NOTE TO USERS

DISSERTATION

THE REGULATION OF INSULIN SIGNALING AND AKT/PKB ACTIVITY BY OBESITY

Submitted by
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In partial fulfillment of the requirements for
the degree of Doctorate of Philosophy
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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY KYLE LEE HOEHN ENTITLED

THE REGULATION OF INSULIN SIGNALING AND AKT/PKB ACTIVITY BY OBESITY

BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Committee on Graduate Work

Adviser

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ABSTRACT OF DISSERTATION

THE REGULATION OF INSULIN SIGNALING AND AKT/PKB ACTIVITY BY OBESITY

Insulin is a key regulator of Akt/Protein Kinase B (PKB), which is a requisite intermediate linking the hormone to postprandial glucose uptake and anabolic metabolism. Increased adiposity, a characteristic feature of obesity, promotes insulin resistance and is linked to the pathogenesis of multiple metabolic diseases. The link between obesity and insulin resistance remains unknown, but is likely to be driven by factors secreted from adipose tissue. Specifically, researchers have identified roles for the adipocyte-derived cytokine tumor necrosis factor-alpha (TNFα) and the sphingolipid ceramide as mediators of insulin resistance that are associated with increased adiposity. However, the molecular mechanisms whereby these factors inhibit insulin signaling are not completely understood.

In this two-part study on the regulation of insulin action and Akt/PKB activity by obesity, we first investigated the control of Akt/PKB activity in yellow-bellied marmots as they progressively became obese and insulin resistant prior to hibernation. Under fasting conditions, we discovered a seasonal and tissue-specific activation of Akt/PKB and glycogen synthase (GS), suggesting the existence of a novel insulin-independent mechanism regulating Akt/PKB activity during periods of marked anabolism. Secondly, we identified Mixed Lineage

Kinase 3 (MLK3) as a novel intermediate in TNFα and ceramide signaling in cultured fat cells. We show that inhibition of MLK3 completely prevents signaling events stimulated by TNFα, and ceramide stimulation of c-Jun N-terminal Kinase, an important regulator of insulin sensitivity.

The first study provides evidence for a novel evolutionary strategy that hibernators use to regulate anabolic metabolism through insulin resistance. Therefore, identifying the mechanism for non-insulin stimulated Akt/PKB and GS activation in these animals could lead to new treatments for metabolic diseases caused by insulin resistance in humans. Secondly, the identification of MLK3 as a required intermediate in TNFα and ceramide signaling identifies a novel therapeutic target for the treatment of insulin resistance.

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ABBREVIATIONS

AS160:	Akt Substrate of 160 kDa
BSA:	Bovine Serum Albumin
Cer:	ceramide
CS:	calf serum
DAG:	diacylglycerol
DMEM:	Dulbecco's Modified Eagle's Medium
DNA-PK:	DNA Protein Kinase
ECL+:	enhanced chemiluminescence plus
EGF:	Epidermal Growth Factor
ERK:	Extracellular Signal Regulated Kinase
FBS:	Fetal Bovine Serum
FFA:	Free Fatty Acid
GFP:	Green Fluorescent Protein
GluT:	Glucose Transporter
GSK3β:	Glycogen Synthase Kinase 3β
HRP:	Horseradish Peroxidase
IKK:	Inhibitor of Kappa B Kinase
ILK:	Integrin Linked Kinase
IR:	Insulin Receptor
IRS:	Insulin Receptor Substrate
JNK:	c-Jun N-terminal Kinase
LPL:	Lipoprotein Lipase
MAPK:	Mitogen Activated Protein Kinase
MCP-1:	Monocyte Chemoattractant Protein-1
MLK3:	Mixed Lineage Kinase 3
mTOR:	mammalian Target of Rapamycin
OA:	Okadaic Acid
PDK1:	Phosphoinositide Dependent Kinase-1
PH:	Pleckstrin Homology domain

PI3K:	Phosphatidylinositol 3-Kinase
PI(4,5)P2:	Phosphatidylinositol (3,4) bisphosphate
PI(3,4,5)P3:	Phosphatidylinositol (3,4,5) trisphosphate
PKA:	Protein Kinase A
PKB:	Protein Kinase B
PKC:	Protein Kinase C
PP1:	Protein Phosphatase 1
PP2A:	Protein Phosphatase 2A
PPAR:	Peroxisome Proliferator Activated Receptor
PRK2:	Protein Kinase C-Related Kinase 2
RAC:	Related to A and C protein kinase
SMase:	Sphingomyelinase
SPT:	Serine Palmitoyltransferase
TLC:	thin layer chromatography
TNFα:	Tumor Necrosis Factor alpha
WAT:	white adipose tissue

CHAPTER 1

Insulin Signaling and the Control of Glucose Homeostasis

1.1 The Control of Glucose Homeostasis

The human body requires a constant supply of energy to ensure survival. Carbohydrates, proteins, and fats are metabolized to glucose, a simple sugar that serves as the primary energy source of most cells in the body. Despite feeding and fasting intervals, glucose concentrations are strictly maintained in a narrow range between 4-7 mM, providing a steady supply of energy to the brain and other tissues [1]. Nutrient deprivation or saturation causes changes to catabolic and anabolic processes, respectively, to maintain equilibrium glucose concentrations. During times of saturation, e.g. after the consumption of a meal, the body removes glucose from the blood and stores it as glycogen. Glycogen stores are mostly found in muscle and liver tissue, but smaller reserves are also found in the kidneys and intestines. Skeletal muscle accounts for 75% of all glucose uptake and can hold up to twice the amount of glycogen as liver; however, the liver has the highest specific content of glycogen and is the major organ responsible for glucose output. During times of fasting, the body increases blood glucose concentrations via catabolism of stored glycogen from the liver and skeletal muscle, and stimulating *de novo* synthesis in the liver and kidneys. This delicate balance in regulation of glucose concentrations occurs through intricate communication between several organs including the pancreas, liver, skeletal muscle, adipose tissue, and the brain.

The pancreas senses changes in blood glucose concentrations and secretes hormones to regulate glucose storage or production accordingly. Glucagon and insulin are two counter-regulatory hormones produced by the α- and β-cells, respectively. These cells reside in the islets of Langerhans, clusters of

specialized cells within in the pancreas. Insulin is the anabolic hormone that regulates nutrient storage and decreases blood glucose concentrations after feeding, while glucagon is a catabolic hormone that stimulates glucose production during fasting. Pancreatic α– and β-cells are especially sensitive to glucose concentrations. For example, β-cells sense rising glucose levels through GluT2 glucose transporter and glucokinase activity, both of which have a low affinity for glucose and trigger the secretion of insulin upon rising glucose levels by stimulating membrane depolarization. Insulin then circulates to promote glucose uptake and storage in insulin-sensitive tissues such as skeletal muscle and adipose tissue, and represses glucose efflux from the liver. Conversely, decreased glucose concentrations result in the production of glucagon from α-cells, which has an opposite effect on these tissues. Insulin and glucagon therefore work in concert to maintain glucose homeostasis throughout feeding and fasting cycles (Figure 1.1).

1.2 Diabetes Mellitus

The inability to maintain glucose homeostasis often results in adverse health effects and contributes to a reduced life expectancy. Hypoglycemia is a dangerous condition when blood glucose concentrations fall below the lower threshold of 4mM, and the body does not have enough energy to perform its basic functions. In contrast, hyperglycemia results from inefficient glucose clearance, causing elevated blood sugar levels. Hyperglycemia is the major defect of diabetes mellitus and leads to significant organ and blood vessel damage to the eyes, kidneys, nervous system, and heart. High blood glucose levels promote glycation, or the

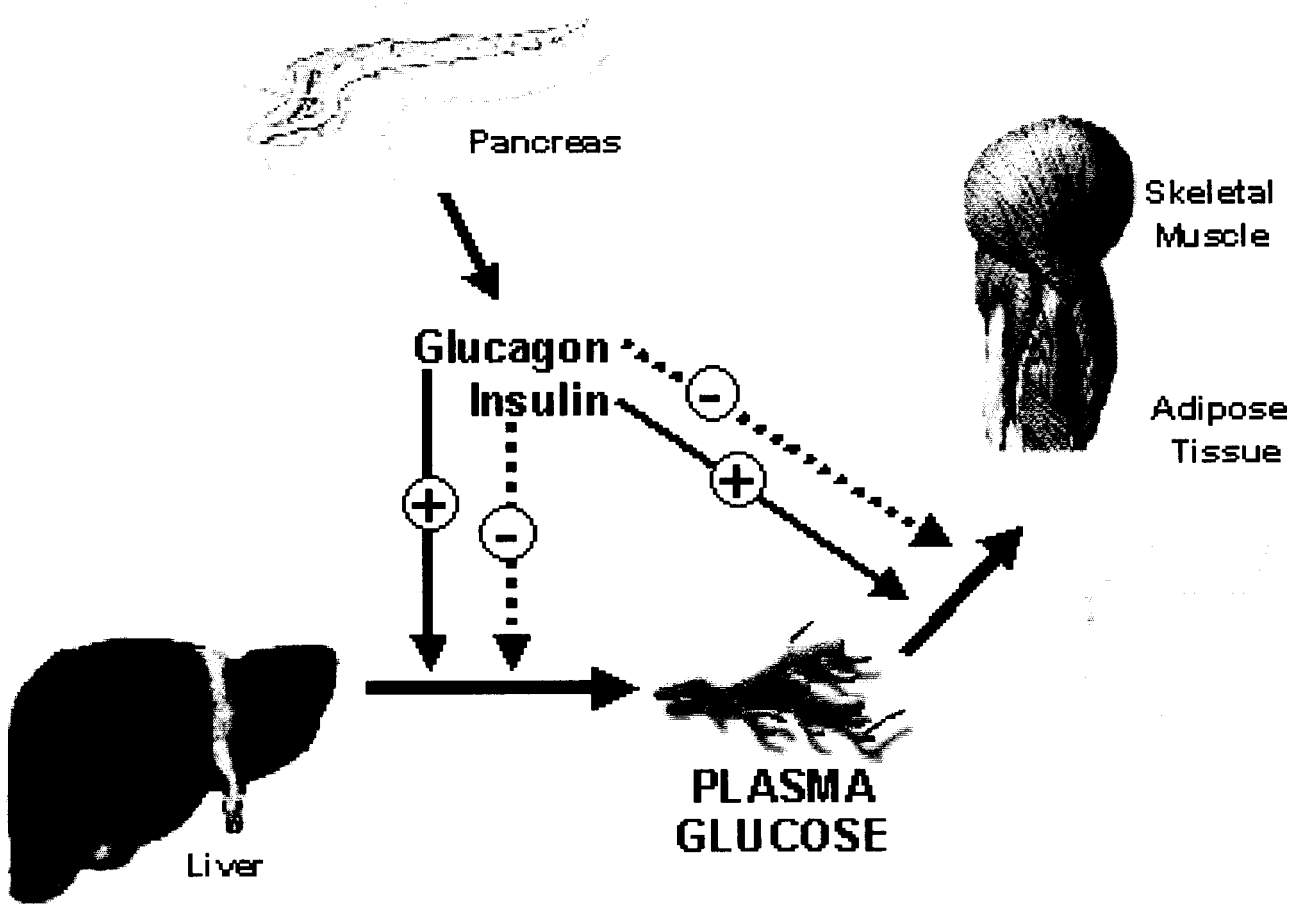

Figure 1.1 The control of glucose homeostasis by insulin and glucagon. Insulin has the net effect of lowering plasma glucose concentrations by decreasing hepatic glucose output and stimulating glucose uptake in adipose and skeletal muscle tissue. Glucagon promotes an increase in glucose production by increasing de novo glucose synthesis in the liver, and stimulating the breakdown of glycogen in the liver, adipose, and skeletal muscle tissues.

attachment of sugar molecules to blood and tissue components. This glycation causes red blood cells to hold onto oxygen and alters the capacity of white blood cells to fight infection. Additionally, excess glucose leads to sorbitol formation, which causes swelling and development of other harmful metabolites.

Diabetes mellitus results from defects in insulin action and/or insulin secretion. Type 1 diabetes, formerly called juvenile-onset or insulin-dependent diabetes, accounts for approximately 5-10% of all cases of diabetes mellitus [2]. Type 1 patients have defective or insufficient insulin secretion due to autoimmune destruction of the insulin producing β-cells. Type 1 is identified in childhood or adolescence, except when specific infections trigger an immune reaction toward the β-cell later in life. Additionally, an artificial type 1 diabetes can result from poisons or tumors that destroy or damage the β-cells sufficiently to reduce insulin production.

Type 2 diabetes mellitus, previously known as maturity-onset or non-insulin-dependent diabetes, accounts for roughly 90-95% of all cases of diabetes mellitus [2]. This type is a progressive disease caused principally by defects in insulin action rather than secretion. An essential feature of type 2 diabetes is ***insulin resistance***; defined as the inability of the body to adequately respond to normal concentrations insulin [3]. Insulin resistance can be compensated in early stages of the disease by increased insulin output from the pancreas. In some individuals, this state of hyperinsulinemia can keep blood glucose levels in check, and they may remain asymptomatic for long periods of time. However, hyperinsulinemia is a precursor to other metabolic abnormalities and the metabolic syndrome [4, 5].

Additionally, when the pancreas fails to produce enough insulin to control blood glucose levels the combination of insulin resistance and progressive β-cell dysfunction leads to hyperglycemia and full-blown type 2 diabetes mellitus.

Type 2 diabetes has a strong genetic and environmental component. Risk factors for insulin resistance include physical inactivity, abdominal obesity, age, and elevated cholesterol levels. Importantly, obesity greatly increases vulnerability to insulin resistance. Greater than 80% of type 2 diabetics are obese or overweight [2]. A possible link between obesity and insulin resistance is the recent identification of the adipocyte as a major source of molecules (hormones, cytokines, lipids, etc) that are antagonistic to insulin signaling (discussed in detail in section 1.8). Less common mechanisms by which type 2 diabetes can develop include long-term steroid use [6, 7], and diseases such as hemochromatosis [8] and polycystic ovary syndrome [9].

A late-developing, hereditary, and usually less severe form of type 1 diabetes mellitus is known as maturity onset diabetes of the youth (MODY), a misnomer. MODY accounts for a small percentage of all diabetes cases, and is principally acquired through inheritance of genetic defects. Currently six types of MODY exist, each of which are slowly progressive (hence the confusion for maturity onset diabetes), but characterized by defects in insulin secretion (type 1). Five types are due to an autosomal dominant gene mutation in a β-cell transcription factor, and one type results from a mutation in glucokinase, part of the glucose sensor in the β-cell.

Diabetes contributes to retinopathy, neuropathy, nephropathy, blood vessel atrophy, and ischemia. These complications make diabetes a leading cause of end-stage renal disease, non-traumatic limb amputations, and blindness, and are major risk factors for stroke and heart disease [10]. Although there is no cure for diabetes, careful blood glucose monitoring and insulin injections significantly help type 1 diabetics maintain normoglycemia. Type 2 diabetics in most early stages do not require insulin, and will benefit from healthy lifestyle changes, exercise, and anti-diabetic drugs, all of which help restore insulin sensitivity and glucose homeostasis.

1.3 Insulin Action and Anabolic Metabolism

As mentioned previously, insulin promotes nutrient uptake and utilization. Insulin plays an integral role in regulating whole-body glucose homeostasis by elaborately coordinating glucose uptake and output by three major organs: skeletal muscle, adipose tissue, and liver. Briefly, the effects of insulin on each tissue are summarized below.

1.3a In Skeletal Muscle

Skeletal muscle is the major site for insulin stimulated glucose storage. Insulin stimulates glucose entry into muscle tissue where it is rapidly converted to glycogen or oxidized for ATP production. Insulin stimulated glucose uptake in muscle is especially important, since ablation of the insulin sensitive glucose transporter (GluT-4) in this tissue results in hyperglycemia, hyperinsulinemia, and

secondary insulin resistance in liver and adipose tissue [11]. Additionally, insulin increases the rate of protein synthesis by activation of the mammalian target of rapamycin (mTOR) and S6-kinase pathway, which promotes mRNA translation, and decreases protein breakdown by inhibiting lysosomes [12].

1.3b In Adipose Tissue

In adipocytes, insulin stimulates glucose uptake through activation of GluT-4 glucose transporters, and promotes glucose storage as glycogen and lipid. Lipids are the primary form of energy storage in adipocytes due to 1) insulin's activation of lipoprotein lipase (LPL), which breaks down triglycerides (TG) to free fatty acids (FFAs) and glycerol, thus allowing their uptake and re-esterification to TG, and 2) increased glucose flux and activation of the lipid synthetic enzymes acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), which form triglyceride from acetyl-CoA and glycerol [13, 14]. These pathways are controlled by the transcription factor sterol regulatory element binding protein (SREBP)-1c, which is regulated by and mediates the effects of insulin [15]. Insulin is also anti-lipolytic in adipocytes by signaling the inactivation of hormone sensitive lipase (HSL) [16], and activation of phosphodiesterase (PDE)-3B. PDE-3B prevents cAMP-activated protein kinase A (PKA) activation [17, 18], consequently preventing PKA-stimulated lipolysis.

Insulin resistance prevents most insulin-stimulated events with the potential exception of anti-lipolysis, which, depending upon the severity of the condition, can still occur at lower insulin concentrations [19]. The observed

increase in lipolysis typical in type 2 diabetics is possibly caused by increased TNFα secretion as described in section 1.8b. Adipose tissue is not particularly important from the standpoint of reducing glucose levels in the blood by glucose uptake, however, GluT-4 ablation in white adipose tissue of mice results in hyperglycemia and leads insulin resistance in liver and muscle tissue [11]. This illustrates that altered metabolism in white fat plays a significant role in whole body insulin sensitivity, and the authors of this study propose that the mechanism is likely through the dysregulation of adipocyte secreted molecules.

1.3c In the Liver

In hepatocytes, insulin does not trigger glucose transport directly, but does increase the rate of glycogen synthesis, which serves as the driving force for liver glucose uptake [20]. Insulin stimulates lipid synthesis in the liver in a similar mechanism to adipocytes-- through FFA absorption and SREBP-1c. Insulin also inhibits hepatic glucose output by shutting down gluconeogenesis (*de novo* generation of glucose from glycerol) and glycogenolysis (breakdown of glycogen to glucose) by inhibiting synthesis of phosphoenolpyruavte carboxykinase and inactivating phosphorylase enzyme, respectively.

1.3d Improper Insulin Action

When the body does not respond to insulin, catabolic processes take over. FFAs and glycerol are released from adipocytes, amino acids are released from muscle, and hepatic glucose output goes unchecked. These molecules can

exacerbate the diabetic phenotype. For example, amino acids and glycerol are substrates for gluconeogenesis by the kidney and liver, respectively. FFA storage in peripheral tissues leads to the formation of lipid metabolites that are antagonistic to insulin action [21-25], and excess FFA metabolism in the liver leads to the formation of ketones [26], which are used for energy by the brain, heart, and skeletal muscle, thereby reducing the utilization of glucose [26]. It was first proposed in 1963, by Randle et al., that changes in FFA metabolism causes altered glucose utilization in the body [27].

1.3e The Randle Hypothesis

Specifically, Randle first postulated that FFA and glucose oxidation are mutually exclusive in skeletal muscle and liver [27]. Randle proposed that an increase in availability of FFAs to liver or muscle tissue would lead to a decrease in glucose oxidation by increasing mitochondrial acetyl-CoA levels that decrease pyruvate dehydrogenase activity and result in an increase in cytosolic citrate, which allosterically inhibits phosphofructokinase. This hypothesis yields an excess of glucose-6-phosphate, preventing glucose phosphorylation by hexokinase, and thus preventing glucose utilization.

The Randle hypothesis posits that FFAs result in defective glucose oxidation; however, later studies show that FFA accumulation interferes directly with insulin signaling and results in reduced glucose transport upstream of Randle's proposed mechanism [28, 29]. Glucose transport defects alone can cause decreased glycogen accumulation and glucose oxidation in muscle,

suggesting the Randle mechanism may have missed the major site of FFA antagonism [30]. In fact, cytosolic citrate also activates Acetyl-CoA Carboxylase (ACC) leading to malonyl-CoA synthesis. Malonyl-CoA inhibits carnitine palmitoyltransferase (CPT)-1, which inhibits fat oxidation. Thus, excess FFAs would block both glucose and FFA oxidation if the Randle hypothesis were correct. A more likely mechanism for the upstream effects may be that *excess* long chain FFAs are abnormally utilized, and stored in muscle. This increase in intramyocellular lipid is directly related to the severity of insulin resistance in non-exercise trained individuals [31], and current data suggest that lipid metabolites such as ceramide or diacylglycerol (DAG) play a role in mediating the antagonistic effects of lipid storage on insulin signaling. Specifically, ceramide directly inhibits Akt/PKB activation [32, 33] and DAG activates Protein Kinase C (PKC) isoforms, which inhibit the insulin receptor, glycogen synthase, and Akt/PKB [34]. These events lead to decreased glucose uptake and metabolism.

1.3f Perspective

Although the precise mechanism is unknown, Randle was the first to correctly hypothesize that FFAs within peripheral tissues contribute to abnormal glucose metabolism, causing insulin resistance. Therefore, it makes sense that a reduction of lipid accumulation in tissues that do not normally store lipids should reinstate insulin action. In fact, the most beneficial treatment for overweight diabetics appears to be a combination of habitual exercise with a healthy low fat diet. Exercise increases the need for energy in the muscle cell, which increases

glycogen consumption and glucose absorption from the blood independent of insulin. Exercise results in increased AMP/ATP and creatine/creatine-phosphate ratios that activate AMP-activated protein kinase (AMPK) [35]. AMPK phosphorylates and inhibits ACC activity, superseding its allosteric activation by citrate. This drops malonyl-CoA levels and increases FFA flux into the mitochondria. In exercise training studies this leads to an increase in fatty acids oxidation, a reduction in ceramide and DAG levels, enhanced glucose uptake, and increased insulin sensitivity [36-38].

1.4 Insulin Signaling

Insulin signaling involves an orderly sequence of protein phosphorylation events that regulate the activity of many pathways involved in cell growth, metabolism, proliferation, and apoptosis. Although insulin regulates many processes involved in proliferation and cell survival etc., I will describe only the pathways involved in anabolic metabolism and glucose homeostasis, as they are important in my study of insulin resistance and type 2 diabetes mellitus.

Insulin circulates systemically until it is bound by specific receptor tyrosine kinases (RTKs) exposed on the cell surface. This ligand binding initiates a signal transduction cascade that diverges at multiple downstream effectors. The insulin receptor (IR), insulin receptor-related receptor (IRR), and insulin-like growth factor receptor-1 (IGFR-1) are all tetrameric RTKs composed of two homodimers, each containing an α– and β–subunit. All of these receptors can heterodimerize with each other and form functional hybrids that maintain various levels of excitability by

insulin. Insulin binding to the exposed α subunit blocks its inhibition of the β subunits' tyrosine kinase activity, leading to activation of the receptor. When stimulated, the receptor will autophosphorylate itself and lead to the activation of at least nine other proteins by direct phosphorylation and docking. Here, the insulin-signaling pathway leading to glucose transport diverges into PI3-kinase dependent and independent paths. Each pathway regulates different cellular processes, yet it remains controversial whether both pathways are required for insulin stimulated glucose uptake.

1.4a PI3-Kinase Dependent Pathway

Directly activated by the insulin RTKs are the Insulin Receptor Substrate (IRS) family of proteins. In mammals, four IRS proteins have been identified, yet only IRS-1 and IRS-2 seem to be important for glucose metabolism based on studies in knockout mice [39-41]. The IR activates IRS proteins through multiple rounds of tyrosine phosphorylation. It is this tyrosine phosphorylation of IRS that recruits and activates the class Ia phosphatidyl-inositol 3 kinase (PI3K).

