THE DEVELOPMENT OF HIGH FAT DIET INDUCED ADIPONECTIN RESISTANCE IN RODENT SKELETAL MUSCLE

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ABSTRACT

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Adiponectin (Ad) is an insulin-sensitizing adipokine, attributable in part to its ability to stimulate skeletal muscle fatty acid (FA) oxidation and prevent intramuscular lipid accumulation and associated lipotoxicity. In established cases of obesity and insulin resistance (IR), resistance to this insulin-sensitizing adipokine has been shown. However, whether a high fat diet, a known contributor to obesity and IR can induce Ad resistance is unknown. Furthermore, whether the type of fat in the diet influences the development of Ad resistance and whether this resistance precedes and contributes to the development of IR is unknown. Finally, the cause of Ad resistance is unknown. It was hypothesized that a high fat diet would induce skeletal muscle Ad resistance by triggering inflammation and this would occur prior to the onset and development of IR. It was also hypothesized that a diet high in saturated fat (SAT) would cause faster changes than a diet high in polyunsaturated fat (PUFA).

From the work presented herein, several novel findings have been made. Importantly, skeletal muscle Ad resistance can be induced by high fat feeding in rats. This resistance is defined as an inability of globular adiponectin (gAd) to acutely stimulate FA oxidation and phosphorylate acetyl CoA carboxylase (ACC) in oxidative

soleus muscle. However, the type of FA in the diet is important in this development. While both PUFA and SAT FA can induce Ad resistance, the induction is more rapid following a SAT diet, occurring within 3 days, while evidence of Ad resistance following a PUFA diet is not apparent until 4 weeks. Critical to note is that SAT FA-induced Ad resistance develops prior to accumulation of intramuscular DAG and ceramide, increased FA transporter content at the plasma membrane and impaired maximally insulin-stimulated glucose uptake. Finally, the underlying cause of Ad resistance remains to be fully elucidated. A change in absolute or phosphorylated inflammatory proteins or total SOCS-3 content in skeletal muscle does not explain the observed development of Ad resistance. Together, this data suggests that Ad resistance may be an important step in the pathogenesis of IR and its cause warrants further investigation.

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Abbreviations

ACC	acetyl CoA carboxylase
Ad	adiponectin
AdipoR1	adiponectin receptor 1
AdipoR2	adiponectin receptor 2
Akt/PKB	Akt/protein kinase B
AMPK	AMP activated protein kinase
APPL1/APPL2	adaptor protein containing plecksin homology domain, phosphotyrosine binding domain, and leucine zipper motif
β-HAD	beta-hydroxyacyl-CoA dehydrogenase
BMI	body mass index
Ca^{++}	calcium
CoA	co-enzyme A
CoxIV	cytochrome c oxidase
CPT1	carnitine palmitoyl transferase
CRP	C reactive protein
CS	citrate synthase
DAG	diacylglycerol
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
FA	fatty acid
FABPpm	fatty acid binding protein, plasma membrane

FAT/CD36	fatty acid translocase
gAd	globular adiponectin
GLUT4	glucose transporter 4
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IKKβ	I kappa B kinase beta
IL-6	interleukin 6
IMTG	intramuscular triglyceride
IR	insulin resistance
IRS-1	insulin receptor substrate 1
Jnk	c-Jun NH(2)- terminal preotein kinase
LCACoA	long-chain fatty acyl-CoA
LCFA	long chain fatty acid
LKB1	serine/threonine kinase 11 (STK11)
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein
mg	milligram
mRNA	messenger RNA
NFκB	nuclear factor kB
PI3-k	phosphoinositide-3-kinase
PKC	protein kinase C
PPARγ	peroxisome proliferator-activated receptor gamma
PUFA	polyunsaturated fatty acid

ROS	reactive oxygen species
SCD1	stearyl coA desaturase
SOCS3	suppressor of cytokine signalling
T2D	type 2 diabetes
TAG	triacylglycerol
TLR4	toll like receptor 4
TNFα	tumor necrosis factor alpha
TZD	thiazolidinediones
UPR	unfolded protein response
ZDF	Zucker diabetic fatty

Introduction and Overview to Thesis:

The strong connection between obesity, insulin resistance (IR) and type 2 diabetes (T2D) is undeniable. Countless associations have been made between high fat diets, increased body fat and these disease conditions. However, the precise mechanism linking increased adiposity to impaired insulin response is not completely understood. It has been proposed that impaired skeletal muscle lipid metabolism, resulting from an imbalance between fatty acid (FA) uptake and oxidation, leads to the accumulation of intramuscular lipid metabolites which directly interfere with insulin signaling. This process, termed lipotoxicity, is likely involved in the pathogenesis of whole body IR.

Adipose tissue is an endocrine organ, secreting a multitude of hormones and peptides termed adipokines. Of these, many act to promote local and systemic insulin resistance and propagate a pro-inflammatory state. In fact, obesity has recently been characterized by low-grade chronic inflammation. How obesity promotes an inflammatory state, and whether inflammation contributes to the development of IR is not fully known. In comparison, a few select adipokines have insulin-sensitizing and anti-inflammatory properties. Adiponectin (Ad) is the most abundant insulin-sensitizing adipokine with peripheral effects in liver, adipose tissue and skeletal muscle. In particular, Ad stimulates FA oxidation and increases glucose uptake in skeletal muscle.

In cases of obesity and IR when body fat stores are increased, the secretory profile of adipose tissue changes and circulating Ad levels are actually decreased. Further to this, evidence of Ad resistance has been shown in genetically obese and diabetic animals and humans. That is, the acute effect of Ad to stimulate FA oxidation in skeletal muscle is lost. However, the ability of a high fat diet, a known contributor to obesity and IR to

induce Ad resistance has not been studied. In addition, whether Ad resistance ***precedes*** and potentially contributes to the development of IR is unknown. Finally, the ***cause*** of Ad resistance remains to be elucidated.

Therefore, the purpose of this thesis is to determine if Ad resistance develops in rodent skeletal muscle following the consumption of high fat diets of varying fatty acid composition. Furthermore, this thesis will examine the time line of changes in skeletal muscle in response to high fat feeding. Lastly this thesis will attempt to uncover if inflammation triggers the onset and development of Ad resistance.

Chapter 1: Review of Literature:

This review of literature will highlight pertinent research contributing to the current understanding of lipid-induced skeletal muscle IR. Special attention is given to the ability of adipokines, namely Ad, to influence skeletal muscle lipid metabolism. Throughout the review, consideration will also be given to the influence of dietary FA type and the potential role of inflammation in modulating skeletal muscle insulin response.

Section 1.1:

Obesity, Inflammation and Insulin Resistance – What is the link?

Obesity is a well-known risk factor in the development of IR and T2D. To date, the mechanism by which increased body fat leads to IR is not fully established. Adipose tissue is a dynamic organ, secreting a multitude of hormones (adipokines) that can influence peripheral tissue lipid and carbohydrate metabolism, insulin sensitivity and inflammation. In particular, adipose tissue abundantly secretes Ad and leptin, two adipokines well-studied for their ability to stimulate FA oxidation and enhance insulin sensitivity in peripheral tissues (177, 228, 230). In contrast, many adipocyte-secreted factors, such as tumor necrosis factor alpha (TNFα) and resistin, act both locally and peripherally to desensitize tissues to insulin and promote an inflammatory state (88, 192).

Clearly, peripheral adipose stores are increased in obesity. Adipocyte structure and dynamics may also be altered, favoring a local and systemic pro-inflammatory environment. For example, observations of elevated TNFα, interleukin-6 (IL-6), monocyte chemoattractant protein -1 (MCP-1) and C- reactive protein (CRP) have been

made in obese, insulin resistant and diabetic rodents and humans (89, 167, 169, 211). However, these changes may be symptomatic of inflammation and not necessarily represent the initial trigger of an inflammatory response. The following section describes contemporary theory by which over-nutrition stimulates inflammation leading to IR.

Chronic over-nutrition results in expanded peripheral adipose tissues stores. A consistent positive energy balance can cause an increase in both adipocyte size and number (55), which has been frequently associated with peripheral IR and T2D (43, 218, 219). In obesity, despite abundant adipose stores, ectopic lipid accumulation also occurs (20), suggesting an impaired ability of peripheral adipose to appropriately sequester lipid. This is supported by studies investigating the insulin sensitizing effect of thiazolidinediones (TZD) in humans, in which a stimulation of PPARγ promotes differentiation of new subcutaneous fat cells and improves insulin sensitivity (3). Furthermore, TZD treatment in *fa/fa* Zucker rats stimulates a remodeling of fat cells, resulting in an increased number of small adipocytes and reduced number of large adipocytes (46). It should be noted that severely compromised peripheral adipose stores (either genetic or acquired lipoatrophy) can also result in impaired insulin sensitivity (178), as ectopic lipid accumulation occurs in liver and skeletal muscle.

As an individual adipocyte increases in volume, there should be a stimulus to recruit pre-adipocytes and make new cells (132). However, if the number of cells cannot be further expanded, (indicative of a failure of adipocyte proliferation and differentiation) an increase in cell volume is the sole method to increase lipid storage (79). It is logical to believe that there is a threshold or upper limit for maximum lipid storage within one adipocyte, but this has yet to be fully characterized. Energy surplus and lipid

accumulation could lead to mechanical stress and an overburdened endoplasmic reticulum (ER), which disrupts normal protein folding patterns resulting in several proteins of the ER becoming unfolded or disorganized (175). This stress, measured as an activation of unfolded protein response (UPR), is increased in subcutaneous adipose biopsies of obese subjects compared to lean controls (17). If the stress of over-nutrition remains, UPR will stimulate the c-Jun NH(2)-terminal protein kinases (Jnk) and I kappa B kinase beta/ nuclear factor κB (IKKβ/NFκB) inflammatory pathways (150) and induce cell death via apoptosis (175). In addition to this mechanical stress there is evidence to suggest that angiogenesis fails to keep pace with adipose tissue expansion. As such, adipocyte hypertrophy is disproportionate to blood supply, resulting is stress signals secondary to tissue hypoxia (45, 196, 231).

Once a critical size or threshold is reached, individual adipocytes send out a 'stress signal' possibly in the form of reactive oxygen species (ROS) or MCP-1, which triggers macrophage infiltration to the site of stress (70, 99). Large adipocytes secrete more MCP-1 compared to smaller adipocytes (180) and macrophage infiltration is directly related to the degree of adiposity and adipocyte size (218). In fact, nearly 90% of macrophages in adipose are localized around large, dead or dying adipocytes (38). If the stress signal persists, eventual apoptosis of the hypertrophied adipocytes can result, with a release of the stored triglyceride into the surrounding extracellular space (231).

As macrophages engulf the hypertrophied adipocytes, they further propagate inflammation by secreting a pro-inflamatory profile (i.e. MCP-1, TNFα, IL-6) (223). These cytokines act in a paracrine fashion, altering the secretory profile from adipocytes (increased FA liberation, decreased Ad, etc), while recruiting more macrophages to the

site of adipose stress (Figure 1.1). A novel macrophage-adipocyte co-culture model has suggested that FA released from adipocytes may act in a paracrine fashion to promote further TNFα and other pro-inflammatory secretions from macrophages (198). As more macrophages infiltrate, they too secrete more inflammatory cytokines until the eventual environment induces a state of local IR, favoring lipolysis over lipogenesis (53). These changes occur prior to increases in systemic insulin concentration (223). The resulting elevated circulating FA may then trigger inflammatory signaling in other peripheral tissues, interfere with glucose clearance and induce skeletal muscle and/or whole body IR.

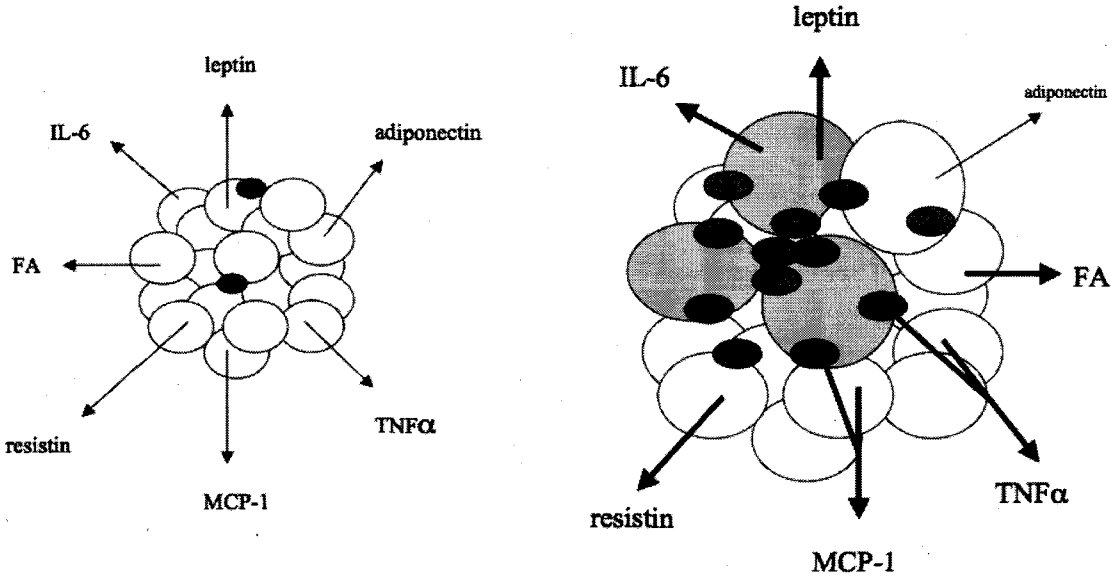

Figure 1.1: Schematic representation of lean and obese adipose tissue and the respective secretory profiles. Adipose tissue and infiltrated macrophages from obese animals and humans secretes a pro-inflammatory profile. Adipocyte = white, apoptotic adipocyte = light grey, macrophage = dark grey.

Taken together, adipocyte hypertrophy, macrophage infiltration, secretion of pro-inflammatory compounds, localized adipose tissue IR and elevated circulating FA all contribute to the development of skeletal muscle IR. While support is mounting that chronic low-grade inflammation is an important etiological mechanism in decreasing insulin signaling in many tissues, sufficient evidence does not yet exist to confirm this as the main route of lipid induced IR. It is unknown whether site-specific IR at the adipocyte always precedes IR at other peripheral tissues such as skeletal muscle. Furthermore, it is poorly understood what effect inflammation and altered adipokine profile have on peripheral tissues. ***This thesis will investigate the role of skeletal muscle adipokine resistance as a potential initiating event in diet-induced skeletal muscle IR, as well as the role of inflammation in the early response/adaptation to high fat feeding.***

Section 1.2:

Skeletal Muscle Fatty Acid Metabolism: Impairments in Obesity Leading to Insulin Resistance

Skeletal muscle is the largest tissue for glucose disposal, responsible for 80% of all insulin stimulated glucose uptake (11). In the post-prandial state, skeletal muscle relies predominantly on fatty acids as a fuel source (110). Several points of control exist in the metabolism of fatty acids both at rest and during exercise, including, but not limited to: fatty acid supply in the circulation, fatty acid uptake across the plasma membrane, movement of fatty acids through the cytosol and across the mitochondrial membrane and subsequent oxidation (85, 184). In lean, healthy individuals, FA supply is matched to the energy demand of the tissue. That is, FA uptake into the muscle increases

when there is a demand for energy, such as during exercise, and remains low when extra energy is not required (184). However, obesity and high fat feeding can alter rates of FA metabolism, correlating strongly with the development of IR (103).

A mismatch of FA uptake in proportion to oxidation, such that FA availability is greater than the rate of oxidation, results in an increased accumulation of lipids as intramuscular triacylglycerol (TAG), diacyglycerol (DAG), ceramide and long-chain fatty acyl-CoA (LCACoA) (2, 90, 93, 96, 152, 194). Some of these lipids (DAG, ceramide, LCACoA) directly interfere with insulin signaling, leading to decreased insulin signal transduction, impaired translocation of GLUT 4 to the plasma membrane and subsequently reduced glucose uptake (34, 135). This situation, termed 'lipotoxicity' is hallmark of the obese, insulin resistant condition (94). The relative importance of increased FA availability, increased rates of FA uptake, decreased FA oxidation or increased rates of esterification as the main site of impairment is currently debatable. It is quite likely that a combination of all of these factors contributes to lipid accumulation. The following sections highlight specific evidence that changes in FA metabolism occur in obesity and/or following high-fat feeding, which lead to the development of skeletal muscle and whole body IR.

1.2a: Circulating fatty acids are elevated following high fat feeding and in obesity.

Fatty acids originating from the diet or liberated through adipose tissue lipolysis travel in the blood combined with albumin. The normal physiological range of circulating fatty acids is 0.2 to 2 mM, with levels increased following prolonged fasting (207), or kept low by insulin suppression of lipolysis following a high carbohydrate meal

(165). Circulating fatty acids are increased post-prandially in obesity and T2D (72, 161) and following high fat feeding (100, 113), which positively correlates with the development of IR. Furthermore, artificial increases in FA levels in lean, healthy volunteers using an Intralipid/heparin infusion, can induce skeletal muscle insulin resistance in as little as three to six hours (96). In comparison, pharmaceutical strategies which lower circulating fatty acids by preventing lipolysis (i.e. overnight Acipimox administration) can improve insulin sensitivity in T2D patients (8), obese non-diabetics (168), and even improve glucose clearance in lean, non-diabetic subjects (20).

Circulating fatty acids may trigger inflammatory signaling in skeletal muscle, which is associated with IR (16, 157). Specifically, saturated fatty acids can act as ligands for toll like receptor 4 (TLR4) (115, 116), a member of the toll like receptor family that has been identified on the plasma membrane of insulin sensitive tissues including skeletal muscle, adipose and liver (118, 119, 157). Upon binding to TLR4, saturated fatty acids trigger activation of the IKK/NFκB signaling cascade (63), which can directly interfere with insulin signal transduction. IKKβ is a serine kinase which can prevent appropriate phosphorylation of IRS-1 (157) or indirectly affect insulin action by up-regulating the transcription of several pro-inflammatory genes (45). Elevated fatty acids are cleared from the circulation to be oxidized to generate ATP, or if the energy demand is low then fatty acids will be esterified. If the potential for adipose tissue expansion is limited, fatty acids may accumulate in peripheral tissues – namely skeletal muscle and liver.

1.2b: Fatty acid transport is up-regulated following high fat feeding and in obesity.

Fatty acid uptake across the plasma membrane occurs by both passive diffusion and active transport (18). A number of proteins have been identified to facilitate long chain fatty acid entry into skeletal muscle cells, including fatty acid translocase, the homolog of human CD36 (FAT/CD36) (1), plasma membrane associated fatty acid binding protein (FABPpm) (195) and a family of fatty acid transport proteins (FATP 1-6), in particular FATP1 and FATP4 (170), however, the relative contributions of each has been debatable. Over-expression of individual transporters in rodent skeletal muscle can increase FA uptake, with effects being most pronounced with increased FAT/CD36 or FATP4, while FA oxidation is increased most by FAT/CD36 of FABPpm over-expression (148).

FAT/CD36 and FABP exist in both cytosolic pools and in the plasma membrane. Conditions of increased energy availability (insulin) and increased energy demand (Ca^{++}) stimulate translocation of FABPpm and FAT/CD36 from the cytosol to the plasma membrane and fatty acid uptake is increased accordingly (19, 122). Whole muscle FAT/CD36 and FABPpm protein content does not differ between lean and obese individuals (20). However, a study by Bonen et al (20) using giant sarcolemmal vesicles, has shown a permanent redistribution of fatty acid transporters from the cytosol to the plasma membrane in overweight, obese and T2D subjects. The use of giant vesicles allows for the assessment of pure FA transport independent of intracellular machinery or the confounding influence of metabolism (123). This altered distribution of FA transporters at the plasma membrane coincides with increased rates of FA uptake and increased intramuscular TAG content (20).

Consumption of a diet high in fat can also alter FA transporter content/distribution. Interventions in sedentary animals that increase plasma membrane FAT/CD36 typically lead to increased intramuscular lipid accumulation that is associated with impaired insulin response (182); conversely, a decrease FA transporter content is associated with reduced intramuscular lipid accumulation and improved skeletal muscle insulin response (73, 182, 187). For example, whole muscle and plasma membrane associated FAT/CD36 are significantly increased in Zucker diabetic fatty (ZDF) rats fed a high-fat diet for 8 weeks compared to chow fed rats (182). However, concurrent aerobic exercise training in these high-fat fed animals prevents the diet-induced increase in fatty acid transporters (normalizing FAT/CD36 protein content to control levels), corresponding with decreased skeletal muscle DAG and ceramide accumulation and improved insulin responsiveness (182). Furthermore, chronic leptin administration to lean rats decreases plasma membrane associated FAT/CD36 protein expression in both red and white muscle, which correlates with decreased rates of FA transport (187). Finally, CD36 null mice are partially protected from high-fat diet induced IR (73).

However, while it is clear that dietary interventions can significantly affect the content and distribution of fatty acid transporters in skeletal muscle, it may be presumptuous to assume that an increase or decrease in fatty acid transporters is always synonymous with an insulin resistant or sensitive state. Rather, an appropriate matching of fatty acid transport to the actual demand to utilize those FA for energy provision may be the most important aspect. For example, high-intensity exercise training in insulin-sensitive humans increases FAT/CD36 protein expression (155). Furthermore, superimposing a five day, very high fat diet in trained cyclists further increases whole

muscle FAT/CD36 protein expression, which is seen as a positive adaptation of skeletal muscle to better utilize an abundant fuel source (30).

More recently, FA transporters have also been identified on rat and human mitochondrial membranes (15, 31, 84), where, in coordination with carnitine palmitoyl trasferase (CPT1), they regulate long chain fatty aid (LCFA) uptake into the mitochondria (171). Blocking FAT/CD36 on isolated mitochondria inhibits palmitate oxidation by nearly 90% (31). In contrast, acute electrical stimulation increases mitochondrial FAT/CD36 content and palmitate oxidation in rodent hindlimb muscles (31). In humans, aerobic exercise causes an increase in mitochondrial FAT/CD36 protein content, which correlates positively with increased palmitate oxidation (84). It is presumable that in obesity, an increased proportion of FAT/CD36 at the plasma membrane, without an increased absolute protein content, results in fewer transporters available at the mitochondrial membrane to promote oxidation. The role of FABPpm with respect to mitochondrial fatty acid oxidation is not completely known, but the current belief is that its main role is to shuttle reducing equivalents across the mitochondrial membrane, as it is identical to aspartate aminotransferase (197).

In summary, FA uptake across the skeletal muscle plasma membrane and mitochondrial membrane is a process regulated in part by translocation of FA transporters FAT/CD36 and FABPpm to their site of need. Both a high fat diet and obesity can modify the quantity and/or distribution of these transporters, favoring increased FA uptake into the muscle. As such, a permanent redistribution of FAT/CD36 to the plasma membrane, coupled with an elevated FA concentration in the circulation, leads to greater rates of FA uptake and leaves the obese muscle susceptible to lipid overload. If

mitochondrial oxidation is insufficient to handle this excess lipid influx, fatty acids will ultimately accumulate within the muscle as TAG, DAG, ceramide or other potentially reactive lipid species.

1.2c: Fatty acid oxidation may be altered by high fat feeding and in obesity

The possibility that FA oxidation is impaired with obesity has been investigated for over 10 years. Initial observations in human muscle showed that obese subjects had lower rates of FA oxidation compared to lean subjects (103, 107). Using whole muscle homogenate from obese and morbidly obese individuals undergoing gastric bypass surgery (BMI range = 28 - 54), FA oxidation was shown to be depressed by approximately 50% compared to lean age matched controls (107). However, these data were obtained predominantly from morbidly obese patients and may not be reflective of a more modestly obese population. Accordingly, a study by Hulver et al (93) demonstrated that in the basal state, FA oxidation in overweight/obese subjects (mean BMI = 30) is not different than that of lean age matched controls, but that rates of basal FA oxidation are decreased in extremely obese subjects (mean BMI = 54), presumably due to a blunted oxidative capacity (i.e.: reduced β-hydroxyacyl dehydrogenase (β-HAD), citrate synthase (CS) and carnitine palmitoyl transferase-1 (CPT1) activity) (103, 107). Interestingly, previously-obese subjects who have undergone gastric bypass surgery and lost significant weight still show diminished rates of FA oxidation, both at rest and during exercise, compared to weight matched never-obese controls (201), suggesting that some obese individuals may have an inherent defect in lipid metabolism that contributed to their development of extreme obesity.

Some controversy exists regarding the underlying cause of impaired whole muscle FA oxidation in obesity. It is possible that 1) mitochondrial content is reduced, 2) existing mitochondria are dysfunctional or, 3) a combination of both fewer and dysfunctional mitochondria are responsible for lower whole muscle FA oxidation in obese individuals. On a whole muscle level, obese subjects show decreased mitochondrial content, evidenced by decreased CS, β-HAD and cytochrome c oxidase (COX IV) protein content (86). In addition, electron microscopy has shown that mitochondria area (longitudinal & transverse measurement) is decreased in obese and T2D subjects (104), while an increase in mitochondria size is associated with improved insulin sensitivity following exercise training and weight loss (77). However, when mitochondria are isolated and compared per mg protein, palmitate oxidation is not different between lean and obese women (86). These data support the conclusion that any impairment in whole muscle oxidation, if one exists, is due to a decreased number of mitochondria, and not to a dysfunction of the mitochondria themselves.

High fat diets have also been studied for their ability to alter rates of FA oxidation. Turner et al (204) have reported that palmitate oxidation, measured both in isolated mitochondria and in whole muscle, is increased in skeletal muscle of rodents fed a high fat diet. In lean, healthy humans fed a high fat diet, basal rates of FA oxidation increase to match the abundant fuel supply (30). This 'metabolic flexibility' (ability to switch between fuel sources to optimize use of abundant fuels) is not evident in obese and formerly obese individuals consuming a high fat diet (6). Again, a 'threshold effect' may be apparent, with acute changes in fat intake promoting a compensation to handle

the excess lipid, but in obesity, decreased mitochondrial content and prolonged exposure to lipids may result in impaired FA oxidation.

It should be noted that it is often difficult to separate obesity and chronic high fat feeding from inactivity, as many obese individuals are self-reported inactive (51, 217). Interestingly, skeletal muscle oxidative capacity is a better predictor of insulin sensitivity than intramuscular lipid content (24). Thus, it is presumable that engaging in regular physical activity while consuming a high fat diet may maintain mitochondrial content and oxidative capacity and provide a better match between FA oxidation and uptake, thereby preventing the development of IR. In support, rodents fed a high fat diet and exercised on a treadmill for 8 weeks were able to maintain oxidative capacity and insulin sensitivity equal to that of animals on a control diet (182). It seems likely that a *mismatch* between FA uptake and oxidation – and not simply a defect of a single process - is the main mechanism contributing to ectopic lipid accumulation and the ensuing IR.

1.2d: Fatty acid accumulation is increased by high fat feeding and in obesity.

Increased IMTG content is positively correlated with obesity, the level of fat in the diet, and IR (20, 152). However, insulin sensitive athletes also have increased IMTG content (68, 209). A constant turnover/recycling of IMTG pools during exercise in trained individuals may circumvent any potential associations of increased lipid and insulin resistance. As such, TAG is thought of as an inert storage form for excess lipid in the muscle, but does not necessarily cause IR or directly interfere with insulin signaling.

In comparison, DAG and ceramide, which are also increased in cases of obesity and insulin resistance, have been shown to directly interfere with insulin signaling (34,

135). Upon insulin binding to the insulin receptor, insulin receptor substrate-1 (IRS-1) is recruited and phosphorylated (Tyr). This recruits phosphoinositide-3-kinase (PI3-k), forming an activated IRS-1/PI3-k complex. In turn, Akt/protein kinase B (PKB) is phosphorylated and activates AS160, causing translocation of GLUT4 to the plasma membrane (Figure 1.2). DAG specifically activates protein kinase C (PKC) and interferes with IRS-1 phosphorylation (135), whereas ceramide has direct inhibitory effects on Akt (34).

