q., Sinha, M., Pories, W. J., Itani, S. I., and Dohm, G. L. (2000) Protein kinase C modulates insulin action in human skeletal muscle. *Am J Physiol Endocrinol Metab* 278, E553-562

123. Tanti, J. F., Gremeaux, T., van Obberghen, E., and Le Marchand-Brustel, Y. (1994) Serine/threonine phosphorylation of insulin receptor substrate 1 modulates insulin receptor signaling. *J Biol Chem* 269, 6051-6057

124. Hannun, Y. A., and Obeid, L. M. (2002) The Ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind. *J Biol Chem* 277, 25847-25850

125. Cutler, R. G., and Mattson, M. P. (2001) Sphingomyelin and ceramide as regulators of development and lifespan. *Mech Ageing Dev* 122, 895-908

126. Adams, J. M., 2nd, Pratipanawatr, T., Berria, R., Wang, E., DeFronzo, R. A., Sullards, M. C., and Mandarino, L. J. (2004) Ceramide content is increased in skeletal muscle from obese insulin-resistant humans. *Diabetes* 53, 25-31

127. Gorski, J., Dobrzyn, A., and Zendzian-Piotrowska, M. (2002) The sphingomyelin-signaling pathway in skeletal muscles and its role in regulation of glucose uptake. *Ann N Y Acad Sci* 967, 236-248

128. Summers, S. A., Garza, L. A., Zhou, H., and Birnbaum, M. J. (1998) Regulation of insulin-stimulated glucose transporter GLUT4 translocation and Akt kinase activity by ceramide. *Mol Cell Biol* 18, 5457-5464

129. Hajduch, E., Balendran, A., Batty, I. H., Litherland, G. J., Blair, A. S., Downes, C. P., and Hundal, H. S. (2001) Ceramide impairs the insulin-dependent membrane recruitment of protein kinase B leading to a loss in downstream signalling in L6 skeletal muscle cells. *Diabetologia* 44, 173-183

130. Zhang, Y., Yao, B., Delikat, S., Bayoumy, S., Lin, X. H., Basu, S., McGinley, M., Chan-Hui, P. Y., Lichenstein, H., and Kolesnick, R. (1997) Kinase suppressor of Ras is ceramide-activated protein kinase. *Cell* 89, 63-72

131. Powell, D. J., Turban, S., Gray, A., Hajduch, E., and Hundal, H. S. (2004) Intracellular ceramide synthesis and PKCzeta activation play an essential role in palmitate-induced insulin resistance in rat L6 skeletal muscle cells. *Biochem J* Pt

132. Salinas, M., Lopez-Valdaliso, R., Martin, D., Alvarez, A., and Cuadrado, A. (2000) Inhibition of PKB/Akt1 by C2-ceramide involves activation of ceramide-activated protein phosphatase in PC12 cells. *Mol Cell Neurosci* 15, 156-169

133. Hawkins, M., Barzilai, N., Liu, R., Hu, M., Chen, W., and Rossetti, L. (1997) Role of the glucosamine pathway in fat-induced insulin resistance. *J Clin Invest* 99, 2173-2182

134. Hebert, L. F., Jr., Daniels, M. C., Zhou, J., Crook, E. D., Turner, R. L., Simmons, S. T., Neidigh, J. L., Zhu, J. S., Baron, A. D., and McClain, D. A. (1996) Overexpression of glutamine:fructose-6-phosphate amidotransferase in transgenic mice leads to insulin resistance. *J Clin Invest* 98, 930-936

135. Choi, C. S., Lee, F. N., and Youn, J. H. (2001) Free fatty acids induce peripheral insulin resistance without increasing muscle hexosamine pathway product levels in rats. *Diabetes* 50, 418-424

136. Saltiel, A. R. (2001) New perspectives into the molecular pathogenesis and treatment of type 2 diabetes. *Cell* 104, 517-529

137. Summers, S. A., Yin, V. P., Whiteman, E. L., Garza, L. A., Cho, H., Tuttle, R. L., and Birnbaum, M. J. (1999) Signaling pathways mediating insulin-stimulated glucose transport. *Ann N Y Acad Sci* 892, 169-186

138. Summers, S. A., Whiteman, E. L., and Birnbaum, M. J. (2000) Insulin signaling in the adipocyte. *Int J Obes Relat Metab Disord* 24 Suppl 4, S67-70.