PI3K is composed of a catalytic (p110) subunit, and a regulatory (p85) subunit that has two SH2 domains. The SH2 domains of the regulatory subunit interact with two tyrosyl-phosphorylated residues on IRS, causing a conformational change resulting in p110 activation. Once activated, p110 phosphorylates phosphatidylinositides in the plasma membrane at the 3' position resulting in phosphatidylinositol 3,4 bisphosphate (PI(4,5)P2) and phosphatidylinositol 3,4,5 trisphosphate (PI(3,4,5)P3). These phosphorylated lipids then serve to regulate

the activity of three main classes of proteins; 1) the cyclic-AMP dependent protein kinase A/ protein kinase G/ protein kinase C (AGC) family of kinases, 2) the Tec kinases, and 3) the guanine nucleotide exchange factors of Rho GTPases.

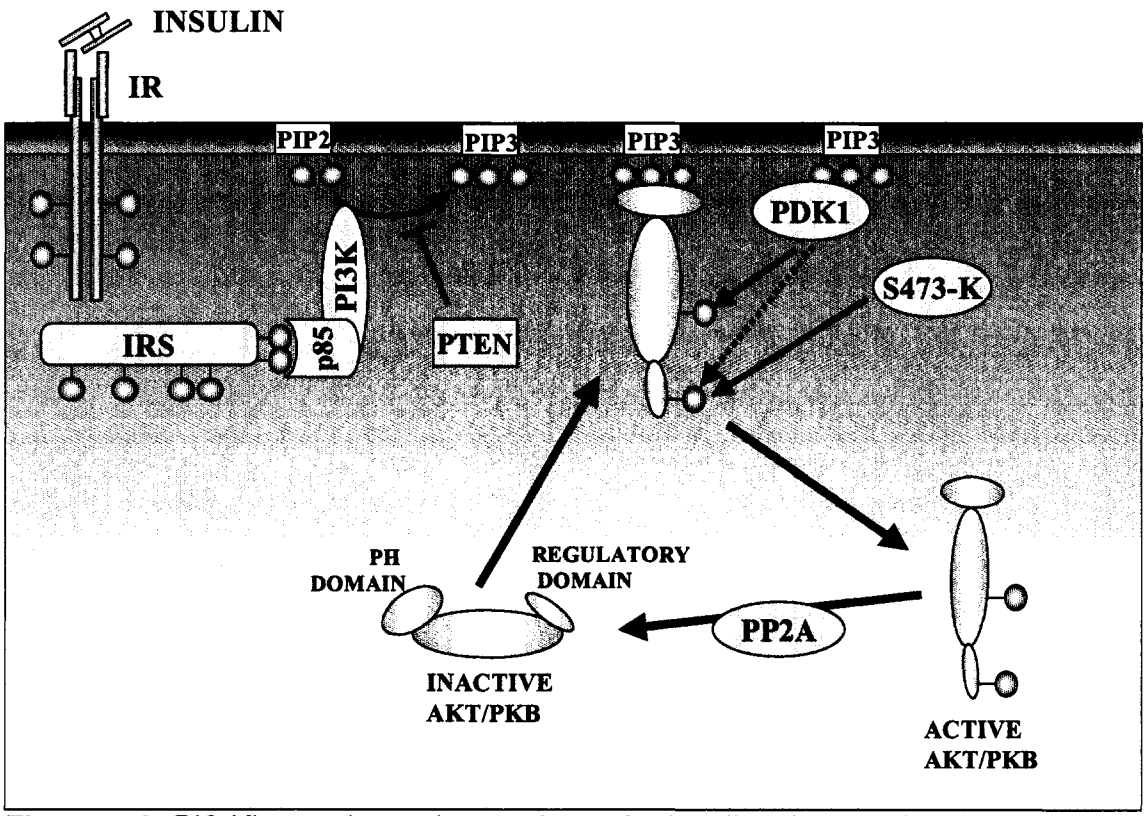

Figure 1.2 PI3-Kinase dependent pathway for insulin stimulated activation of Akt/PKB. Akt/PKB is activated by translocation to the plasma membrane where it is phosphorylated at two sites. Akt/PKB activity is downregulated by protein phosphatase 2A, which removes phosphates from each site. Small grey circles represent phosphorylations.

PI(3,4,5)P3 is the predominant molecule created by insulin stimulated p85 activity, and recruits pleckstrin homology (PH) domain containing proteins to the plama membrane. The AGC serine/threonine kinases Phosphoinositide Dependent Kinase-1 (PDK1) and Akt/Protein Kinase B (Akt/PKB) co-localize to the plasma membrane where PDK1 phosphorylates Akt/PKB on a threonine-308

residue in the catalytic domain [42]. An elusive serine 473-kinase phosphorylates Akt/PKB on a serine residue in its c-terminal regulatory motif, and both phosphorylations are required for full activation of Akt/PKB (Figure 1.2). Putative serine 473-kinase candidates include MAPKAP kinase 2, Akt/PKB, PDK1, ILK, DNA-PK, and PRK2 (reviewed in [43, 44]). Akt/PKB is an obligate intermediate in most of insulin's actions, including GluT-4 externalization and anabolic metabolism [45]. Akt/PKB's role in insulin action is described in detail in section 1.6.

1.4b PI3-Kinase Independent Pathway

The insulin stimulated PI3-kinase independent pathway is necessary for MAP kinase activation, and is therefore responsible for the majority of insulin regulated gene transcription. Recently, this pathway has been hypothesized to be required for glucose transport only in adipocytes, yet its necessity is currently at the heart of much debate. The proposed pathway results in tyrosine phosphorylation of Casitas B-lineage Lymphoma ubiquitin ligase (Cbl) by the insulin receptor. Cbl is often associated with the adapter protein, cbl-associated protein (CAP). After the CAP-Cbl complex is phosphorylated, it moves to lipid rafts via CAP interaction with flotillin. CrkII is another adapter protein that binds phospho-cbl and associates with the guanine nucleotide exchange factor C3G. Once recruited to the membrane, C3G exchanges GTP for GDP on the G-protein TC10. This exchange activates TC10, which has been described to provide a necessary, but not sufficient, role in GluT-4 translocation to the plasma membrane (Figure 1.3). Several groups claim this pathway is necessary for GluT-4 translocation [46-49],

yet others have shown that c-Cbl ablation in mice [50], or the inhibition of this pathway by gene knockdown of CAP, CrkII, and c-Cbl plus Cbl-b, still results in normal glucose transport and GluT-4 trafficking [51, 52] in adipocytes.

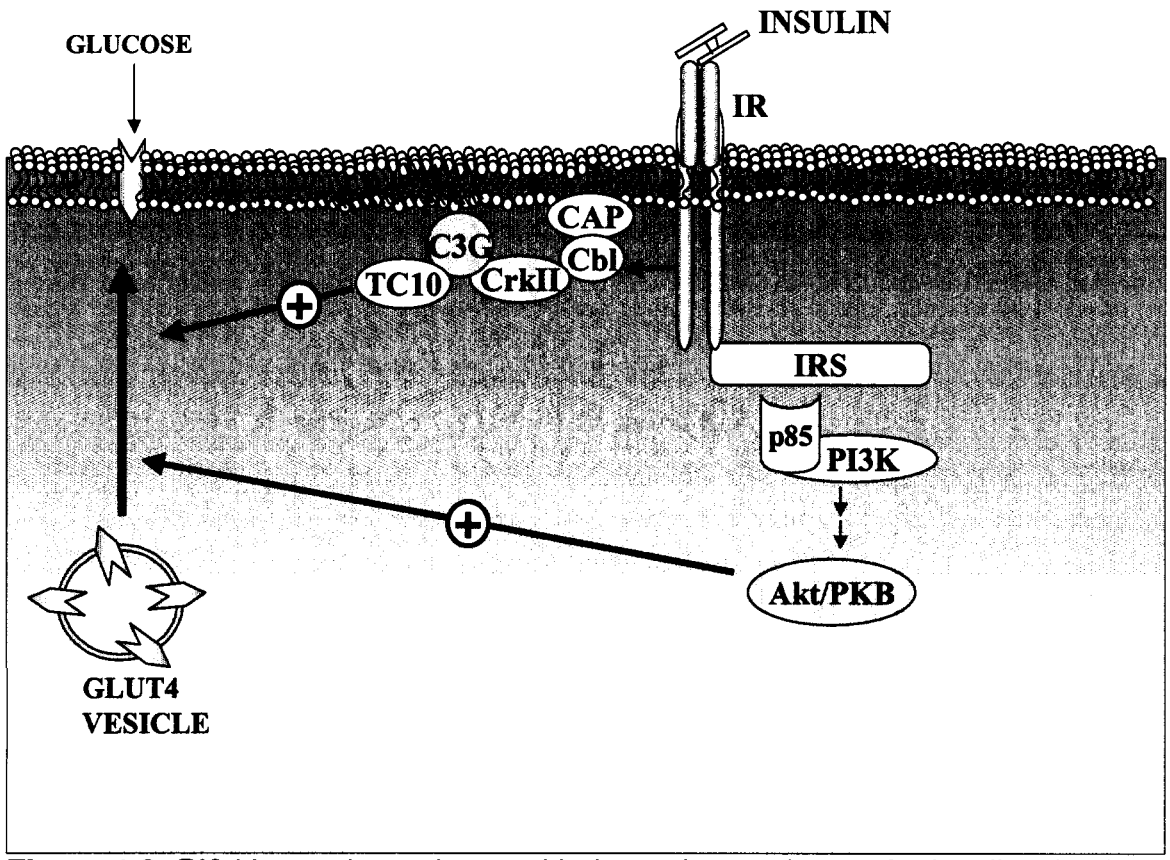

Figure 1.3 PI3-kinase dependent and independent pathways for insulin stimulated glucose uptake.

1.5 Glucose Transporter Trafficking

Mammals have at least 13 different types of GluTs: Class I GluTs (GluTs 1-4) are glucose transporters, Class II GluTs (GluTs 5, 7, 9, and 11) are fructose transporters (a misnomer), and Class III GluTs (GluTs 6, 8, 10, 12 and HMIT-1) are atypical members of this family. Of the Class I GluTs, GluT-1 is found ubiquitously expressed and is responsible for basal glucose uptake in most tissues; GluT-2 is

found in the β-cells and liver and is important for glucose sensing; GluT-3 is found in brain; and GluT-4 is the insulin sensitive glucose transporter found in adipose, cardiac, and skeletal muscle tissue.

GluT-4 transporters cycle between the plasma membrane and intracellular compartments at a basal rate. However, upon stimulation by insulin, GluT-4 containing vesicles rapidly rush to the cell surface resulting in a 5 to 40-fold increase in glucose transport velocity, depending upon cell/fiber type. There are three major steps involved in insulin stimulated glucose transport; translocation and tethering, docking, and vesicle fusion with the membrane (see Figure 1.4). Although it is unknown how insulin-stimulated signaling events converge to drive glucose transport, we do know many of the fundamental steps regulating this process.

Vesicle tethering is facilitated by small Rab GTPases, which attach to transport vesicles and recruit cytosolic tethering molecules that bind these vesicles to docking sites on the plasma membrane. This is followed by the formation of a stable ternary complex between the vesicle soluble N-ethylmaleimide-sensitive factor attachment protein receptor (v-SNARE) vesicle-associated membrane protein-2 (VAMP-2) containing vesicles (which are enriched in GluT-4, and thus also known as GluT-4 vesicles) with the target-SNAREs (t-SNAREs) syntaxin-4 and SNAP-23 at the plasma membrane. This interaction is highly specific and is facilitated by Sec1-like/Munc-18 (SM) accessory proteins, which have both positive and negative roles in SNARE complex formation. The last step of GluT-4 externalization is vesicle fusion with the membrane. Fusion is facilitated by N-

ethylmaleimide sensitive factor (NSF) and SNAP in all SNARE dependent membrane fusions, yet the precise roles of these proteins remain elusive for GluT-4 vesicles (see Figure 1.4 in section 1.6).

1.6 Akt/PKB

Akt/PKB is a serine/threonine protein kinase of the AGC family that plays a major role in insulin and growth factor-induced signal transduction pathways. Akt/PKB was discovered nearly simultaneously by four groups; one group identified it as the transforming oncogene of the Akt8 retrovirus, naming it Akt [53], the other groups named it Protein Kinase B (PKB) [54, 55] and Related to A and C kinase (RAC) [56], based on its homology to protein kinases A and C. There are three known isoforms of Akt/PKB in mammals: Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ. Isoforms are from distinct genes and their expression varies by tissue. Akt1/PKBα and Akt2/PKBβ are primarily found in insulin sensitive tissues and have important roles in growth and metabolism, respectively [57, 58], while Akt3/PKBγ appears to be necessary for the regulation of brain size only [59].

1.6a Structure and Regulation of Akt/PKB by Insulin

Akt/PKB has an N-terminal PH domain, followed by a central kinase domain, and a C-terminal regulatory domain containing a hydrophobic motif. The PH domain is important for Akt/PKB translocation to the plasma membrane upon PI3K-induced generation of 3' phosphoinositides, especially phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3) and PI(3,4)P2. The Akt/PKB catalytic domain is very

similar to other AGC kinases [44] and contains a central threonine residue in the activation loop (T308 in Akt1) that must be phosphorylated for activity. Phosphorylation of this residue is sufficient for partial activation of Akt/PKB, and full activation results when a serine residue (S473 in Akt1) is phosphorylated in the hydrophobic motif of the regulatory domain. Phosphorylation of S473 alone has little effect on Akt/PKB activity.

1.6b PI3K-Independent Activation of Akt/PKB

PI3K activation and PI(3,4,5)P3 generation is the predominant mechanism of Akt/PKB activation, however there are reports that Akt/PKB can be activated independent of PI3K activity. Although counter-intuitive, cyclic-AMP (cAMP) elevation and subsequent activation of protein kinase A (PKA) can lead to threonine-308 phosphorylation, and activation of Akt/PKB [60-62]. Although PKA has been proposed to directly mediate this phosphorylation, the precise mechanism remains unknown. Akt/PKB activation by cAMP elevating agents is resistant to the PI3K inhibitor wortmannin, suggesting that PI3K is not involved. Heat shock, cell stress, and β-adrenergic agonists can also activate Akt/PKB in a wortmannin insensitive manner [63-65]. The potential for this PI3K-independent regulation of Akt/PKB in metabolism is important as we found a similar pattern of Akt/PKB activation in hibernating animals (see Chapter 3).

1.6c Akt/PKB in Carbohydrate Metabolism- Regulation of Glycogen Synthase

Akt/PKB is a required intermediate in insulin's regulation of carbohydrate metabolism [45, 66-70]. Insulin signals Akt/PKB to stimulate an influx of glucose for storage as glycogen. Glycogen synthase (GS) is the enzyme that catalyzes the addition of UDP-glucose to the non-reducing end of existing glycogen strands in liver, skeletal muscle, and adipose tissue. GS can be phosphorylated at many residues by multiple kinases, but glycogen synthase kinase-3 (GSK-3) and PKA are the major upstream kinases. GS activity is inversely proportional to the number of phosphorylations it has [71], nevertheless, it is especially sensitive to allosteric activation by glucose-6-phosphate (caused by an increase in glucose flux), which overrides inhibitory phosphorylations [72]. Akt/PKB promotes glycogen synthesis by inactivation of GSK-3 isoforms through serine phosphorylation (Figure 1.4). This prevents GSK-3 from phosphorylating and inhibiting GS [69]. Additionally, insulin promotes the inhibition of PKA and the activation of protein phosphatase (PP)-1 through Akt/PKB independent mechanisms to further promote the dephosphorylation of GS [73].

1.6d Akt/PKB in Carbohydrate Metabolism- AS160 and GluT-4 Translocation

Akt/PKB is necessary for insulin stimulated glucose uptake; however, the precise mechanism for the involvement of Akt/PKB in this process is still unknown. RNA interference studies and knockout mouse models illustrate the importance for Akt2/PKBβ, but not other isoforms, in GluT-4 trafficking [52, 67]. Another study has

recently shown that a low temperature (19°C) block will prevent Akt/PKB activation and GluT-4 vesicle fusion to the plasma membrane [74]. The low temperature block does not inhibit PI3K activity, and does not prevent movement of GluT-4 vesicles toward the plasma membrane. This suggests there are Akt/PKB-independent processes by which GluT-4 vesicles move toward the cell membrane, and Akt/PKB-dependent vesicle docking or fusion with the membrane.

A noteworthy new Akt/PKB substrate, named Akt Substrate of 160 kilodaltons (kDa) (AS160), was identified in adipocytes as a necessary link between Akt/PKB and GluT-4 trafficking [75-78]. Insulin stimulation leads to up to five direct phosphorylations of AS160 by Akt/PKB. This phosphorylation is shown to be required for GluT-4 translocation and glucose transport. AS160 contains a Rab-GTPase activating protein (Rab-GAP) domain and may be the point of connection between insulin signaling to Akt/PKB and glucose transport. Recall from section 1.5 that Rab GTPase activity is necessary for tethering of GluT-4 vesicles to the plasma membrane. Thus, when active, AS160 keeps Rab-GTPases in the GDP bound, and inactive form. Upon insulin stimulation, Akt/PKB inactivates AS160 allowing Rab-GTPase activity and consequently promotes GluT-4 vesicle tethering, docking, and fusion (Figure 1.4). The major hurdle for confirming this hypothesis has been identifying the Rab-GTPase that AS160 recognizes.

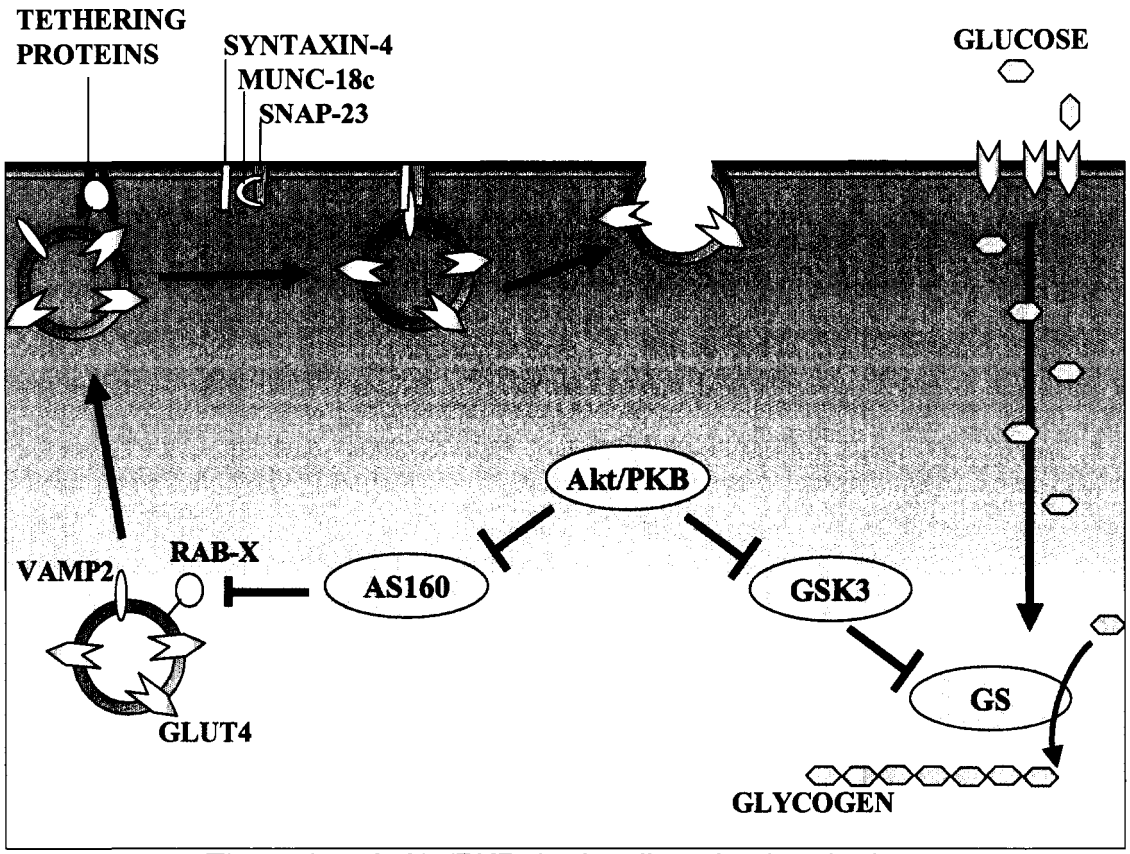

Figure 1.4 The role of Akt/PKB in insulin stimulated glucose transport and glycogen synthesis. Akt/PKB inactivates AS160, thereby preventing its inhibition of Rab proteins necessary for GluT-4 vesicle tethering, and thus docking and fusion into the plasma membrane. Akt/PKB also inactivates GSK3, preventing its repression of GS and promoting glycogen synthesis.

1.6e Akt/PKB Knockout Mice

In an effort to determine the specific role of each Akt/PKB isoform in mammals, Morris Birnbaum and colleagues created mice with a targeted disruption in each Akt/PKB isoform. They showed that Akt2/PKBβ null mice [58] were incapable of regulating blood glucose concentrations. The effects in these mice stemmed largely from defective insulin signaling in liver and skeletal muscle tissue, leading to insulin resistance and diabetes mellitus-like syndrome. More

specifically, insulin sensitivity and glucose uptake was decreased in muscle, and insulin was unable to suppress hepatic glucose output. This led to hyperglycemia and non-compensatory hyperinsulinemia characteristic of type 2 diabetes mellitus. The knockout of Akt2/PKBβ did not affect the expression of Akt1/PKBα or Akt3/PKBγ.

In contrast to Akt2/PKBβ deficient mice, Akt1/PKBα null mice displayed normal glucose homeostasis and insulin stimulated glucose disposal [57]. However, a major defect of these mice was a dramatic decrease in total organism size at birth, which persisted throughout life. Akt3/PKBγ null mice [59], like Akt1/PKBα deficient mice, demonstrate normal glucose disposal and homeostasis, but also had growth defects. Akt1/PKBα deficient mice showed a universal 14% decrease in organ size, while the Akt3/PKBγ deficient mice exhibited a 20% decrease in brain size only. Although the brains are approximately the same size in both types of null mice, the Akt3/PKBγ deficient mice have brains with smaller cells, and exhibit defects in signaling to cascades involved in protein synthesis and metabolism. It seems reasonable that Akt/PKB isoforms do not completely compensate for one another and suggests that they have different functions and cellular substrates

Despite 80% sequence conservation between Akt/PKB isoforms, there appears to be a divergence in isoform specificity and function. Due to the importance of Akt/PKB in overall survival, it is likely that Akt/PKB isoforms do compensate sufficiently to keep these knockout animals alive. Double knockout, Akt1/PKBα and Akt2/PKBβ null animals [79], survive to birth but die shortly

thereafter and show more obvious defects in growth. These double knockouts are unable to activate the transcription factor, peroxisome proliferator activated receptor-gamma (PPARγ), which is necessary for adipogenesis. This fact suggests that isoforms 1 and 2 may compensate for this defect in single knockout animals, although there are different roles for each Akt/PKB isoform. Akt1/PKBα is important for organism growth and proliferation as a whole; Akt3/PKBγ is important for growth and protein synthesis only in the brain; and Akt2/PKBβ is critical for whole body insulin sensitivity and glucose homeostasis.

1.7 Down-Regulation of Insulin Signaling

The negative regulation of insulin signaling is complicated and can be blocked by feedback or cross talk inhibition from multiple signals. The IR, IRS, and PI3-kinase are significant molecules targeted for regulation early in the insulin stimulated signal transduction cascade.

1.7a At the Insulin Receptor

Insulin receptor activity is tightly regulated by protein tyrosine phosphatases (PTP), especially PTP-1B. These tyrosine phosphatases negatively regulate IR activity by removing phosphates from tyrosine residues exposed in the cytosol. Ablation of PTP1B in mice results in increased insulin sensitivity, energy expenditure, resistance to obesity, and improved glucose tolerance [80]. Additionally, suppressor of cytokine signaling (SOCS)-1 and -3

have been shown to bind to the IR and inhibit its capacity to act on substrates [81, 82].