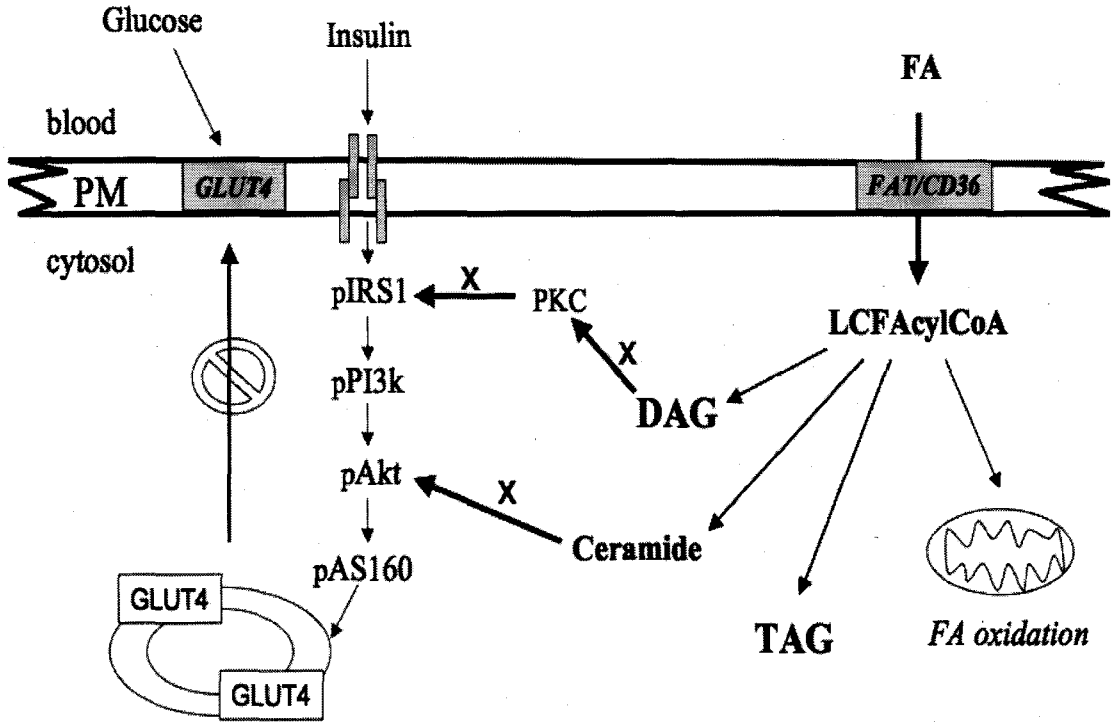

Figure 1.2: Schematic representation of skeletal muscle lipotoxicity. Excess FA taken into the cell can accumulate as reactive lipid species. Specifically, DAG and ceramide have been shown to directly interfere with insulin signaling and glucose transport.

Not only does the specific lipid pool influence insulin sensitivity, but the type of FA in the diet can also influence which lipid pool it incorporates into (113). Saturated fatty acids are esterified as reactive DAG and ceramide (34, 35, 64, 113, 114, 135), while polyunsaturated fatty acids (PUFA) are preferentially stored as relatively inert TAG (64, 113, 135). Much of the comparison of metabolic fates of saturated vs. unsaturated fatty acids has been made in cell culture models (34, 35, 64, 135) and more studies are needed to address this issue in animal or human models. However, studies would suggest that a diet high in saturated fats would be more detrimental to skeletal muscle lipid metabolism and insulin sensitivity compared to a diet high in PUFA (113). This hypothesis has not been extensively studied with proper control. Furthermore, whether diets of differing fatty acid type influence adipokine action at skeletal muscle has not been evaluated.

It is important to clarify that fatty acid esterification is not simply a passive pathway for the removal of excess fatty acids. Several lipogenic enzymes, including stearoyl CoA desaturase 1 (SCD1), actively regulate intramuscular lipid esterification and turnover. It has been hypothesized that channeling fatty acids into TAG pools actually serves a metabolically protective role, repartitioning fatty acids away from more bioactive lipid pools (DAG and ceramide) (199). Surprisingly, lipogenic enzymes are not increased in skeletal muscle of obese women compared to lean controls (199). It is possible that this is evidence of an insufficient lipogenic response in obesity and an inability to appropriately handle excess lipid, as suggested by the increased ceramide content measured in obese muscle (199).

In summary, skeletal muscle lipid metabolism is significantly altered in obesity and following high fat feeding. An increased availability of fatty acids, combined with

increased rates of fatty acid uptake and insufficient levels of fatty acid oxidation and IMTG esterification leaves the muscle susceptible to ectopic lipid accumulation and IR. Strategies that act to restore balance between fatty acid uptake and oxidation should improve insulin sensitivity.

Section 1.3:

High fat diets and lipid surplus: Importance of fatty acid type, concentration and length of exposure on the development of insulin resistance.

As previously discussed, increased post-prandial plasma FA levels are frequently observed in obesity and following high fat feeding and have been proposed to play a central role in the pathophysiology of peripheral IR and T2D (72, 100, 113, 161). However, the precise mechanism by which elevated fatty acids lead to these conditions is not fully characterized. Cell culture models, *in vitro* skeletal muscle incubations, acute lipid infusion as well as chronic high fat feeding have all 'successfully' induced IR in an attempt to understand the underlying mechanisms by which excess lipid causes metabolic impairments. However, to state that fatty acids cause IR is oversimplified and not always accurate, as the amount, type and length of FA exposure, as well as the metabolic "state" (i.e. training status) of the muscle all affect the physiological response to increased FA exposure. The following sections will discuss various techniques used to assess lipid-induced IR, as well as specific challenges of each model.

1.3a: Cell Culture.

Exposure of C2C12 cells to palmitic acid (i.e: 6+hours) impairs insulin stimulated glucose uptake in a dose dependant manner (83). Palmitic acid exposure in this model has been shown to decrease insulin receptor gene expression and activity (48), increase IRS-1 ser phosphorylation, activate PKC and thus impair down stream insulin signal transduction (159).

A potential weakness of many experiments is the use of only a single type of fatty acid; e.g. palmitate, a saturated fatty acid (48). While being one of the most abundant fatty acids in the plasma (~20%), it is still important to consider the effects of other fatty acid types (e.g. oleate abundance in the plasma is ~ 40%), to determine if they have similar effects on glucose uptake and insulin signaling. Hirabara et al (82) incubated rat C2C12 myocytes with 0.1 mM of saturated fatty acid (palmitic and stearic) or 0.1 mM of PUFA (oleic, linoleic, EPA and DHA) for 24 hours. Saturated fatty acid exposure impaired glucose metabolism and mitochondrial function, while there was no effect of the PUFA exposure confirming that the type of FA is a critical factor when considering the impact of FA on skeletal muscle metabolism and insulin sensitivity.

Another limitation of the cell culture model (and of any isolated *in vitro* model) is that it examines skeletal muscle cells in isolation, devoid of interaction with adipocytes or macrophages. Skeletal muscle of obese, insulin resistant individuals is characterized by increased macrophage infiltration (218) as well as a greater amount of inter-muscular adipose tissue (69). Secretions from these infiltrated cells influence the metabolism and signaling in skeletal muscle and therefore these cells should be considered in combination. A limited number of studies have examined the cross talk between

adipocytes and myocytes using a co-culture model (49, 50). Co-incubation of human myocytes with human adipocytes impaired insulin stimulated IRS-1 tyrosine phosphorylation, despite sufficient IRS-1 protein content (49). The co-culture medium did not have elevated levels of TNFα or IL-6, suggesting that some other fat derived factor, potentially liberated fatty acids, was responsible for the observed impairment (49). Furthermore, insulin stimulated Akt phosphorylation was impaired, and IKK activation was increased following adipocyte-myocyte co-culture (50), suggesting that fatty acids liberated from adipose tissue may have increased inflammation in the skeletal muscle and interfered with insulin signaling. The interaction between adipocytes and macrophages has also been examined in co-culture (124). Activated macrophage conditioned medium (i.e. macrophages were stimulated with lipopolysaccharide (LPS), a TLR4 ligand) has been shown to decrease insulin stimulated glucose uptake, IRS-1 and Akt phosphorylation in 3T3-L1 adipocytes (124).

Cell culture models are valuable for identifying specific signaling targets of fatty acids in a tightly controlled environment without confounding variables, such as hormonal changes common to whole body animal and human studies. However, the concentrations of substrates, hormones, drugs etc. applied to the cells far exceed physiologically relevant levels, making meaningful application of findings to a whole body level difficult. Furthermore, the development of contractile proteins, generation of fibre types and establishment of subcellular domains are compromised in the frequently used C2C12 and L6 satellite cell lines (145). As such, observations made in cultured cells may not directly reflect metabolic changes in mature skeletal muscle.

1.3b: *In vitro* incubations.

In vitro incubation of skeletal muscle has the advantage of examining metabolism and the onset of lipid induced IR in an intact tissue under more physiologically relevant conditions. Studying an intact tissue *in vitro* also allows for paracrine signaling and a feedback system compared to isolated cells, while still controlling for potentially confounding factors such as hormonal environment and local blood supply.

Incubation of isolated skeletal muscle from a rodent (soleus or epitrochlearis) or human (rectus abdominus) have been used to examine the effect of FA exposure (up to 24 hr) on insulin sensitivity and FA metabolism *in vitro* (4, 101, 200). Insulin stimulated glucose transport and palmitate oxidation are both reduced in rodent skeletal muscle after 6 hours of *in vitro* palmitate exposure, and both became progressively worse after 12, 18 and 24 hours of exposure (4). Furthermore, this susceptibility to FA induced IR appears to be greater in obese individuals, as 4 hours of 2 mM palmitate exposure impaired insulin stimulated glucose transport in isolated rectus abdominus muscle from obese but not lean individuals (200).

In addition, the *in vitro* model has provided evidence that adipokines have differing effects on skeletal muscle lipid metabolism depending on the local fatty acid environment. Exposure of isolated soleus muscle strips to the adipokine resistin for 4 hours impairs maximal insulin stimulated glucose transport, but only in the presence of 2 mM palmitate (101). Palmitate exposure alone is not sufficient to induce IR in this time frame. That is, the interaction between fatty acids and adipokines may be important when considering lipid induced IR. *In vitro* incubations provide an excellent opportunity to study lipid metabolism in mature skeletal muscle in a controlled hormonal environment, and to elucidate the mechanism of individual adipokines without confounding hormonal profiles.

1.3c: Lipid Infusion.

Acute lipid infusion is another technique commonly used to induce insulin resistance in animals and humans (60, 71, 96, 137). Plasma free fatty acids are acutely increased via lipid/heparin infusion lasting approximately 3-8 hours. It is important to note that during the lipid infusions, plasma free fatty acid levels attained are within physiological ranges (1.5 - 2 mM), albeit at the upper range (60, 96). Therefore infusions provide a rapid method of assessing lipid induced IR in vivo.

In rats, acute Intralipid infusion increases plasma free fatty acids, impairs skeletal muscle insulin signaling and induces whole body IR (60, 71). Pre-treatment with anti-inflammatory (106) or anti-diabetic drugs (81) protect against lipid induced IR in rodents. Interestingly, there is some evidence of a sex difference in response to lipid infusion. Female rats administered an identical Intralipid infusion to male rats show no signs of peripheral IR; however insulin-stimulated glucose disposal rate in male rats is markedly impaired (80).

In humans, 6 hr of lipid infusion induces IR in healthy males, corresponding to an increase in intramuscular DAG accumulation and inflammation (96). Prior moderate exercise training improves insulin sensitivity and insulin production during an Intralipid challenge in older men (183). Again, the sex difference in susceptibility to FA induced IR is apparent in humans, with females being at least partially protected from Intralipid induced IR (61).

One caveat of the Intralipid/heparin infusion protocol is that it is only commercially available as one formula; soybean oil, a PUFA. Other formulas of saturated or mixed fatty acids are not commercially available. Identifying this limitation,

a few research groups have created their own unique lipid emulsions. In male Wistar rats, infusing PUFA, oleate (monounsaturated FA) or lard oil (saturated FA) for 7 hours results in similar degrees of peripheral IR (74). Furthermore, limited evidence in T2D patients has shown that n-3 supplementation during a 4 hr Intralipid infusion failed to affect insulin sensitivity or insulin secretion (137). That is, insulin sensitivity was not maintained despite the n-3 enrichment. Greater accessibility to lipid infusates of different FA composition would allow for a better understanding of the influence of FA type on rapidly inducible IR.

1.3d: High Fat Diets.

High fat diets are one of the most commonly utilized means to induce insulin resistance in rodents, yet there is very little standardization between laboratories and treatments protocols. Most high fat diets range in duration from 4 weeks to several months (16, 108, 113, 176, 186, 190, 229), with the earliest evidence of diet-induced insulin resistance occurring after 3 weeks (220). Diets also vary significantly with respect to the percentage of calories derived from fat, ranging from 40% to 60% (16, 108, 113, 176, 186, 190, 220, 229). Frequently, high fat diets are administered ad libitum (16, 108, 113, 157, 229) while some studies closely monitor daily caloric consumption and pair feed animals to minimize differences in body mass gain (186, 190, 220). Pure saturated and pure n-6 unsaturated diets are common (113, 186, 190, 229), as are mixtures of different fatty acid types (108, 220). Still, many dietary interventions do not specify the details of their preparation (16, 157, 176). The dietary composition is of important consideration, as saturated fatty acids are generally considered to have negative

health consequences i.e. promote inflammation and insulin resistance (113, 135), whereas high PUFA diets have been shown to increase (113) and decrease (220) insulin sensitivity. Since different fatty acid types can have very different metabolic and inflammatory responses, it is insufficient to simply state that high fat diets induce insulin resistance. The current thesis carefully examines the influence of saturated vs. polyunsaturated fatty acids on the development of skeletal muscle IR, inflammation and Ad resistance.

Section 1.4:

The role of inflammation in skeletal muscle insulin resistance

Inflammation is now recognized as an important step in the etiology of lipid-induced obesity and T2D. It is likely that macrophage infiltration into white adipose tissue and the ensuing production of pro-inflammatory adipokines play a critical step in the propagation of the inflammatory state. Inflammatory signaling in skeletal muscle is receiving more attention as a potential contributor to lipid-induced skeletal muscle IR.

1.4a: Toll Like Receptors and IKK/NFκB Signaling Axis.

Classically studied as part of the innate immune system, toll like receptors are a family of receptors located on macrophages and other inflammatory cell types, which respond robustly to activation by viral and bacterial components, including LPS, an endotoxin secreted by gram negative bacteria (45). The lipid A moitiey of LPS is specifically required for complete recognition (158). Fairly recently, members of the TLR family, specifically TLR4, have been identified on insulin sensitive tissues,

including skeletal muscle and adipose tissue (13, 157), and can be activated by dietary saturated fatty acids (115, 157, 176). Interestingly, PUFA do not appear to activate TLR4 (115).

Fatty acids are also able to activate the IKK/NFκB pathway (Figure 1.3), a proximal target of TLR4 (16, 96, 162). The IKKB/NFκB axis is commonly measured as an index of skeletal muscle inflammatory signaling. Specifically, NFκB is a transcription factor sequestered by IκBα in the cytoplasm under non-stimulated conditions. Upon saturated fatty acid - TLR4 binding, IKKβ becomes phosphorylated, which in turn activates/phosphorylates IκBα. This causes degradation of IκBα and the release of NFκB to the nucleus where it up-regulates pro-inflammatory gene transcription, including TNFα (97, 114). A decrease in IκBα is indicative of skeletal muscle inflammation. Furthermore, IKK is a serine kinase, which can directly interfere with IRS-1/PI3-k activation and insulin signal propagation (106, 179).

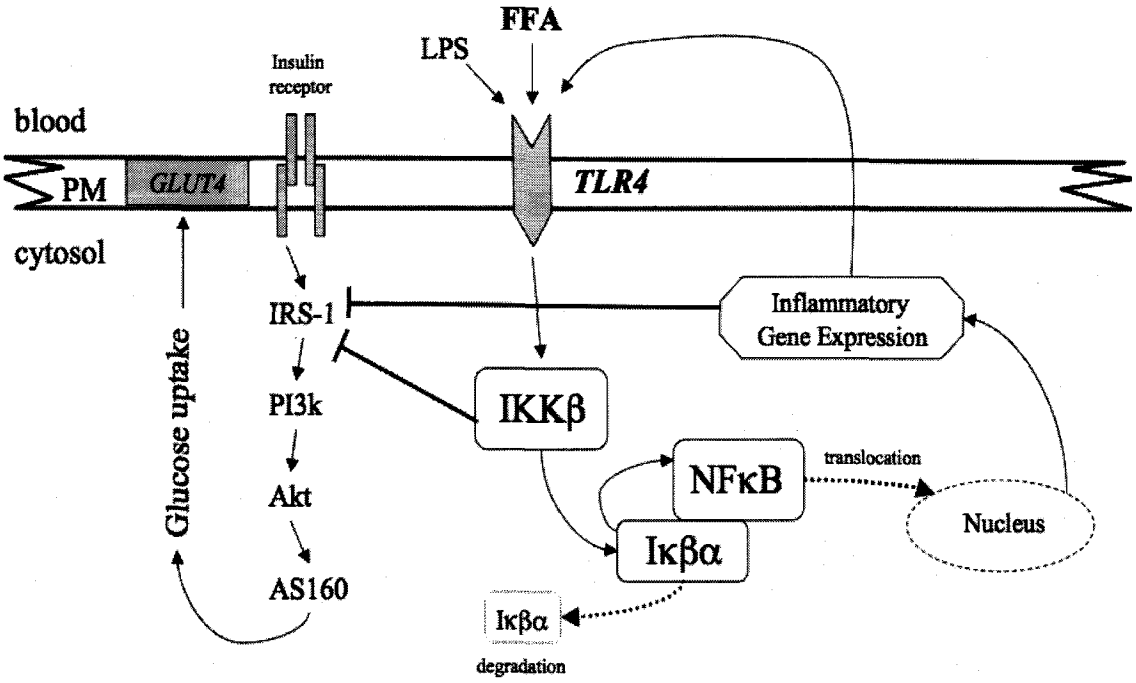

Figure 1.3: Schematic representation of skeletal muscle inflammatory signaling. Circulating FA bind to TLR4 and activate the IKK/NFκB signaling axis, causing an up-regulation of several pro-inflammatory genes which can further interfere with insulin signaling. IKKβ is also a serine kinase which can directly inhibit insulin-stimulated phosphorylation of IRS-1.

1.4b: Inflammatory Signaling and Insulin Resistance

A role for IKK/NFκB signaling has been implicated in the development of lipid induced IR. Hyperlipidemia results in activation of this pathway (16, 157, 176, 179), while blocking the pathway improves insulin sensitivity (106, 157, 176, 179, 235). Specifically, 6 hours of palmitate incubation induces insulin resistance in L6 myotubes, as measured by a decrease in insulin stimulated glucose uptake, but blocking the translocation of NFκB to the nucleus can prevent this (179). In rodents, 6 weeks of high

fat feeding, or a 6 hr lipid infusion (both previously shown to induce insulin resistance) decreases IκBα activity in skeletal muscle, indicating an increase in NFκB activity (16). Furthermore, complimentary studies by Yuan et al (235) and Kim et al (106) examined the role of IKKβ in the development of whole body insulin resistance. Both studies used an IKKβ+/- heterozygous mouse model (homozygous genotype was embrionically lethal), as well as high dose anti-inflammatory salicylate treatment in an attempt to prevent lipid induced insulin resistance. Aspirin and other salicylates have been shown to directly inhibit IKKβ activity both *in vitro* and *in vivo* (232), thereby preventing NFκB signaling. Insulin resistance induced by lipid infusion (106) or high fat feeding (235) can be prevented by both aspirin treatment and IKKβ down regulation. The TLR4 knock out mouse is also protected against insulin resistance induced via lipid infusion, high fat feeding or *in vitro* palmitate incubation (157, 176). Similar findings have also been evidenced in humans. Itani et al (96) reported a 70% decrease in IκBα activity following 6 hours of Intralipid/heparin infusion in healthy humans, corresponding with an increase in skeletal muscle DAG content and decreased glucose clearance. Furthermore, TLR4 protein is increased and IκBα is decreased in skeletal muscle of obese, diabetic humans (162).

It should be noted the impact of IKK/NFκB signaling in lipid induced IR is not always definitive. For example, Shi et al (176) reported that TLR4 deletion improved insulin sensitivity in high fat fed mice, but only in females. Further still, a TLR4 knock out model (157) protected against acute lipid induced IR (by lipid infusion), but those animals fed a high fat diet for 21 weeks (of an undisclosed FA type and content) still developed IR to the same magnitude as wild type animals. In addition, while activation

of NFκB is evident in many insulin resistant states, over expression of NFκB in skeletal muscle had no detrimental effect on glucose disposal rate during an insulin clamp (156). That is, local activation of IKK/NFκB is not sufficient to induce insulin resistance. Lastly, Bhatt et al (16) reported that 6 weeks of high fat feeding decreased IκBα in fast-twitch glycolytic vastus lateralis, but not in oxidative soleus, evidence of a fibre specific effect.

Clearly there is a role for inflammation in lipid induced insulin resistance, but currently the extent of which is unknown. Even with its proposed role as an initiating event in the development of lipid-induced insulin resistance, it is unknown how rapidly inflammation occurs at the muscle in response to high fat feeding and whether this response (if any) depends on the type of FA ingested. Also, how pharmacological prevention of inflammatory signaling affects lipid metabolism is poorly understood. Finally it is completely unknown if inflammatory signaling interferes with adipokine signaling and contributes to the development of adipokine resistance. The current thesis will investigate the potential role of inflammation in skeletal muscle metabolic impairments associated with high fat feeding.

Section 1.5.

Adiponectin: Influence on skeletal muscle metabolism

Mounting evidence from cell culture, animal models and human interventions strongly implicate lipid overload and inflammation as contributors to the onset and development of insulin resistance. Adiponectin is an insulin-sensitizing adipokine, an effect likely attributable in part to its ability to stimulate skeletal muscle FA oxidation.

The following section will review the discovery, basic biology and insulin-sensitizing effects of Ad. Also, the concept of adipokine resistance, in particular Ad resistance, will be examined.

1.5a: Discovery and Basic Biology

Adiponectin is a secretory protein expressed predominantly by adipocytes. It was first identified in 1996 by four independent laboratories using various approaches (91, 125, 143, 172). Its basic structure consists of a carboxyl-terminal globular domain and an amino terminal collagen domain. In circulation, adiponectin exists in a wide range of multimeric complexes. Full length adiponectin assembles via its collagen domain into a trimer, which can then associate into oligomers of low, medium or high molecular weight (LMW, MMW, HMW) (151). It has been suggested that the relative proportion of each multimeric compound is of importance for overall adiponectin signaling and function. In particular, the HMW complex is thought to be the most biologically active full-length form of Ad (215). A ratio of HMW:total Ad is known as the Ad sensitivity index (151).

Cleavage of full length Ad in activated monocytes or neutrophils (212) yields a smaller proteolytic fragment termed globular adiponectin (gAd), which accounts for approximately 1% of total Ad (62, 126, 212). Some controversy exists as to the biological importance of this globular fragment in the circulation, since detection of gAd in plasma has proven difficult, and to date, measurement via commercially available methods is unavailable. However, laboratories that have developed their own antibodies for detection of gAd have been able to quantify its presence in the plasma and confirmed that it is biologically active (92).

Under normal physiological conditions, Ad circulates in the plasma at very high concentrations, (0.5-30 mg/ml), nearly 100 times that of other adipokines (eg: TNFα, resistin, leptin, IL-6) whose plasma concentration are in the ng/ml range (5). Adiponectin expression and secretion from adipocytes is regulated by several factors, including insulin (56), other adipokines such as TNFα (28), sex hormones (40, 222) as well as dietary intervention (58, 141, 142) and pharmaceuticals (42, 127). Insulin and other adipokines can decrease Ad expression, acting through a negative feedback loop to keep levels within a tight range (28, 56). Production and secretion of Ad by adipose tissue is increased by diets high in n-3 fatty acids (58), soy protein (142) and linoleic acid (141), but Ad levels (total or multimerization) do not appear to be impacted by a single meal or short term fasting (95). Adiponectin levels are also increased by anti-diabetic drugs in the TZD family (42, 127), a potential mechanism underlying their insulin-sensitizing effects. It is also interesting to note that females have higher circulating levels compared to males, which may in part account for the decreased risk of developing T2D in females (40, 222). Counter-intuitively, Ad levels vary inversely with BMI; that is, when adipose tissue mass increases, Ad levels decrease (5). Adiponectin is the only adipokine to exhibit this pattern of response. Interestingly, exercise does not appear to alter circulating levels of Ad (94), unless there is a corresponding change in body mass, in which case Ad levels increase with weight loss (54).

Two distinct Ad receptors have been identified in peripheral tissues; AdipoR1 and AdipoR2 (225). Both receptors are integral membrane proteins with 7 trans-membrane domains; the N terminus is internal to the cell and the C-terminus is external, and they share approximately 67% homology (225). AdipoR1 is ubiquitously expressed, but is

concentrated highly in skeletal muscle and preferentially activated by gAd. AdipoR2 is located predominantly in liver (and to a lesser extent on skeletal muscle) but is activated by full-length Ad (62, 226). This suggests that the globular form likely accounts for the majority of adiponectin's insulin-sensitizing effects in skeletal muscle while the full-length form has a more important role in the liver. It should be noted that it is very difficult to presume that AdipoR1 would exist as a receptor specific to the globular head (gAd) if this form does not exist *in vivo*. Adiponectin receptors have also been identified on adipocytes, the pancreas, in the hypothalamus and on arterial walls (102, 224), but detailed signaling events and the mechanism of action in these tissues is beyond the scope of this review. The current thesis examines specifically the role of gAd and interaction with AdipoR1 on skeletal muscle.

Upon binding to AdipoR1, gAd triggers a series of signaling events. Most studied is gAd's ability to phosphorylate and activate AMPK, which in turn phosphorylates and inactivates ACC. However, there is some controversy surrounding this signaling cascade, as gAd stimulated AMPK phosphorylation is consistently observed in glycolytic, but not always in oxidative muscle (202). Whether gAd can directly phosphorylate ACC and bypass AMPK in oxidative muscle is unknown. Furthermore, the phosphorylation of AMPK appears to be transient, returning to baseline within 60 minutes, whereas ACC remains phosphorylated following 60 min of gAd exposure (202). Despite fibre type specificity or transient activation of AMPK, gAd has consistently been shown to phosphorylate ACC. This decreases the production of malonyl CoA, thereby relieving inhibition of CPT1, allowing for LCFAs to enter the mitochondria and increase

FA oxidation (Figure 1.4) (202, 226). This mechanism of action is very similar to that of leptin, another insulin-sensitizing adipokine discovered several years prior (189).

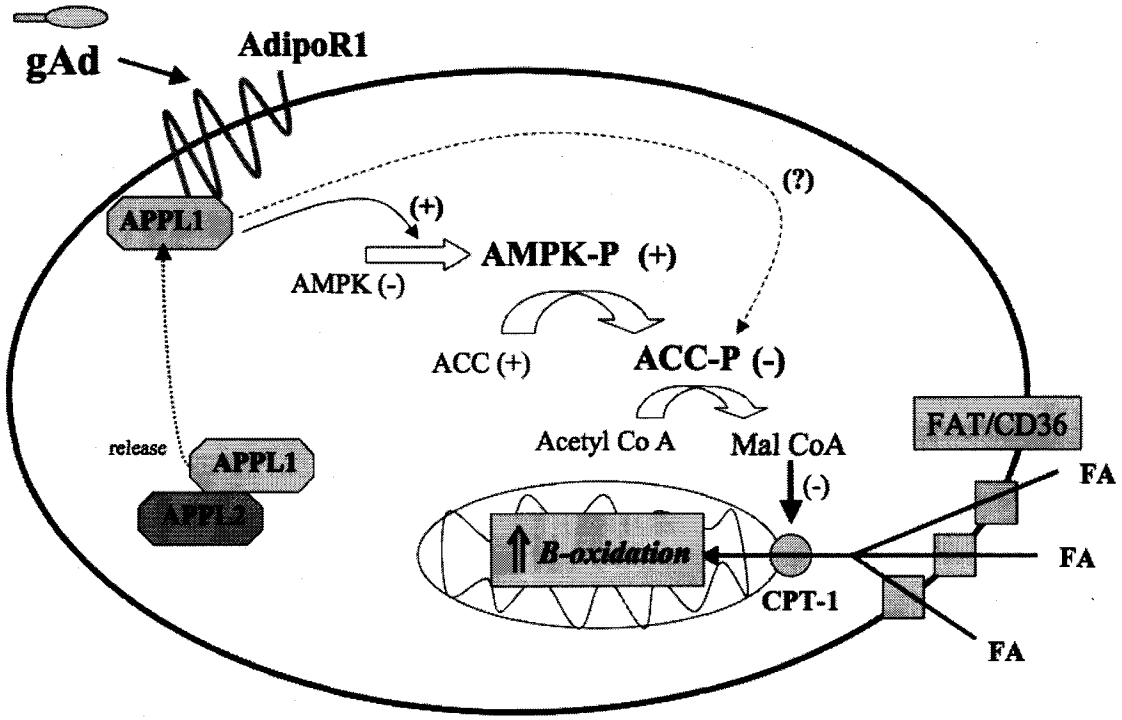

Figure 1.4: Schematic representation of gAd signaling in skeletal muscle. Globular Ad binds to AdipoR1 in skeletal muscle, and initiates intracellular signaling, a process that requires APPL1. Globular Ad signaling increases FA oxidation in skeletal muscle, but whether this requires AMPK phosphorylation or acts directly through ACC is controversial and may be fibre type specific.

