Abstract

Benjamin T. Bikman. Modulation of IKKβ with AMPK Improves Insulin Sensitivity in Skeletal Muscle. (Under the direction of Dr. G. Lynis Dohm) Department of Physiology and Exercise and Sport Science, October 2008.

Activation of the insulin receptor substrate (IRS)-1 is necessary for proper transduction of the insulin signal. IRS-1 serine$^{312(human)/307(rodent)}$ phosphorylation, however, results in disruption of this signal and subsequent insulin resistance. Inflammatory mediators, such as the inhibitor of κB kinase β (IKKβ), interrupt insulin signaling within insulin-sensitive tissues, such as skeletal muscle. In contrast, AMP-activated protein kinase (AMPK), a key metabolic enzyme, increases insulin sensitivity. To study the association of IKKβ and AMPK, muscle biopsies were taken from post-bypass patients and control subjects. Post-bypass patients displayed similar levels of IKKβ action and IRS1-pSer^{312} as the lean subjects and reduced levels compared with weight-matched control and morbidly obese subjects, which supports evidence that IKKβ phosphorylates IRS-1 Ser^{312} and reduces the insulin signal, resulting in reduced whole-body insulin sensitivity. However, these findings did not correlate with baseline differences in AMPK phosphorylation.

To explore the role of AMPK in attenuating IKKβ activity and ameliorating insulin resistance, lean and obese Zucker rats were treated with metformin, an AMPK activator. Despite no differences at baseline, AMPK action was increased with metformin treatment in a fiber-type specific (white muscle) manner with a reduction in IKKβ activity and IRS1-pSer^{307} in the muscle of obese rats only.

To explore this relationship further, myotubes from lean and morbidly obese humans were exposed to the saturated fatty acid palmitate in the presence or absence of AICAR to activate AMPK. In contrast to the lean, obese myotubes demonstrated no depression in insulin signaling with lipid exposure. Co-incubation with AICAR prevented the lipid-induced decay of the insulin signal in the lean and improved the signal in the obese. AICAR-stimulated AMPK activation for the final 4 h of lipid exposure was not sufficient to restore phosphorylation of AS160.

These data support the hypothesis that lipids induce insulin resistance via lipid-sensitive serine kinases that attenuate insulin signaling. Whereas AMPK activity does not appear to play a role in baseline differences of IKKβ action and insulin signaling, we present findings that the lipid-induced decay in the insulin signal can be prevented by acute activation of AMPK. These results provide valuable information explaining differences in skeletal muscle function in the lean and obese, as well as elucidating a potential mechanism for insulin-sensitizing, AMPK-activating agents.

Modulation of IKKβ with AMPK Improves Insulin Sensitivity in Skeletal Muscle

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Modulation of IKKβ with AMPK Improves Insulin

Sensitivity in Skeletal Muscle

by

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Abbreviations

AICAR,	5-amino-4-imidazole riboside
AMP,	adenosine monophosphate
AMPK,	adenosine monophosphate-activated protein kinase
ANOVA,	analysis of variance
AS160,	akt substrate of 160 kDa
ATP,	adenosine triphosphate
BMI,	body mass index
BSA,	bovine serum albumin
DAG,	diacylglycerol
ECL,	enhanced chemiluminescence
GLUT4,	glucose transporter 4
HFD,	high-fat diet
HOMA,	homeostasis model assessment
IκB	inhibitor of κB
IKK,	inhibitor of κB kinase
IMCL,	intramyocellular lipid
IP,	immunoprecipitation
IR,	insulin receptor
IRS-1,	insulin receptor substrate 1
IVGTT,	intravenous glucose tolerance test
JNK,	c-Jun-N-Terminal Kinase

kDa,	kilodalton
LCACoA,	long-chain acyl-coenzyme A
LPS,	lipopolysaccharide
NF-κB,	nuclear factor κB
OGTT,	oral glucose tolerance test
PDK,	phosphoinositide-dependent kinase
PI3K,	phosphatidylinositol-3 kinase
PIP_3,	phosphatidylinositol 3,4,5-trisphosphate
PKC,	protein kinase C
PTB,	phosphotyrosine binding
RG,	red gastrocnemius
SE,	standard error
S_I,	insulin sensitivity index
TG,	transgenic
TNF,	tumor necrosis factor
TZD,	thiazolidinedione
WG,	white gastrocnemius

Chapter One

Introduction

As a result of our modern western lifestyle, people are suffering from unique infirmities rarely seen in previous generations. Among these is obesity—the second leading cause of preventable death in the country and a problem that is becoming increasingly prevalent in our society. Over 66% of adults in the U.S. are considered overweight or obese, with 4.8% being extremely obese—a category that did not exist previously. Additionally, among children and adolescents, over 17% are considered overweight with over 33% 'at risk' (Ogden et al., 2006). Moreover, an overweight child has a 70% chance of being an overweight or obese adult (*The Surgeon General's Call To Action To Prevent and Decrease Overweight and Obesity*, 2007). Once thought to be a malady unique to western, wealthy societies, obesity is now a concern in less affluent countries as well (Popkin, 1998; Vainio, Kaaks, & Bianchini, 2002).

Obesity results in a host of health complications, including cardiovascular disease (CVD) (Sjostrom, Lissner, Wedel, & Sjostrom, 1999), which accounts for more deaths in the U.S. than any other disease, hypertension (Wilson, D'Agostino, Sullivan, Parise, & Kannel, 2002), osteoarthritis (Hart & Spector, 1993), and type 2 diabetes mellitus (Flegal, Graubard, Williamson, & Gail, 2007). More specifically, being obese increases the risk of developing diabetes to a relative risk of 40 and 60.9 compared to lean individuals in women and men, respectively (Chan, Rimm, Colditz, Stampfer, & Willett, 1994; Colditz, Willett, Rotnitzky, & Manson, 1995; Malnick & Knobler, 2006). Moreover, approximately

half of obese and overweight people have significant insulin resistance, a hallmark of type 2 diabetes mellitus (McLaughlin et al., 2001).

Research within the last decade has revealed that one possible link between obesity and insulin resistance is the NF-κB pathway and its associated inflammatory cytokines that stems from the chronic and excessive lipid circulation and accumulation in non-adipose tissue sites, which is a prominent feature of obesity (Wellen & Hotamisligil, 2005). In contrast to the insulin-resistant effects of excessive lipid, AMP-activated protein kinase (AMPK), a key metabolic enzyme, acts to improve insulin sensitivity, potentially counter-acting the deleterious effects of lipid excess. Inasmuch as skeletal muscle is the main consumer of glucose *in vivo*, studying the effects of lipid-induced inflammatory cytokines on insulin-mediated glucose uptake in skeletal muscle, and the role of AMPK in possibly mitigating this response, holds great potential for addressing the obesity-diabetes relationship.

Insulin Signaling Pathway

The process through which insulin binds to a receptor and ultimately leads to the uptake of glucose into the cell has been the focus the extensive research for decades, yet despite this research, the precise pathway is not fully understood. Glucose diffuses across the plasma membrane very slowly and inasmuch as glucose is the primary fuel for most cells of the body, it is imperative that the cell have a way whereby it can rapidly allow glucose to enter the cell. As such, many cell types in the body, including myocytes, red blood cells,

adipocytes, and hepatocytes, possess glucose transporters, with glucose transporter-4 (GLUT4) being the key insulin-responsive transporter. Whereas the intermediates in the insulin signaling pathway are not fully understood, the primary step, the binding of insulin to its receptor, and the final step, the translocation of GLUT4 from intracellular vesicles to the plasma membrane, are well characterized (Bryant, Govers, & James, 2002).

The insulin signal pathway begins with the insulin receptor, which is a transmembrane heterotetrameric glycoprotein. Two of the receptor's subunits protrude into extracellular space with two intracellular subunits that contain tyrosine residues (Ullrich et al., 1985). The insulin receptor is capable of autophosphorylation at these tyrosine residues and they subsequently phosphorylate the insulin receptor substrate (IRS)-1, among others, which is a pivotal step in the transduction of the activation of the insulin signal. Upon activation, phosphorylated IRS-1 recruits a number of effectors, including phophoinositol 3-kinase (PI3K), which is a heterodimer of a catalytic (p110) and regulatory (p85) subunit. The p85 subunit interacts with IRS-1, leading to increased PI3K activity, and PI3K, in turn, causes the production of phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 recruits Akt to the plasma membrane by supplying docking sites that place Akt in close proximity to its upstream activator, phosphatidylinositol-dependent kinase-1 (PDK-1)(Bryant et al., 2002). Until very recently, the activation of Akt was thought to be the concluding step prior to GLUT4 translocation. However, a substrate of Akt has

been identified that offers a link between Akt and GLUT4 mobilization. This link, an Akt substrate of 160 kDa (AS160), is a GAP protein—a family of GTPases that regulate numerous membrane trafficking processes (Deneka, Neeft, & van der Sluijs, 2003). The proposed role for AS160 in regards to GLUT4 is that phosphorylation of AS160 inhibits its GAP activity; consequently, the GTP form of a Rab(s) required for GLUT4 translocation is elevated, and thus translocation is triggered (Miinea et al., 2005; Sano et al., 2003). Finally, upon reaching the plasma membrane, GLUT4 proteins form an aqueous pore across the membrane through which glucose can move.

Insulin Receptor Substrate (IRS)-1 Dysfunction

Due to its location at the proximal end of the pathway, any dysfunction in the action of IRS-1 can result in disruption of the entire cascade. Hence, this area has received a great deal of attention in recent years. Yamauchi et al. (Yamauchi et al., 1996) observed that skeletal muscle from IRS1-deficient rodents exhibited reduced insulin-stimulated glucose uptake. Further, it has been shown using short interfering (si) RNA-mediated ablation of IRS-1 that the loss of IRS-1 function results in reduced insulin-stimulated glucose uptake, Akt phosphorylation, and GLUT4 translocation (Huang, Thirone, Huang, & Klip, 2005).

A unique characteristic of IRS-1 is the presence of multiple tyrosine phosphorylation sites (Thirone, Huang, & Klip, 2006). Whereas tyrosine phosphorylation of insulin-receptor substrate (IRS)-1 by the insulin receptor is

necessary for normal signaling, the phosphorylation of other sites may disrupt normal function. In particular, serine phosphorylation of IRS-1 has been implicated in inhibiting insulin signal transduction (Cai et al., 2005; Yuan et al., 2001) and the IRS-1 $Ser^{312\ (Human)/307(rodent)}$ residue has received particular interest given its proximity to the phosphotyrosine-binding (PTB) domain in IRS-1. IRS-1 undergoes a conformational change as a result of serine phosphorylation that decreases its ability to interact with the insulin receptor, making it a poor substrate for the insulin receptor (Aguirre et al., 2002).

Inflammatory Mediators Inhibit Insulin Signaling

An interesting area of research has developed in recent years, which explores the role of inflammatory mediators in metabolic functions. Whereas the immune and metabolic systems were once thoughts of as distinct, they appear to work jointly in maintaining healthy function. However, such a realization should come as no surprise given that these systems represent the most indispensable abilities within an organism—the ability to defend itself against infection and heal and the ability to store energy for times of low nutrient availability and high energy demand. For example, several hormones and cytokines not only mediate both metabolic and immune processes, but also operate through similar cellular mechanisms. As such, these systems are interdependent and work synergistically to maintain homeostasis.

The first molecular link between inflammation and metabolic function was the discovery that tumor necrosis factor α (TNFα) is overexpressed in adipocytes

of rodent models of obesity approximately 15 years ago (Hotamisligil, Shargill, & Spiegelman, 1993). Subsequent research has revealed similar results in human adipose tissue (Festa et al., 2001), as well as in rodent and human skeletal muscle (Borst & Conover, 2005; de Alvaro, Teruel, Hernandez, & Lorenzo, 2004; Jove et al., 2006). However, it has become clear that the presence and overexpression of TNFα is among a host of results that stem from the activities of a key player in the inflammatory, immune response, specifically the nuclear factor κB (NF-κB).

NF-κB has garnished a great deal of attention in recent years due to its newfound role in regulating metabolic activities. NF-κB was first discovered over 20 years ago and was thought to be unique to B cells (Sen & Baltimore, 1986), though now it is known to be ubiquitous. Within the cell NF-κB is retained in the cytoplasm in an inactive form bound to specific inhibitors, the IκB proteins. When bound in the cytosol by its inhibitors NF-κB is in an inactive form, however, upon degradation of IκB, which is an essential step in this process, NF-κB translocates to the nucleus and elicits transcription of a host of cytokines and other inflammatory mediators. The signal is eventually terminated through the export and cytoplasmic resequestration of NF-κB, which is contingent on the newly synthesized IκBα isoform entering the nucleus, binding NF-κB and causing its dissociation from the DNA strand (Arenzana-Seisdedos et al., 1997). Because IκBα, the best characterized isoform, is so critical to the NF-κB pathway, the study of its regulation revealed an upstream kinase named the inhibitor of IκB

kinase (IKK) (J. A. DiDonato, Hayakawa, Rothwarf, Zandi, & Karin, 1997). In fact, IKK is considered to be the key to activating the canonical NF-κB pathway (Karin & Ben-Neriah, 2000).

Of IKK's three subunits (α, β, γ), the function of the catalytic β subunit is the most thoroughly elucidated due to its interaction with and phosphorylation of IκBα (Klement et al., 1996). For IKKβ to induce the degradation of IκBα, IκBα must be phosphorylated at two serine residues, 32 and 36, which results in its polyubiquitinylation and subsequent degradation by the 26S proteosome (J. DiDonato et al., 1996; J. A. DiDonato, Mercurio, & Karin, 1995). Indeed, the presence or absence of IκBα is so reflective of IKKβ activity, and is so vital in this process, that it is frequently measured as indicator of IKKβ and NF-κB pathway activity (Bikman et al., 2008; Coll et al., 2006; Jove, Planavila, Laguna, & Vazquez-Carrera, 2005; Jove et al., 2006; Schenk & Horowitz, 2007).

Interestingly, IKKβ exacerbates insulin resistance through two known pathways. First, through activation of NF-κB, which stimulates the synthesis of multiple cytokines and activation of multiple kinases that disrupt insulin signal transduction (Bastard et al., 2006; Ropelle et al., 2006). Second, IKKβ is a serine kinase that has been implicated in causing deficient insulin signal transduction through its phosphorylation of IRS-1 $Ser^{312/307}$ (Arkan et al., 2005; Cai et al., 2005; McLaughlin et al., 2001; Yuan et al., 2001).

Excess Lipid Activates IKKβ and Inhibits Insulin Signaling

In addition to having multiple pathways of action, IKKβ itself is activated by multiple upstream events (Figure 1.1). Unsurprisingly, cytokines trigger IKKβ, but, interestingly, IKKβ is also activated by lipid accumulation and lipid metabolites, such as diacylglycoerol (DAG) (Itani, Ruderman, Schmieder, & Boden, 2002; Lee et al., 2006; C. Yu et al., 2002), and ceramide (Chavez et al., 2003; Schmitz-Peiffer, 2002; C. Yu et al., 2002), which have all been shown to be higher in insulin resistant states, such as obesity (Itani et al., 2002). While a tremendous amount of research has revealed an association between intramuscular triglyceride accumulation and insulin resistance, triglycerides are more and more perceived as the metabolically inert (Schenk & Horowitz, 2007) acquaintances of the more favored suspects just mentioned (DAG, ceramide). Evidence also supports the role of the activated form intracellular fatty acid—long-chain acyl-coenzyme A (LCACoA)(Nakamura & Nishizuka, 1994).

Recent work has effectively established the role of inflammatory cytokines in lipid-induced insulin resistance by preventing the inflammatory response. For example, *Ikk*$\beta^{+/-}$ mice have lower fasting glucose and insulin concentrations compared to *Ikk*$\beta^{+/+}$ littermates when on high-fat diets, demonstrating that a reduction in the *Ikk*β gene may protect against insulin resistance (Yuan et al., 2001). Additionally, IKKβ KO mice experience no decrement in insulin sensitivity in response to lipid infusion compared to control mice (Kim et al., 2001). Finally,

lipid infusion in humans resulted in significantly reduced levels of IκB, the NF-κB inhibitor that is degraded by IKKβ, during a 6-hr time course (Itani et al., 2002).

The excess lipid that results from the obese state may explain the observation that obesity is associated with an increased low-grade inflammatory tone, as evidenced by increased levels of proinflammatory cytokines and NF-κB activity (Bastard et al., 2006; Festa et al., 2001; Permana, Menge, & Reaven, 2006; Warnberg & Marcos, 2008). Our group has previously shown that obesity is associated with reduced insulin-stimulated glucose uptake (Dohm et al., 1988) and that weight loss, through both lifestyle intervention (Houmard et al., 2004) and surgery (Bikman et al., 2008; Gray, Tanner, Pories, MacDonald, & Houmard, 2003; Houmard et al., 2002), reduces intramuscular lipid and improves insulin sensitivity in humans. Moreover, inflammation is reduced with weight loss (Bastard et al., 2006).

AMPK Activation Improves Insulin Sensitivity through Improved Signaling and Reduced Inflammatory Activity

Whereas inflammatory cytokines and NF-κB pathway intermediates inhibit insulin signaling, AMPK plays a key role in increasing insulin sensitivity. In fact, a deficiency in AMPK activity has been implicated in the onset of type 2 diabetes (Winder & Hardie, 1999). In skeletal muscle, AMPK directs lipid and glucose metabolism in an effort to restore cellular levels of ATP, regulating energy balance within the cell (Carling, 2004; Hardie, 2004). As such, AMPK is potently activated by reduced ATP levels within the cell. AMPK can also be covalently

activated by upstream kinases. AMPK's role in the cell is evidently so vital to proper metabolic function that it is highly conserved in all eukaryotic species and ubiquitous in mammalian cells (Hardie, Scott, Pan, & Hudson, 2003).

Regardless of the stimulus, upon activation AMPK phosphorylates several downstream substrates with the general trend of upregulating ATP-generating pathways and inhibiting ATP-consuming pathways with the end result being a restoration of the AMP/ATP ratio (Winder, 2001). A host of insulin-sensitizing drugs have been shown to act, in part, through inhibition of NF-κB pathway activity, including salicylate (McLaughlin et al., 2001), thiazolidinediones (TZDs)(Ghanim et al., 2001; LeBrasseur et al., 2006; Todd, Watt, Le, Hevener, & Turcotte, 2007), and metformin (Cleasby et al., 2004; G. Zhou et al., 2001). Interestingly, evidence indicates that both TZDs and metformin increase activity of AMPK (LeBrasseur et al., 2006; Lessard et al., 2006; Ye et al., 2006). Two of the primary consequences of AMPK activation are an increase in glucose uptake by AMPK-driven phosphorylation of AS160 (Thong, Bilan, & Klip, 2007), which facilitates the insulin signal, and stimulating mitochondrial import and oxidation of fatty acids by disinhibiting carnitine palmitoyl transferase-1 (Ferre, Azzout-Marniche, & Foufelle, 2003; McGarry, 2002). Through this process AMPK likely prevents the accumulation of lipid metabolites that activate NF-κB pathway intermediates and disrupts insulin signal transduction (Fediuc, Pimenta, Gaidhu, & Ceddia, 2008). However, AMPK has also been shown to prevent cytokine-induced NF-κB pathway stimulation and result in reduced IKKβ action (Cacicedo,

Yagihashi, Keaney, Ruderman, & Ido, 2004; Cleasby et al., 2004; Hattori, Suzuki, Hattori, & Kasai, 2006; Hattori et al., 2008), suggesting a more direct effect of AMPK on the NF-κB pathway.

AMPK and Inflammatory Intermediates in Endothelial Cells and Liver

The initial research investigating the novel role of AMPK as a mediator in inflammation was conducted with endothelial cells. This research began in endothelial cells likely due to the interest in inflammation-induced atherosclerosis. As a result, it was determined that AMPK inhibits both fatty acid- and TNFα-induced increases in NF-κB in cultured endothelial cells (Cacicedo et al., 2004). When endothelial cell cultures were incubated with palmitate, inflammatory markers, i.e. VCAM-1, increased, but this response was attenuated in the presence of AICAR. Moreover, AICAR acted to prevent NF-κB activation in the presence of TNFα. This provides strong evidence in support of AMPK's role in attenuating inflammation. Additionally, metformin, an AMPK-activating drug, dose-dependently inhibited TNFα-induced NF-κB activation. Further, small interfering RNA for AMPKα1 attenuated metformin- and AICAR-induced inhibition of NF-κB activation by TNFα, further supporting a role for AMPK (Hattori et al., 2006). In a study with macrophages, cells were pretreated with AICAR for one hour followed by LPS infusion. The LPS insult failed to increase TNFα when preceded by AICAR incubation (Jhun et al., 2004).

Additionally, research involving metformin has displayed anti-IKKβ properties in the liver. Cleasby et al. (Cleasby et al., 2004) observed elevated

levels of IκBα in liver from metformin-treated rodents, providing indirect evidence that AMPK may be serving as an IKKβ inhibitor in an insulin sensitive tissues. These findings provide the rationale for exploring the potential role of AMPK as an IKKβ inhibitor in skeletal muscle inasmuch as muscle represents the main site of insulin-dependent glucose uptake.

AMPK and Inflammatory Intermediates in Skeletal Muscle

In skeletal muscle from sedentary rats, AICAR (an AMPK activator) and insulin co-incubation was shown to induce a twofold greater glucose uptake compared to insulin alone (Fisher, Gao, Han, Holloszy, & Nolte, 2002). Moreover, muscle contraction was shown to increase AMPK activity similar to that induced by AICAR, shedding light on potential mechanisms of contraction-mediated glucose uptake. Similarly, AICAR-perfused rat hindlimbs have been shown to increase glucose uptake compared with controls (Merrill, Kurth, Hardie, & Winder, 1997). In addition, reductions in AMPK activity are associated with diet-induced insulin resistance (Bonnard, Durand, Vidal, & Rieusset, 2008) and ablation of AMPK in skeletal muscle was observed to exacerbate lipid-induced insulin resistance (Fujii et al., 2008).

The idea of AMPK attenuating inflammatory activity in skeletal muscle has only recently been investigated and, due to a lack of research, a consensus has yet to be reached. For example, Steinberg et al. (Steinberg et al., 2006) observed that muscle cells with constitutively active AMPK were protected from TNFα-induced suppression of insulin-stimulated glucose uptake (TNFα has been

shown to elicit an increase in IKKβ activity). In *ob/ob* mice they found that AMPK activity was significantly reduced compared with lean controls and that TNFα neutralization in *ob/ob* mice restored AMPK activity to that of lean controls. Furthermore, obese mice exhibited reduced fatty acid oxidation, a defect that was not observed following TNFα neutralization. Lastly, *ob/ob* mice lacking a functional TNFα receptor (*TNF* $^{-/-}$) enjoy greater insulin sensitivity than control *ob/ob* mice. It was observed that AMPK activity was higher in obese *TNF* $^{-/-}$ relative to obese controls. Whereas the evidence provided by Steinberg et al. (Steinberg et al., 2006) places inflammatory mediators upstream of AMPK, the work by Hattori et al. (Hattori et al., 2006) in endothelial cells offer the opposite perspective—that AMPK inhibits NF-κB pathway activity. Interestingly, AICAR treatment in human myotubes has also been found to decrease cytokine production (Lihn, Pedersen, Lund, & Richelsen, 2008).

The role of AMPK in the etiology of insulin resistance has more recently been explored by observing the effects of HFD in mice expressing a muscle-specific transgenic ablation of AMPKα2 (Fujii et al., 2008). In addition to having a 50% reduction in insulin-stimulated glucose transport compared with wild type mice, muscle from the transgenic (TG) mice had reduction in insulin receptor β, IRS-1, and Akt. Moreover, whereas muscle DAG concentrations were not different between wild type and TG mice on the control diet, HFD caused an elevation in DAG levels in the TG mice only. Though not measured, it is

tempting to conclude that HFD TG mice might suffer from elevated IKKβ activity and inhibited insulin signaling.

AMPK Activity is Unclear in Obesity

Interestingly, recent studies suggest that there may be defective AMPK action in the presence of excessive lipid accumulation in obese subjects. This discovery may represent a mechanism by which lipid-induced insulin resistance and inflammation occur (Bruce, 2005; Chen, Liu, Sun, Tang, & Deng, 2005).

Despite obesity being causally linked with inflammation, the sparse amount of data linking obesity with reduced levels or activity of AMPK in humans are conflicting. Steinberg et al. (2004) and Bruce et al. (2005) observed no difference between lean and obese levels of skeletal muscle AMPK subunit mRNA as well as basal AMPK activity (and p-ACC). In contrast, the study by Bandyopadhyay et al. (2006) revealed very different results. In this study, obese subjects had far less total basal p-AMPK level and activity compared to lean subjects. Lastly, p-ACC was also dramatically reduced in obese compared to lean. It is clear that more research is warranted.