1.7b At the Insulin Receptor Substrates

Tyrosine phosphorylation of IRS proteins by the IR is stimulatory for IRS function and signal propagation. IRS also has between 30 and 50 potential serine/threonine phosphorylation sites that can either serve to promulgate or inhibit IRS activity depending on the location. Activated Akt/PKB is shown to serine phosphorylate IRS in an excitatory positive-feedback manner. However, insulin induced activation of several other kinases including JNK, PKC, mTOR, S6K, IKK, and ERK negatively regulate IRS1 function by phosphorylation at minimally, Ser-307, Ser-612, and Ser-632 (of rat IRS-1). Additional known serine phosphorylation sites that may enhance or inhibit IRS activity include Ser-302, Ser-318, Ser-719, and Ser-1101. Other mechanisms of IRS inhibition include proteosomal degradation by the 26S proteasome and *O*-linked glycosylation (Reviewed in [83]).

1.7c At PI3-Kinase

As PI(3,4,5)P3 is critical for Akt/PKB activation, its levels are tightly regulated by two phosphoinositide phosphatases. Protein phosphatase and tensin homologue deleted on chromosome 10 (PTEN) has PI-3′ phosphatase activity [84-86], and SH2-domain-containing inositol phosphatase (SHIP)-2 has PI-5′ phosphatase activity [87-89]. PTEN reduces PI(3,4,5)P3 to PI(4,5)P2, which leads to repression of Akt/PKB translocation and activity. SHIP-2 produces PI(3,4)P2,

through which the effect on Akt/PKB repression is less established as Akt/PKB has binding capacity to this product [52, 90].

1.8 The Role of Obesity in the Antagonism of Insulin Signaling

The disparity between nutrient intake and energy expenditure can have detrimental effects on one's health. The excessive intake and storage of nutrients causes weight gain and obesity. This excessive adiposity is a serious risk factor for the development of insulin resistance syndrome (also called metabolic syndrome X) which includes atherosclerosis, hypertension, impaired glucose tolerance, dyslipidemia, and hypercoagulability [91].

The United States is currently in the midst of twin epidemics of obesity and diabetes. The correlation between obesity and type 2 diabetes mellitus is well established; therefore it is not surprising that the diabetes epidemic is running concurrent with the obesity epidemic. Obesity is defined as an excessively high amount of body fat or adipose tissue in relation to lean body mass [92]. A standard calculation for determining obesity is referred to as the body mass index (BMI). BMI is expressed as weight to height^2 ratio (kg/m^2), where being overweight equates to a BMI of 25-29.99, and obesity equals a BMI of 30 or greater.

Within the past ten years, white adipose tissue (WAT), particularly visceral or abdominal fat, which has a higher metabolic capacity than subcutaneous fat [93], has proven to be an important organ for communicating the nutritional status of the body. Since the cloning of the obesity gene (leptin) in mice in 1995,

numerous new signaling molecules have been identified in adipocytes. WAT plays a very important role in relaying information about the adipose reserves to the brain and other tissues and plays a central role in regulating whole body fuel homeostasis. WAT controls fuel utilization in peripheral tissues such as liver and muscle through the secretion of numerous biologically active protein hormones (adipokines), inflammatory cytokines (e.g. resistin, adiponectin, IL-6, leptin, PAI-1, and tumor necrosis factor-alpha (TNFα)), and nutritional metabolites such as free fatty acids, glycerol, and triglycerides (Reviewed in [94]). Secretagogues such as leptin and adiponectin work together to increase insulin sensitivity in liver and skeletal muscle; however, adiponectin secretion decreases with increasing adipocyte size [95]. Of the major negative regulators of insulin action secreted from adipocytes, TNFα and FFAs are secreted in direct proportion to fat cell size [96], and antagonize insulin signaling in not only WAT, but also peripheral tissues as well. Thus, obesity and subsequent increases in fat cell size result in alterations in adipocyte-derived secretagogues, leading to altered insulin sensitivity and energy expenditure.

1.8a Obesity and Inflammation

Obesity is commonly associated with a limited low-grade inflammation within adipose tissue [97]. Total fat mass, and adipocyte size and location regulate insulin sensitivity and promote inflammation. Although the mechanisms that trigger the inflammatory response are not well understood, this local inflammation appears to be a contributing factor in insulin resistance. As obesity

progresses and adipocytes enlarge, there is an increase in macrophage infiltration into WAT [98, 99]. The progressive infiltration of immune cells is hypothesized to be due to increased secretion of pro-inflammatory molecules such as leptin, TNFα, angiotensin II, and interleukin-6 from adipocytes. In particular, TNFα stimulates monocyte chemoattractant protein (MCP)–1 release from preadipocytes and reticuloendothelial cells, leading to macrophage recruitment and activation. Activated macrophages then exacerbate the inflammatory response by secreting more TNFα, triggering signal transduction cascades that lead to fat cell lipolysis, and the inhibition of insulin signaling in multiple tissues.

1.8b TNF-alpha

TNFα is a pro-inflammatory molecule expressed on the exofacial surface of white adipose and macrophage cells as a 51kDa homotrimer. Upon cleavage by TNFα Converting Enzyme (TACE), the truncated 17kDa TNFα becomes soluble and circulates systemically where it acts upon WAT and other peripheral tissues that express one or more of the two classes of TNF receptors (TNFR). Although soluble TNFα acts primarily on TNFR1, it has limited ability to activate TNFR2 [100]. Adipose cells express both classes of TNF receptors [101] and are especially sensitive to the autocrine/paracrine effects of TNFα signaling that retard insulin action. Due to the correlation between the increased presence of TNFα with obesity and its negative effects on insulin action, TNFα is considered to be a contributing factor in obesity induced insulin resistance [102-106].

Studies supporting a role for TNFα in the development of insulin resistance *in vivo* include: 1) the administration of TNFα to otherwise healthy rats will generate insulin resistance [107]; 2) genetically obese insulin resistant animals [108] and centrally obese insulin resistant humans demonstrate elevated levels of TNFα [109, 110]; and 3) TNFα or TNFR knockout mice are protected from the obesity-related decline of insulin signaling in muscle and fat tissues and exhibit improved insulin sensitivity with lower levels of circulating free fatty acids, even when made obese [111-113].

Obese individuals have plentiful energy reserves, thus it seems natural that the body would signal to promote a reduction in the excess energy storage with increased adiposity. One such signal is TNFα, which is secreted from fat tissue and activated macrophages. TNFα reduces nutrient storage by stimulating their release and negatively regulating pathways that promote their accumulation (i.e. insulin signaling). Insulin sensitive tissues are especially sensitive to the effects of TNFα.

The precise mechanism through which TNFα induces insulin resistance is unknown, but as obesity progresses there is a direct increase in TNFα production [114]. This decreases glucose uptake by downregulating GluT-4 transcription [115], increases lipolysis by stimulating HSL expression [116], inhibits LPL activity [117], and decreases phosphorylation of IR and IRS proteins. TNFα attenuates insulin signaling by activating mitogen activated protein (MAP) kinase and inhibitor of kappa B kinase (IKK) cascades which 1) directly inactivate insulin signaling intermediates by phosphorylation and 2) directly activate transcription factors (e.g.

c-Jun and NFκB) that alter the expression of insulin signaling intermediates. TNFα stimulates ceramide accumulation via *de novo* synthesis, and the activation of sphingomyelinases (SMases) [118], which hydrolyze sphingomyelin to ceramide.

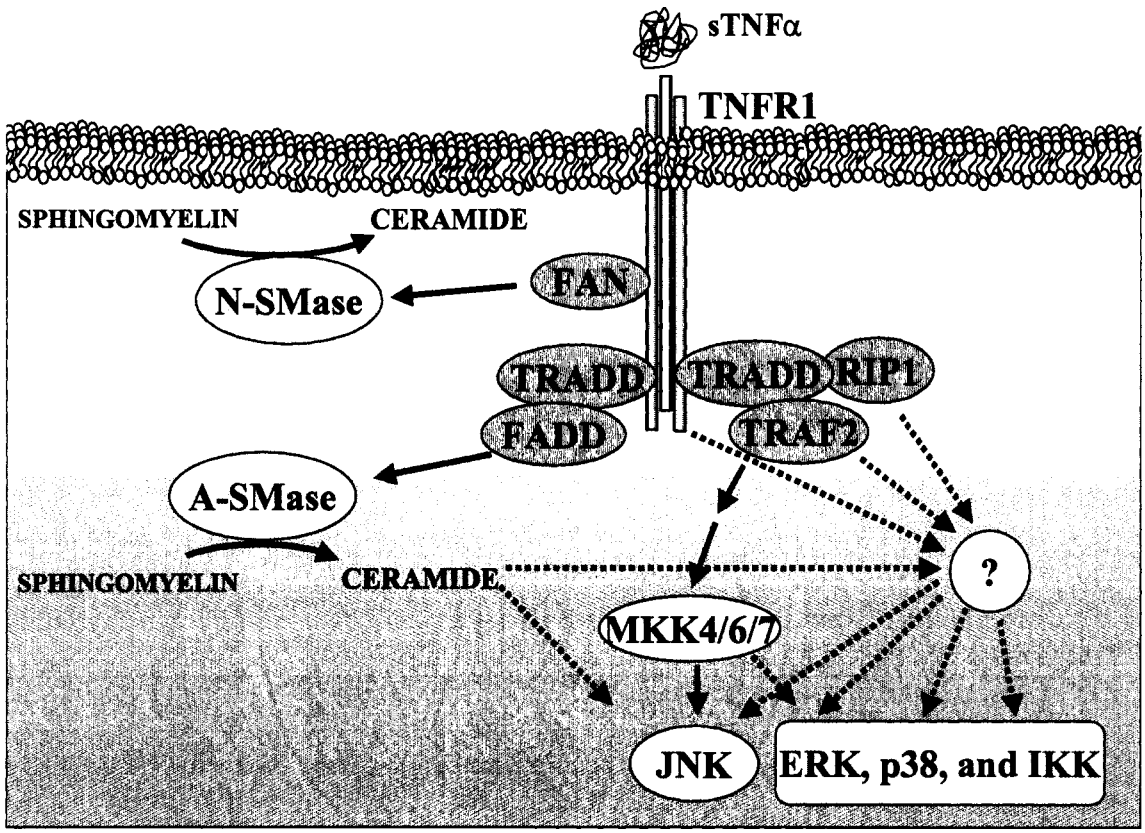

Figure 1.5 Soluble TNFα signaling through TNFR1. Dotted lines indicate pathways that are unknown or poorly defined.

Despite much attention to the downstream effects of TNFα, the signaling pathways initiated by TNFα are cell type specific and not fully characterized. Specifically, TNFα activation of MAPK pathways is poorly defined [100]. TNFα initiates TNFR trimerization, which activates a complex signaling network to propagate the TNFα signal. The TNFα pathway involves a series of

phosphorylation and dephosphorylation events, activation of lipases (ex. SMases), activation of MAP kinases, IKK, and transcription factors, and leads to production of reactive oxygen species. Some of the known and hypothesized pathways for TNFα signaling are shown in Figure 1.5.

1.8c Ceramide

Ceramide is a lipid second messenger antagonist of Akt/PKB activation that inhibits many of the anabolic and pro-survival events stimulated by insulin [25, 32, 33, 119-121]. The sphingolipid ceramide is synthesized *de novo* in the endoplasmic reticulum from the precursor molecules palmitate, serine, and another FFA. Additionally, ceramide is generated by SMases named for their location in the cell. Acid-SMases are localized in lysosomal compartments and neutral-SMases are localized in the plasma membrane and mitochondria. TNFα induces ceramide production by activation of the p55 TNFR-1. The time course for the emergence of ceramide depends on the cell type and concentrations of TNFα. Commonly, there are early ceramide accumulation events (triggered by SMase activation) and a delayed but sustained increase in ceramide due to *de novo* synthesis. The mechanism for induction of *de novo* ceramide synthesis is unresolved, but it has been shown that *de novo* ceramide synthesis inhibitors prevent TNFα from stimulating the long-term ceramide accumulation [122, 123].

Alternatively, the increased availability of long chain FFAs under the conditions of obesity and/or TNFα stimulated lipolysis for example, leads to an increase in FFA uptake in peripheral tissues. If these FFAs are not oxidized they

are stored or converted to lipid metabolites such as diacylglycerol (DAG) and ceramide, both of which have been shown to be antagonistic to insulin signaling [25, 34].

Studies by Chavez et al. [25] show that incubation of cultured myotubes with saturated (but not unsaturated) FFAs increases ceramide levels, but not DAG accumulation, and leads to the antagonism of insulin action on these cells. Inhibitors of *de novo* ceramide synthesis prevented the inhibition of insulin signaling by saturated FFAs, providing evidence that ceramide is the mediator of insulin resistance by increased availability of saturated FFAs.

Short chain ceramide species, C2- or C6-ceramides, are cell membrane permeable, and used in cell culture studies to determine the effects of ceramide on insulin signaling [32, 124]. Studies by Stratford et al. [33] revealed that C2-ceramide blocks Akt/PKB activation by insulin via two distinct mechanisms in cultured 3T3-L1 preadipocytes. First, ceramide blocks Akt/PKB translocation to the plasma membrane; and second, ceramide triggers the dephosphorylation of Akt/PKB by activation of PP-2A. In these studies, ceramide had no role upstream of Akt/PKB, as insulin stimulated PI3-kinase activity and PI(3,4,5)P3 levels were unchanged, and PDK-1 translocation and activity were intact in the presence of ceramide. Moreover, the inhibition of either ceramide mechanism individually was not sufficient to prevent its antagonism of Akt/PKB.

Insulin sensitivity appears to be inversely proportional to the amount of ceramide at hand. A moderate (about 2-fold) elevation in skeletal muscle ceramide levels has been observed in obese, insulin resistant humans [125] and

animals [126, 127]. Furthermore, exercise training reduces ceramide content in skeletal muscle [37, 38] and improves insulin sensitivity and glucose uptake. This suggests that ceramide accumulation is a major contributor to FFA induced insulin resistance.

1.9 Summary

Obesity induced insulin resistance and type 2 diabetes mellitus are associated with defects in a complicated array of cellular signaling pathways. Together, these defects result in dysfunctional regulation of glucose homeostasis and communication between tissues. Thus far, it is clear that no single molecule or pathway is fully responsible for all the defects in insulin sensitivity, and it is still unclear how exactly obesity is linked to insulin resistance. There are many details of the mechanisms involved that need to be solved before we can fully understand the pathophysiology of this chronic and progressive disease. Ultimately, the goal of my research has been to investigate the mechanisms of obesity induced insulin resistance.

The first study performed herein utilized yellow-bellied marmots as a model for obesity induced insulin resistance. Yellow-bellied marmots are hibernators that predictably become obese and insulin resistant over the summer in preparation for winter hibernation. We analyzed protein levels and activity of central regulators of anabolic metabolism, Akt/PKB and GS, during the months accompanying maximal weight gain and entry into hibernation. Our studies showed that there was not only a seasonal, but also a tissue specific (muscle vs. adipose tissue) regulation of

Akt/PKB and GS activity by a novel PI3-kinase independent mechanism. This study provides evidence of alternative mechanisms for the regulation of Akt/PKB during periods of obesity, hyperinsulinemia, and insulin resistance.

The second study investigated the role of a ceramide activated protein kinase, mixed lineage kinase (MLK)-3, in TNFα signaling in 3T3-L1 adipocytes. This study was conducted in adipocytes, as they are especially sensitive to the autocrine/paracrine effects of TNFα signaling that retard insulin action. We have identified MLK3 as potential intermediate required for TNFα mediated signal transduction in fat cells. We show that over-expression of MLK3 mimics TNFα signaling, and that inhibiting MLK3 prevents TNFα signaling. These data suggest that MLK3 could be a potential drug target for protection from TNFα mediated antagonism of insulin signaling.

CHAPTER 2

Methods for Assaying Akt/PKB Enzymatic Activity

This chapter describes work published in Methods in Molecular Medicine

(Hoehn and Summers, 2003)

Hoehn and Summers, Methods in Molecular Medicine 2003; 83:137-44.

2.1 Introduction

Akt/Protein Kinase B (PKB) is a serine/threonine kinase that mediates many of the anabolic actions of insulin [24], as well as the growth-promoting and/or anti-apoptotic effects of other growth factors, cytokines, or transforming oncogenes [44]. These agonists all stimulate Akt/PKB by promoting its phosphorylation on two regulatory residues (e.g. S473 and T308 for the Akt1 isoform), an event dependent upon the prior activation of a signaling pathway initiated by the lipid kinase phosphatidylinositol 3-kinase. Once Akt/PKB is activated, it phosphorylates numerous different substrates, including transcription factors (e.g. FKHR1, others), anti-apoptotic enzymes (e.g. Bad, caspase 9), and metabolic enzymes (e.g. glycogen synthase kinase 3β), to regulate this diverse array of biological processes. This chapter describes a method for measuring the catalytic activity of cytoplasmic Akt/PKB isolated from cell or tissue extracts by quantifying its ability to catalyze phosphate incorporation into an exogenous substrate (outlined in Figure 2.1). The text below illustrates techniques for immunoprecipitating Akt/PKB, choosing an appropriate substrate for the reaction, and optimizing the assay conditions.

Although recombinant Akt/PKB can be prepared using baculovirus [128], most researchers are interested in measuring the activity of Akt/PKB in tissues or cultured cells. Akt/PKB is typically isolated from these tissues by immunoprecipitation, and a variety of antibodies raised against the Akt/PKB regulatory domain can be used to extract Akt/PKB from cell and tissue lysates without affecting its catalytic activity. Alternatively, many researchers choose to

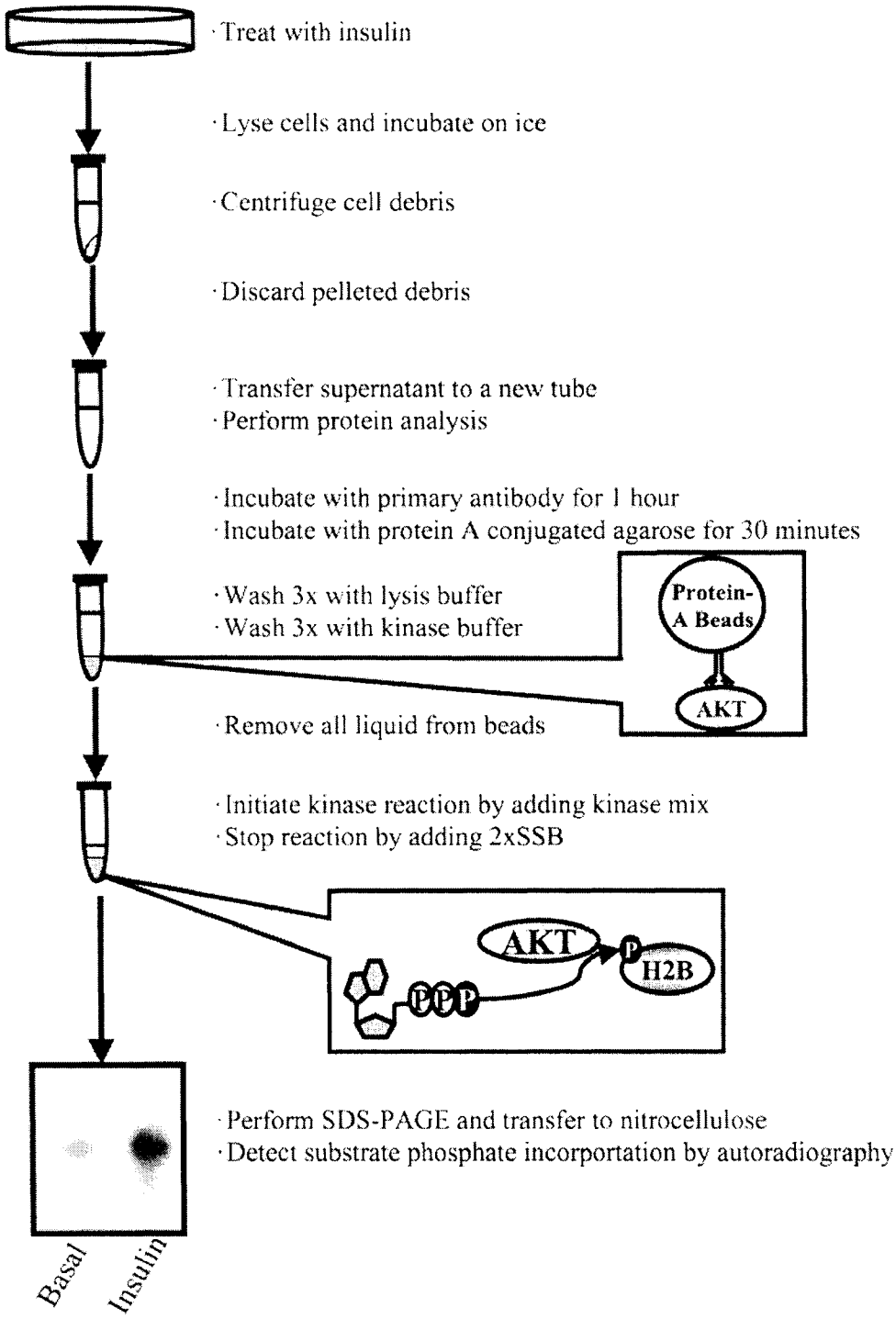

Figure 2.1 Schematic diagram depicting the major steps in the Akt/PKB kinase assay.

evaluate the activity of wild type or mutant forms of Akt/PKB that have been overexpressed in different cell lines. For example, Akt/PKB can be epitope tagged on either its amino or carboxyl terminus, and can then be immunoprecipitated with antibodies recognizing the epitope [129, 130].

Akt/PKB phosphorylates a wide variety of cellular proteins *in vivo*, and can phosphorylate histone H2B and several engineered peptides *in vitro.* The choice of substrate for the Akt/PKB kinase reactions depends largely upon the method to be used for detecting phosphate incorporation. One can monitor *in vitro* Akt kinase activity by quantifying the amount of radioactive phosphate transferred from [γ^{32}P] ATP into the substrate protein, or alternatively, by immuno-detecting newly phosphorylated Akt substrates with phospho-specific antibodies. The radioactive method will work with multiple different substrates. Histone-2B (H2B) was one of the first proteins used to measure Akt/PKB's catalytic activity, and is used frequently because it is commercially available and relatively inexpensive. However, H2B is not exclusively phosphorylated by Akt/PKB, which could prove problematic should contaminants, such as cAMP or cGMP activated protein kinases, co-precipitate alongside Akt/PKB. As shown below, more specific substrates, such as glycogen synthase kinase-3β (GSK3β), work equally well in the radioactive assay (Figure 2.2). Alternatively, if using GSK3β or Bad as a substrate, one can detect the phosphorylated product using commercially available antibodies recognizing the phosphorylated form. Immunodetection with a phospho-specific antibody precludes the necessity of using radioactive ATP for

detecting phosphate incorporation. Regardless of which substrate is used, sufficient quantities must be added to keep the reaction in the linear range of enzyme activity. Under the conditions described below, most of the substrate can be phosphorylated within 10 minutes of the addition of ATP.

