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The role of adiponectin and the adipocyte in energy metabolism and inflammation

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THE ROLE OF ADIPONECTIN AND THE ADIPOCYTE IN ENERGY METABOLISM AND INFLAMMATION

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With love and appreciation to my parents and family;

Mom and Dad

Julie and Gary, Kaylyn, Garrett, and Nathan

Debbie

You all have helped me walk through this journey!.

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LIST OF ABBREVIATIONS

ACC	acetyl CoA carboxylase
adipoR1	Adiponectin receptor 1
adipoR2	Adiponectin receptor 2
ADP	Adenosine di-phosphate
AICAR	5-Aminoimidazole-4-carboxamide ribonucleoside
AMP	Adenosine mono-phosphate
AMPK	AMP-activated protein kinase
AP-1	activator protein-1
ATP	Adenosine tri-posphate
C/EBP	CAAT/enhancer-binding protein
COX 2	Cyclooxygenase 2
CPT-1	carnitine palmitoyltransferase
FA	fatty acids
FFA	Free fatty acids
Foxo1	Forkhead transcription factor
g	Gram
gAdn	Globular adiponectin
GLUT4	glucose transporter protein 4

GSK	glycogen synthase kinase
h	Hour
HIV	human immunodeficiency virus
ICAM-1	endothelin-1 intracellular adhesion molecule-1
IGF	insulin-like growth factor
IL-12	Interleukin 12
IL-1β	Interleukin 1β
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
IRR	insulin receptor-related receptor
IRS	Insulin receptor substrate
IRS-1	insulin receptor substrate-1
IκB	nuclear factor kappa B inhibitory factor
kg	Kilogram
L	Liter
LPS	lipopolysaccaride
mg	Milligram
min	Minute
mL	Milliliter
MMP-9	matrix metalloproteinase
mRNA	Messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NFκB	nuclear factor kappa B

pAdn	porcine adiponectin
PGF1α	6-keto platelet growth factor 1α
PI3-kinase	phosphoinositide-3 kinase
pmol	Picomoles
PPAR	peroxisome proliferators-activated receptor
rel	Relish
SH2	Src-homology-2
TG	triglyceride
Thr-172	threonine-172
TNFR1	tumor necrosis factor α receptor 1
TNFR2	tumor necrosis factor α receptor 2
TNFα	tumor necrosis factor α
TZD	thiazolidinediones
VCAM-1	vascular cell adhesion molecule-1
WAT	white adipose tissue
µg	Micro-gram
µL	Micro liter

ABSTRACT

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Adipocytes play an important role in the regulation of whole body energy balance. They synthesize and secrete many hormones which communicate with the diverse physiological systems of the body. However, when adipose tissue physiology is in a state of imbalance the communication between adipocytes and the rest of the body is compromised and can lead to metabolic disease. The adipocyte derived hormone adiponectin is efficacious in reversing insulin resistance in rodent models of lipodystrophy and obesity. Therefore, a series of experiments were conducted to characterize the autocrine role of adiponectin in modulating fatty acid metabolism and inflammation in the pig. In the first study, we cloned and sequenced the porcine adiponectin open reading frame and evaluated the regulation of adiponectin, in vitro and in vivo. The porcine sequence shares approximately 88, 86, 85 and 83% homology with the dog, human, cow and mouse adiponectin, respectively, and 79-83% similarity with dog, human, cow and mouse proteins at the amino acid level, based on the translated porcine sequence and GenBank submissions for the other species. Analysis of serum from very lean vs. a substantially fatter line of pigs indicated that relative circulating

adiponectin concentrations are higher ($P < 0.01$) in lean pigs than in the fatter line, and that the difference is established relatively early in the growth curve. Incubating pig adipocytes for 6 hours with recombinant pig adiponectin also resulted in an approximate 30% reduction ($P < 0.05$) in lipogenesis compared with adipocytes under basal conditions and with those incubated in the presence of insulin.

Based on in vivo and in vitro data showing adiponectin is positively correlated with leanness and reduces lipogenesis in porcine adipocytes, experiment investigated adipocyte lipid metabolism. In a series of cell culture studies for experiment two, we further investigated the role of adiponectin in modulating lipid metabolism by measuring mRNA abundance of genes associated with fatty acid synthesis and oxidation in porcine adipocytes. Adiponectin transiently increased (2-6.5 fold, $P < 0.05$) the expression of acetyl Co-A carboxylase (ACC), AMP activated kinase (AMPK), acyl Co-A oxidase (ACO) and peroxisome proliferator activated receptor-α (PPARα). After the initial increase in mRNA abundance, there was either a return to initial levels of expression (PPARα), a significant reduction (AMPK), or a cyclic pattern of expression (ACC, ACO). The abundance of the uncoupling protein-3 (UCP3) transcript was not influenced by adiponectin until the final measure at 360 minutes, at which time it was increased 3.8-fold ($P < 0.05$). Collectively, these data indicate that adiponectin acts through autocrine/paracrine signaling pathways to coordinately regulate lipid metabolism in the adipocyte, and may thus be a determinant of adipocyte size and overall lipid accumulation.

Experiment three was designed to examine anti-inflammatory properties of adiponectin contributing to its insulin sensitizing effects through regulation of the

adiponectin receptors, adipoR1 and adipoR2. Cells were incubated ± TNFα or IL-6 (30 ng/mL), with or without pretreatment of 10 μM AG490, in hyper-glycemic or normo-glycemic conditions for 6 h. There was no effect of cytokines at 5.5 mM glucose for R1 expression. However, there was a trend for a down-regulation of R2 by AG490-TNFα ($P < 0.07$). At 25 mM glucose, there was an increase in R1 by AG490-TNFα treatment ($P = 0.06$). R2 was marginally reduced by IL-6, but there was a reduction ($P < .01$) with AG490 plus IL-6. High glucose caused a reduction of both receptors, whereas TNFα increased both in the high glucose media. R1 ($P < 0.1$) and R2 ($P < 0.05$) were further elevated by AG490 plus TNFα in high glucose. Interestingly, IL-6 and AG490 plus IL-6 ($P < 0.05$) reduced R1 and R2 expression, but only in the high glucose media. Collectively, these data indicate that the effects of TNFα and IL-6 on adiponectin receptor expression are influenced by glucose concentration, and that the JAK-STAT pathway may be a determinant of adiponectin receptor expression.

In the final experiment, the effects of hyperglycemia on insulin sensitivity in pig adipocytes, and 3T3-L1 adipocytes were examined. In pig adipocytes, concentrations of 25 and 40 mM glucose inhibited insulin stimulated glucose uptake ($P < 0.05$), that could not be reversed by addition of adiponectin to the culture media. To examine if hyperglycemic conditions were associated with impaired insulin signaling pig adipocytes were cultured in euglycemic and hyperglycemic conditions and insulin receptor and Akt phosphorylation were measured by semiquantitative Western blots. However, insulin resistance in primary pig adipocytes could not be attributed to changes in protein phosphosphorlation of the insulin receptor or its down-stream target Akt ($P > 0.05$). To examine glycemic regulation of insulin-induced glucose uptake for longer duration the

3T3-L1 mouse derived adipocytes were used. Similar to the effects seen in acute porcine adipocyte cultures hyperglycemia inhibited insulin stimulated 2-deoxyglucose uptake in 3T3-L1 adipocytes ($P < 0.05$), and 24 hour treatment with adiponectin could not reverse the insulin resistance. Again, difference in phosphorylation of the insulin receptor and Akt could not be detected in hyperglycemic culture cells compared with control cells. Interestingly, although adiponectin did not appear to impact insulin signaling pathways in under hyperglycemic conditions it did have an effect on inflammatory pathways in the adipocyte. Adiponectin reduced the production of intracellular ROS in 3T3-L1 adipocytes cultured in hyperglycemic conditions ($P < 0.05$). Taken together these data implicate adiponectin suppressing intracellular inflammation during hyperglycemia, but was not able to reverse hyperglycemia induced insulin resistance effect on glucose uptake.

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CHAPTER 1. INTRODUCTION

1.1 Introduction

Animal growth, development and maintenance require complex integration of signals from peripheral tissues. Thus, communication among physiologic systems and tissues is critical to homeostasis. Recent advances in adipocyte physiology have changed the face of white adipose tissue (WAT) by defining the adipocyte as an active endocrine cell, rather than a simple passive reservoir of surplus energy. The adipocyte is now recognized to have pleiotropic functions involved in energy metabolism, inflammation and metabolic disease states. The adipocyte synthesizes and secretes many molecules that regulate processes involved in fat storage, feed intake, reproduction, immune function, and energy balance and metabolism.

Understanding the role of the adipocyte in integrated systems physiology is of paramount importance due to its recent implications in inflammatory states and immune modulation (Fantuzzi, 2005; Xu et al., 2003b). First, the development of obesity is associated with chronic states of positive energy balance which has also been linked to chronic inflammation (Chandra, 1996). Interestingly, ablation of adipose tissue in animal models of lipodystrophy is also associated with chronic immune stimulation (Gavrilova et al., 2000). There is evidence that adipocytes are required for the maintenance of the

immune system, and both starvation (Chandra, 1996; Nova et al., 2002) and excessive adipose accumulation lead to impaired immune function and increased disease susceptibility (Klasing, 1998; Marti et al., 2001). These and other data clearly indicate a link between WAT, regulation of energy balance, and the immune system.

1.2 Adipocytes and energy balance

Understanding the integral role of the adipocyte in regulating energy storage and expenditure is a key component of gaining a comprehensive view of energy balance. Historically, humans consumed food for survival and maintained energy balance because their energy input was similar to their energy expenditure. However, as times have advanced food has become plentiful and lifestyles have become more sedentary. These changes favor a positive energy balance, and therefore an increased prevalence of obesity.