More recently, APPL1 (adaptor protein containing plecksin homology domain, phosphotyrosine binding domain, and leucine zipper motif) has been identified as an adaptor protein required for Ad signal transduction (130). APPL1 associates with the

inner membrane terminus of AdipoR1 upon gAd stimulation. This association allows for downstream kinases, including Akt, LKB1 (upstream kinase to AMPK) and AMPK to become activated.

In addition, it has been proposed that APPL1 association with AdipoR1 is regulated by a second protein, APPL2 (214). In an inactivated state, APPL2 forms a dimer with APPL1, sequestering it in the cytoplasm, thereby preventing its interaction with AdipoR1. APPL2 can also associate with the inner N terminus of AdipoR1, essentially blocking the binding site of APPLI. Upon gAd binding to AdipoR1, APPL2 releases from the inner region of AdipoR1, opening up a binding spot for APPL1, while simultaneously releasing the dimerized APPL1 it had sequestered within the cytoplasm, allowing it to move to the AdipoR1 binding site (214).

1.5b: Adiponectin is an insulin-sensitizing adipokine

The insulin-sensitizing effect of Ad was first identified in 2001 by several groups through administration of Ad to various genetic animal models of IR (12, 62, 228). Using KKAy mice as a model of the metabolic syndrome, Yamauchi et al (228) first induced IR in these animals by feeding them a high-fat diet and observed that plasma Ad levels decreased. Subsequent administration of exogenous Ad ameliorated whole body IR and hypertriglyceridemia caused by the diet. Scherer's group (12) reported that an acute increase in Ad lowered basal glucose concentration in both wild type and T2D mice, at least in part by depressing hepatic glucose production. Finally, the laboratory of Lodish et al (62) reported that gAd administration increased rates of FA oxidation in skeletal muscle, lowered plasma glucose and induced weight loss in mice. This evidence

prompted generation of both Ad transgenic and Ad deficient mice to investigate the chronic effects of Ad on insulin sensitivity *in vivo*. Over expression of gAd in ob/ob mice partially restores insulin sensitivity (227), while over-expression of full length Ad suppressed insulin mediated endogenous glucose production (41). In comparison, mice lacking the Ad gene showed phenotypes ranging from mild to severe IR when administered standard or high fat diets respectively (111, 126).

To date, the majority of studies examining the direct effect of Ad on peripheral tissues have been performed in rodent models. In general, most human studies only report association/correlations between changes in Ad concentration and various metabolic parameters in disease states or subsequent to lifestyle interventions. For example, abnormalities in the Ad gene, specifically single nucleotide polymorphisms at locus 276, are associated with increased risk of IR, metabolic syndrome and T2D in several populations (76, 133, 210). In a cohort of diabetic women, those with the best glycemic control and most favourable lipid profiles also had the highest total plasma Ad concentration (129). Furthermore, 3 months of TZD treatment improved whole body insulin sensitivity and increased Ad levels in obese and diabetic subjects (234). Plasma Ad levels are also shown to increase following diet and exercise interventions that cause significant fat loss and improve insulin sensitivity (9, 136). To compliment this body of work, the use of isolated muscle strips *in vitro* has allowed for direct assessment of Ad signaling in human skeletal muscle (26). Acute incubation of isolated human rectus abdominal strips with gAd increased skeletal muscle FA oxidation and glucose uptake (26). Taken together, this information strongly supports the role of Ad as an insulin sensitizer in skeletal muscle, liver and whole body. Interventions that increase Ad levels

or Ad signaling serve to improve insulin response, whereas conditions of low plasma Ad are generally associated with IR.

1.5c: Evidence of Adiponectin Resistance

Prior to the identification of Ad, leptin was the best-studied adipokine with insulin-sensitizing and anti-obesity properties. Leptin and Ad have very similar mechanisms of action, as both stimulate FA oxidation in skeletal muscle (189, 202, 226). Chronic leptin administration to lean rodents increases basal and insulin-stimulated glucose uptake and FA oxidation, and decreases plasma membrane FA transporter content (FAT/CD36, FABPpm) and esterification rate (185, 187). Leptin also has a significant central effect, not evidenced with Ad, acting at the hypothalamus to regulate food intake (191). As such, leptin was termed the 'anti-obesity' hormone. Paradoxically, in cases of obesity, leptin concentration is increased, even when corrected for body mass (121), suggesting the development of peripheral leptin resistance. Since this initial observation, direct evidence of leptin resistance has been demonstrated in humans and rodents through a series of experiments. Leptin resistance (or resistance to any adipokine) is analogous to insulin resistance, where a blunted peripheral tissue response is observed in response to a given amount of leptin (or other adipokine). Four weeks of PUFA feeding (60% kcal from fat) induces skeletal muscle leptin resistance, as evidenced by impaired leptin stimulated FA oxidation (186). Both dietary supplementation with n-3 fatty acids or concurrent aerobic exercise have been shown to at least partially prevent the development of leptin resistance otherwise induced by high fat feeding in rodent skeletal muscle (190). In lean humans, acute leptin exposure

increases FA oxidation and decreases FA esterification, but this stimulatory effect is lost in skeletal muscle from obese subjects (189).

In contrast to what is observed with leptin, circulating levels of Ad are decreased in obesity (5). Similarly, evidence of an impaired peripheral response to Ad has been demonstrated in obese mice and humans. In ob/ob mice, binding of gAd in muscle is decreased, and the stimulatory effect of gAd on AMPK is lost (203). Furthermore, the mRNA content of AdipoR1 in skeletal muscle is reduced in this animal model (203). Additionally, our laboratory has demonstrated a blunted increase in FA oxidation in response to gAd in skeletal muscle from obese humans compared to lean controls (26).

Evidence of Ad resistance in established models of obesity or IR, while useful for characterizing these conditions, is not sufficient to properly understand disease prevention. Rather, studying the development of Ad resistance using an inducible model is key for determining whether this is a potential target for prevention of IR and associated metabolic diseases. The ability of a high fat diet, a known contributor to obesity and IR, to induce skeletal muscle Ad resistance has not been examined. For the purpose of this thesis, Ad resistance is defined as a diminished ability of gAd to stimulate FA oxidation in skeletal muscle. It is logical to assume that the development of resistance to insulin-sensitizing adipokines such as leptin and Ad would lead to deranged lipid metabolism (i.e. impaired oxidation, increased esterification) and IR. However, whether adipokine resistance precedes and contributes to the initial development skeletal muscle IR has not been investigated.

Furthermore, the cause of adipokine resistance in obesity is not completely understood. Evidence suggests that suppressor of cytokine signaling 3 (SOCS-3) may be

involved in the development of leptin resistance. SOCS-3 acts in a negative feedback loop to regulate cytokine (ex: TNFα) signaling (45). SOCS-3 mRNA is elevated in skeletal muscle from T2D patients (164) and high fat fed rats (190), and very recently, SOCS-3 protein content has also been shown to be elevated in skeletal muscle of high fat fed rats (229). SOCS-3 has been shown to interfere with leptin signaling by inhibiting leptin-stimulated phosphorylation of AMPK in cultured myotubes from obese humans (188). Furthermore, over expression of SOCS-3 in lean myotubes to similar levels measured in obese myotubes can induce leptin resistance (188). It is logical to think that SOCS-3 may interfere with Ad signaling, as leptin and Ad share common pathways to stimulate FA oxidation. To date, the role of SOCS-3 in Ad signaling has been completely untested.

In summary, despite adiponectin's insulin-sensitizing effects, in obesity it appears as though these stimulatory effects are lost. The cause of Ad resistance and its physiological significance remain to be elucidated. Since obese, insulin resistant, high fat fed animals exhibit signs of inflammation, adipokine resistance and elevated SOCS3 levels, it is possible that these presentations are inter-related. Whether acute dietary interventions trigger inflammation or an up-regulation of SOCS3 sufficient to explain the development of adipokine resistance in skeletal muscle is completely unknown. Furthermore, whether PUFA and saturated FA differently affect the onset of these conditions has not been adequately evaluated. Together the studies of the current thesis will address the role of high fat diets, FA type and inflammation in the initiation and development of skeletal muscle Ad resistance.

Chapter 2: Statement of the problem

The primary objective of the current thesis is to understand the role of Ad resistance in the development of lipid-induced IR in skeletal muscle. An impaired peripheral response to insulin-sensitizing adipokines (leptin and Ad) has been shown in established cases of obesity and IR (26, 189, 203). In addition, leptin resistance can be induced by 4 weeks of high PUFA feeding in rats (186). However, at the time of this thesis, the development of resistance to Ad, the most abundant adipokine in circulation, in skeletal muscle was not well studied.

Specifically, this thesis sought to determine the following:

1) Is Ad resistance inducible in rodent skeletal muscle following 4 weeks of high fat feeding?
2) Does the type of FA in the diet (PUFA vs SAT) influence the development of Ad resistance and IR?
3) Does Ad resistance precede the development of skeletal muscle IR?
4) Does lipid induced inflammation play a role in the development of Ad resistance?

It was hypothesized that:

1) Ad resistance could be induced by 4 weeks of HF feeding in oxidative soleus muscle, as assessed by an inability of gAd to stimulate FA oxidation.
2) Ad resistance and IR would occur earlier in SAT fed animals compared to PUFA fed animals.

3) Ad resistance would precede changes in lipid metabolism, (FA transporters, intramuscular DAG and ceramide), blunted insulin signaling and impaired maximally insulin-stimulated glucose transport.
4) A high fat diet would promote inflammation in skeletal muscle and thereby interfere with Ad signaling, causing Ad resistance. Inflammation would be more pronounced in SAT fed animals compared to PUFA fed animals.

Chapter 3: Globular adiponectin resistance develops independently of impaired insulin-stimulated glucose transport in soleus muscle from high-fat-fed rats.

As published, with minor revisions:

Mullen KL, Smith AC, Junkin KA, and Dyck DJ. Globular adiponectin resistance develops independently of impaired insulin-stimulated glucose transport in soleus muscle from high-fat-fed rats. ***Am J Physiol Endocrinol Metab*** **293: E83-90, 2007.**

Introduction:

Over consumption of a high fat (HF) diet results in an increased risk of developing obesity, insulin resistance (IR) and Type 2 diabetes (163). However, there is some controversy regarding the impact of the type of fat in the diet. While it is well accepted that diets high in saturated fat are detrimental to health i.e. result in impaired glucose and lipid metabolism (113, 131, 135), the metabolic effects of diets high in unsaturated fat are less conclusive. Some studies suggest that high polyunsaturated fat (PUFA) diets lead to IR (220), while others suggest that animals maintain (135), or even improve (113) their insulin sensitivity.

Following HF feeding for as little as 3 weeks, impairments in carbohydrate and lipid metabolism are apparent, even when differences in body mass are controlled (113, 220). Decreased fatty acid (FA) oxidation coupled with excess FA uptake and esterification results in the accumulation of intramuscular lipid stores, which negatively correlate with insulin sensitivity (103, 105). Additionally, skeletal muscle from genetically (206) or dietary-induced (112) obese animals shows decreased rates of insulin-stimulated glucose uptake. Several adipocyte-secreted proteins such as leptin,

adiponectin, resistin and TNF-α have been suggested as potential links between HF feeding, changes in adiposity and alterations in skeletal muscle metabolism.

Both leptin and adiponectin (Ad) are stimulatory cytokines known to stimulate FA oxidation, reduce lipid storage and improve insulin sensitivity in human and rodent skeletal muscle (26, 140, 189, 228). This repartitioning of FA towards oxidation appears to be due to the activation of AMP-activated protein kinase (AMPK), which results in the deactivation of acetyl coenzyme-A carboxylase (ACC), leading to a decrease in malonyl CoA content and a relief of inhibition at carnitine palmitoyltransferase I (CPT I) (32, 202, 226). Activation of AMPK has also been shown to play a role in the recruitment of GLUT 4 to the plasma membrane, thereby contributing to increased glucose uptake (32).

In cases of obesity, elevated levels of circulating leptin are common, even when normalized for body fat, suggesting the development of central and/or peripheral leptin resistance (121). Previous work in our laboratory has demonstrated that consumption of a high-unsaturated fat diet for 4 weeks resulted in the loss of leptin's effects on muscle lipid metabolism in rodents (186), the first direct evidence of the development of leptin resistance in this tissue. In contrast, low levels of circulating Ad characterize most cases of obesity (5). Adiponectin exists in the plasma primarily as aggregates of a full-length form. However, the smaller proteolytic fragment (globular head, gAd) specifically interacts with muscle (62, 126). While the paradoxical decrease in total circulating Ad concentration may not immediately suggest a peripheral resistance to Ad, there is recent evidence to support this possibility. In genetically obese diabetic mice, the acute stimulatory effect of gAd on AMPK in muscle is lost. This may be due, at least in part, to the observed reduction in skeletal muscle Ad R_1 receptors (203). Furthermore, our

laboratory has also demonstrated that the stimulatory effect of gAd on both glucose transport and FA oxidation is impaired in skeletal muscle from obese humans (26). However, the ability of a HF diet, a known contributor to obesity and diabetes, to induce skeletal muscle Ad resistance, has not been examined.

Therefore, the purpose of the present study was to examine the effect of gAd on skeletal muscle FA metabolism and glucose transport following a four-week feeding trial with diets of varying fat composition. Specifically, we aimed to determine 1) if gAd-stimulated FA oxidation in skeletal muscle is impaired in response to HF feeding of either a high PUFA or high-saturated fat diet; 2) if gAd stimulated phosphorylation of AMPK and ACC is impaired, 3) if the development of impaired maximal insulin-stimulated glucose transport occurs concurrently with the loss of gAd's effects, and 4) if these responses differ depending on the type of fat consumed. We hypothesized that the stimulatory effects of gAd on FA oxidation would be blunted in rats fed both HF diets, but that impaired insulin stimulated glucose transport would only develop in animals fed a high-saturated fat diet. Furthermore, since gAd may exert its acute stimulatory effects on FA oxidation by activating AMPK, we hypothesized that gAd will phosphorylate AMPK and ACC in control, but not HF-fed rats.

Methods:

Animals and Diets.

Upon arrival, female Sprague-Dawley rats (140-145g, Charles River, Quebec, Canada) were assigned to individual cages in a controlled environment with a reverse 12h light-dark cycle and *ad libitum* access to Purina standard rodent chow and water.

Following a 3-day acclimation period, rats were randomly assigned to one of three dietary interventions for 4 weeks (Research Diets, New Brunswick, NJ); control diet (12% kcal from fat, CON), high-unsaturated fat diet (60% kcal from safflower oil, SAFF) or high saturated fat diet (60% kcal from lard, LARD) (Table 3.1). High fat fed animals were pair-fed to CON rats (fed ad libitum) with respect to caloric intake on a daily basis and body mass was recorded 3 times per week. After 4 weeks on the diets, the animals were overnight fasted prior to experimental procedures. Ethical consent for all procedures used was obtained from the Animal Care Committee at the University of Guelph.

Blood Collection and Analyses.

One-milliliter blood samples were collected from the tail artery of each animal under anesthetic with isofluorane inhalant, prior to starting the dietary intervention. A terminal blood collection was also made at the completion of the treatment via cardiac puncture after first excising skeletal muscles for incubation. A glucometer reading (Bayer Elite XL, Toronto, ON, Canada) of whole blood glucose was made at each blood collection. All blood samples were collected after an overnight fast in heparinized tubes, centrifuged at 9300 x g for 5 min at 4°C, and the plasma was removed for analyses of insulin (sensitive rat RIA kit, Linco, St Charles, MS) and adiponectin (mouse RIA kit, Linco, St Charles, MS).

Muscle Incubations.

Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (6 mg/100 g body mass) and the soleus (Sol) muscle was carefully dissected into

longitudinal strips from tendon to tendon using a 27-gauge needle. Two strips from each Sol were then incubated under various conditions for measurement of glucose transport, fat metabolism or Western blot analysis (phosphorylated AMPK and ACC). Due to the number of conditions in these experiments, one animal was used for determination of glucose transport (4 Sol strips), and another animal was used for measurement of both lipid metabolism (2 Sol strips) and Western blot analysis (2 Sol strips). Soleus muscle was chosen due to its oxidative potential and capacity to oxidize FA, and because we have used this muscle in our previous experiments demonstrating diet-induced leptin resistance (186).

Glucose Transport.

Individual strips were randomly assigned to one of 4 conditions; 1) basal, 2) gAd (2.5 μg/ml; Peprotech, ON, Canada), 3) insulin (10mU/mL) or 4) insulin + gAd. These conditions were maintained in all buffers for the duration of the experiments. Immediately after excision from the animal, Sol strips were pre-incubated in a shaking water bath at 30°C for 30 min in 2 mL pregassed (95%O_2/5% CO_2) Krebs-Henseleit buffer (KHB) containing 0.1 % FA free BSA, 8 mM glucose and 32 mM mannitol. Then, strips underwent two 10 min incubations in glucose-free KHB containing 4mM pyruvate and 36mM mannitol. Finally, strips were transferred into a third KHB-based buffer containing 8 mM 3-O-[^{3}H]methyl-D-glucose (0.5 μCi/mL) and 32 mM [^{14}C]mannitol (0.2 μCi/mL) and incubated for 20 min (insulin-stimulated) or 40 min (basal). After incubation, muscles were blotted of excess fluid, trimmed of tendons and weighed. Strips were digested in 1mL 1M NaOH at 95°C for 10 min and 200 μl was then sampled in

duplicate for liquid scintillation counting. Glucose transport was analyzed as accumulation of intracellular 3-O-[^{3}H]methyl-D-glucose, as described previously. (213)

FA Metabolism and AMPK/ACC Signaling.

Soleus strips were placed in 20 mL glass scintillation vials containing 2 mL of warmed, (30°C) pre-gassed, (95% O_2/5% CO_2) KHB containing 4% FA-free BSA, 5 mM glucose and 0.5 mM palmitate, with or without gAd (2.5 ug/ml). After a 30 min equilibration, muscles were transferred to a new vial containing 2 mL of the same medium, with the addition of 0.5 μCi/ml of [1-^{14}C] palmitate (Amersham, Oakville ON, Canada) for an additional 60 min incubation. This permitted the monitoring of exogenous palmitate oxidation and incorporation of palmitate into endogenous triacylglycerol (TAG) and diacylglycerol (DAG) lipid pools as previously described (52).

After the incubations, muscles were blotted of excess liquid, trimmed of tendons, weighed and placed in a 14 mL centrifuge tube containing 5 mL of ice-cold 2:1 chloroform methanol. Muscles were homogenized using a handheld polytron (PT1200, Brinkman Institute, Mississauga, ON, Canada) and centrifuged at 10 000 x g for 10 min. The supernatant was transferred to a clean 14 mL centrifuge and 2 mL of distilled water was added. Samples were shaken for 10 min and centrifuged as before to separate the aqueous and lipophilic phases. One milliliter of the aqueous phase was quantified by liquid scintillation counting to determine the amount of ^{14}C-labelled oxidation intermediates resulting from isotopic fixation. The chloroform phase, containing the total lipids extracted from the muscle, was gently evaporated under a stream of N_2 and re-dissolved in 100 μl of 2:1 chloroform-methanol containing small amounts of dipalmitin

and tripalmitin to facilitate identification of lipid bands on the silica plates. Fifty microliters of each sample was spotted on to an oven dried silica gel plate (Fisher Scientific Canada, Mississauga, ON, Canada) and placed into a sealed tank containing solvent (60:40:3, heptane:isopropyl-ether: acetic acid) for 50 min. Plates were allowed to dry and were sprayed with dichlorofluorescein dye (0.2% w/vol in ethanol) and visualized under long-wave ultra-violet light. The individual lipid bands were scraped into vials for liquid scintillation counting.

Gaseous $^{14}CO_2$ produced during the incubation was trapped by 250 µl benzethonium hydroxide contained in a microcentrifuge tube placed in the sealed 20 mL scintillation vial for the course of the 60 min chase phase. In addition, $^{14}CO_2$ remaining in the buffer following the incubation was released by immediately transferring 1 mL of buffer into a sealed flask and acidifying with 1mL of 1M sulfuric acid to then be captured in benzethonium hydroxide. The tubes containing benzethonium hydroxide and trapped $^{14}CO_2$ were counted using standard liquid scintillation counting techniques.

The remaining two Sol strips were incubated in the presence or absence of gAd (2.5 µg/ml) for 30 minutes in KHB containing 4% FA-free BSA, 5 mM glucose and 0.5 mM palmitate for the determination of pAMPK and pACC protein content. These measurements were repeated in Sol strips from additional CON animals at a 10 minute time point. After incubation, the strips were immediately frozen and stored in liquid nitrogen until subsequent analyses.

Western Blot Analysis.

Soleus muscles (~50 mg) were homogenized (5000 μl/g tissue, 1:5 dilution) in ice-cold buffer suitable for protein extraction and preserving phosphorylation states of proteins, containing 50 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM Na pyrophosphate, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 2 mg/mL leupeptin, 2 mg/mL aprotinin, 2 mg/mL pepstatin, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 20,000 x *g* for 20 min at 4°C and the supernatant was removed and protein content was determined using BSA as standards. Fifty micrograms of this whole tissue lysate protein was solubilized in 4x Laemmeli's buffer and boiled at 95°C for 5 min, resolved by SDS-PAGE and wet transferred to PVSF membranes (1.5 hours, 100V for pAMPK, 8 hours, 30V for pACC). The membranes were blocked for one hour and then incubated with the specific primary antibodies for Thr172 phosphorylated AMPK (pAMPK, Cell Signaling, Danvers, MA) or Ser-79 phosphorylated ACC (pACC, Cell Signaling, Danvers, VA). After incubation with the appropriate secondary antibody and final wash, the immune complexes were detected using the ECL method and immunoreactive bands were quantified with densitometry. Equal loading was confirmed by non-specific protein staining with Ponceau-S.

Calculations and Statistics.

All data are reported as mean ± SE. Results were analyzed using a randomized block design 2-way ANOVA and a Student Newman Keuls's post hoc test was used to test significant differences revealed by the ANOVA. One-way ANOVA was used for body mass and blood measurements. Significance was accepted at $p \leq 0.05$.

The quantity of palmitate esterified and oxidized was calculated from the specific activity of labeled palmitate in the incubation medium (i.e. ratio of labeled to total palmitate in the incubation medium). Total palmitate uptake was calculated by summing the incorporation of labeled palmitate into lipid pools plus oxidation.

Results:

Body composition and blood measurements:

There was no significant difference between diet groups with respect to pre-trial or terminal body mass (Table 3.2). Diet groups did not differ with respect to any measured blood parameter prior to commencing the experimental diets (data not shown). There was no significant difference in fasting blood glucose or insulin between groups following the 4 week trial. Plasma Ad was significantly reduced in both HF-fed groups compared to CON by 4 weeks ($p \leq 0.05$; Table 3.2).

Skeletal muscle glucose transport:

Basal glucose transport was not different among the 3 dietary groups (Figure 3.1A). However, the LARD rats showed significantly reduced rates of insulin-stimulated glucose transport (Figure 3.1B) compared to CON and SAFF (+68%, vs. +172%, +184% $p \leq 0.001$). That is, only rats fed a high saturated fat diet developed skeletal muscle insulin resistance, while rats fed a high unsaturated-fat or control low-fat diet did not. Glucose transport was unaltered by the addition of gAd to the incubation medium in basal (CON, 0.11 ± 0.01; SAFF, 0.12 ± 0.01; LARD, 0.10 ± 0.01 umol/g/5 min) and insulin-stimulated conditions (CON, 0.26 ± 0.02; SAFF, 0.25 ± 0.03; LARD, 0.20 ± 0.02 umol/g/5 min), regardless of the dietary treatment.

Skeletal muscle lipid metabolism:

FA oxidation: Basal FA oxidation rate was significantly higher in SAFF (49.8 ± 3.5 nmol/g/hr) and LARD (46.4 ± 3.4 nmol/g/hr) groups compared to CON

(34.4±2.8 nmol/g/hr, p≤0.05), suggesting a diet-induced adaptation by the SAFF and LARD rats to the higher fat availability. Adiponectin significantly increased FA oxidation in Sol from CON rats (+28%, p≤0.05), but had no stimulatory effect on FA oxidation in either HF group (Figure 3.2A).

FA esterification: FA esterification into TAG was greater in both HF groups compared to CON, (67.9±5.8 nmol/g/hr, SAFF, 66.8±3.4 nmol/g/hr, LARD vs. 47.6±3.3 nmol/g/hr, p≤0.05), but gAd did not have a significant effect within dietary treatments (Figure 3.2B). FA esterification into DAG was not different between the 3 dietary treatments, and was unaffected by gAd (Figure 3.2C).

Oxidation:TAG esterification ratio: There was no difference in the ratio of palmitate oxidized to esterified between dietary groups under basal conditions. However, the ratio was significantly greater in the CON group (0.93±0.1) compared to both SAFF and LARD (0.56±0.05, 0.68±0.07, p≤0.05) following exposure to gAd.

Total palmitate uptake: Basal rates of total palmitate uptake tended to be greater in both high fat groups (131±10 nmol/g SAFF, 120±7 nmol/g LARD) compared to CON (101±7 nmol/g, p=0.058). Treatment with gAd did not change total FA uptake in any group (107±4 nmol/g, CON; 126±9 nmol/g, SAFF; 120±13 nmol/g, LARD).

Influence of Adiponectin on pAMPK and pACC:

Basal pAMPK and pACC (Figure 3.3) were similar across all 3 diet groups. There was no significant effect of acute gAd exposure (30 min, Figure 3.3A; 10 min, Figure 3.4A) on pAMPK in any group. Phosphorylation of ACC was significantly elevated

after 30 min gAd exposure (Figure 3.3B) in CON rats only (+ 25%, $p \leq 0.01$). There was no effect of gAd on pACC after 10 min (Figure 3.4B).

Table 3.1: Composition of Experimental diets

	CON		SAFF		LARD	
	gm	**kcal**	**gm**	**kcal**	**gm**	**kcal**
Protein	19.2	20	26.2	20	26.2	20
Carbohydrate	67.3	70	26.3	20	26.3	20
Fat	4.2	10	34.9	60	34.9	60
Total		100		100		100
kcal/gm	3.85		5.24		5.24	
Ingredient						
Casein, 80 mesh	200	800	200	800	200	800
L-Cystine	3	12	3	12	3	12
Cornstarch	506.2	2025	0	0	0	0
Maltodextrin 10	125	500	125	500	125	500
Sucrose	68.8	275	68.8	275	68.8	275
Cellulose, BW200	50	0	50	0	50	0
Lard	0	0	0	0	270	2430
Safflower Oil, USP	45	405	270	2430	0	0
Mineral Mix S10026	10	0	10	0	10	0
DiCalcium Phosphate	13	0	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0	5.5	0
Potassium Citrate, 1H2O	16.5	0	16.5	0	16.5	0
Vitamin Mx V10001	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0
Total	1055.05	4057	773.85	4057	773.85	4057

CON= control diet, SAFF = safflower oil diet, LARD = lard diet

Table 3.2 Body mass and blood measurements

	CON	SAFF	LARD
Body mass - pre trial (g)	144 ± 2	145 ± 2	143 ± 1
Body mass -post trial (g)	242 ± 4	240 ± 3	246 ± 4
Body mass gain (g)	98 ± 4	95 ± 3	103 ± 3
Glucose (mmol/L)	6.6 ± 0.2	6.8 ± 0.2	6.8 ± 0.2
Insulin (ng/mL)	2.7 ± 0.3	2.1 ± 0.5	3.2 ± 0.7
Adiponectin (μg/mL)	4.5 ± 0.5	2.6 ± 0.2*	3.5 ± 0.2*

CON= control diet, SAFF = safflower oil diet, LARD = lard diet

Values are means ± SE; n = 12 animals

* Significantly different from control; $p \leq 0.05$.