In contrast to humans, research investigating the same relationship in rodents is far more conclusive, establishing a clear reduction in AMPK activity with obesity (Barnes et al., 2002; Sriwijitkamol et al., 2006; Ye et al., 2006; X. Yu et al., 2004). In particular, obese insulin-resistant Zucker rats have reduced AMPK activity and related signaling abnormalities in skeletal muscle (Barnes et al., 2002; Bergeron et al., 2001).

In addition to elucidating the relationship between obesity and AMPK activity, it is worthwhile to explore the effects of high-fat feeding on AMPK activity. In one study, high-fat fed rodents experienced a significant decline in both level and activity of AMPK (Liu, Wan, Guan, Gao, & Zhao, 2006). *In vitro* studies have demonstrated that LCACoAs inhibit AMPK activity (Taylor, Ellingson, Lamb, Chesser, & Winder, 2005). Moreover, high-fat–fed rats have accumulated tissue LCACoAs, further suggesting an inverse relationship between high-fat diet and AMPK activity (Ye et al., 2003). However, in a separate study AMPK levels were not significantly different between high-fat- and chow-fed rats (Martin et al., 2006). Despite the lack of research exploring the relationship between high-fat feeding and AMPK activity in humans, the relationship between high-fat-diet induced elevations in inflammation is well established (Borst & Conover, 2005; Morin, Eckel, Marcel, & Pagliassotti, 1997).

Statement of the Problem

AMPK's role in healthy metabolic function is vital and the question of whether AMPK affects the IKKβ response to lipid accumulation in muscle is essential to understanding lipid-induced insulin resistance. Due to the prevalence of obesity in our society and the fact that obesity is associated with elevated free fatty acids and increased incidence of type 2 diabetes mellitus, one method to elucidate the role of AMPK in improving lipid-induced insulin resistance via attenuation of the inflammatory pathway is to observe this relationship in obese, lean, and weight-loss subjects. If AMPK activity is altered between obese, lean, and weight-loss subjects, it may offer some explanation concerning the deleterious consequences of obesity. Moreover, considering that metformin is such a widely prescribed anti-diabetic drug and AMPK activator despite its precise mechanisms remaining elusive, it seems worthwhile to explore its role in conditions that mimic the lifestyles of the majority of those who suffer from diabetes, diet-induced obesity. Further, recent and convincing data from endothelial cells and liver have uncovered a significant effect of AMPK to inhibit the lipid-induced increase in inflammatory cytokine activity. However, AMPK's ability to counter the deleterious consequences of elevated lipids has not been explored in muscle, which represents a truly meaningful area of research considering skeletal muscle's vital role in healthy insulin responsiveness. Hence, we feel it is crucial to determine the effect of AMPK manipulation in skeletal muscle in attenuating lipid-induced insulin resistance via inhibition of IKKβ.

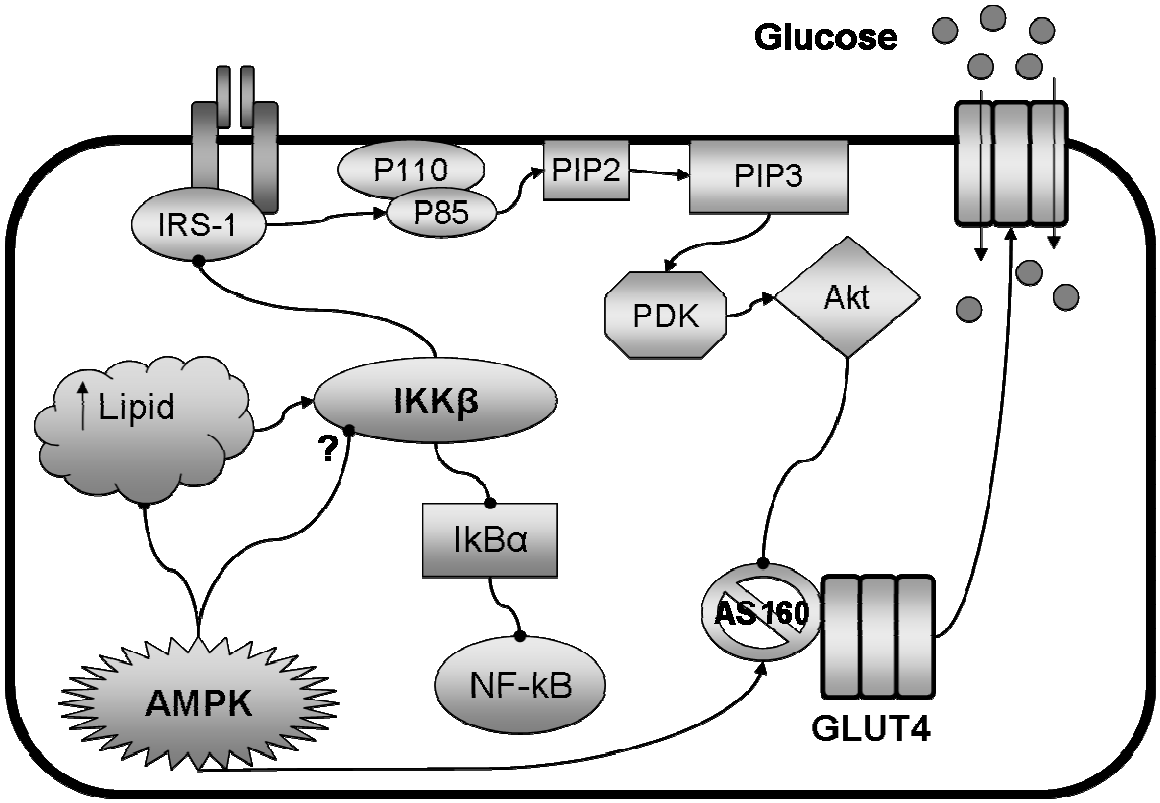

Figure 1.1. Proposed pathway through which AMPK potentially inhibits IKKβ activity and improves insulin signaling in skeletal muscle.

Chapter Two

Mechanisms for Improved Insulin Sensitivity after Gastric Bypass Surgery

Abstract

Surgical treatments of obesity have been shown to induce rapid and prolonged improvements in insulin sensitivity. To investigate the effects of gastric bypass surgery and the mechanisms that explain the improvement in insulin sensitivity we employed a cross-sectional, non-randomized, controlled study. This study was conducted jointly between the Departments of Exercise Science and Physiology at East Carolina University in Greenville, NC. Subjects were recruited into four groups: 1) lean (BMI<25; n=93), 2) weight-matched (BMI=25 to 35; n=310), 3) morbidly obese (BMI>35; n=43), and 4) post-surgery patients (BMI≈30 kg/m^2; n=40). Post-surgery patients were weight stable one year post surgery. Whole-body insulin sensitivity, muscle glucose transport, and muscle insulin signaling were assessed as markers of glucose uptake and insulin sensitivity. Post-surgery subjects had insulin sensitivity index values that were similar to the lean and higher than morbidly obese and weight-matched control subjects. Glucose transport was higher in the post-surgery vs. morbidly obese and weight-matched groups. IRS1-pSer^{312} in the post-surgery group was lower than morbidly obese and weight-matched groups. IκBα was higher in skeletal muscle from the post-surgery patients when compared to the morbidly obese and weight-matched controls, indicating reduced IKKβ activity. Insulin sensitivity and glucose transport were greater in the post-surgery patients than predicted from the weight-matched group, suggesting improved insulin sensitivity after bypass is due to something other than, or in addition to, weight loss. We conclude that

improved insulin sensitivity following gastric bypass surgery is related to reduced IKKβ activity and enhanced insulin signaling in muscle.

Introduction

Gastric bypass surgery has been employed as a tool to assist in weight loss in morbidly obese individuals. For over 20 years, our group has been performing gastric bypass surgery, which consists of surgical reduction in the size of the stomach and bypassing a portion of the proximal small intestine. We were the first to report that gastric bypass surgery results in rapid and long term improvement in insulin sensitivity and an ultimate reversal of diabetes (Friedman et al., 1992; Pories, 1992; Pories et al., 1992). More recently, Dixon et al (Dixon et al., 2008) observed that laparoscopic adjustable gastric banding (LAGB) resulted in a higher remission rate of type 2 diabetes compared with conventional medical treatment. Additionally, the reversal of diabetes by various surgical treatments of obesity was confirmed by meta-analysis, wherein gastric bypass surgery and LAGB were found to resolve diabetes in approximately 84% and 48% of post-surgery patients, respectively (Buchwald et al., 2004).

Although an increase in whole-body insulin sensitivity after gastric bypass surgery is well established, the mechanisms involved are not fully understood. A potential signaling defect in the insulin signaling cascade in the muscle of obese and type 2 diabetics may be a cause for reduced insulin sensitivity. We have shown previously that insulin-stimulated muscle glucose transport is blunted in obese individuals and patients with type 2 diabetes and that insulin-receptor tyrosine kinase activity is diminished in obese and diabetic skeletal muscle (Dohm et al., 1988; Q. Zhou, Dolan, & Dohm, 1999). Serine phosphorylation of

the insulin receptor substrate (IRS)-1 has been implicated in inhibiting insulin signal transduction (Cai et al., 2005; Yuan et al., 2001) and while there are many potential serine phosphorylation sites on IRS-1 (Morino, Petersen, & Shulman, 2006), the IRS1 $Ser^{312\ (Human)}$/$Ser^{307(rodent)}$ residue is of particular interest given its proximity to the phosphotyrosine-binding (PTB) domain in IRS-1. The inhibitor of κB kinaseβ (IKKβ), a serine kinase, has been suggested as a potential culprit that results in deficient insulin signal transduction given that it has been shown to phosphorylate IRS-1 on $Ser^{312/307}$ (Arkan et al., 2005; Cai et al., 2005; Kim et al., 2001; Yuan et al., 2001). Moreover, IKKβ is activated by fatty acid metabolites, such as diacylglycoerol (DAG) and ceramide (Schmitz-Peiffer, 2002; C. Yu et al., 2002), which have been shown to be higher in insulin resistant states, such as obesity (Itani et al., 2002).

Obesity is associated with an increased low-grade inflammatory tone, as evidenced by increased levels of proinflammatory cytokines as well as greater activity of the inflammatory factor, NF-κB (Bastard et al., 2006; Festa et al., 2001; Permana et al., 2006; Warnberg & Marcos, 2008). NF-κB is a nuclear transcription factor that is responsible for the transcription of a multitude of cytokines (including TNF-α and IL-1β) (Granger, Vowinkel, & Petnehazy, 2004; Piva, Belardo, & Santoro, 2006). NF-κB is prevented from reaching the nucleus by an inhibitor, known as inhibitor κBα (IκBα). IκBα is phosphorylated by IKKβ, which results in the ubiquitination and degradation of IκBα and NF-κB is free to migrate into the nucleus. In addition to regulating NF-κB activity, IKKβ has been

shown to directly inhibit insulin signaling (Cai et al., 2005; Ghanim et al., 2001; Yuan et al., 2001). To highlight the role of NF-κB pathway activity in inhibiting insulin signaling, thiazolidinediones, a known insulin-sensitizing drug, inhibit NF-κB activity (Ghanim et al., 2001; Todd et al., 2007). Similarly, Yin et al. (Yin, Yamamoto, & Gaynor, 1998) discovered that the mechanism of action for salicylate in improving insulin sensitivity was its inhibitory actions on IKKβ.

We are interested in the causes of insulin resistance in muscle of obese individuals and the mechanisms that improve insulin resistance after patients have gastric bypass surgery. The purpose of this study was to investigate gastric bypass surgery-induced increases in insulin sensitivity at the whole body and muscle level. We also sought to determine a potential mechanism for the rapid and sustained improvement in insulin sensitivity as a result of gastric bypass surgery.

Materials and Methods

Subjects and study design

To investigate the reversal of insulin resistance after bypass surgery three experiments were performed. In the first experiment whole-body insulin sensitivity was measured in post-surgery patients and non-surgical controls by an intravenous glucose tolerance test (IVGTT) as outlined below. The second experiment involved glucose transport measured in rectus abdominis muscle strips from patients having elective surgery. These values were compared to previous data published for gastric bypass patients after weight loss (Friedman et al., 1992). The activity of IKKβ and IRS-1 serine^{312} phosphorylation was assayed in vastus lateralis muscle biopsies of post-surgery patients and non-surgery controls in the third experiment. All subjects were female and considered to be in good health after filling out a medical history questionnaire. Subjects were informed of potential risks associated with the study and signed an informed consent document, which was approved by the East Carolina University Institutional Review Board.

Whole-body insulin sensitivity experiment

The first experiment consisted of 128 female subjects who were divided into four groups. One group consisted exclusively of post-gastric bypass surgery patients who were weight stable at least 12 mo after surgery (BMI ≈ 30). The other three groups of non-surgery subjects were divided according to BMI: 1) lean (BMI < 25), 2) weight-matched to post-surgery patients (BMI = 25 to 35),

and 3) morbidly obese (BMI > 35). As mentioned, this first cohort of subjects was used to obtain insulin sensitivity data, including fasting insulin and glucose, as well as insulin sensitivity with a 3-h intravenous glucose tolerance test (IVGTT) (outlined below).

Muscle glucose transport experiment

In the second experiment glucose transport was measured in incubated muscle strips obtained during elective abdominal surgery. Subjects were recruited into similar BMI groups, namely, lean (BMI < 25), weight-matched controls to post-surgery patients (BMI = 25-35), and morbidly obese (BMI >35). The data from these groups were then compared to data previously reported (Friedman et al., 1992) for five post-surgery subjects in order to compare the given groups with post-surgery patients.

IKKβ activity and IRS1-Ser^{312} experiment

The third experiment included 36 subjects who were evenly recruited into four groups, similar to the BMI groups mentioned above. This cohort of subjects had a muscle biopsy (vastus lateralis) that was used to obtain all muscle protein data.

Roux-en-Y gastric bypass surgery

The gastric-bypass surgery was performed as described previously (Pories et al., 1992). Briefly, Roux-en-Y gastric bypass surgery consists of surgical reduction in the size of the stomach and bypassing of a portion of the proximal small intestine (foregut).

Anthropometrics

Body mass was measured to the nearest 0.1 kg on a digital electronic scale. Height was measured to the nearest 0.5 cm with a stadiometer and BMI calculated [mass (kg)/height^2 (m^2)].

IVGTT

Insulin sensitivity was determined by a minimal model analysis using a 3-h IVGTT (Bergman, Finegood, & Ader, 1985), as described previously (Houmard et al., 1995). After fasting samples were obtained, glucose (50%) was injected into a catheter placed in an antecubital vein at a dose of 0.3 g/kg body mass. Insulin, at a dose of 0.025 U/kg body mass was injected at minute 20. Blood samples were obtained at minutes 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 19, 22, 25, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, and 180 and the samples were then centrifuged, and plasma transferred and frozen at -80°C for the subsequent determination of insulin and glucose. Insulin was determined with immunoassay (Access Immunoassay System, Beckman Coulter, Fullerton, CA) and glucose was determined with an oxidation reaction (YSI model 2300 Stat Plus, Yellow Springs Instruments, Yellow Springs, OH). Insulin sensitivity index (S_I) was calculated by using the minimal model (Bergman et al., 1985); a higher S_I indicates greater insulin sensitivity.

Muscle strip incubations

Human rectus abdominis muscle strip preparation and incubation was performed according to the procedure previously described (Dohm et al., 1988).

Muscle strips were incubated in the presence (100 nM) or absence of insulin. 2-deoxy-D-glucose was used at a concentration of 5 mM for 60 min and glucose transport was measured as described previously (Dohm et al., 1988). 2-[1,2-^{3}H(N)]deoxy-D-glucose (30.2 Ci/mmol) was obtained from Du Pont-NEN (Boston, MA).

Muscle IRS-1, pSer^{312}, and IκBα protein content

A muscle sample was obtained from the vastus lateralis with the percutaneous needle biopsy technique. Biopsy samples were immediately frozen in liquid nitrogen. Frozen muscle samples were homogenized in ice-cold lysis buffer [50 mM HEPES, 50 mM Na^{+} pyrophosphate, 100 mM Na^{+} fluoride, 10 mM EDTA, 10 mM Na^{+} orthovanadate, and protease and phosphatase (1 and 2) inhibitor cocktails (Sigma, St. Louis, MO)], followed by addition of 1% triton and brief sonication. After centrifugation for 1 h at 45,000 *g* at 4°C, supernatants were extracted (cytosolic portion) protein content was detected using a BCA protein assay (Pierce, Rockford, IL) and individual homogenate volumes were adjusted so that precisely 50 µg of protein were loaded into each lane. For IRS1-Ser^{312} and total IRS-1 analysis, 200 µg of homogenates were subjected to 10 µl IRS-1 monoclonal IP antibody (Santa Cruz Biotech, Santa Cruz, CA) overnight then coupled to protein A sepharose beads and rotated for 2 hours at 4°C (Amersham Biosciences, Uppsala Sweden) and washed. After addition of sample buffer, samples were separated by SDS-PAGE using 7.5% or 10% Tris·HCl gels and then transferred to PVDF membranes for probing by the

following antibodies: rabbit polyclonal IRS-1 and IRS-1 phospho serine^{312} purchased from Millipore (Billerica, MA) and IκBα antibodies were purchased from Cell Signaling (Beverly, MA). Following incubation with primary antibodies, blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. Horseradish peroxidase activity was assessed with ECL solution (Thermo Scientific, Rockford, IL), and exposed to film. The image was scanned and band densitometry was assessed with Gel Pro Analyzer software, version 4.2 (Media Cybernetics, Silver Spring, MD). Content of phospho-proteins (using phospho-specific antibodies) was calculated from the density of the band of the phospho-protein divided by the density of the protein using the appropriate antibody.

Statistics

All data are shown as mean ± SE. Comparisons between the groups with insulin sensitivity, muscle protein levels, and muscle glucose transport rates were made using one-way ANOVA, followed by post hoc analyses where appropriate. Correlations between variables were determined by Dunnette T3 post hoc analysis.

Results

Subject characteristics are presented in Table 2.1. None were taking medications that would interfere with metabolism, and all were free from known cardiovascular disease and diabetes mellitus, and a few were glucose tolerance impaired (fasting glucose > 110 mg/dL; two in the lean group, two in the obese group, and five in the weight-matched group). The mean weight of all gastric bypass subjects decreased from 136.6±21.5 to 81.9±13.2 kg (mean±SD, $P<0.001$), resulting in a mean weight loss of 54.7±18.7 kg. Similarly, BMI fell from 48.6±5.7 to 28.8±4.6 kg/m^2 ($P=0.001$). The BMI of the post-surgery groups was not significantly different than the weight-matched control groups.

Fasting glucose, insulin and insulin resistance (HOMA)

Average plasma glucose concentration for the post-surgery subjects (76±10.2 mg/dl) was lower when compared with all non-surgery control subjects (95.3±13.8 mg/dl) (Figure 2.1A; $P<0.001$). There was no significant change in fasting glucose with increasing BMI in either control or post-surgery groups. Fasting insulin concentration and BMI were positively correlated ($r=0.598$, $P<0.001$) for all non-surgery control subjects. In contrast, the same correlation in post-surgery subjects revealed no significant relationship ($r = 0.304$, $P= 0.169$; Figure 2.1B). Fasting glucose and insulin values were used to calculate homeostasis model assessment (HOMA) values, an index of insulin resistance. HOMA values were significantly related with BMI in the non-surgery subjects

(P<0.01, r = 0.283, Figure 2.2A), whereas the post-surgery patients revealed no significant correlation.

Whole-body insulin sensitivity

The post-surgery group had insulin sensitivity index (S_I) values that were 361% higher than the morbidly obese and 47% higher than weight-matched groups (P<0.005 for both; Figure 2.2B). The post-surgery group's S_I levels were not significantly different from the lean group, despite having a greater BMI. The S_I value of the morbidly obese was 68% lower than the weight-matched controls, suggesting an inverse relationship between whole-body insulin sensitivity and BMI (P<0.05). This is consistent with the positive correlation between BMI and HOMA from all non-surgery subjects (Figure 2.2A; P<0.01, r = 0.283).

Muscle glucose transport

We previously reported that muscle glucose transport was significantly lower in morbidly obese patients than in lean controls (Dohm et al., 1988; Friedman et al., 1992). Additionally, in five gastric bypass patients who subsequently had elective abdominal surgery after weight loss, muscle glucose transport was not different than that of lean subjects, despite the post surgery group having an average BMI of 31 (Friedman et al., 1992). To determine how post-surgery muscle glucose transport compared to a weight-matched control group, we retrieved muscle glucose transport measurements from a database of our previous experiments (Figure 2.3A). Previously published post-surgery glucose transport values (Friedman et al., 1992) are shown in figure 2.3A for

comparison. Insulin-stimulated glucose transport values were lower in the weight-matched group and morbidly obese groups compared to the lean control groups ($P<0.05$). There were no differences in insulin-stimulated glucose transport between the morbidly obese and weight-matched groups. This is in agreement with our previous report that muscle glucose transport is related to BMI up to a BMI of 30, after which there is no further change (Elton, Tapscott, Pories, & Dohm, 1994); i.e., muscle insulin resistance is maximal at a BMI of 30. Post surgery glucose transport values were similar to the lean group and greater than morbidly obese and weight-matched subjects.

We previously reported that insulin-stimulated glucose transport was depressed in muscle of morbidly obese patients, compared to lean controls (Dohm et al., 1988). With the larger number of observations in the present data set we extend this observation by reporting that basal glucose transport (in the absence of insulin) is also lower in muscle of morbidly obese patients. Also, a significant positive relationship was found between the rates of basal and insulin-stimulated glucose transport ($P<0.001$, $r=0.726$, Figure 2.3B). This suggests that the factors that regulate basal glucose might also be those that regulate the rate in the presence of insulin. This was unexpected as we had predicted that insulin resistance might only have an effect on insulin-stimulated glucose transport.

Insulin sensitivity and IRS1-Ser^{312}

Inasmuch as serine^{312} phosphorylation of the IRS-1 protein has been implicated in inhibiting insulin signaling, this parameter was investigated in vastus lateralis muscle biopsies of lean, weight-matched, morbidly obese, and post-surgery subjects (group 3 in Table 2.1). Ser^{312} phosphorylation of IRS-1 was significantly greater in the morbidly obese and weight-matched subjects versus the lean and post-surgery subjects (P<0.05). IRS1-Ser^{312} phosphorylation followed a similar pattern to the insulin-stimulated glucose transport data. In particular, IRS1-pSer^{312} did not differ between the morbidly obese and weight-matched groups (Figure 2.4A). Moreover, IRS1-Ser^{312} phosphorylation in post-surgery subjects was significantly reduced by 47% compared with morbidly obese (P=0.04) and tended to be lower when compared with the weight-matched subjects (P=0.057).

IκBα and IRS1-Ser^{312}

Due to the implicated role of IKKβ as a serine kinase of IRS1-Ser^{312}, muscle levels of IκBα, a substrate of IKKβ that is rapidly ubiquitinated and degraded upon phosphorylation, were measured in an effort to determine IKKβ activity (de Alvaro et al., 2004). Greater IKKβ activity would be evident in reduced levels of IκBα. Similar to our observations with IRS1-phospho-Ser^{312} and insulin-stimulated glucose uptake in muscle, IκBα levels in the post-surgery subjects were similar to lean, and were significantly elevated by 44% compared with the morbidly obese (P<0.05) and 47% compared with the weight-matched

controls (P<0.05), which demonstrated increased IKKβ activity in the weight-matched and morbidly obese compared to the lean and post-bypass subjects (Figure 2.4B). With correlational analysis, we discovered a highly significant inverse relationship between IκBα and phospho-Ser^{312} levels (P=0.003, r =-0.47, Figure 2.4C).

Table 2.1. Characteristics of patients/subjects in the three experiments.

Subject characteristics			
Experiment	**Age (y)**	**BMI (kg/m^2)**	**Number of observations (n)**
Experiment 1: Whole-body insulin sensitivity, fasting glucose and insulin			
Lean BMI<25 (non-surgery)	38.9 ± 15.0	23.4 ± 1.5	33
Weight-matched BMI 25-35 (non-surgery)	41.8 ± 14.1	28.5 ± 1.7	50
Morbidly obese BMI>35 (non-surgery)	40.3 ± 16.1	47.7 ± 9.5	14
Post-surgery	43.5 ± 9.8	29.4 ± 4.9	31
Experiment 2: Muscle glucose transport from rectus abdominis muscle taken during surgery			
Lean BMI<25	39.3 ± 6.4	22.4 ± 2.1	51
Weight-matched BMI 25-35	42.6 ± 19.0	28.9 ± 2.8	251
Morbidly obese BMI>35	41.6 ± 4.2	46.2 ± 4.0	20
Experiment 3: Vastus lateralis muscle biopsy for IRS1-Ser^{312} and IκBα			
Lean BMI<25 (non-surgery)	35.1 ± 14.4	21.7 ± 1.9	9
Weight-matched BMI 25-35 (non-surgery)	35.5 ± 10.1	30.9 ± 2.7	9
Morbidly obese BMI>35 (non-surgery)	32.8 ± 13.3	44.6 ± 4.2	9
Post-surgery	40.8 ± 8.1	29.9 ± 4.1	9

Mean ± SD. There is no statistical difference in BMI between the weight-matched and post-surgery groups.