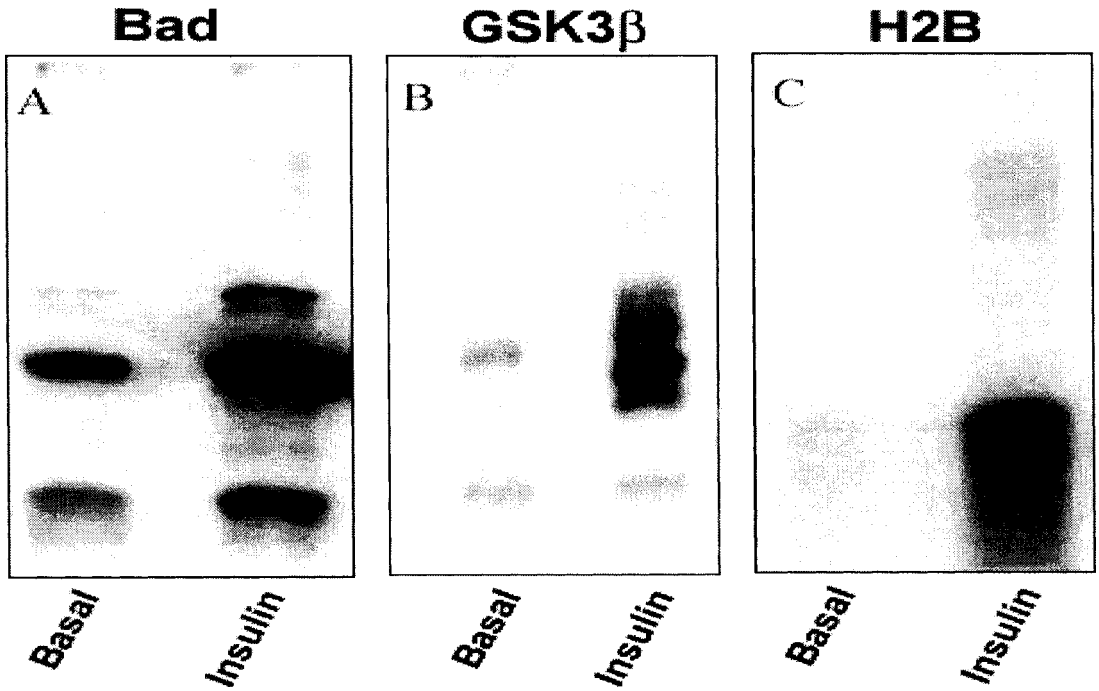

Figure 2.2 Example kinase assay done using three different Akt/PKB substrates. Assays were performed as described in the text using 3T3-L1 preadipocytes treated with or without insulin as indicated. (A) Assay was performed using Bad as a substrate, and detection was performed using the phospho-Bad antibody. The top band is the full length recombinant 31kDa protein, and the lower band is degradation product. (B) Recombinant GSK3β was used as a substrate, and the amount of phosphate transferred into GSK3β from [γ^{32}P]-ATP was determined by autoradiogram. (C) Histone H2B was used as a substrate, and the amount of phosphate transferred into H2B from [γ^{32}P]ATP was determined by autoradiogram.

Wherever possible, the *in vitro* assay described herein should be complemented with studies assessing Akt/PKB regulation and/or activity *in vivo.*

Specifically, the phosphorylation state of Akt/PKB can be assessed by immuno-blotting cell lysates with phospho-specific antibodies against either regulatory residue (antibodies available from New England Biolabs, Beverly, MA; Biosource International, Camarillo, CA; and others). Alternatively, the activity of Akt/PKB towards particular substrates can be assessed by immunoblotting with phospho-specific antibodies that recognize various Akt substrates (e.g. phospho-BAD, phospho-GSK3β, and phospho-Akt-substrate panel antibodies from New England Biolabs).

2.2 Materials

1. Equipment: Equipment required for the assay includes a Labquake tube rotator from VWR Scientific (West Chester, PA), a gel electrophoresis unit from Amersham Pharmacia (San Francisco, CA), and a Storm 860 phosphorimager from Molecular Dynamics, Inc. (Sunnyvale, CA).
2. Cell Culture Reagents: The protocol below describes the measurement of Akt/PKB kinase activity in cultured 3T3-L1 adipocytes, a model cell line used for studying insulin-stimulated signal transduction and metabolism. The following reagents are used for the maintenance and differentiation of the 3T3-L1 line. Dubelcco's modified Eagle's-H21 medium, Leibovitz L-15 medium, fetal bovine serum, and calf serum can be obtained from Invitrogen (Carlsbad, CA). Dexamethasone can be prepared as a 4mg/ml stock solution in ethanol; this is stable for several months if stored at -20°C. Isobutylmethylxanthine can be prepared as a 5mM stock solution in

isotonic saline; this will require boiling to dissolve, but can be filter sterilized and then stored at -20°C until use.

3. Insulin Solution: A stock insulin solution can be prepared by diluting enough insulin in 0.005N HCl to make a ~300 μM stock solution. The final insulin concentration should be determined by UV absorbance. This solution may be stored for several months at 4°C.
4. Lysis Buffer: The lysis buffer (pH 7.2) used herein is comprised of the following reagents: 20mM Tris-HCl, 150mM sodium chloride, 10% glycerol, 1% Igepal, 10mM Sodium fluoride, 30mM sodium pyrophosphate, and 1mM EDTA. This solution can be stored for 6 months when filter sterilized and kept at 4°C. The following protease and phosphatase inhibitors must be added fresh: 1mM Na_3VO_4, 1mM PMSF, 10μg/mL leupeptin, and 2μg/mL aprotinin (see note 1).
5. Protein assays: Protein concentrations should be determined using a method that is unaffected by detergents found in the lysis buffer. We use the bicinchoninic acid (BCA) protein assay kit from Pierce Chemical Company (Rockford, IL).
6. Antibodies: Isoform specific anti-Akt antibodies generated against a regulatory domain near the carboxyl terminus are available from Upstate Biotechnology, Inc. (Lake Placid, NY). New England Biolabs also sells antibodies recognizing different Akt/PKB isoforms, as well as phospho-specific antibodies recognizing the phosphorylated form of the enzyme [129, 131]. Agarose conjugated protein-A and/or secondary antibodies

used to extract the antibody bound Akt from the lysate are available from Santa Cruz Biotechnology (Santa Cruz, CA) (see note 2).

7. Substrate: Histone H2B can be purchased from Roche Applied Science (Indianapolis, IN) and Bad can be purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). GSK3β can be produced as a recombinant fusion protein with glutathione S-transferase using standard techniques. Substrates can be stored in 50% glycerol at –20°C. If using histone H2B, include 25 μg/reaction. For GST-GSK3β or Bad use 15 μg or 3 ug per reaction, respectively.
8. Kinase Reaction Solutions: Prepare a 20x *Kinase Buffer* (pH 7.2) containing 400 mM Hepes and 100 mM $MgCl_2$. This solution can be stored for several months at 20°C. Prepare the following *Kinase Mix* the day of the experiment: 1 mM DTT, 10 μM MgATP, 200 μM EGTA, 2 μg protein kinase inhibitor, and the appropriate amount of substrate. Ten μl of this kinase mix containing 5 μCi of 6000 Ci/mMol [γ^{32}P] ATP is added to each reaction (see note 3). The DTT and EGTA can be kept as 100X stock solutions, and the MgATP can be frozen as a 100X stock. All reagents can be obtained from Sigma (St. Louis, MO).
9. Sample Solubilization Buffer (SSB): Prepare a 2x stock of SSB (pH 6.8) comprised of the following: 125mM Tris-HCl, 5.5% SDS, 20% glycerol, 10% β- mercaptoethanol, and 0.006% bromophenol blue.

2.3 Methods

The following protocol describes the measurement of Akt/PKB activity in insulin-stimulated 3T3-L1 adipocytes (Figure 2.1), but is applicable to studies evaluating Akt/PKB activity in numerous cells or tissues treated with a variety of different cellular agonists.

2.3a Cell Treatment

1. Split 3T3-L1 preadipocytes into 6-cm dishes in Dubelcco's modified Eagle's-H21 medium supplemented with 10% calf serum. Two days post confluence, differentiate the cells into adipocytes by replacing the media with Dubelcco's modified Eagle's-H21 medium supplemented with 10% fetal bovine serum, 1 μg/ml dexamethasone, and 112 μg/ml isobutylmethylxanthine. After 3 days, replace the media with Dubelcco's modified Eagle's-H21 medium supplemented with 10% fetal bovine serum. Thereafter, replace the media at least once/week. The cells can be used for the Akt/PKB assays up to 35 days post-differentiation.
2. Use a 6-cm plate of differentiated 3T3-L1 adipocytes for each treatment condition. Serum deprive the cells by washing them in PBS and then incubating them for two hours at 37°C in Leibovitz L-15 buffer supplemented with 0.2% BSA.
3. Stimulate selected dishes of cells with insulin (100 nM final concentration) for 10 minutes. Quickly wash the cells twice with ice cold phosphate buffered saline (PBS).

2.3b Cell Lysis

1. Lyse the cells by adding 1 ml of ice-cold lysis buffer containing protease inhibitors.
2. Scrape the cells off the plate, transfer them to a microcentrifuge tube, and incubate them on ice for 10 minutes.
3. Centrifuge the lysate for 10 minutes at 4°C at top speed in a microcentrifuge (20,800xg). Transfer the supernatant to new microcentrifuge tubes.
4. Perform a protein assay while keeping all samples on ice (see note 4).

2.3c Immunoprecipitation

1. Perform immunoprecipitations on lysates normalized to contain equal quantities of cellular protein. Aliquot lysate containing ~300 μg protein into a labeled microcentiruge tube, and then dilute with lysis buffer containing protease inhibitors to bring the lysate up to a total volume of 500 μl.
2. Start the immunoprecipitation by adding the amount of antibody suggested by the manufacturer for immunoprecipitation. Rotate the tubes at 4°C for at least one hour.
3. For each treatment condition, aliquot 50μL of a 1:1 slurry of protein-A conjugated to agarose beads (or secondary antibody conjugated to agarose beads) into a newly labeled microcentrifuge tube.
4. Wash the beads three times with 1mL of ice-cold lysis buffer containing protease inhibitors. Pellet the beads by centrifuging in a bench top picofuge

after the addition of each wash. After the last wash, remove most of the fluid, leaving a very small volume above the beads.

5. Add the cell lysates containing the primary antibody to each of the new tubes containing the agarose-conjugated protein A. Rotate this mixture at 4°C for at least 30 minutes.
6. During the incubation with the protein A, thaw out the 20x kinase buffer. Prepare at least 3mL of 1x kinase buffer and 25μL of 1.5x kinase buffer for each reaction being performed.
7. After the 30-minute incubation is complete, pellet the beads by centrifuging at 6,000xg for 30 seconds. Remove the supernatant.
8. Wash the beads three times with ice cold lysis buffer containing protease inhibitors, then three times with 1x kinase buffer. Use ~1 ml. for each wash. Completely remove all liquid after the last wash (see note 5).
9. Add 20 μL of 1.5x kinase buffer to the beads.

2.3d Kinase Reaction

1. Start the reaction by adding 10 μl of kinase mix containing the 5μCi [γ^{32}P] ATP to each reaction tube (see note 3). This effectively dilutes the 1.5x kinase buffer to 1x. Suspend the beads by tapping the bottom of the tube.
2. Allow the reaction to proceed for 5 minutes at 30°C then stop the reaction by adding 30μL of 2x SSB (see note 6).
3. Incubate the samples at 95°C for 2 minutes, then spin at maximum speed (~20,800xg) in a microcentrifuge.

2.3e Electrophoresis

1. Load half of the reaction on a 12.5% SDS-PAGE gel (see note 7). Run the gel until the dye front is at the bottom of the gel. If you are doing a radioactive assay, do not let the dye front run off of the bottom of the gel; this contains the free [^{32}P] ATP. Cut the dye front off of the gel using a razor blade.
2. Transfer the proteins in the gel to nitrocellulose (see note 8).
3. If doing the radioactive assay, quantify ^{32}P incorporation into the substrate by autoradiography using a phosphorimager. If doing the non-radioactive assay, detect phosphate incorporation with the phospho-specific antibody by immunoblotting. We use the enhanced chemiluminescence kit from Amersham Biosciences (Piscataway, NJ) for visualization of the secondary antibody.

2.4 Notes

1. The protease inhibitors aprotinin, leupeptin, and PMSF can be replaced with a commercial protease inhibitor cocktail.
2. For non-radioactive detection of phosphate incorporation into Bad, we use a phospho-specific antibody against the S136 phosphorylation site from Upstate Biotechnologies, Inc (Lake Placid, NY). For non-radioactive detection of phosphate incorporation into GSK3β, we use a phospho-specific antibody against the serine-9 phosphorylation site from Cell Signaling (Beverly, MA).

3. If you have chosen to detect substrate phosphorylation by the non-radioactive method (i.e. using a phospho-specific antibody), you do not need to add the [^{32}P] labeled ATP.
4. If necessary, one can stop at this point and store the extracts at –20°C without losing much kinase activity. If this is to be done, snap-freeze the cell lysates in liquid nitrogen.
5. To remove all of the liquid from the beads after the last wash, attach a small gauge needle (27G½) to the vacuum apparatus and insert the needle down the side of the tube into the beads. This will remove all excess liquid, but will not disturb the beads. Quickly add the 20μL of 1.5x kinase buffer (so that the beads do not dry out) and proceed to the kinase reaction.
6. If you are performing the assay on many samples, consider starting and stopping the reactions at twenty-second intervals to keep the timing consistent. The Akt/PKB reaction proceeds quickly, and it is thus important to determine that the Akt/PKB is not phosphorylating all of the substrate in the mixture before completion of the reaction. The timing may vary depending upon source of Akt/PKB, amount of enzyme precipitated, and the substrate used. Under the conditions described above, the reaction proceeds rather quickly; five minutes is sufficient to put the kinase reaction in the middle of the linear range for H2B phosphorylation.
7. When loading the gel it is best to skip a lane between each sample so that the signals do not overlap. Also, when loading the samples, try not to load the beads onto the gel.

8. We find that we obtain less background signal if we transfer the blot to nitrocellulose, rather than drying the gel.

CHAPTER 3

Seasonal, Tissue-Specific Regulation of Akt/Protein Kinase B and Glycogen Synthase in Hibernators

This chapter describes work published in the American Journal of Physiology (Hoehn *et.al.,* 2003).

Hoehn KL, Hudachek SF, Summers SA, Florant GL. Am J Physiol Regul Integr Comp Physiol. 2004 Mar;286(3):R498-504

3.1 Abstract

Yellow-bellied marmots (*Marmota flaviventris*) exhibit a circannual cycle of hyperphagia and nutrient storage in the summer followed by hibernation in the winter. This annual cycle of body mass gain and loss is primarily due to large-scale accumulation of lipid in the summer, which is then mobilized and oxidized for energy during winter. The rapid and predictable change in body mass makes these animals ideal for studies investigating the molecular basis for body weight regulation. In the study described herein, we monitored seasonal changes in the protein levels and activity of a central regulator of anabolic metabolism, the serine-threonine kinase Akt-Protein Kinase B (Akt/PKB), during the months accompanying maximal weight gain and entry into hibernation (June-November). Interestingly, under fasting conditions, Akt/PKB demonstrated a tissue-specific seasonal activation. Specifically, although Akt/PKB levels did not change, the activity of Akt/PKB (isoforms 1/$\alpha$and 2/β) in white adipose tissue (WAT) increased significantly in July. Moreover, glycogen synthase, which lies downstream of Akt/PKB on a linear pathway linking the enzyme to the stimulation of glycogen synthesis, demonstrated a similar pattern of seasonal activation. By contrast, Akt/PKB activity in skeletal muscle peaked much later (i.e., September). These data suggest the existence of a novel, tissue-specific mechanism regulating Akt/PKB activation during periods of marked anabolism.

3.2 Introduction

Mammals that hibernate use an evolutionary strategy that allows them to survive winter food shortages and extremely low temperatures. The yellow-bellied marmot (*Marmota flaviventris*), for example, increases nutrient storage significantly during the summer months, but then utilizes the accumulated nutrients during its long winter hibernation [132]. The seasonal exaggerated increase in body mass and adiposity in these animals makes them an ideal model for studying the molecular basis for rapid weight gain leading to obesity.

In the study described herein, we evaluated the seasonal regulation of Akt/protein kinase B (PKB), a serine-threonine kinase that is a primary regulator of nutrient storage in white adipose tissue (WAT) and skeletal muscle tissue. Mammals contain three highly homologous isoforms of the Akt/PKB enzyme, each of which are expressed in all tissues [133]. Studies in cultured adipocytes and/or myotubes strongly implicate the enzyme as a key stimulus of nutrient storage: *1*) the expression of constitutively active forms of Akt/PKB (CA-Akt/PKB) in adipocytes increases rates of glucose uptake by both affecting a redistribution of the GLUT4 glucose transporter from intracellular stores to the plasma membrane and simultaneously increasing the expression of GLUT1 [134-137]; *2*) CA-Akt/PKB expression stimulates rates of lipogenesis [138, 139] and simultaneously increases the rate of fatty acid synthase transcription [140]; *3*) CA-Akt/PKB expression in either adipocytes or muscle markedly stimulates rates of glycogen synthesis by inactivating glycogen synthase kinase-3β, an upstream inhibitor of the rate-limiting enzyme regulating glucose incorporation into

glycogen [i.e., glycogen synthase (GS)] [69, 137, 141, 142]; and *4*) CA-Akt/PKB expression stimulates protein synthesis by activating the mammalian target of rapamycin (mTOR) to regulate pp70S6-kinase and the translational repressor PHAS1/4EBP1 [137, 143]. Constitutively active forms of the enzyme additionally promote the differentiation of preadipocytes into adipocytes [134, 144] and stimulate the secretion of leptin [145], an adipokine that suppresses appetite in rodents. Collectively these studies implicate Akt/PKB as a critical regulator of many of the biological processes controlled so precisely in hibernators as they prepare for winter torpor. Interestingly, it has also recently been shown that Akt/PKB protein levels are reduced during torpor in WAT of the little brown bat (*M. lucifugus*) [146].

Numerous hormones, cytokines, and transforming oncogenes activate Akt/PKB, which, in addition to regulating metabolism, is a key stimulator of cell size and survival. In WAT and skeletal muscle, insulin is a key regulator of Akt/PKB, which is a requisite intermediate linking the hormone to postprandial stimulation of glucose uptake and anabolic metabolism [45, 66-70]. The aforementioned ligands activate Akt/PKB by promoting its phosphorylation on two regulatory residues (Thr308 and Ser473 for the Akt1/PKBα isoform; reviewed in Ref. [133]). Insulin-stimulated Akt/PKB phosphorylation requires the activation of phosphatidylinositol 3-kinase (PI3-kinase), a lipid kinase that phosphorylates phosphoinositides to generate phosphatidylinositol-3,4-bisphosphate or phosphatidylinositol-3,4,5-trisphosphate. These phosphoinositides activate Akt/PKB by directly binding a pleckstrin homology domain on the enzyme, which

exposes the regulatory phosphorylation sites. Akt/PKB can also be activated by PI3-kinase independent mechanisms, for example in response to growth hormone [147], cellular stresses such as heat shock and hyperosmolarity [65, 148], or increases in intracellular Ca^{2+} or cAMP [62, 149].

We evaluated changes in Akt/PKB expression and activity from June to November, which covered the periods of maximal lipid accumulation, peak body mass, and the beginning of the hibernation season. The findings obtained revealed that the activity of Akt/PKB isoforms 1 and 2 in WAT, measured under fasting conditions, increased markedly during the weight-gaining lipogenic months (i.e., July) and decreased substantially during the months immediately preceding hibernation. The increase in Akt/PKB activity was mirrored by an increase in both the activity and the expression of GS, an anabolic enzyme that lies downstream of Akt/PKB in the signaling cascade linking insulin to the regulation of glycogen synthesis. These changes in signaling did not correlate with circulating insulin concentrations. Interestingly, in another insulin-responsive cell type, skeletal muscle, Akt/PKB activity peaked much later than it did in WAT (i.e., peak activity in September), confirming that the increase in fasting Akt/PKB activity in WAT was independent of circulating insulin concentrations. Collectively these data suggest that hibernators possess a unique intrinsic regulatory mechanism for activating Akt/PKB, perhaps as a means for selectively accelerating rates of anabolic metabolism in particular tissues.

3.3 Experimental Procedures

3.3a Animals

Four yellow-bellied marmots (3 females, 1 male) were trapped in the Rocky Mountains of Gunnison County, CO. The animals were transported to the laboratory of animal resources at Colorado State University, caged individually, and housed under a 14:10-h light/dark photoperiod and ambient temperature of 20 ± 3°C until September. Animals were provided with Purina rodent chow (5001) and water ad libitum. In the first week of September, all food was removed and the animals were moved to a cold room (5 ± 2°C) kept in constant darkness (DD). In October, animals demonstrated all of the behavioral changes associated with preparation for hibernation, although we did not measure body temperature. In April, the animals were moved from the cold room back to the laboratory and maintained under the same conditions as described above. Colorado State University's Animal Care and Use Committee approved all animal protocols.

3.3b Sample collection and preparation.

After initial trapping, the animals were maintained at Colorado State University for one year. Beginning in the last week of June 2001, monthly blood, WAT, and muscle samples were collected. All subsequent samples were collected during the last week of each month. Twenty-four hours before surgery, all animals were transferred to a holding facility (at room temperature) and fasted overnight. Animals were euthermic during surgery. The animals were then anesthetized by intramuscular injection of ketamine-acepromazine and

maintained under halothane anesthesia during the sterile surgical procedure. Before tissue collection, body mass data were obtained and percent body lipid was determined using an EM scan body composition analyzer (model SA-3000, EM SCAN, Springfield, IL) and the species-specific calibration equation described previously [150]: total lipid mass (LM) = 2,101 + 0.7 body mass-10.3 EM2-258.4 foot length-20.5 tail length, R^2 = 0.9997. Blood was then collected and centrifuged. The serum was flash frozen in liquid nitrogen. A sample of WAT was taken from the gonadal fat pads, and a sample of the skeletal muscle was taken from the gastrocnemius (mixed fiber types), alternating legs each month. Both WAT and muscle samples were flash frozen in liquid nitrogen. All samples were kept at -80°C until analysis. The serum samples were then thawed and analyzed for insulin using a human insulin ELISA kit from Linco Research per protocol. The interassay variation was <5%. Briefly, samples were homogenized in ice-cold lysis buffer [50 mM HEPES, pH 7.2, 2 mM EDTA, 30 mM sodium pyrophosphate, 1% Triton X-100, 10% glycerol, 10 mM NaF, 150 mM NaCl, 2 mM Na_3VO_4, and protease inhibitor cocktail (Sigma)]. Lysates were then spun at 10,000 *g* for 10 min at 4°C. The supernatant was collected, and protein amounts were determined via a bicinchoninic acid assay kit (Pierce).

3.3c Western Blotting

Crude lysate protein (45 or 100 µg) was prepared for SDS-PAGE by the addition of 2x Laemmli sample buffer and was boiled for 2 min. The samples, along with molecular weight markers (BioRad), were loaded on an 8% polyacrylamide gel. Resolved samples were transferred to nitrocellulose

membrane (Protran) for 2 h at 500 mA at 4°C. The membranes were rinsed in PBS and blocked in 5% nonfat dry milk in PBS with 1% Tween (PBST) for 60 min, then were rinsed in PBST and incubated for 90 min in anti-Akt1 (Santa Cruz), anti-Akt2 (courtesy of Dr. M. J. Birnbaum, University of Pennsylvania), anti-GS (courtesy of Dr. J. Lawrence, University of Virginia), anti-p-Akt1 Thr308 (Cell Signaling), or anti-p-Akt1 Ser 473 (Santa Cruz). The membranes were washed in PBST and incubated in horseradish peroxidase-coupled goat anti-rabbit, rabbit anti-chicken, or donkey anti-goat antibody for 30 min. The membranes were then washed again in PBST, and the resolved bands were detected using ECL Plus (Amersham Pharmacia Biotech) and a STORM phosphorimager (Molecular Dynamics, Sunnyvale, CA). Band intensity was analyzed using ImageQuant software (Molecular Dynamics), and values obtained were well within the linear range of the detection method

3.3d Akt kinase assays

Marmot WAT and muscle tissue were analyzed for Akt kinase activity using methods described previously [151] with slight modifications. Briefly, 100 µg of crude lysate was diluted to 300 µl in lysis buffer. Akt isoform 1 was immunoprecipitated with 30 µl of mouse anti-Akt1 agarose conjugated beads (Santa Cruz) at 4°C for 90 min. Akt isoform 2 was immunoprecipitated with 5 µl of a rabbit anti-Akt2 antibody for 1 h at 4°C, and then 50 µl of preequilibrated protein-A conjugated agarose beads (Santa Cruz) were added for 30 min. The immune complexes were then washed three times in cold lysis buffer and three times in 1x kinase buffer (20 mM HEPES pH 7.2 and 5 mM $MgCl_2$). The kinase

reaction was then started by the addition of 30 µl of kinase mix [10 µM Mg^{2+} ATP, 1 mM DTT, 200 µM EGTA, 25 µg histone H2B (Boehringer Mannheim), and 5 µCi [γ-^{32}P]-γ-ATP (Amersham Pharmacia)] per reaction. The reactions were incubated at 30°C for 25 min (this being a median time point in the linear range of the assay) before being stopped by addition of 30 µl of 2x Laemmli sample buffer. The samples were then heated to 90°C for 2 min and the beads were pelleted. Thirty microliters of each reaction was then loaded onto a 12.5% SDS-PAGE gel, electrophoresed, and transferred to nitrocellulose. The membrane was then analyzed for substrate histone phosphorylation using a STORM phosphorimager and ImageQuant software. Background kinase assays were performed identically as described above except without the presence of protein in the lysis buffer. Background levels of phosphorylated substrate were minimal and were subtracted from each experiment.