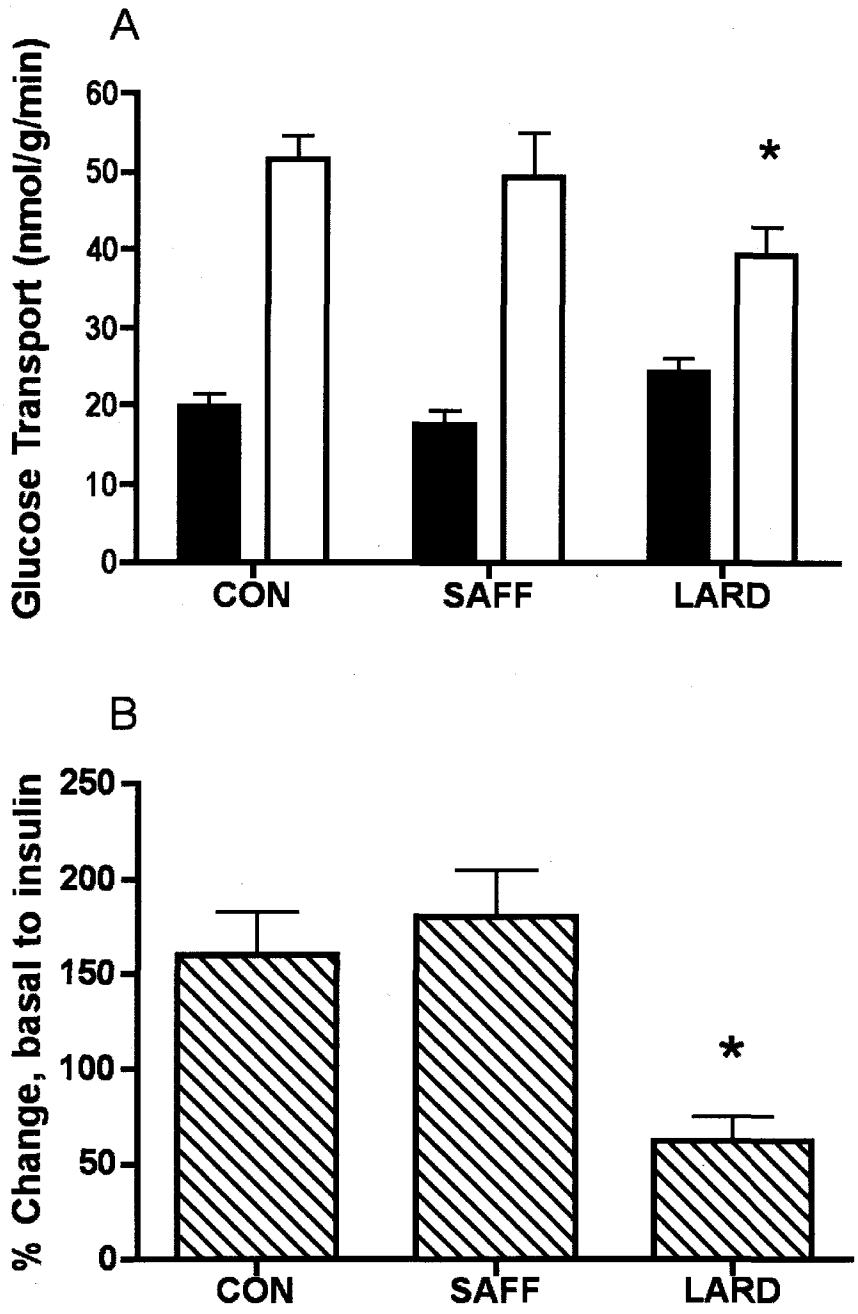

Figure 3.1. A: Basal and insulin stimulated glucose transport in skeletal muscle from CON, SAFF and LARD rats. B: The change in glucose transport rate from basal in response to insulin. Data were calculated by subtracting respective rates of basal uptake from the insulin response. Data are mean ± SE, n=12, *, significantly different from all other groups, $p \leq 0.001$. Closed bars basal, Open bars = insulin stimulated. CON = control diet, SAFF = safflower oil diet, LARD = lard diet

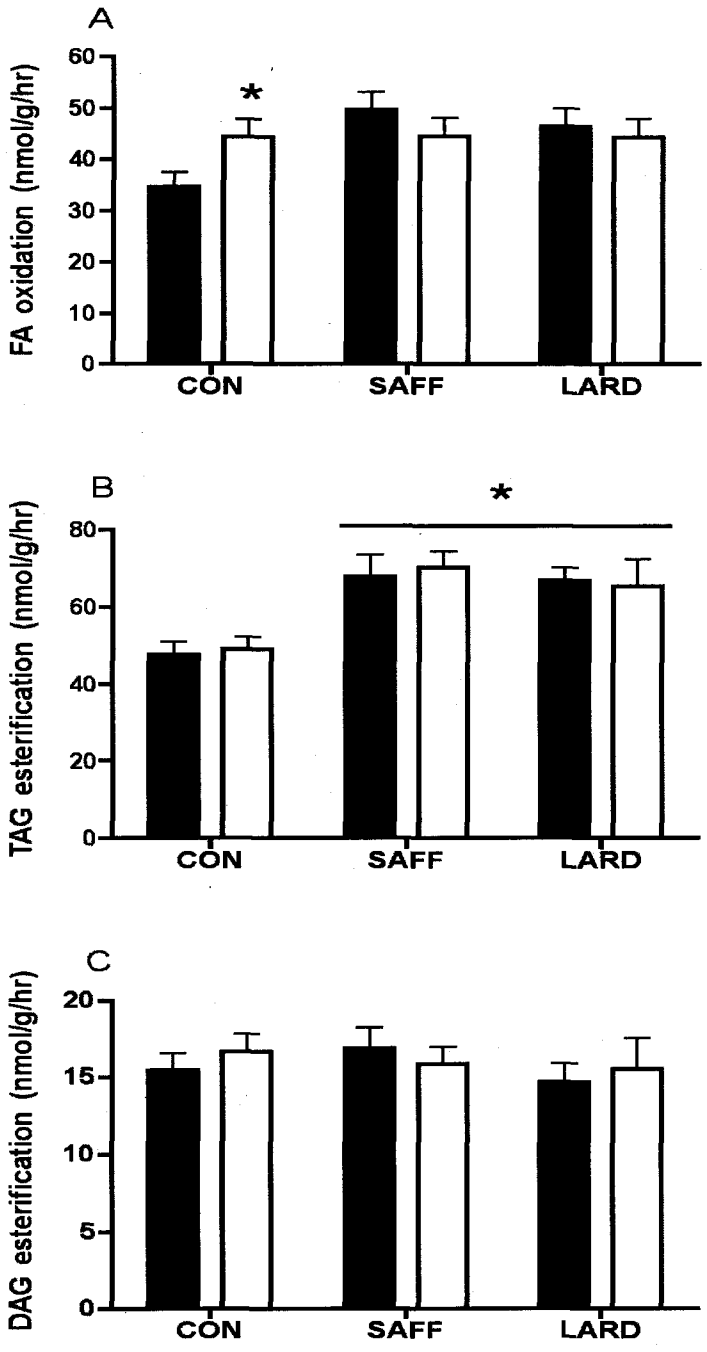

Figure 3.2. The effect of gAd on FA metabolism in skeletal muscle from CON, SAFF and LARD rats. A: The rate of FA oxidation in muscle strips in the absence or presence of gAd (2.5μg/ml). B: Palmitate incorporation into TAG. C: Palmitate incorporation into DAG. Data are mean ± SE, n=12, *, significantly different from control, $p \leq 0.05$. Closed bars = basal, Open bars = gAd stimulated. CON = control diet, SAFF = safflower oil diet, LARD = lard diet

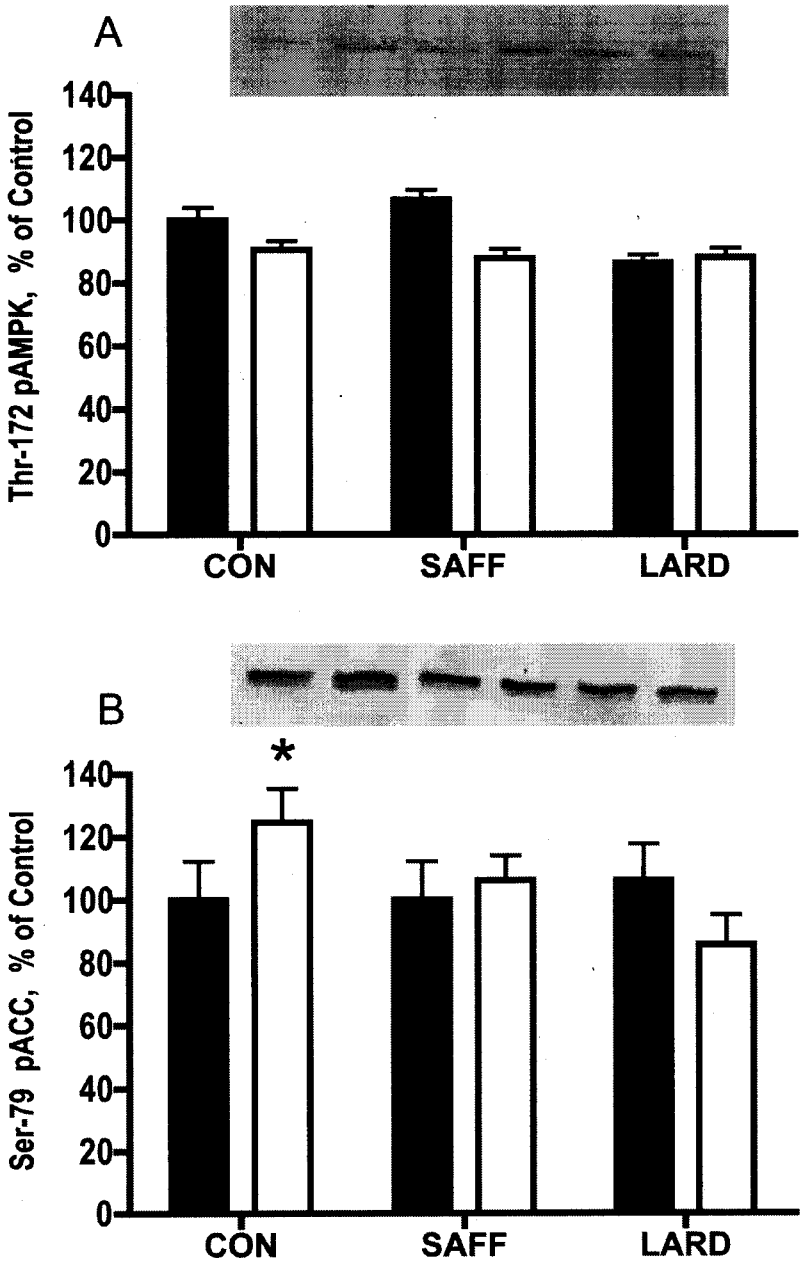

Figure 3.3. The effect of 30 min gAd exposure on A: pAMPK and B: pACC Data are mean ± SE, n=12, *, significantly different from basal, $p \leq 0.01$. Closed bars = basal, Open bars = gAd stimulated. CON = control diet, SAFF = safflower oil diet, LARD = lard diet

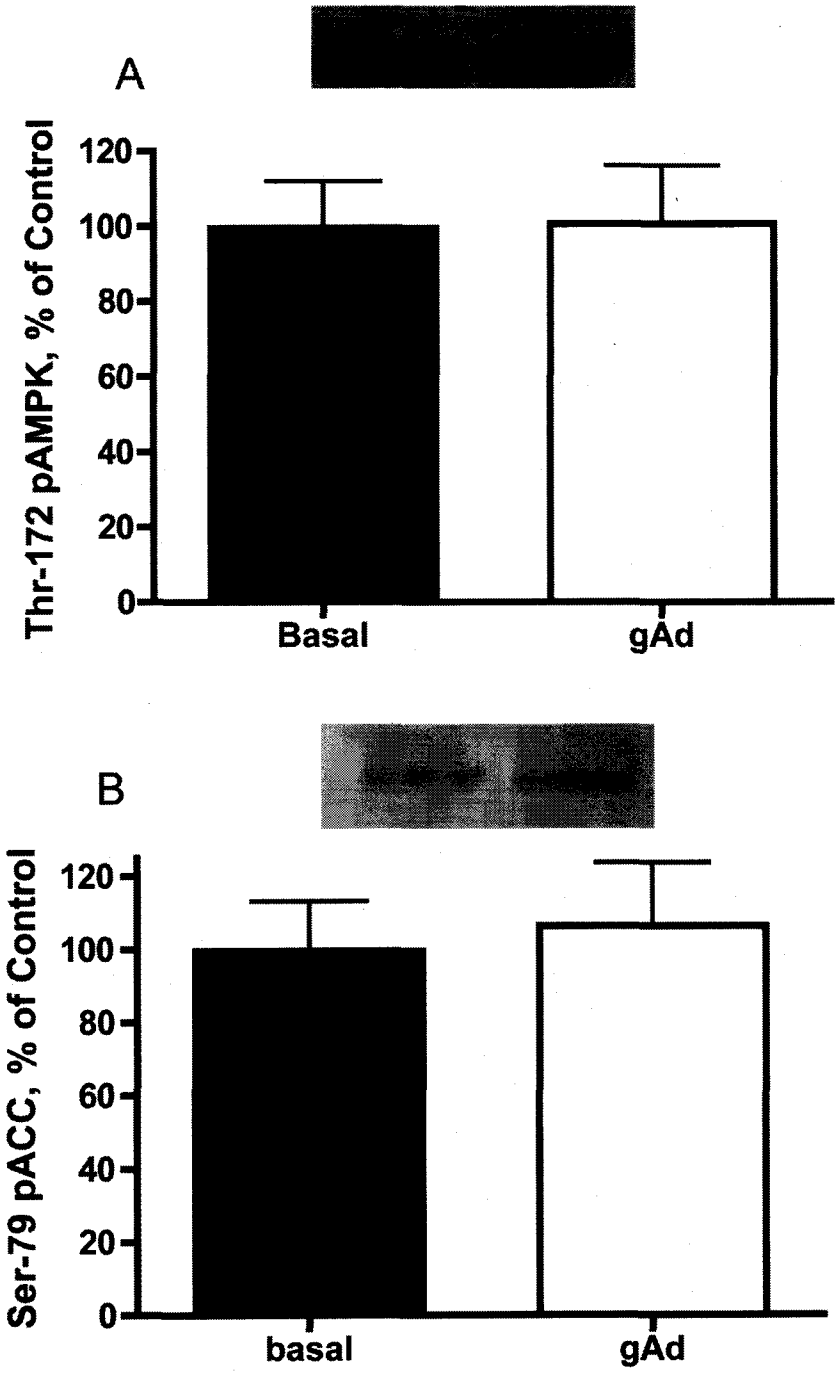

Figure 3.4. The effect of 10 min gAd exposure on A: pAMPK and B: pACC in control rats.Data are mean ± SE, n=10. Closed bars = basal, Open bars = gAd stimulated.

Discussion:

Adiponectin is an adipocyte-derived cytokine with anti-diabetic properties, mediated predominantly by its effects on muscle and liver FA and glucose metabolism. However, low circulating levels of Ad characterize most cases of obesity (5), and recently, suggestions of an impaired peripheral response to this adipokine have been reported in genetically obese mouse models (203), and in obese humans (26). In the present study, we used isolated Sol muscle to determine the direct effects of gAd on maximal insulin-stimulated glucose transport, and FA metabolism in skeletal muscle from rats following 4 weeks of HF feeding. More specifically, our primary goal was to determine whether the consumption of a diet high in saturated or unsaturated fat led to the loss of gAd's stimulation of FA oxidation in skeletal muscle, and if this coincided with the development of impaired insulin-stimulated glucose transport. Furthermore, we wanted to determine if consumption of a HF diet would interfere with the acute stimulatory effect of gAd on the AMPK-ACC axis. Several novel observations were made in the present study; 1) both HF diets resulted in the loss of gAd's ability to stimulate FA oxidation, 2) the lack of stimulation of FA oxidation in response to gAd is supported by the lack of ACC phosphorylation in these dietary groups, 3) the development of gAd resistance did not necessarily coincide with the development of impaired glucose transport, as both conditions were manifested only in the LARD group and 4) we confirmed previous findings in rodent muscle that gAd results in the phosphorylation of ACC independently of AMPK.

Effect of diet on blood parameters:

As expected for a brief dietary intervention, plasma glucose levels did not differ between groups. However, somewhat surprisingly, there was also no change in insulin levels. It is possible that 4 weeks is not long enough to see whole body changes in insulin levels, even though impairment of insulin-stimulated glucose uptake in Sol muscle was evident with the LARD diet. However, the insulin response assessed in Sol may not be representative of other muscle types; additionally, blood samples were taken from fasted animals. As such, it is possible that post-prandial insulin responses may differ between groups. Lastly, both HF groups had significantly decreased plasma total Ad concentrations compared to CON, consistent with previous findings in obese models (5). The physiological significance of gAd, which was utilized in this study, is unclear. It has been reported that the globular form may represent ~1% of the total circulating Ad (62), while other reports have failed to detect it (151). However, even as little as 1% of the total circulating Ad may be significant, as Ad is present in the circulation in much higher concentrations than other adipokines. Teleologically, it is also difficult to reconcile the presence of the Adipo R1 receptor in skeletal muscle, which specifically binds the gAd form, if there is no gAd present in the circulation. Regardless of this controversy, it seems clear that the globular form does have significant effects on muscle FA metabolism and insulin sensitivity, and might be of important therapeutic value.

Effect of diet and gAd on skeletal muscle lipid metabolism:

Basal adaptations to increased fat availability:

Animals in both HF groups demonstrated several adaptations to the increased fat availability in their diet. Despite differences in the metabolic handling of saturated FA vs. PUFA, both HF-fed groups displayed similar compensatory increases in basal palmitate oxidation and esterification into TAG, similar to previous HF feeding trials (37, 174, 186).

gAd stimulated changes in fat metabolism:

Of greater interest to the current study are the differences between dietary groups with respect to lipid metabolism under gAd-stimulated conditions. As expected, gAd stimulated FA oxidation by approximately 30% in CON rats. This is in agreement with our previous findings in human muscle (26), and the findings of others using rodents (62, 202), which show increases in FA oxidation ranging from 10 to 60% in isolated muscle strips incubated with gAd. However, to the best of our knowledge, we are the first group to demonstrate that gAd does not stimulate FA oxidation in Sol muscle following the consumption of a HF diet, indicating the development of gAd resistance. Similar to our previous observations in rodents (186), baseline FA oxidation rates were elevated in HF fed rats, potentially complicating the interpretation of a lack of further stimulation by gAd in these animals i.e. FA had already reached maximal capacity. However, this seems quite unlikely given that only 0.5 mM palmitate was present in the incubation buffer. Nevertheless, in light of this finding, we confirmed the ability of gAd to further stimulate FA oxidation when Sol muscles from CON-fed animals were incubated at a higher concentration of palmitate (0.75 mM), which elicits a basal rate of oxidation

similar to that observed in the HF fed rats. Under these conditions, gAd still stimulated FA oxidation (76.6 $\pm$ 4.1 vs. 65.6 $\pm$ 3.4 nmol/g/h; $p<0.05$). Thus, we interpret the inability of gAd to further stimulate palmitate oxidation above basal levels in HF rats as an indicator of resistance to the effect of adiponectin. Finally, the inability of gAd to stimulate FA oxidation in the HF-fed rats is also supported by a lack of ACC phosphorylation.

In addition to increasing the amount of palmitate oxidized, gAd has been previously shown to decrease intramuscular lipid accumulation in murine muscle (228). Contrary to our expectations, treatment with gAd did not acutely decrease lipid accumulation in either DAG or TAG lipid pools in any dietary group. However, the ratio of oxidation:TAG esterification was significantly greater in muscle from CON rats when incubated with gAd. This suggests a protective repartitioning of FA towards oxidation and away from storage as potentially active lipid moieties. While not immediately apparent in a short-term resting muscle preparation, over time this beneficial repartitioning by gAd may reduce the lipid content in muscle, a possible mechanism by which adiponectin improves insulin sensitivity. It is also possible that gAd may not have been able to decrease TAG and DAG in the absence of a stimulus that promotes esterification, such as insulin or TNFα. This hypothesis is supported by the findings of a previous study by Bruce et al. (25), where leptin decreased DAG accumulation only in the presence of TNFα, a known promoter of lipid storage. Furthermore, it is also possible that changes may have occurred in other lipid pools, such as ceramide or long chain fatty acyl CoA, which we did not measure.

Effect of diet and gAd on skeletal muscle glucose transport:

One of the major findings of the current study was that a diet high in saturated fat resulted in impaired insulin-stimulated glucose transport, but a diet containing 60% unsaturated fat did not. It should be noted that all animals were pair-fed to a control; thus, changes in muscle responsiveness to insulin in LARD rats cannot be attributed to increases in body mass relative to the other two groups. Recently, there has been some controversy regarding the development of IR after consuming HF diets of varying composition. It has been previously shown that a diet high in PUFAs can induce liver and skeletal muscle IR, and partial substitution with n-3 FA is necessary for reversal (193, 220). However, more recently, studies have shown that while a diet high in saturated fat leads to decreased whole body insulin sensitivity, a diet high in n-6 PUFA sustains insulin sensitivity (113). Direct examination of muscle cells *in vitro* indicate that once taken up in skeletal muscle, saturated and unsaturated fats are metabolized differently. Unsaturated fats are more readily oxidized (64, 117) and are stored preferentially as TAG (64), whereas saturated fats are not readily oxidized and are stored as DAG (135) or ceramides (173), which can directly interfere with insulin signaling (34, 233).

Taken together with our previous findings that a high SAFF diet can induce leptin resistance (186), it is noteworthy that this diet did not lead to a loss of maximal insulin-stimulated glucose uptake. However, it is important to note that in our study, rodents were overnight fasted, which has previously been shown to ameliorate diet-induced muscle insulin resistance (149). Therefore, it is possible that our SAFF diet may have also led to impaired insulin-stimulated muscle glucose transport, which was reversed by

the overnight fast. Nevertheless, the impairment in insulin-stimulated glucose transport clearly persisted in the LARD fed animals, as did the resistance to gAd in both HF fed groups, and were not reversed by a brief fast. Whether prolonged HF feeding (i.e. greater than 4 weeks) would result in the eventual development of impaired soleus glucose transport in the high SAFF group, persisting beyond an overnight fast, warrants further examination, as would future studies examining the time course of adipokine/insulin resistance in soleus muscle.

A recent study by Ceddia *et al.* using L6 myotubes suggests that adiponectin exerts a stimulatory effect on skeletal muscle glucose transport by enhancing GLUT 4 translocation to the cell surface via AMPK activation (32). We were unable to show an effect of gAd on basal or insulin-stimulated glucose transport. This is also in contrast to our work in humans (26) in which glucose transport was stimulated by gAd in both lean and obese subjects. However, our results are in support of Tomas et al. (202) in which gAd-stimulated increases in glucose transport occurred in the presence of AMPK activation (glycolytic extensor digitorum longus muscle), but not in its absence (oxidative Sol muscle). We, too, did not observe a gAd-stimulated activation of AMPK in Sol muscle. Thus, it is possible that gAd stimulates glucose transport in a fibre-type specific manner, exerting its effects through AMPK in fast twitch glycolytic muscles, but having no effect in slow oxidative muscle where this acute phosphorylation is absent. However, it should be noted that Yamauchi *et al.* do show an increase in AMPK activation in Sol muscle (226).

Effect of diet and gAd on AMPK and ACC:

Acutely, gAd has been shown to stimulate FA oxidation in muscle due, at least in part, to the phosphorylation, (i.e. activation) of AMPK. Consequently, ACC is phosphorylated (deactivated) leading to decreased malonyl CoA content, relieving the inhibition on CPT-1 and allowing for increased FA oxidation (32, 202, 226).

There is some controversy regarding AMPK activation by gAd in various muscle types. Stimulation of the AMPK-ACC axis has been shown in L6 myotubes (32), and glycolytic muscle (202). However, while Yamauchi *et al.* (226) have reported AMPK activation in oxidative muscle, Tomas et al (202) have reported no change in pAMPK in oxidative Sol muscle. Still, Tomas *et al.* (202) did report an increase in pACC in oxidative muscle. The activation of AMPK by gAd has been shown to be transitory, with peak activation occurring at ~5 min, and returning to baseline values by 60 min (32, 202, 226). In comparison, pACC levels have been shown to increase later and remain sustained longer (202). In agreement with Thomas *et al.* (202), our results showed that acute gAd exposure had no effect on Sol pAMPK in any dietary group, but did cause a significant increase in pACC in CON rats. Since this observation was consistent at both 10 and 30 min of incubation, it is highly unlikely that the transitory increase in pAMPK was simply not detected. Therefore, it is possible that in Sol muscle gAd phosphorylates ACC, without acting through AMPK. As such, the phosphorylation of ACC in CON rats supports our findings that CON rats are sensitive to the stimulatory effects of gAd, while HF rats show signs of gAd resistance.

Summary

In conclusion, we suggest that 4 weeks of HF feeding results in the development of skeletal muscle Ad resistance and this occurs independent of impaired capacity for insulin-stimulated glucose transport. Moreover, this reduced response to gAd in HF-fed rats is likely due to a decreased phosphorylation of ACC. Thus, it appears as though a general HF diet can induce Ad resistance, but the source of fat is important for the development of insulin resistance.

Chapter 4: Adiponectin resistance precedes the accumulation of skeletal muscle lipids and insulin resistance in high-fat-fed rats.

As published, with minor revisions:

Mullen KL, Pritchard J, Ritchie I, Snook LA, Chabowski A, Bonen A, Wright D, and Dyck DJ. Adiponectin resistance precedes the accumulation of skeletal muscle lipids and insulin resistance in high-fat-fed rats. *Am J Physiol Regul Integr Comp Physiol* 296: R243-251, 2009.

Introduction

Consumption of a high-fat (HF) diet and obesity are well known contributors to the development of insulin resistance (IR) and Type 2 Diabetes. High fat diets may induce IR in part by altering lipid metabolism in skeletal muscle (113, 139, 220). Intramuscular lipid accumulation in untrained humans and rodents is consistently associated with skeletal muscle IR, although the cause of this accumulation is controversial. Elevated lipids may be attributable to increased fatty acid (FA) uptake (78), decreased oxidation (105, 107), increased esterification (113) or likely an imbalance among all of these. Impaired rates of FA oxidation and oxidative capacity have been reported in skeletal muscle of obese, IR and diabetic subjects (105, 107). Recently, increased FA uptake into the cell as a result of increased FA transporters (FAT/CD36, FABPpm) at the plasma membrane has been suggested as an important cause of HF diet induced intramuscular lipid accumulation (75, 204).