Traditionally adipose tissue has been viewed as only a passive energy storage depot. However, there is substantial evidence indicating that body fat content is controlled, at least partially, by the metabolism of adipose tissue itself. First, human genome scans for obesity-related candidate genes identified several genes which play critical roles in adipocyte biology (Arner, 2000). Second, through the use of transgenic mouse models, it is clear that modifications to adipose tissue result in a high incidence of obesity prone or resistant animals, depending upon the genetic modification (Kopecky et al., 2001). For example, adipose-specific glucose transporter protein 4 (GLUT4) knockout mice develop insulin resistance in skeletal muscle and liver (Abel et al., 2001), and adipose-specific insulin receptor knockout mice (FIRKO) have low fat mass, loss of

relationship between plasma leptin and body weight, and are protected against age-related and hypothalamic lesion-induced obesity (Bluher et al., 2002). The metabolic changes induced in adipose tissue in these transgenic models were pivotal to the altered accretion of body fat and the development of metabolic disorders. It has been clearly demonstrated that insulin resistance is a commonality of obesity and the pathogenesis of metabolic syndrome-associated disorders (Rossmeisl et al., 2003; Xu et al., 2003). In models of obesity, it is thought the heightened deposition of lipid in peripheral tissues results in insulin resistance and this increased lipid accumulation in muscle and liver is due to an increased release of fatty acids (FA) from hypertrophic fat cells (Perseghin et al., 2003). Moreover, the hypertrophied adipocyte becomes resistant to insulin, resulting in lower plasma triglyceride (TG) clearance and increased FA release from WAT. In contrast, others have reported that insulin resistance is a function of a deficiency of WAT (Gavrilova et al., 2000; Moitra et al., 1998). In these conditions, circulating FA are elevated due to the lack of capacity of the adipocyte to buffer circulating FA concentrations. As a result, other organs become reservoirs for lipid and develop insulin resistance (Kim et al., 2000). In addition to fatty acids, other nutrients have also been implicated in the development of cellular insulin resistance (Patti, 1999). The infusion of amino acids or prolonged hyperglycemia are both implicated in sustained insulin resistance.

In addition to nutrient regulation of energy balance, adipocyotokines have a pronounced endocrine impact on peripheral tissues and a paracrine/autocrine regulation of adipocyte cellular energy balance. Some adipocytokines of particular interest are adiponectin, leptin, tumor necrosis factor α (TNFα), and interleukin-6 (IL-6), all of which

have been implicated in insulin sensitivity, cellular energy metabolism and whole body energy balance (Fantuzzi, 2005). Thus, adipocytes and adipose tissue play a distinct and critical role in energy balance.

1.3 Adipocytes and AMPK

One of the key enzymes involved in cellular energy balance is AMP-activated protein kinase (AMPK). This kinase is a conserved sensor of cellular energy status, and is implicated as a central component in regulation of cellular energy metabolism (Kahn et al., 2005). It is a downstream component of a kinase cascade which is activated by a drop in energy status of the cell. If ATP is consumed faster than its rate of synthesis, ATP levels fall and AMP accumulates. The increase in the AMP:ATP ratio triggers the activation of AMPK, and leads to the activation of a large number of downstream targets. The overall effects of AMPK activation are to switch off energy-consuming pathways and switch on energy-producing processes, to restore the energy balance within the cell (Hardie *et al*, 2003). Because the reaction catalyzed by adenylate cyclase ($2ADP \leftrightarrow ATP + AMP$) is always held near equilibrium, the cellular AMP:ATP ratio varies roughly as the square of the ADP:ATP ratio, making the AMP:ATP ratio a more sensitive index of energy status (Kahn et al., 2005).

AMPK exists as a heterotrimeric enzyme comprised of a catalytic α-subunit and regulatory β and γ subunits. The complexity of regulation and control which this enzyme exerts on cellular metabolism is evident by the fact each subunit is encoded by multiple genes (Hardie, 2003) with splice variants and alternative promoters in mammals. There

is also allosteric regulation of the enzyme complex by AMP which promotes activation by phosphorylation of the kinase domain at threonine-172 (Thr-172) in the activation loop (Kahn et al., 2005). Dephosphorylation of AMPK at Thr-172 is also inhibited by AMP. The regulation of phosphorylation and dephosphorylation by AMP seems to be due to binding of ligand to substrate, AMPK, and not a change in activity of upstream kinases and phosphatases. The extreme sensitivity of AMPK to AMP:ATP ratios is demonstrated by how AMP activates the enzyme complex by three mechanisms (allosteric activation, phosphorylation, and inhibition of dephosphorylation) and all three mechanism are antagonized by high cellular ATP (Hardie, 2003).

Until the last several years, understanding the regulation of AMPK was focused on acute cellular energy balance. However, AMPK responds to the adipocyte-derived hormones leptin (Minokoshi et al., 2002) and adiponectin (Yamauchi et al., 2002), and evidence is mounting that AMPK is linked directly to whole body energy metabolism. Leptin activates AMPK in skeletal muscle to increase fatty acid oxidation (Minokoshi et al., 2002). Adiponectin activates AMPK in skeletal muscle, isolated adipocytes, and liver to increase fatty acid metabolism, glucose utilization and inhibit glucose production, respectively (Berg et al., 2001; Wu et al., 2003; Yamauchi et al., 2002).

The interactions among lipid metabolism, energy balance and inflammation in abnormal metabolic states are unique relationships which could tie the role of nutrient excess seen in obesity and related metabolic conditions to the low level inflammatory state. Recently Lihn et al. (2004) reported that 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), a synthetic compound that mimics the actions of AMP, can

activate AMPK and dose-dependently increase adiponectin gene expression in human adipose tissue. They also demonstrated AICAR attenuates the release of TNFα and IL-6 from human adipose tissue, and the reduced concentrations of the cytokines lead to increased levels of adiponectin. Taken together these results indicate AMPK activity might play a role in the insulin-sensitizing effects of AICAR and adiponectin. This is consistent with findings that adiponectin attenuates the translocation of nuclear factor kappa B (NFκB) to the nucleus of adipocytes stimulated with lipopolysacharide (LPS) (Ajuwon et al., 2004). Attenuation of NFκB translocation is coupled with an expected decrease in production of pro-inflammatory cytokines.

1.4 Adipocytes, inflammation and interleukin-6

Interleukin-6 is produced in many cell types and seems to have pro-inflammatory and anti-inflammatory effects, depending on the cell type, assay system and timing of exposure (Ohshima et al., 1998; Tilg et al., 1994). In the recent years, studies show that adipose tissue is a major site of IL-6 secretion and accounts for as much as 15-35% of circulating levels (Fried et al., 1998; Mohamed-Ali et al., 1997). Maachi et al. (2004) demonstrated circulating IL-6 concentrations are highly correlated with adipose mass. However, concentrations within different adipose tissue depots vary depending on the depot. Fried et al. (1998) reported 3 times as much IL-6 release from the omental adipose than the subcutaneous depot, and also suggested the origin of the IL-6 was only 10% derived from adipocytes and 90% from stromal vascular cells.

Increasing circulating IL-6 levels are highly correlated with insulin resistant states such as obesity, impaired glucose tolerance and type II diabetes (Fernandez-Real et al., 2001; Kado et al., 1999; Mohamed-Ali et al., 1997; Muller et al., 2002; Pickup et al., 1997; Straub et al., 1996). The physiological importance of IL-6 may be closely related to its high correlation with body mass index (BMI), percent fat mass, systolic and diastolic blood pressure, and fasting insulin levels (Fernandez-Real et al., 2001; Mohamed-Ali et al., 1997). Additionally, IL-6 is associated with increased risk for development of type II diabetes, independent of percent of body fat (Pradhan et al., 2001).

Regulation of IL-6 secretion from the adipocyte is quite complex. Stimulation of the adipocyte with β-agonists (Fasshauer et al., 2003; Vicennati et al., 2002), IL-6 (Fasshauer et al., 2003; Lagathu et al., 2003), interleukin-1β (Flower et al., 2003), TNFα (Fasshauer et al., 2003) and endotoxin (Lin et al., 2000) result in IL-6 secretion. Weigert et al. (2004) also reported that free fatty acids modulate IL-6 secretion from the adipocyte. This is intriguing, and raises the question as to whether adipocytokine secretion profiles can be modulated by dietary components and metabolic substrates. In states of metabolic imbalance, such as obesity and lipodystrophy, the metabolic profiles are associated with elevated plasma fatty acids and glucose. Although adipocytokine secretion from the adipocyte is complex, it is intriguing that IL-6 secretion is regulated *in vitro* by other pro-inflammatory cytokines. Thus, a greater amplification of inflammation during states of metabolic imbalance likely exacerbates insulin resistance.

The study of IL-6 gene expression in other cell types is quite extensive and is regulated by several transcription factors. The most prominent of these response elements studied to date is NFκB (Libermann and Baltimore, 1990). This transcription

factor is composed of homodimeric and heterodimeric proteins from the relish (rel) family (Ghosh et al., 1998); review). Cellular NFκB is found in one of two states: the inactive state where it is bound to its cellular inhibitor, IκB, or the active state where phosphorylated IkB disassociates from NFkB and it translocates to the nucleus where it binds its response element. The regulation of this process is dependent upon phosphorylation of IκB, its ubiquination and ultimate degradation, which frees NFκB for translocation. This IL-6 transcription factor is probably the best studied because its activation seems to be essential for final activation of the IL-6 gene promoter (Vanden Berghe et al., 2000).

There are other transcription factors involved in IL-6 gene expression, and Matsusaka et al. (1993) suggested multiple transcription factors are required for maximal stimulation of IL-6 gene expression. By contransfection of NF-IL-6 (C/EBP) and NFκB p65, these researchers found synergistic activation of the IL-6 promoter reporter construct. The complexity of the molecular regulation of IL-6 transcription is apparent in that others have found that activation of NFκB alone does not always lead to IL-6 expression (Legrand-Poels et al., 2000). Nonetheless, the adipocyte expresses significant levels of NFκB (Ajuwon et al., 2004; Ruan et al., 2002), C/EBPβ and C/EBPδ (Tang et al., 2004), and activator protein-1 (AP-1). Therefore, this cell certainly has the molecular machinery needed to produce significant levels of IL-6, and it is not surprising that the adipocyte contributes to a chronic inflammatory state seen in abnormal metabolic states.

1.5 Adipocytes, inflammation and tumor necrosis factor α

TNFα is synthesized as a 26-kD transmembrane pro-cytokine, which is cleaved to yield a 17 kD soluble molecule. Both of these proteins are capable of creating a metabolic and inflammatory response (Sethi and Hotamisligil, 1999). TNFα has two receptors type 1 (TNFR1) and type 2 (TNFR2), which are expressed ubiquitously, and dimerize upon ligand binding. Receptor subtype seems to have unique signaling properties in the adipocyte (Fruhbeck et al., 2001). Understanding the signaling properties of TNFα in adipocytes and other cells could help discern the pathogenesis of metabolic diseases associated with insulin resistance since tissue expression and circulating TNFα are elevated during this physiological state (Maachi et al., 2004).