3.3e GS assays

Marmot muscle and WAT were analyzed for GS activity using methods described previously [152]. Briefly, 50 µg of crude lysate protein was diluted to 30 µl in GS extraction buffer (100 mM NaF, 10 mM EDTA, 1 mM benzamidine, and 50 mM Tris·HCl, pH 7.8). The sample was then added to 60 µl of assay solution [100 mM NaF, 10 mM EDTA, 1% glycogen, 150 µM UDP-D-glucose, 0.22 µCi UDP-D-[^{3}H]glucose, and 50 mM Tris·HCl (pH 7.8)]. The reactions were performed in duplicate in the presence of 1 or 10 mM glucose-6-phosphate (G6P) for 20 min at 37°C. Tubes were then placed on ice and 75 µl aliquots of the mixture were transferred to 1 x 1-in.^{2} filter papers (Whatman #3), allowed to dry,

and then were washed in an ice-cold 66% ethanol solution overnight. Papers were then dried, and the radioactivity was measured in a liquid scintillation counter. The ratio of active GS was calculated by dividing the activity measured in the presence of 1 mM G6P by the activity measured in the presence of 10 mM G6P. Background GS assays were performed as described above except without the presence of protein in the lysis buffer. Background levels of ^{3}H were minimal and were subtracted from each experiment.

3.3f Statistical Analysis

All statistical comparisons were done using ANOVAs (SAS program, SAS Institute). The analysis used was a repeated-measures design over months, blocking on marmots. The SAS procedure, Proc Mixed, fit the model with an autoregressive error structure, which has an exponentially decreasing correlation between months, meaning that responses for months closer together in time are more highly correlated than months farther apart in time. Also, the more correlation there is between pairs of months, the smaller the differences that can be detected as statistically significant. Differences in mean were considered significant if $P < 0.05$.

3.4 Results

3.4a Body mass and percent lipid change in marmots

As these animals were fed ad libitum and had been in the laboratory for one year, their body and lipid masses increased very rapidly during the spring and summer. In the marmots used for this study, mean body mass peaked in July

(3.7 ± 0.2 kg), decreased gradually through hibernation, and reached nadir in February (1.7 ± 0.2 kg; Figure 3.1*A*). A large percentage of their summer caloric intake was converted to triglyceride stores as total body lipid mass increased from 0.19 ± 0.02 kg in March to 0.73 ± 0.05 kg in July (Figure 3.1*B*). Total body adiposity likewise increased from 9.9 ± 0.4% in March to 19.4 ± 0.4% in July (Figure 3.1*C*). These data reveal that marmots increase triglyceride accumulation during the summer, with maximum lipid gain in June and July (in this case), then switch to a lipolytic state before hibernation in early October. Wild marmots do not experience the same abundance of food and thus peak in body mass months later and switch to a lipolytic state immediately preceding hibernation [153].

3.4b Regulation of Akt/PKB in WAT

To evaluate whether Akt/PKB activity was differentially regulated during the months preceding hibernation, we excised and analyzed gonadal WAT from fasted animals monthly from June through November. Although Akt/PKB protein levels did not change (data not shown), the activities of both Akt1/PKBα and Akt2/PKBβ were significantly higher in July when compared with the later months ($P < 0.05$) (Figure 3.2, *A* and *B*). The increase in activity during these summer months was not associated with an increase in Akt/PKB phosphorylation on Ser473 (Figure 3.3*B*), but Thr308 phosphorylation increased significantly during the early months in the study period (Figure 3.3*A*).

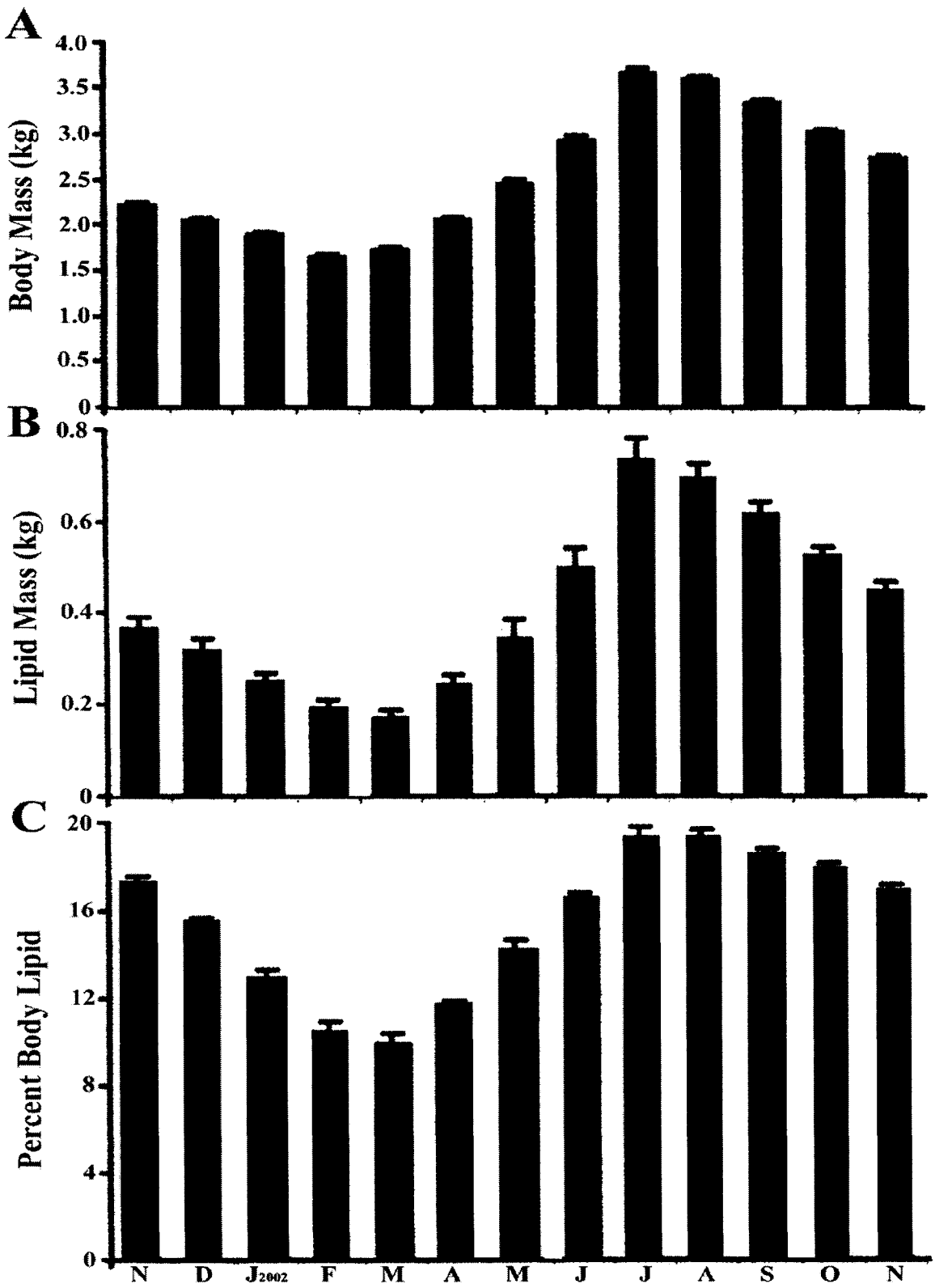

Figure 3.1 Average body mass (*A*), lipid mass (*B*), and percent body lipid (*C*) observed from November 2001 to November 2002 for the 4 marmots used in this study. Data are represented as means ± SE, $n = 4$.

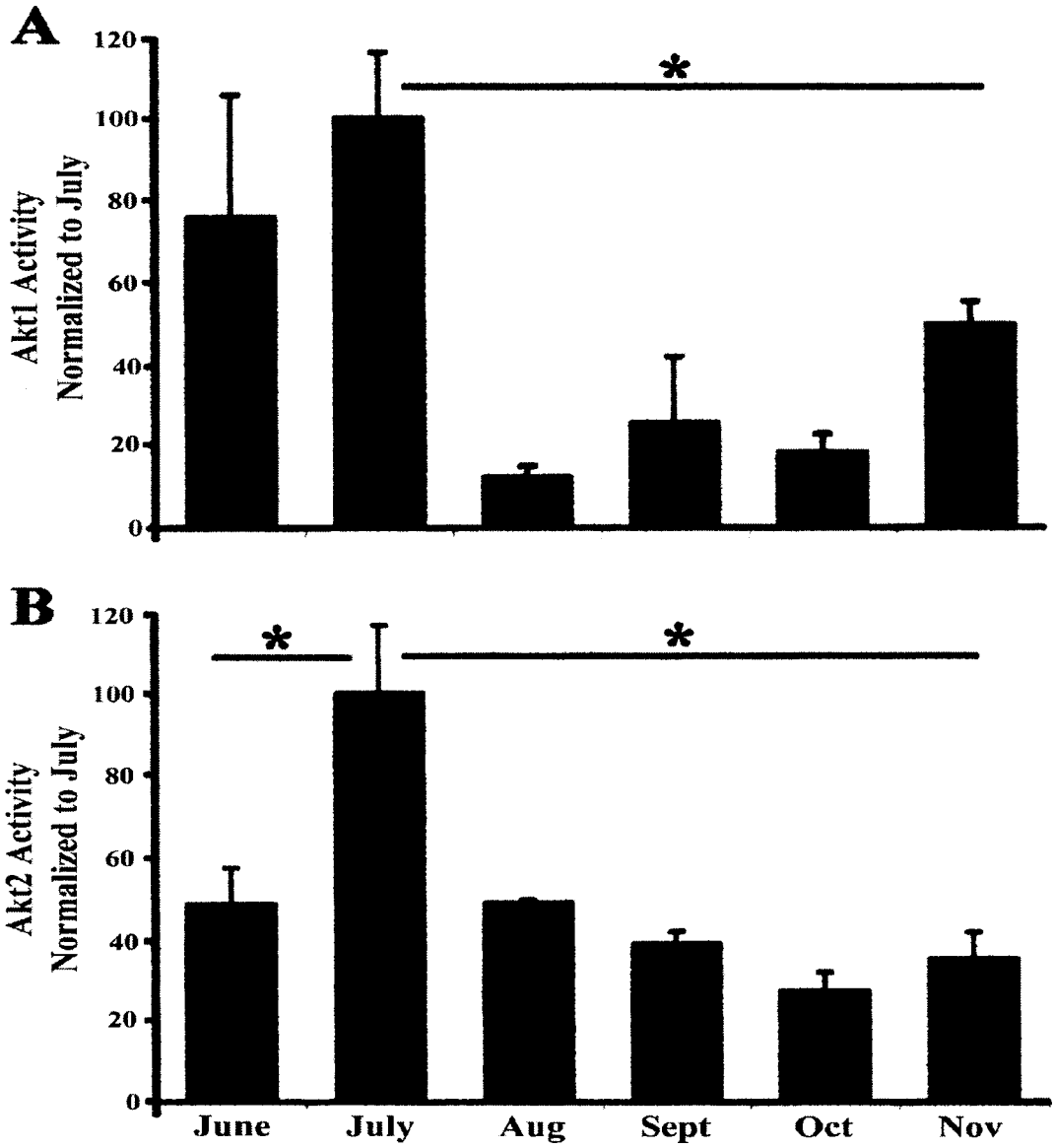

Figure 3.2 Mean ± SE for Akt1 (*A*) and Akt2 (*B*) activity in white adipose tissue (WAT) from June to November. Animals were fasted overnight before removal of gonadal adipose tissue. Akt activity was determined by in vitro kinase assay. $^{*}P < 0.05$, $n = 3$.

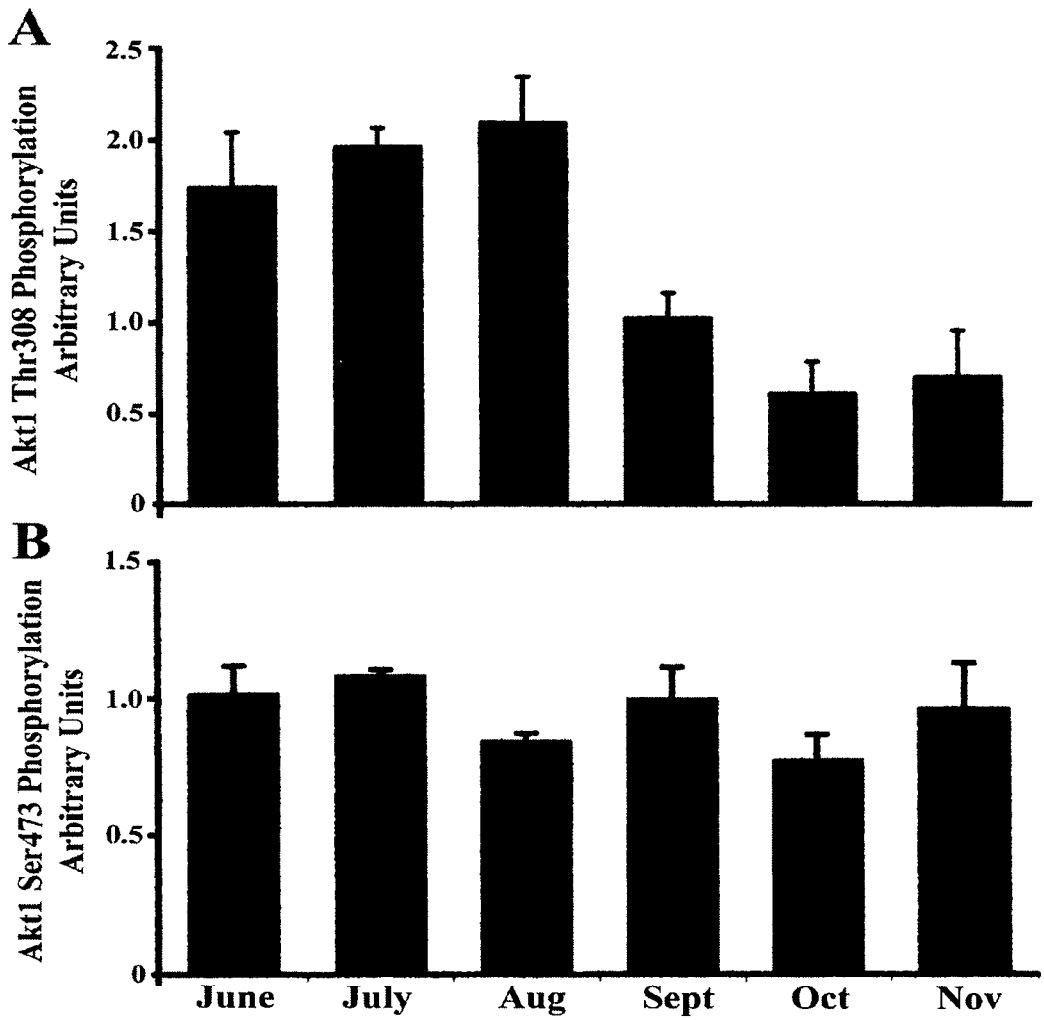

Figure 3.3 Akt1 threonine 308 (*A*) and serine 473 (*B*) phosphorylation in WAT from June to November. Data are quantified from Western blots and are expressed as means ± SE, $n = 3$.

3.4c Regulation of Glycogen Synthase in WAT

Glycogen synthase kinase 3β (GSK3β) is an Akt/PKB substrate that inhibits glycogen synthesis by phosphorylating the metabolic enzyme GS. Under anabolic conditions, Akt/PKB phosphorylates and inhibits GSK3β to increase

rates of glucose incorporation into glycogen. Therefore we hypothesized that GS would be activated in parallel with the increase in fasting Akt/PKB activity. We performed a GS assay each month using two different concentrations of glucose-6-phosphate (G6P). G6P is an allosteric activator of GS and will override any inhibitory phosphorylation. Under conditions of low G6P, only the weakly or non-phosphorylated forms of the GS enzyme will participate in glycogen synthesis; by contrast, in the presence of high G6P, all GS that is present in the extract will be activated. The percent of active GS found in each extract under basal conditions was expressed as a ratio of the activity obtained under low/high G6P concentrations (Figure 3.4*A*). These assays showed that WAT GS activity correlated with Akt2/PKBβ activity, as both peak in July ($P < 0.02$). Interestingly, in July the total amount of GS activity in the lysate was also high, as indicated by increased activity in the presence of high G6P (Figure 3.4*B*). This suggested that WAT contained elevated quantities of the enzyme, which we subsequently confirmed by Western blot (Figure 3.4*C*).

3.4d Circulating Insulin Concentrations

Insulin is a primary circulating factor regulating Akt/PKB activity in WAT and skeletal muscle. We quantified insulin levels in serum samples obtained at the same time that the tissues were excised. As shown previously [154], fasting insulin levels increased twofold in months preceding hibernation (July through September; Figure 3.5). This increase is likely to be the result of the insulin

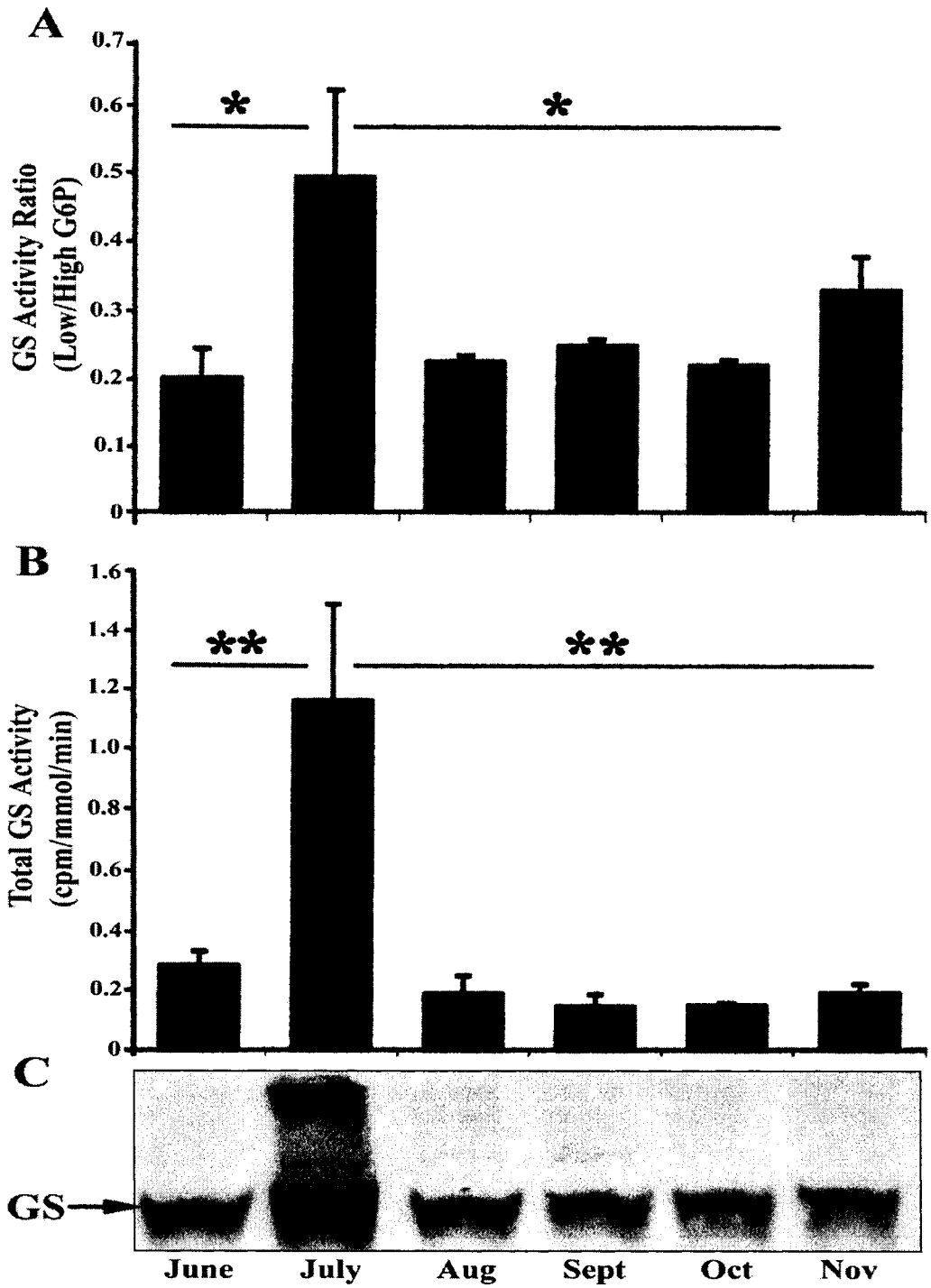

Figure 3.4 Mean ± SE for percent of active glycogen synthase (GS; *A*) and total GS activity (*B*), and a representative Western blot (*C*) of GS from June to November in WAT. GS is seen at ~85 kDa, and the larger molecular weight band is unknown but was seen at similar levels in all animals only in July. *$P < 0.02$, **$P < 0.01$, $n = 3$.

resistance that accompanies weight gain in these animals [132] and is unlikely to account for the sudden increases in Akt/PKB and GS activity seen herein.