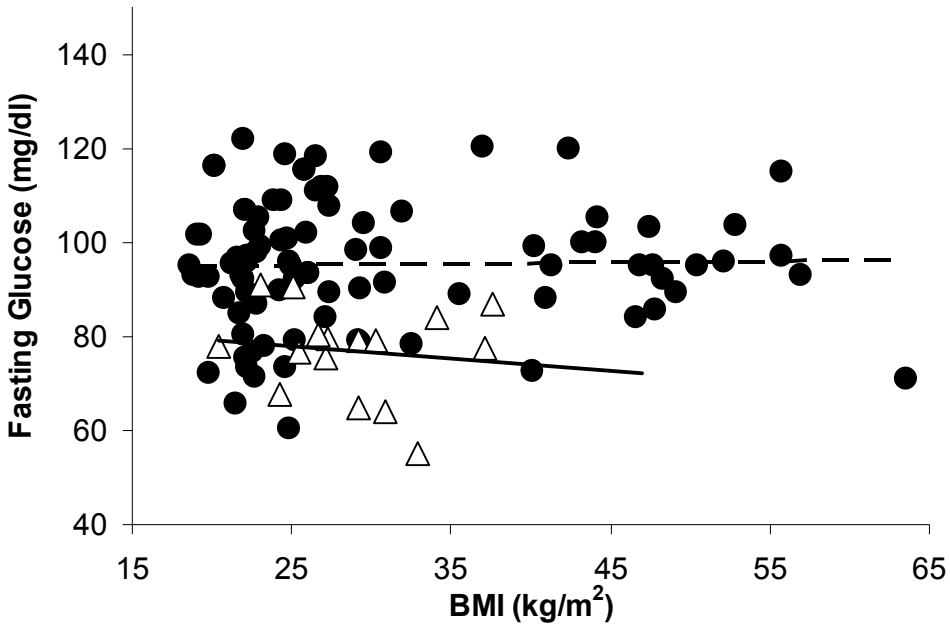

Figure 2.1A. The correlations between BMI and glucose for non-surgery control subjects (black circles) and post-surgery patients (white triangles). The data are from experiment 1 patients (see Table 2.1). The comparison of fasting glucose values between non-surgery control subjects (n=97) and post-surgery subjects (n=31).

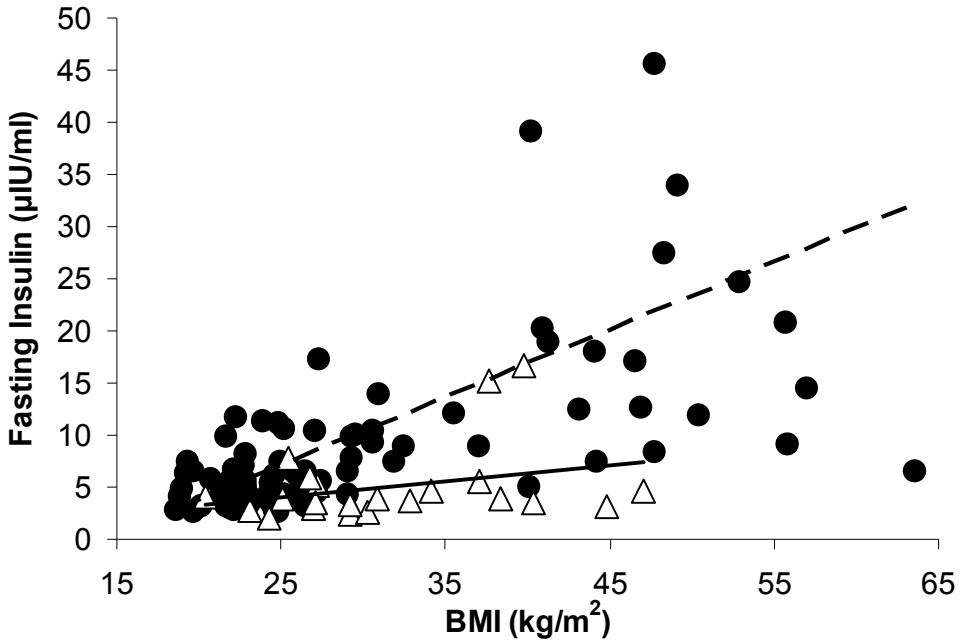

Figure 2.1B. The correlations between BMI and insulin for non-surgery control subjects (black circles) and post-surgery patients (white triangles). The data are from experiment 1 patients (see Table 2.1). Fasting insulin values between non-surgery subjects and post-surgery subjects. A positive and significant correlation was found (P<0.001, r = 0.598) for the non-surgery subjects. The same correlation in post-surgery subjects revealed no significant relationship (P = 0.169, r = 0.304). Data are presented as mean ± SD.

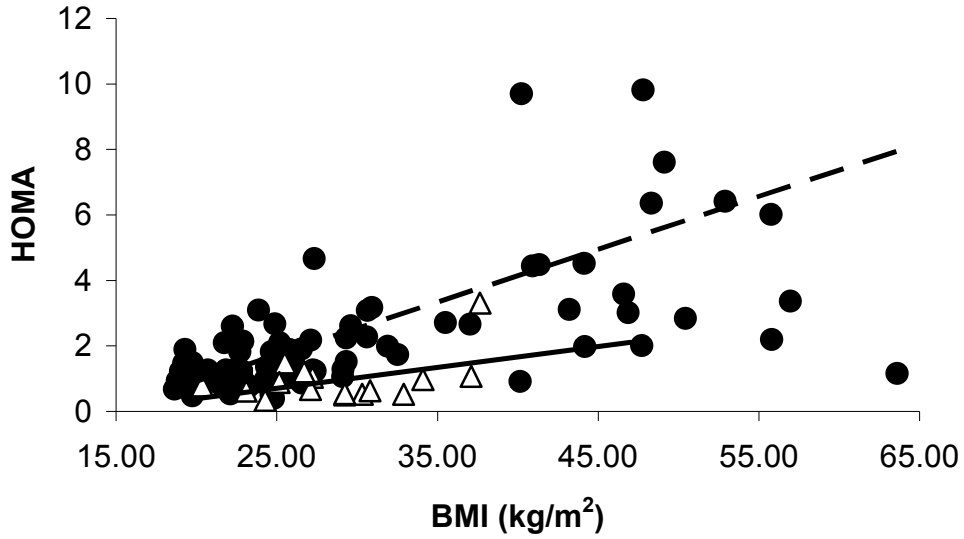

Figure 2.2A. Whole-body insulin sensitivity determined by HOMA correlated with BMI. The data for the HOMA analysis were taken from experiment 1 (see Table 2.1). The comparison of HOMA values for non-surgery control subjects (black circles) and post-surgery patients (white triangles). A positive and significant correlation between HOMA and BMI was found ($P<0.01$, $r = 0.283$) for the non-surgery subjects. The same correlation in post-surgery subjects revealed no significant relationship ($P = 0.083$, $r = 0.446$).

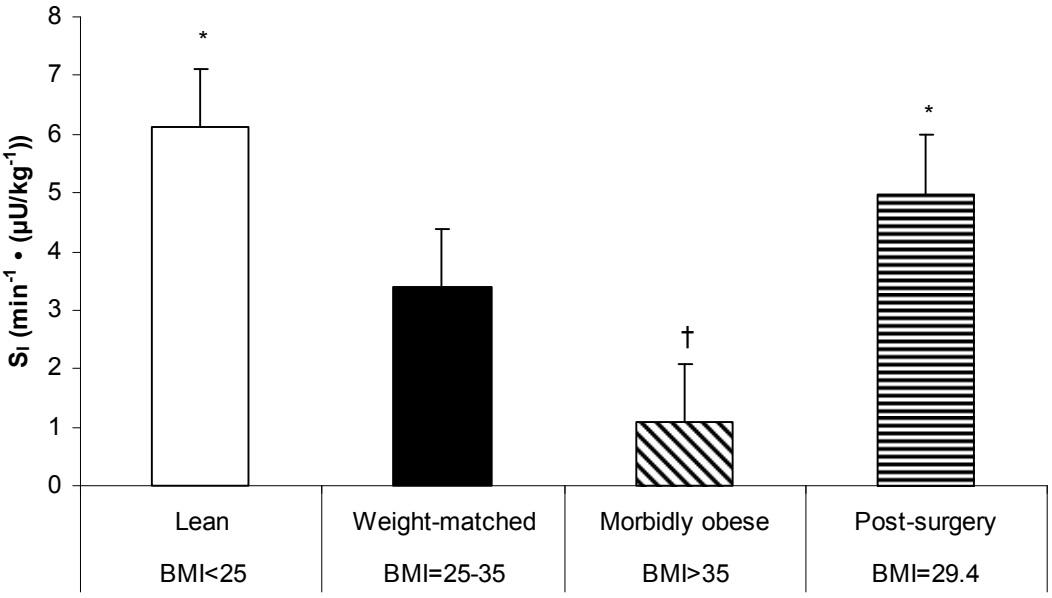

Figure 2.2B. Whole-body insulin sensitivity determined by IVGTT for four BMI groups. The data for insulin sensitivity analysis were taken from experiment 1 (see Table 2.1). The difference in whole-body insulin sensitivity between BMI groups. *The post-surgery and lean groups are statistically higher (P<0.005) than the morbidly obese and weight-matched groups. †P<0.05 for the comparison between the morbidly obese and all other groups. Data are presented as mean ± SD.

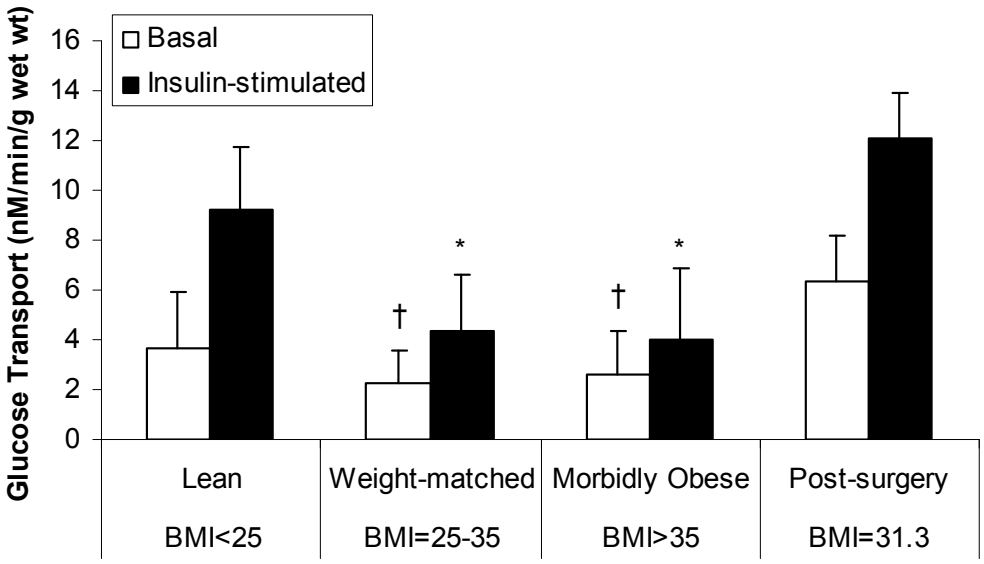

Figure 2.3A. 2-Deoxy-D-glucose uptake in incubated muscle strips in the presence (100 nM) or absence of insulin. The data were obtained from subjects in experiment 2 (see Table 2.1). A comparison of basal (empty bars) and insulin-stimulated (filled bars) glucose transport. *Insulin-stimulated glucose transport in the morbidly obese and weight-matched groups was statistically lower (P<0.05) than the lean and post-surgery groups. †Basal glucose transport in the morbidly obese and weight-matched groups was statistically lower (P<0.05) than the lean and post-surgery groups. Basal and insulin-stimulated glucose transport in muscle from morbidly obese subjects did not differ from the weight-matched subjects.

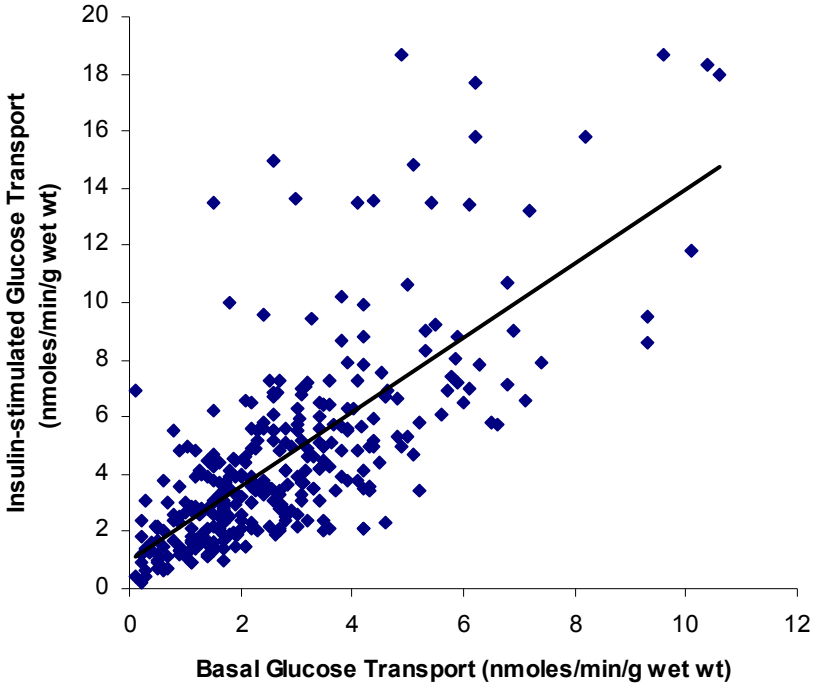

Figure 2.3B. The correlation between basal and insulin-stimulated glucose transport in skeletal muscle strips. The data were obtained from subjects in experiment 2 (see Table 2.1). The correlation between basal and insulin-stimulated glucose transport revealed a highly significant relationship ($P<0.001$; $r=0.726$). Data are presented as mean ± SD.

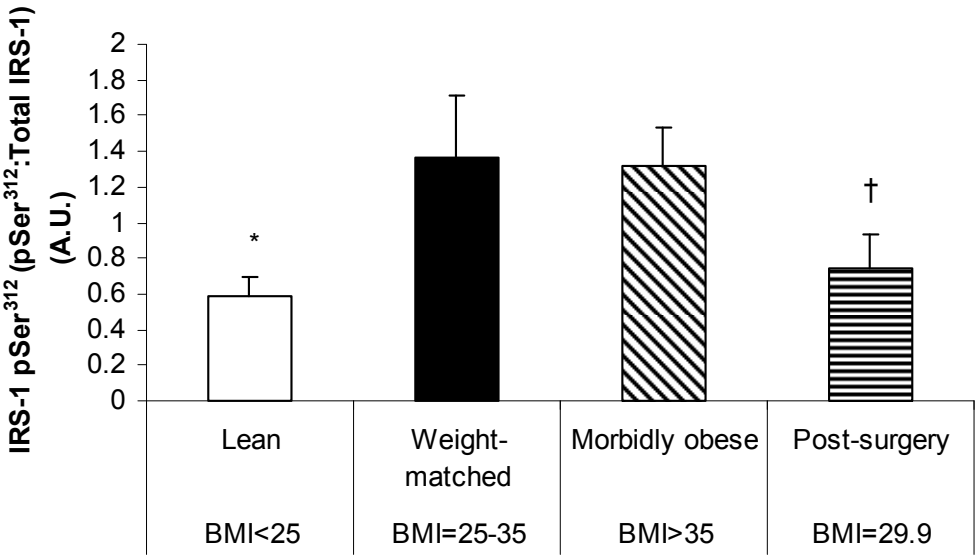

Figure 2.4A. Muscle levels of IRS1-phospho-Ser^{312}. Non-surgery control subjects and post-surgery patients were from experiment 3 (see Table 2.1). Western blot analysis of serine^{312} phosphorylation of IRS1. *P<0.05 for the comparison between the lean group and the morbidly obese and weight-matched. †P<0.05 for the comparison between the post-surgery and the morbidly obese groups. The post-surgery group tended to have a lower level compared with the weight-matched group (P =0.057).

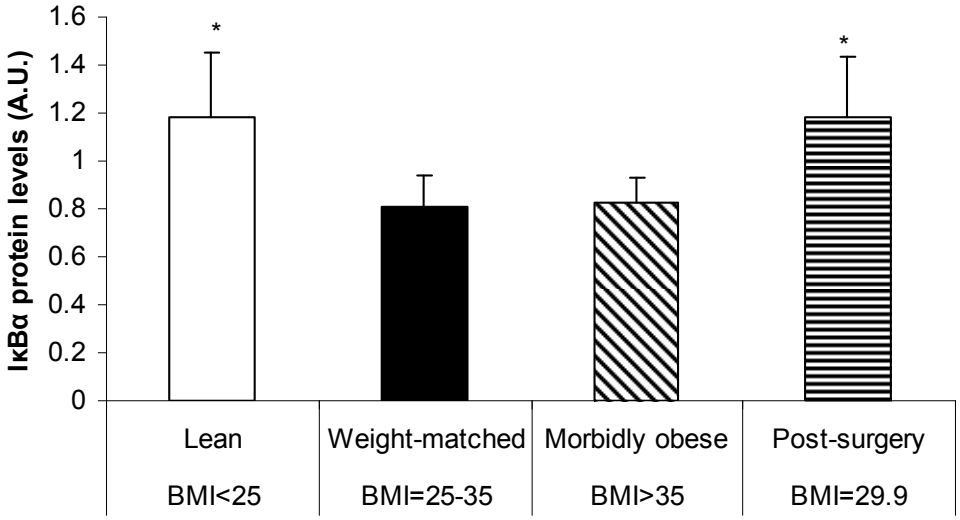

Figure 2.4B. Muscle levels of IκBα. Non-surgery control subjects and post-surgery patients were from experiment 3 (see Table 2.1). Western blot analysis of IκBα in skeletal muscle between groups. *P<0.05 for the observation that the lean and post-surgery groups were statistically higher than the morbidly obese and weight-matched groups. IκBα levels were similar between post-surgery subjects and lean subjects.

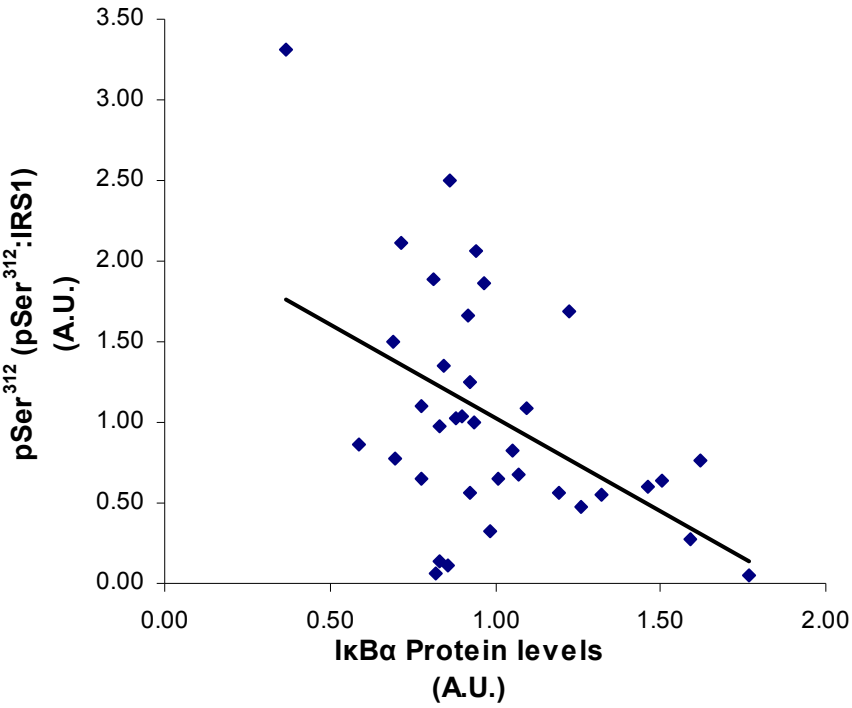

Figure 2.4C. The correlation between IRS1-pSer^{312} and IκBα in skeletal muscle. Non-surgery control subjects and post-surgery patients were from experiment 3 (see Table 2.1). The correlation between IκBα and IRS1-phospho-Ser^{312} levels between non-surgery and post-surgery subjects (P=0.003, r =-0.47). Data are presented as mean ± SD.

Discussion

The primary effect of gastric-bypass surgery in the obese is significant weight loss, but an important side effect of the procedure is the dramatic and rapid rescuing of insulin sensitivity. We observed lower fasting glucose and insulin values from the post-surgery group compared with all other subjects. Further, we found that whole-body insulin sensitivity, as measured by IVGTT, is significantly greater in the post-surgery subjects compared with BMI-matched control subjects. Additionally, despite having a significantly higher BMI than the lean subjects, post-surgery subjects enjoy comparable S_I values.

We have previously shown that muscle insulin resistance in obese individuals is related to depressed insulin signal transduction (Q. Zhou et al., 1999). Since phosphorylation of the IRS1-$Ser^{312(human)/307(rodent)}$ residue has been implicated in attenuating insulin signal transduction, we measured IRS1-$pSer^{312}$ and IκBα protein levels, as a surrogate for IKKβ activity, and found that both IκBα and IRS1-phospho-Ser^{312} protein levels in skeletal muscle from post-surgery patients were similar to that seen in muscle from lean subjects despite the post-surgery group having a significantly higher BMI (Figure 2.4A and 2.4B). The NF-κB pathway has garnished a great deal of attention recently in regards to its novel effects on metabolic function and substrate utilization. In particular, IKKβ has been studied in a variety of cells, including endothelial, hepatic, and skeletal muscle. Research into the role of the NF-κB pathway in endothelial cells has been conducted due, in part, to the pathological consequences of inflammation in

the vascular system (Cacicedo et al., 2004; Hattori et al., 2006; Partridge et al., 2007; Zhang & Frei, 2001). Recent work by Wu et al. (X. Wu et al., 2007) determined that activation of IKKβ by TNF-α is suppressed by adiponectin. This is particularly interesting due to adiponectin's ability to improve insulin sensitivity (Yamauchi et al., 2001). Further, studying the role of NF-κB pathway proteins in the liver, Cai et al. (Cai et al., 2005) revealed that diet-induced obesity in mice lead to increased NF-κB pathway signaling in the liver. Mice with a liver-specific constitutively active IKKβ transgene suffered from systemic insulin resistance and further analysis revealed a dose-dependent relationship between NF-κB activity in the liver and the onset of system insulin resistance. Moreover, in human hepatocytes, Gao et al. (Gao et al., 2002) treated cells with TNF-α and found increased IRS1 phosphorylation at Ser^{312}. This IRS1-Ser^{312} phosphoryltaion correlated with the disappearance of IκBα, which represents increased IKKβ activity.

Since skeletal muscle is the main depot for insulin-stimulated glucose uptake, studying the effects of IKKβ on insulin signaling in this tissue is worthwhile. A study by de Alvaro et al. (de Alvaro et al., 2004) reported that IKKβ activation in rat skeletal muscle cell culture resulted in a significant increase in IRS1-Ser^{307} phosphorylation. They also explored the role of a pharmacological IKKβ inhibitor, salicylate, in preventing IKKβ-induced insulin resistance. Targeted disruption of the *Ikk*β locus in mice results in lower fasting glucose and insulin concentrations, as well as preventing insulin resistance from a high-fat

diet. Similar results have been reported with salicylate treatment (Yuan et al., 2001). Additionally, Kim et al. (Kim et al., 2001) employed lipid infusions in rodents to cause acute insulin resistance. They observed that this effect was attenuated with pretreatment with salicylates. Further, lipid infusions performed in *Ikkb* heterozygous knockout mice revealed improvements in insulin sensitivity during hyperinsulinemic-euglycemic clamps when compared to controls.

IKKβ activation, and subsequent IRS1-Ser^{312} phosphorylation, has been shown to be a result of the accumulation of excess fatty acids within the muscle that are subsequently partitioned toward storage or the formation of fatty acid metabolites, resulting in insulin resistance (Itani et al., 2002; Lee et al., 2006; Schmitz-Peiffer, 2002; Todd et al., 2007; C. Yu et al., 2002). We have previously shown that intramyocellular lipid (IMCL) levels are significantly reduced following gastric bypass surgery (Gray et al., 2003). Moreover, Greco et al. (Greco et al., 2002) determined that surgery-induced weight loss results in a greater-than-expected reduction in IMCL when compared with diet-induced weight loss alone. IMCL depletion is certainly a likely explanation for the improvements we observed in post-surgery patients in regards to levels of IκBα and IRS1-pSer^{312} due to their role in mediating lipid-induced insulin resistance and it may partly explain the differences in whole-body insulin sensitivity.