Understanding the autocrine and paracrine effects of adipocyte-derived TNFα may present a clearer picture of whole body energy balance, given that this cytokine plays an important role in lipid metabolism (Sethi and Hotamisligil, 1999). First, treatment of tumor-bearing (cachexic) rodents with anti- TNFα antibodies protects against lipid metabolism abnormalities (Carbo et al., 1994). Second, in obesity models elevated TNFα seems to stimulate lipolysis (Ramsey, 1996), thus increasing free fatty acids (FFA) in circulation, which is a hallmark of metabolic diseases associated with insulin resistance. Moreover, $TNF\alpha^{-/-}$ mice are characterized by lower levels of circulating concentrations of FFA and TG (Uysal et al., 1997; Ventre et al., 1997). Finally, treatment of cultured adipocytes with TNFα stimulates lipolysis, as indicated by the release of FFA in to the culture media (Green et al., 1994; Kawakami et al., 1987). This is substantial evidence that TNFα promotes the hyperlipidemia that is common in patients with insulin resistance.

The mechanisms by which TNFα is involved in the development of insulin resistance are still being unraveled, and are likely to involve complex intracellular communications. There are indications that TNFα directly impairs insulin signaling in the adipocyte by down-regulating insulin-stimulated glucose uptake (Hotamisligil et al., 1994). Stephens and Pekala (1992) reported TNFα reduces insulin receptor tyrosine kinase activity at low concentrations, and at higher levels, will decrease insulin receptor, insulin receptor substrate-1 (IRS-1) and GLUT4 expression. Furthermore, TNFα increases serine^{307} phosphorylation of IRS-1, which causes conformational changes in the protein which inhibit binding to the insulin receptor to attenuate downstream signaling (Rui et al., 2001). Collectively, these data indicate that TNFα plays a direct role in impairing nutrient clearance by adipocytes and other peripheral tissues to perpetuate the hyperglycemia and hyperlipidemia associated with insulin resistance.

1.6 Adiponectin: adipocyte derived protein combating insulin resistance

Adiponectin is a relatively new adipocytokine that was cloned independently by four separate groups in the mid-1990s (Scherer et al, 1995; Maeda et al, 1996; Hu et al, 1996; Nakano et al, 1996). Since that time, it has become clear adiponectin plays an important role in regulation of insulin sensitivity, glucose homeostasis, and lipid metabolism. Adiponectin mRNA in fat and plasma hormone concentrations are suppressed with the development of insulin resistance and obesity (Hu et al, 1996; Hotta et al, 2000; Weyer et al, 2001). In agreement with these data, adiponectin expression increases with weight loss and improved insulin sensitivity (Yang et al, 2001; Milan et al, 2002; Hulver et al, 2002). Treatment with thiazolidinediones (TZDs) *in vitro* and *in vivo*

increases synthesis and secretion of adiponectin (Yamauchi *et al*, 2001; Maeda et al, 2001; Yu et al, 2002; Yang et al, 2002; Motoshima et al, 2002). However, the mechanisms by which adiponectin is regulated remain somewhat unclear. Since insulin sensitivity is highly correlated with adiponectin concentrations it might be expected that insulin regulates adiponectin. Fasting plasma insulin concentrations are negatively correlated with plasma adiponectin, and chronic treatment of 3T3-L1 adipocytes with insulin also have reduced adiponectin mRNA expression (Yu et al, 2002; Fasshauer et al, 2002; Bluher et al, 2002). In contrast, others have found that treatment of human visceral adipocytes and mouse brown adipocytes with insulin increases adiponectin mRNA expression (Halleux et al, 2001; Viengchareun et al, 2002). These data suggest there is differential regulation of adiponectin across species and adipose tissue depots.

Hormones involved in insulin resistance seem to play an antagonistic role on adiponectin synthesis and secretion. Fasshauer et al. (2001) demonstrated β-adrenergic agonists inhibit adiponectin mRNA expression in 3T3-L1 adipocytes. Other researchers have found similar results in human adipocytes, and found down regulation at the protein and mRNA levels (Delporte et al, 2002). Glucocorticoid treatment of 3T3-L1 and human adipocytes also inhibits adiponectin mRNA expression (Halleux et al, 2001; Fasshauer et al, 2002), and correlates with inhibition of protein secretion (Fasshauer et al, 2003). The *in vitro* data coincide with results showing that adrenalectomized *ob/ob* mice have increased adiponectin mRNA in fat, and increased circulating plasma adiponectin (Makimura et al, 2002). Additionally, adipose tissue-specific over-expression of 11β-hydroxysteroid dehydrogenase type 1 decreases adiponectin mRNA (Masuzaki et al, 2001).

The pro-inflammatory cytokines highly associated with the development of insulin resistance also down regulate adiponectin expression. Tumor necrosis factor-α inhibits adiponectin mRNA expression and protein synthesis in 3T3-L1 and human adipocytes (Kappes & Loffler, 2000; Maeda et al, 2001). Also, Fasshauer et al. (2003) reported that treatment of 3T3-L1 adipocytes with IL-6 inhibits adiponectin mRNA expression and protein secretion. This finding was substantiated by a recent report of IL-6 as a paracrine regulator of adipose tissue. Sopasakis et al. (2004) demonstrated a higher interstitial fluid concentration of IL-6 than plasma concentration, and when adipocytes were treated *in vitro* with concentrations of IL-6 found in interstitial fluid, adiponectin expression was inhibited. Patients who are being treated for human immunodeficiency virus (HIV)-associated lipodystrophy syndrome (HALS) have elevated levels of TNFα, IL-6, and interleukin 8 due to the pro-inflammatory response from active anti-retroviral therapy (Lihn *et al*, 2003). These individuals also have reduced adiponectin mRNA expression and plasma adiponectin levels. These findings concur with *in vitro* data from Bruun et al. (2003) who determined that TNFα, IL-6, and IL-8 in the presence of its soluble receptor inhibit adiponectin mRNA expression. Taken together, it seems that pro-inflammatory responses are major regulators of adiponectin expression and secretion.

Whereas pro-inflammatory cytokines regulate adiponectin expression, there seems to be a regulatory loop which allows adiponectin to exert anti-inflammatory effects on macrophages and adipocytes. Yokota et al. (2000) were the first to report adiponectin exerts anti-inflammatory effects in activated human macrophages. These researchers demonstrated that adiponectin suppresses pro-inflammatory cytokine production from

macrophages. Since these data were reported, Wulster-Radcliffe et al. (2004) determined that adiponectin attenuates TNFα and IL-6 production in activated pig macrophages. Our lab has also found that adiponectin exerts similar anti-inflammatory effects on porcine adipocytes which have been stimulated with lipopolysaccharide to induce IL-6 expression and release (Ajuwon et al, 2004). This information is extremely intriguing given the recent information linking obesity to a chronic state of inflammation, which includes a marked infiltration of macrophages in adipose tissue (Weisberg et al, 2003; Xu et al, 2003). It is thus not too far reaching to conceptualize how adipose tissue, which expresses and secretes inflammatory cytokines, and, an anti-inflammatory hormone could be a key determinant of energy balance.

1.7 Metabolic functions of adiponectin

To date there is limited information as to the regulation of energy metabolism in the adipocyte by adiponectin. Most of the metabolic studies of adiponectin have been focused on its relationship to insulin resistance and obesity with an emphasis on skeletal muscle and liver. Some of the first clues about the metabolic effects of adiponectin were reports that adiponectin concentrations are lower in obese individuals and negatively correlated with insulin resistance (Arita et al, 1999). This information was particularly interesting due to the contrast with leptin, which circulates at high concentrations during obesity.

The first direct evidence for the metabolic effects of adiponectin were reported by Fruebis et al. (2001) who showed that adiponectin regulates lipid metabolism and body composition. These researchers used recombinant adiponectin and a carboxyl terminal

peptide produced by trypsin cleavage of the recombinant protein (gAdn) to identify metabolic implications of adiponectin. First, mice consuming a high-fat, sucrose diet, or infused i.v. with a fat emulsion, had suppressed post-prandial surges in plasma triglyceride, free fatty acids, and glucose concentrations when infused with gAdn compared to control animals. Second, fatty acid oxidation was increased in muscle preparations and C2C12 myotubes cultured with gAdn. Lastly, the authors examined the effect of adiponectin on growth and weight change in mice. Both growing and mature mice were fed a high-fat, sucrose diet while body weight was monitored. Immature mice infused with gAdn had suppressed weight gain verses controls, and mature mice showed appreciable and sustained weight loss. This work suggests that adiponectin acts to promote fat utilization, rather than storage, in growing and mature rodents.

We recently published that adiponectin acts directly on the porcine adipocyte to suppress lipogenesis (Jacobi et al, 2004), and other groups have provided insight as to how this occurs. Yamauchi et al. (2002) and Tomas et al. (2002) independently determined that adiponectin controls fatty acid oxidation and triglyceride storage in skeletal muscle by activation of 5'AMPK. Activated AMPK phosphorylates acetyl CoA carboxylase (ACC) to deactivate it. Inactivation of ACC allows depletion of cytosolic malonyl CoA, which in turn relieves the allosteric repression of carnitine palmitoyltransferase (CPT-1). This enhances fatty acid transport and oxidation. Given this mechanism, it seems possible that adiponectin could work similarly in adipocytes to relieve obesity and modulate lipid metabolism.

1.8 Adiponectin receptors

Understanding the mechanisms by which adiponectin functions to regulate energy balance and inflammation will require a delineation of how the adiponectin receptor is regulated. Recently, Yamauchi et al. (2003) reported cloning of two adiponectin receptors, adipoR1 and adipoR2. AdipoR1 is predominantly expressed in skeletal muscle, whereas adipoR2 is predominantly found in liver (Yamauchi *et al*, 2003). However, recent reports document expression of both receptors in adipose tissue and macrophages (Chinetti *et al*, 2004; Fasshauer *et al*, 2004). The insulin-sensitizing effects of adiponectin seems to be mediated by increased fatty acid oxidation via phosphorylation of AMPK (Tomas *et al*, 2002; Yamauchi *et al*, 2002) and PPARα (Yamauchi *et al*, 2001; Fruebis *et al*, 2001; Yamauchi *et al*, 2003) which may be associated by regulation of the adiponectin receptors.