139. Rosen, O. M. (1987) After insulin binds. *Science* 237, 1452-1458

140. Keller, S., and Lienhard, G. (1994) Insulin signalling the role of insulin receptor substrate 1. *Trends Cell Biol* 4, 115-119

141. Okada, T., Kawano, Y., Sabakibara, T., Hazeki, O., and Ui, M. (1994) Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. Studies with a selective inhibitor wortmannin. *J Biol Chem* 269, 3568-3573

142. Kanai, F., Ito, K., Todaka, M., Hayashi, H., Kamohara, S., Ishii, K., Okada, T., Hazeki, O., Ui, M., and Ebina, Y. (1993) Insulin-stimulated GLUT4 translocation is relevant to the phosphorylation of IRS-1 and the activity of PI3-kinase. *Biochem Biophys Res Comm* 195, 762-768

143. Sharma, P. M., Katsuya, E., Huang, Y., Martin, J. L., Huvar, I., Boss, G. R., and Olefsky, J. M. (1998) Inhibition of phosphatidylinositol 3-kinase activity by adenovirus-mediated gene transfer and its effect on insulin action. *Journal of Biological Chemistry* 273, 18528-18537

144. Yao, R., and Cooper, G. M. (1995) Requirement for phosphatidylinositol-3-kinase in the prevention of apoptosis by nerve growth factor. *Science* 267, 2003-2006

145. Kulik, G., Klippel, A., and Weber, M. J. (1997) Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. *Molecular & Cellular Biology* 17, 1595-1606

146. Kapeller, R., and Cantley, L. (1994) Phosphatidylinositol 3-kinase. *Bioessays* 16, 565-576

147. Coffer, P. J., Jin, J., and Woodgett, J. R. (1998) Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem J* 335, 1-13

148. Marte, B. M., and Downward, J. (1997) PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. *Trends in Biochemical Sciences* 22, 355-358

149. Franke, T. F., Kaplan, D. R., and Cantley, L. C. (1997) PI3K: downstream AKTion blocks apoptosis. *Cell* 88, 435-437

150. Stratford, S., DeWald, D. B., and Summers, S. A. (2001) Ceramide dissociates 3'-phosphoinositide production from pleckstrin homology domain translocation. *Biochem J* 354, 359-368

151. Kellerer, M., Mushack, J., Seffer, E., Mischak, H., Ullrich, A., and Haring, H. U. (1998) Protein kinase C isoforms alpha, delta and theta require insulin receptor substrate-1 to inhibit the tyrosine kinase activity of the insulin receptor in human kidney embryonic cells (HEK 293 cells). *Diabetologia* 41, 833-838.

152. Danielsen, A. G., Liu, F., Hosomi, Y., Shii, K., and Roth, R. A. (1995) Activation of protein kinase C alpha inhibits signaling by members of the insulin receptor family. *J Biol Chem* 270, 21600-21605.

153. Lin, Y., Itani, S. I., Kurowski, T. G., Dean, D. J., Luo, Z., Yaney, G. C., and Ruderman, N. B. (2001) Inhibition of insulin signaling and glycogen synthesis by phorbol dibutyrate in rat skeletal muscle. *Am J Physiol Endocrinol Metab* 281, E8-E15.

154. Motley, E. D., Kabir, S. M., Eguchi, K., Hicks, A. L., Gardner, C. D., Reynolds, C. M., Frank, G. D., and Eguchi, S. (2001) Protein kinase C inhibits insulin-induced Akt activation in vascular smooth muscle cells. *Cell Mol Biol (Noisy-le-grand)* 47, 1059-1062.

155. Summers, S. A., Lipfert, L., and Birnbaum, M. J. (1998) Polyoma middle T antigen activates the Ser/Thr kinase Akt in a PI3-kinase-dependent manner. *Biochem Biophys Res Commun* 246, 76-81

156. Gorski, J., Nawrocki, A., and Murthy, M. (1998) Characterization of free and glyceride-esterified long chain fatty acids in different skeletal muscle types of the rat. *Mol Cell Biochem* 178, 113-118.

157. Rivellese, A. A., De Natale, C., and Lilli, S. (2002) Type of dietary fat and insulin resistance. *Ann N Y Acad Sci* 967, 329-335.