Not surprisingly, in control subjects fasting insulin levels and insulin resistance (HOMA) have positive relationships with BMI. However, fasting insulin and insulin resistance are not associated with BMI in the post-surgery

subjects. BMI was used as a global assessment of body fat and weight loss in the current study. Whereas BMI is not an accurate indicator of body fat in certain populations with relatively greater muscle mass, given that all subjects in the current study were sedentary, we feel it is a legitimate measurement. Moreover, in regards to the weight loss and reduction in BMI associated with bypass surgery, it has been shown that the tremendous fat loss associated with gastric bypass surgery is mostly a result of a reduction in truncal fat (Olbers et al., 2006).

A possible concern may be the possible confounding effect of a change in food intake as a result of gastric bypass surgery, which our group has previously explored (Sinha, Perdomo, Brown, & O'Doherty, 2004). While patients experience an acute and meaningful decrease in caloric intake following surgery that results in significant weight loss, the outcomes we explored in the current study were measured approximately 12 mos following surgery in patients who were weight stable on a eucaloric diet, which would seemingly negate any dietary effect.

We interpret our data to suggest that the improvement in insulin sensitivity after gastric bypass is not exclusively due to weight loss and may implicate an effect of the surgery itself (Pories, 1992). A comparison of various surgical interventions lends support for this hypothesis. Namely, gastric banding surgery restricts food intake but does not change the channel of food flow. The remission rate for gastric banding is 48% while that for gastric bypass is 84% (Buchwald et al., 2004). In addition, Dixon et al. (Dixon et al., 2008) reported that the

remission of diabetes was correlated with the degree of weight loss, which is in contrast with the data we present here for gastric bypass. Moreover, Lafèrrere et al. (Laferrere et al., 2008) compared subjects who experienced comparable levels of weight loss due to hypocaloric diet and surgery. Whereas both interventions result in improved diabetes control, they found that patients who underwent bypass surgery enjoyed a better clinical outcome and no longer required diabetes medication.

If the improvement in insulin sensitivity after gastric bypass is not related to weight loss, an attractive alternative hypothesis is that increased insulin sensitivity and remission of diabetes after gastric bypass is due to restructuring of the gastrointestinal tract (Pories, 1992). According to this hypothesis, bypassing a portion of the duodenum and jejunum causes either: 1) decreased secretion of a diabetes-inducing peptide (anti-incretin) from the foregut or 2) increased secretion of an anti-diabetes peptide (incretin) from the hindgut. This “foregut bypass hypothesis” was first suggested Pories (Pories, 1992) but has only recently received attention by Rubino (Rubino, 2008), who, with his colleagues, has extensively studied this hypothesis in a non-obese animal model of diabetes (GK rat). They found that a duodenal-jejunal bypass, which does not result in food restriction or weight loss, leads to improved glucose tolerance in the diabetic GK rat.

In summary, whole-body insulin sensitivity, as measured by IVGTT, and muscle glucose transport following gastric bypass are greater than predicted

from the weight-matched control group. We interpret these findings to suggest that the improvement in insulin sensitivity is not a result of weight loss, but is more likely related to bypass of the foregut. Based on our results, we believe that IRS1-Ser^{312} phosphorylation is a potential cause for the insulin resistance seen in the weight-matched and morbidly obese compared to the lean group, and that gastric bypass appears to result in reduced IRS1-Ser^{312} phosphorylation to levels similar to lean controls. Finally, we have implicated IKKβ, an NF-κB pathway serine kinase, as a potential culprit responsible for phosphorylating IRS1-Ser^{312}, and that bypass surgery may indirectly result in reduced IKKβ activity in human skeletal muscle.

Addendum

Whereas inflammatory cytokines and NF-κB pathway intermediates inhibit insulin signaling, AMPK plays a key role in increasing glucose uptake. In fact, a deficiency in AMPK activity has been implicated in the onset of type 2 diabetes (Winder & Hardie, 1999). AMPK has been shown to prevent cytokine-induced NF-κB pathway stimulation and result in reduced IKKβ action (Cacicedo et al., 2004; Cleasby et al., 2004; Hattori et al., 2006; Hattori et al., 2008), suggesting a more direct effect of AMPK on the NF-κB pathway. Interestingly, AICAR treatment in human myotubes has also been found to decrease cytokine production (Lihn et al., 2008). The purpose of this study was to determine whether baseline differences in AMPK activity may account for the differences observed in insulin sensitivity between post-bypass patients and control subjects.

To investigate the reversal of insulin resistance after bypass surgery, in phosphorylation state of AMPK was assayed in vastus lateralis muscle biopsies of post-surgery patients and non-surgery controls, as described above. Western blot analysis was performed as described above, and antibodies against phospho-AMPK and total AMPK were purchased from Cell Signaling Technologies (Beverly, MA). Levels of AMPK phosphorylation were controlled for by total AMPK. AMPK phosphorylation was significantly greater in the post-bypass group compared with all other groups ($P<0.05$; Figure 2.5).
Our hypothesis that baseline differences in AMPK activity explain differences in IKKβ action was not supported from the data. However, the observation that

post-bypass patients exhibit elevated AMPK phosphorylation is intriguing and worth highlighting.

A unique characteristic of the post-bypass group is their trend to mirror the lean group regarding IKKβ action, IRS1-pSer^{312}, and whole-body insulin sensitivity, despite being overweight/obese. However, in contrast to all previous measurements between these groups, AMPK activity, as measured by its phosphorylation state, differed drastically in the post-bypass group compared to all others. This finding may reveal a potential pathway through which the muscle from post-bypass patients becomes remarkably insulin sensitive.

The ability of AMPK to prevent lipid-induced insulin resistance has been recently explored in skeletal muscle (Fujii et al., 2008). Namely, Fujii et al. (2008) observed reduced insulin receptor-β subunit, IRS-1, and Akt in muscle-specific transgenic (TG) mice expressing an inactive form of AMPKα2 when fed a high-fat diet compared with wild type controls fed the same diet. Further, whereas muscle levels of DAG did not differ between the mice on control diet, DAG levels were significantly elevated in TG mice versus control when fed a high-fat diet. Moreover, these observations correlated with a reduction in insulin-stimulated glucose uptake in isolated soleus of TG mice.

The disparity in phospho-AMPK seen in the post-bypass group may be a function of the varying role of AMPK-stimulating hormones that accompany the surgery and the subsequent tremendous weight loss. In particular, adiponectin is a hormone released from the adipocytes that has been shown to stimulate AMPK

activity in skeletal muscle (Hardie, 2004) and circulating levels of adiponectin have been shown to be elevated following gastric bypass (de Carvalho et al., 2008; de la Torre et al., 2008). Moreover, lifestyle intervention in individuals suffering from type 2 diabetes also elicits a significant improvement in adiponectin levels. Together these findings possibly suggest that increased adiponectin, and subsequent AMPK stimulation, may be an adaptive mechanism whereby formerly insulin-resistant muscle becomes more insulin sensitive.

An alternative explanation regarding the elevations in phospho-AMPK observed in the post-bypass group may be a function of reduced stomach volume and a heightened sensitivity to fasting in the post-bypass group. AMPK has been shown to mediate a host of adaptations in skeletal muscle during fasting to promote fatty acid oxidation, including elevated expression of CD36 and CPT1, which would encourage fatty acid flux into the cell and shuttling into the mitochondira (Long et al., 2005; Long & Zierath, 2008). Muscle biopsies were pulled from subjects following an overnight fast. Due to the drastically reduced stomach volume of the post-bypass patients, it is highly possible that these subjects eat relatively less during the final meal on the night preceding the biopsy and are subsequently in a greater state of nutrient deprivation the following day. Further, this heighted sensitivity to fasting may be sensed in skeletal muscle and AMPK is activated in response.

In summary, we provide data that AMPK phosphorylation is elevated in the muscle of post-bypass patients compared with control subjects. We believe

this may be an adaptive mechanism that is partly responsible for the improvements in insulin signaling and glucose tolerance. Moreover, we offer the explanations that this effect may result from elevated circulating adiponectin and/or heighted sensitivity to fasting, both of which stimulate AMPK activity in skeletal muscle.

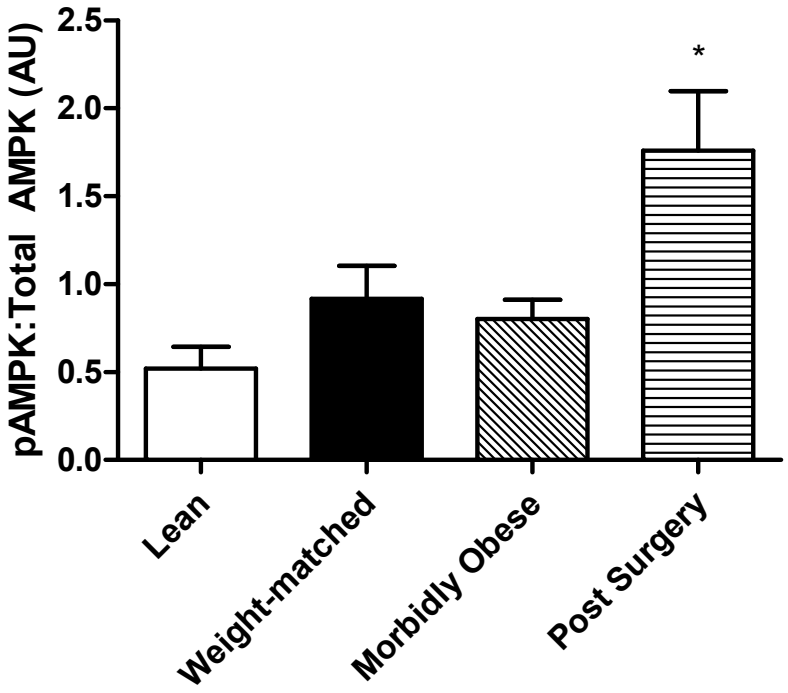

Figure 2.5. Differences in pAMPK:AMPK in post-surgery patients and controls. Post-surgery patients had significantly greater pAMPK levels than all other groups. *$p<0.05$ for the comparison between Post Surgery and all other groups.

Chapter Three

Metformin Improves Insulin Signaling in Obese Rats via Reduced IKKβ Action in a Fiber-type Specific Manner

Abstract

Metformin is a widely used insulin-sensitizing drug, though its mechanisms are not fully understood. Metformin has been shown to activate AMPK in a variety of tissue, including skeletal muscle; however, its effects on the inhibitor of κB kinaseβ (IKKβ) in this same tissue are unknown. The aim of this study was to 1) determine the ability of metformin to attenuate IKKβ, 2) determine whether changes in AMPK activity are associated with changes in IKKβ action in skeletal muscle, and 3) examine whether changes in AMPK and IKKβ are consistent with increased insulin sensitivity and reduced phosphorylation of IRS1-Ser^{307}. Lean and obese Zucker male rats received either vehicle or metformin by oral gavage daily for four weeks (four groups of eight) followed by an oral glucose tolerance test (OGTT). All proteins were measured in white gastrocnemius (WG), red gastrocnemius (RG), and soleus. Metformin treatment reduced OGTT area under the curve for both glucose and insulin values in the obese rodents only (P<0.05), indicating improved insulin sensitivity. AMPK activation, determined by phosphorylation state, increased (P<0.05) in WG in both lean (57%) and obese (106%). Further, metformin increased IκBα levels in both WG (150%) and RG (67%) of obese rats, indicative of reduced IKKβ activity (P<0.05), and was associated with reduced IRS1-pSer^{307} (30%) in the WG of obese rats (P<0.02). From these data we conclude that metformin treatment appears to exert an inhibitory influence on skeletal muscle IKKβ activity, as evidenced by elevated

IκBα levels and reduced IRS1-Ser^{307} phosphorylation in a fiber-type specific manner.

Keywords: insulin signaling, AMPK, IKKβ, Zucker rat

Introduction

Insulin resistance is considered a characteristic feature of a clustering of diseases referred to often as the metabolic syndrome (Grundy, Brewer, Cleeman, Smith, & Lenfant, 2004). Such associated pathologies include not only cardiovascular disease but also Type II Diabetes Mellitus and, more recently, inflammatory-related metabolic diseases. In this regard, obesity is often associated with insulin resistance, notably in skeletal muscle (Arkan et al., 2005; Perseghin, Petersen, & Shulman, 2003). This has immense clinical and economic significance as obesity has reached epidemic proportions not only in the U.S., but in Western Society at large (Mokdad, Marks, Stroup, & Gerberding, 2004). Although increased physical activity and nutritional interventions have been used to reduce the progression or reverse obesity (and hence improve insulin action), it has often been met with limited success due to low, long-term patient compliance. These circumstances leave pharmacological interventions as an attractive adjunct or alternative therapeutic intervention not only for treating obesity but also associated type II diabetes. Currently, the insulin-sensitizing drug metformin, a member of the biguanide drug class, is widely prescribed. Despite its widespread prescription however, its basic mechanism of action is poorly understood, slowing the development of even more effective insulin-sensitizing molecules.

The link between obesity and insulin resistance can be partly explained by the NF-κB pathway and its associated upstream kinases, whose activity is

stimulated as a result of the chronic and excessive lipid circulation and accumulation in non-adipose tissue sites, a prominent feature of obesity (Kahn & Flier, 2000; Wellen & Hotamisligil, 2005). Lipid accumulation in skeletal muscle has been shown to be associated with insulin resistance (McGarry, 2002), and weight loss appears to reverse this effect (Gray et al., 2003). Ectopic fat deposition can lead to activation of the inhibitor of κB kinase β (IKKβ) and induction of its downstream substrate, the inflammatory transcription factor NF-κB (Arkan et al., 2005). Specifically, NF-κB is sequestered in the cytoplasm in an inactive state while complexed with the inhibitor protein called inhibitor κBα (IκBα). Upon stimulation by IKKβ, which is itself stimulated by both cytokines and lipids (Hotamisligil, 2006), IκBα is phosphorylated and degraded, resulting in the liberation of NF-κB, which migrates into the nucleus and activates transcription of inflammatory cytokine genes. Interestingly, in addition to activating NF-κB, IKKβ has also been shown to *directly* phosphorylate serine$^{312\ (Human)/307(rodent)}$ on insulin receptor substrate (IRS)-1, leading to a decrease in insulin signal transduction (Cai et al., 2005; Ghanim et al., 2001; Perseghin et al., 2003; Yuan et al., 2001).

In contrast to the insulin-resistant effects of excessive IKKβ activity, AMP-activated protein kinase (AMPK), a prominent metabolic enzyme, acts to improve insulin sensitivity, potentially counter-acting the deleterious effects of lipid excess. Additionally, many commonly prescribed anti-diabetic medications, such as metformin and the thiazolidinediones, are known to increase AMPK activity

(Lessard et al., 2006; Suwa, Egashira, Nakano, Sasaki, & Kumagai, 2006). In the past few years, research has revealed the role of AMPK as an inhibitor of IKKβ activity in certain cell types. AMPK has been shown to inhibit both fatty acid- and TNFα-induced increases in IKKβ activity in cultured endothelial cells (Cacicedo et al., 2004; Hattori et al., 2006), macrophages (Jhun et al., 2004), and astrocytes (Giri et al., 2004). Furthermore, metformin treatment has also displayed anti-IKKβ properties in the liver and has been shown to prevent lipid-induced insulin resistance. Namely, Cleasby et al. (Cleasby et al., 2004) observed elevated levels of IκBα, indicative of reduced IKKβ activity, in liver from metformin-treated rodents.

Inasmuch as skeletal muscle is the main consumer of glucose *in vivo*, studying the effects of metformin on IKKβ activity in skeletal muscle, and the role of AMPK in possibly mediating this response, holds great potential for addressing the obesity-diabetes relationship in skeletal muscle. Metformin has been shown to upregulate AMPK activity in both human and rat skeletal muscle (Musi, 2006; Suwa et al., 2006). Work in endothelial cells and liver has shown that metformin can reduce IKKβ activity, raising the possibility that activation of AMPK may be a mechanism by which metformin improves insulin sensitivity in skeletal muscle. Therefore, the aim of this study was to 1) determine whether metformin attenuates IKKβ activity in skeletal muscle of obese rats, 2) determine whether changes in AMPK are associated with reduced IKKβ activity in skeletal muscle, and 3) examine whether a decrease in IKKβ activity coincides with reduced

phosphorylation of Ser^{307} on IRS1 in skeletal muscle and whether this translates into increased glucose tolerance.

Materials and Methods

Materials

All antibodies used in this investigation were obtained from commercial sources. Anti-rabbit monoclonal IRS-1 IP antibody for immunoprecipitation was purchased from Santa Cruz Biochtech (Santa Cruz, CA). IRS-1 and Phospho-IRS1 Ser^{307} antibodies were purchased from Millipore (Billerica, MA). Antibodies for phospho-AMPKα, and AMPKα were purchased from Cell Signaling Technologies (Beverly, MA) and IκBα from Santa Cruz Biochtech (Santa Cruz, CA). Horseradish peroxidase-conjugated anti rabbit secondary antibodies were purchased from Cell Signaling Technologies (Beverly, MA).

Animals and housing

All protocols for animal use were approved by the Animal Care and Use Committee at East Carolina University. Lean and obese male Zucker rats were obtained from Harlan (Indianapolis, IN) and were housed under controlled temperature (23 °C) and lighting (12 hours of light, 0600-1800 hours; 12 hours of dark; 1800-0600 hours) with free access to water and standard rat chow. Animals were fasted 10 h before the oral glucose tolerance tests (OGTT).

Metformin treatment and oral glucose tolerance testing

Obese and lean Zucker rats were randomly assigned to receive either control (saline) or metformin (320 mg/kg/day) by daily gavage for four weeks (N=8 per group) with the final dose given 4 h prior to sacrifice. At the end of the fourth week, an OGTT (2 g/kg BW by oral gavage of dextrose) was performed on

each animal. Blood glucose (glucose oxidase method, One Touch Ultra glucose analyzer; Lifescan, Milpitas, CA) and insulin levels (ELISA kit, Linco Research, St. Charles, MO) were determined in the fasting condition and at time 30, 60, and 120 min after dextrose administration (data was expressed as area under each curve). On experimental days, rats were anesthetized with 0.1 ml/100 g body wt of a mixture containing 90 mg/ml ketamine and 10 mg/ml xylazine. With blood flow intact, white gastrocnemius (WG), red gastrocnemius (RG), and soleus muscles were harvested from hind limbs. Samples were rapidly dissected, cleaned, and frozen within seconds in liquid nitrogen and stored at -80º C until analysis.

Preparation of skeletal muscle homogenates

Frozen muscle samples (50-80 mg) were homogenized in ice-cold lysis buffer [50 mM HEPES, 50 mM Na^+ pyrophosphate, 100 mM Na^+ fluoride, 10 mM EDTA, 10 mM Na^+ orthovanadate, 1% Triton X-100, and protease and phosphatase (1 and 2) inhibitor cocktails (Sigma, St. Louis, MO)]. Homogenates were sonicated for 10 sec then rotated for 2 h at 4 C. After centrifugation for 25 min at 15,000 *g*, supernatants were extracted and protein content was detected using a BCA protein assay (Pierce, Rockford, IL) and individual homogenate volumes were separated into 50 µg of protein before being frozen in liquid nitrogen and stored at -80 C until used for immunoblotting.

Immunoblotting

For IRS-1, homogenates were subjected to 10 µl IRS-1 monoclonal IP antibody (Santa Cruz Biotech, Santa Cruz, CA) overnight then coupled to protein A sepharose beads and rotated for 2 hours (Amersham Biosciences, Uppsala Sweden) and eluted with sample buffer. Samples were separated by SDS-PAGE using 7.5% or 10% Tris·HCl gels and then transferred to PVDF membranes for probing by appropriate antibodies. Following incubation with primary antibodies, blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. Horseradish peroxidase activity was assessed with ECL solution (Thermo Scientific, Rockford, IL), and exposed to film. The image was scanned and band densitometry was assessed with Gel Pro Analyzer software (Media Cybernetics, Silver Spring, MD). Content of phospho-proteins (using phospho-specific antibodies) was calculated from the density of the band of the phospho-protein divided by the density of the protein using the appropriate antibody.

Statistical analysis

All data are presented as means ± SEM. Two-way analysis of variance (ANOVA) was used to compare group and treatment (SPSS, Chicago, IL). Where an interaction was observed, post hoc analysis (Bonferroni and Tukey's HSD) was used to determine significance. Correlation analysis was performed by the Pearson product-moment method. Significance level was established *a priori* at $P \leq 0.05$.

Results

Glucose tolerance

OGTTs were performed to determine glucose tolerance before and after treatment and area under the curve (AUC) was used to compare results. No difference in glucose or insulin AUC was observed in the lean control versus metformin-treated rodents. However, both glucose and insulin AUC were significantly reduced in the obese rats as a result of the 4-week metformin treatment (Figure 3.1; $P<0.05$).

Metformin and AMPK activation

AMPK activation was determined by measuring phosphorylation of AMPK and its substrate acetyl-CoA-carboxylase (ACC) while controlling for total levels of both proteins. No difference in AMPK activation was observed in the controls between lean and obese in any of the three muscles. Moreover, chronic metformin treatment did not significantly affect AMPK phosphorylation in RG or soleus in either the lean or obese rodents (Figure 3.2A), and this was further evident by no change in levels of pACC in these muscles (Figure 3.2B). In contrast, metformin resulted in a significant increase in both phosphorylation of AMPK and ACC in both the lean (57% for pAMPK and 525% for pACC) and obese (106% for pAMPK and 710% for pACC) rats in WG compared with control animals (Figures 3.2A and B; $P<0.05$).

Metformin and IκBα

Inasmuch as metformin has been shown to reduce IKKβ activity in various tissues, IκBα was measured as an indicator of IKKβ activity. No differences in IκBα levels were observed between controls animals in either soleus or RG and chronic metformin treatment resulted in no significant effect in soleus from the lean or obese animals (Figure 3.3A). However, IκBα levels were significantly lower in WG of obese when compared with lean. Further, metformin treatment increased IκBα levels in both RG and WG of obese animals by 67% and 150%, respectively, to a level similar to that seen in lean (P<0.05 for RG, P<0.005 for WG). When combining all data from WG, IκBα showed a strong correlation to AMPK phosphorylation (r =0.755, P<0.005, Figure 3.3B).

IRS1-Serine^{307} phosphorylation

Due to the implicated role of serine phosphorylation of IRS1 in inhibiting insulin signaling, we measured IRS1-pSer^{307} and controlled for total IRS1. Similar to our observation with IκBα, there was no significant effect of treatment on pSer^{307} levels soleus in either lean or obese rodents (Figure 3.4A). Similarly, no differences were observed in RG, although pSer^{307} in obese tended to be lower in the metformin-treated vs control animals (P=0.061). In contrast, pSer^{307} levels in control animals were significantly higher in obese WG compared with lean (P<0.05). Moreover, pSer^{307} levels were significantly reduced (30%) with metformin treatment in WG from obese rodents (P<0.05), which follows results from the OGTT. Inasmuch as IκBα and pSer^{307} appeared to follow similar trends

in WG, we performed a correlation analysis between the two variables. When comparing $pSer^{307}$ and IκBα in all groups, no significant correlation was observed (R=-0.536, *P*=0.067). However, in WG alone the correlation between $pSer^{307}$ and IκBα reached significance (*r* =-0.789, *P*<0.01), suggesting an inverse relationship in WG (Figure 3.4B).

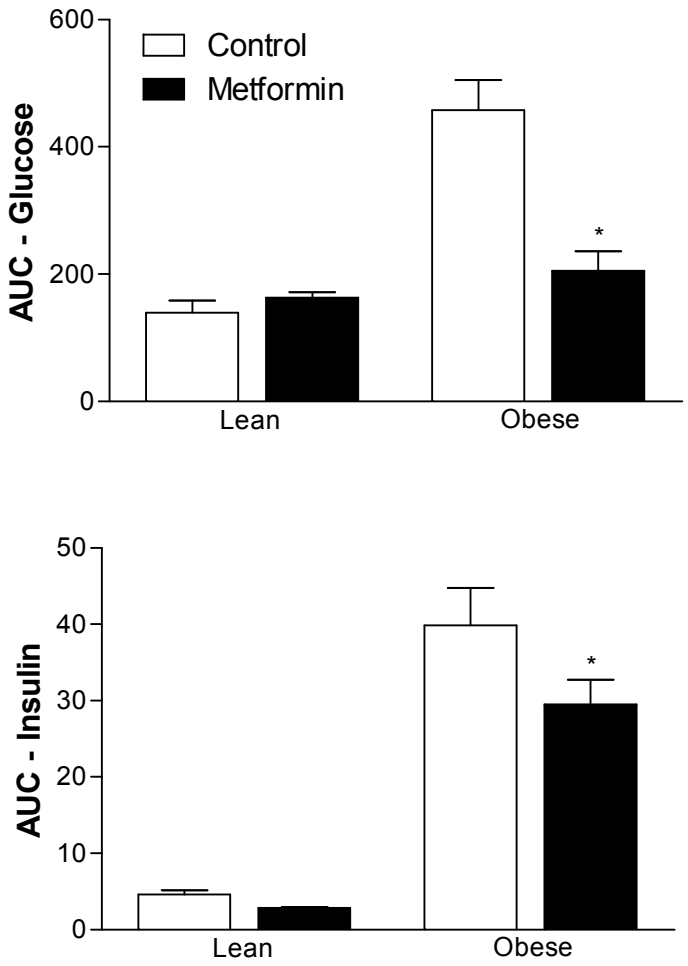

Figure 3.1. Area under the curve (AUC) from an oral glucose tolerance test performed on obese and lean male Zucker controls following 4 weeks of metformin treatment. **P*<0.05. Metformin treatment in the obese rats improved glucose tolerance compared with control.