There are currently two reports which address the regulation of these receptors in different physiological states. Debard et al. (2004) reported adipoR1 and adipoR2 expression were not altered in the muscle of type II diabetic patients compared with healthy subjects, and that adipoR1 was up-regulated in patients with type II diabetes following a 3 hour hyperinsulinemic-euglycaemic clamp. However, Tsuchida et al. (2004) demonstrated insulin levels are negatively correlated with adipoR1 and adipoR2 expression and with adiponectin sensitivity *in vivo* and *in vitro* in muscle, liver and adipocytes. The down regulation of adipoR1 and adipoR2 occurs by a PI3-kinase/Foxo1 dependent mechanism (Tsuchida *et al*, 2004). Through the use of binding experiments researchers demonstrated a down-regulation of adiponectin receptors in skeletal muscle membrane from *ob/ob* mice versus wild-type counterparts (Tsuchida *et al*, 2004). When

ob/ob mice were infused with adiponectin (50 μg/10g body weight) the animals were resistant to the activation of AMPK in their skeletal muscle, suggesting that adiponectin resistance may occur by down-regulation of the adiponectin receptors in states of insulin resistance. Development of metabolic syndrome and eventually insulin resistance is highly correlated with chronic inflammation in adipose tissue (Xu et al, 2003). Recently, Tsuchida et al. (2005) reported there is a link between adiponectin, adiponectin receptors, PPARs and inflammation. They reported a novel mechanism by which there is a balance between activation of PPARα and PPARγ that tightly controls obesity-induced insulin resistance. Their proposed mechanism begins with the activation of PPARα suppressing inflammation in WAT by suppressing adipocyte hypertrophy. Second, when there is activation of PPARγ, as well as PPARα, the former allows for an increase in high molecular weight form of adiponectin and the latter allows increase adiponectin receptor expression in WAT. Therefore, it would appear there is an elegant feedback system in adipocyte hyperplasia and hypertrophy regulated by PPAR transcription factors that tightly controls adiponectin, adiponectin receptors, inflammation and energy balance.

1.9 PPARs and inflammation

The peroxisome proliferator-activated receptors (PPARs) are members of a nuclear receptor superfamily consisting of three different genes, PPARα, PPARβ/δ, and PPARγ. All three PPARs are widely expressed; however, their relative levels differ greatly between tissues and are reflective of their distinct biological functions (Chinetti et al., 2003). PPARs are ligand-dependent transcription factors. Some or their known ligands are fatty acids, eicosanoids, and oxidized fatty acids, as well as, specific

pharmacological compounds. The lipid lowering fibrates and the antidiabetic glitazones are synthetic ligands for PPARα and PPARγ, respectively (Chinetti et al., 2000). PPARs influence gene expression either by activation or repression of specific promoter/transcription factors of targeted genes. The different isoforms play distinctive roles in the regulation of lipid metabolism, glucose metabolism, and cell differentiation, and recently have been implicated in obesity-related inflammation (Moller and Berger, 2003).

The first evidence for a potential role for PPARs in inflammation came from Devchand et al. (1996) who observed PPARα-deficient mice have prolonged inflammation in response to leukotriene B4. Interestingly, others have implicated PPARα as inhibitor of inflammatory molecules such as endothelin-1 intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells and tissue factor, matrix metalloproteinase (MMP)-9 and TNFα in macrophages (reviewed by Chinetti et al., (2000)). Staels et al. (1998) demonstrated in human aortic smooth muscle cells (SMC), PPARα activation by fibrates inhibits IL-1 stimulated IL-6 secretion and 6-keto platelet growth factor $(PGF)_{1\alpha}$ production by decreasing IL-6 and cyclooxygenase (COX)2 gene transcription. Additionally, splenocytes from PPARα-deficient mice have increased IL-6 and interleukin 12 (IL-12) secretion in response to LPS than cells from wild-type mice (Delerive et al., 2001). With the current evidence demonstrating the adipocyte functions in inflammation it was not surprising when Tsuchida et al. (2005) showed PPARα agonist decrease inflammatory genes in WAT from obese diabetic KKAy mice. Altogether, these observations substantiate the role of PPARα in the inflammatory response.

The role of PPARγ in inflammatory response is more controversial because the PPARγ null mouse is a lethal mutation (Zhu et al., 2000). Therefore, researchers must use a heterozygous knockout model or a tissue specific knockout to study the genetic impact of PPARγ on inflammation. Chinetti et al. (2001) demonstrated PPARγ ligands can inhibit mRNA abundance of TNFα, IL-6 and IL-1β in monocytes, and in macrophages. PPARγ ligands also suppress inducible nitric oxide synthase (iNOS) and MMP-9 expression and increase IL-1 receptor antagonist (Meier et al., 2002). PPARγ activation also has decreased IFN-γ and IL-12 secretion in splenocytes (Cunard et al., 2002). Ajuwon and Spurlock (2004) demonstrated that IFN-γ suppressed the activation of PPARγ2 induction by adiponectin in LPS-challenged adipocytes. These data implicate PPARs as a regulator of inflammation and a modulator of energy status.

The recent reports of chronic inflammation in WAT by macrophage infiltration may cause whole-body insulin resistance in obese diabetic animals (Xu et al., 2003). Activated macrophages that infiltrate into WAT secrete cytokines which can impair adipocyte insulin sensitivity. Adipocytes stimulated by pro-inflammatory cytokines secrete chemokines that can contribute to macrophage infiltration. This vicious cycle impairs adipocyte insulin signaling and may eventually cause system insulin resistance (reviewed by Fantuzzi, 2005). Moreover, inflammatory markers such as C-reactive protein, MCP-1, and MSF-1 are associated with insulin resistance, adiposity, and type II diabetes in human subjects. Therefore, it has become important to investigate the mechanisms of PPARs, insulin sensitization, energy metabolism, and the regulation of inflammation.

1.10 Insulin resistance

Regulation of glucose metabolism is tightly controlled in normal individuals even during fasting and feeding within a very narrow range. The tight control is directed by the balance between glucose absorption from the intestine, production of glucose by the liver, uptake and metabolism by peripheral tissues. Insulin helps modulate glucose concentrations by stimulating glucose uptake in muscle and fat and inhibits hepatic glucose production, thus serving as the principal regulator of blood glucose concentration. Insulin also promotes cell growth and differentiation, and enhances the storage of substrates in fat, liver and muscle by promoting lipogenesis, glycogen and protein synthesis, and inhibiting lipolysis, glycogenolysis and protein breakdown. Hyperinsulinemic conditions associated with insulin resistance produce an overwhelming dysregulation of these processes, and produces elevations in fasting and postprandial glucose and lipid levels.

Insulin stimulates glucose uptake in cells by activating a signaling cascade leading to the translocation of the glucose transporter, GLUT4, from intracellular vesicles to the cell surface. Skeletal muscle is responsible for up to 75% of insulin-stimulated glucose disposal, while adipose tissue accounts for only a small portion (Klip et al., 1990). Despite skeletal muscle being the dominant tissue for glucose disposal the importance of adipose tissue is substantiated in the literature. Mice with an insulin receptor knockout in muscle have normal glucose tolerance (Bruning et al., 1998), while those with a knockout of the insulin-sensitive GLUT4 in fat have impaired glucose tolerance, apparently due to induction of insulin resistance in muscle and liver (Abel et al., 2001). Both obesity and lipoatrophy cause insulin resistance and a predisposition for associated diseases,

demonstrating that adipose tissue is critical in regulating metabolism (Gavrilova et al., 2000).

1.11 The insulin receptor and signaling pathways

The insulin receptor belongs to a subfamily of receptor tyrosine kinases (Patti and Kahn, 1998). These receptors are tetrameric proteins consisting of two α- and two β-subunits that function as allosteric enzymes in which the α-subunit inhibits the tyrosine kinase activity of the β-subunit. When insulin binds to the α-subunit it enables the kinase activity in the β-subunit followed by transphosphorylation of the β-subunits and a conformational change that further increases kinase activity (Patti and Kahn, 1998).

The signaling cascade initiated by the induction of tyrosine phosphorylation of the insulin receptor can activate at least nine intracellular substrates (Saltiel and Kahn, 2001). Four of these proteins belong to the insulin receptor substrate (IRS) family (White and Yenush, 1998). Some of the alternative substrates include Gab-1, $p60^{dok}$, Cbl, APS and isoforms of Shc (Pessin and Saltiel, 2000). Phosphorylation of tyrosine molecules in the substrates provides docking sites for proteins with Src-homology-2 (SH2) domains. The SH2 containing proteins can have different functions. They can function as adaptor proteins which activate further proteins in the cascade, as kinases and phosphatases which propagate the signaling cascade, and/or regulate their own function and subcellular location (Saltiel and Kahn, 2001).

The IRS proteins have high homology but their functions do not appear to be redundant but rather complimentary to each other. In fact, mice lacking IRS-1 function have general pre- and post-natal growth retardation and develop insulin resistance in

peripheral tissues (Araki et al., 1994; Tamemoto et al., 1994). Whereas, IRS-2 deficient mice have tissue specific growth retardation in the areas of the brain, islets, and retina, but these animals also develop peripheral tissue insulin resistance (Kido et al., 2000; Withers et al., 1998). However, the IRS-3 and IRS-4 knockout mice display mild aberrations to near normal growth and metabolic development (Fantin et al., 2000). The actual metabolic importance of IRS-3 and -4 is not well understood but there is some thought that IRS-3 and 4 may play a role as negative regulators of IRS-1 and 2 (Tsuruzoe et al., 2001).

The insulin receptor and insulin receptor substrates are activated by tyrosine phosphorylation but they can be negatively regulated by serine phosphorylation allowing negative feedback on insulin signaling and enabling crosstalk with other pathways which may be involved in the development of insulin resistance {(Saltiel and Kahn, 2001). Some of the key kinases implicated in this regulation are PI3K, Akt, glycogen synthase kinase (GSK)-3 and mammalian target of rapamycine (mTOR). The PI3K, Akt/PKB, and PKC seem to play integral parts in regulation of glucose metabolism and therefore glycogen, lipid and protein synthesis. Inhibition of a cell to regulate glucose and/or lipid metabolism has become tightly aligned with obesity and the development of related metabolic disorders.

The complexities by which insulin signaling and insulin resistance are regulated still remain a complex story to be unraveled. The pleiotropic condition is a combination of genetic and environmental factors which impact the metabolic state of a person. There are different thresholds of insulin resistance and once an individual reaches a certain degree there is decreased glucose tolerance and increased hyperglycemic conditions.

Perpetuation of this state can lead to sustained insulin insensitivity in multiple tissues of the body.