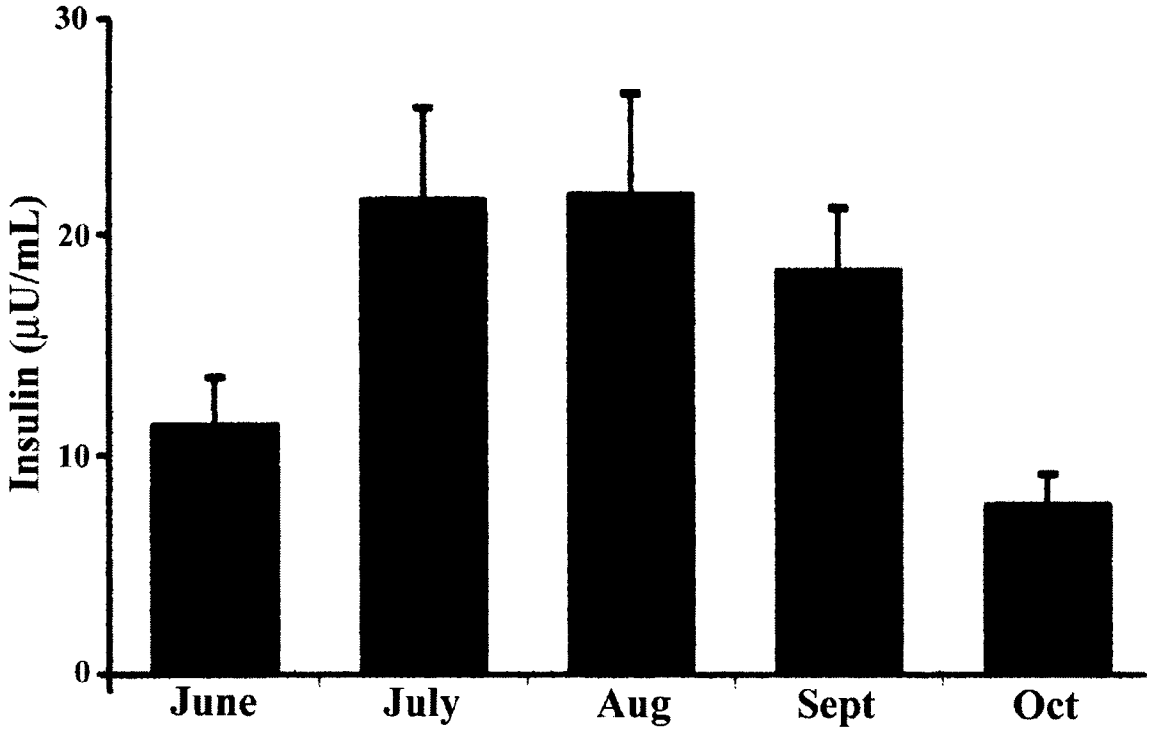

Figure 3.5 Mean ± SE for average serum insulin levels from June to October. *n* = 3.

3.4e Regulation of Akt/PKB in skeletal muscle.

Hibernators also increase protein and glycogen accumulation in skeletal muscle during the months preceding hibernation [155, 156]. We investigated Akt/PKB activity in skeletal muscle tissue obtained during the same surgeries that we collected the WAT samples. As with WAT, neither Akt1/PKBα nor Akt2/PKBβ protein levels changed in skeletal muscle during the study period (data not shown). Under fasting conditions, Akt1/PKBα activity again demonstrated a

seasonal change in activity. However, peak activity did not match that found in WAT. Specifically, this activity was significantly higher in September (3 wk after the animals were moved to constant darkness) than it was in the other 4 mo ($P < 0.03$; Figure 3.6). This shows that fasting Akt1/PKBα activity increases not only seasonally, but also in a tissue-specific manner. We also investigated Akt2/PKBβ activity in skeletal muscle from July through November and, although a similar pattern was observed, no statistically significant differences were found (data not shown).

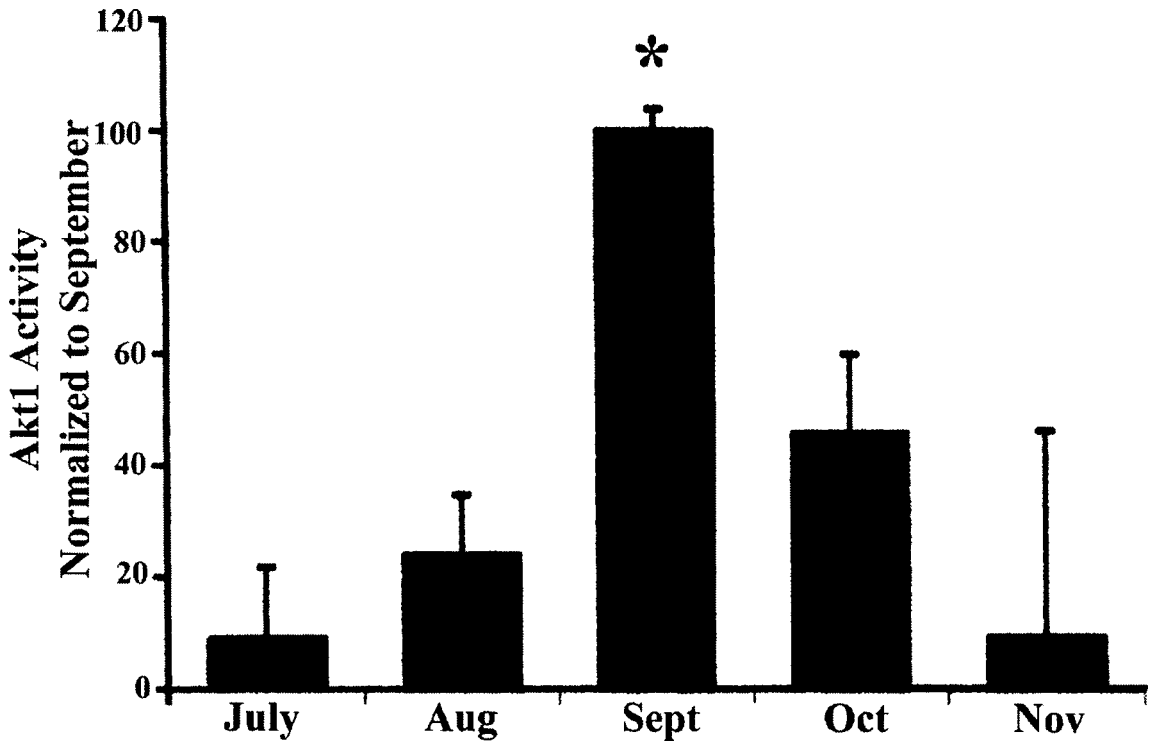

Figure 3.6 Mean ± SE for muscle Akt1 activity from July to November. Akt activity was determined by an in vitro kinase assay. $^{*}P < 0.03$.

3.5 Discussion

After emerging from hibernation in March, the marmots used in this study more than doubled their body mass and body adiposity by July in preparation for hibernation in October (Figure 3.1). This increase in body mass occurs earlier when compared with field animals that are not able to accumulate lipid stores until nearly June. Thus the body mass cycles of our lab animals are shifted forward as are food intake and other physiological mechanisms that produce fat accumulation. The peak in mass and adiposity at such an early time suggests that the change in metabolism may be regulated by nutrient availability and accumulation. We obtained monthly biopsies of skeletal muscle and gonadal fat during this period bracketing the peak rate of weight gain (i.e., June to July) and entry into hibernation (i.e., September to October). Importantly, Akt/PKB activity increased in a seasonal, tissue-specific manner. Specifically, in July, fasting Akt1/PKBα and Akt2/PKBβ activity increased several-fold in WAT compared with later months in the study period (Figure 3.2).

Although insulin is a primary regulator of Akt/PKB activity in this tissue, it was unlikely to account for the increased activity obtained in these samples. First, the samples were obtained under fasting conditions, when a postprandial insulin surge would not occur. Direct measurement of fasting insulin concentration confirmed that the increase in Akt/PKB activity did not correlate with circulating insulin concentrations, which increased slightly (i.e., 2-fold) in July, but remained elevated through September (Figure 3.5). Second, another insulin responsive tissue (i.e., skeletal muscle) demonstrated a dissimilar pattern of Akt/PKB

activation. Specifically, although muscle biopsies were obtained at the same time as the WAT biopsies and these tissue samples also demonstrated a seasonal increase in Akt/PKB activity, the peak activity in muscle occurred much later in the year (i.e., September) than it did in WAT (Figure 3.6). These findings suggest the existence of an intrinsic, tissue-specific mechanism for increasing fasting Akt/PKB activity. Interestingly, prior studies reveal the existence of a PKA-dependent, PI3-kinase-independent mechanism leading to Akt/PKB activation resulting exclusively from Thr308 phosphorylation [62]. Future studies will evaluate the contribution of PKA to the stimulation of fasting Akt/PKB activity in these tissues.

The seasonal changes in Akt/PKB activity suggest that marmots selectively increase fuel storage first in adipose tissue and, secondly, in skeletal muscle, during the months preceding hibernation. As shown in Figure 3.4, the increase in Akt/PKB activity corresponded with increased activity of GS, the rate-limiting enzyme governing incorporation of glucose into glycogen. In addition to regulating the acute storage of nutrients after consumption of a meal, however, Akt/PKB also controls the expression of several molecules involved in nutrient absorption or deposition. Specifically, Akt/PKB stimulates the expression of transporters for glucose (i.e., GLUT1) [134], amino acids [157], low-density lipoprotein [157], and iron [157]. GLUT1 permanently resides in the plasma membrane of many tissues to increase cell permeability to glucose. We also found that the expression of constitutively active forms of Akt/PKB in 3T3-L1 adipocytes stimulates the transcription of GS (data not shown), which is

consistent with the increased levels of GS seen in WAT containing elevated fasting Akt/PKB activity (Figure 3.4*B*). Additionally, Akt/PKB also controls rates of transcription of fatty acid synthase [139, 140], the enzyme that catalyzes all reactions for synthesis of palmitate from acetyl-CoA and malonyl-CoA. This observation is consistent with our prior studies showing that the levels of fatty acid synthase increase during the summer months [158].

Akt/PKB is also implicated in the differentiation of preadipocytes into adipocytes [134, 144] and is required for the expression of the adipogenic transcription factor PPARγ [79]. Although it is tempting to speculate that the increase in fasting Akt/PKB activity contributes to the recruitment of preadipocytes into the fat pad, thus increasing storage space for the large lipid reserves necessary for survival through the winter, prior studies reveal that lipid accumulation in adipose tissue results primarily from adipocyte enlargement rather than an increased number of cells [159, 160]. The size of the adipose cells in the animals evaluated herein nearly tripled in size during the study period, with peak size achieved in July and August, when Akt/PKB activity was maximal. Interestingly, Akt/PKB is strongly implicated in the regulation of cell size. In 1999, Verdu et al. [161] determined that ectopic expression of Akt/PKB in *Drosophila* increased cell and organ size without affecting cell-fate determination, apoptosis, or proliferation in imaginal discs. Subsequent studies in several different mammalian cell and tissue types confirm a role for the enzyme in cell growth independently of changes in cell number. For example, overexpression of Akt1/PKBα induced a twofold increase in the size of H4IIE rat hepatoma cells

[143], the interleukin-3-dependent FL5.1 cell line [157], pancreatic β-cells [162], and cardiac myocytes [163]. The importance of Akt/PKB in cell growth is underscored in knockout mice lacking the Akt1/PKBα isoform, which display significant impairment in organismal growth [58, 79].

In summary, yellow-bellied marmots prepare for hibernation by selectively increasing fuel stores in WAT months before a subsequent accumulation in skeletal muscle. We found that this unique tissue-specific accumulation of nutrients is likely due to an intrinsic, and thus non-insulin-dependent mechanism, by which these animals regulate the fasting activity and phosphorylation of Akt/PKB, a critical regulator of lipid and glycogen synthesis, and GS. Future studies will address the mechanisms underlying this profound regulatory event in this animal model.

3.6 Acknowledgements

We thank Dr. J. R. ZumBrunnen, the Associate Director of the statistics department at Colorado State University, for contributions to the statistical analyses. This work was supported by National Institutes of Health Grants (R01-DK58784 to S. A. Summers and R21-DK60676 to. G. L. Florant) and a beginning grant-in-aid from the American Heart Association (to S. A. Summers).

CHAPTER 4

The Role of Mixed Lineage Kinase 3 in TNFα and Ceramide Signaling in 3T3-L1 Adipocytes

4.1 Abstract

Tumor necrosis factor alpha (TNFα) and ceramide are two major antagonists of insulin signaling that are elevated with adiposity and are implicated in the development of obesity-induced insulin resistance. These molecules antagonize insulin action through direct effects on Akt/PKB activity, and stimulation of IKK and MAPK signal transduction cascades. Mixed Lineage Kinase 3 (MLK3) is a MAP3K activated by both TNFα and ceramide in Jurkat cells, leading to the activation of JNK MAP kinase. In other cell types, MLK3 activates IKK and the three major classes of MAP kinases. In the study herein, we investigated the role of MLK3 in TNFα and ceramide induced signal transduction in cultured 3T3-L1 adipocytes. We show that MLK3 overexpression mimics many of the effects of TNFα and ceramide treatment including similar patterns of signal transduction, and inhibition of insulin signaling. Inhibition of MLK3 completely prevents TNFα signaling pathways leading to MAPK and IKK activation and prevents TNFα–induced ceramide synthesis. Additionally, MLK3 blocks exogenous ceramide activation of JNK, but not other MAPKs or IKKs. Consequently, we identify MLK3 as a necessary intermediate linking TNFα to the activation of MAPK/IKK signaling and ceramide accumulation, and ceramide signaling to JNK in adipocytes. Additionally, we have identified the necessity of MEK1/ERK in IKK activation by several agonists in this cell type.

4.2 Introduction

Visceral white adipose tissue (WAT) serves as the body's primary lipid storage depot, and a major endocrine organ that communicates its nutritional status and energy reserves with the rest of the body. With overnutrition, lipid storage increases, and adipocytes swell in diameter. The increase in adipocyte size is directly proportional to the accumulation and/or secretion of several molecules that repress food intake, reduce adipocyte size, and prevent further nutrient storage [114, 164]. Researchers hypothesize that this is an evolutionary mechanism to reduce excess energy stores during times of nutrient oversupply. Leptin is the primary adipocyte-derived hormone (adipokine) that signals satiety [165, 166], while free fatty acids (FFAs) and TNFα are major secretagogues that thwart nutrient storage [25, 102, 104, 111, 167, 168].

TNFα is a pro-inflammatory cytokine secreted from enlarged visceral white adipocytes that antagonizes insulin action through many mechanisms. These include triggering lipid secretion from fat cells, activating *de novo* ceramide synthesis, and stimulating mitogen activated protein kinase (MAPK) and inhibitor kappa B kinase (IKK) signaling cascades. This cytokine is shown to inhibit insulin action when administered to both animals [107] and cultured cells [116, 169, 170], and inhibition of TNFα production or action by ablation of TNFα [105, 112] or its receptors (TNFRs) [113, 171] in insulin resistant mice increases insulin sensitivity and protects them from obesity-induced insulin resistance.

In adipocytes, TNFα activates inhibitor kappa-B kinases (IKKs), and the three major classes of mitogen activated protein kinases (MAPKs): the

extracellular-signal regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and the p38 MAP kinases. The activation of these serine/threonine kinases results in a large array of subsequent phosphorylation events that lead to the inhibition of insulin signaling intermediates and the regulation of several transcription factors resulting in the underexpression of IRS-1 and GluT4. Specifically, JNK and IKK have been identified as primary mediators of the inhibitory effects of TNFα on insulin action [172]. JNK and IKK promote the serine phosphorylation of IRS proteins, making them poor substrates for tyrosine phosphorylation by the insulin receptor [106, 173, 174]. Other studies have shown the necessity of p38 in the activation of IKK and the inhibition of insulin signaling by TNFα [175], and that TNFα requires the ERK pathway to inhibit insulin signaling in cultured adipocytes [169].

Furthermore, TNFα induces ceramide accumulation, which inhibits Akt/PKB. Akt/PKB is an obligatory intermediate in insulin signaling [25, 33, 124], and a study by Teruel et al., showed ceramide accumulation mediates TNFα-induced insulin resistance in brown adipocytes by keeping Akt/PKB in an inactive dephosphorylated state [176]. Similarly, a study by Grigsby and Dobrowsky [177] showed that inhibition of TNFα-stimulated ceramide accumulation reverses TNFα-induced insulin resistance in cultured white adipocytes. From these data it is unclear which pathways (p38, ERK, JNK, IKK, or ceramide) are necessary or sufficient to mediate the inhibitory effects of TNFα on insulin signaling in the adipocyte.

TNFα induces ceramide accumulation through the hydrolysis of sphingomyelin and by *de novo* synthesis. The cytokine triggers sphingomyelin hydrolysis by activation of neutral and acidic classes of sphingomyelinases (SMases), however, *de novo* ceramide synthesis occurs through an unresolved mechanism. Ceramide accumulation occurs in two stages in fat cells; the first is a rapid, but small, increase in ceramide production triggered by SMase activation, and the second is a sustained *de novo* accumulation that significantly elevates ceramide levels beginning at about twelve hours after initial exposure [122].

The signaling pathways leading from TNFα to MAPK activation, IKK activation, and *de novo* ceramide accumulation remain incompletely understood despite substantial interest. TNFα activates MAPKs through a three-tiered phospho-relay involving MAPK kinase kinases (MAP3Ks) and MAPK kinases (MAP2Ks). Recently, both TNFα and ceramide were shown to activate mixed-lineage kinase 3 (MLK3) [178], a MAP3K and IKK-kinase. MLK3 directly activates IKK in HeLa cells [179], and interacts with several scaffolding proteins [180-183] to activate the MAP2Ks; SEK1, MEK1, and MKK3/6 [180, 184, 185], in a cell type specific manner. These MAP2Ks activate JNK, ERK, and p38, respectively (Figure 4.1). Additionally, in fibroblasts, MLK3 underexpression by RNA interference was shown to suppress TNFα activation of all three classes of MAPK [186].

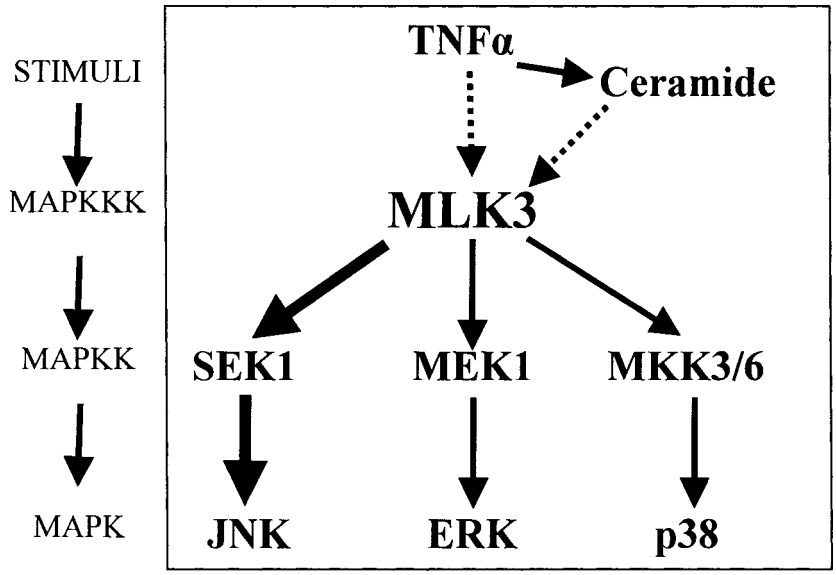

Figure 4.1. Proposed MAP kinase signaling downstream of MLK3. Dotted lines indicate unknown or indirect mechanisms of activation.

MLK3 is a serine/threonine kinase comprised of a N-terminal SH3 domain; a central region containing a kinase domain, two leucine zippers, and a CRIB domain; and a C-terminal pro/ser/thr-rich region. The leucine zippers facilitate the formation of a MLK3 homodimer, which is not mandatory for activity, but enhances autophosphorylation, which is stimulatory [185, 187]. Additionally, MLK3 has at least 11 known phosphorylation sites, yet the role of these sites in the regulation of the enzyme are not fully understood [188].

As stated above, TNFα and ceramide activate MLK3, and similarly activate a comparable pattern of signal transduction in different cell types. Therefore, we evaluated the role of MLK3 as a putative intermediate in TNFα and ceramide signaling in cultured 3T3-L1 adipocytes. In the study described herein, we report that MLK3 overexpression results in nearly identical signaling

cascades, and repression of insulin signaling as seen with TNFα treatment. Additionally, we show that the MLK3 inhibitor CEP-11004 blocks ceramide signaling to JNK, and TNFα signaling completely. Thus we show that MLK3 is a necessary intermediate in TNFα stimulated signal transduction and ceramide stimulated JNK activation in adipocytes.

4.3 Methods

4.3a Antibodies and Reagents

Unless otherwise stated, all polyclonal antibodies directed against the phosphorylated and unphosphorylated forms of IKK, mitogen-activated protein kinases, and Akt/PKB were from Cell Signaling Technology, Inc. (Beverly, MA). Protein-A agarose beads, total MLK3, total Akt, hemagglutinin (HA), and secondary anti-rabbit antibodies coupled to horseradish peroxidase were from Santa Cruz Biotechnology, Inc. C_2-ceramide and C_6-ceramide were from EMD biosciences - Calbiochem. Porcine insulin was from Sigma.

4.3b Cell Culture

3T3-L1 preadipocytes over-expressing the coxsackie and adenovirus receptor (CAR) were obtained from Dr. Jim Bamburg, who acquired these cells from David Orlicky et al. [189]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum until five days post-confluence when they were differentiated into adipocytes by addition of DMEM supplemented with 10% fetal bovine serum, 1 μg/ml dexamethasone, 3.33μg/ml

Ciglitizone, and 0.5mM isobutylmethylxanthine. After 3 days, cells were maintained in DMEM supplemented with 10% fetal bovine serum.

4.3c PI3-Kinase Assays

PI3-kinase assays were performed according to the methods described by Wang and Summers [190].

4.3d Western Blot Analyses

Adipocytes were serum-deprived (DMEM with 0.2% BSA) for two hours before all treatments with insulin as described. Cells were washed twice with ice-cold phosphate-buffered saline and lysed in 100 µl of 66 mM Tris (pH 8.0) containing 2% SDS. Samples were sonicated and insoluble material was pelleted by 20 minutes of centrifugation at 20,000xg. Protein concentrations were determined using the bicinchoninic protein assay kit (Pierce), and 60µg of total protein was loaded into each well of an 8.5% polyacrylamide gel. Proteins were transferred to nitrocellulose, blocked with 5% non-fat milk in Tris-buffered saline with 1% Tween (TBST), and probed with the antibodies at the recommended concentrations in TBST. The blots were developed with enhanced chemiluminescence plus (Amersham Biosciences) and scanned with a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

4.3e MLK3 Kinase Assays

3T3-L1-CAR adipocytes were lysed in 0.3 ml of lysis buffer (150 mM NaCl, 20 mM Tris-HCl (pH 7.2), 1 mM EDTA, 10% glycerol, 1% Igepal, 10mM NaF, 30mM NaPP, 1 mM phenylmethylsulfonyl fluoride, 50 IU/ml aprotinin, 2 µg/ml leupeptin), incubated on ice for 15 min, and then centrifuged for 10 min. The supernatant was collected, diluted to 300µg protein per reaction, and was transferred to new tubes and rocked with 15 µl of rabbit anti-MLK3 or anti-HA antibodies for 1 hour at 4°C. 30µL of Protein A-agarose beads were washed in lysis buffer, centrifuged, and incubated with the supernatant for 1 hour at 4 °C. Beads were then washed three times with lysis buffer and twice more with 1x kinase buffer (20 mM HEPES pH 7.2 and 5 mM $MgCl_2$). The kinase reaction was then started by the addition of 30 µl kinase mix (10 µM Mg^{2+} ATP, MKK7-(KA) substrate, and 5 µCi [γ-^{32}P]-ATP (Amersham Pharmacia)) per reaction. Samples were incubated at 30 °C for 30 min, and the reaction was stopped by the addition of 50 µl Laemmli buffer. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and visualized on a STORM PhosphorImager.

4.3f Ceramide Assays

Adipocytes were lysed in 0.25 mL ice-cold 1 M NaCl and transferred to glass tubes containing 0.75mL ice-cold $CHCl_3$:CH_3OH (1:2, v/v). The mixture was vortexed for 30 seconds, 0.25 mL of 1 M NaCl and 0.25 mL of $CHCl_3$ was added, and the samples were vortexed again and incubated on ice for 15 minutes. The organic phase was separated from the aqueous phase by

centrifugation at 2000x*g* for 5 min. The lower organic phase containing ceramide and diacylglycerol (DAG) was removed and labeled with DAG kinase and [γ-^{32}P]-ATP using a DAG assay kit (Amersham Biosciences, Piscataway, NJ) as described previously [191]. The labeled ceramide-1-phosphate and phosphatidic acid were then separated by thin layer chromatography with chloroform/acetone/methanol/acetic acid/water (40:16:8:8:4, v/v) as the developing solvent. The corresponding spots were quantitated using a STORM PhosphorImager.