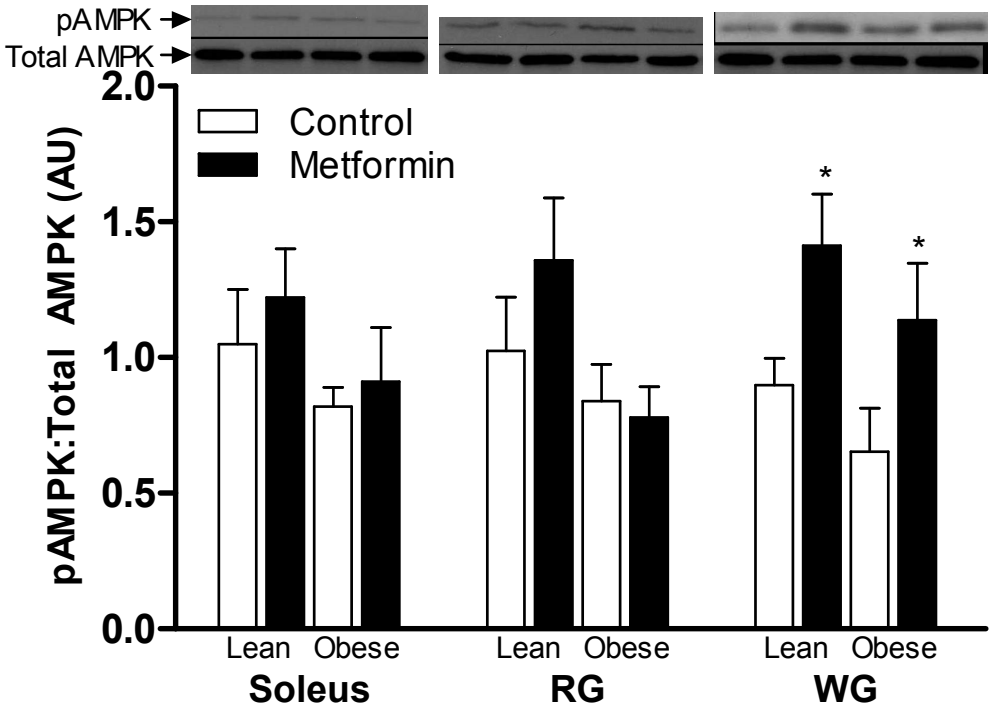

Figure 3.2A. The effect of vehicle (open bars) or metformin (filled bars) on AMPK phosphorylation in soleus, RG, and WG. *P<0.05. AU, arbitrary units.

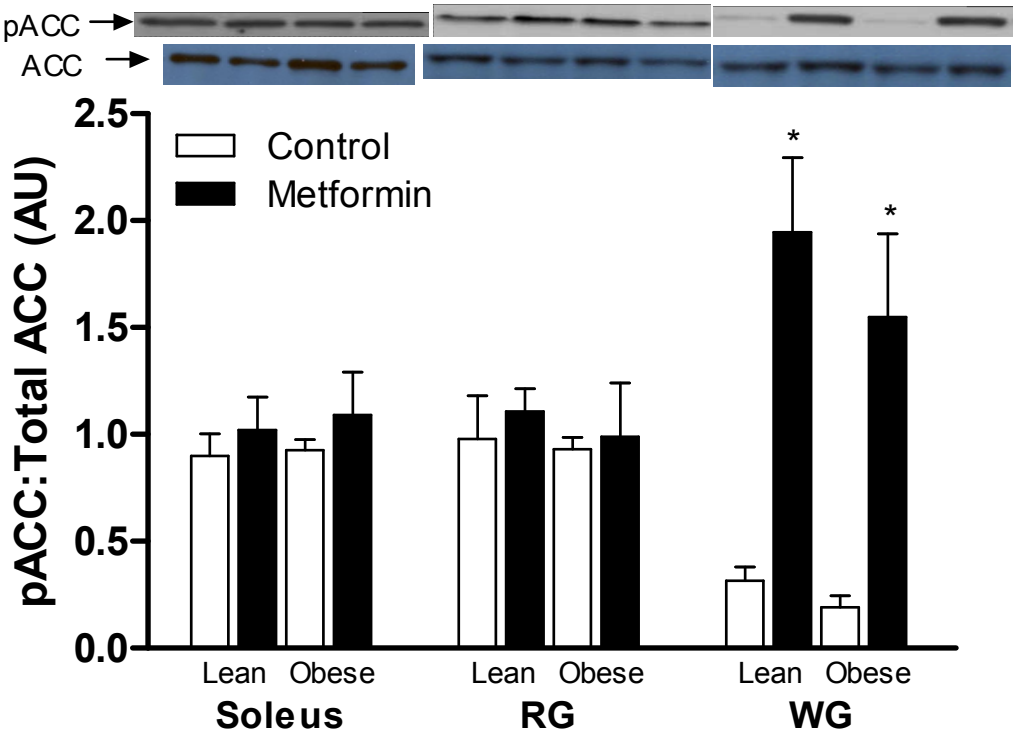

Figure 3.2B. The effect of vehicle (open bars) or metformin (filled bars) on ACC phosphorylation in soleus, RG, and WG. **P*<0.05. AU, arbitrary units.

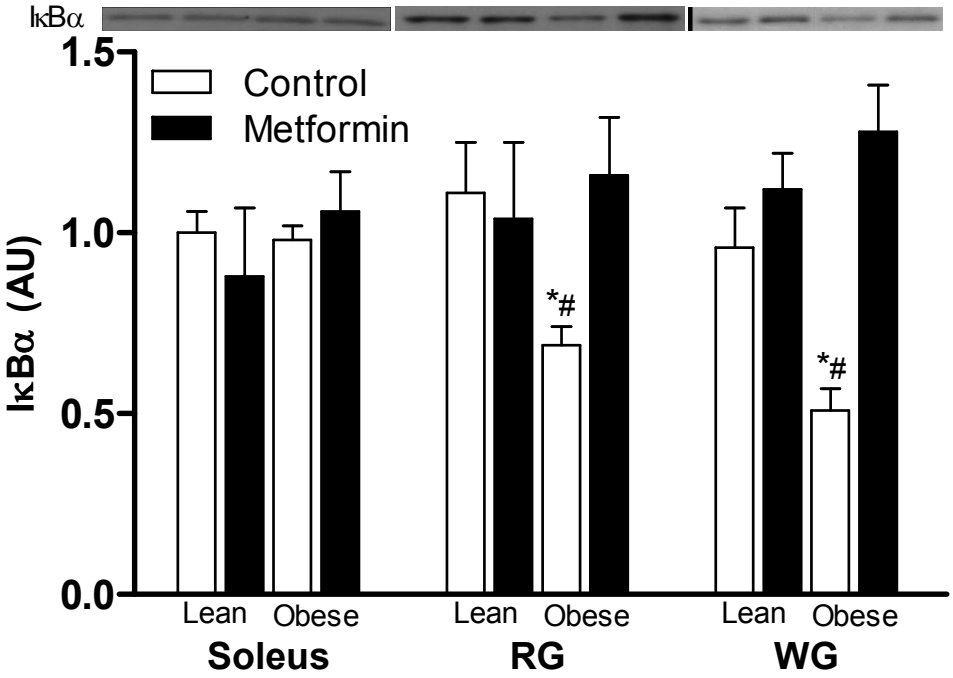

Figure 3.3A. The effect of vehicle (open bars) or metformin (filled bars) on IκBα levels in soleus, RG, and WG. $P<0.05$ for vehicle vs. metformin treatment within same phenotype (*) and lean vs. obese within a given treatment (#).

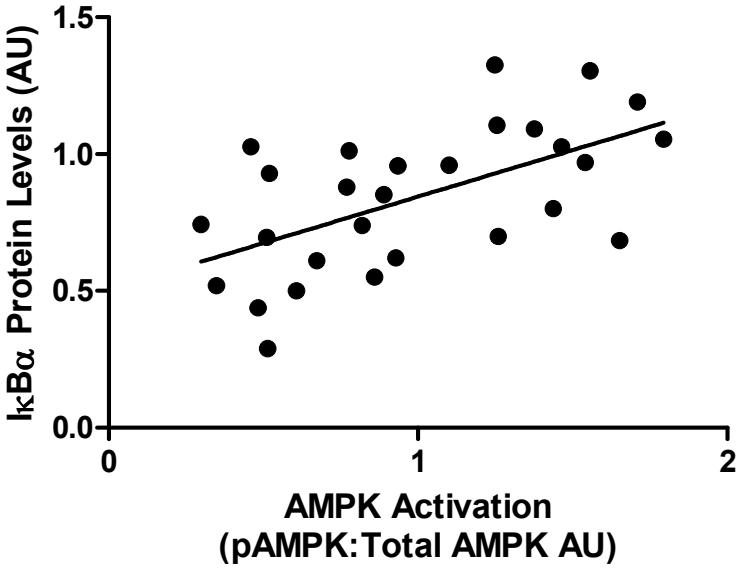

Figure 3.3B. The correlation between AMPK and IκBα in WG from obese and lean male Zucker rats. AMPK activation and IκBα levels were significantly correlated in WG (r =0.755, *P*<0.005).

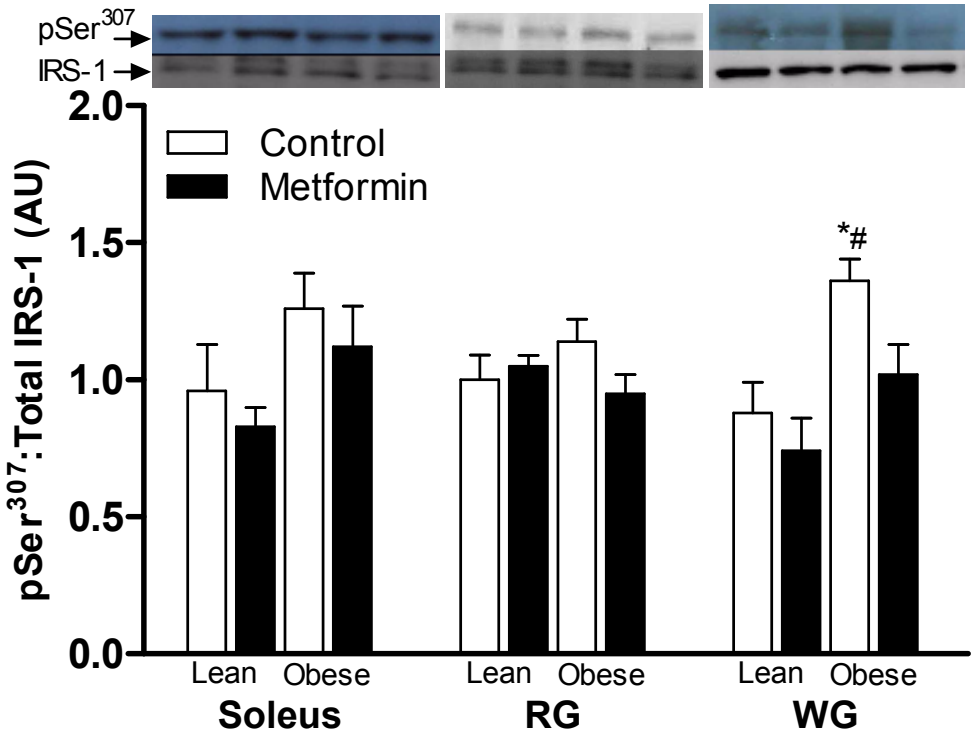

Figure 3.4A. The effect of vehicle (open bars) or metformin (filled bars) on IRS1-pSer^{307} in soleus, RG, and WG. $P<0.05$ for vehicle vs. metformin treatment within same phenotype (*) and lean vs. obese within a given treatment (#).

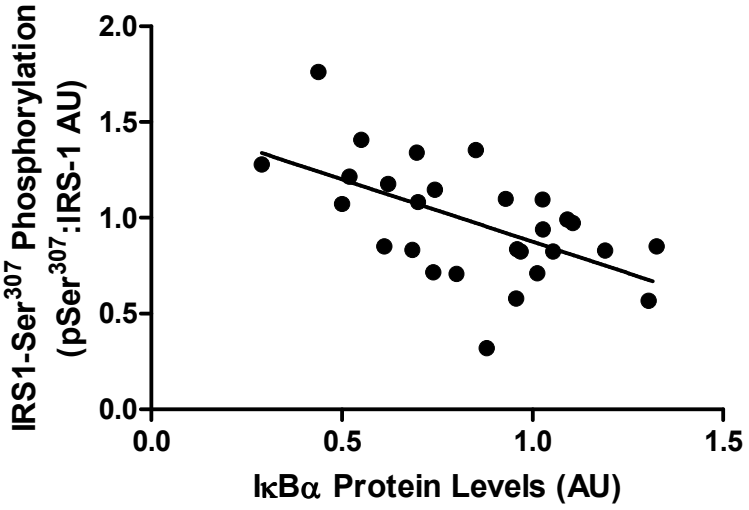

Figure 3.4B. The correlation between IκBα and IRS1-pSer^{307} in WG from obese and lean Zucker male rats. IκBα and IRS1-pSer^{307} levels were significantly related in WG (R=-0.789, *P*<0.01).

Discussion

The main finding of the present study is that the beneficial effects of metformin treatment on glucose tolerance in obese, insulin resistant rodents is associated not only with an elevation in AMPK activity, but also IκBα levels in WG from obese, insulin-resistant rats. This finding is an indication of reduced IKKβ activity and suggests that metformin treatment is able to reduce IKKβ activity and restore IκBα protein levels within the muscle. Moreover, these observations are associated with improved insulin signaling, as evidenced by reduced IRS1-pSer^{307} levels. Finally, levels of IκBα and IRS1-Ser^{307} were significantly and inversely correlated with metformin-treated obese rats, but only in white muscle, suggesting a fiber-type specific action of metformin on the IKKβ signaling pathway.

Recent work has effectively established the role of IKKβ as an inhibitor of insulin action that results in insulin resistance. In particular, salicylate is known to inhibit the activity of IKKβ, and pretreatment of salicylate in lipid-infused rats rescues glucose tolerance back to similar levels seen in control rats when compared with lipid infusion alone (Yin et al., 1998). Further, Yuan et al. (Yuan et al., 2001) showed that *Ikk*β$^{+/-}$ mice have lower fasting glucose and insulin concentrations compared to *Ikk*β$^{+/+}$ littermates when on high-fat diets. Additionally, IKKβ KO mice experience no decrement in insulin sensitivity in response to lipid infusion compared to control mice (Kim et al., 2001).

Collectively, these findings demonstrate that activation of IKKβ is associated with a negative impact on insulin sensitivity.

Contrary to the reduced insulin sensitivity associated with IKKβ activity, AMPK plays a key role in improving glucose handling and increasing insulin action. In muscles from sedentary rats, co-incubation with insulin and the compound 5-aminoimidasole-4-carboxamide-1-β-D-ribofuranoside (AICAR; an AMP-mimetic and AMPK activator) has been shown to induce a twofold greater glucose uptake compared to insulin alone (Fisher et al., 2002). Similarly, AICAR-perfused rat hindlimbs have been shown to increase glucose uptake compared with controls (Merrill et al., 1997).

Research investigating the novel role of AMPK as a mediator in IKKβ's ability to inhibit IRS-1 function in endothelial cells has revealed that AMPK inhibits both fatty acid- and TNFα-induced increases in NF-κB in cultured endothelial cells (Cacicedo et al., 2004). When endothelial cell cultures were incubated with palmitate, inflammatory markers increased, but this response was attenuated in the presence of AICAR. Moreover, AICAR acted to prevent NF-κB activation in the presence of TNFα. This provides strong evidence in support of a role for AMPK in attenuating inflammation and associated cellular metabolic dysfunction. Additionally, metformin, which leads to AMPK activation, dose-dependently inhibits TNFα-induced NF-κB activation, whereas blocking signaling through AMPKα1 (via small interfering RNA) attenuates metformin- and AICAR-

induced inhibition of NF-κB activation by TNFα, further supporting a role for AMPK attenuating the inflammation response (Hattori et al., 2006).

Research involving metformin has also displayed anti-IKKβ properties in the liver. Cleasby et al. (Cleasby et al., 2004) observed elevated levels of IκBα in liver from metformin-treated rodents, providing indirect evidence that AMPK may be serving as an IKKβ inhibitor in an insulin sensitive tissue. These findings provide the rationale for exploring the potential role of AMPK as an IKKβ inhibitor in skeletal muscle inasmuch as muscle represents the main site of insulin-dependent glucose uptake.

The idea of AMPK attenuating inflammatory activity in skeletal muscle has only recently been investigated and, due to a scarcity of data, a consensus has yet to be reached. For example, Steinberg et al. (Steinberg et al., 2006) observed that muscle cells with constitutively active AMPK were protected from TNFα-induced suppression of insulin-stimulated glucose uptake (TNFα has been shown to elicit an increase in IKKβ activity). In *ob/ob* mice, it was found that AMPK activity was significantly reduced compared with lean controls and that TNFα neutralization in *ob/ob* mice restored AMPK activity to that of lean controls. Furthermore, obese mice exhibited reduced fatty acid oxidation, a defect that was not observed following TNFα neutralization. Lastly, *ob/ob* mice lacking a functional TNFα receptor (*TNF* $^{-/-}$) enjoy greater insulin sensitivity than control *ob/ob* mice. It was observed that AMPK activity was higher in obese *TNF* $^{-/-}$ relative to obese controls. Whereas the evidence provided by Steinberg et al.

(Steinberg et al., 2006) places inflammatory mediators upstream of AMPK, the work by Hattori et al. (Hattori et al., 2006) offer the opposite perspective—that AMPK inhibits NF-κB pathway activity. Moreover, the observations of the current study extend the work by Hattori et al. (Hattori et al., 2006) in that we provide evidence that AMPK may attenuate IKKβ activity in skeletal muscle. In conjunction with improved glucose tolerance, we observed an increase in AMPK phosphorylation, an increase in IκBα levels (suggesting reduced IKKβ activity), and, finally, reduced levels of IRS1-pSer^{307} in white muscle from metformin-treated obese rats.

In contrast, Ho et al. (Ho et al., 2005) explored the effects of AICAR-stimulated AMPK activation in rats *in vivo* and found no reduction in IKKβ phosphorylation 60 min following an intraperitoneal injection of AICAR despite a robust increase in AMPK activity in skeletal muscle. Further, they did not observe any change in IKKβ phosphorylation in isolated rat EDL muscles treated with AICAR. These findings appear to indicate that AMPK does not directly regulate IKKβ activity; however, the acute nature of the study design must be considered. The findings of the current study utilized a chronic treatment intervention and support the possibility that metformin-mediated activation of AMPK attenuates IKKβ activity, as has been established in hepatic tissue (Cleasby et al., 2004).

Interestingly, metformin-mediated activation of AMPK occurs in the absence of any changes in ATP/ADP ratio, indicating that a decrease in cellular

energy charge is not the link between metformin and AMPK activation (Hawley, Gadalla, Olsen, & Hardie, 2002). In this regard, synergistic or independent mechanisms should also be considered. For example, metformin has also been shown to partially inhibit mitochondrial complex I and subsequent free radical production (Owen, Doran, & Halestrap, 2000), raising the possibility that an additional, unique effect of metformin in improving IκBα and IRS1-pSer^{307} levels may be related to its effects on mitochondrial function in addition to that of AMPK activation. An association between free radical production, IKKβ activity and insulin sensitivity has been established and might prove to be a fruitful area of investigation on this topic.

It is noteworthy that we observed neither an increase in AMPK activity nor a reduction in IKKβ activity or IRS1-pSer^{307} in soleus with metformin treatment. Similarly, no differences were noted in either AMPK or IRS1-pSer^{307} levels in RG, although IκBα levels increased in obese RG with metformin treatment. The singular change in IκBα levels without an accompanying reduction in IRS1-Ser^{307} phosphorylation in RG was unexpected, though the comparison of IRS1-pSer^{307} levels between metformin-treated and control obese animals did approach statistical significance (P=0.061). Future studies seem warranted then to determine if both statistical and physiological significance would be realized in red muscle following a longer treatment time as would be expected in human subjects under chronic metformin prescription.

In contrast to the observations in soleus and RG, AMPK, IκBα, and IRS1-pSer^{307} levels were all affected by chronic metformin treatment in WG from the obese rats. Specifically, metformin treatment increased AMPK activity and was associated with both an increase in IκBα protein levels and a reduction in IRS1-pSer^{307}. In accordance with these findings, it should be recognized that differences in the IKKβ signaling pathway between muscle fiber types have been noted by other investigators. For example, Bhatt et al. (Bhatt, Dube, Dedousis, Reider, & O'Doherty, 2006) observed a reduction in IκBα levels (increase in IKKβ activity) with diet-induced obesity in rat skeletal muscle in a fiber-type dependent manner. Specifically, obesity was associated with decreased levels of IκBα in superficial vastus (white, fast twitch-glycolytic), whereas the soleus (red, slow twitch-oxidative) appeared to be protected from such an effect. Additionally, Iglesias et al. (Iglesias, Furler, Cooney, Kraegen, & Ye, 2004) observed an AICAR-induced improvement in glucose uptake in white muscle that was not evident in red muscle. Inasmuch as metformin may exert its action by altering mitochondrial function, it is possible that given red muscle's prevalence of mitochondria, a greater metformin dose is required to elicit the same response as observed in white muscle.

In summary, these findings demonstrate that IRS1-Ser^{307} phosphorylation is elevated in certain muscle types of obese, highly insulin resistant Zucker rats. Moreover, metformin treatment improves glucose tolerance in the obese Zucker rats, and this treatment effect is associated with increased AMPK activity and

reduced IKKβ activity, at least in white muscle. These findings are novel, in that they offer support for the hypothesis that AMPK may be upstream of IKKβ and that under the experimental conditions present in the current study, AMPK appears to exert an inhibitory effect on IKKβ in a fiber-type dependent manner which contributes to the change in muscle insulin signaling and whole-body glucose tolerance. Future studies investigating dose and treatment time for metformin seem warranted when considering potential effects on the IKKβ signaling pathway in muscles of red fiber type composition.

Chapter Four

Lipid-induced Insulin Resistance is Prevented in Lean and Obese Myotubes by AICAR Treatment

Abstract

The molecular mechanisms of obesity-associated insulin resistance are becoming increasingly clear and the effects of various lipid molecules, such as diacylglycerol and ceramide, on the insulin signal are being actively explored. To better understand the divergent response to lipid exposure between lean and obese, we incubated primary human muscle cell cultures from lean (BMI < 25 kg/m^2) and morbidly obese (BMI > 40 kg/m^2) subjects with the saturated fatty acid palmitate. Additionally, given that AMPK-activating drugs are widely prescribed for their insulin-sensitizing effects, we sought to determine whether AICAR-stimulated AMPK activity could prevent or reverse the deleterious effects of lipid on insulin signaling. We found that palmitate incubation for 1 h in lean myotubes reduced (p<0.05) IκBα mass, elevated IRS-1 Ser^{312} phosphorylation, and decreased insulin-stimulated phosphorylation of Akt and AS160 and that AICAR inclusion prevented these effects. Moreover, in contrast to the lean, we observed that myotubes from morbidly obese individuals appear to be largely resistant to the detrimental effects of 16-h lipid exposure as was evident by the absence of a reduction in insulin-stimulated IRS-1 Tyr phosphorylation, phospho-Akt, and phospho-AS160 (p<0.05). Further, lipid exposure significantly reduced IκBα levels and increased IRS1-$pSer^{312}$ in lean myotubes only (p<0.05). Despite a divergent response to lipid between lean and obese myotubes, AICAR inclusion improved insulin signaling in myotubes from lean and morbidly obese subjects. These findings suggest an important role for regular exercise in

addition to offering a potential mechanism of action for oral AMPK-activating agents, such as TZDs and metformin.

Introduction

Skeletal muscle is the main site of insulin-mediated glucose uptake and it has been shown that skeletal muscle is the principal site for peripheral insulin resistance (Shulman et al., 1990). Moreover, it is well established that intramyocellular lipid accumulation is associated with insulin resistance (McGarry, 2002). Our group has previously demonstrated that obesity is associated with reduced insulin sensitivity (Dohm et al., 1988) and that weight loss, through both lifestyle intervention (Houmard et al., 2004) and surgery (Bikman et al., 2008; Gray et al., 2003; Houmard et al., 2002), reduces intramuscular lipid and improves insulin sensitivity in humans. Additionally, lipid metabolites such as ceramide and diacylglycerol (DAG) have been shown to inhibit insulin signaling in a variety of tissue, including liver (Kolak et al., 2007) and skeletal muscle (Chavez et al., 2003; C. Yu et al., 2002).