Despite controversies as to the method of accumulation, various lipids have been shown to interfere with insulin signal transduction and subsequent translocation of GLUT 4 to the plasma membrane. Specifically, diacylglycerol (DAG) has been shown to

activate protein kinase C, thereby impairing IRS-1 and PI3k activation (135), and ceramide has been shown to inhibit phosphorylation/activation of Akt, thus diminishing Akt's stimulatory effect on GLUT 4 translocation to the plasma membrane (34). Collectively, the imbalance between FA uptake and oxidation, resultant intramuscular lipid accumulation, diminished insulin signal transduction and impaired glucose transport are hallmarks of skeletal muscle lipotoxicity which can be induced by HF feeding. Adiponectin (Ad) is an insulin-sensitizing cytokine known to stimulate FA oxidation (26, 62, 139) and glucose uptake into skeletal muscle (32). More specifically, the globular head, gAd, which has been shown to circulate in small quantities, exerts these effects in skeletal muscle by binding to its receptor AdipoR1 (62, 126, 202). Globular Ad stimulates AMPK, which in turn phosphorylates and inactivates ACC, thereby decreasing malonyl CoA formation and relieving inhibition on CPT-1, allowing FA to enter the mitochondria to be oxidized (202, 226). Circulating Ad levels are decreased in obesity (5) and evidence of Ad resistance has also been shown in peripheral tissues of obese humans and animals. In genetically obese diabetic mice, the acute stimulatory effect of Ad on AMPK in muscle is lost. This may be due, at least in part, to an observed reduction in skeletal muscle AdipoR1 mRNA (203). In obese humans, the stimulatory effect of gAd on FA oxidation in skeletal muscle is also blunted (26, 36). Recently, we demonstrated that following 4 weeks of feeding rats a diet high in saturated or polyunsaturated fat, gAd no longer stimulated FA oxidation; that is, both HF diets induced Ad resistance (139). However, only the animals fed the saturated diet became IR by 4 weeks, while polyunsaturated fed animals were insulin responsive at this time point.

Thus, while a HF diet can induce Ad resistance, it is unclear whether Ad resistance precedes intramuscular lipid accumulation and the development of IR.

Surprisingly, there has been virtually no examination of the early changes in muscle lipid metabolism and response to insulin sensitizing adipokines during the development of diet induced IR. Therefore, the purpose of the current study was to elucidate the time-course over which diet-induced Ad resistance develops to determine if it precedes impairments in insulin response, and identify ensuing changes in lipid metabolism and insulin signaling which may contribute to this relationship. We chose to employ a high saturated fat diet as we have previously shown this to produce both Ad resistance and impaired insulin response within a 4 week period (139). We hypothesized that 1) Ad resistance would occur first, attributable in part to decreased AdipoR1 content, followed by 2) greater FA transporter abundance at the plasma membrane, leading to 3) increased IM DAG and ceramide content, 4) impaired activation of the insulin-signaling cascade and ultimately, 5) impaired skeletal muscle glucose transport under maximal insulin stimulated conditions.

Methods:

Animals and Diets.

Upon arrival, female Sprague-Dawley rats (140-145g, Charles River, Quebec, Canada) were assigned to individual cages in a controlled environment with a reverse 12h light-dark cycle with *ad libitum* access to Purina standard rodent chow and water. Following a 3-day acclimation period, rats were randomly assigned to a control (12% kcal from fat, CON), or high-fat diet (60% kcal from lard, HF) (Research Diets, New

Brunswick, NJ) for 3 days, 2 weeks or 4 weeks. High-fat fed animals were pair-fed to CON rats (fed *ad libitum*) with respect to caloric intake on a daily basis and body mass was recorded 3 times per week. After the pre-determined length on the diets, the animals were overnight fasted prior to experimental procedures. Ethical consent for all procedures used was approved by the Animal Care Committee at the University of Guelph.

Muscle and Blood Sampling.

Soleus Muscle

Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (6 mg/100 g body mass) and the soleus (Sol) muscle was carefully dissected into longitudinal strips from tendon to tendon using a 27-gauge needle. The two outside strips from each Sol were then incubated under various conditions for measurement of i) glucose transport, ii) fat metabolism or iii) key signaling proteins. Due to the number of conditions in these experiments, one animal was used for metabolism measures (leg 1: glucose transport, leg 2: fat metabolism), and a second animal was used for assessment of signaling proteins (leg 1: insulin-stimulated signaling, leg 2: gAd-stimulated signaling). The remaining piece of Sol from each leg was immediately frozen in liquid N_2, without incubation, to determine the effects of the chronic dietary intervention on AdipoR1 protein levels or total muscle lipid content. For analysis of whole muscle lipid content, one Sol strip was subsequently freeze-dried, powdered, and cleaned of any visible connective tissue, and individual lipids (DAG and ceramide) were extracted and measured using gas-liquid chromatography as described previously (27).

Blood

Terminal blood collection was made at the completion of the treatment via cardiac puncture after first excising skeletal muscles for incubation. A glucometer reading (Bayer Elite XL, Toronto, ON, Canada) of whole blood glucose was also made. All blood samples were collected in heparinized tubes after an overnight fast, centrifuged at 9300 x g for 5 min at 4°C, and the plasma removed for analyses of insulin (sensitive rat RIA kit, Linco, St Charles, MS) and total adiponectin (mouse RIA kit, Linco, St Charles, MS).

Preparation of Giant Sarcolemmal Vesicles

Red gastrocnemius (RG) and red tibialis anterior (RTA) muscles from each animal were pooled to prepare giant sarcolemmal vesicles as described previously (33). These representative oxidative muscles have previously been used as surrogates for the oxidative soleus when tissue is limited (153, 182). The vesicles were frozen at –80°C until analyzed for sarcolemmal membrane-associated FA transport protein expression (fatty acid translocase (FAT)/CD36, plasma membrane-bound fatty acid binding protein (FABPpm)).

Fatty Acid Metabolism.

Soleus strips were equilibrated in 2 mL of pregassed (95% O_2-5% CO_2) Krebs Henseleit buffer (KHB; 4% BSA, 30°C), with 0.5 mM palmitate and 5 mM glucose for 30 min. Muscles were incubated for an additional 60 min with the addition of 0.5 μCi/mL [1-^{14}C]palmitate (Amersham, ON, Canada) and in the absence or presence of gAd (2.5 μg/mL; Peprotech, ON, Canada) to determine palmitate oxidation and incorporation into endogenous triacylglycerol (TAG) and DAG lipid pools as outlined previously (52).

After the incubations, muscles were blotted of excess liquid, trimmed of tendons, weighed and treated to separate the aqueous and lipophilic phases. One milliliter of the aqueous phase was quantified by liquid scintillation counting to determine the amount of ^{14}C-labelled oxidation intermediates resulting from isotopic fixation. Muscle lipids were re-dissolved in 100 μL of 2:1 chloroform-methanol, spotted on to an oven dried silica gel plate (Fisher Scientific Canada, Mississauga, ON, Canada) and placed into a sealed tank containing 60:40:3, heptane:isopropyl-ether: acetic acid for 50 min. Plates were dried, sprayed with dichlorofluorescein dye (0.2% w/vol in ethanol) and visualized under long-wave ultra-violet light. The individual lipid bands were scraped into vials for liquid scintillation counting. $^{14}CO_2$ accumulated in the buffer was released by transferring 1 mL of buffer into a sealed flask and acidifying with 1 mL of 1M sulfuric acid and captured in benzethonium hydroxide. The trapped $^{14}CO_2$ were counted using standard liquid scintillation counting techniques. Total palmitate uptake was calculated by summing the incorporation of labeled palmitate into lipid pools plus oxidation.

Glucose Transport.

Soleus strips were equilibrated for 30 min in 2 mL of pregassed (95% O_2-5% CO_2) KHB (0.1% BSA, 30°C) containing 8 mM glucose and 32 mM mannitol, in the absence or presence of insulin (10 mU/mL, maintained in all subsequent steps), in a gentle shaking bath. Muscle strips were washed (2 x 10 min) with glucose-free KHB (4 mM pyruvate, 36 mM mannitol). Soleus strips were then incubated for 20 min (insulin) or 40 min (basal) in KHB [4 mM pyruvate, 8 mM 3-*O*-[^{3}H]methyl-D-glucose (800 μCi/mmol), 28 mM [^{14}C]mannitol (60 μCi/mmol)]. Muscles were blotted, weighed, and

digested (95°C, 10 min, 1 mL of NaOH). Glucose transport was calculated from a 200 μL aliquot of muscle digest to quantify intracellular 3-*O*-[^{3}H]methyl-D-glucose as described previously (213).

AMPK/ACC and Insulin Signaling Proteins.

Soleus strips were incubated in KHB containing 4% FA-free BSA, 5 mM glucose, and 0.5 mM palmitate for either 1) 30 min in the presence or absence of gAd (2.5 μg/mL) for the determination of total and phosphorylated AMPK and ACC protein content, or ii) for 10 min in the presence or absence of insulin (10 mU/mL) for the determination of total and phosphorylated insulin signaling proteins. After incubation, the strips were immediately frozen and stored in liquid N_2 until subsequent Western Blot analyses.

Western Blot Analyses.

Muscle tissue (~ 50 mg Sol) was homogenized (5,000 μL/g tissue, 1:5 dilution) in ice-cold buffer suitable for protein extraction and preserving phosphorylation states of proteins as described previously (181). Homogenates were centrifuged at 20,000 x *g* for 20 min at 4°C and the supernatant was removed and protein content was determined using BSA as standards. Fifty micrograms of this whole tissue lysate protein or 10 μg of giant sarcolemmal vesicle protein were solubilized in 4x Laemmli's buffer, boiled (95°C, 5 min), resolved by SDS-PAGE, and wet-transferred to polyvinylidene difluoride membranes [1–1.5 h, 100 V; total and Ser^{79}-phosphorylated acetyl-CoA carboxylase: 8–15 h, 25–40 V, 4°C]. The membranes were blocked for 1 hour and then incubated overnight at 4°C with the specific primary antibodies for total and Thr^{172} phosphorylated AMPK (tAMPK, pAMPK, Cell Signaling, Danvers, MA), total and Ser^{79} phosphorylated

ACC (tACC, Cell Signaling; pACC, Upstate, Billerica MA), sarcolemmal FABPpm (gift from Dr. J. Calles-Escandon, Wake Forest University School of Medicine), FAT/CD36 (gift from Dr. N. N. Tandon, Otsuka Maryland Medicinal Laboratories), AdipoR1 (AbCAM, Cambridge, MA), total, Ser^{473} and Thr^{308} phosphorylated Akt, (tAkt, Upstate; serAkt, thrAkt, Santa Cruz Biotechnology, Santa Cruz, CA), total and Thr^{642} phosphorylated Akt substrate 160 (tAS160, Upstate; pAS160, Medicorp, Montreal, QC, Canada) total insulin receptor substrate 1 (tIRS-1, Upstate) and p85 subunit of phosphoinositide-3 kinase (PI3k, Upstate). After incubation with appropriate secondary antibody for 1 hour, the immune complexes were detected using the enhanced chemiluminescence method (Syngene Chemigenius2; PerkinElmer, Waltham, MA), and quantified with densitometry (Gene Tools software, PerkinElmer). Equal loading was confirmed using nonspecific protein staining with Ponceau-S stain (Sigma Aldrich, Oakville, ON, Canada).

Calculations and Statistics.

All data are reported as mean ± SE. Results were analyzed using a randomized block design 2-way ANOVA and a Student Newman Keuls's post hoc test was used to test significant differences revealed by the ANOVA. For all muscle measurements, no differences were found between CON animals at the 3 time points, therefore the CON groups were combined. Similarly, for Western blots of total protein content, no differences were found between basal and acute stimulated conditions; therefore these groups were also combined. One-way ANOVA was used to analyze dietary and/or time effects when no additional treatment was present. Significance was accepted at $p \leq 0.05$.

Results:

Body mass and blood measurements:

There was no significant difference in pre-trial or terminal body mass of CON and HF animals at any time point (Table 4.1). Fasting blood glucose and plasma insulin did not differ between CON and HF animals at any time point (Table 4.1). Fasting plasma Ad was significantly lower in HF animals compared to CON animals at each time point ($p \leq 0.05$, Table 4.1).

Lipid metabolism:

FA oxidation: Basal FA oxidation rate did not differ between CON and HF rats at any time point. Adiponectin significantly increased FA oxidation in Sol from CON rats (+28%; $p \leq 0.05$), but had no stimulatory effect on FA oxidation in HF animals at any time point (Figure 4.1A).

FA esterification: FA esterification into TAG was greater in HF animals by 2 and 4 weeks compared to CON (+23%, +34% respectively, $p \leq 0.05$), but gAd did not have a significant acute effect within dietary treatments (Figure 4.1B). Fatty acid esterification into DAG was not different between the dietary treatments at any time point, and was unaffected by gAd (range, 13.1 ± 0.9 to 14.5 ± 0.5 nmol/g/hr).

Total palmitate uptake: Total palmitate uptake was greater in HF fed rats after 2 and 4 weeks compared to CON (+13% for both, $p \leq 0.05$). Treatment with gAd did not change total FA uptake in any group (Figure 4.1C).

Adiponectin stimulated signaling proteins and receptor:

AMPK and ACC Signaling: There was no significant effect of diet or acute gAd exposure on tAMPK, pAMPK (Figure 4.2A) or tACC protein levels. Adiponectin acutely increased pACC in CON rats (+34%; $p \leq 0.05$), but failed to do so in HF rats at any time point (Figure 4.2B).

AdipoR1: There was no significant effect of diet or time on AdipoR1 protein content in Sol muscles (Figure 4.3).

Sarcolemmal fatty acid transporters and muscle lipid content:

Transporters: FAT/CD36 (Figure 4.4A) was significantly increased in the PM of red muscle (RG and RTA) of rats after 2 and 4 weeks of chronic HF feeding when compared to CON (+31% and +34% respectively; $p \leq 0.05$). FABPpm (Figure 4.4B) was elevated after 4 weeks of HF feeding compared to CON (+57%; $p=0.06$).

Muscle Lipid: Total DAG and ceramide content was not different between 3d HF and CON. However, total DAG (+19% to +25%; $p \leq 0.05$) and ceramide (+23% to +26%; $p \leq 0.05$) content were increased in Sol following 2wk and 4wk HF feeding compared to CON (Figure 4.4C, D).

Insulin signaling proteins:

Total Proteins: High fat feeding had no significant effect on the total amount of IRS-1, p85 PI3k, Akt or AS160 protein in Sol muscle (data not shown).

Phosphorylated Proteins: Insulin-stimulated phosphorylation of Akt Ser^{473} and Thr^{308} was blunted in HF animals compared to CON after only 3 days, and remained

blunted at 2 and 4 weeks (Figure 4.5A, B). There was a trend (p=0.08) for insulin-stimulated Thr^{642} phosphorylation of AS160 to be blunted in HF animals at 2 and 4 weeks compared to CON (Figure 4.5C).

Skeletal muscle glucose transport:

Basal glucose transport was not different among the dietary groups at any time point. Insulin increased glucose transport above basal levels in all groups; this stimulation was significantly blunted only in the 4wk HF compared to all other groups (Figure 4.6).

Table 4.1: Body mass and blood measurements

	CON 3d	HF 3d	CON 2wk	HF 2wk	CON 4wk	HF 4wk
Pre-trial body mass, g	184 ± 2	186 ± 3	195 ± 2	191 ± 3	191 ± 2	188 ± 2
Terminal body mass, g	188 ± 3	189 ± 2	234 ± 3	237 ± 3	265 ± 5	272 ± 4
Fasting blood glucose, mmol/l	7.5 ± 0.3	8.1 ± 0.2	7.5 ± 0.3	8.8 ± 0.3	7.6 ± 0.5	8.2 ± 0.4
Fasting plasma insulin, ng/ml	1.6 ± 0.1	1.5 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	1.7 ±0.1
Fasting plasma Ad, µg/ml	4.3 ± 0.6	2.9 ± 0.4*	4.9 ± 0.5	3.6 ± 0.3	4.6 ± 0.4	3.6 ± 0.2*

Data are mean ± SE, n = 8-12 rats. CON = control diet; HF = high fat diet; Ad = adiponectin

* $p \leq 0.05$, significantly different from control

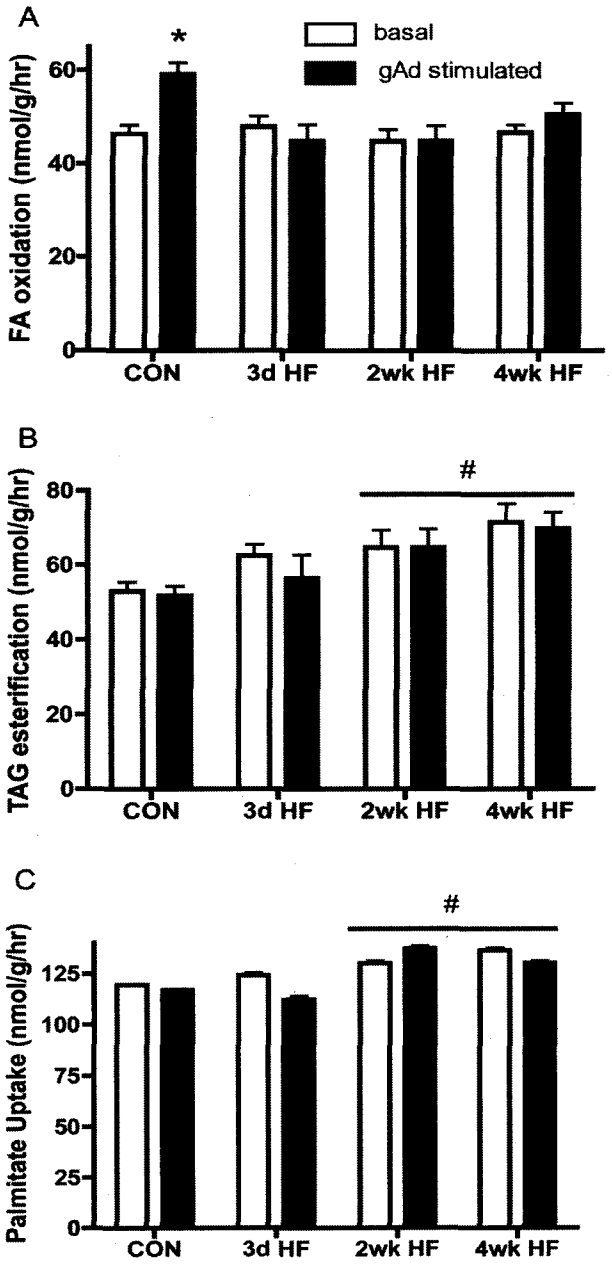

Figure 4.1: The effect of gAd on skeletal muscle FA metabolism. (A) The rate of FA oxidation. (B) Palmitate incorporation into TAG. (C) The rate of palmitate uptake calculated as palmitate oxidation plus DAG and TAG esterification. Data are mean ± SE, n=12, *, significantly different from basal, $p \leq 0.05$, #, significantly different from CON conditions, $p \leq 0.05$. Open bars = basal, Closed bars = gAd stimulated (2.5μg/ml). CON = control diet, 3d HF = 3 day high fat diet, 2wk HF = 2 weeks high fat diet, 4wk HF = 4 weeks high fat diet.

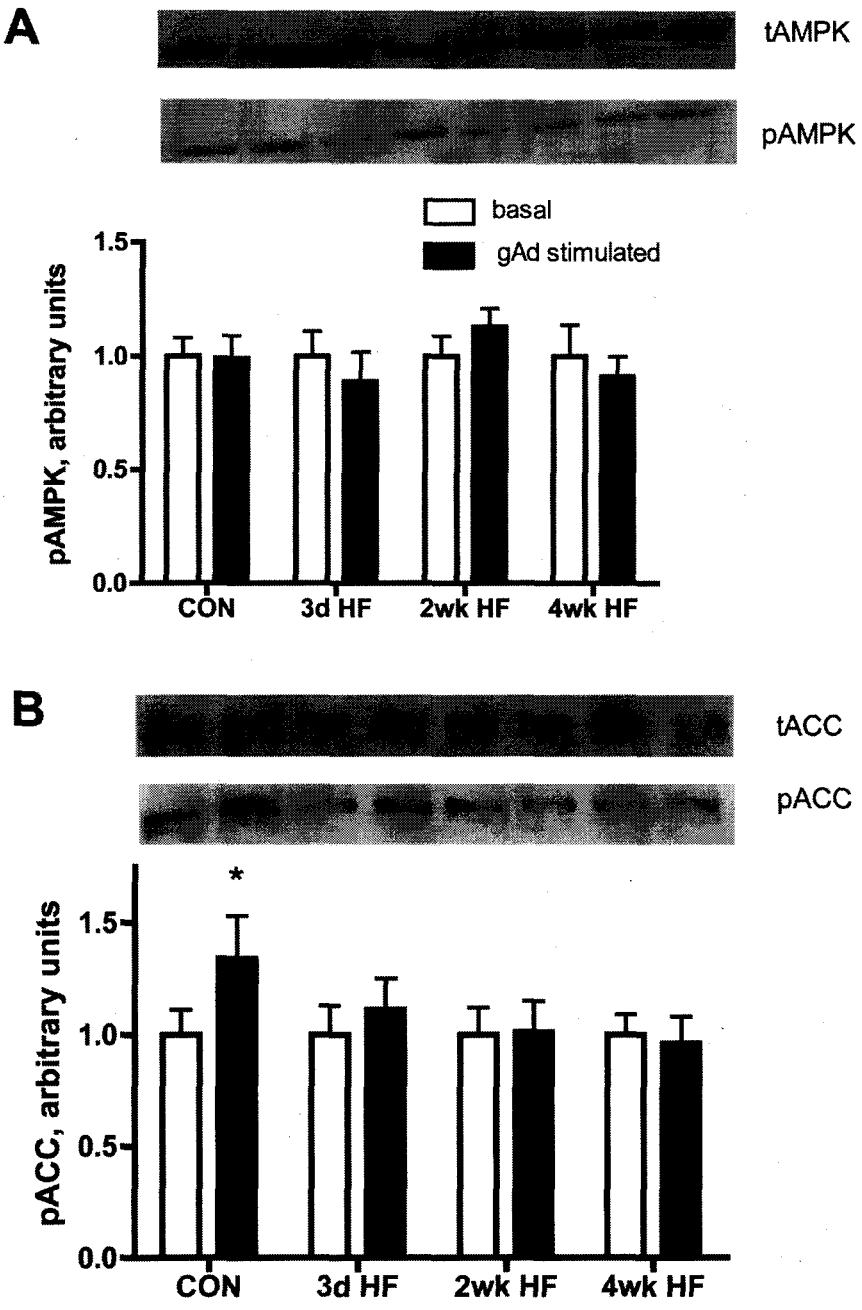

Figure 4.2: The acute effect of 30 min gAd exposure on protein content of (A) tAMPK and pAMPK and (B) tACC and pACC in soleus muscle. Data are mean ± SE, n=10, *, significantly different from basal, $p \leq 0.05$, Open bars = basal, Closed bars = gAd stimulated (2.5μg/ml). CON = control diet, 3d HF = 3 day high fat diet, 2wk HF = 2 weeks high fat diet, 4wk HF = 4 weeks high fat diet.

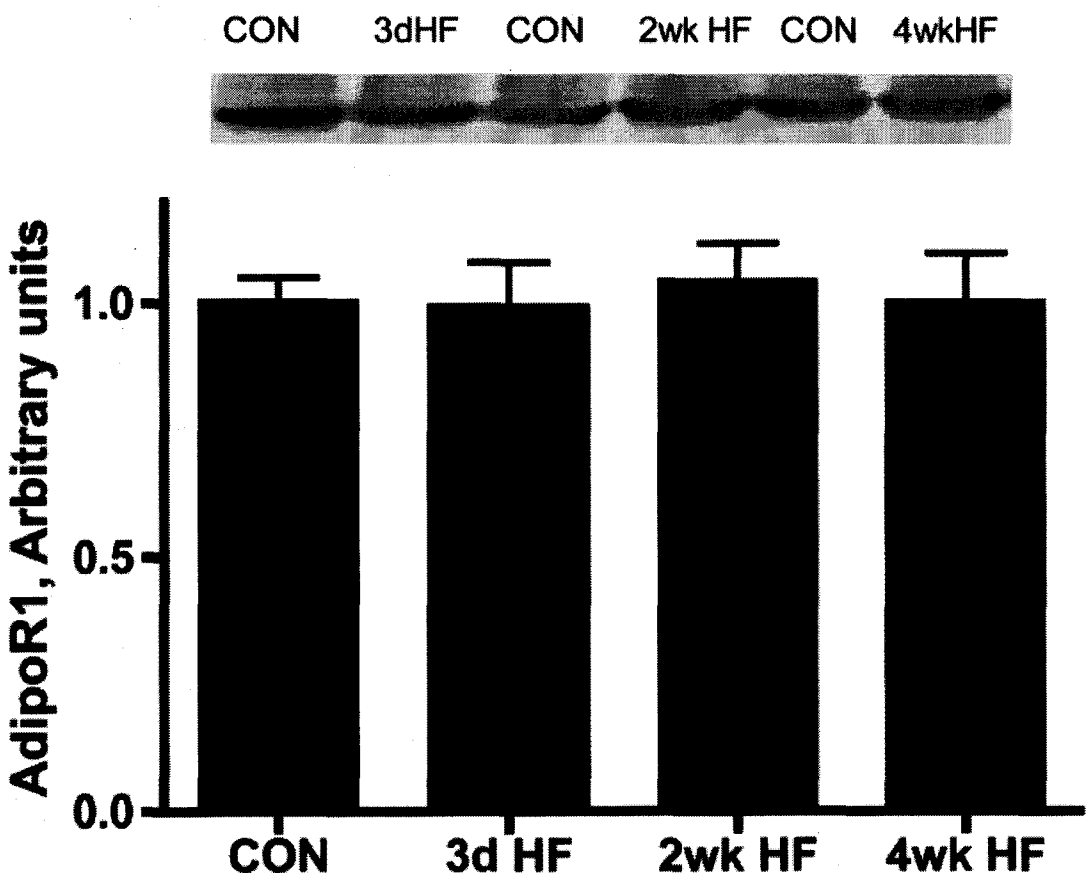

Figure 4.3: The effect of chronic HF feeding on soleus muscle AdipoR1 protein content. Data are mean ± SE, n=12 CON = control diet, 3d HF = 3 day high fat diet, 2wk HF = 2 weeks high fat diet, 4wk HF = 4 weeks high fat diet.

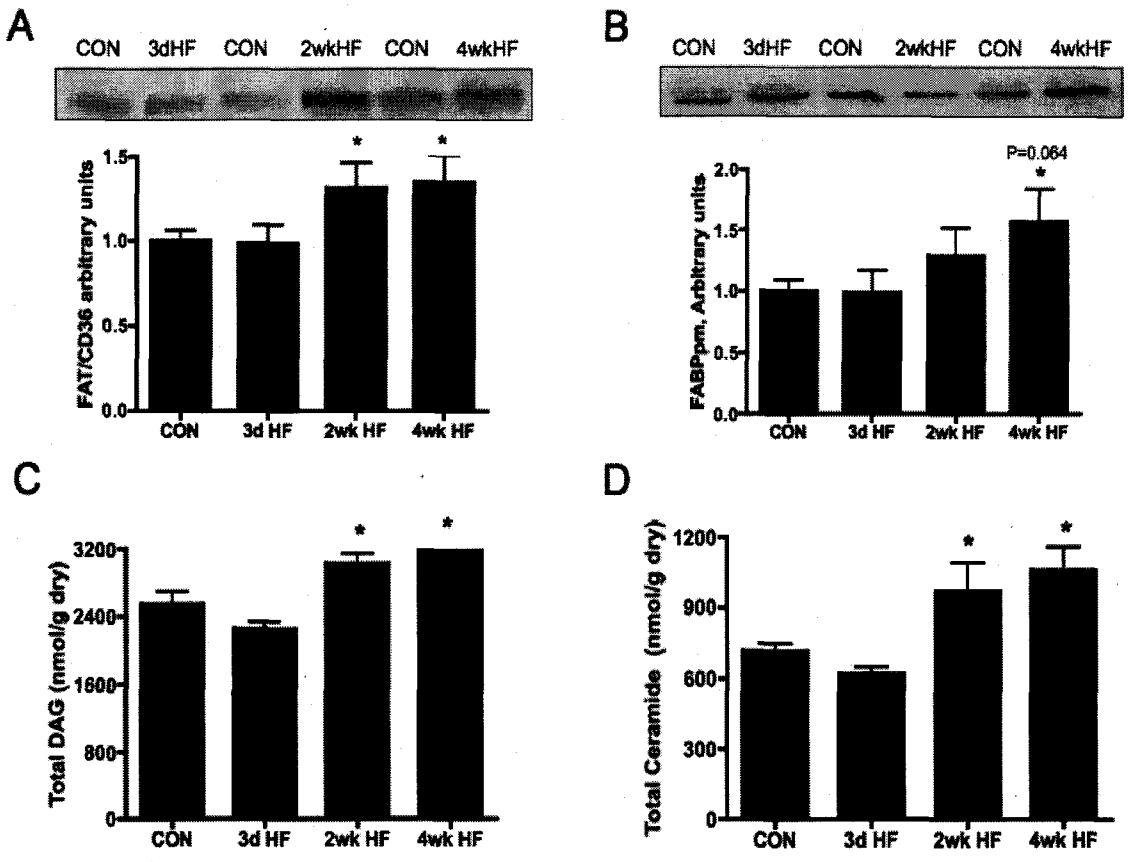

Figure 4.4: The effect of chronic HF feeding on PM-associated FA transporter protein content and intramuscular lipid content. (A) Plasma membrane associated FAT/CD36 and (B) Plasma membrane associated FABPpm measured in giant sarcolemmal vesicles. (C) Total DAG and (D) Total ceramide measured in soleus muscle. Data are mean ± SE; A,B: n=12, C,D: n=6-10; *, significantly different from CON, $p \leq 0.05$, unless otherwise stated. CON = control diet, 3d HF = 3 day high fat diet, 2wk HF = 2 weeks high fat diet, 4wk HF = 4 weeks high fat diet.