158. Glenn, K. C., Shieh, J. J., and Laird, D. M. (1992) Characterization of 3T3-L1 storage lipid metabolism: effect of somatotropin and insulin on specific pathways. *Endocrinology* 131, 1115-1124

159. Perry, D. K., Bielawska, A., and Hannun, Y. A. (2000) Quantitative determination of ceramide using diglyceride kinase. *Methods Enzymol* 312, 22-31

160. Reaven, G. M., Hollenbeck, C., Jeng, C. Y., Wu, M. S., and Chen, Y. D. (1988) Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM. *Diabetes* 37, 1020-1024

161. Hall, S. E., Saunders, J., and Sonksen, P. H. (1979) Glucose and free fatty acid turnover in normal subjects and in diabetic patients before and after insulin treatment. *Diabetologia* 16, 297-306

162. Merrill, A. H., Jr. (2002) De novo sphingolipid biosynthesis: a necessary, but dangerous, pathway. *J Biol Chem* 277, 25843-25846.

163. Shimabukuro, M., Higa, M., Zhou, Y. T., Wang, M. Y., Newgard, C. B., and Unger, R. H. (1998) Lipoapoptosis in beta-cells of obese prediabetic fa/fa rats. Role of serine palmitoyltransferase overexpression. *J Biol Chem* 273, 32487-32490

164. Wakelam, M. J. (1998) Diacylglycerol--when is it an intracellular messenger? *Biochim Biophys Acta* 1436, 117-126.

165. Charles, M. A., Eschwege, E., Thibult, N., Claude, J. R., Warnet, J. M., Rosselin, G. E., Girard, J., and Balkau, B. (1997) The role of non-esterified fatty acids in the deterioration of glucose tolerance in Caucasian subjects: results of the Paris Prospective Study. *Diabetologia* 40, 1101-1106

166. Hunnicutt, J. W., Hardy, R. W., Williford, J., and McDonald, J. M. (1994) Saturated fatty acid-induced insulin resistance in rat adipocytes. *Diabetes* 43, 540-545

167. Montell, E., Turini, M., Marotta, M., Roberts, M., Noe, V., Ciudad, C. J., Mace, K., and Gomez-Foix, A. M. (2001) DAG accumulation from saturated fatty acids desensitizes insulin stimulation of glucose uptake in muscle cells. *Am J Physiol Endocrinol Metab* 280, E229-237

168. Bachmann, O. P., Dahl, D. B., Brechtel, K., Machann, J., Haap, M., Maier, T., Loviscach, M., Stumvoll, M., Claussen, C. D., Schick, F., Haring, H. U., and Jacob, S. (2001) Effects of intravenous and dietary lipid challenge on intramyocellular lipid content and the relation with insulin sensitivity in humans. *Diabetes* 50, 2579-2584

169. Sinha, R., Dufour, S., Petersen, K. F., LeBon, V., Enoksson, S., Ma, Y. Z., Savoye, M., Rothman, D. L., Shulman, G. I., and Caprio, S. (2002) Assessment of skeletal muscle triglyceride content by (1)H nuclear magnetic resonance spectroscopy in lean and obese adolescents: relationships to insulin sensitivity, total body fat, and central adiposity. *Diabetes* 51, 1022-1027

170. Neschen, S., Moore, I., Regittnig, W., Yu, C. L., Wang, Y., Pypaert, M., Petersen, K. F., and Shulman, G. I. (2002) Contrasting effects of fish oil and safflower oil on hepatic peroxisomal and tissue lipid content. *Am J Physiol Endocrinol Metab* 282, E395-401

171. Ferreira, L. D., Pulawa, L. K., Jensen, D. R., and Eckel, R. H. (2001) Overexpressing human lipoprotein lipase in mouse skeletal muscle is associated with insulin resistance. *Diabetes* 50, 1064-1068

172. White, M. F. (1998) The IRS-signalling system: a network of docking proteins that mediate insulin action. *Mol Cell Biochem* 182, 3-11

173. Shepherd, P. R., Withers, D. J., and Siddle, K. (1998) Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. *Biochem J* 333 (Pt 3), 471-490