The concept of excess nutrients impacting insulin sensitivity is not far reaching, because obesity is associated excess energy intake and there is a high correlation of hyperglycemia and hyperlipidemia with the development of insulin resistance. This concept has been substantiated by Tang et al., (2001), who demonstrated in adipocytes a combination of hyperglycemia and hyperinsulinemia reduced insulin-stimulated glucose uptake. The mechanism was in part due to decreased insulin receptor dephosphorylation. Others have also shown low and high glucose levels in 3T3-L1 adipocytes effect insulin-mediated IRS-1 phosphorylation (Gagnon and Sorisky, 1998). The reduced capacity of the cell to respond to insulin in times of nutrient excess has been linked to the development of reactive oxygen species (ROS) in the cell. Lu et al. (2001) demonstrated rat adipocytes under hyperglycemic conditions have decreased insulin signaling and increase in intracellular ROS. Additionally, adipocytes isolated from high fat diet-induced diabetic animals have elevated intracellular ROS which can be normalized by incubating the cells in low glucose media (Talior et al., 2003). In this study, there was an increase in basal glucose uptake, however, a reduction in insulin-stimulated glucose uptake. The increase in basal glucose uptake may increase the flux of glucose through the aldose reductase pathway leading to elevated mitochondrial ROS and induction of inflammation in WAT. This hypothesis was further investigated by Scherer's lab (2005), who demonstrated 3T3-L1 adipocytes under hyperglycemic conditions have a reduced ability for insulin-stimulated glucose uptake. This effect is mediated by an induction of serine phosphorylation of IRS-1 and reduction in the insulin signaling cascade compared

to euglycemic conditions. In addition, the researchers' demonstrated hyperglycemia causes an induction of mitochondrial proteins and ROS in adipocytes. The *in vitro* induction of ROS in adipocytes was substantiated by a pro-inflammatory response in animals under euinsulinemic hyperglycemic clamp condition (Lin et al., 2005). Interestingly, they also demonstrated lowering of the mitochondrial membrane potential in adipocytes can significantly reduce ROS levels. Combined, the induction of insulin resistance by nutrient excess in the adipocyte is partially mediated by an increase in ROS that leads to an inflammatory state.

1.12 Putting it together: adipocytes, nutrient excess, inflammation and adiponectin

Our published data provide clear evidence that adiponectin suppresses LPS-induced activation of the NFkB transcription factor in macrophages and adipocytes (Wulster-Radcliffe et al., 2004; Ajuwon et al., 2004). This is of particular interest because adiponectin activates AMPK in adipocytes (Wu et al., 2003), and because recent publications indicate that 5-amino-4-carboxamide-1-β-4-ribofuranoside (AICAR), a chemical activator of AMPK, disrupts LPS-induced activation of NFkB and suppresses pro-inflammatory cytokine production in rodent glial cells (Giri et al., 2004). Consistent with these findings, AICAR also activates AMPK in human adipose explants and suppresses pro-inflammatory cytokine release (Lihn et al., 2004). Consequently, the first objective is to determine whether adiponectin activates AMPK in adipocytes. We hypothesize that adiponectin will activate AMPK, and that this activation is critical to the anti-inflammatory activity of this hormone in these cells by causing a reduction in size of

the adipocyte by inhibition of adipocyte lipogenesis and induction of oxidation in the cells.

To further the hypothesis of adipocyte energy metabolism being an integral part in whole body energy homeostasis the following diagram implicates the role of excess nutrients in perpetuating chronic inflammation during obesity. In the following figure we hypothesize that over consumption of energy increases adipocyte size. As adipocyte size increases, an inflammatory signal, possibly IL6, is generated to initiate a local inflammatory response which results in the expression of macrophage recruitment and maturation factors (MCP-1 and M-CSF). As inflammation progresses, recruited macrophages are activated and adiponectin and/or its receptors in macrophages and adipocytes are down regulated, resulting in adiponectin resistance. Thereafter, systemic inflammation develops, adiponectin receptors (R1 and R2) are down-regulated in skeletal muscle, and the ability of adiponectin to activate the AMPK is diminished. Consequently, insulin resistance in skeletal muscle is induced and the metabolic syndrome is exacerbated.

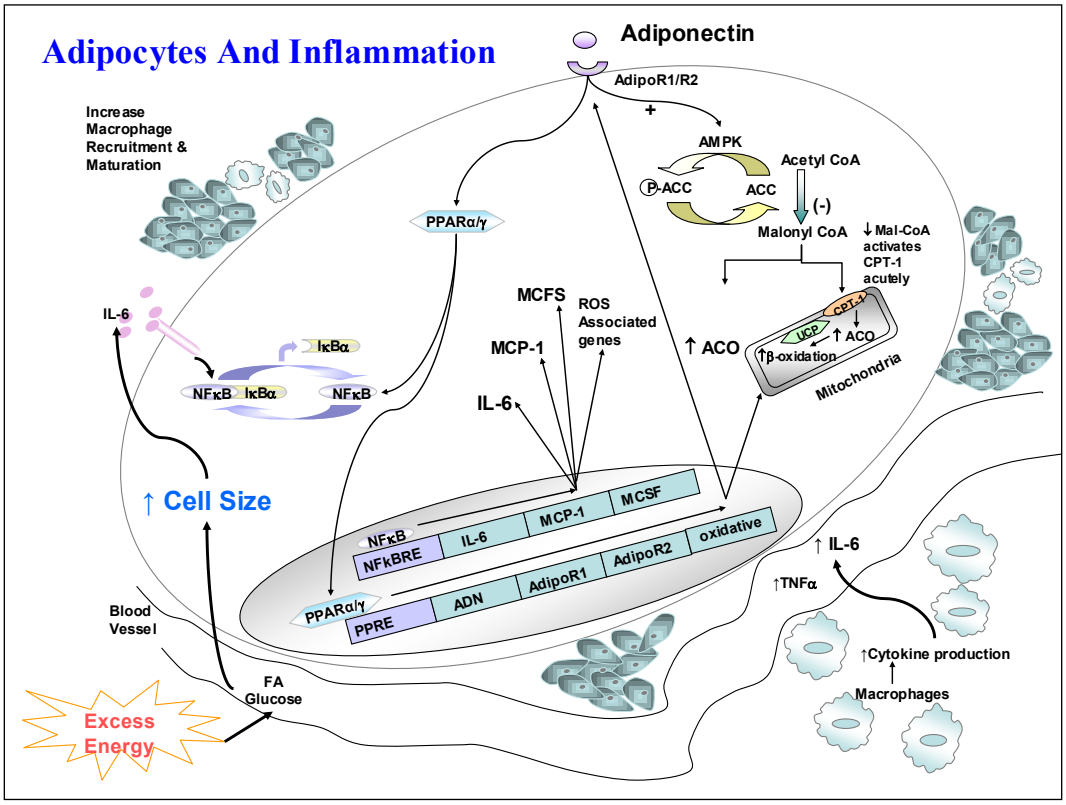

Figure 1.1 Hypothesis for the autocrine role of adiponectin in maintaining energy balance in the adipocyte and inhibiting inflammation in adipose tissue

1.13 References

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CHAPTER 2. CLONING AND EXPRESSION OF PORCINE ADIPONECTIN, AND ITS RELATIONSHIP TO ADIPOSITY, LIPOGENESIS, AND THE ACUTE PHASE RESPONSE

2.1 Abstract

Adiponectin is an adipocyte-derived hormone that has been implicated recently in the regulation of inflammation in immunocytes, and in lipid metabolism and glucose homeostasis in liver, skeletal muscle, and adipocytes. However, information in non-rodent models is limited. We have cloned and sequenced the porcine adiponectin open reading frame (ORF) and evaluated the regulation of adiponectin, *in vitro* and *in vivo*. The porcine sequence shares approximately 88, 86, 85 and 83% homology with the dog, human, cow and mouse adiponectin, respectively, and 79-83% similarity with dog, human, cow and mouse proteins at the amino acid level, based on the translated porcine sequence and GenBank submissions for the other species. Short term (4-8 hours) treatment of isolated pig adipocytes with the pro-inflammatory cytokines, TNF-α and IL-6, and the β-adrenergic agonist, isoproterenol, had no effect on the abundance of adiponectin mRNA. Likewise, relative serum adiponectin concentrations were not altered in pigs infused with E. coli, and mRNA expression in adipose tissue was not responsive to lipopolysaccharide. However, analysis of serum from very lean vs. a substantially fatter line of pigs indicated that relative circulating adiponectin

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concentrations are higher ($P < 0.01$) in lean pigs than in the fatter line, and that the difference is established relatively early in the growth curve. Also, incubating pig adipocytes for 6 hours with recombinant pig adiponectin resulted in an approximate 30% reduction ($P < 0.05$) in lipogenesis compared with adipocytes under basal conditions and with those incubated in the presence of insulin. This is the first report in any species that adiponectin antagonizes the incorporation of glucose carbon into lipid in the adipocyte, and provides additional evidence that adiponectin acts as an autocrine regulatory factor to regulate energy metabolism.

2.2 Introduction

Adiponectin is a relatively new "adipocytokine" that circulates in the blood as the mature protein (Arita et al., 1999), and as an apparent carboxyl terminal proteolytic cleavage peptide that has marked structural similarity to tumor necrosis factor-α (Fruebis et al., 2001). In humans, relatively high concentrations of adiponectin (2-20 μg per mL) are found in the circulation (Arita et al., 1999). Early findings indicated an anti-inflammatory role for adiponectin. Specifically, recombinant human adiponectin reduced TNF-α expression and secretion by macrophages stimulated with lipopolysaccharide, and also reduced their phagocytic activity and stimulated apoptosis of certain myeolomonocytic cell populations (Yokota et al., 2000). Apart from the anti-inflammatory activity, recent developments have also implicated adiponectin as a regulator of insulin sensitivity, glucose homeostasis and lipid metabolism. Both intact adiponectin and the globular cleavage peptide regulate energy metabolism in skeletal

muscle (Fruebis et al., 2001; Tomas et al., 2002; Yamauchi et al., 2002) and liver (Berg et al., 2001; Combs et al., 2001; Yamauchi et al., 2001), albeit with different potencies; adiponectin causes weight loss, up-regulates fatty acid transporters, and stimulates fatty acid oxidation in skeletal muscle, and augments the suppression of glucose production by insulin in the liver. Furthermore, Wu et al. (2003) have shown a direct stimulatory effect of globular adiponectin on glucose uptake in primary rat adipocytes, and provided compelling evidence that this peptide attenuates the inhibition of insulin-stimulated glucose uptake by TNF-α. Collectively, these regulatory actions across skeletal muscle, liver and adipose tissue would abrogate the insulin insensitivity and hyperglycemia common in rodent models of Type II diabetes.

In contrast with leptin, which is increased with body fat accretion and obesity, plasma concentrations of adiponectin are decreased in a number of altered metabolic states, including obesity (Arita et al., 1999), dyslipidemia (Matsubara et al., 2002), and Type II diabetes and coronary artery disease (Hotta et al., 2000). Recently, Fruebis et al. (2001) hypothesized that the reduction in circulating adiponectin that is associated with obesity is causally related to the excessive accumulation of body fat. Furthermore, Yamauchi et al. (2001) discovered that leptin and adiponectin were additive in their enhancement of the inhibition of glucose production by insulin in the liver of lipotrophic mice. Thus, the relationship between circulating adiponectin, leptin, and adiposity in the pig, a rapidly-emerging research model, is of considerable interest.