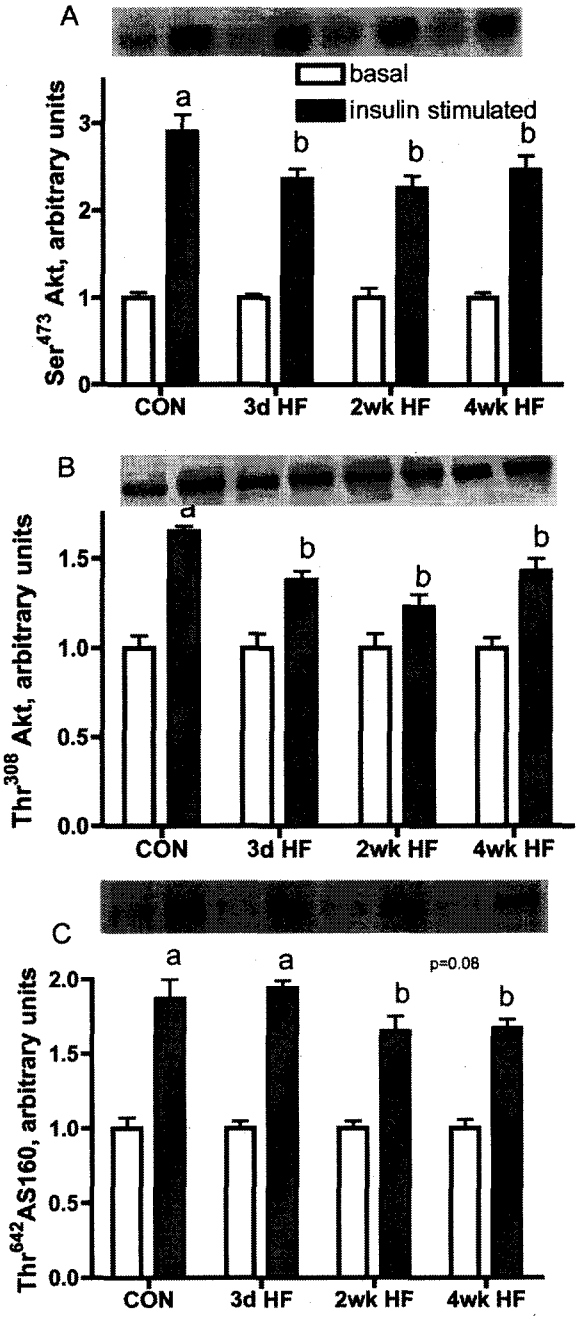

Figure 4.5: The acute effect of 10 min insulin exposure on protein phosphorylation of (A) ser^{473} Akt, (B) thr^{308} Akt, (C) thr^{642} AS160 in soleus muscle. Data are mean ± SE, n=10, bars not sharing a letter are significantly different, $p \leq 0.05$, unless otherwise stated. Open bars = basal, Closed bars = insulin stimulated. (10mU). CON = control diet, 3d HF = 3 day high fat diet, 2wk HF = 2 weeks high fat diet, 4wk HF = 4 weeks high fat diet.

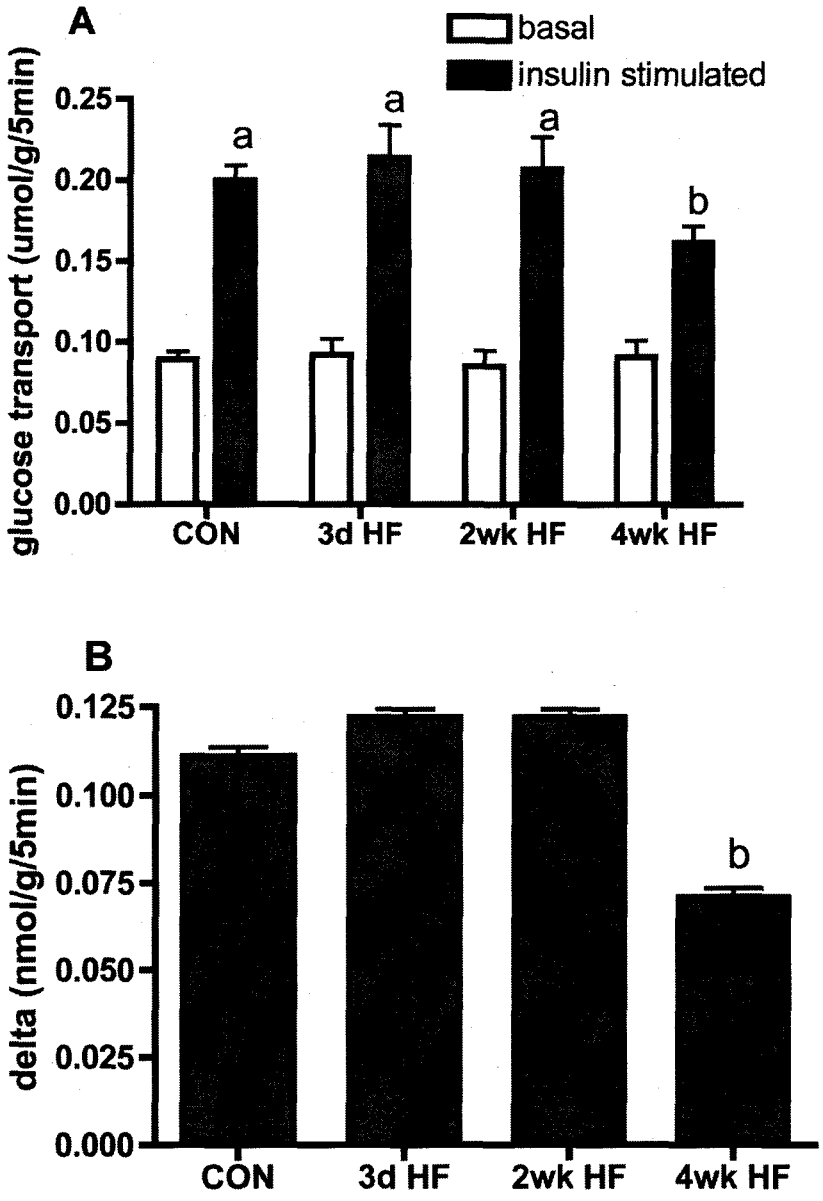

Figure 4.6: (A) Basal and insulin-stimulated glucose transport in soleus muscle. (B) The change in glucose transport rate from basal in response to insulin. Data were calculated by subtracting respective rates of basal uptake from the insulin response. Data are mean ± SE, n=12, bars not sharing a letter are significantly different, $p \leq 0.05$. Open bars = basal, Closed bars = insulin stimulated (10mU). CON = control diet, 3d HF = 3 day high fat diet, 2wk HF = 2 weeks high fat diet, 4wk HF = 4 weeks high fat diet.

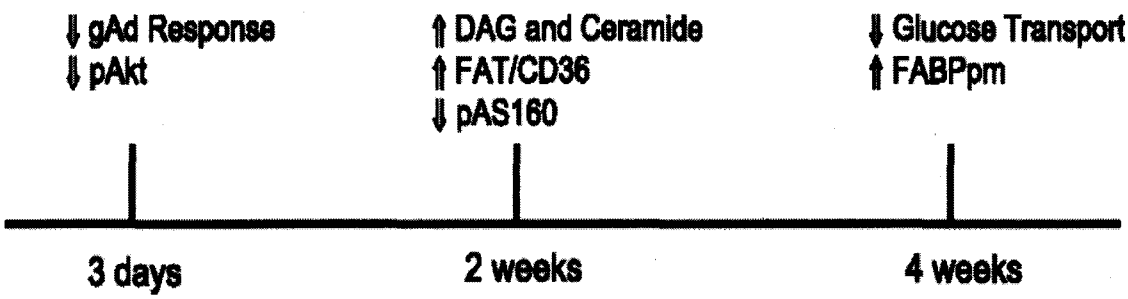

Figure 4.7: Timeline of events occurring in skeletal muscle of HF fed rats. All changes observed were maintained through subsequent time points.

Discussion

Evidence of Ad resistance in skeletal muscle has been shown in diabetic mice (203), obese humans (26, 36) and following HF feeding in rats (139). However, whether Ad resistance precedes and potentially contributes to intramuscular lipid accumulation and IR is unknown. Therefore, we conducted a time course HF feeding trial in rats to determine the onset of Ad resistance and to identify the ensuing changes in lipid metabolism and insulin signaling leading to IR. Here we have shown that Ad resistance occurs very rapidly, after only 3 days of HF (saturated) feeding and is sustained, as determined by a failure of gAd to acutely stimulate FA oxidation and phosphorylate ACC in skeletal muscle of all HF fed groups. However, this was not due to a decrease in AdipoR1 protein content. By 2 weeks of HF feeding, we observed increased FAT/CD36 at the PM of skeletal muscle, accompanied by increased total FA uptake into muscle and intramuscular ceramide and DAG accumulation. Furthermore, blunted insulin-stimulated phosphorylation of both Akt and AS160 was apparent by 2 weeks, whereas impaired maximally insulin-stimulated glucose transport did not occur until 4 weeks (Figure 4.7). Taken together, our results suggest that the early loss of gAd's stimulation of FA oxidation, coupled with a subsequent increase in FA transport, is associated with the accumulation of reactive DAG and ceramide lipid species and impaired insulin response.

High fat feeding induces adiponectin resistance prior to increases in intramuscular lipid content and impaired insulin response.

Similar to our previous study (139), we have shown that a 60% saturated fat diet can induce skeletal muscle Ad resistance, as evidenced by a failure of gAd to increase FA

oxidation or phosphorylate ACC above basal levels in intact, isolated soleus muscles. However, we have now identified that this resistance occurs extremely rapidly, preceding intramuscular lipid accumulation and impairment of maximal-insulin stimulated glucose transport. Adiponectin is known to increase FA oxidation in skeletal muscle by the inactivation of ACC, and reducing inhibition of CPT1 by malonyl CoA, leading to increased FA uptake into the mitochondria (202, 226). Whether this requires the activation of AMPK is controversial (202). As with our previous study (139), we did not show an effect of 30 min gAd exposure on pAMPK in soleus muscle of any group, but did observe a 30% increase in gAd-stimulated pACC in CON animals. This suggests that the loss of gAd-stimulated FA oxidation in HF animals may be attributable to a lack of phosphorylation of ACC, independent of changes in AMPK phosphorylation.

The decreased gAd response in HF fed animals was not attributable to a decrease in receptor content. Several studies that have examined muscle AdipoR1 in response to HF feeding have only examined mRNA levels and have reported no change (14, 21) or an increase (10, 29) following 1 to 5 months of HF feeding. Moreover, AdipoR1 mRNA is reported to be decreased in genetically obese diabetic mice (203). Interestingly, a recent study by Weigert et. al. (216) reported increased AdipoR1 mRNA levels in monocytes from type 2 diabetic subjects, whereas AdipoR1 protein content was actually decreased, re-enforcing the concern that changes in protein do not always parallel changes in mRNA. In the current study we did not observe any changes in whole muscle AdipoR1 protein content. Therefore, a change in receptor protein content does not appear to be a likely cause of Ad resistance. However, we cannot rule out the possibility that AdipoR1 sensitivity, conformation, or association with the PM or other required molecules could

have been altered by the HF diet and contributed to the observed resistance. Furthermore, the signaling events between gAd binding to AdipoR1 and AMPK-ACC phosphorylation are not well established. Suppressor of cytokine signaling 3 (SOCS3) has been shown to inhibit leptin activation of AMPK in cultured human myotubes and contribute to leptin resistance observed in obese subjects (188). Since leptin and Ad stimulate FA oxidation through similar mechanisms, it is possible that SOCS3 may interfere with intracellular gAd signal transduction as well. Further research is required to determine the effects of SOCS3 on gAd signaling in skeletal muscle.

High fat feeding increases fatty acid transporter and intramuscular lipid content by 2 weeks.

Total palmitate uptake was increased above CON by 2 and 4 weeks of HF feeding, as was the rate of esterification into TAG, and total muscle DAG and ceramide contents. A likely explanation for the increased palmitate uptake is a parallel increase in FA transporters at the PM. Specifically, PM associated FAT/CD36 protein content was increased above CON in 2 and 4wk HF and FABPpm protein content was increased above CON in 4wk HF. Thus, by 2 weeks of HF feeding, membrane associated FAT/CD36 and palmitate uptake into the muscle was increased, which, together with a diminished stimulation of FA oxidation by Ad, likely contributed to the observed accumulation of DAG and ceramide.

Clearly, the temporal data in this study cannot prove that the early development of Ad resistance is causative in the accumulation of intramuscular lipids and IR. Indeed, we (86) and others (75, 204) have recently questioned the notion that impaired

mitochondrial FA oxidation is necessarily an initiating cause of lipid accumulation and the ensuing impairment in insulin-stimulated glucose transport. However, this certainly does not exclude FA oxidation as an important factor in these events. Thus, it may be possible that a loss or blunted response of FA oxidation in vivo, to such adipokines as Ad and leptin, coupled with an increased capacity to take up and store FA, results in a relatively rapid accumulation of muscle lipids. Indeed we have also noted a loss of leptin's ability to stimulate FA oxidation within 3 days of HF feeding (unpublished data).

High fat feeding impairs insulin signaling prior to impairing glucose transport.

Numerous studies have correlated an increase in intramuscular lipids with IR (205). Specifically, elevated TAG has been associated with IR, although, this is now recognized as a marker of elevated intramuscular lipids rather than a direct cause of IR. Here we have shown that accumulation of DAG and ceramide precede functional impairments in maximally insulin-stimulated glucose transport into muscle. Ceramide and DAG have both been shown to directly interfere with insulin signal transduction. Specifically, ceramide can impair phosphorylation of Akt, thereby preventing Akt's stimulatory effect on GLUT 4 translocation (34, 173). Blunted Akt phosphorylation has been observed in skeletal muscle of HF fed animals (144, 154, 166). In the current study, we see early impairments of insulin-stimulated Akt phosphorylation, coinciding with increased ceramide accumulation by 2 and 4 wks in HF-fed animals. Somewhat surprisingly, insulin-stimulated Akt phosphorylation was also blunted at 3d HF compared to CON although ceramide content was not yet elevated.

In comparison, impaired insulin-stimulated AS160 phosphorylation was not evident until 4wk of HF feeding. Nascimento et al (144) has reported blunted insulin stimulation of AS160 following 7 weeks of a HF diet. As AS160 is a downstream substrate of Akt, it is possible that impaired Akt activation does not translate into impaired AS160 activation until later on. To the best of our knowledge, it is unknown whether ceramide directly interferes with AS160 phosphorylation, independent of its effects on Akt.

While intramuscular lipid accumulation may partially explain the blunted phosphorylation of Akt, it is interesting that this reduction in insulin signaling occurs well before the reduction in glucose transport. To the best of our knowledge, ours is one of the first papers to show this timeline. Akt is a known intermediate necessary for GLUT 4 translocation (128). However, a recent study by Ng et al (147) proposed the idea of Akt spareness; that is, minimal activation of Akt is sufficient to elicit a maximum effect on glucose transport and AS160 phosphorylation. Ng et al showed that the dose response of insulin-stimulated 2-deoxyglucose uptake in 3T3-L1 adipocytes paralleled the dose-response activation of AS160 phosphorylation, but not that of Akt itself. Therefore, in the current study, although Akt signaling is impaired early on, it is possible that glucose transport can be maintained, and it is not until subsequent AS160 impairment that functional impairments in glucose transport are seen. Why AS160 phosphorylation is retained longer, and the physiological significance of early impairments of insulin stimulated Akt phosphorylation remain to be determined.

Unlike the rapid development of Ad resistance, maximal insulin-stimulated glucose transport was not impaired until 4 weeks of HF feeding. While a 4 week HF

feeding period is still relatively brief, we have clearly demonstrated that several significant impairments in lipid metabolism and insulin signaling occur much earlier (i.e. after 3 days), and likely contribute to the development of lipid induced IR. Although we cannot discount that impaired glucose transport may have developed prior to 4 weeks, our results confirm that following 2 weeks on a 60% high saturated diet, skeletal muscle is still fully responsive to a maximal dose of insulin. It is possible, however, that we may have observed decreased insulin sensitivity to a sub-maximal insulin dose prior to this.

Summary and Perspectives

The present study utilized a time course high saturated fat feeding model to determine the early metabolic events in skeletal muscle leading to IR. We propose that a rapid loss of gAd's stimulatory effect on FA oxidation leaves the muscle less able to adequately respond to the excess lipid it is exposed to during HF feeding. The actual cause of Ad resistance remains unknown, although a decrease in receptor number does not appear to be the cause. Regardless of the specific cause, it seems plausible that the rapid development of resistance to an insulin-sensitizing cytokine, such as Ad, may be a contributing factor to the ensuing development of IR. This inability to appropriately stimulate FA oxidation, coupled with increased FA transporters at the plasma membrane and increased rate of FA uptake, results in intramuscular lipid accumulation. Specifically, ceramide accumulation is likely involved in the observed impaired phosphorylation of Akt and AS160, eventually resulting in impaired glucose transport. It should be recognized that these findings were restricted to a high saturated fat diet, and may not represent the course of events with the feeding of a polyunsaturated fat diet.

Future studies should determine the specific cause of Ad resistance to serve as a potential therapeutic target for the treatment of insulin resistance.

Chapter 5: Skeletal muscle inflammation is not responsible for the rapid impairment in adiponectin response with high fat feeding in rats.

Under revisions:

Mullen, KL, Tishinsky, JM, Robinson, LE, and Dyck, DJ. Skeletal muscle inflammation is not responsible for the rapid impairment in adiponectin response with high fat feeding in rats. ***Am J Physiol Regul Integr Comp Physiol***

Introduction:

Adiponectin (Ad) is an insulin-sensitizing adipokine. In skeletal muscle, the binding of Ad's globular fragment (gAd) to its receptor, AdipoR1, triggers an intracellular signaling cascade, initiated by the association of APPL1 with AdipoR1 and subsequent phosphorylation of ACC, resulting in the stimulation of fatty acid oxidation (62, 130, 226). The potential roles of other upstream kinases, (LKB1 and AMPK) in this pathway are somewhat controversial and may depend on fiber type (202, 226). This gAd stimulated increase in skeletal muscle fatty acid oxidation may act to prevent increases in intramuscular lipid accumulation and thus maintain or improve skeletal muscle insulin sensitivity (62, 228). However, in cases of obesity, circulating Ad levels are decreased (5). Furthermore, we and others have shown evidence of Ad resistance in both obese animals and humans (26, 203) as demonstrated by a loss or blunting of the acute stimulatory effect of gAd on FA oxidation in skeletal muscle.

We have also shown that Ad resistance is inducible; that is, muscle from rats fed a diet high in either saturated (SAT) or polyunsaturated fat (PUFA) for 4 weeks becomes completely resistant to gAd's ability to stimulate FA oxidation (139). Of particular interest is our most recent finding that gAd resistance develops very rapidly, in as little as

3 days on a high saturated fat diet and prior to the derangement in muscle FA metabolism and onset of insulin resistance (138). This suggests that the development of Ad resistance may be an initiating event in lipid-induced skeletal muscle insulin resistance. Whether the same rapid induction occurs following a diet high in PUFA has not yet been examined. Furthermore, the mechanism underlying the cause of lipid-induced Ad resistance is unknown.

Obesity is characterized by a state of chronic, low-grade inflammation (89, 169, 211). Conditions that induce insulin resistance by increasing lipid exposure stimulate inflammatory signaling (16, 96); conversely, blocking key components of the inflammatory cascade can protect against lipid-induced insulin resistance (157, 179, 235). Specifically, toll like receptor 4 (TLR4), a component of the innate immune response, has been identified on skeletal muscle and is activated by saturated fatty acids, causing phosphorylation of IKKβ (157), resulting in nuclear factor κB (NFκB) translocation to the nucleus and the up-regulation of pro-inflammatory gene expression (65, 157, 179). Triggering TLR4 can also induce suppressor of cytokine signalling-3 (SOCS-3) expression in innate immune cells and brain tissue (7, 23, 120), but has yet to be demonstrated in skeletal muscle. There is strong evidence supporting the role of SOCS-3 in the development of skeletal muscle leptin resistance, as it interferes with leptin-stimulated phosphorylation of AMPK (188). Since leptin and Ad have similar mechanisms of action in muscle (134, 202), it is possible that SOCS-3 may also interfere with Ad signaling, although this is unknown. Recently, Yaspelkis et al (229) reported increased SOCS-3 protein in skeletal muscle of rats following 12 weeks of high fat feeding, corresponding with decreased IRS-1 tyrosine phosphorylation. However, the

role of SOCS-3 in the early adaptations to high-fat feeding is currently unknown. Furthermore, whether a short-term high-fat diet will induce changes in skeletal muscle inflammatory signaling, and whether this depends on the type of fatty acid ingested has not been examined.

Activation of the inflammatory signaling cascade can directly interfere with insulin signal transduction, as IKKβ phosphorylates IRS-1 at ser^{307} thereby preventing further insulin signal propagation (232). Aspirin, (acetylsalisilic acid, ASA) a common anti-inflammatory drug, has been shown to be a weak inhibitor of IKKβ (232). In higher doses (i.e. grams/day), ASA has been shown to improve insulin sensitivity, decrease circulating FA levels and decrease plasma glucose and insulin, effects that are at least partially attributable to its actions at IKKβ (106, 235). Despite its widespread usage, literature examining the role of ASA on skeletal muscle lipid metabolism and adipokine response is virtually non-existent.

The objectives of the current study were to determine whether: i) Ad resistance develops in skeletal muscle in only 3 days on a high PUFA diet, similar to a high SAT diet; ii) inflammatory markers are altered after 3 days of HF feeding and are dependent on the type of fat consumed; and iii) blocking inflammation with ASA supplementation can maintain Ad response. It is hypothesized that, i) a diet high in PUFA will not cause Ad resistance in 3 days, ii) a high fat diet will cause inflammatory changes in skeletal muscle in as little as 3 days and the changes will be more pronounced in SAT fed rats, ii) Ad sensitivity will be maintained in skeletal muscle of high fat fed animals by using an anti-inflammatory drug (ASA).

Methods:

Animals, Diets and Aspirin supplementation.

Upon arrival, female Sprague-Dawley rats (140-145 g, Charles River, Quebec, Canada) were assigned to individual cages in a controlled environment with a reverse 12 h light-dark cycle with *ad libitum* access to a low-fat control diet and water.

Following the 3-day acclimation period, half of the rats began a 7-day ASA supplementation period, with 100 mg ASA per kg body mass mixed into their control diet daily. Following this 7 day priming period, both ASA-supplemented and non-supplemented animals were further divided into one of 3 dietary groups for an additional 3 days, low fat control (12% kcal from fat, CON), high saturated fat (60% kcal from lard, SAT) or high polyunsaturated fat (60% kcal from safflower oil, PUFA) (Research Diets, New Brunswick, NJ). This 3 day SAT diet protocol has previously been shown to induce Ad resistance (138). ASA supplemented animals continued to receive 100 mg/kg ASA in their experimental diets. There was a total of 6 experimental groups: 1) CON, 2) CON + ASA, 3) PUFA, 4) PUFA + ASA, 5) SAT, 6) SAT + ASA. High-fat fed animals were pair-fed to CON rats (fed *ad libitum*) with respect to caloric intake on a daily basis and body mass was recorded 3 times per week.

After the 3 days of dietary treatment, animals were overnight fasted prior to experimental procedures. Ethical consent for all procedures used was obtained from the Animal Care Committee at the University of Guelph.

Muscle and Blood Sampling

Soleus Muscle

Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (6 mg/100 g body mass) and the soleus (Sol) muscle was carefully dissected into longitudinal strips from tendon to tendon using a 27-gauge needle. The two outside strips from each Sol were then incubated under basal or gAd stimulated (2.5 μg/ml; Peprotech, Rocky Hill, NJ) conditions for measurement of i) fatty acid metabolism or ii) key signaling proteins. The remaining piece of Sol from each leg was immediately frozen in liquid N_2, without incubation, to determine the chronic effects of the dietary/drug intervention on inflammatory signaling proteins.

Blood

Terminal blood collection was made at the completion of the treatment via cardiac puncture after first excising skeletal muscles for incubation. A glucometer reading (Bayer Elite XL, Toronto, ON) of whole blood glucose was also made. All blood samples were collected in heparinized tubes after an overnight fast, centrifuged at 9300 x g for 5 min at 4°C, and the plasma removed for analyses of ASA (salicylate enzyme assay, Cambridge Life Sciences, Cambridgeshire, UK), total Ad (mouse RIA kit, Linco, St Charles, MS), C reactive protein (mouse/rat - single plex, Millipore, Billerica, MA), insulin, leptin, TNFα, MCP-1 and IL-6 (rat serum adipokine panel, Millipore, Billerica, MA).

Fatty Acid Metabolism.

Soleus strips were equilibrated in 2 mL of pre-gassed (95% O_2-5% CO_2) Krebs

Henseleit buffer (KHB; 4% BSA, 30°C), with 0.5 mM palmitate and 5 mM glucose for 30 min. Muscles were incubated for an additional 60 min with the addition of 0.5 μCi/mL [1-^{14}C]palmitate (Amersham, ON, Canada) and in the absence or presence of gAd (2.5 μg/ml) to determine palmitate oxidation and incorporation into endogenous triacylglycerol (TAG) and diacylglycerol (DAG) lipid pools as outlined previously (52).

Briefly, after the incubations, muscles were blotted of excess liquid, trimmed of tendons, weighed and treated to separate the aqueous and lipophilic phases. One milliliter of the aqueous phase was quantified by liquid scintillation counting to determine the amount of ^{14}C-labelled oxidation intermediates resulting from isotopic fixation. Muscle lipids were re-dissolved in 100 μL of 2:1 chloroform-methanol, spotted on to an oven dried silica gel plate (Fisher Scientific Canada, Mississauga, ON, Canada) and placed into a sealed tank containing 60:40:3, heptane:isopropyl-ether: acetic acid for 50 min. Plates were dried, sprayed with dichlorofluorescein dye (0.2% w/vol in ethanol) and visualized under long-wave ultra-violet light. The individual lipid bands were scraped into vials for liquid scintillation counting. $^{14}CO_2$ accumulated in the buffer was released by transferring 1 mL of buffer into a sealed flask and acidifying with 1 mL of 1M sulfuric acid and captured in benzethonium hydroxide. The trapped $^{14}CO_2$ were counted using standard liquid scintillation counting techniques. Total palmitate uptake was calculated by summing the incorporation of labeled palmitate into lipid pools plus oxidation.

Adiponectin Stimulated Signaling Proteins.

Soleus strips were incubated in KHB containing 4% FA-free BSA, 5 mM glucose, and 0.5 mM palmitate for 30 min in the presence or absence of gAd (2.5 μg/mL) for the

determination of total APPL1 and total and phosphorylated LKB1, AMPK and ACC protein content. After incubation, the strips were immediately frozen and stored in liquid N_2 until subsequent Western Blot analyses.

Western Blot Analyses.

Muscle tissue (~ 35 mg Sol) was homogenized in ice-cold buffer (1:9 w/v dilution) suitable for whole cell protein extraction and preserving phosphorylation states of proteins. Homogenates were sonicated for 5 seconds to ensure the nuclear membrane was completely broken, centrifuged at 1500 x *g* for 15 min at 4°C and the supernatant was removed and protein content was determined using BSA as standards.