4.4 Results

4.4a MLK3

This study on the role of MLK3 in TNFα and ceramide signaling was performed in 3T3-L1 adipocytes over-expressing the coxsackie and adenovirus receptor (CAR). Adipocytes are an ideal cell line for studying TNFα signaling and its role in insulin resistance as they are highly sensitive to both insulin and TNFα. When we sampled these CAR adipocytes for MLK3 expression we found endogenous MLK3 at a relatively low abundance by Western blotting (Figure 4.2). To study the role of MLK3 in TNFα signaling, we used recombinant adenovirus to over-express wild-type HA-MLK3 into terminally differentiated CAR adipocytes. To our surprise, MLK3 over-expression led to constitutive phosphorylation and activity while in minimal media without serum or growth factors (Figures 4.2 and 4.6B). Furthermore, we could not reduce this basal activity, and two known agonists of MLK3 (ceramide and TNFα) did not

significantly increase MLK3 phosphorylation or activity above this level (data not shown).

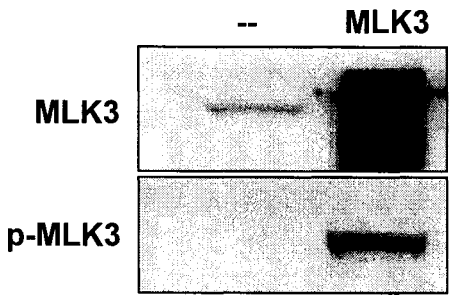

Figure 4.2 MLK3 expression and phosphorylation in CAR adipocytes. CAR adipocytes were uninfected or infected with adenovirus expressing the wt-HA-MLK3 transgene (MLK3) as indicated above.

4.4b TNFα, ceramide, and MLK3 activate similar signaling cascades

In adipocytes, TNFα, exogenous short chain ceramide, and MLK3 overexpression all led to the activation of ERK, p38, JNK, and IKK (Figure 4.3). With TNFα addition, these phosphorylation events were rapid, but transient, with a peak in activation at 10 minutes (Figure 4.3A). Ceramide stimulation was seen with N-acetyl (C2), and N-hexanoyl (C6), cell permeable ceramide species. C2-ceramide was more potent than C6- ceramide, and maximal signal transduction stimulation was seen from one-two hours post treatment (Figure 4.3B). Longer or shorter treatments showed no activity. Overexpression of MLK3 led to significant phosphorylation of these kinases after an overnight infection with the adenovirus (Figure 4.3C). In fact, MLK3 over-expression mimicked all of the signaling effects seen with TNFα and ceramide. MLK3 is an upstream kinase that activates IKK and three classes of MAPK in adipocytes, illustrating its likelihood of being an intermediate in TNFα and/or ceramide signaling.

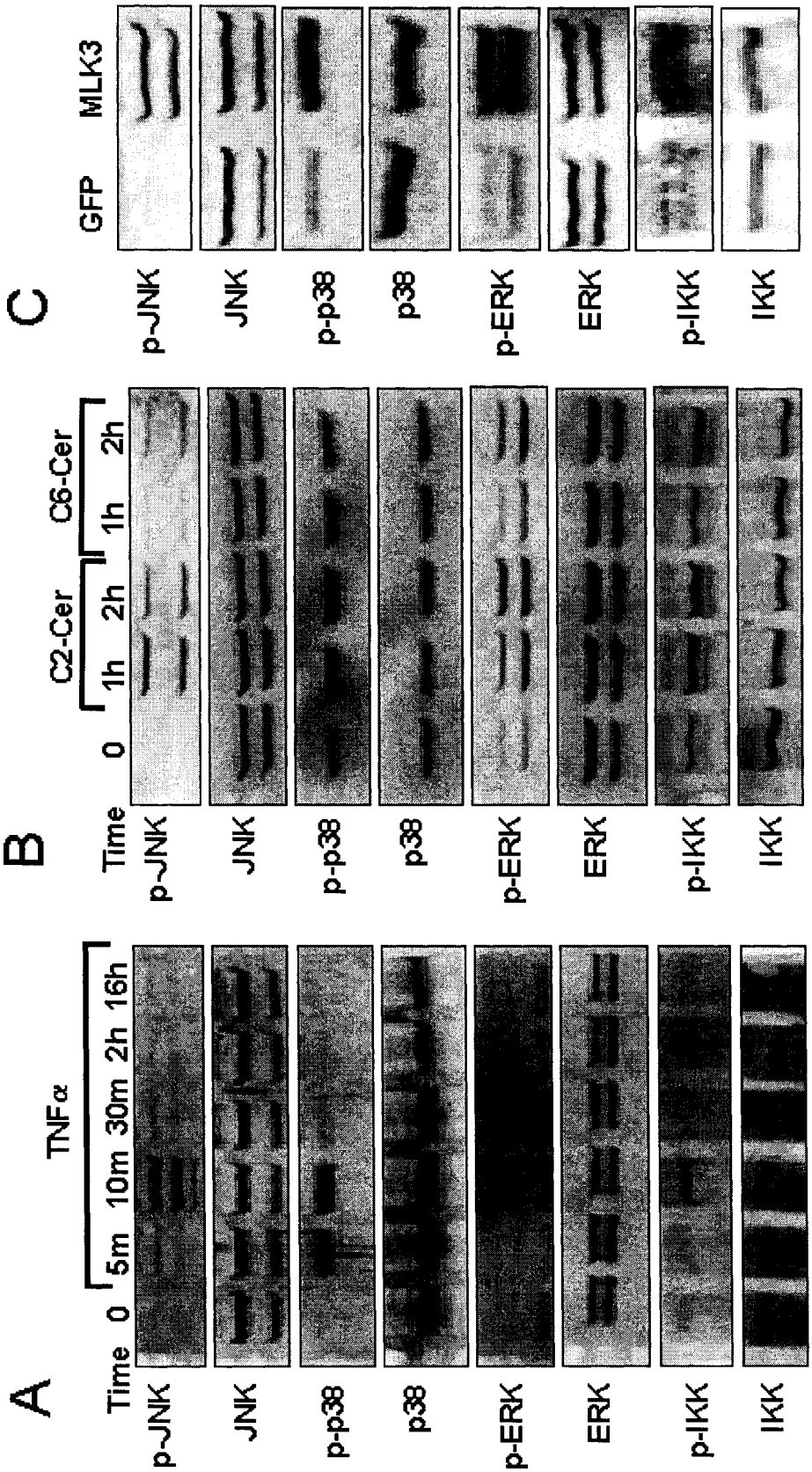

Figure 4.3 Activation of IKK, and p38, JNK, and ERK MAP kinases in cultured 3T3-L1 CAR adipocytes by TNFα, ceramide, and MLK3. Adipocytes were treated with (A) 20ng/mL TNFα or (B) 100μM n-acetyl (C2) or n-hexanoyl (C6) ceramide for the times indicated above. In (C) green fluorescent protein (GFP) or wild-type HA-tagged MLK3 were over-expressed in adipocytes for 24h and the signaling cascades were mapped. Cultures were maintained in DMEM + 10%FBS media until 16h before harvest when they were changed to serum free DMEM + 0.2% BSA. Western blots are representative of at least three separate experiments.

4.4c MLK3 and TNFα increase ceramide accumulation

Prolonged exposure to TNFα leads to the accumulation of ceramide species in a time dependent fashion in adipocytes (Figure 4.4A). Similarly, overexpression of MLK3 mimicked this effect. We over-expressed GFP or MLK3 in adipocytes for 36h, extracted the lipids, and performed a diacylglycerol (DAG)/ceramide assay (Figure 4.4B) to determine ceramide levels. We found that both TNFα and MLK3 led to significant increases in ceramide accumulation (Figure 4.4C) without having an effect on DAG levels (not shown).

4.4d MLK3 and TNFα act through MEK1 to activate IKK

To analyze the mechanism of the observed IKK activation by MLK3 we investigated the roles of all three MAP kinases by utilizing specific inhibitors to each. Specifically, we used PD98059 for MEK1, and thus ERK inhibition; SP600125 for JNK inhibition; and SB203580 for p38 inhibition. Subsequently, we found that inhibition of the MEK1/ERK pathway with PD98059 prevented MLK3 from activating IKK (Figure 4.5A). This shows that MLK3 is not an IKK kinase in this cell type, as was described in HeLa cells. Furthermore, we found that PD98059 prevented ceramide and TNFα activation of IKK (Figure 4.5B), suggesting that they also signal through MEK1 to IKK in adipocytes.

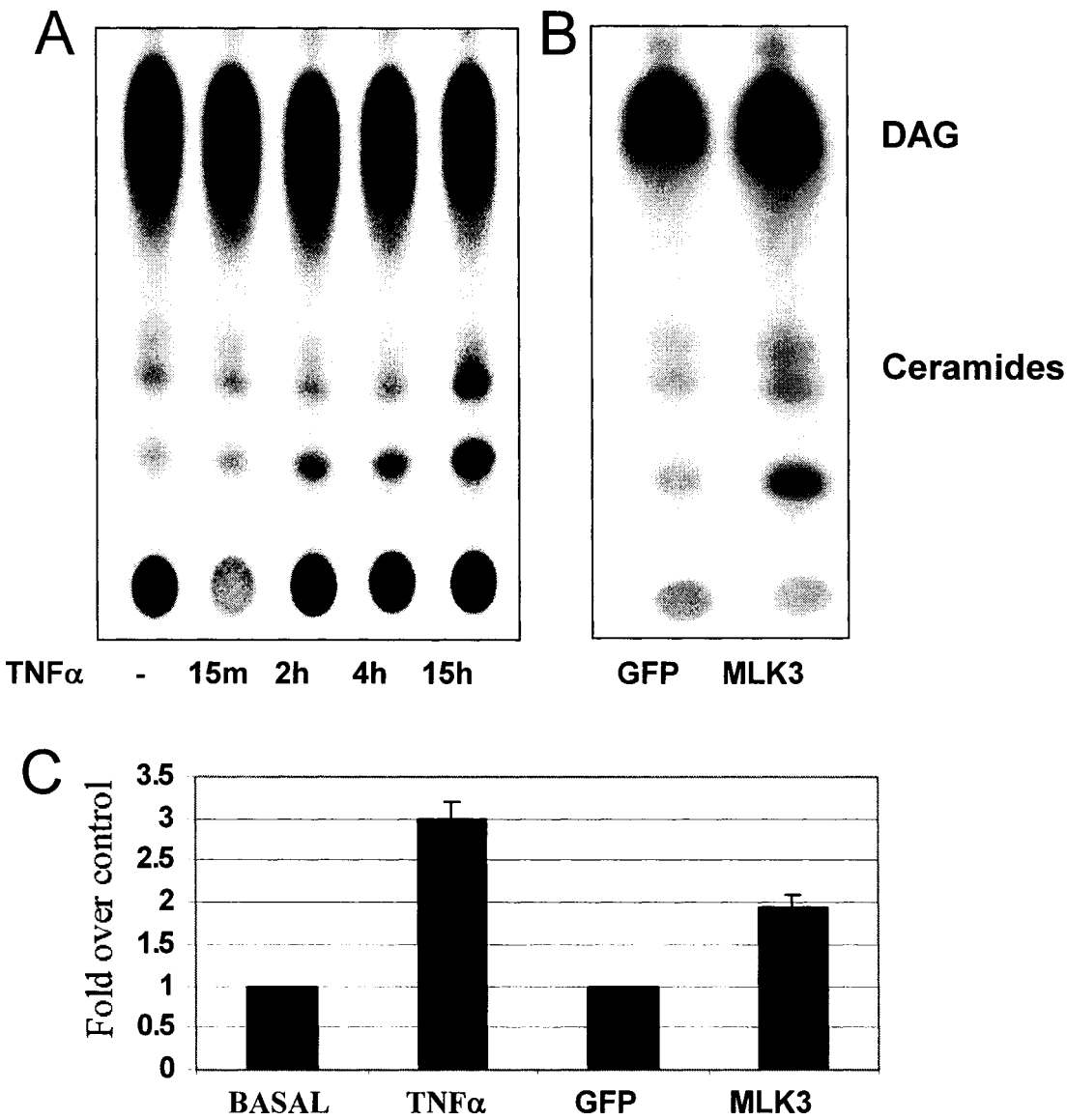

Figure 4.4 Ceramide and DAG assays showing (A) a time course for 20ng/mL TNFα exposure and (B) the effect of MLK3 overexpression on ceramide, but not DAG accumulation. (C) Represents the average fold ceramide induction for 16h of TNFα treatment and 24h MLK3 overexpression respectively. Error bars represent standard deviation of three independent experiments.

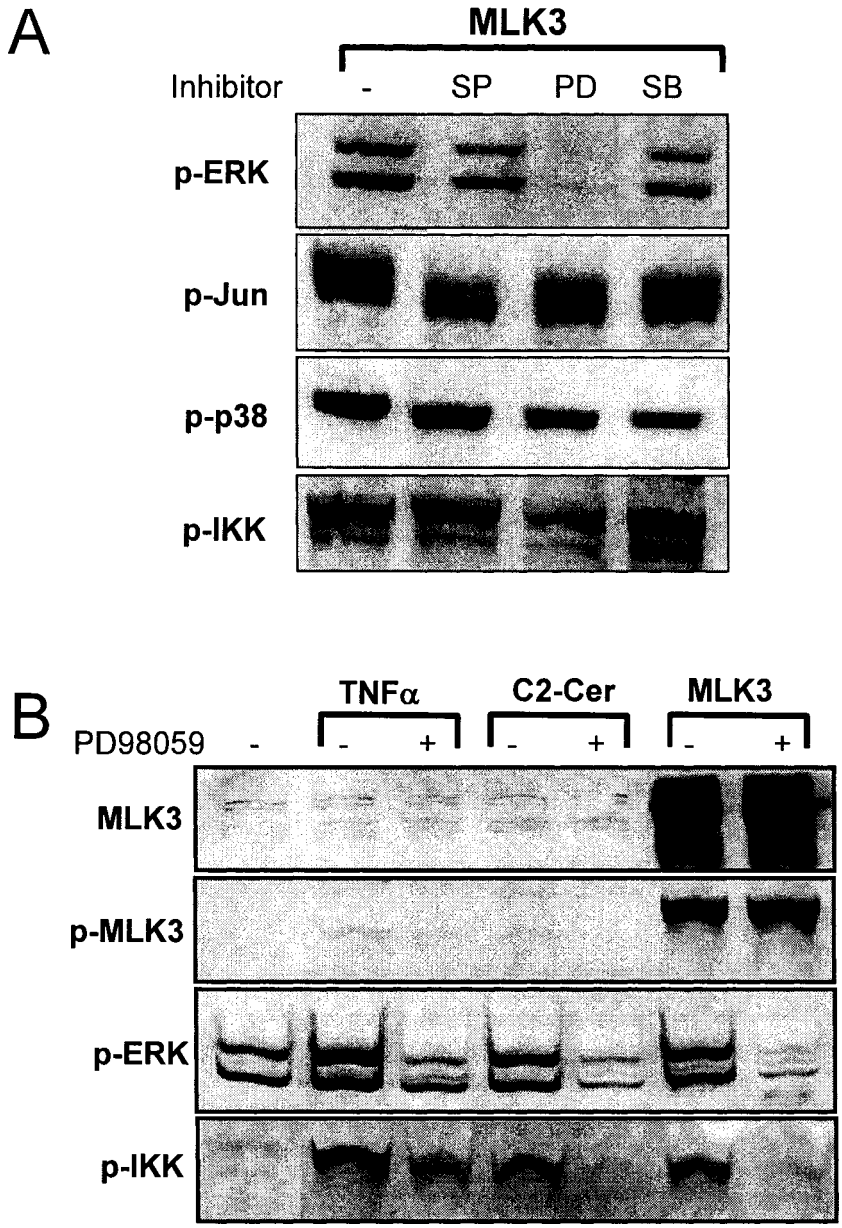

Figure 4.5 (A) Specific JNK (SP), MEK1 (PD), and p38 (SB) inhibitors prevent their activation by MLK3. (B) The MEK1 inhibitor prevented MLK3, TNFα, and ceramide induced activation of IKK illustrating the requirement for MEK1 in IKK activation by these stimuli. MLK3 was over-expressed 24h, ceramide was administered for 90 minutes at 100μM, and TNFα was administered for 10 minutes at 20ng/mL. The inhibitors were given three hours before harvest at the following concentrations: SP600125 (SP), 50μM; PD98059 (PD), 30μM; and SB203580 (SB), 16μM. Data is representative of three similar experiments.

4.4e Inhibiting MLK3 prevents TNFα and ceramide signaling

CEP-11004 is a drug derivative of the indolocarbazole natural product (+)K-252a [192]. CEP-11004 prevents cell death in neurons by preventing JNK activation through the inhibition of mixed lineage kinases [193]. CEP-11004, has been used successfully for the inhibition of MLK3 in cultured cells [194, 195]. Recent studies reveal that CEP-11004 prevented TNFα induced activation of JNK, but not p38 or ERK, in Jurkat cells [178]. We utilized CEP-11004 to determine the role of MLK3 in TNFα-mediated events in adipocytes.

In CAR adipocytes over-expressing MLK3, CEP-11004 prevented MLK3 phosphorylation and activity in a dose dependent manner (Figure 4.6A and B). In adipocytes with no transgene, CEP-11004 prevented TNFα from activating p38, ERK, JNK, and IKK phosphorylation (Figure 4.7A). Interestingly, the drug also blocked ceramide stimulation of JNK, but not p38, ERK, or IKK (Figure 4.7B). To provide evidence that the effects seen with CEP-11004 were not due to the inhibition of molecules upstream of the MAP kinases or IKK, we treated adipocytes with epidermal growth factor (EGF) in the presence of CEP-11004. We found that CEP-11004 had no effect on EGF stimulated ERK or IKK phosphorylation (Figure 4.7C). Paired with the specificity seen with ceramide stimulation and inhibition of signaling only to JNK, this suggests that CEP-11004 is not preventing generalized MAPK and IKK activation. These data show that MLK3 is a necessary intermediate linking TNFα to the activation of the IKK and MAPK cascades. Additionally, we show that CEP-11004 prevented full ceramide accumulation induced from TNFα treatment (Fig 4.7D).

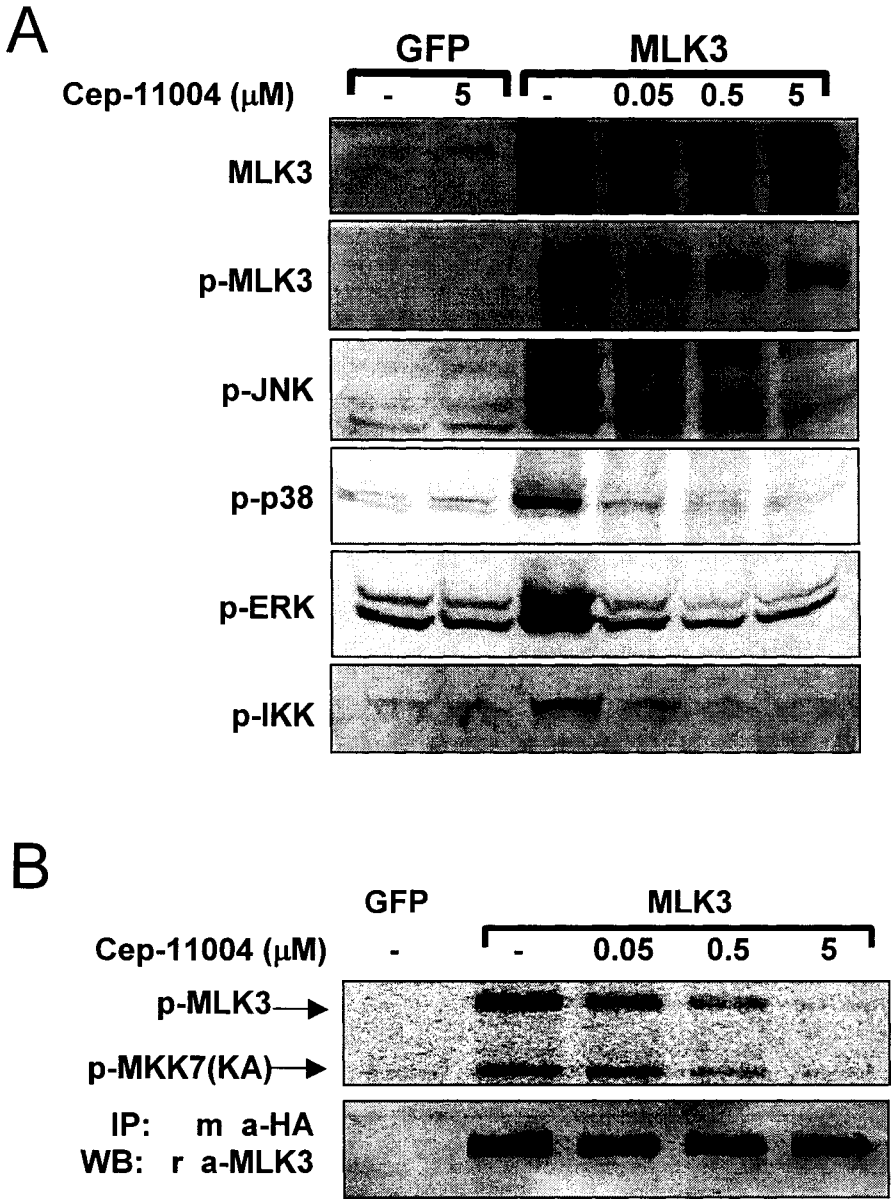

Figure 4.6 CEP-11004 prevents MLK3 phosphorylation (A) and activity (B) in a dose dependent manner. In (A), the MLK3 inhibitor reduced MLK3 phosphorylation and prevented its activation of MAPK and IKK pathways. In (B), MLK3 kinase activity was inhibited by CEP-11004 in a dose dependent manner.