With these considerations in mind, our primary objectives were to test the hypotheses that 1) adiponectin exerts an anti-lipogenic action directly upon the pig

adipocyte, and 2) that serum adiponectin concentrations reflect differences in adiposity in lean vs. a fatter line of pigs, and that it is reciprocally regulated with leptin. Also, the anti-inflammatory actions of adiponectin indicate that the short-term regulation of adiponectin may be important during the acute phase response, and may relate to the regulation of metabolism and immune response pathways by pro-inflammatory cytokines. Consequently, our third objective was to determine whether serum adiponectin or mRNA expression is altered by bacterial infection or endotoxin challenge, and to determine whether specific cytokines act acutely on the adipocyte to regulate adiponectin mRNA abundance.

2.3 Materials and Methods

Animals and adipocyte isolation

Adipose tissue was obtained from male castrates weighing approximately 90-110 kg, and consuming a nutritionally-complete diet ad libitum. The pigs were maintained according to protocols approved by the Purdue University Animal Care and Use Committee, and killed in the Purdue University abattoir according to USDA regulations. Subcutaneous adipose tissue was removed from the dorsal depot located over the cervical spine, and transported in buffered saline (37^{o}C, 0.15 M NaCl, 10 mM HEPES, pH 7.4) to the laboratory. Adherent skeletal muscle was removed, and the tissue was cubed and sliced with a Stadie-Riggs microtombe. Approximately 6-7 g of sliced tissue was minced, rinsed with saline, and transferred to a flask containing 15 mg collagenase (100 U/mL final concentration; Worthington BioChemical, Lakewood, NJ USA). Following a

30-35 minute digestion period, isolated adipocytes were washed through a 290 μm screen with warmed Krebs Ringer bicarbonate buffer (pH 7.4) containing 10 mM HEPES, 5 mM glucose, and 3% bovine serum albumin (Fraction V, essentially fatty acid free, Invitrogen life technologies, Carlsbad, CA USA) to remove all other cell debris. The cells were resuspended in culture media (DMEM Sigma D6780; St. Louis, MO USA) with 3% BSA and 10 mM HEPES at a final concentration of approximately 2 x 10^5 cells per mL and aliquoted immediately into 15 mL polypropylene scintillation vials for incubation.

RNA isolation

Total RNA was extracted from cells as described by Chomczynski and Sacchi (1987). Briefly, cells were homogenized in guanidium thiocyanate, followed by the addition of sodium acetate. The samples were then extracted sequentially with water-saturated phenol and chloroform:isoamyl alcohol, and the aqueous fractions precipitated with isopropanol. After the second ethanol precipitation, the RNA pellets were resuspended in Tris-HCl buffer (pH 8.0) and quantitative and qualitative measures obtained spectrophotometrically. In addition, the actual RNA concentration of sample was determined using the RiboGreen® assay (Molecular Probes, Eugene, OR USA) and the manufacturer's protocol.

Cloning

Degenerative primers from highly conserved regions of mouse and human adiponectin sequences were designed to amplify porcine adiponectin cDNA using long distance PCR (forward primer: 5'-CTGTCAATTTCAGGGCTCAGG-3'; reverse primer: 5'- GGCGCAGAGGAAACA AATCAGACTAG-3'). The cDNA was cloned by

amplifying a reverse-transcribed product of RNA extracted from isolated adipocytes from 90 kg pigs. A cDNA clone of about 1100 bp contained a 732 bp open reading frame coding a 243 amino acid protein. The 1100 bp product was cloned into the PCR®II-TOPO vector (Invitrogen life technologies; Carlsbad, CA USA) for sequencing.

Protein expression and purification

The protein expression and purification was carried out in a commercial facility (C&P Biotech Corp.,Thornhill, Ontario, Canada). Briefly, DNA containing the coding region of mature porcine adiponectin was amplified by PCR, then subcloned into pET15b (Novagen; Madison, WI USA) sites (NdeI, and BamHI/BglII) to introduce an N-terminal His-tag and thrombin cleavage site. Once the insertion of the gene was verified by DNA sequencing, the plasmid was transformed into BL21 (DE3) cells (Stratagene; La Jolla, CA USA). The cells were grown in LB broth with 50 µg per mL of ampicillin in shaker flasks at 37^{o}C, 200 rpm, until the OD600 was 0.7. Thereafter, 1 mM IPTG was added for induction, and after two hours, the cells were harvested by centrifugation at 5,000 rpm for 15 minutes, and stored at –80^{o} C. The cells were lysed in 50 mM Tris pH 8.0, 500 mM NaCl, 1 µM phenylmethysulfonyl fluoride (PMSF), and sonicated with power level 8, 30% duty cycle for 3 minutes with a Sonifer brand sonicator (VWR International; Bristol, CT USA). The supernatant fraction from the lysate was harvested by centrifugation at 15,000 rpm for 30 minutes, and it was loaded onto a Ni-NTA agarose column (Qiagen; Valencia, CA USA). The column was washed with 50 mM Tris pH 8.0, 500 mM NaCl, 10 mM immidazole, and then the protein was eluted with 250 mM immidazole in the same buffer. Fractions containing the protein of interest were pooled and dialyzed overnight at 4^{o}C into 25 mM Tris, 50 mM NaCl, 2 mM $CaCl_2$, pH7.5, containing 1U

Thrombin (Calbiochem; San Diego, CA USA) per mg of protein. The protein sample was further purified over an S75 column (Amersham Pharmacia Biotech; Piscataway, NJ USA) with PBS to remove the thrombin and His-tag. The fractions containing the protein were pooled and dialyzed into PBS overnight. Prior to use, the recombinant protein was concentrated by evaporative drying. Amino terminal sequencing of the recombinant protein was carried out to confirm the identity of the expressed protein. The globular domain was cloned and expressed similarly. Amino terminal sequencing of this peptide confirmed its identity. The N-terminal sequence of the recombinant cleavage product was obtained to confirm the identity of the expressed peptide. Endotoxin analysis was performed by a commercial laboratory (Cambrex Inc., Walkersville, MD USA) to verify the purity of the preparation.

Northern analysis and treatments

Total RNA was extracted as described above from isolated adipocytes which were incubated for 4 and 8 hours under 5% CO_2- 95% air with the following treatments: basal control, 10 ng/mL TNF-α, 10 ng/mL IL-6 and 10^{-6} M isoproterenol. Adiponectin mRNA was measured by Northern blot analysis with a porcine adiponectin cDNA corresponding to a ~320 bp region from the ORF of porcine adiponectin. The porcine adiponectin cDNA template was obtained by reverse transcription-polymerase chain reaction (RT-PCR) procedures, and the PCR product was confirmed by sequence analysis. The antisense primer of adiponectin was added to the T7 promoter sequence (5'-GGATCCTAAT ACGACTCACT ATAGGGAGG-3') to allow northern probe transcription under the control of T7 polymerase. Radiolabeling of the probe was accomplished by in vitro transcription with T7 RNA polymerase in the presence of ^{32}P-

UTP (NEN/Perkin Elmer; Boston, MA USA). The probe was gel purified on a 5 % acrylamide-8 M Urea gel and eluted from gel with probe elution buffer (Ambion; Austin, TX USA) overnight. Northern blots were performed with 3 μg of total adipocyte RNA. Samples were transferred to nitrocellulose membranes (Perkin Elmer Life Sciences Inc., Boston, MA USA) cross-linked with UV light and baked for 2 hours at 80^{o}C. Membranes were probed overnight, washed and exposed to x-ray film overnight. Autoradiographs were quantitated by image analysis. Following autoradiography, the membranes were stripped in .1 X SSC (1.5 M NaCl, .15 M sodium citrate, pH 7.0), .5% SDS at 95^{o}C to remove probe and rehybridized with a RNA probe corresponding to 18S rRNA. The abundance of the 18S rRNA was used to standardize adiponectin mRNA abundance.

Lipogenesis

For lipogenic determinations, six 2-mL aliquots of cell suspension were added to 15 mL of polypropylene scintillation vials, gassed with 5% CO_2- 95% air and incubated for 6 hours at 37^{o}C in an environmentally controlled shaking incubator. Cells were treated with no hormone, 30 μg/mL full length porcine adiponectin, 30 μg/mL cleaved porcine adiponectin or 1 mU/ml porcine insulin (Sigma I- 5532; St. Louis, MO USA). Vials also contained 0.75 μCi [U-^{14}C]-glucose (NEN/Perkin Elmer; Boston, MA USA) and incubation media was the same as described above. After the appropriate incubation periods, the reactions were terminated with the addition of 100 μl 17% perchloric acid. Total lipid was extracted with Dole's reagent. Briefly, 5 mL of Dole's reagent, 3 mL hexane, and 4 mL distilled H_2O were added and mixed in each vial. The phases were allowed to separate and the upper phase recovered and evaporated at 70^{o}C overnight.

Thereafter, 5 mL scintillation cocktail was added to vials the counts determined for calculation of lipogenic activity.

SDS-PAGE and Immunoblotting

Sample proteins were separated by discontinuous SDS-PAGE (Laemmli, 1970) using a 3.75% stacking gel and 12.5% resolving gels and then transferred to nitrocellulose membranes. For all experiments in which serum adiponectin was quantified on a relative basis, the adiponectin protein was detected using a monoclolnal anti-human adiponectin antibody (ANOC 9108) which has been described previously (Okamoto et al., 2000; Ouchi et al., 2000). Preliminary experiments established a range of dilutions for pig serum (1:5 to 1:40) over which adiponectin signal intensity was linear with our colorimetric detection system and image analysis. Furthermore, the antibody was validated for use with pig serum by competitive displacement of signal when a range of antibody dilutions (1:1000 to 1:20,000) were preincubated with an excess of recombinant pig protein. Colorimetric visualization was accomplished via alkaline phosphatase-linked secondary antibody (goat anti-mouse IgG; American Qualix; La Mirada, CA), which was used at a dilution of 1:2,000. Western blotting with the monoclonal antibody indicated a primary band at approximately 30 kDa. This band was quantified as adiponectin in all experiments based on the fact that the antibody is monoclonal and binds recombinant pig adiponectin, and on our determination that the serum signal is competitively displaced with excess recombinant pig adiponectin.