The precise mechanism through which lipid interrupts the insulin signaling cascade is unclear, however a likely candidate is serine phosphorylation of the insulin receptor substrate (IRS)-1 (Cai et al., 2005; Yuan et al., 2001) possibly through the actions of novel lipid-sensitive protein kinase C isoforms (Griffin et al., 1999; Schmitz-Peiffer, 2002; C. Yu et al., 2002), c-jun-N-terminal kinase (JNK) (Aguirre et al., 2002; de Alvaro et al., 2004; Ropelle et al., 2006) and the inhibitor of κB kinase β (IKKβ) (Arkan et al., 2005; Cai et al., 2005; Kim et al., 2001; Sinha et al., 2004; Yuan et al., 2001), which are activated by lipid metabolites (Schmitz-Peiffer, 2002; C. Yu et al., 2002).

Both obesity and insulin resistance are associated with an increased low-grade inflammatory tone, as evidenced by increased levels of proinflammatory cytokines as well as greater activity of the inflammatory factor, NF-κB (Bastard et al., 2006; Festa et al., 2001; Permana et al., 2006; Warnberg & Marcos, 2008), which is responsible for the transcription of multiple cytokines (including TNF-α and IL-1β) (Granger et al., 2004; Piva et al., 2006). IKKβ is also a prominent mediator in the NF-κB pathway through its actions on the NF-κB inhibitor IκBα. Briefly, IκBα is phosphorylated by IKKβ, which results in the rapid degradation of IκBα and subsequent migration of NF-κB into the nucleus.

A host of insulin-sensitizing drugs have been shown to act, in part, through inhibition of NF-κB pathway activity, including salicylate (Kim et al., 2001), thiazolidinediones (TZDs)(Ghanim et al., 2001; LeBrasseur et al., 2006; Todd et al., 2007), and metformin (Cleasby et al., 2004; G. Zhou et al., 2001). Interestingly, evidence indicates that both TZDs and metformin increase activity of the AMP-activated protein kinase (AMPK) (Fryer, Parbu-Patel, & Carling, 2002; LeBrasseur et al., 2006; Lessard et al., 2006; Ye et al., 2006). AMPK is ubiquitous in mammalian cells and is considered the 'fuel gauge' of the cell, responding when cellular energy status is threatened, such as a decrease in ATP concentration, by induction of catabolic ATP-generating processes and inhibition of synthetic (ATP-consuming) processes (Winder, 2001). By stimulating the mitochondrial oxidation of fatty acids (Ferre et al., 2003; McGarry, 2002), AMPK likely prevents the accumulation of lipid metabolites that activate NF-κB pathway

intermediates and disrupts insulin signal transduction (Fediuc et al., 2008). However, AMPK has also been shown to prevent cytokine-induced NF-κB pathway stimulation and result in reduced IKKβ action (Cacicedo et al., 2004; Cleasby et al., 2004; Hattori et al., 2006; Hattori et al., 2008; Lihn et al., 2008), suggesting a more direct effect of AMPK on the NF-κB pathway.

Our lab has previously shown that incubation with saturated fatty acid evokes insulin resistance via reduced signaling in primary muscle cells cultured from lean individuals (Bell et al., unpublished data). In addition, we observed that insulin signaling was blunted in cultured muscle cells from morbidly obese individuals, compared to lean controls (Bell et al., unpublished data). In the present study we more deeply explore the result of various palmitate incubation periods in human myotubes from lean and morbidly obese subjects in regards to its effect on insulin signaling. Additionally, given the widely prescribed use of oral AMPK activators in diabetes treatment, we examine whether AICAR-induced AMPK activation mitigates and/or prevents the deleterious effects of lipid on insulin signaling.

Materials and Methods

Subjects

Lean (n=8; BMI < 25 kg/m^2) and morbidly obese (n=8; BMI > 40 kg/m^2) Caucasian women were recruited for participation in the current study. All subjects were considered sedentary, weight stable, and in good health after filling out a medical history questionnaire. None suffered from metabolic disease, such as type 2 diabetes mellitus. Subjects were informed of potential risks associated with the muscle biopsy and signed an informed consent document, which was approved by the East Carolina University Institutional Review Board.

Primary human muscle cell cultures

Following a 12-h overnight fast, skeletal muscle from the vastus lateralis of subjects was obtained by percutaneous biopsy (Evans, Phinney, & Young, 1982). Approximately 40-50 mg of muscle sample was transferred to refrigerated low-glucose Dulbecco's Modified Eagle's Medium [DMEM; media and media supplements were purchased from Gibco (Invitrogen Corporation, Carlsbad, CA) unless otherwise stated] and all visible connective and adipose tissue was removed prior to culture. Myoblasts were suspended in growth media (GM) [DMEM supplemented with 10% fetal bovine serum (FBS), 0.5 mg/ml BSA, 0.5 mg/ml fetuin, 20 ng/ml human epidermal growth factor, 0.39 µg/ml dexamethasone, and 50 µg/ml gentamicin/amphotericin B (Lonza Inc., Walkersville, MD)], and cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95 O_2. After reaching 80-90% confluence, myoblasts from lean and morbidly

obese subjects were separately pooled into two banks then subcultured onto T-75 type I collagen-coated plates (Fisher Scientific, Waltham, MA) followed by transfer onto 6-well type I collagen-coated plates (Fisher Scientific). After achieving 80-90% confluence, cells were differentiated by replacing GM with low-serum differentiation media (DM) [DMEM, 2% heat-inactivated horse serum, 0.5 mg/ml BSA, 0.5 mg/ml fetuin, and 50 µg/ml gentamicin/amphotericin B]. Media was changed every 1-2 days. Media-containing lipid was prepared by dissolving palmitate in ethanol and diluted in DMEM containing 1% BSA and 1 mM carnitine. On the treatment day (day 6), mature myotubes were serum starved with fasting media (FM) (DMEM, 0.2% BSA) for 4 h prior to actual treatment.

The *initial* experiment involved mytotubes from the lean cell bank exposed to 1-h incubations with 0.45 mM palmitate in the presence or absence of 2 mM AICAR (Calbiochem, San Diego). The *secondary* experiment involved myotubes from lean and obese cell banks exposed to a 16-h incubation in one of two conditions using DMEM with 1% BSA and 1) no supplementation or 2) supplemented with 0.45 mM palmitate. The *final* experiment similarly employed myotubes from both lean and obese cell banks. Following serum starvation, cells were incubated in one of four 16-h treatments using DMEM with 1% BSA and 1) no supplementation (control), 2) supplemented with 0.45 mM palmitate, 3) supplemented with 0.45 mM palmitate and 2 mM AICAR, and 4) supplemented with 0.45 mM palmitate with 2 mM AICAR added for the final 4 h. During the final 10 min of treatment cells were incubated in the presence or absence of 100 nM

insulin and subsequently rinsed with DPBS and harvested in 400 µl of ice-cold lysis buffer ([50 mM HEPES, 50 mM Na^+ pyrophosphate, 100 mM Na^+ fluoride, 50 mM Na^+ pyrophosphate, 10 mM Na^+ orthovanadate, 10 mM EDTA, 1% Triton X-100, and protease and phosphatase (1 and 2) inhibitor cocktails (Sigma, St. Louis, MO)] per well and sonicated for 5 sec, followed by a 2-h rotation at 4°C. Cell viability was determined by comparison with control before and after treatment by trypan blue cell exclusion.

Immunoblotting

Total protein content was measured using a BCA protein assay (Pierce, Rockford, IL) and individual homogenate volumes were aliquoted into 20 µg and 200 µg of protein before being frozen in liquid nitrogen and stored at -80°C until used for immunoblotting. For IRS-1, IRS1-pSer^{312} and IRS1-phosphotyrosine, 200 µg homogenates were subjected to 10 µl IRS-1 monoclonal IP antibody (Santa Cruz Biotech, Santa Cruz, CA) overnight then coupled to protein A sepharose beads (Amersham Biosciences, Uppsala Sweden)(for IRS-1 and pSer^{312}) or to 40 µl anti-phosphotyrosine-agarose antibody (Sigma-Aldrich, Inc., St. Louis, MO), respectively, then rotated for 2 hours at 4°C and eluted with a 1:10 solution of Bond Breaker TCEP (Pierce, Rockford, IL) and Laemmli Sample Buffer (Biorad, Hercules, CA). Samples were separated by SDS-PAGE using 7.5% or 10% Tris·HCl gels and then transferred to PVDF membranes for probing by appropriate antibodies. Following incubation with primary antibodies, blots were incubated with appropriate HRP-conjugated secondary antibodies. IRS-1

and Phospho-IRS1 Ser^{307} antibodies were purchased from Millipore (Billerica, MA). Antibodies for phospho-AMPKα (Thr172), AMPKα, phospho-ACC, ACC, phospho-Akt-Substrate, and pan-AS160 were purchased from Cell Signaling Technologies (Beverly, MA) and phospho-Akt, Akt, IκBα, and actin from Santa Cruz Biotech (Santa Cruz, CA). Horseradish peroxidase activity was assessed with ECL solution (Thermo Scientific, Rockford, IL), and exposed to film. The image was scanned and band densitometry was assessed with Gel Pro Analyzer software (Media Cybernetics, Silver Spring, MD). Equal loading of proteins was ensured by probing for actin. Content of phospho-proteins (using phospho-specific antibodies) was calculated from the density of the band of the phospho-protein divided by the density of the total protein using the appropriate antibody.

Statistics

Data are expressed as means ± SEM of four separate experiments. Statistical analyses were performed by one-way ANOVA followed by Tukey's post-hoc test where necessary. Significance was set at $P < 0.05$.

Results

1-h Treatment in Lean Myotubes

In control media, insulin stimulation was associated with reduced pAMPK levels ($p<0.05$; Figure 4.1A), a finding confirmed in other studies (Fediuc et al., 2008). Additionally, one hour of lipid incubation had no significant effect on pAMPK levels or its substrate, pACC (Figure 4.1B). In contrast, when AICAR was included in the incubation, phosphorylation of both AMPK and ACC increased dramatically ($p<0.001$). As expected, insulin stimulation significantly elevated Akt and AS160 phosphorylation and, whereas levels of pAMPK were seemingly unaffected by lipid incubation alone, insulin-stimulated phosphorylation of Akt and AS160 was significantly diminished with lipid incubation ($p<0.001$; Figure 4.1C and D). However, AICAIR co-incubation prevented the lipid-induced decrement in Akt and AS160 activation. Inasmuch as IKKβ has been implicated in reducing insulin signaling through its inhibitory actions on IRS-1, IκBα, a downstream substrate and inverse indicator of IKKβ, was determined as a measure of IKKβ action. Lipid treatment significantly reduced IκBα mass ($p<0.05$), though this effect was prevented with AICAR co-incubation (Figure 4.1E). Moreover, while there was a tendency for insulin stimulation to reduce IkBα in control media, this disparity increased with lipid treatment ($p<0.05$). Finally, insulin stimulation had no significant effect on IRS1-Ser^{312} phosphorylation in lean myotubes in control media, although palmitate incubation was associated with elevated levels of insulin-stimulated IRS1-pSer^{312}

($p<0.05$; Figure 4.1F). Conversely, AICAR inclusion significantly reduced Ser^{312} phosphorylation in both basal and insulin-stimulated cells in comparison to both control and lipid incubation alone ($p<0.01$).

16-h Lipid Treatment in Lean and Obese Myotubes

We were further interested in comparing myotubes from lean and obese subjects and determining whether they exhibit a divergent response to palmitate incubation. Firstly, no baseline differences in pAMPK or pACC were observed between lean and obese cells (Figure 4.2A and B). However, lipid incubation appeared to increase the disparity between basal and insulin-stimulated pAMPK levels as evident in a significant decrease in pAMPK levels with insulin stimulation in lipid treated lean cells ($p<0.05$). As a marker of the proximal insulin signal, IRS-1 Tyrosine phosphorylation was determined. Insulin increased IRS1-pTyr levels in lean cells ($p<0.01$), but not obese in control media, and insulin-stimulated IRS1-pTyr levels were greater in lean myotubes compared with obese ($p<0.01$; Figure 4.2C). However, lipid treatment significantly reduced the ability of insulin to stimulate IRS-1 Tyrosine phosphorylation in the lean myotubes.

In exploring downstream markers of insulin signaling, phosphorylation states of both Akt and Akt Substrate of 160 kDa (AS160) were determined. Insulin-stimulated pAkt was significantly elevated in all treatments in both lean and obese myotubes (Figure 4.2D). Interestingly, this effect was diminished with lipid incubation in lean myotubes only. In fact, the difference in insulin-stimulated pAkt levels between the lean and obese myotubes in control media ($p<0.05$)

were removed upon inclusion of palmitate such that there was no significant difference between the two with lipid. Further, pAS160 levels in the lean myotubes followed a similar pattern to pAkt in that insulin resulted in pAS160 increasing significantly ($p<0.01$) as well as the effect of palmitate in reducing the magnitude of change (Figure 4.2E). In contrast, insulin stimulation had no effect on pAS160 levels in myotubes from obese subjects despite differences in pAkt, suggesting an interruption between Akt and AS160 in the obese.

Baseline differences in IκBα mass were observed between myotubes from lean and obese subjects ($p<0.01$; Figure 4.2F). Additionally, lipid treatment was associated with reduced basal IκBα levels in lean myotubes and the disparity between basal and insulin-stimulated levels increased with lipid incubation ($p<0.05$). Further, IRS1-Ser^{312} phosphorylation differed drastically between lean and obese myotubes in control media ($p<0.001$; Figure 4.2G). In contrast to the increase in IRS1-pSer^{312} with lipid incubation in lean myotubes ($p<0.05$), no change was observed in the obese.

16-h Lipid and AICAR Treatment in Lean and Obese Myotubes

Upon differences between lean and obese myotubes being observed with regards to lipid exposure, a follow-up experiment was performed to determine whether AICAR-induced AMPK activation prevents the decrease in insulin signaling with lipid exposure and whether lean and obese myotubes respond different to AICAR. When AICAR was included in the palmitate incubation for either the final 4 h or the full 16 h treatment period, a robust increase in pAMPK

and pACC levels were observed compared to lipid treatment alone in both lean and obese myotubes ($p<0.001$; Figures 4.3A and B). IRS1-pTyr levels followed a similar trend to pAMPK in that AICAR significantly elevated IRS1-pTyr in both lean ($p<0.001$) and obese ($p<0.05$) myotubes compared with lipid alone (Figure 4.3C). Moreover, AICAR inclusion at both time points was associated with restoring differences between basal and insulin-stimulated pTyr levels in the lean myotubes ($p<0.05$). However, despite an increase ($p<0.05$), AICAR stimulation in the obese myotubes did not elevate IRS1-pTyr to similar levels to that seen in the lean.

In looking downstream, insulin-stimulated pAkt increased with AICAR inclusion at both time points in the lean and obese myotubes ($p<0.01$; Figure 4.3D). In contrast, pAS160 levels only tended to increase with the 4-h AICAR incubation in lean myotubes ($p=0.071$; Figure 4.3E) while this same 4-h treatment significantly elevated basal pAS160 in obese myotubes when compared with lipid alone ($p<0.05$). Nevertheless, full co-incubation with both lipid and AICAR significantly increased both basal and insulin-stimulated pAS160 levels in lean and obese versus lipid alone ($p<0.01$), though insulin-stimulated pAS160 levels in obese myotubes were lower compared with lean in the same treatment ($p<0.05$).

IκBα levels were similar between lean and obese myotubes when incubated with lipid alone and insulin caused a decrease in IκBα mass in lean myotubes ($p<0.05$; Figure 4.3F). At both 4 and 16 h of AICAR incubation with

lipid, IκBα increased in lean ($p<0.01$) and obese ($p<0.05$), though the effect was not as robust in the obese myotubes and a difference was observed compared with lean ($p<0.05$). Additionally, AICAR treatment significantly reduced the increase in IRS1-Ser^{312} phosphorylation induced by lipid incubation in insulin-stimulated cells in the lean ($p<0.05$; Figure 4.3G). Similarly, AICAR inclusion significantly reduced IRS1-pSer^{312} levels in obese myotubes ($p<0.01$), and IRS1-Ser^{312} phosphorylation was significantly elevated in the obese compared with the lean in all treatments.

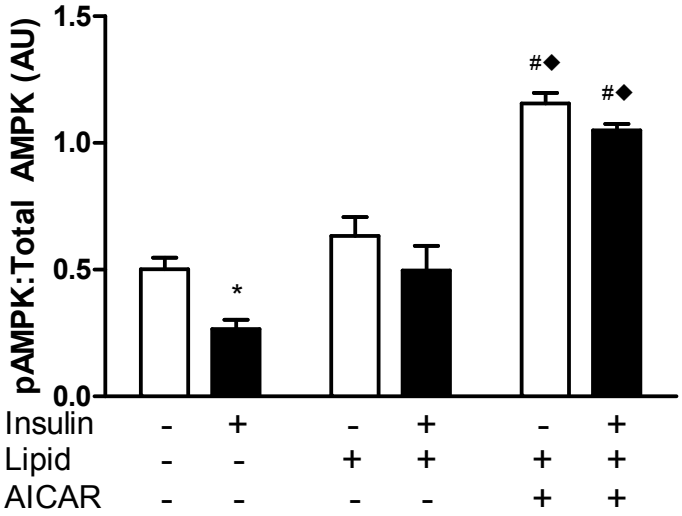

Figure 4.1A. The effects of 1 h of 0.45 mM palmitate with and without AICAR inclusion on pooled lean (BMI < 25 m/kg^2) myotubes on pAMPK. Myotubes were incubated in control media, 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine, or lipid + AICAR, for 1 h, in the presence (black bars) or absence (white bars) of 10 min insulin (100 nM) and probed for pAMPK. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). *p<0.05 for basal vs. insulin stimulation within a given treatment. #p<0.05 for lipid or co-incubation (lipid + AICAR) vs. control. ♦p<0.05 for co-incubation vs. lipid alone.

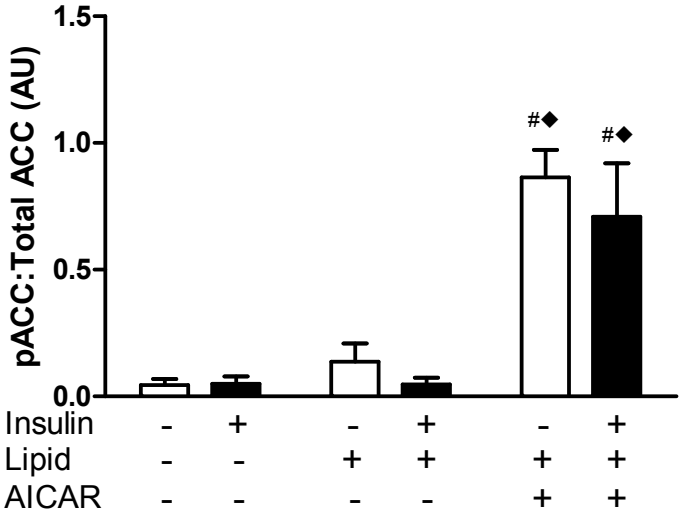

Figure 4.1B. The effects of 1 h of 0.45 mM palmitate with and without AICAR inclusion on pooled lean (BMI < 25 m/kg^2) myotubes on pACC. Myotubes were incubated in control media, 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine, or lipid + AICAR, for 1 h, in the presence (black bars) or absence (white bars) of 10 min insulin (100 nM) and probed for pACC. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). *p<0.05 for basal vs. insulin stimulation within a given treatment. #p<0.05 for lipid or co-incubation (lipid + AICAR) vs. control. ♦p<0.05 for co-incubation vs. lipid alone.

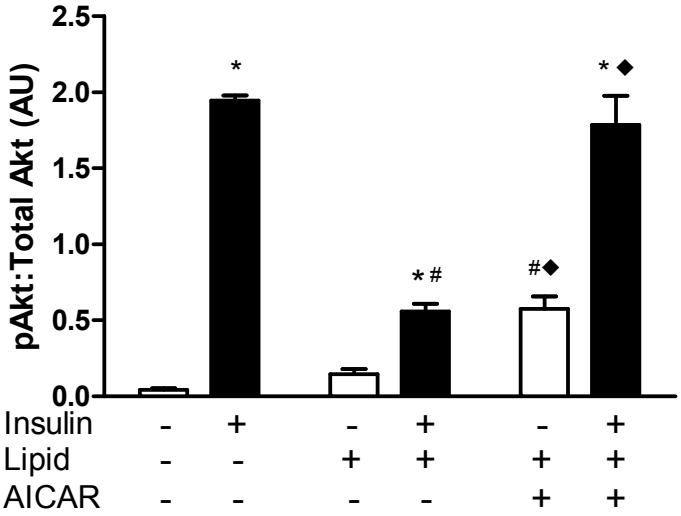

Figure 4.1C. The effects of 1 h of 0.45 mM palmitate with and without AICAR inclusion on pooled lean (BMI < 25 m/kg^2) myotubes on pAkt. Myotubes were incubated in control media, 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine, or lipid + AICAR, for 1 h, in the presence (black bars) or absence (white bars) of 10 min insulin (100 nM) and probed for pAkt. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). *p<0.05 for basal vs. insulin stimulation within a given treatment. #p<0.05 for lipid or co-incubation (lipid + AICAR) vs. control. ♦p<0.05 for co-incubation vs. lipid alone.

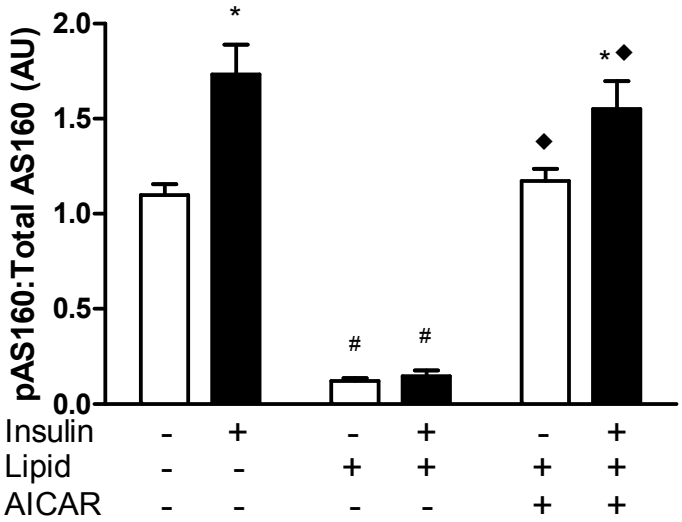

Figure 4.1D. The effects of 1 h of 0.45 mM palmitate with and without AICAR inclusion on pooled lean (BMI < 25 m/kg^2) myotubes on pAS160. Myotubes were incubated in control media, 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine, or lipid + AICAR, for 1 h, in the presence (black bars) or absence (white bars) of 10 min insulin (100 nM) and probed for pAS160. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). *p<0.05 for basal vs. insulin stimulation within a given treatment. #p<0.05 for lipid or co-incubation (lipid + AICAR) vs. control. ♦p<0.05 for co-incubation vs. lipid alone.

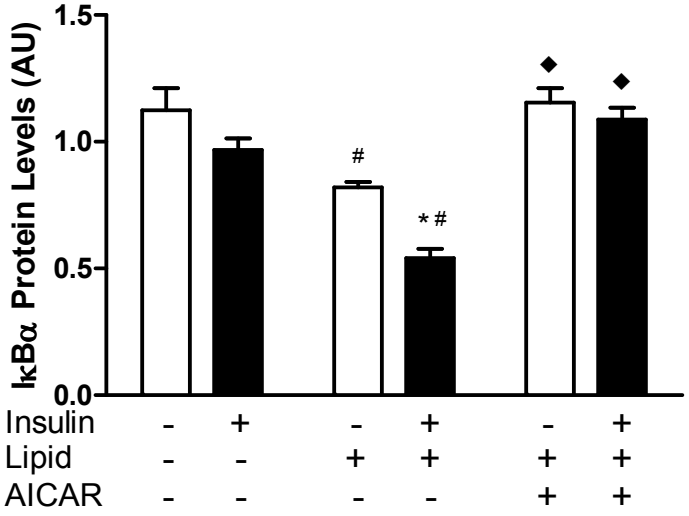

Figure 4.1E. The effects of 1 h of 0.45 mM palmitate with and without AICAR inclusion on pooled lean (BMI < 25 m/kg^2) myotubes on IκBα. Myotubes were incubated in control media, 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine, or lipid + AICAR, for 1 h, in the presence (black bars) or absence (white bars) of 10 min insulin (100 nM) and probed for IκBα. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). *p<0.05 for basal vs. insulin stimulation within a given treatment. #p<0.05 for lipid or co-incubation (lipid + AICAR) vs. control. ♦p<0.05 for co-incubation vs. lipid alone.