Fifty micrograms of whole cell lysate protein (or 100 μg for pIKKα/β and pIκBα) was solubilized in 4x Laemmli's buffer, boiled (95°C, 5 min), resolved by SDS-PAGE, and wet-transferred to polyvinylidene difluoride membranes [1–1.5 h, 100 V; total and Ser^{79} phosphorylated acetyl-CoA carboxylase: 8–15 h, 25–40 V, 4°C]. The membranes were blocked for 1 hour and then incubated overnight at 4°C with the specific primary antibodies for AdipoR1 (AbCAM, Cambridge, MA), APPL1, total and Ser^{428} phosphorylated LKB1, total and Thr^{172} phosphorylated AMPK, total and Ser^{79} phosphorylated ACC (pACC, Upstate, Billerica MA), TLR4, total and $Ser^{176/180}$ phosphorylated IKKα/β, total and Ser^{32} phosphorylated IκBα, total and Ser^{536} phosphorylated NFκB, total and $Thr^{183/185}$, $Thr^{221/223}$ phosphorylated Jnk (Millipore, Lake Placid, NY) and SOCS-3. All antibodies were purchased from Cell Signaling (Danvers, MA) unless otherwise stated. After incubation with appropriate secondary antibody for 1 hour, the immune complexes were detected using the enhanced chemiluminescence

method (Syngene Chemigenius2; PerkinElmer, Waltham, MA), and quantified with densitometry (Gene Tools software, PerkinElmer). Equal loading was confirmed by probing for α-tubulin (AbCAM, Cambridge, MA) and using nonspecific protein staining with Ponceau-S staining (Sigma Aldrich, Oakville, ON, Canada)

Calculations and Statistics

All data are reported as mean ± SE. Results were analyzed using a randomized block design 2-way ANOVA and a Student Newman Keuls's post hoc test was used to test significant differences revealed by the ANOVA. A one-tailed paired t-test was used to compare basal vs. gAd stimulation within a dietary treatment for FA oxidation and ACC phosphorylation as well as to compare plasma ASA levels of supplemented vs. non-supplemented animals. Significance was accepted at $p \leq 0.05$.

Results:

Body Mass and Blood Measurements

Body Mass: There were no significant differences in final body mass between any groups (range, 237-245g). All animals consumed an average of 68 kcal/day (range, 60-75 kcal), and this did not differ between groups.

Blood: The ASA supplemented animals had significantly elevated plasma ASA levels (2.05 mM ± 0.2, $p \leq 0.05$) compared to non-supplemented animals (non-detectable). There was no difference in ASA levels between groups. Adiponectin levels were significantly decreased in PUFA animals ($p \leq 0.05$) and tended to be lower in SAT animals ($p=0.06$) compared to CON animals (Table 5.1). ASA supplementation significantly increased plasma Ad levels in all dietary groups, such that it normalized low levels in the high fat fed animals and further increased Ad levels in CON fed animals. Fasting plasma glucose was not different between groups and there was no significant effect of diet on insulin concentration, but ASA tended to decrease fasting insulin in all 3 dietary groups (Table 5.1). ASA tended to decrease leptin concentration in all 3 dietary groups. Neither dietary intervention nor ASA supplementation significantly altered plasma CRP or MCP-1 levels (Table 5.1). Circulating TNFα and IL-6 were below detectable limits of <4.9 pg/ml and <12.2 pg/ml respectively, in all groups.

Skeletal muscle lipid metabolism

Fatty acid oxidation: Basal rates of fatty acid oxidation did not differ between any groups. Acute gAd exposure significantly increased FA oxidation in CON (+41%, $p \leq 0.05$) and PUFA (+35%, $p \leq 0.05$) fed animals, but failed to do so in SAT fed animals.

ASA supplementation resulted in a loss of the stimulatory effect of gAd on FA oxidation in all dietary groups (Figure 5.1).

Fatty acid esterification: Fatty acid esterification into TAG and DAG was not different between dietary treatments and was unaffected by ASA supplementation or acute gAd exposure (range, TAG: 92.8 ± 3.8 to 116.9 ± 7.8 nmol/g/hr; DAG: 13.3 ± 0.5 to 18.9 ± 1.4 nmol/g/h).

Total uptake: Total fatty acid uptake into skeletal muscle was unaffected by diet, ASA supplementation or acute gAd exposure (range: 142.8 ± 7.3 to 175.0 ± 11.8 nmol/g/hr).

Adiponectin stimulated signaling

Total proteins: There was no significant difference in total protein content of AdipoR1, APPL1, LKB1 or ACC between any treatment groups (Figure 5.2). However, ASA supplementation significantly decreased total AMPK protein in all diet groups (Figure 5.2).

Phosphorylated proteins: Adiponectin significantly increased the phosphorylation of ACC in CON and PUFA fed animals, but not in SAT fed animals (Figure 5.3). Adiponectin, diet and ASA had no significant effect on LKB1 or AMPK phosphorylation (Figure 5.3).

Skeletal muscle inflammatory signaling (non-stimulated)

Total protein: There was no significant effect of diet or ASA treatment on total protein content of TLR4, IKKα/β, IκBα, NFκB, SOCS-3 and JNK (Figure 5.4).

Phosphorylated proteins: Three days of high fat feeding (SAT and PUFA) and 10 days of ASA supplementation did not significantly alter phosphorylation of any measured inflammatory signaling proteins (Figure 5.5).

Table 5.1: Body Mass and Plasma Measurements

	CON	CON + ASA	PUFA	PUFA + ASA	SAT	SAT + ASA
Body Mass (g)	239 ± 5	243 ± 8	241 ± 3	237 ± 8	237 ± 5	245 ± 9
Adiponectin (μg/ml)	4.1 ± 0.7	6.2 ± 0.6 ^	2.5 ± 0.3 *	4.2 ± 0.4 ^	2.8 ± 0.2* p=0.06	4.6 ± 0.5 ^
Glucose (mmol)	8.1 ± 0.8	7.6 ± 0.3	8.0 ± 0.5	8.1 ± 0.4	8.7 ± 0.6	8.0 ± 0.5
Insulin (pg/ml)	1557 ± 191	1268 ± 329 #	1124 ± 114	935 ± 150 #	1624 ± 257	1153 ± 194 #
Leptin (pg/ml)	2868 ± 475	2427 ± 450 ^	2173 ± 410	1297 ± 105 ^	2863 ± 325	1507 ± 281 ^
MCP-1 (pg/ml)	81 ± 21	83 ± 41	103 ± 15	101 ± 25	80 ± 10	57 ± 15
CRP (μg/ml)	543 ± 35	484 ± 35	480 ± 36	497 ± 36	415 ± 31	501 ± 28

Data are mean ± SE, n = 7-12

^ = significantly different from non ASA-supplemented $p \leq 0.05$

= significantly different from non ASA-supplemented $p \leq 0.1$

* = significantly different from CON diet $p \leq 0.05$

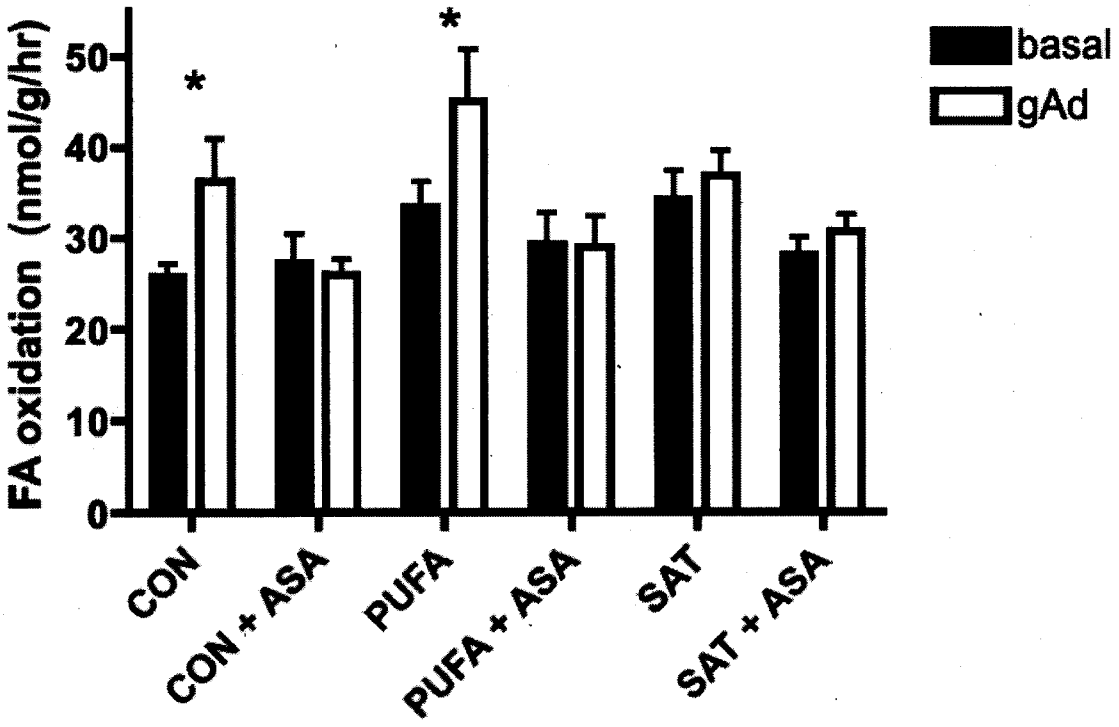

Figure 5.1: The effect of acute gAd exposure on soleus muscle fatty acid oxidation. Data are mean ± SE, n=12, *, significantly different from basal, $p \leq 0.05$, Closed bars = basal, Open bars = gAd stimulated (60 min, 2.5μg/ml). CON = control diet, PUFA = high polyunsaturated fat diet, SAT = high saturated fat diet, ASA = aspirin

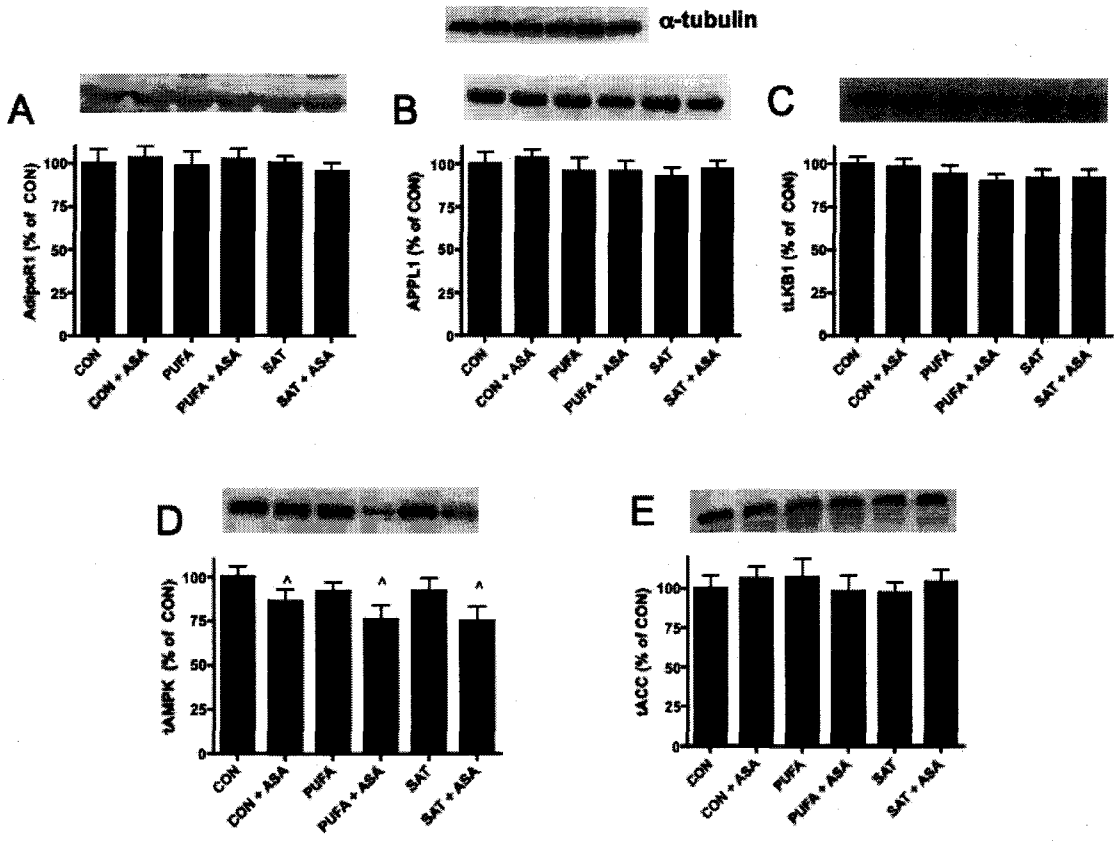

Figure 5.2: The effect of high fat feeding and ASA supplementation on total protein content of Ad signaling intermediates in soleus muscle. A) Total AdipoR1, B) Total APPL1 C) Total LKB1, D) Total AMPK and E) Total ACC under non-stimulated conditions for each treatment group (CON, CON + ASA, PUFA, PUFA + ASA, SAT, SAT + ASA). Alpha tubulin was used as a loading control. ^, significantly different from non-ASA supplemented. CON = control diet, PUFA = high polyunsaturated fat diet, SAT = high saturated fat diet, ASA = aspirin

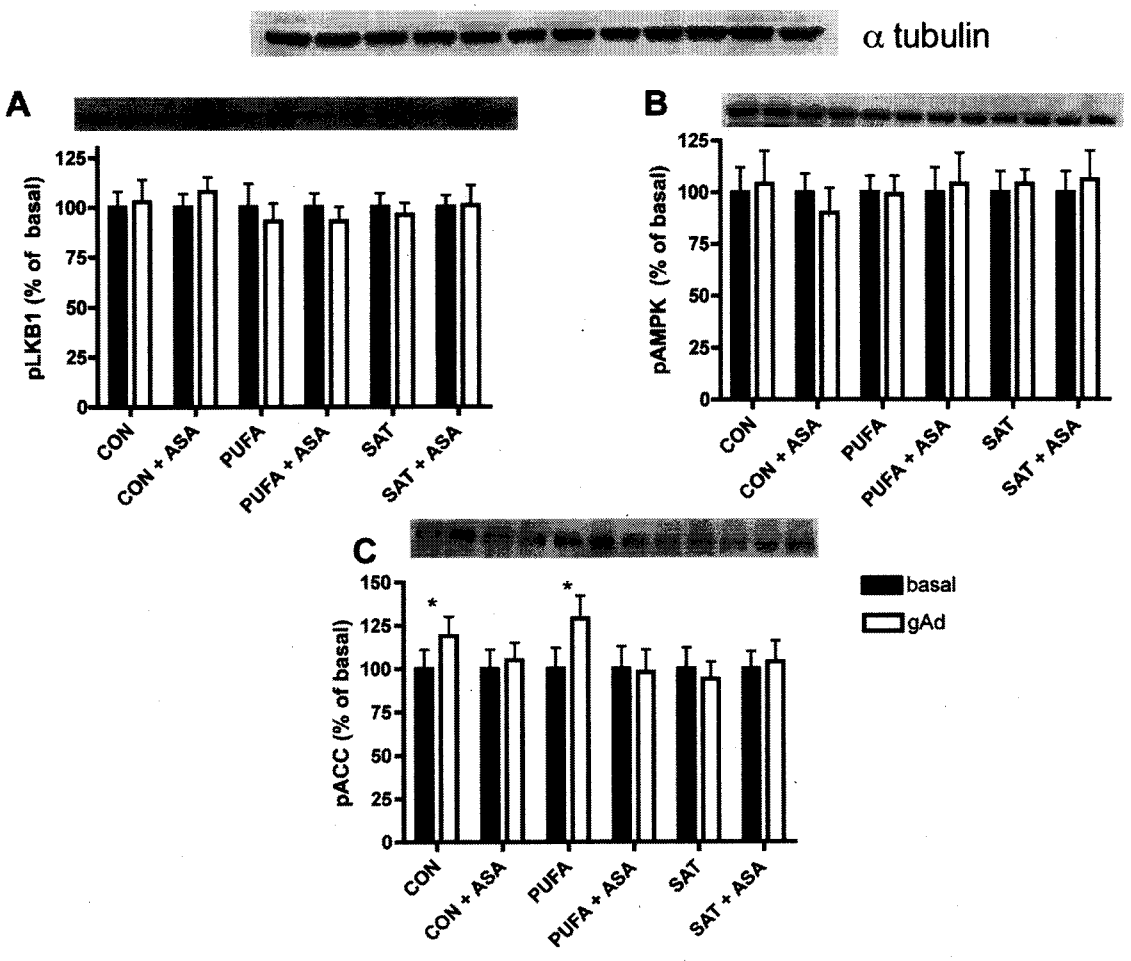

Figure 5.3: The effect of high fat feeding and ASA supplementation on phosphorylated Ad signaling intermediates in soleus muscle. A) Ser^{428} LKB1, B) Thr^{172} AMPK and C) Ser^{79} ACC under basal and gAd stimulated conditions for each treatment group (CON, CON + ASA, PUFA, PUFA + ASA, SAT, SAT + ASA). Alpha tubulin was used as a loading control. Data are mean ± SE, n=12, *, significantly different from basal, $p \leq 0.05$, Closed bars = basal, Open bars = gAd stimulated (30 min, 2.5μg/ml). CON = control diet, PUFA = high polyunsaturated fat diet, SAT = high saturated fat diet, ASA = aspirin

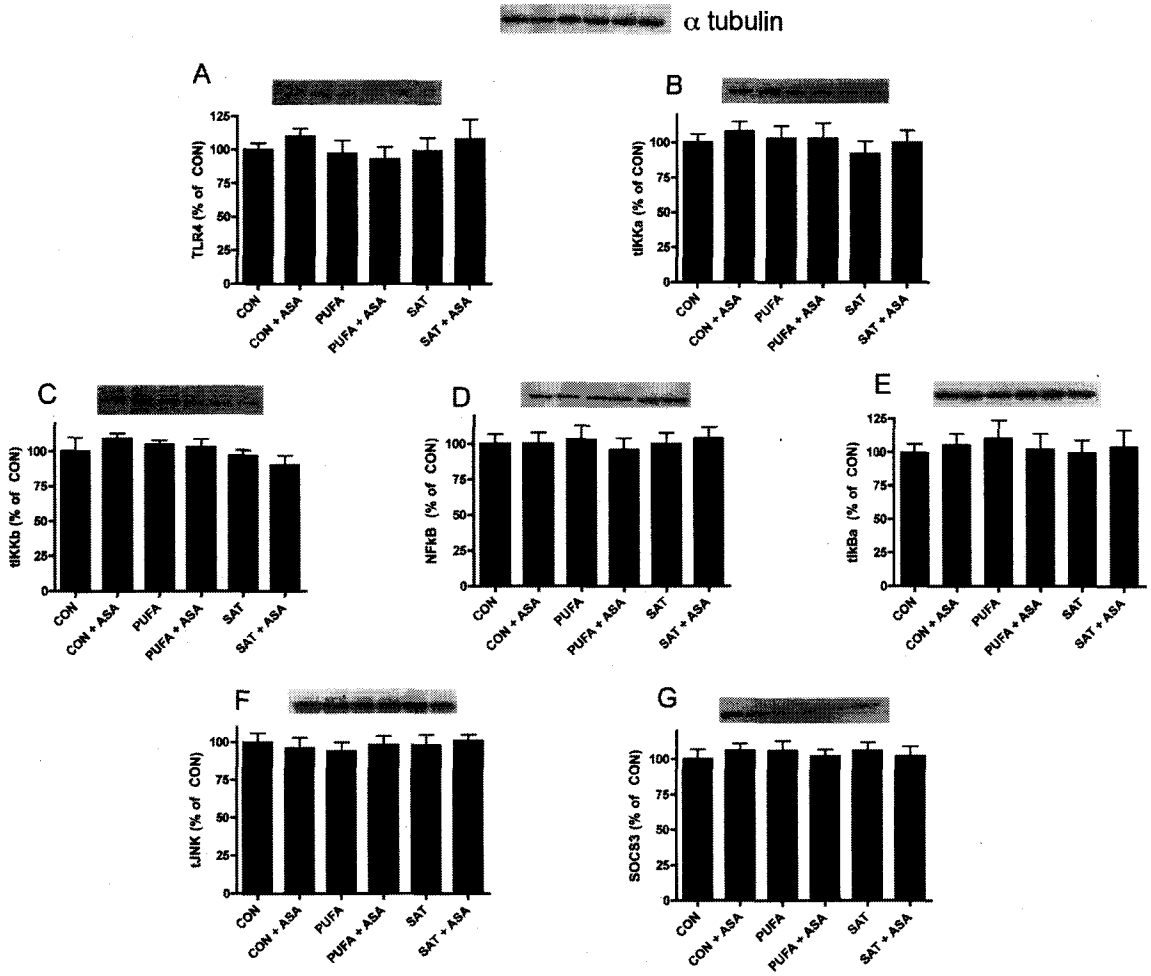

Figure 5.4: The effect of high fat feeding and ASA supplementation on total protein content of inflammatory signaling intermediates in soleus muscle. A) Total TLR4, B) Total IKKα, C) Total IKKβ, D) Total NFκB, E) Total IκBα, F) Total Jnk and G) Total SOCS-3 under non-stimulated conditions for each treatment group (CON, CON + ASA, PUFA, PUFA + ASA, SAT, SAT + ASA). Alpha tubulin was used as a loading control. n=12 CON = control diet, PUFA = high polyunsaturated fat diet, SAT = high saturated fat diet, ASA = aspirin

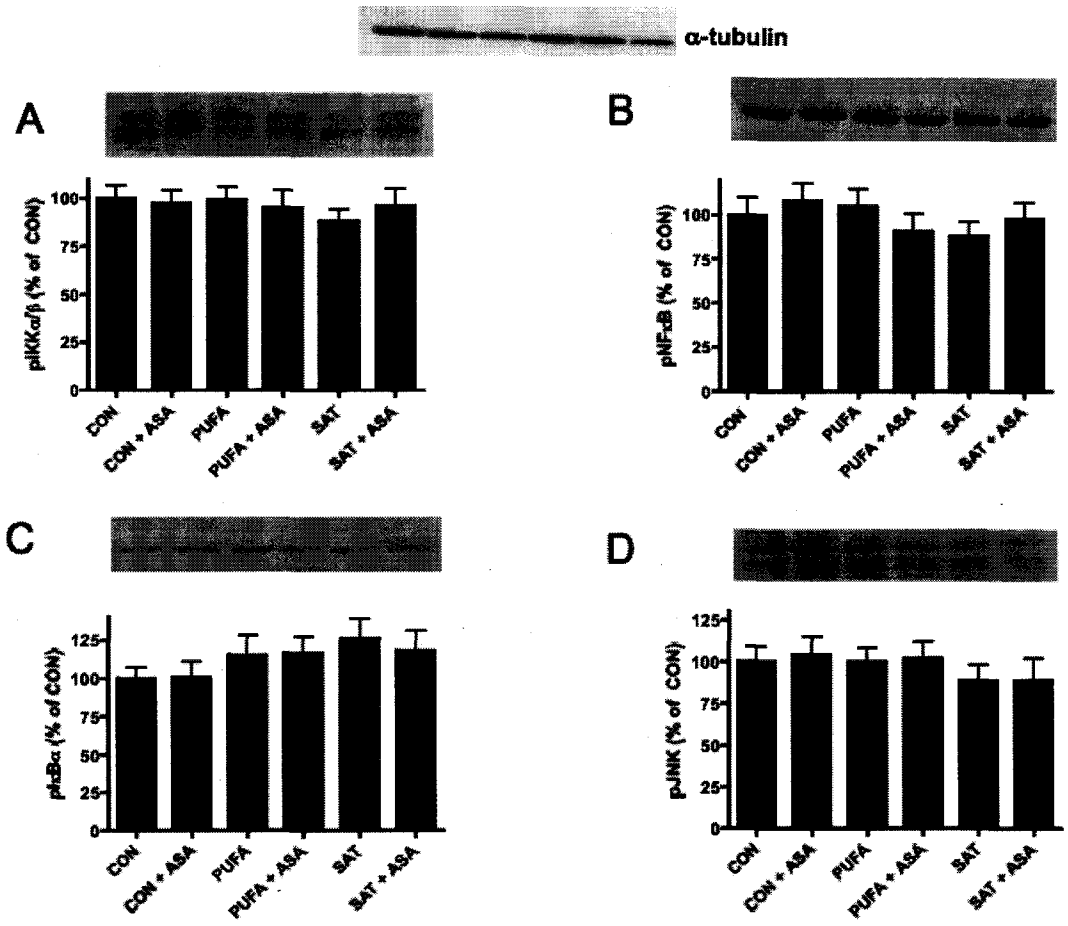

Figure 5.5: The effect of high fat feeding and ASA supplementation on phosphorylated inflammatory signaling intermediates in soleus muscle. A) $Ser^{176/180}$ IKKα/β, B) Ser^{536} NFκB C) Ser^{32} IκBα, and D) $Thr^{183/185}$, $Thr^{221/223}$ Jnk under non-stimulated conditions for each treatment group (CON, CON + ASA, PUFA, PUFA + ASA, SAT, SAT + ASA). Alpha tubulin was used as a loading control. n=12 CON = control diet, PUFA = high polyunsaturated fat diet, SAT = high saturated fat diet, ASA = aspirin

Discussion:

In the current study we show that animals fed a high PUFA diet remained Ad responsive as evidenced by increased skeletal muscle ACC phosphorylation and FA oxidation in the presence of gAd; whereas SAT fed rats became resistant and did not respond to gAd. However, regardless of the type of dietary FA, 3 days of high fat feeding did not alter muscle or blood markers of inflammation. Surprisingly, ASA supplementation decreased total AMPK protein and prevented gAd stimulated increases in FA oxidation. Neither APPL1 nor LKB1 were affected by diet or ASA, suggesting that absolute changes in APPL1 and LKB1 content/phosphorylation are not required for altered Ad response.

Adiponectin response differs depending on the type of fatty acid ingested.

Saturated and polyunsaturated fatty acids are known to differentially affect insulin sensitivity and inflammation (115, 131, 135, 220). To the best of our knowledge, we are the first to study i) the effects of PUFA vs. SAT feeding on adiponectin response in skeletal muscle, and ii) the very early (i.e. 3 day) adaptation of skeletal muscle to high fat diets of differing FA composition. In support of our hypothesis, muscle from PUFA fed animals remained Ad sensitive, as determined by the ability of gAd to acutely increase FA oxidation and pACC, while SAT fed animals became Ad resistant. In our earlier study (139), we showed that although 4 weeks of PUFA feeding did induce Ad resistance, these animals remained insulin sensitive, whereas SAT fed animals showed impaired insulin stimulated glucose transport in skeletal muscle following 4 weeks of their high fat diet. Thus, if Ad sensitivity can be maintained longer (as is the case when

PUFA are the main source of the diet) then the development of insulin resistance appears to be delayed. Together, these studies support the initiating role of Ad resistance in the development of insulin resistance.

Prior to commencing the current study, we hypothesized that a SAT diet would promote inflammation, which in turn would interfere with Ad signaling, while a PUFA diet would not cause inflammation and thus preserve Ad signaling. However, the results of the current study do not support this hypothesis. While the metabolic response to Ad was different between diets, there were no changes in muscle or blood markers of inflammation to explain this difference. Therefore, while it is clear that Ad response varies based on the type of FA ingested, it remains unclear why these differences exist.

Three days of high fat feeding is not sufficient to induce inflammation.

We hypothesized that a diet high in saturated fat would induce a state of elevated inflammation (i.e. increased muscle pIKKβ, pIκBα, pNFκB and plasma CRP, TNFα, MCP-1) since SAT FA have been reported to trigger inflammation is several tissues and cell types (157, 162, 208, 229). Radin et al (157) have shown that SAT FA stimulate TLR4 on the plasma membrane of skeletal muscle and trigger activation of the downstream IKK/NFκB signaling cascade, as indicated by an increased phosphorylation of IKK and IκBα. Yaspelkis et al (229) have recently shown that IκBα and IKKβ phosphorylation is significantly increased in skeletal muscle following 12 weeks of high SAT feeding. In the current study, we did not show increased total protein content or phosphorylation state of any measured inflammatory protein following 3 days of high fat feeding.