Genotype Comparison

To determine whether relative serum adiponectin concentrations reflect the differences in body fat in lean vs. a fatter genotype of pigs, two genetic lines were compared across several ages. The pigs were maintained, and killed at the appropriate time, according to a protocol approved by the Purdue University Animal Care and Use Committee. Females were housed in individual pens and allowed ad libitum intake of diets that were nutritionally complete with respect to the age of the pig. Actual body weight was recorded and measures of subcutaneous backfat depth were taken at the 10^{th} rib approximately 2 cm off the midline in the pigs killed at the selected time points (28, 56, 90 and 165 days of age). Relative serum adiponectin concentrations were determined by western blot analysis with the monoclonal antibody as described above. Additionally, serum leptin was quantified by a pig-specific RIA that is based on a polyclonal antibody generated against a synthetic peptide designed per the amino acid sequence derived from translation of the pig leptin ORF, as we have reported previously (Bidwell et al., 1997). The peptide (LQGALQDMLRQLDLSPGC) was complexed to keyhole limpet hemocyanin and used to immunize rabbits. The antibody was affinity purified, validated by western blot analysis of recombinant pig leptin and pig serum, and used for the RIA of serum leptin. Recombinant pig leptin (4 μg) was radiolabeled (^{125}-I) using Chloramine-T and a 22 second reaction period. For the RIA, 200 μL samples were assayed in duplicate. The range of accurate quantification was from 0.25 to 16 ng per mL. Excellent regression was obtained from 94% to 7% binding (based on the 0 standard), and the standard curve has excellent correlation (0.99) over the range of 0-16 ng leptin per mL. Additionally, the recovery of a leptin standard (also prepared with recombinant pig

leptin) that was added to a pooled internal standard averaged 112.8%, well within the acceptable range. Parallelism was confirmed by assaying different volumes of a pooled internal serum standard (25, 50, 100, 150 and 200 μL per tube) which produced concentration estimates that were acceptably similar (coefficient of variation of 11.4%). The intra- and inter-assay coefficients of variation were 9.7% and 11.6%, respectively. The data were analyzed using a mixed model analysis which tested the effects of genetic line and age, and the interaction of line and age. Mean comparisons were established using the pdiff procedure of SAS when protected by a significant F-test.

E. coli challenge

The pigs used in this study were obtained from a commercial genetics company (Pig Improvement Company, Inc., Franklin, KY) and housed at the Purina Mills, Inc. Research Center, Gray Summit, MO. The protocol was approved by the corporate animal care and use committee. Briefly, individually-penned pigs (male castrates weighing approximately 70 kg) were allotted to 3 treatment groups consisting of a control group that was allowed ad libitum feed intake, a feed-deprived group, and a group deprived of feed and infused i.v. with an E. coli isolate (5×10^9 cfu) that was cultured from pig feces and provided by the Veterinary Services Department, Purina Mills, Inc., St. Louis, MO. The control and feed-deprived groups were infused i.v. with the same volume (10 mL) of sterile medium used to deliver the E. coli to the challenged group. Blood samples were obtained by jugular venipuncture from all pigs just prior to infusion, and at 6, 12 and 24 hours following infusion. Feed consumption and rectal temperatures were obtained at the time of blood sampling to confirm the induction of an innate immune response to the E. coli challenge. Relative serum adiponectin

concentrations were assessed by western blot analysis as described above and the data quantified by image analysis. Statistical analysis was performed using a mixed model analysis in which the effects of time (age) and treatment were tested, as was the interaction of time and treatment.

2.4 Results

Cloning of porcine adiponectin ORF

Using RT-PCR and adipocyte RNA, we cloned the porcine adiponectin ORF. The resulting nucleotide sequence, aligned with available sequences from selected species, is shown in Figure 2.1A, and the translated amino acid sequence is presented in Figure 2.1B. The ORF for porcine adiponectin was determined to be 732 bp in length, and when translated, yields a protein of 243 amino acids. The identity between the porcine nucleotide sequence and the rhesus monkey, human, dog, cow, mouse and rat is 86, 86, 88, 85, 83, and 82 percent, respectively (Table 2.1), and at the amino acid level, the identity was 79-83%, based on the GenBank translations of the mouse, cow, human, and monkey adiponectin sequences. Northern blot analysis of adiponectin expression in adipose tissue and isolated adipocytes is shown in Figures 2.2A and B, respectively. In adipose tissue, we detected a predominant transcript of approximately 3.0 kb, and transcripts of much lesser abundance at approximately 1.7, 1.3, and .9 kb. In isolated adipocytes, the three smaller transcripts are absent, with the exception of one pig in which the smallest transcript was the predominant band, but the 3.0 and 1.3 kb transcripts

were detected. Using Northern blot analysis and RT-PCR, we also confirmed that adiponectin expression is limited to the adipose tissue of the pig (data not shown).

Western blot analyses of pig serum with the monoclonal anti-human adiponectin antibody indicated the presence of two major proteins of approximately 30 and 35 kDa (Figure 2.3). The most abundant 30 kDa protein is similar in size to the single band detected by the antibody in control human serum, and the less abundant, 34-35 kDa protein is similar that reported previously for mice (Yoda-Murakami et al., 2001).

Anti-lipogenic effect of adiponectin on porcine adipocytes

The effect of adiponectin on lipogenesis and lipolysis were measured in isolated adipocytes in six-hour incubations. Basal and insulin-stimulated lipogenesis, which were based on the incorporation of labeled glucose into extractable lipid, were reduced 28% and 30%, respectively, ($P < 0.05$) by the full-length recombinant adiponectin (Figure 2.4). However, the recombinant molecule representing the apparent cleavage peptide was ineffective at the same concentration (i.e., 30 μg per mL). Neither the intact protein nor cleavage peptide altered lipolysis, as assessed based on glycerol release from incubated adipocytes (data not shown).

Regulation of serum adiponectin and mRNA expression in vivo and in vitro

The northern blot analyses of adiponectin expression in the in vitro adipocyte experiments are summarized in Figure 2.5. The abundance of adiponectin mRNA in adipocytes incubated for 4 or 8 hours with 10 ng per mL of TNF-α or IL-6, or 10^{-6} M isoproterenol, was not influenced by either pro-inflammatory cytokine, or by isoproterenol. Likewise, relative serum adiponectin was not influenced in growing pigs

infused i.v. with an E. coli isolate to induce septicemia (Figure 2.6). The latter finding was confirmed at the mRNA level using E. coli-derived lipopolysaccharide to mimic infection. In these pigs, the abundance of the adiponectin was not changed 6 hours after a second injection of LPS that was administered 12 hours after the initial injection, despite the induction of a transient febrile response and obvious sickness behavior. It is also notable that exogenous leptin did not alter the expression of adiponectin in pig adipose tissue ($P > 0.5$), whether administered alone or in combination with the lipopolysaccharide (data not shown).

Serum Adiponectin and Leptin in Lean vs. Fatter Lines of Pigs

Serum samples were collected from pigs of two genetic lines with very different potentials for fat accretion, and subjected to western blot analysis. As shown in Figure 2.7A, relative serum adiponectin concentrations were similar in samples collected at 28 and 56 days of age. However, by 90 days of age, differences in adiposity (backfat depth, Figure 2.7B) were reflected in serum adiponectin concentrations, with the leaner pigs having less backfat but higher ($P < 0.05$) relative adiponectin concentrations at 90 and at 165 days of age. In contrast, serum leptin concentrations were lower ($P < .01$) in the lean line by 56 days of age (data not shown), and the magnitude of the difference between lines increased with time (Line X Time, $P < .01$). Identical differences were detected in the abundance of leptin mRNA in the adipose tissue of these two lines (not shown).

2.5 Discussion

Herein we report for the first time the sequence of the porcine adiponectin ORF, and provide the amino acid sequence of the protein inferred from translation of the nucleotide sequence. Both the coding region and the inferred amino acid sequence share substantial homology with those of other species. The adiponectin transcript in pig adipocytes is approximately 3.0 kb, whereas there are three additional transcripts faintly detected in pig adipose tissue. Other publications have documented the presence of three similar transcripts in rat adipose tissue (Bensaid et al., 2003; Hu et al., 1996), whereas mouse adipose tissue expresses a single transcript (Hu et al., 1996). The identities of the smaller, lesser abundant transcripts are not known.

Also consistent with previous findings (Scherer et al., 1995), the mRNA for adiponectin seems to be limited to the adipose tissue (adipocytes) of pigs. Although others have reported that adiponectin expression is induced in the liver of mice exposed to carbon tetrachloride, and in HepG2 cells cultured with IL-6 (Yoda-Murakami et al., 2001), we did not detect adiponectin mRNA in the liver of pigs injected with lipopolysaccharide (Northern blot analysis), or in primary pig hepatocytes stimulated with IL-6, even using RT-PCR (unpublished observations). A monoclonal anti-human adiponectin antibody detected proteins of approximately 30 and 35 kDa in pig serum. This smaller (30 kDa) protein is similar in size to the single protein detected in human serum by the monoclonal antibody, and was quantified in all experiments for which western blot analysis was used to establish relative differences in circulating concentrations.

Understanding the regulation of adiponectin expression in the adipocyte is central to understanding the relationship of this protein to inflammation, metabolism, and insulin sensitivity. Previous publications (Bruun et al., 2003; Fasshauer et al., 2003; Kappes and Loffler, 2000) indicated that both TNF-α and IL-6 alter the expression or secretion of adiponectin from adipocytes (or adipose explants) in vitro. In our experiments, neither cytokine altered the relative adiponectin mRNA abundance. However, the duration of our experiments (8 hours or less) was shorter than that used for other model systems described in the publications cited above. Fasshauer et al. (2003) showed that IL-6 acted within 8 hours, and was apparently not dependent on the presence of its soluble receptor, as it was in human adipose explants (Bruun et al., 2003). It is possible that adiponectin mRNA abundance would have been reduced in pig cells had we added the soluble IL-6 receptor, but we believe it likely that the pig adipocytes require a longer duration of cytokine exposure for changes in adiponectin mRNA abundance to occur. A similar explanation for the lack of a response to the β-agonist (i.e., reduced mRNA expression in response to isoproterenol) seems plausible.

Yokota et al. (2000) provided convincing evidence that adiponectin acted directly upon macrophages to attenuate pro-inflammatory cytokine (TNF-α) expression and secretion into the medium, and to antagonize phagocytosis. Accordingly, we hypothesized initially that the acute regulation of adiponectin by inflammatory mediators in vivo would be such that transient increases in expression and secretion of adiponectin would potentiate its anti-inflammatory and metabolic actions by increasing the circulating protein concentration. In the lipopolysaccharide and E. coli infusion models that we used, there were clear indications of the innate immune response (anorexia, fever, vomiting),

and Webel et al. (1997) and Carroll et al. (2003) reported that the TNF-α, IL-6, and cortisol responses to lipopolysaccharide injection in the pig are relatively acute (i.e., peak within 4-6 hours). Thus, our collective results at the mRNA and serum protein level indicate that the expression and secretion of adiponectin are not acutely regulated in vivo to alter the circulating protein concentration during infection. Given the transient nature of the increases in the circulating concentrations of pro-inflammatory cytokines caused by these challenges in the pig (Webel et al., 1997), this finding in vivo is consistent with the absence of an effect of TNF-α and IL-6 in adipocytes exposed in vitro, as discussed above. Recently, Tsao et al. (2002) determined that the oligomerization state of adiponectin regulates the biological activity and effective concentration of adiponectin. Consequently, it seems likely that the anti-inflammatory activity of adiponectin is controlled through oligomerization, rather than through alterations in expression and secretion. It will also be of considerable importance to determine whether either of the adiponectin receptors identified by Yamauchi et al. (2003) are regulated in immune cells or peripheral tissues during the innate immune response.