174. Kitamura, T., Ogawa, W., Sakaue, H., Hino, Y., Kuroda, S., Takata, M., Matsumoto, M., Maeda, T., Konishi, H., Kikkawa, U., and Kasuga, M. (1998) Requirement for activation of the serine-threonine kinase Akt (protein kinase B) in insulin stimulation of protein synthesis but not of glucose transport. *Mol Cell Biol* 18, 3708-3717

175. Boden, G., and Shulman, G. I. (2002) Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction. *Eur J Clin Invest* 32 Suppl 3, 14-23

176. Teruel, T., Hernandez, R., and Lorenzo, M. (2001) Ceramide mediates insulin resistance by tumor necrosis factor-alpha in brown adipocytes by maintaining Akt in an inactive dephosphorylated state. *Diabetes* 50, 2563-2571

177. Wang, C. N., O'Brien, L., and Brindley, D. N. (1998) Effects of cell-permeable ceramides and tumor necrosis factor-alpha on insulin signaling and glucose uptake in 3T3-L1 adipocytes. *Diabetes* 47, 24-31

178. Shmueli, E., Alberti, K. G., and Record, C. O. (1993) Diacylglycerol/protein kinase C signalling: a mechanism for insulin resistance? *J Intern Med* 234, 397-400

179. Chen, K. S., Heydrick, S., Kurowski, T., and Ruderman, N. B. (1991) Diacylglycerol-protein kinase C signalling in skeletal muscle: a possible link to insulin resistance. *Trans Assoc Am Physicians* 104, 206-212

180. Takayama, S., White, M. F., and Kahn, C. R. (1988) Phorbol ester-induced serine phosphorylation of the insulin receptor decreases its tyrosine kinase activity. *J Biol Chem* 263, 3440-3447.

181. Minamide, L. S., Shaw, A. E., Sarmiere, P. D., Wiggan, O., Maloney, M. T., Bernstein, B. W., Sneider, J. M., Gonzalez, J. A., and Bamburg, J. R. (2003) Production and use of replication-deficient adenovirus for transgene expression in neurons. *Methods Cell Biol* 71, 387-416

182. Raatz, S. K., Bibus, D., Thomas, W., and Kris-Etherton, P. (2001) Total fat intake modifies plasma fatty acid composition in humans. *J Nutr* 131, 231-234

183. van Blitterswijk, W. J., van der Luit, A. H., Veldman, R. J., Verheij, M., and Borst, J. (2003) Ceramide: second messenger or modulator of membrane structure and dynamics? *Biochem J* 369, 199-211

184. Rani, C. S., Abe, A., Chang, Y., Rosenzweig, N., Saltiel, A. R., Radin, N. S., and Shayman, J. A. (1995) Cell cycle arrest induced by an inhibitor of glucosylceramide synthase. Correlation with cyclin-dependent kinases. *J Biol Chem* 270, 2859-2867

185. Schubert, K. M., Scheid, M. P., and Duronio, V. (2000) Ceramide inhibits protein kinase B/Akt by promoting dephosphorylation of serine 473. *J Biol Chem* 275, 13330-13335

186. Yang, S. I., Lickteig, R. L., Estes, R., Rundell, K., Walter, G., and Mumby, M. C. (1991) Control of protein phosphatase 2A by simian virus 40 small-t antigen. *Mol Cell Biol* 11, 1988-1995.

187. Chin, J. E., Dickens, M., Tavare, J. M., and Roth, R. A. (1993) Overexpression of protein kinase C isoenzymes alpha, beta I, gamma, and epsilon in cells overexpressing the insulin receptor. Effects on receptor phosphorylation and signaling. *J Biol Chem* 268, 6338-6347.

188. Chin, J. E., Liu, F., and Roth, R. A. (1994) Activation of protein kinase C alpha inhibits insulin-stimulated tyrosine phosphorylation of insulin receptor substrate-1. *Mol Endocrinol* 8, 51-58.