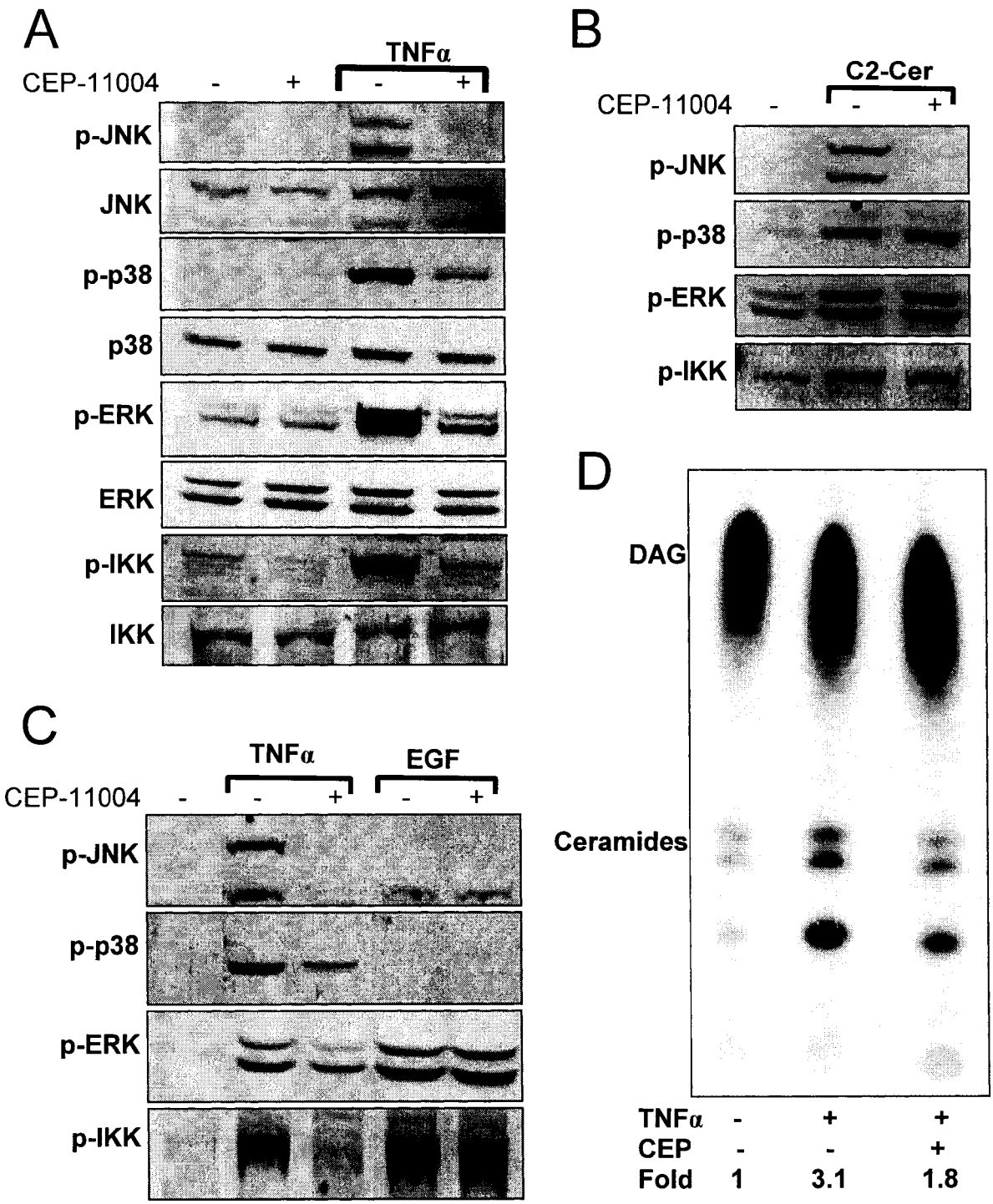

Figure 4.7 CEP-11004 blocks TNFα stimulated signaling to all MAPK and IKK (A), and ceramide signaling to JNK but not p38, ERK, or IKK (B). Additionally, CEP-11004 does not prevent EGF stimulated ERK or IKK activation (C). CEP-11004 also partially prevents ceramide accumulation by TNFα (D). TNFα was administered at 20ng/mL for 10m in (A) and (C), and 16h in (D). EGF was administered for 5m at 5nM in (C), and ceramide was administered for 90m at 100μM in (B). CEP-11004 was used at 0.5μM and was administered 3h before harvest, except in (D) when it was added for 18h.

4.4f MLK3 inhibits insulin signaling similar to TNFα

TNFα and ceramide inhibit insulin signaling to Akt/PKB through different mechanisms. TNFα's antagonism of insulin signaling starts as far upstream as reduced tyrosine phosphorylation of the insulin receptor, with subsequently reduced activity of all intermediates leading to Akt/PKB (Figure 4.8A). C2-ceramide induced antagonism of insulin signaling occurs through a different mechanism as it prevents Akt/PKB phosphorylation, but does not interfere with insulin signaling upstream of Akt/PKB (Figure 4.8B). We show here that MLK3 overexpression inhibits insulin signaling by a mechanism similar to TNFα (Figure 4.8C). In an effort to determine the mechanism of MLK3 induced antagonism of insulin signaling, we utilized inhibitors to determine if MAP kinases were necessary for the repressive effects of MLK3 on insulin signaling. We found that MEK1 inhibition was necessary for MLK3 induced antagonism of insulin signaling to Akt/PKB (Figure 4.9).

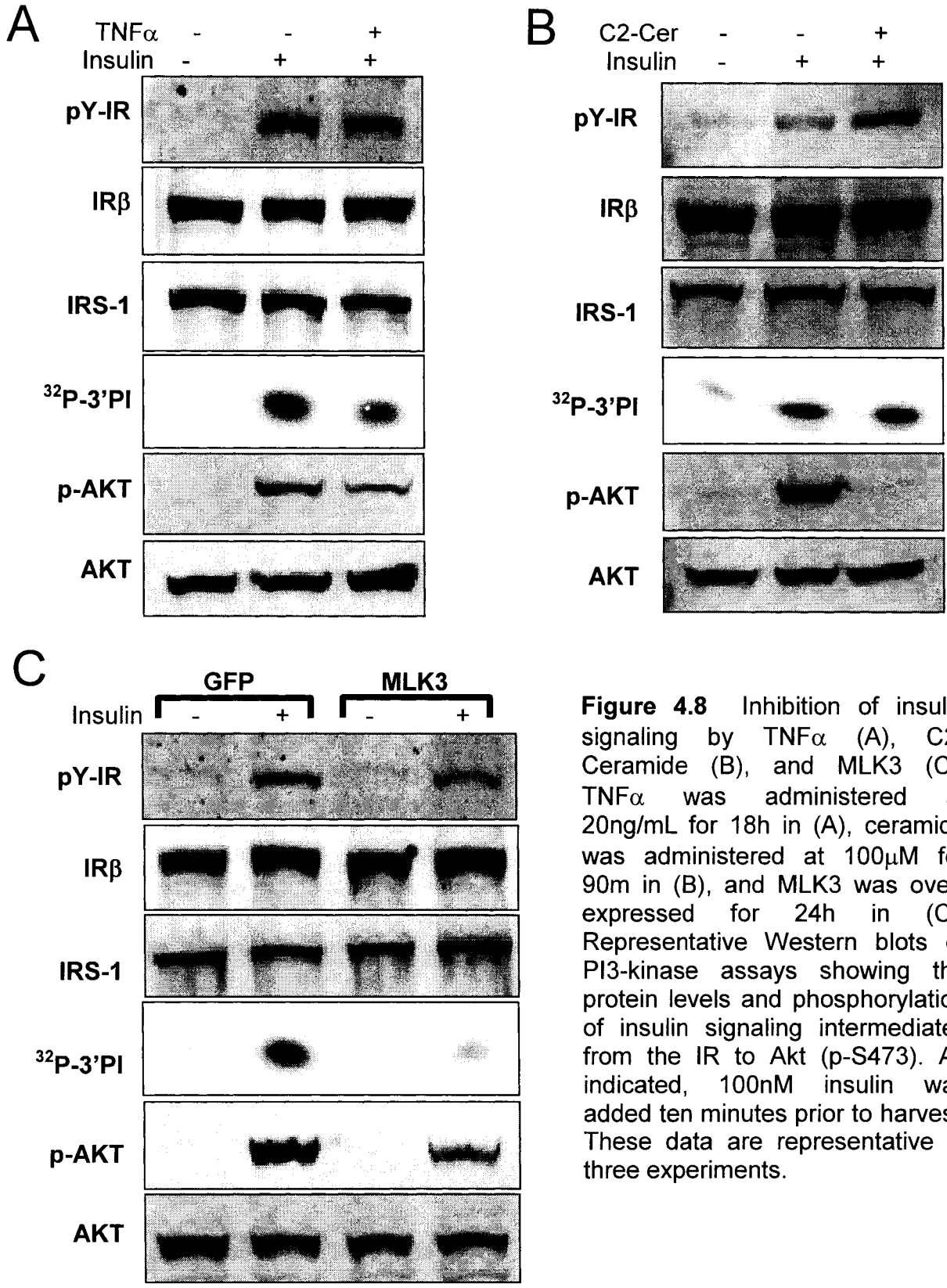

Figure 4.8 Inhibition of insulin signaling by TNFα (A), C2-Ceramide (B), and MLK3 (C). TNFα was administered at 20ng/mL for 18h in (A), ceramide was administered at 100μM for 90m in (B), and MLK3 was over-expressed for 24h in (C). Representative Western blots or PI3-kinase assays showing the protein levels and phosphorylation of insulin signaling intermediates from the IR to Akt (p-S473). As indicated, 100nM insulin was added ten minutes prior to harvest. These data are representative of three experiments.

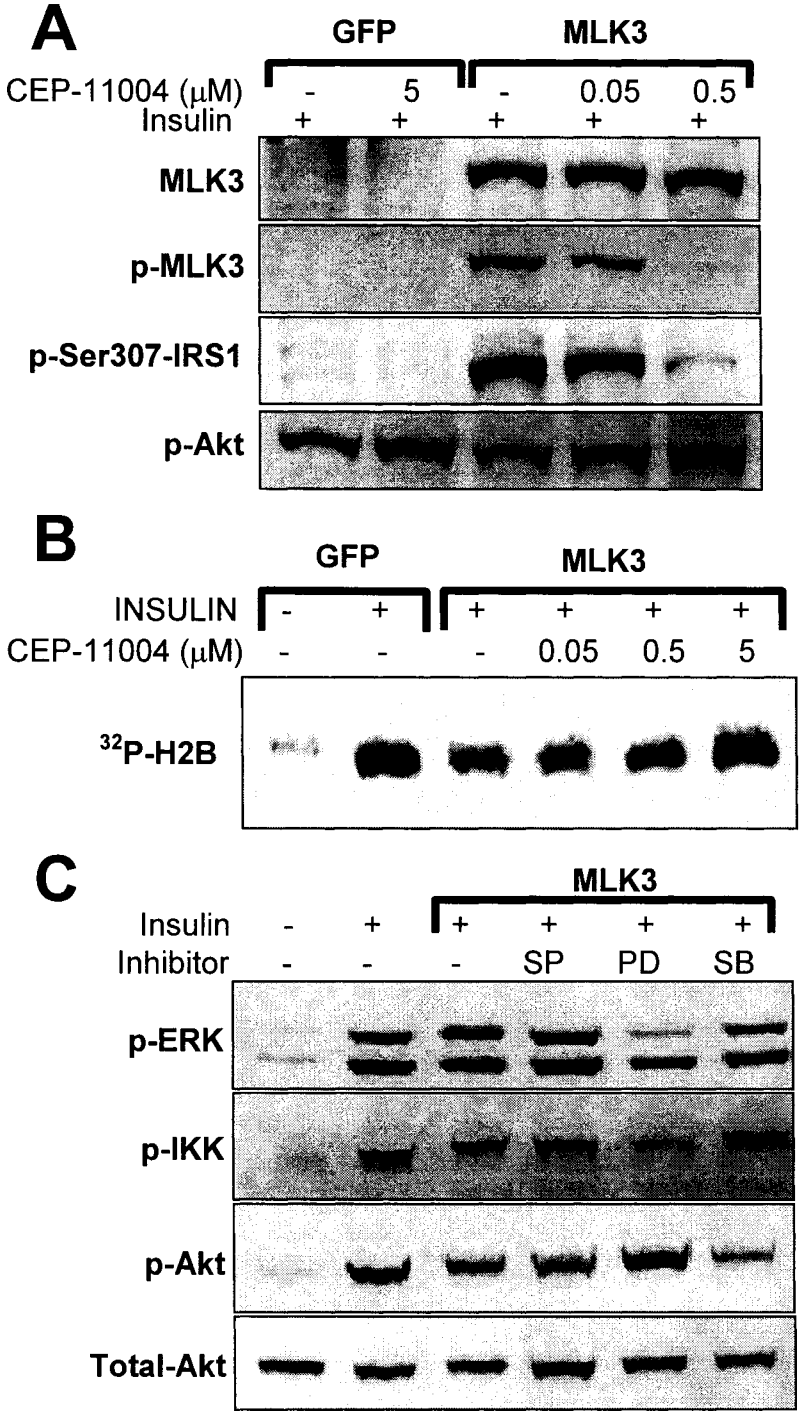

Figure 4.9 CEP-11004 prevents inhibitory MLK3-induced serine 307 phosphorylation of IRS-1, re-establishing insulin signaling to Akt (A) and restoring Akt kinase activity (B). Additionally, MAP kinase inhibitors were used with MLK3 overexpression for 20h at the concentrations mentioned previously. We discovered that MEK1 inhibition with PD98059 prevented MLK3's antagonism of insulin signaling to Akt/PKB (C). These data are representative of two experiments.

4.5 Discussion

The growing epidemic of obesity is a mounting concern as it is a metabolic disease associated with insulin resistance and the pathogenesis of type 2 diabetes, metabolic syndrome X, hypertension, dyslipidemia, and cardiovascular disease, etc. The association between insulin resistance and increased adiposity is well established, and the pro-inflammatory molecule TNFα is a major factor that appears to be a link between obesity and insulin resistance.

An abundance of data implicates TNFα in insulin resistance, but the molecular mechanisms underlying its inhibitory effects and precise signaling pathways remain elusive. In an effort to dissect the pathway between TNFα and MAPK/IKK activation, we focused our attention on MLK3, a MAP3K and IKK kinase activated by TNFα and ceramide in other cell types. We analyzed MLK3 overexpression and inhibition in order to determine its role in TNFα and ceramide signaling in the adipocyte.

In this study, we show that MLK3 overexpression mimics the effects seen with TNFα stimulation. Our data show that inhibition of MLK3 prevented TNFα mediated signaling, and partially prevented TNFα-induced *de novo* ceramide accumulation. The observed portion of ceramide accrual not repressed by MLK3 inhibition is possibly due to SMase activity, as MLK3 is not an intermediate linking TNFα to SMase activity. Additionally, the lack of SMase activation by MLK3 is likely why we observed less ceramide accumulation with MLK3 overexpression than with TNFα stimulation. MLK3 induced ceramide accrual occurs through the *de novo* synthesis of ceramide, as inhibition of this pathway

with the serine palmitoyltransferase inhibitor, cycloserine, prevented MLK3 induced ceramide accumulation completely (data not shown). Thus, the observed inhibition of TNFα-induced ceramide accumulation by CEP-11004 is likely due to the inhibition of MLK3, and prevention of the *de novo* ceramide pathway.

Interestingly, the MLK3 inhibitor selectively prevented ceramide signaling to JNK, but not to other MAP kinases or IKK, suggesting that ceramide has redundant pathways to stimulate p38, ERK, and IKK activation, but requires MLK3 to activate JNK. Significantly, this suggests that ceramide accumulation is not the mechanism responsible for TNFα-induced MLK3 activation since the MLK3 inhibitor prevents all TNFα signaling events.

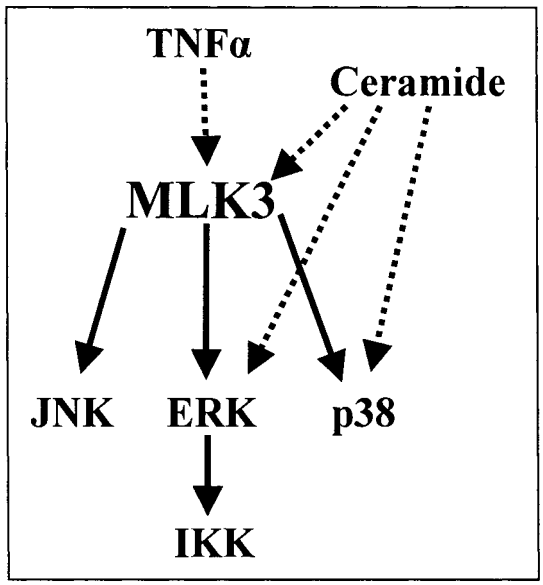

Figure 4.11 Proposed pathways of MAPK and IKK activation by ceramide and TNFα. Dotted lines indicated incompletely understood mechanisms of activation.

We also illustrate that MLK3, TNFα, ceramide, and insulin all require MEK1/ERK pathway for IKK activation (Figure 4.11). Additionally, we show that MEK1 inhibition prevents MLK3-induced antagonism of insulin signaling to Akt/PKB. However, because MEK1 is requisite for IKK, it remains to be shown whether ERK or IKK is responsible for MLK3 induced insulin antagonism. Other studies have shown that TNFα signaling was sensitive to MEK1 inhibition [169], yet this study is the first to show that MEK1 is required for IKK activation in adipocytes.

Interestingly, TNFα did not affect insulin signaling during peak MAPK or IKK activation. In fact, J. A. Engelman, et al. [169] also observed the necessity of prolonged TNFα exposure in 3T3-L1 adipocytes for the inhibition of insulin signaling. In our study, we found that TNFα was only effective in inhibiting insulin signaling during prolonged exposure of 12-16 hours or more. This argues that transient activation of MAPKs, IKKs, and SMase-induced ceramide accumulation are not sufficient to block insulin action in this cell type. Therefore, changes in gene expression and/or *de novo* ceramide accumulation may be the mechanisms by which TNFα creates insulin resistance in white adipocytes.

In May 2005, D. Brancho et al. published the first study on MLK3 knockout mice [196], showing that MLK3 is required for TNFα signaling to JNK in embryonic fibroblasts (MEFs). Adipocytes were not evaluated directly, and therefore this study does not exclude MLK3 as a required intermediate for TNFα stimulated pathways other than JNK in adipocytes. Moreover, this phenotype would be expected if TNFα induced significant sphingomyelin hydrolysis, as we

show that ceramide signals exclusively through MLK3 to activate JNK, yet has alternative pathways to activate p38, ERK, and IKK.

4.6 Summary

We have shown that the over-expression of MLK3 mimics many of the effects of TNFα exposure to adipocytes, and that the inhibition of MLK3 prevented all TNFα signaling events. We also observed that the mechanism of MLK3 induced antagonism of insulin signaling occurs through MEK1 activity, and additionally that MEK1 is a required intermediate for IKK activation by many stimuli. These data suggest that MLK3 is a necessary intermediate for TNFα stimulation of MAPK and IKK pathways, and we speculate that MLK3 inhibitors are promising drugs for the antagonism of TNFα-induced inhibition of insulin signaling.

4.7 Acknowledgements

This work was supported by the National Institutes of Health (R01-DK58784 to SAS), the American Diabetes Association (Career Development Award to SAS), and the American Heart Association (Predoctoral Fellowship to KLH).

We would like to thank Dr. Phil Barker (McGill University, Canada) for the adenovirus expressing HA-MLK3 and Dr. Kathleen Gallo for kindly providing the kinase inactive MLK3 substrate MKK7-KA. The MLK3 inhibitor CEP-11004 was a generous gift from Cephalon, Inc (West Chester, PA).

CHAPTER 5

Conclusions and Future Perspectives

Signaling pathways regulating anabolic metabolism are altered by obesity. The studies described above evaluated how these pathways are affected during weight gain in an animal model of obesity, and investigated the pathway linking TNFα, a fat derived antagonist of insulin signaling, to insulin resistance. The studies summarized below highlight our conclusions thus far, and propose future studies to build upon these findings.

5.1 Novel Mechanism for Akt/PKB Regulation in Hibernators

The first study, which evaluated anabolic metabolism in marmots as they progressively increased in adiposity, gave surprising results. We performed this study based on the hypothesis that as these animals undergo a doubling of body mass in the fall, largely in the form of fat, they would develop insulin resistance and display altered regulation of anabolic metabolism. In fact, the animals do exhibit many characteristics of peripheral insulin resistance associated with weight gain, including hyperinsulinemia, and they do show seasonal alterations in basal activity of Akt/PKB, a major regulator of anabolic metabolism. However, the surprising part of this study was that the seasonal changes in Akt/PKB activity were also tissue specific. In particular, Akt/PKB activity in WAT peaked concurrent with a climax in body mass and adipocyte size, while, Akt/PKB activity in skeletal muscle peaked two months later, just before the animals entered hibernation and concurrent with their conversion to a lipolytic and fasting state.

The tissue specific activation of Akt/PKB suggests that a universal circulating factor that acts indiscriminately does not control its activity under

fasting conditions. This argues for the existence of either an intrinsic or a tissue specific mechanism for increasing fasting Akt/PKB activity. Therefore, the potential exists for novel circulating factor(s) that lead to Akt/PKB activation in targeted tissues despite insulin resistance.

Forthcoming studies will evaluate the role of adipocytes in this novel mechanism. Our collaborators have endeavored to find novel molecules that are upregulated in the hibernating vs. pre-hibernating marmot through suppression subtractive hybridization techniques. The first studies will be performed in adipocytes, as they are major endocrine factories with roles in the regulation of anabolic metabolism. Additionally, the evaluation of other recognized PI3-Kinase independent mechanisms of Akt/PKB activation will be performed in these tissues. Specifically, molecules that elevate PKA activity have been shown to activate Akt/PKB only on the Thr-308 residue, similar to our observations in this study. Previous studies showing PKA activation of Akt/PKB have been performed in COS (African Green Monkey kidney) cells, therefore, it will be interesting to determine if PKA stimulates Akt/PKB in muscle or adipose tissue.

5.2 Linking TNFα to MLK3 in Adipocytes

This study was initiated due to the discovery that TNFα and ceramide were both agonists of a common protein, MLK3. MLK3 is a MAPKKK and IKKK in specific cell types, and is thus upstream of MAPKs and IKKs, which are important regulators of insulin sensitivity. Due to these studies, we hypothesized that MLK3 plays a role in TNFα and ceramide induced antagonism of insulin

signaling. Therefore, we investigated a role for MLK3 in signaling events triggered by these molecules in adipocytes. Our studies show that MLK3 overexpression mimics TNFα and ceramide signaling events nearly exactly in these cells. Additionally, we show that pharmacological inhibition of MLK3 completely blocked TNFα-stimulated signaling events. However, the inhibitor selectively blocked ceramide signaling to JNK, but not to the other classes of MAPK or IKK. These data show that 1) the MLK3 inhibitor is not non-specifically blocking the ERK, p38, or IKK effects seen with TNFα stimulation; 2) ceramide has alternative pathways to activate ERK, p38, and IKK in fat cells, but requires MLK for signaling to JNK; 3) MLK3 is an obligate intermediate in TNFα signaling to all MAPK and IKK molecules; and 4) ceramide is not likely to be the mechanism for TNFα stimulation of MLK3 in adipocytes.

Impending studies will be performed to confirm the inhibitor results above. Specifically, we will evaluate TNFα and ceramide signaling in 1) adipocytes underexpressing MLK3 by RNA interference, using a new electroporation method; and 2) in differentiated MLK3 -/- adipocytes derived from a MLK3 knockout mouse. Other studies will be centered on identifying the mechanism for MLK3 activation by TNFα in adipocytes. We have previously explored the possibility that TNFα induced SMase activity leads to a specific ceramide pool that activates MLK3, however, inhibitors for SMases failed to prevent downstream TNFα signaling. An alternative pathway worth evaluating is Cdc42 activation of MLK3. Both TNFα and ceramide activate Ras-GTP in fibroblasts [197]. This stimulates Ras binding to PI3K, triggering the activation of Rac and

Cdc42 [198]. Cdc42 is an allosteric activator of many p21-activated kinases, including MLK3 [199].

5.3 Determining the roles of TNFα and MLK3 in *de novo* ceramide accretion

We also observed an effect of MLK3 on ceramide accumulation. It appears that this effect arises from *de novo* ceramide synthesis, as cycloserine, an inhibitor of serine palmitoyltransferase (SPT, the rate limiting enzyme in *de novo* ceramide synthesis), blocks MLK3 induced ceramide accumulation. Further studies will determine how TNFα and MLK3 stimulate *de novo* ceramide synthesis, and if MAPK or IKK inhibitors block this effect. Specifically, we will measure ^{3}H-palmitate incorporation into ceramide to confirm *de novo* synthesis, and to verify earlier results that inhibition of MLK3 prevents TNFα induced *de novo* ceramide accumulation.

5.4 Identifying a Role for MLK3 in Insulin Resistance

Studies evaluating the role of MLK3 in TNFα and ceramide induced antagonism of insulin signaling are currently being conducted. We have identified that the MLK3 inhibitor, only when combined with a PP2A inhibitor will prevent ceramide antagonism of Akt/PKB (data not shown). This shows that MLK3 is not involved in ceramide's stimulation of PP2A activity, but likely has a role in defective Akt/PKB translocation to the plasma membrane. Future studies will be conducted to evaluate this possibility. Additionally, the data remain

inconclusive for whether inhibition of MLK3 blocks TNFα antagonism of Akt/PKB. Further experiments will be performed by RNA interference of MLK3 expression in adipocytes, and through the use of cells derived from MLK3 knockout mice. Added studies will also evaluate the necessity of MLK3 in TNFα and ceramide antagonism of anabolic events such as glucose uptake.

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