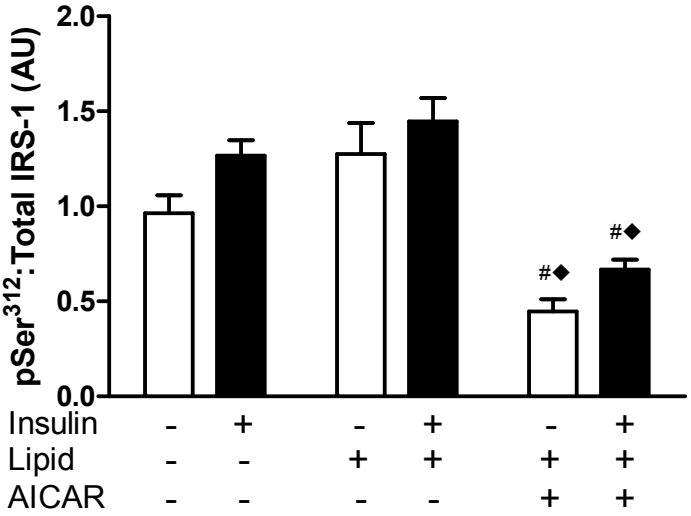

Figure 4.1F. The effects of 1 h of 0.45 mM palmitate with and without AICAR inclusion on pooled lean (BMI < 25 m/kg^2) myotubes on pSer^{312}. Myotubes were incubated in control media, 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine, or lipid + AICAR, for 1 h, in the presence (black bars) or absence (white bars) of 10 min insulin (100 nM) and probed for IRS1-pSer^{312}. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). *p<0.05 for basal vs. insulin stimulation within a given treatment. #p<0.05 for lipid or co-incubation (lipid + AICAR) vs. control. ♦p<0.05 for co-incubation vs. lipid alone.

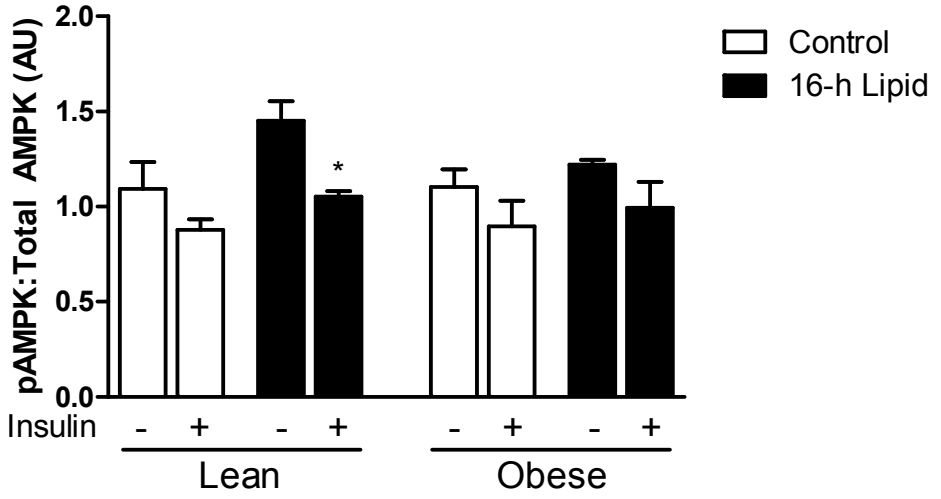

Figure 4.2A. The effects of 16 h of 0.45 mM palmitate exposure on pooled myotubes from lean (BMI < 25 m/kg^2) and morbidly obese (BMI > 40 m/kg^2) humans on pAMPK. Myotubes were incubated in control media (white bars) or 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine (black bars), in the presence or absence of 10 min insulin (100 nM) and probed for pAMPK. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). **p*<0.05 for basal vs. insulin stimulation within a given treatment. #*p*<0.05 for lipid vs. control. ♦*p*<0.05 for obese vs. lean within a given treatment.

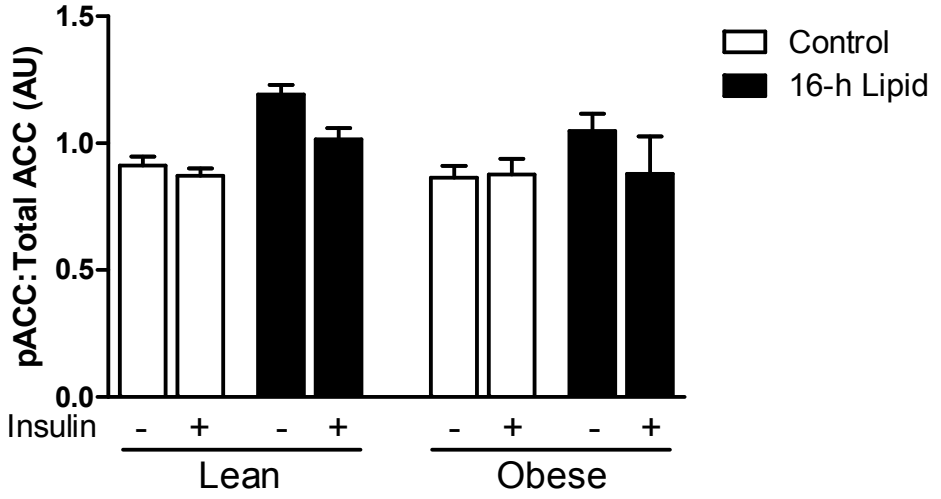

Figure 4.2B. The effects of 16 h of 0.45 mM palmitate exposure on pooled myotubes from lean (BMI < 25 m/kg^2) and morbidly obese (BMI > 40 m/kg^2) humans on pACC. Myotubes were incubated in control media (white bars) or 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine (black bars), in the presence or absence of 10 min insulin (100 nM) and probed for pACC. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). **p*<0.05 for basal vs. insulin stimulation within a given treatment. #*p*<0.05 for lipid vs. control. ♦*p*<0.05 for obese vs. lean within a given treatment.

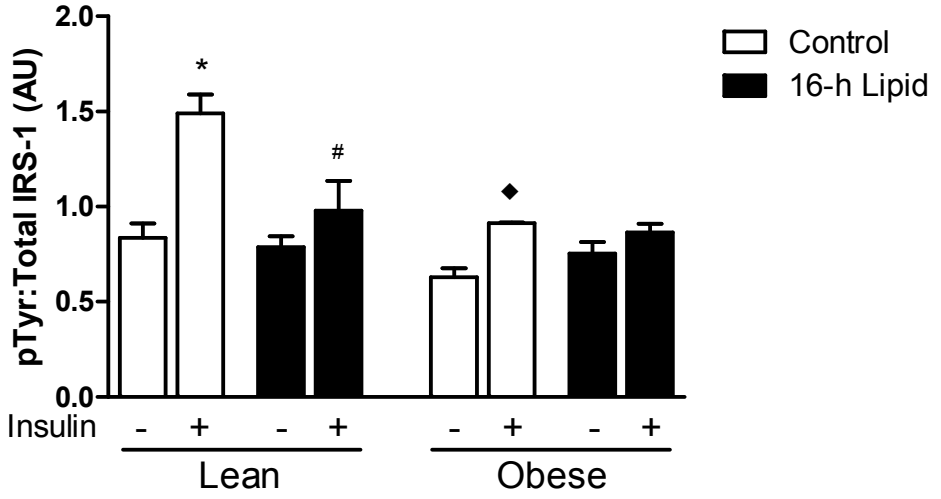

Figure 4.2C. The effects of 16 h of 0.45 mM palmitate exposure on pooled myotubes from lean (BMI < 25 m/kg^2) and morbidly obese (BMI > 40 m/kg^2) humans on IRS1-pTyr. Myotubes were incubated in control media (white bars) or 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine (black bars), in the presence or absence of 10 min insulin (100 nM) and probed for IRS1-pTyr. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). *p<0.05 for basal vs. insulin stimulation within a given treatment. #p<0.05 for lipid vs. control. ♦p<0.05 for obese vs. lean within a given treatment.

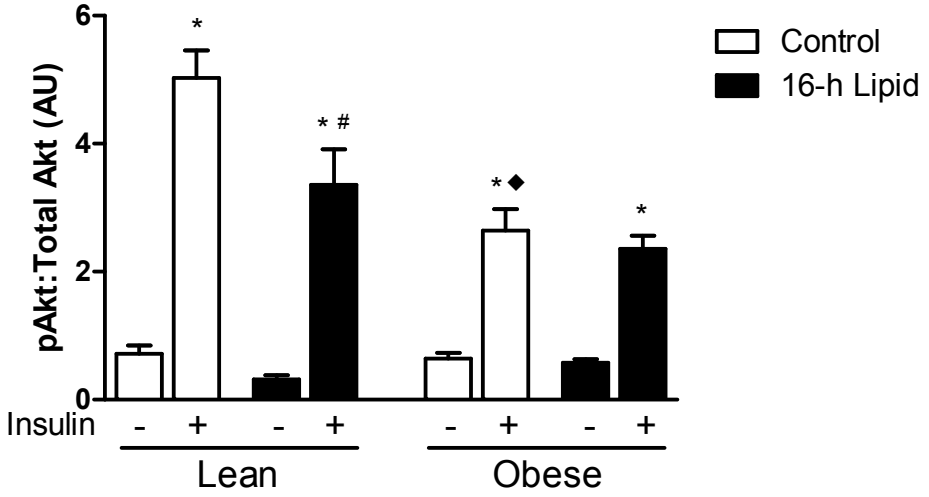

Figure 4.2D. The effects of 16 h of 0.45 mM palmitate exposure on pooled myotubes from lean (BMI < 25 m/kg^2) and morbidly obese (BMI > 40 m/kg^2) humans pAkt. Myotubes were incubated in control media (white bars) or 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine (black bars), in the presence or absence of 10 min insulin (100 nM) and probed for pAkt. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). *p<0.05 for basal vs. insulin stimulation within a given treatment. #p<0.05 for lipid vs. control. ♦p<0.05 for obese vs. lean within a given treatment.

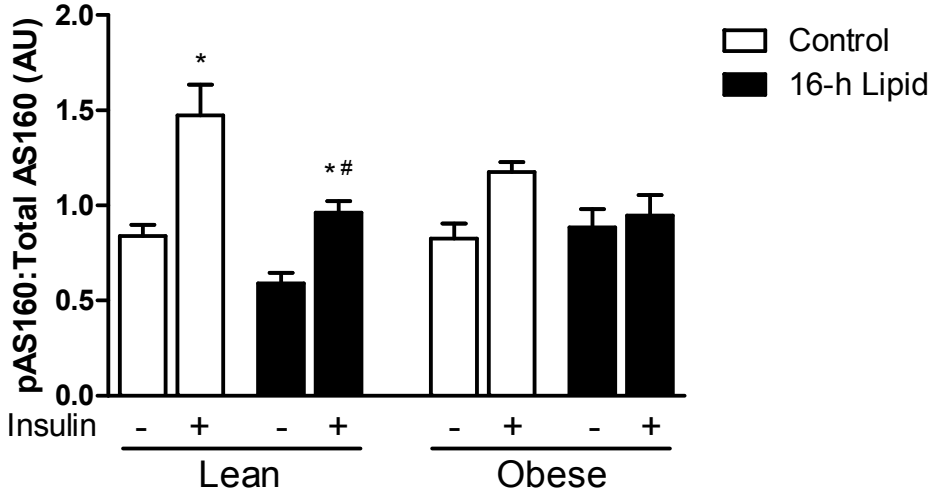

Figure 4.2E. The effects of 16 h of 0.45 mM palmitate exposure on pooled myotubes from lean (BMI < 25 m/kg^2) and morbidly obese (BMI > 40 m/kg^2) humans pAS160. Myotubes were incubated in control media (white bars) or 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine (black bars), in the presence or absence of 10 min insulin (100 nM) and probed for pAS160. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). *p<0.05 for basal vs. insulin stimulation within a given treatment. #p<0.05 for lipid vs. control. ♦p<0.05 for obese vs. lean within a given treatment.

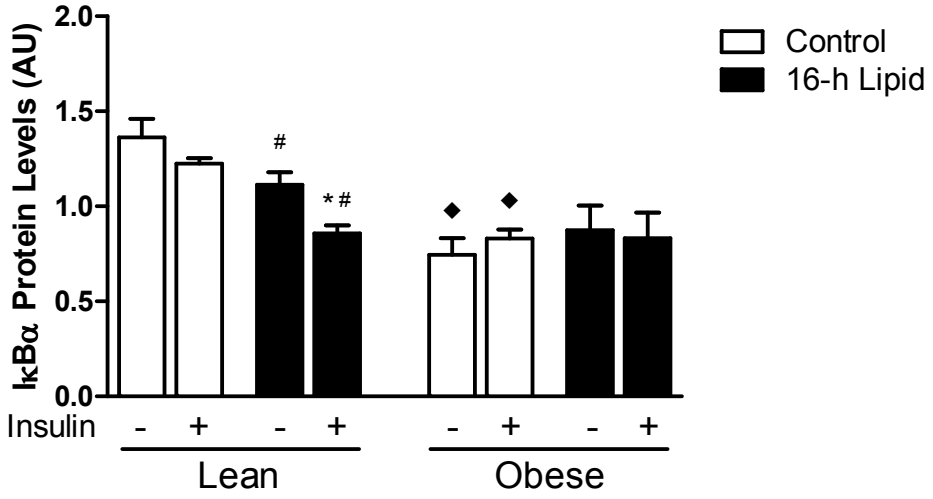

Figure 4.2F. The effects of 16 h of 0.45 mM palmitate exposure on pooled myotubes from lean (BMI < 25 m/kg^2) and morbidly obese (BMI > 40 m/kg^2) humans on IκBα. Myotubes were incubated in control media (white bars) or 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine (black bars), in the presence or absence of 10 min insulin (100 nM) and probed for pIκBα. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). *p<0.05 for basal vs. insulin stimulation within a given treatment. #p<0.05 for lipid vs. control. ♦p<0.05 for obese vs. lean within a given treatment.

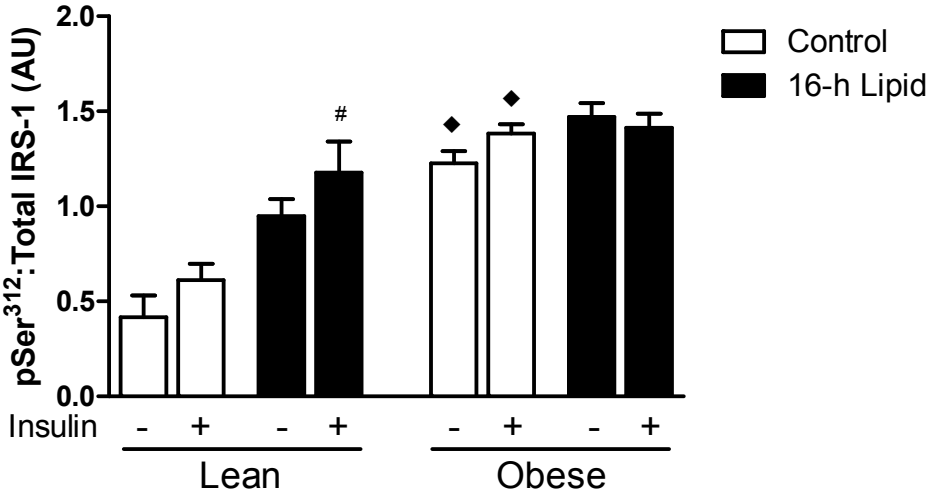

Figure 4.2G. The effects of 16 h of 0.45 mM palmitate exposure on pooled myotubes from lean (BMI < 25 m/kg^2) and morbidly obese (BMI > 40 m/kg^2) humans on pSer^{312}. Myotubes were incubated in control media (white bars) or 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine (black bars), in the presence or absence of 10 min insulin (100 nM) and probed for IRS-1 pSer^{312}. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). **p*<0.05 for basal vs. insulin stimulation within a given treatment. #*p*<0.05 for lipid vs. control. ♦*p*<0.05 for obese vs. lean within a given treatment.

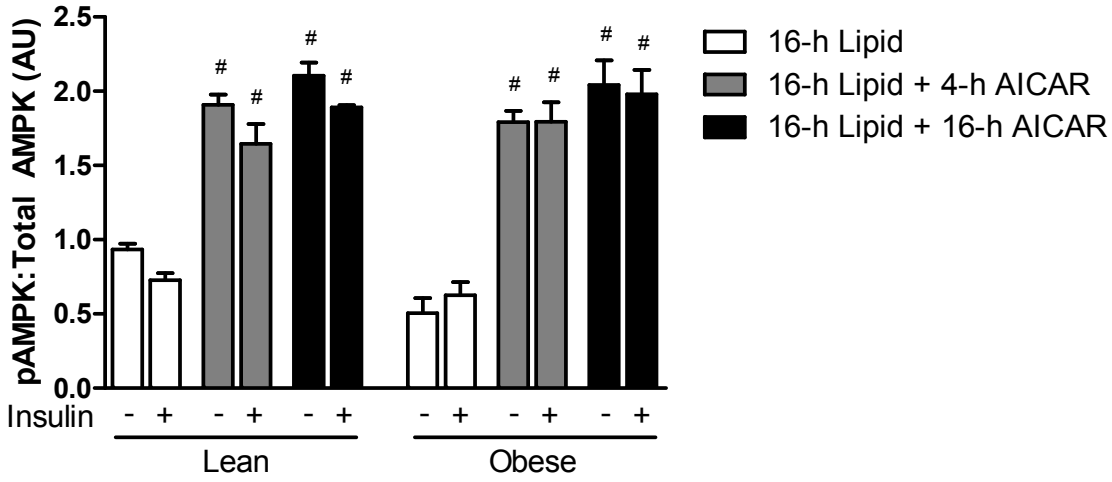

Figure 4.3A. The effects of 16 h of 0.45 mM palmitate and AICAR exposure on pooled myotubes from lean (BMI < 25 m/kg^2) and morbidly obese (BMI > 40 m/kg^2) humans on pAMPK. Myotubes were incubated in 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine (white bars), lipid + 4-h AICAR (grey bars), or lipid + 16-h AICAR, in the presence or absence of 10 min insulin (100 nM) and probed for pAMPK. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). *p<0.05 for basal vs. insulin stimulation within a given treatment. #p<0.05 for co-incubations (4 and 16 h) vs. lipid alone within a give group. ♦p<0.05 for obese vs. lean within a given treatment.

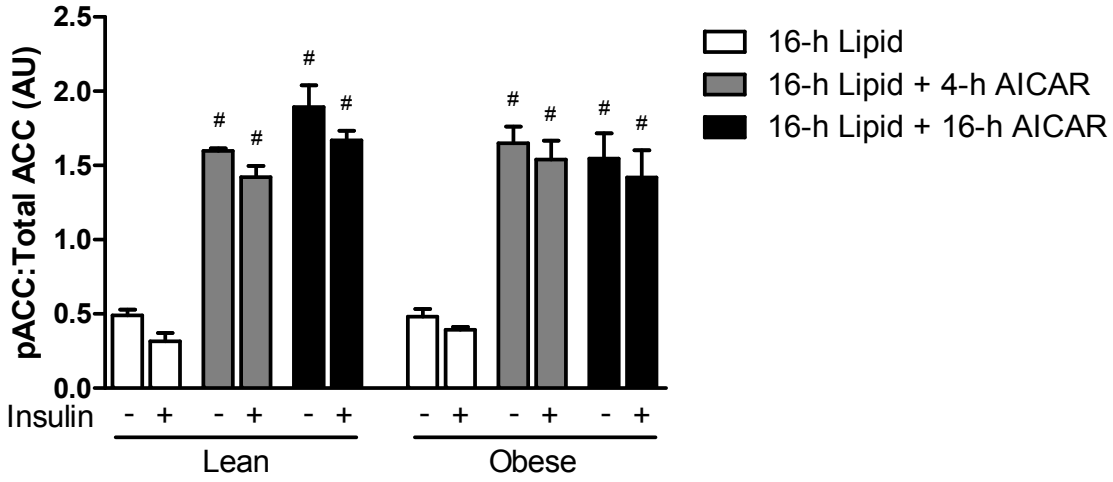

Figure 4.3B. The effects of 16 h of 0.45 mM palmitate and AICAR exposure on pooled myotubes from lean (BMI < 25 m/kg^2) and morbidly obese (BMI > 40 m/kg^2) humans on pACC. Myotubes were incubated in 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine (white bars), lipid + 4-h AICAR (grey bars), or lipid + 16-h AICAR, in the presence or absence of 10 min insulin (100 nM) and probed for pACC. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). *p<0.05 for basal vs. insulin stimulation within a given treatment. #p<0.05 for co-incubations (4 and 16 h) vs. lipid alone within a give group. ♦p<0.05 for obese vs. lean within a given treatment.

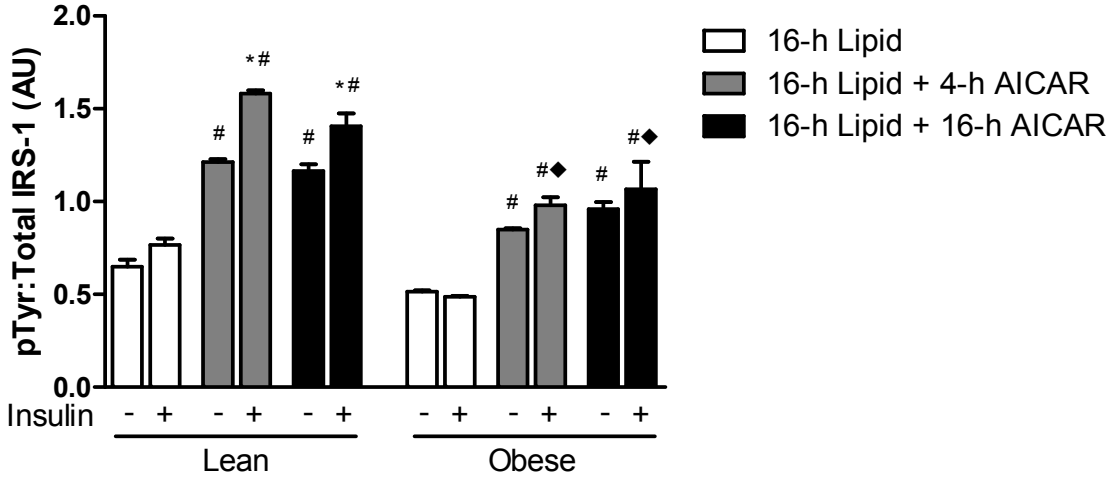

Figure 4.3C. The effects of 16 h of 0.45 mM palmitate and AICAR exposure on pooled myotubes from lean (BMI < 25 m/kg^2) and morbidly obese (BMI > 40 m/kg^2) humans on IRS1-pTyr. Myotubes were incubated in 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine (white bars), lipid + 4-h AICAR (grey bars), or lipid + 16-h AICAR, in the presence or absence of 10 min insulin (100 nM) and probed for IRS1-pTyr. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). **p*<0.05 for basal vs. insulin stimulation within a given treatment. #*p*<0.05 for co-incubations (4 and 16 h) vs. lipid alone within a give group. ♦*p*<0.05 for obese vs. lean within a given treatment.

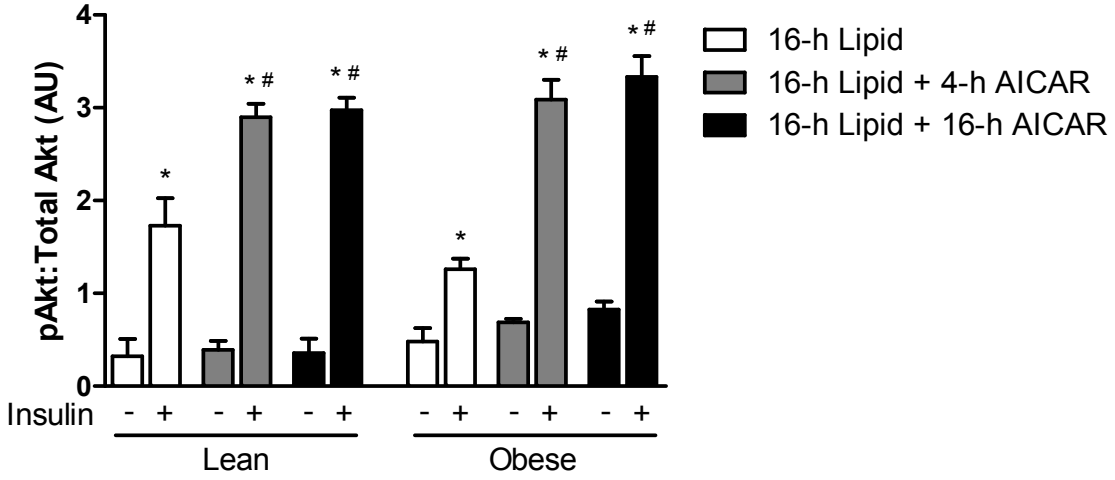

Figure 4.3D. The effects of 16 h of 0.45 mM palmitate and AICAR exposure on pooled myotubes from lean (BMI < 25 m/kg^2) and morbidly obese (BMI > 40 m/kg^2) humans on pAkt. Myotubes were incubated in 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine (white bars), lipid + 4-h AICAR (grey bars), or lipid + 16-h AICAR, in the presence or absence of 10 min insulin (100 nM) and probed for pAkt. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). **p*<0.05 for basal vs. insulin stimulation within a given treatment. #*p*<0.05 for co-incubations (4 and 16 h) vs. lipid alone within a give group. ♦*p*<0.05 for obese vs. lean within a given treatment.