189. Bossenmaier, B., Mosthaf, L., Mischak, H., Ullrich, A., and Haring, H. U. (1997) Protein kinase C isoforms beta 1 and beta 2 inhibit the tyrosine kinase activity of the insulin receptor. *Diabetologia* 40, 863-866

190. Schmitz-Peiffer, C. (2002) Protein kinase C and lipid-induced insulin resistance in skeletal muscle. *Ann N Y Acad Sci* 967, 146-157

191. Resjo, S., Goransson, O., Harndahl, L., Zolnierowicz, S., Manganiello, V., and Degerman, E. (2002) Protein phosphatase 2A is the main phosphatase involved in the regulation of protein kinase B in rat adipocytes. *Cell Signal* 14, 231-238

192. Zinda, M. J., Vlahos, C. J., and Lai, M. T. (2001) Ceramide induces the dephosphorylation and inhibition of constitutively activated Akt in PTEN negative U87mg cells. *Biochem Biophys Res Commun* 280, 1107-1115

193. Zhou, H., Summers, S. A., Birnbaum, M. J., and Pittman, R. N. (1998) Inhibition of Akt kinase by cell-permeable ceramide and its implications for ceramide-induced apoptosis. *J Biol Chem* 273, 16568-16575

194. Bourbon, N. A., Sandirasegarane, L., and Kester, M. (2002) Ceramide-induced inhibition of Akt is mediated through protein kinase Czeta: implications for growth arrest. *J Biol Chem* 277, 3286-3292

195. Cazzolli, R., Carpenter, L., Biden, T. J., and Schmitz-Peiffer, C. (2001) A role for protein phosphatase 2A-like activity, but not atypical protein kinase Czeta, in the inhibition of protein kinase B/Akt and glycogen synthesis by palmitate. *Diabetes* 50, 2210-2218

196. Vessby, B., Aro, A., Skarfors, E., Berglund, L., Salminen, I., and Lithell, H. (1994) The risk to develop NIDDM is related to the fatty acid composition of the serum cholesterol esters. *Diabetes* 43, 1353-1357

197. Ho, R. C., Davy, K. P., Hickey, M. S., Summers, S. A., and Melby, C. L. (2002) Behavioral, metabolic, and molecular correlates of lower insulin sensitivity in Mexican-Americans. *Am J Physiol Endocrinol Metab* 283, E799-808

198. Turcotte, L. P., Swenberger, J. R., Zavitz Tucker, M., and Yee, A. J. (2001) Increased fatty acid uptake and altered fatty acid metabolism in insulin-resistant muscle of obese Zucker rats. *Diabetes* 50, 1389-1396

199. Dobrzyn, A., and Gorski, J. (2002) Ceramides and sphingomyelins in skeletal muscles of the rat: content and composition. Effect of prolonged exercise. *Am J Physiol Endocrinol Metab* 282, E277-285

200. Ahima, R. S., and Flier, J. S. (2000) Adipose tissue as an endocrine organ. *Trends Endocrinol Metab* 11, 327-332

201. Birnbaum, M. J. (2001) Diabetes. Dialogue between muscle and fat. *Nature* 409, 672-673

202. Goodpaster, B. H., and Kelley, D. E. (2002) Skeletal muscle triglyceride: marker or mediator of obesity-induced insulin resistance in type 2 diabetes mellitus? *Curr Diab Rep* 2, 216-222

203. Chavez, J. A., and Summers, S. A. (2003) Characterizing the effects of saturated fatty acids on insulin signaling and ceramide and diacylglycerol accumulation in 3T3-L1 adipocytes and C2C12 myotubes. *Arch Biochem Biophys* 419, 101-109

204. Hegarty, B. D., Furler, S. M., Ye, J., Cooney, G. J., and Kraegen, E. W. (2003) The role of intramuscular lipid in insulin resistance. *Acta Physiol Scand* 178, 373-383

205. Straczkowski, M., Kowalska, I., Nikolajuk, A., Dzienis-Straczkowska, S., Kinalska, I., Baranowski, M., Zendzian-Piotrowska, M., Brzezinska, Z., and Gorski, J. (2004) Relationship between insulin sensitivity and sphingomyelin signaling pathway in human skeletal muscle. *Diabetes* 53, 1215-1221

206. Chavez, J. A., Knotts, T. A., Wang, L. P., Li, G., Dobrowsky, R. T., Florant, G. L., and Summers, S. A. (2003) A role for ceramide, but not diacylglycerol, in the antagonism of insulin signal transduction by saturated fatty acids. *J Biol Chem* 278, 10297-10303

207. Shimabukuro, M., Zhou, Y. T., Levi, M., and Unger, R. H. (1998) Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes. *Proc Natl Acad Sci U S A* 95, 2498-2502