It is possible that the absolute amount or the phosphorylation state of individual proteins may not be the sole or best indicator of an altered inflammatory state. Cellular location or co-localization with other proteins may be equally important determinants of a protein's action. In support of this, Yaspelkis et al (229) reported increased SOCS-3 co-localization with IRS-1 following 12 weeks of high fat feeding, resulting in impaired insulin signal transduction. It is possible that SOCS-3 may co-localize with proteins in the Ad signaling cascade and prevent gAd signal propagation.

It is also quite plausible that 3 days of HF feeding is simply not long enough to cause a detectable increase in the inflammatory markers measured in this study. Previous HF feeding studies that report increased inflammatory signaling in skeletal muscle have ranged from 6 weeks to several months in duration (16, 106, 157, 176, 229, 235). Although 3 days may be a short time period to observe inflammatory changes, there are clear and sustained changes in Ad stimulated lipid metabolism after only 3 days of high fat feeding. Furthermore, stimulation of TLR4 in macrophages can increase SOCS-3 mRNA in as little as 1 hour (44), and 3 days of palmitate incubation can significantly up-regulate TLR4 protein content in human myotubes (162). Therefore, it is reasonable to presume that inflammatory changes might have occurred within 3 days, although the results of the current study do not support this.

It should be noted that in the current study, all animals were fasted for 12 hours prior to the measurement of inflammatory markers in plasma and skeletal muscle. Therefore it remains possible that an acute increase in inflammatory proteins occurred following ingestion of HF diet, but subsided during the subsequent 12 hour fast. Even if

such is the case, the impaired response of skeletal muscle to Ad stimulation still remained following an overnight fast, in the absence of any alteration in inflammatory markers.

Total AdipoR1 and APPL1 may not predict Ad response

AdipoR1 protein content was not affected by diet, in agreement with our previous work (138), or by ASA, and thus does not explain the differences in Ad response between dietary and ASA treatments. Also, as expected based on our previous findings (138, 139), changes in pACC mirrored changes in FA oxidation and thus Ad response. However contrary to our hypothesis, there was no difference in APPL1 or total and phosphorylated LKB1 protein content between animals who were Ad responsive or resistant, suggesting that changes in the content of these Ad signaling proteins are not necessarily required to alter Ad response.

Recently, Wang et al (214) identified APPL2, a novel protein involved in the regulation of APPL1 association with AdipoR1. In a basal state, APPL2 sequesters APPL1 in the cytoplasm, and also occupies the APPL1-AdipoR1 intracellular binding site. Upon binding of gAd to AdipoR1, APPL2 releases APPL1 and simultaneously dissociates from AdipoR1, thereby facilitating APPL1-AdipoR1 interaction. Thus, it is possible that while the total quantity of APPL1 and AdipoR1 did not change, a greater proportion of APPL1 remained sequestered in the cytoplasm and did not associate with the receptor upon gAd binding. Due to soleus muscle tissue limitations, we were unable to measure co-localization of APPL1 with AdipoR1. However, future studies should examine the potential role of APPL2 and APPL1 co-localization in the development of Ad resistance.

Aspirin decreases total AMPK protein and prevents Ad stimulated fatty acid oxidation

A surprising finding of the current study was that ASA supplementation decreased total AMPK protein and prevented gAd stimulation of FA oxidation in soleus muscle. This does not necessarily mean that ASA interferes specifically with Ad signal transduction. We speculated that ASA might decrease mitochondrial content (oxidative capacity) of the muscle, rendering it incapable of responding to a stimulus of oxidation above basal levels. Very limited evidence in patients with Reyes Syndrome suggests that aspirin may inhibit palmitate oxidation in skin fibroblasts from both control and disease patients (67). To this end, we measured two markers of mitochondrial content (citrate synthase, β-HAD protein content), but did not show any decrease in their content in soleus muscle from ASA supplemented animals (data not shown).

We are not the first group to show potentially undesirable metabolic responses to ASA supplementation. Recently, Xiao et al (221) reported that one week of ASA supplementation did not prevent lipid-induced insulin resistance in overweight and obese non-diabetic men as assessed by the insulin sensitivity index. In fact, ASA supplementation decreased insulin sensitivity below that seen in non-supplemented men (221). Additional conflicting results have been reported in humans following aspirin and salicylate derivative supplementation. Evidence of improved (59), unchanged (57, 109, 160) and decreased (7, 22, 66, 146) insulin sensitivity have all been reported following salicylate supplementation at doses (1-7g/day in adult humans) and time periods (3 days to 4 weeks) similar to the current study. In the current study, plasma ASA levels in supplemented animals averaged ~2 mM, consistent with previously reported levels in humans and animals (range 1-5mM) and well below toxic levels (232, 235).

Summary and Perspectives:

Taken together, these results demonstrate that Ad resistance develops rapidly in skeletal muscle in response to high fat feeding, but is dependent on the type of dietary fatty acid. Saturated fatty acids induce Ad resistance while PUFAs maintain Ad response, at least in the short term. Adiponectin's stimulation of FA oxidation is not dependant on changes in total content or phosphorylation of other Ad signaling intermediates (AdipoR1, APPL1). In comparison, 3 days of high fat feeding, whether PUFA or SAT is insufficient to cause chronic changes in inflammatory proteins in the muscle or plasma. Therefore, we must conclude from the current findings that skeletal muscle inflammation is not responsible for the rapid diet-induced Ad resistance. Furthermore, the current results provide support for other recent findings demonstrating negative metabolic consequences to ASA administration.

Chapter 6: General Discussion

Obesity and T2D are recognized as chronic, low-grade inflammatory diseases and are characterized by impaired lipid metabolism (87, 103). Lipotoxicity in skeletal muscle results from an imbalance between FA uptake and oxidation leading to intramuscular lipid accumulation as DAG and ceramide, which can interfere with insulin signal transduction and glucose uptake (94). Furthermore, conditions of lipid surplus have been shown to trigger tissue specific inflammation and promote a systemic pro-inflammatory state (16, 157, 176). Adiponectin is an insulin-sensitizing adipokine, known to stimulate skeletal muscle FA oxidation and protect against lipid-induced IR (12, 62, 228). However, in established cases of obesity, resistance to Ad has been shown in peripheral tissues (26, 203).

Prior to this thesis it was unknown 1) whether Ad resistance is inducible, 2) whether Ad resistance contributes to or results from lipid-induced IR and 3) what causes Ad resistance. Therefore, the primary objectives of the current thesis were 1) to determine if Ad resistance develops in rodent skeletal muscle following the consumption of high fat diets of varying FA composition 2) to determine the time line of changes in skeletal muscle Ad response, lipid metabolism and insulin response following high fat feeding, and 3) to determine if inflammation triggers the onset and development of Ad resistance.

High fat diets induce Ad resistance.

The first objective of this thesis was to determine if high fat feeding, a known contributor to the development of obesity and insulin resistance, would induce Ad

resistance. As PUFA and saturated FA have been suggested to differently affect insulin response (113), our secondary objective was to determine if the type of FA in the diet influenced the development of both Ad resistance and IR. Results of the first study clearly demonstrate that Ad resistance is inducible by both PUFA and SAT diets, but this coincided with impaired maximally insulin-stimulated glucose transport in SAT fed animals only. That is, Ad resistance and IR can be divorced. It is important to clarify that this observation was made at a single time point (ie: after 4 weeks) and as such, it could not be concluded whether Ad resistance preceded IR, resulted from IR or was unrelated to the development of IR. The finding that a PUFA diet induced Ad resistance without yet causing IR strongly suggested that Ad resistance might be an initiating event contributing to an impaired insulin response. However, this conclusion could not definitively be drawn without an appropriate time course study. We chose to focus solely on the time course development of SAT induced IR since we knew that both Ad resistance and IR would develop in a 4 week time period. It remains to be determined if a longer period of PUFA feeding would result in IR, but we suspected this to be true.

Ad resistance is rapidly induced by SAT feeding and precedes the development of IR.

The use of a time course study design allowed us to determine that Ad resistance is very rapidly induced, evident after only 3 days of SAT feeding. Importantly, this resistance preceded an increased FA transporter content at the plasma membrane, increased DAG and ceramide accumulation and impaired maximally insulin-stimulated glucose transport. Furthermore, additional pilot experiments show that leptin resistance is also inducible following very short term SAT feeding (Appendix A). It seems logical

to presume that resistance to insulin-sensitizing adipokines would be a detrimental adaptation of skeletal muscle to high fat feeding. These results are interpreted to show that in cases of high fat feeding the development of adipokine resistance leaves the muscle with a diminished capacity to appropriately partition FA towards oxidation and away from storage. Accordingly, as FA transporter content at the plasma membrane increases promoting a greater influx of FA into the cell, these FA become esterified. Accumulation of DAG and ceramide in turn can interfere with appropriate insulin signal transduction, leading to impaired insulin-stimulated glucose transport.

Interestingly, the earliest signs of impaired insulin signaling coincided with the onset of Ad resistance, well before an impairment in maximally insulin stimulated glucose transport. That is, after only 3 days, insulin stimulated Akt phosphorylation was slightly blunted in SAT fed animals. It is important to note that this impairment occurred prior to increases in ceramide content, the lipid metabolite known to interfere with Akt phosphorylation, suggesting that gAd and its downstream signaling targets may stimulate insulin-signaling intermediates and facilitate glucose transport directly. A recent report by Deepa and Dong (47) shows that APPL1, the molecule required for gAd signaling through AdipoR1 also plays a role in Akt phosphorylation. Therefore, it is possible that if a SAT diet interferes with gAd signaling through APPL1, it might also prevent APPL1 facilitated phosphorylation of Akt. At the time of publication for the second study of this thesis, this evidence was unknown. However, these recent advances stimulate re-evaluation of this seemingly incongruent result from the time course work and supports that Ad responsiveness may have a greater metabolic significance than

simply preventing intramuscular lipid accumulation by stimulating FA oxidation i.e. a direct effect on insulin signaling.

Furthermore, all measures of glucose transport in the current studies were made under maximally insulin-stimulated conditions (i.e. response), and thus do not reflect insulin sensitivity. Had sub-maximal insulin concentrations been used, it is possible that impaired insulin sensitivity would have been evident earlier that 4 weeks and more closely matched the time frame in which impairments of insulin signaling intermediates were observed. It is also possible that 4 weeks of PUFA feeding, while not sufficient to impair maximally insulin stimulated glucose transport, may have decreased insulin sensitivity. Similarly, a single maximal gAd dose was used in all studies. Had a sub-maximal Ad dose been administered, (i.e.: had Ad sensitivity been assessed), we hypothesize that decreased Ad sensitivity would have been observed in the PUFA fed animals earlier than the 4 week time point. Due to tissue limitations of the soleus muscle, we were unable to assess multiple insulin or gAd doses, but acknowledge that this is an important question and warrants future investigation.

The underlying cause of Ad resistance remains to be determined.

After determining that Ad resistance is rapidly inducible and precedes the development of IR, we set to determine how a HF diet causes Ad resistance. Furthermore, we questioned whether a PUFA diet would have similar immediate results to those observed with SAT feeding. We hypothesized that dietary SAT FA would stimulate TLR4 and increase skeletal muscle inflammatory signaling, which would result in an up-regulation of SOCS-3 protein expression. In turn, SOCS3 would interfere with

Ad signal transduction, similar to reported evidence with leptin resistance (188). This hypothesis was prompted for several reasons. An inflammatory response, by its nature must be rapid and thus could explain the very rapid onset of Ad resistance. Also, dietary FA have been shown to activate TLR4, but PUFA and SAT FA are suggested to have differing inflammatory properties (115) perhaps explaining their opposite influence on IR. Furthermore, HF diets have been shown to up-regulate inflammatory profiles in multiple tissues (157, 176) and obesity and IR have been characterized as chronic low-grade inflammatory conditions (87). Therefore, we fed rats a PUFA or SAT diet for 3 days to induce Ad resistance and supplemented the animals with aspirin, a common anti-inflammatory drug and weak inhibitor of IKKβ previously shown to protect animals from lipid induced insulin resistance.

Contrary to our hypothesis, 3 days of PUFA or SAT feeding did not cause a sustained elevation in SOCS3 or any inflammatory proteins measured, nor did aspirin supplementation have any protective effect. However, PUFA feeding for 3 days protected against the rapid development of Ad resistance that was confirmed in SAT fed animals. This finding supports our general hypothesis that Ad resistance is an initiating and a potentially contributing event in the development of IR. That is when Ad resistance is delayed, as in the case of PUFA feeding it appears as though IR is delayed as well, whereas early development of Ad resistance with SAT feeding is matched with earlier evidence of impaired insulin stimulated glucose transport. Further studies would be required to definitively state if Ad resistance is necessary in the development of IR, but the results presented in the current thesis support this logic.

We are cautious to say that inflammation does not have a role in diet induced Ad resistance. As previously discussed, in Chapter 5, total and phosphorylated whole muscle protein content is not the sole or even necessarily the best index of an inflammatory state. It is still possible that existing SOCS3 protein or existing inflammatory proteins co-localized with intermediates in the Ad signaling cascade and interfered with gAd signal transduction, without a significant change in total amount of protein in the muscle. Furthermore, all proteins were measured in muscle from fasted rats, and can only provide a snapshot in time. Through pilot work examining the influence of chronic TNFα infusion on insulin response, it was discovered that the half-life of TNFα is relatively short and its concentration is drastically reduced within several of hours (Appendix B). It is possible some inflammatory proteins may have increased immediately following ingestion of the diet, but had returned to baseline values during the hours between the last 'meal' and terminal muscle sampling on the following morning. Future studies should closely examine the localization of inflammatory proteins, as well as thoroughly investigate the signaling events that may occur in the post-prandial state, a condition more representative of human metabolism.

Alternatively, it is possible that dietary FA themselves interfere with Ad signaling in skeletal muscle. Despite no change in AdipoR1 protein content following HF feeding, we cannot discount the potential that the diet reduced the affinity of AdipoR1 for gAd or altered AdipoR1 position/location in the membrane. The FA composition of membranes has been shown to mimic the type of FA consumed in the diet and could theoretically affect membrane associated receptors (98). We hypothesize that any change in AdipoR1 function, if one exists, would be attributable to circulating FA and not an accumulation of

FA as lipid metabolites within the cell (DAG, ceramide) since blunted Ad signaling is evident prior to accumulation of these lipids.

Future directions and considerations.

Experiments aimed at identifying the cause of Ad resistance should still be a priority. Once the trigger of Ad resistance is identified, it can be manipulated in order to conclusively determine if Ad resistance is necessary and/or sufficient to cause IR. We suspect that Ad resistance alone may not be sufficient to cause IR without compensatory changes in lipid status (i.e. lipid surplus and increased FA transporter content). Rather, Ad resistance likely acts as an accelerant, facilitating lipotoxic skeletal muscle lipid accumulation under conditions of lipid excess. If Ad response can be maintained, it should serve to prevent or at least delay the development of IR.

It would be interesting to evaluate the physiological consequences of Ad deficiency or Ad receptor deficiency in genetically altered animals, analogous to the leptin deficient (*ob/ob)* or leptin receptor deficient (*db/db)* mouse. These models are well documented to have an obese phenotype and rapidly show signs of hyperlipidemia, hyperglycemia and IR (39). It is surprising that comparable Ad deficient models are not as well studied. Generation of tissue specific AdipoR1 or AdipoR2 knock out animals, or neutralizing antibodies specific to gAd or full-length Ad could serve to identify which form of Ad and which target tissue is most important for Ad's whole body insulin-sensitizing abilities. Although the focus of this thesis was on the effects of gAd on skeletal muscle metabolism, as identified in chapter 1, gAd constitutes only 1% of total Ad and full-length multimers comprise the majority of circulating Ad. Future studies

should also consider the role of full length Ad in the liver to understand the entire scope of Ad's insulin-sensitizing effects. Furthermore, whether an impaired Ad response in one tissue can be compensated by Ad action in another tissue and preserve whole body insulin sensitivity should be determined.

The unexpected finding that aspirin supplementation for 10 days decreased total AMPK protein and prevented gAd stimulated FA oxidation, although not central to the objectives of the current thesis, still raises several worthy questions. First, it should be questioned whether aspirin specifically blocked gAd signaling, or rather prevented the ability of skeletal muscle to respond to a stimulus of FA oxidation. We measured total protein content of several markers of oxidative capacity in aspirin supplemented and non-supplemented animals but found no significant difference between groups. Future studies should assess AICAR stimulation of FA oxidation in aspirin supplemented and non-supplemented animals to assess if aspirin prevents general stimulation of FA oxidation. In addition, other inhibitors of IKKβ could be applied to skeletal muscle to determine if IKKβ inhibition specifically causes the decrease in total AMPK protein or if it was a generic effect of aspirin. The underlying mechanism for this effect of ASA supplementation is unknown.

Since aspirin is a crude anti-inflammatory agent, it is perhaps not surprising that some unforeseen adaptations occurred. Future studies should employ more specific inhibitors of inflammation in order to better understand the role of inflammation in FA induced Ad resistance and IR. TLR4 knock out mice are commercially available and have been used to study the role of inflammation in lipid induced IR. As discussed in Chapter 1, some controversy exists with respect to sex differences and method of lipid

overload (diet vs. infusion) in these animals (157, 176). Importantly, the evaluation of adipokine response and function has yet to be measured in this model. Furthermore, TIP (TIRAP inhibitor peptide) is an anti-inflammatory agent shown to specifically block TLR-4 activation (157). This synthetic agent could be used in cell culture or in vitro models with long-term lipid exposure to understand the inflammatory mechanisms (if any) behind lipid induced adiponectin resistance and IR.

All measurements in the current thesis were made in the oxidative soleus muscle because it is a highly suitable tissue to measure FA oxidation and had been used previously to demonstrate diet-induced leptin resistance (186). Furthermore, the soleus muscle is unique in that it allows for multiple strips to me made from a single muscle, thereby increasing control in all experiments. However, we acknowledge that a primarily oxidative muscle is not representative of mixed or glycolytic muscle fibres and may exhibit different metabolic properties. For example, as previously discussed in Chapters 1 and 3, gAd is consistently shown to phosphorylate AMPK in glycolytic but not always in oxidative muscle (202). In addition, dietary fatty acid induced inflammation has been shown to occur in glycolytic but not oxidative muscle (16). Thus, it is possible that a lack of AMPK phosphorylation or a lack of skeletal muscle inflammation in the studies of this thesis are due to our choice of oxidative muscle and may have presented differently in a mixed or oxidative muscle fibres. We contend that an impaired gAd response in oxidative muscle is a novel finding, but future work should consider fibre type differences in metabolic response to high fat feeding.

The use of isolated rodent skeletal muscle and extreme high fat feeding in the current studies provided an opportunity to tightly regulate FA intake and allowed for the

investigation of mechanisms responsible for Ad resistance. However, caution must be exercised before translating the findings into recommendations for humans. The diets used in the current studies were very high in fat (60% of total kcal) and consisted exclusively of one FA type (n-6 PUFA from safflower oil or SAT FA from lard). Future studies should examine the ability of a more physiologically relevant intervention, namely a mixed FA diet or a diet of lower total fat content (~40%), to cause changes in Ad response and skeletal muscle lipid metabolism. Furthermore, omega-3 FA were not included in any of the experimental diets in order to specifically examine the metabolic consequence of exposure to a single FA type. Partial substitution of a PUFA diet with omega-3 FA has been shown to modestly protect against high fat diet induced leptin resistance (186). Similar studies are currently being conducted in our laboratory to determine if partial replacement of SAT FA with 12% menhaden oil (omega-3 FA) can both prevent and restore Ad resistance. It is worthwhile to note that in the studies of the current thesis, all high fat fed animals were pair fed to match the caloric intake of control animals. Pair feeding, while useful to control for the confounding influence of increased body weight, artificially restricts the high fat fed animals and may not be representative of the normal physiological consequences associated with over-nutrition. Future studies may consider *ad libitum* access to all dietary treatments and re-evaluate the influence on Ad resistance progression.

In addition to dietary interventions, the ability of concurrent or supplemental exercise to prevent or reverse Ad resistance should be explored. Concurrent aerobic exercise has been shown to partially prevent diet induced leptin resistance (190), while supplemental exercise imposed after the development of leptin resistance can restore

skeletal muscle leptin response and insulin response (Ritchie, unpublished data). Interesting to note is that supplemental exercise after high fat feeding restored maximally insulin stimulated glucose transport in just one week, but leptin stimulated FA oxidation required two weeks of exercise to be restored. Contrary to our theory, this finding suggests that adipokine resistance may not be critical in the development of IR or restoration of insulin response. However, it is possible that the maximal insulin dose administered in this study was sufficient to stimulate glucose transport, and does not reflect insulin sensitivity. That is, it is possible that animals were still insulin insensitive for longer than one week. To date, no published data exist on the ability of exercise to prevent or restore dietary induced Ad resistance. Investigation into the ability of moderate aerobic exercise to restore Ad and insulin response in skeletal muscle after administering a HF diet is currently underway in our laboratory. Together, these new and intriguing findings will provide the necessary framework in order to design and recommend new strategies to both treat and prevent lipid induced IR.

Summary

In summary, the current thesis presents a framework to explain the role of gAd resistance in high fat diet induced IR. Dietary FA, in particular SAT FA, rapidly induce a state of skeletal muscle Ad resistance, leaving the muscle with a diminished capacity to stimulate FA oxidation. This resistance precedes and likely acts as an accelerant in the development of lipid induced IR. As the high fat diet persists, FA transporters relocate to the plasma membrane, resulting in increased FA uptake. Without a sufficient stimulation of FA oxidation, lipids accumulate as inert TAG but also as metabolically active DAG

and ceramide. These lipids then can directly interfere with insulin signaling, resulting in a lower rate of insulin stimulated glucose uptake by muscle. The cause of Ad resistance is currently unknown, but should remain the focus of future studies. The role of diet induced inflammatory changes should not yet be discounted.

We acknowledge that *in vivo,* many overlapping and redundant pathways act in accordance to regulate metabolism and may compensate for Ad resistance. However, early development of adipokine resistance (Ad and leptin) may accelerate the progression of lipid induce IR. Therefore, we propose adipokine resistance as a novel target for therapeutic strategies in the battle against diet induced IR. We hypothesize that dietary, lifestyle or pharmaceutical interventions that serve to maintain adipokine response should prevent or at least delay the onset of lipotoxicity and IR and are therefore worthy of continued research and attention.

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Appendix A

Pilot work: Leptin resistance following short term high fat feeding.

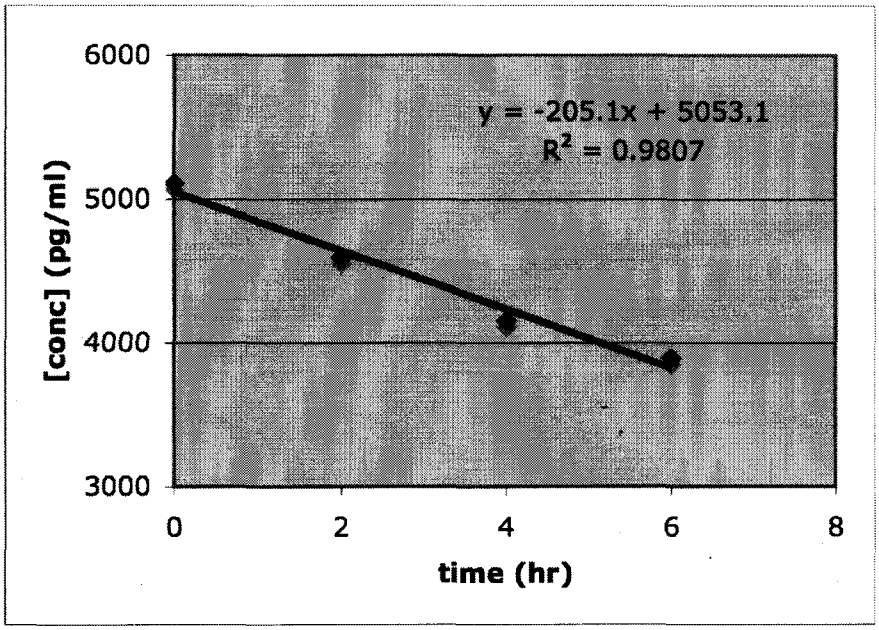
Basal and leptin-stimulated FA oxidation in soleus muscle from control (CON) and high saturated fat (HF) fed (3 days or 7 days) female sprague dawley rats. Evidence of leptin resistance presents after 3 days of HF feeding.

	CON basal	CON leptin	3d HF basal	3d HF leptin	7d HF basal	7d HF leptin
	57.64	71.87	59.18	96.03	30.51	45.15
	70.20	57.69	55.89	69.13	34.40	39.64
	25.41	81.67	55.23	36.30	36.95	39.99
	46.64	52.29	39.32	52.49	69.30	33.03
	49.52	71.87	61.64	48.32	55.41	40.94
	42.37	57.69	63.06	42.53	73.50	65.58
	40.95	81.67			34.20	54.80
	24.91	52.29			59.10	52.10
Mean nmol/g/hr	**44.71**	**65.88**	**55.72**	**57.47**	**49.17**	**46.40**
SE	5.4	4.4	3.5	8.9	6.1	3.7
% change from basal		**47%**		**3%**		**-6%**
p=		0.01		0.43		0.34

Appendix B

Pilot work: TNFα degradation

Vial prepared with TNFα at a concentration of 5000pg /ml. Vial incubated at 37°C for 6 hours, with samples being taken every 2 hours. TNFα concentration assessed by Cytelisa 5000 kit.



Time point	TNFα pg/ml
Pre	5110.36
2h	4597.03
4hr	4152.45
6hr	3891.20
Delta	**-23.86%**
TNF-a degrades by ~ 25% over 6 hr	

Appendix C

Western Blot Conditions

Protein	MW	gel	block	1'Ab	wash	2'Ab	wash	Detect
TLR4	110	10%	5% BSA	1:250 5% BSA 20 hrs	TBST	1:2000 rabbit	TBST	5x1 min
IKKα	85	10%	5% BSA	1:1000 5% BSA	TBST	1:2000 rabbit	TBST	5x1 min
IKKβ	87	10%	5% BSA	1:1000 5% BSA	TBST	1:2000 rabbit	TBST	5x1 min
pIKKα/β	85/87	10%	5% BSA	1:500 5% BSA (48hrs)	TBST	1:2000 rabbit	TBST	5x2 min
NFκB (p65)	65	10%	5% BSA	1:2000 5% BSA	TBST	1:2000 rabbit	TBST	5x1 min
pNFκB	65	10%	5% BSA	1:1000 5% BSA	TBST	1:2000 rabbit	TBST	5x1 min
IκBα	39	10%	5% NFM	1:500 5% NFM	TBST	1:2000 mouse	TBST	5x1 min
pIκBα	41	10%	5% BSA	1:500 5% BSA (48 hrs)	TBST	1:2000 rabbit	TBST	5x2 min
JNK	54/45	10%	7.5% BSA	1:500 7.5% BSA	TBST	1:3000 rabbit	TBST	5x1 min
pJNK	55/46	10%	7.5% BSA	1:500 7.5% BSA	TBST	1:1000 rabbit	TBST	3x2 min
SOCS3	26	10%	5% BSA	1:1000 5% BSA	TBST	1:2000 rabbit	TBST	5x1 min
AdipoR1	42	10%	2.5% NFM	1:1000 2.5% NFM	TBST	1:2000 goat	TBST	5x1 min
APPL1	82	10%	5% NFM	1:1000 5% NFM	TBST	1:2000 rabbit	TBST	1x2 min
LKB1	54	10%	5% BSA	1:1000 5% BSA	TBST	1:2000 rabbit	TBST	5x1 min
pLKB1	54	10%	5% BSA	1:1000 5% BSA	TBST	1:2000 rabbit	TBST	5x3 min
AMPK	64	10%	5% BSA	1:1000 5% BSA	TBST	1:2000 rabbit	TBST	5x30 sec
pAMPK	64	6%	5% BSA	1:1000 5% BSA	TBST	1:2000 rabbit 5% NFM PBST	PBST	5x30 sec
ACC	260	6%	2.5% BSA	1:1000 2.5 % BSA	TBST	1:2000 rabbit	TBST	5x1 min
pACC	260	6%	2.5% BSA	1:1000 2.5% BSA	TBST	1:2000 rabbit PBS no tween	PBST	5x1min