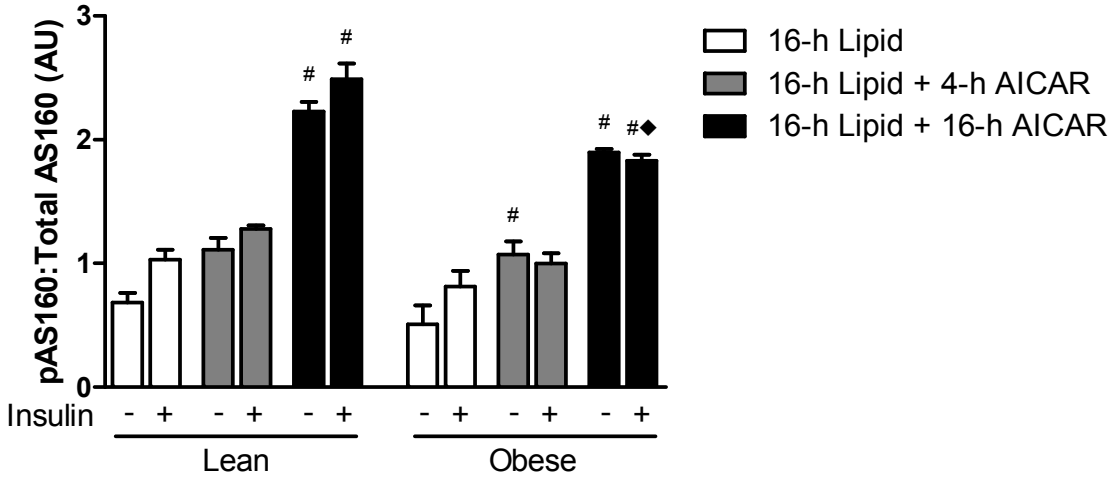

Figure 4.3E. The effects of 16 h of 0.45 mM palmitate and AICAR exposure on pooled myotubes from lean (BMI < 25 m/kg^2) and morbidly obese (BMI > 40 m/kg^2) humans on pAS160. Myotubes were incubated in 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine (white bars), lipid + 4-h AICAR (grey bars), or lipid + 16-h AICAR, in the presence or absence of 10 min insulin (100 nM) and probed for pAS160. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). *p<0.05 for basal vs. insulin stimulation within a given treatment. #p<0.05 for co-incubations (4 and 16 h) vs. lipid alone within a give group. ♦p<0.05 for obese vs. lean within a given treatment.

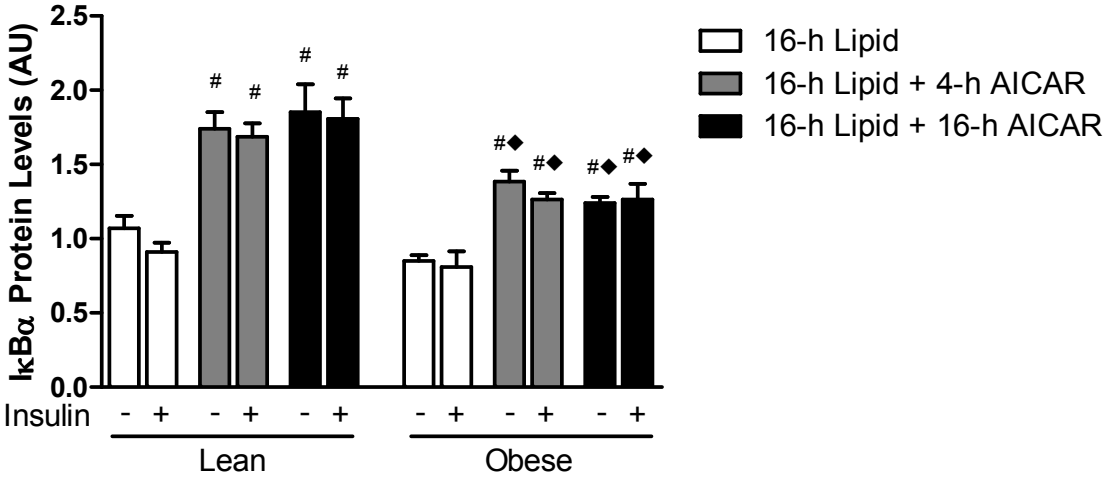

Figure 4.3F. The effects of 16 h of 0.45 mM palmitate and AICAR exposure on pooled myotubes from lean (BMI < 25 m/kg^2) and morbidly obese (BMI > 40 m/kg^2) humans on IκBα. Myotubes were incubated in 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine (white bars), lipid + 4-h AICAR (grey bars), or lipid + 16-h AICAR, in the presence or absence of 10 min insulin (100 nM) and probed for IκBα. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). **p*<0.05 for basal vs. insulin stimulation within a given treatment. #*p*<0.05 for co-incubations (4 and 16 h) vs. lipid alone within a give group. ♦*p*<0.05 for obese vs. lean within a given treatment.

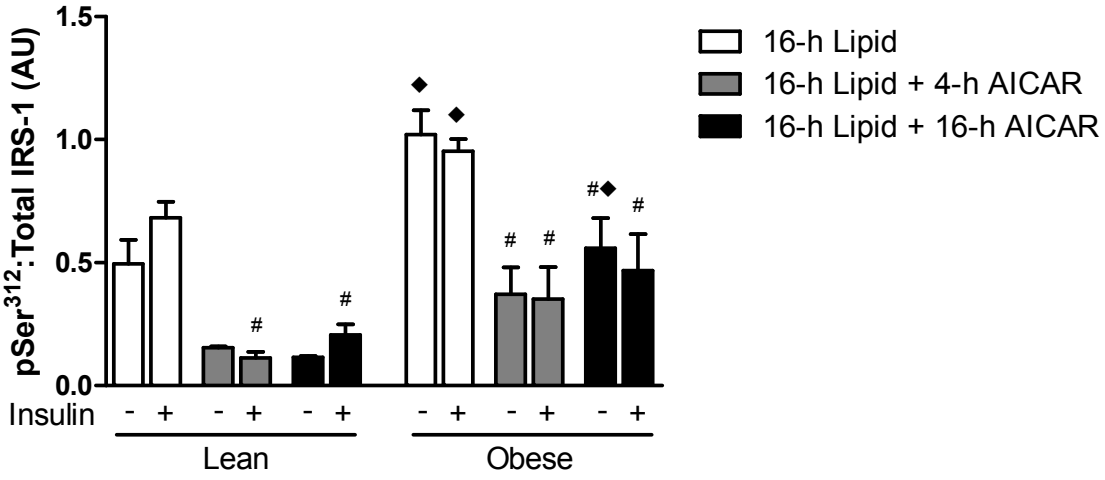

Figure 4.3G. The effects of 16 h of 0.45 mM palmitate and AICAR exposure on pooled myotubes from lean (BMI < 25 m/kg^2) and morbidly obese (BMI > 40 m/kg^2) humans on pSer^{312}. Myotubes were incubated in 0.45 mM palmitate complexed to 1% BSA in low-glucose DMEM with 1 mM carnitine (white bars), lipid + 4-h AICAR (grey bars), or lipid + 16-h AICAR, in the presence or absence of 10 min insulin (100 nM) and probed for IRS1-pSer^{312}. All proteins were compared with either the total of its respective unphosphorylated protein or actin (not shown). *p<0.05 for basal vs. insulin stimulation within a given treatment. #p<0.05 for co-incubations (4 and 16 h) vs. lipid alone within a give group. ♦p<0.05 for obese vs. lean within a given treatment.

Discussion

The findings from the current study support the mounting evidence that saturated fatty acids induce insulin resistance in skeletal muscle via decay of the insulin signal. Specifically, we found that a 1- or 16-h incubation of the saturated fatty acid palmitate with myotubes from lean humans elicited a significant reduction in IκBα mass and insulin-stimulated phosphorylation of Akt and AS160 as well as an elevation in IRS-1 Ser^{312} phosphorylation and that the effects of lipid incubation were completely prevented with AICAR inclusion. In agreement with our previous findings (Bell et al., unpublished data), the current evidence supports the conclusion that myotubes from morbidly obese individuals are resistant to insulin-stimulated signal transduction as well as the detrimental effects of lipid exposure on this signal. This was evident with a 16-h lipid treatment, in contrast to the lean, by the absence of a reduction in insulin-stimulated IRS-1 Tyr phosphorylation, levels of phospho-Akt, and phospho-AS160. Further, whereas lipid exposure significantly reduced IκBα levels and increased IRS1-$pSer^{312}$ in lean myotubes, no effect was observed in the obese. However, despite a divergent response to lipid between lean and obese myotubes, AICAR inclusion restored insulin signaling at every measured step in the lean and improved the signal in obese.

The concentration and type of fatty acid treatment used in this study follows the work of many others (Chavez et al., 2003; Coll et al., 2008; Fediuc et al., 2008; Jove et al., 2005; Jove et al., 2006; Montell et al., 2001; Pimenta et al.,

2008; Weigert et al., 2004; Wu, Song, Xu, Zhang, & Zou, 2007). Palmitate is the major saturated fatty acid within the body and circulating levels of free fatty acids in obese humans have been found to be as high as 800 μM (Roden, 2004). Additionally, saturated fatty acids such as palmitate have been shown to exhibit a particularly striking contrast in regards to its ability to activate lipid-sensitive kinases and reduce both the proximal (IRS-1) and distal (Akt, AS160) insulin signal when compared with unsaturated fats (Coll et al., 2006; Y. Wu et al., 2007).

Recent advances in the area of obesity and diabetes research have revealed a role for various lipid-stimulated kinases that act at IRS-1 and result in attenuation of the insulin signal, namely IKKβ, PKCθ, and JNK1 (Holland et al., 2007). We previously reported differences in IκBα protein levels, an inverse measure of IKKβ action, and IRS1-Ser^{312} phosphorylation in vastus lateralis of lean and morbidly obese humans (Bikman et al., 2008) and herein report a retention of this disparity in cultured myotubes. Moreover, in the lipid- and insulin-sensitive lean myotubes we observed a significant decrease in IκBα levels when exposed to lipid and insulin concurrently in both the 1- and 16-h treatments. Lipid-induced reductions in IκBα levels have been observed as early as 30 min after lipid inclusion (Weigert et al., 2004) and our findings of a decrease in IκBα after 10 min were somewhat unexpected. However, a key difference in the current study is the presence of insulin. Insulin's anabolic role encourages the rapid influx and storage of fatty acids in skeletal muscle (Pimenta

et al., 2008) and the decrement in IκBα after 10 min of insulin treatment in the current study may be a function of the synergistic effects of lipid and insulin resulting in a rapid uptake of lipid in the absence of a comparable increase in oxidation.

In support for our findings implicating a role for IKKβ in mediating lipid-induced insulin resistance in muscle, Sinha et al. (2004) observed that 6 h of palmitate exposure in L6 myotubes resulted in a significant decrease in insulin-stimulated glucose uptake, as well as reduced IκBα mass and phospho-Akt levels. However, when IKKβ action was prevented with inclusion of the IKKβ-specific inhibitor salicylate, signaling was rescued. Additionally, IKKβ inhibition through salicylate treatment in rodents has been shown to be associated with improved glucose tolerance and improved lipid profile (Kim et al., 2001; Yuan et al., 2001). Also, heterozygous *IKK*$\beta^{+/-}$ mice fed a high-fat diet have been found to have lower fasting insulin and glucose values compared with *IKK*$\beta^{+/+}$ littermates (Yuan et al., 2001). Further, Kim et al. (2001) observed that IKKβ knock out mice did not display any decrement in skeletal muscle insulin signaling with lipid infusion.

Additionally, an unexpected observation was the effect of AICAR inclusion on phosphorylation of Akt. Specifically, lipid incubation significantly reduced the ability of insulin to stimulate Akt phosphorylation in the lean (Figure 2D), however, compared to lipid alone, AICAR dramatically increased insulin-stimulated phospho-Akt in both lean and obese (Figure 3D). However, AMPK

has been shown to induce glucose uptake through activation of AS160, which is downstream of Akt (Thong et al., 2007). Additionally, the observation that in the absence of insulin Akt phosphorylation was unchanged with or without AICAR suggests, in contrast to AS160 phosphorylation, that AMPK is not acting directly on Akt, but is rather facilitating the ability of insulin to stimulate Akt phosphorylation. With this in mind, it is tempting to speculate that AICAR-stimulated AMPK activation attenuates the lipid-induced decrement in upstream insulin signaling (Figure 4). Regarding the latter option, research in various cell types has revealed a role for AMPK in inhibiting NF-κB pathway activity (Cacicedo et al., 2004; Giri et al., 2004; Hattori et al., 2006) though evidence of this effect in skeletal muscle is scarce and contrasting (Ho et al., 2005; Lihn et al., 2008; Steinberg et al., 2006). Considering that AICAR treatment in human myotubes has also been found to decrease cytokine production (Lihn et al., 2008), we feel that these observations, in addition to our own findings that AICAR-stimulated AMPK activation restores IκBα levels in the lean and elevates levels above normal in the morbidly obese, suggest a potential role for AMPK in preventing activation of IKKβ in response to lipid.

As mentioned, in exploring the distal insulin signal we observed an unexpected incongruity between Akt and AS160 phosphorylation. AICAR treatment increased insulin-stimulated Akt phosphorylation in both basal and insulin-stimulated myotubes at both 4 and 16 h of incubation. However, AICAR treatment elicited an increase in phospho-AS160 even in the absence of insulin,

offering support for AMPK's direct action on AS160 (Thong et al., 2007). In addition, we observed that AICAR-stimulated AMPK activity only increased phospho-AS160 with full AICAR + lipid co-incubation and that inclusion of AICAR in the final 4 h of treatment elicited a far less dramatic effect. This difference may represent an inability of AICAR-stimulated AMPK activity to fully *restore* the decay of the insulin signal following a prolonged period of lipid incubation alone, whereas AICAR inclusion at the onset of lipid incubation can *prevent* this decay. The ability of AMPK action to prevent lipid-induced insulin resistance has been recently explored in skeletal muscle. Fujii et al. (Fujii et al., 2008) found reduced insulin receptor-β subunit, IRS-1, and Akt protein levels in muscle-specific transgenic (TG) mice expressing an inactive form of AMPKα2 when fed a high-fat diet compared with wild type controls fed the same diet. Further, whereas muscle levels of DAG did not differ between the mice on control diet, DAG levels were significantly elevated in TG mice versus control when fed a high-fat diet. These observations correlated with a reduction in insulin-stimulated glucose uptake in isolated soleus of TG mice.

In summary, the present study shows a divergent response to palmitate incubation between myotubes from lean and morbidly humans. Palmitate incubation in lean myotubes was associated with reduced IκBα protein mass, reduced phosphorylation of IRS1-Tyr, Akt, and AS160, and elevated IRS-1 Ser^{312} phosphorylation, while the myotubes from the morbidly obese did not appear to be significantly affected. In AICAR + lipid co-incubation, AICAR-stimulated

AMPK activity prevented the decay in insulin signaling observed with lipid alone, though AICAR inclusion in the final 4 h of lipid incubation was not sufficient to restore AS160 phosphorylation. Additionally, AMPK activation was associated with reduced IKKβ action. We conclude that AMPK activation prevents lipid-induced insulin resistance in both lean and obese myotubes possibly through the inhibition of lipid-stimulated serine kinases. These findings suggest an important role for regular exercise in addition to offering a potential mechanism of action for oral AMPK-activating agents, such as TZDs and metformin.

Chapter Five

Summary and Conclusions

Exploration into the etiology of type 2 diabetes mellitus has discovered the vital role of the insulin receptor substrate proteins (IRS) in insulin-sensitive tissue like skeletal muscle. In particular, IRS-1 has received a great deal of attention due to the various factors that play a role in impeding its function, primarily lipid-sensitive kinases like the inhibitor of κB kinase β (IKKβ). In contrast, mounting evidence supports the role of AMPK as a vital component in known insulin-sensitizing agents, although the mechanism through which AMPK facilitates the insulin signal is unknown (Figure 1.1). In addition to further exploring the various molecules that have been found to mediate IRS-1 function, we feel that elucidating the role of AMPK in preventing the development of insulin resistance and treating overt diabetes may be very beneficial.

The purpose of this project was to i) determine whether baseline differences in AMP-activated protein kinase (AMPK) activity account for the disparity in insulin sensitivity between post-bypass patients and control subjects; ii) determine the effect of chronic metformin administration (an AMPK activator) on whole-body insulin sensitivity and skeletal muscle insulin signaling in lean and obese Zucker rats; and iii) explore the ability of AICAR-stimulated AMPK activity to prevent and/or reverse the deleterious consequences of palmitate exposure on insulin signaling in myotubes from lean and morbidly obese humans.

Collectively, these studies serve to i) confirm previous findings regarding the presence of insulin signaling defects in skeletal muscle, particularly the role of IKKβ and IRS1-pSer$^{312/307}$; ii) reveal a potential mechanism for the dramatic

improvement in insulin sensitivity following surgery-induced weight loss; iii) establish the differential effects of metformin in stimulating AMPK activity and improving insulin signaling in muscle fiber types and obesity state; iv) confirm previous findings of the retention in cultured myotubes of insulin signaling defects evident in vivo; and v) offer a novel finding of the ability of AICAR-stimulated AMPK activation to prevent lipid-induced insulin resistance via preservation of the insulin signal.

Firstly, we measured IRS1-pSer^{312} and IκBα protein levels, as a surrogate for IKKβ activity, as well as AMPK phosphorylation in the vastus lateralis of post-bypass patients and control subjects and found that IκBα mass and IRS1-phospho-Ser^{312} protein levels in skeletal muscle from post-surgery patients were similar to that seen in muscle from lean subjects despite the post-surgery group having a significantly higher BMI. Additionally, we observed a significant elevation in AMPK phosphorylation in the muscle of post-bypass patients compared with all other groups. While this observation did not support our hypothesis that baseline differences in insulin signaling may be accounted for by various rates of AMPK activity, we feel it may nonetheless assist in explaining the improvements in insulin signaling in the post-bypass group. The disparity in phospho-AMPK seen in the post-bypass group compared with the control subjects may be a function of the varying role of AMPK-stimulating hormones that accompany the surgery and the subsequent tremendous weight loss. In particular, adiponectin is a hormone released from the adipocytes that has been

shown to stimulate AMPK activity in skeletal muscle (Hardie, 2004) and circulating levels of adiponectin have been shown to be elevated following gastric bypass (de Carvalho et al., 2008; de la Torre et al., 2008). Moreover, lifestyle intervention in individuals suffering from type 2 diabetes also elicits a significant improvement in adiponectin levels (Kadoglou et al., 2007). Alternatively, the elevations in phospho-AMPK observed in the post-bypass group may be a function of reduced stomach volume and a heightened sensitivity to fasting in the post-bypass group. AMPK has been shown to mediate a host of adaptations in skeletal muscle in response to fasting to promote fatty acid oxidation, including elevated expression of CD36 and CPT1, which would encourage fatty acid flux into the cell and shuttling into the mitochondria (Long et al., 2005; Long & Zierath, 2008). Muscle biopsies were pulled from subjects following an overnight fast and due to the drastically reduced stomach volume of the post-bypass patients, it is highly possible that these subjects eat relatively less during the final meal on the night preceding the biopsy and are subsequently in a greater state of nutrient deprivation the following day. This heighted sensitivity to fasting (being in a more severe state of nutrient deprivation) may be sensed in the skeletal muscle of post-bypass patients and AMPK is activated in response.

Secondly, we investigated the correlation between AMPK and IKKβ activity in the muscle of lean and obese Zucker rats and, similar to our previous findings, observed no correlation between baseline differences insulin sensitivity and signaling and AMPK phosphorylation. However, in exploring the beneficial

effects of metformin treatment on metabolic function, we found not only an elevation in AMPK action, but also IκBα levels in WG of obese, insulin-resistant rodents. This finding is an indication of reduced IKKβ activity and suggests that metformin treatment is able to stimulate AMPK and reduce IKKβ activity within the muscle. Moreover, these observations were associated with improved insulin signaling, as evidenced by reduced IRS1-pSer^{307} levels. Finally, levels of IκBα and IRS1-Ser^{307} were significantly and inversely correlated with metformin-treated obese rats, but only in white muscle, suggesting a fiber-type specific action of metformin on the IKKβ signaling pathway. The fiber-type specific effect of metformin may be a function of two possibilities. First, the white muscle in the obese rats may contain higher-than-normal levels of lipid due to the gross obesity associated with the fat Zucker rat, and, not being able to handle such high levels of lipid, unlike red muscle, lipid metabolite levels may be higher and, hence, lipid-sensitive kinases may be in a greater state of activation. In fact, muscle levels of the lipid metabolite diacylglycerol (DAG) and PKC activity have been found to be higher in obese Zucker rats compared with lean controls (Qu, Seale, & Donnelly, 1999). Alternatively, given metformin's actions on mitochondrial function, due to the relatively reduced abundance of mitochondria within white muscle compared to red muscle, it is possible that the given dose and time period of treatment of metformin was sufficient to elicit an observable effect in white muscle, though insufficient to evoke the same response in the mitochondria-rich red muscle.

Lastly, we found that a 1-h incubation of the saturated fatty acid palmitate with myotubes from lean humans elicited a significant reduction in insulin-stimulated phospho-Akt and IκBα mass as well as an elevation in IRS-1 Ser^{312} phosphorylation and that the effects of lipid incubation were completely prevented with AICAR inclusion. Moreover, we offered evidence that myotubes from obese individuals appear to be largely resistant to the detrimental effects of lipid exposure. This was evident, in contrast to the lean, by the absence of a reduction in insulin-stimulated IRS-1 tyrosine phosphorylation with a 16-h lipid treatment, as well as no decrement in levels of phospho-Akt and phospho-AS160. Further, whereas lipid exposure significantly reduced IκBα levels and increased IRS1-$pSer^{312}$ in lean myotubes, no effect was observed in the obese. However, despite a divergent response to lipid between lean and obese myotubes, AICAR inclusion restored insulin signaling at every measured step in the lean and improved the signal in obese. From this it appears that AMPK activation prevents lipid-induced insulin resistance in both lean and obese myotubes possibly through the inhibition of lipid-stimulated serine kinases. These findings suggest an important role for regular exercise in addition to offering a potential mechanism of action for oral AMPK-activating agents, such as TZDs and metformin.

The findings of this project support the mounting evidence in favor of the inhibitory effect of intracellular lipid species on insulin signal transduction. In particular, lipid-sensitive kinases, such as IKKβ, have been shown to reduce

insulin signaling and exacerbate skeletal muscle insulin resistance by serine phosphorylation of IRS-1. Due to its location at the proximal end of the pathway, any dysfunction in the action of IRS-1 can result in disruption of the entire cascade. Yamauchi et al. (Yamauchi et al., 1996) observed that skeletal muscle from IRS1-deficient rodents exhibited reduced insulin-stimulated glucose uptake. Further, it has been shown using short interfering (si) RNA-mediated ablation of IRS-1 that the loss of IRS-1 function results in reduced insulin-stimulated glucose uptake, Akt phosphorylation, and GLUT4 translocation (Huang et al., 2005).

An ever-increasing amount of research offers support for the role of inflammatory cytokines and NF-κB pathway intermediates, such as IKKβ, to inhibit insulin signaling. Interestingly, research into the role of AMPK has revealed its presence and function in more and more cellular processes, from glucose uptake to gene expression. Whereas the immune and metabolic systems were once thought of as distinct, they appear to work jointly in maintaining healthy function. These systems represent the most indispensable abilities within an organism—the ability to defend itself against infection and heal and the ability to store energy for times of low nutrient availability and high energy demand (Wellen & Hotamisligil, 2005). As such, these systems are interdependent and work synergistically to maintain homeostasis. Moreover, it is tempting to seek a relationship between two of the more prominent players in their respective systems, namely IKKβ in the immune system and AMPK in the metabolic system. Hence, we feel it is valuable and potentially beneficial to

determine the effect of AMPK manipulation in skeletal muscle in attenuating lipid-induced insulin resistance via inhibition of IKKβ.

In summary, we have demonstrated that IRS-1 Ser^{312} phosphorylation and IKKβ action differ in the skeletal muscle of lean and obese individuals and that this disparity is resolved following surgery-induced weight loss and accompanied by an increase in AMPK phosphorylation. Moreover, through the use of AMPK-activating agents both *in vivo* and in cell culture, we have attempted to establish AMPK's ability, albeit indirectly, to mediate proximal insulin signaling events possibly through the inhibition of lipid-sensitive kinases. These findings present a valuable contribution to the understanding and treatment of insulin resistance associated with type 2 diabetes mellitus and human obesity.

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