208. Gatt, S. (1963) Enzymic Hydrolysis and Synthesis of Ceramides. *J Biol Chem* 238, 3131-3133

209. el Bawab, S., Mao, C., Obeid, L. M., and Hannun, Y. A. (2002) Ceramidases in the regulation of ceramide levels and function. *Subcell Biochem* 36, 187-205

210. Sugita, M., Dulaney, J. T., and Moser, H. W. (1972) Ceramidase deficiency in Farber's disease (lipogranulomatosis). *Science* 178, 1100-1102

211. Koch, J., Gartner, S., Li, C. M., Quintern, L. E., Bernardo, K., Levran, O., Schnabel, D., Desnick, R. J., Schuchman, E. H., and Sandhoff, K. (1996) Molecular cloning and characterization of a full-length complementary DNA encoding human acid ceramidase. Identification Of the first molecular lesion causing Farber disease. *J Biol Chem* 271, 33110-33115

212. Olivera, A., Barlow, K. D., and Spiegel, S. (2000) Assaying sphingosine kinase activity. *Methods Enzymol* 311, 215-223

213. Edsall, L. C., and Spiegel, S. (1999) Enzymatic measurement of sphingosine 1-phosphate. *Anal Biochem* 272, 80-86

214. Cohen, P., Nimmo, H. G., and Proud, C. G. (1978) How does insulin stimulate glycogen synthesis? *Biochem Soc Symp*, 69-95

215. Strelow, A., Bernardo, K., Adam-Klages, S., Linke, T., Sandhoff, K., Krèonke, M., and Adam, D. (2000) Overexpression of acid ceramidase protects from tumor necrosis factor-induced cell death. *J Exp Med* 192, 601-612

216. Seelan, R. S., Qian, C., Yokomizo, A., Bostwick, D. G., Smith, D. I., and Liu, W. (2000) Human acid ceramidase is overexpressed but not mutated in prostate cancer. *Genes Chromosomes Cancer* 29, 137-146

217. Nava, V. E., Cuvillier, O., Edsall, L. C., Kimura, K., Milstien, S., Gelmann, E. P., and Spiegel, S. (2000) Sphingosine enhances apoptosis of radiation-resistant prostate cancer cells. *Cancer Res* 60, 4468-4474

218. Nelson, D. H., and Murray, D. K. (1986) Sphingolipids inhibit insulin and phorbol ester stimulated uptake of 2-deoxyglucose. *Biochem Biophys Res Commun* 138, 463-467

219. Murray, D. K., Hill, M. E., and Nelson, D. H. (1990) Inhibitory action of sphingosine, sphinganine and dexamethasone on glucose uptake: studies with hydrogen peroxide and phorbol ester. *Life Sci* 46, 1843-1849

220. Robertson, D. G., DiGirolamo, M., Merrill, A. H., Jr., and Lambeth, J. D. (1989) Insulin-stimulated hexose transport and glucose oxidation in rat adipocytes is inhibited by sphingosine at a step after insulin binding. *J Biol Chem* 264, 6773-6779

221. Mandon, E. C., Ehses, I., Rother, J., van Echten, G., and Sandhoff, K. (1992) Subcellular localization and membrane topology of serine palmitoyltransferase, 3-dehydrosphinganine reductase, and sphinganine N-acyltransferase in mouse liver. *J Biol Chem* 267, 11144-11148

222. Li, C. M., Hong, S. B., Kopal, G., He, X., Linke, T., Hou, W. S., Koch, J., Gatt, S., Sandhoff, K., and Schuchman, E. H. (1998) Cloning and characterization of the full-length cDNA and genomic sequences encoding murine acid ceramidase. *Genomics* 50, 267-274

223. Li, C. M., Park, J. H., Simonaro, C. M., He, X., Gordon, R. E., Friedman, A. H., Ehleiter, D., Paris, F., Manova, K., Hepbildikler, S., Fuks, Z., Sandhoff, K., Kolesnick, R., Schuchman, E. H., and Hepbiloikler, S. (2002) Insertional mutagenesis of the mouse acid ceramidase gene leads to early embryonic lethality in homozygotes and progressive lipid storage disease in heterozygotes. *Genomics* 79, 218-224