Both adiponectin and the cleavage peptide regulate body weight and energy metabolism in skeletal muscle (Tomas et al., 2002, Yamauchi et al., 2002) and liver (Berg et al., 2001, Yamauchi et al., 2001), albeit with different potencies. Furthermore, Arita et al. (1999) determined that circulating adiponectin concentrations are lower in obese individuals, and in association with non-insulin-dependent diabetes, than in individuals with normal body composition and insulin sensitivity. Others have recently confirmed this finding in adolescent obesity (Weiss et al., 2003). We have extended these findings in two significant areas. First, we determined that serum adiponectin

concentrations also reflect adiposity in the pig, a rapidly emerging biomedical research model, and that the lower serum adiponectin in the fatter line coincides with marked increases in serum leptin and in leptin mRNA in adipose tissue. This is of particular interest because the ratio of leptin to adiponectin has recently been proposed as an index of obesity in monkeys (Chen et al., 2003), and may have similar utility in other animal models. Secondly, we have shown for the first time in any species that intact adiponectin acts directly upon the adipocyte to suppress lipogenesis. Collectively, these findings support the hypothesis advanced by Fruebis et al. (2001) that the lower concentration of adiponectin observed in obese individuals is causally related to the excessive lipid accumulation. It is also notable that leptin administration did not alter the expression of adiponectin, despite the substantial increase (3-fold) in circulating leptin that is achieved within 24 hours using the injection regimen and dose of leptin used in this study (Ajuwon et al., 2003). Although not the primary objective of this experiment, to our knowledge, this is the first experiment to evaluate the regulation of adiponectin mRNA abundance by leptin.

It is potentially of considerable importance that adiponectin reduced lipogenic activity in incubated adipocytes. It should be noted that the response of pig adipocytes to insulin is typically small (Mills, 1999) versus other species, and that adiponectin reduced lipogenic activity relative to both basal and insulin-stimulated conditions. As regards the potential mechanism by which intact adiponectin regulates lipogenesis, recent findings are quite pertinent. Yamauchi et al. (2002) and Tomas et al.(2002) reported that activation of the 5'-AMP-activated protein kinase (AMPK) is central to the regulation of fatty acid oxidation and acetyl Co-A carboxylase activity by adiponectin in skeletal

muscle. More recently, Wu et al. (2003) reported that globular adiponectin stimulated glucose transport in primary rat adipocytes cultures. This response was dependent upon the activation (phosphorylation) of the AMPK, and was independent of critical factors in the insulin signaling pathway, including IRS-1 or Akt. Additionally, the increased activity of the AMPK caused a deactivation of acetyl Co-A carboxylase, which would be expected to attenuate lipogenic activity. In our experiments, the cleavage peptide did not reduce lipogenesis, as did the intact protein. It is possible that our recombinant cleavage peptide is less biologically active, and that higher concentrations are required to suppress lipogenesis.

In summary, we have confirmed herein that relative circulating adiponectin concentrations reflect differences in adiposity in the pig, and report for the first time that adiponectin acts directly upon isolated pig adipocytes to attenuate lipogenesis. Thus, the regulation of fat accretion by adiponectin may also include the direct regulation of de novo lipogenesis in the pig adipocytes. The mechanism by which adiponectin regulates lipogenesis, and the mechanisms which culminate in lower concentrations of adiponectin in obese individuals may offer new targets for alleviating obesity. We have also shown that adiponectin is not acutely regulated by pro-inflammatory cytokines, in vitro, or during the acute phase response in vivo. These findings indicate that the anti-inflammatory actions of adiponectin are not regulated by transient changes in expression and secretion of adiponectin.

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Table 2.1 Identity of porcine adiponectin compared to other species.
Percent identity of Porcine adiponectin with selected species at the nucleotide and amino acid level.

Species	**Nucleotide % identity**	**Amino Acid % Similarity**
Dog	88	---
Human	86	83
Rhesus Monkey	86	83
Cow	85	81
Mouse	83	79
Rat	82	---

(A)

```
                1                                                                                80
porcine_adn  .......... .......... .........A TGCTGTTGTT GGGAGCTGTT CTACTGCTAC TAGCCCTGCC CAGTCTCGGC
  human_adn  ...CTGATTC CATACCAGAG GGGCTCAGGA TGCTGTTGCT GGGAGCTGTT CTACTGCTAT TAGCTCTGCC CGGGCATGAC
 monkey_adn  .......... .......... .......... ..ATGTTGCT GGGAGCTGTT CTACTGCTAT TAGCTCTGCC CAGTCATGGC
  mouse_adn  TGGATCTGAC GACACCAAAA GGGCTCAGGA TGCTACTGTT GCAAGCTCTC CTGTTCCTCT TAATCCTGCC CAGTCATGCC

                81                                                                              160
porcine_adn  CAGGAA.... ..ACCACCGA GAA...GC.. .CTGGAGCAC TACTGCCCGT GCCCAAGGGG GCCTGCGCAG GCTGGATGGC
  human_adn  CAGGAA.... ..ACCACGAC TCAAGGGC.. .CCGGAGTCC TGCTTCCCCT GCCCAAGGGG GCCTGCACAG GTTGGATGGC
 monkey_adn  CAGGAT.... ..ACCACAAC TCAAGGGC.. .CCGGAGTCC TGCTTCCTCT GCCCAAGGGG GCCTGCACAG GTTGGATGGC
  mouse_adn  GAAGATGACG TTACTACAAC TGAAGAGCTA GCTCCTGCTT TGGTCCCTCC ACCCAAGGGA ACTTGTGCAG GTTGGATGGC

                161                                                                             240
porcine_adn  GGGTATCCCA GGGCATCCTG GCCACAACGG TACCCCAGGC CGTGATGGCA GAGATGGCGT CCCTGGCGAG AAGGGTGAGA
  human_adn  GGGCATCCCA GGGCATCCGG GCCATAATGG GGCCCCAGGC CGTGATGGCA GAGATGGCAC CCCTGGTGAG AAGGGTGAGA
 monkey_adn  AGGCATCCCA GGGCATCCAG GCCATAATGG GGTCCCAGGT CGTGATGGCA GAGATGGCAC CCCTGGCGAG AAGGGTGAGA
  mouse_adn  AGGCATCCCA GGACATCCTG GCCACAATGG CACACCAGGC CGTGATGGCA GAGATGGCAC TCCTGGAGAG AAGGGAGAGA

                241                                                                             320
porcine_adn  AAGGAGATAC AGGTCTTACT GGTCCTAAGG GTGACACTGG GGAATCTGGA GTGACTGGGG TTGAAGGTCC CCGAGGTTTC
  human_adn  AAGGAGATCC AGGTCTTATT GGTCCTAAGG GAGACATCGG TGAAACCGGA GTACCCGGGG CTGAAGGTCC CCGAGGCTTT
 monkey_adn  AAGGAGATCC AGGTCTTATC GGTCCTAAGG GAGACACTGG TGAAACTGGA GTAACCGGGG CTGAAGGTCC CCGAGGCTTT
  mouse_adn  AAGGAGATGC AGGTCTTCTT GGTCCTAAGG GTGAGACAGG AGATGTTGGA ATGACAGGAG CTGAAGGGCC ACGGGGCTTC

                321                                                                             400
porcine_adn  CCAGGAATCC CGGGCAGAAA AGGAGAACCT GGAGAAAGCG CCTATGTCTA CCGTTCAGCA TTCAGTGTGG GCCTGGAGAC
  human_adn  CCGGGAATCC AAGGCAGGAA AGGAGAACCT GGAGAAGGTG CCTATGTATA CCGCTCAGCA TTCAGTGTGG GATTGGAGAC
 monkey_adn  CCGGGAATCC AAGGCAGGAA AGGAGAACCT GGAGAAGGTG CCTATGTATA CCGCTCAGCA TTCAGTGTGG GATTGGAGAC
  mouse_adn  CCCGGAACCC CTGGCAGGAA AGGAGAGCCT GGAGAAGCCG CTTATGTGTA TCGCTCAGCG TTCAGTGTGG GGCTGGAGAC

                401                                                                             480
porcine_adn  TCGGGTCACT GCCCCTAACA TGCCCATTCG CTTTACCAAG ATCTTCTACA ATCAGCAAAA CCACTATGAT GTCACCACTG
  human_adn  TTACGTTACT ATCCCCAACA TGCCCATTCG CTTTACCAAG ATCTTCTACA ATCAGCAAAA CCACTATGAT GGCTCCACTG
 monkey_adn  CTACGTTACT GTCCCCAACA TGCCCATTCG CTTTACCAAG ATCTTCTACA ATCAGCAAAA CCACTATGAT GGCTCCACTG
  mouse_adn  CCGCGTCACT GTTCCCAATG TACCCATTCG CTTTACTAAG ATCTTCTACA ACCAACAGAA TCATTATGAC GGCAGCACTG

                481                                                                             560
porcine_adn  GCAAATTCCA CTGCAACATT CCTGGGCTGT ACTACTTCTC CTTCCACATC ACGGTCTACT TGAAGGATGT GAAGGTCAGC
  human_adn  GTAAATTCCA CTGCAACATT CCTGGGCTGT ACTACTTTGC CTACCACATC ACAGTCTATA TGAAGGATGT GAAGGTCAGC
 monkey_adn  GTAAATTCCA CTGCAACATT CCTGGGCTGT ACTACTTTGC CTACCACATC ACAGTCTATA TGAAGGATGT GAAGGTCAGC
  mouse_adn  GCAAGTTCTA CTGCAACATT CCGGGACTCT ACTACTTCTC TTACCACATC ACGGTGTACA TGAAAGATGT GAAGGTGAGC

                561                                                                             640
porcine_adn  CTCTACAAGA AGGACAAGGC TGTACTCTTC CCCTACGACC AGTACCAGGA CAAGAATGTG GACCAGGCCT CTGGCTCTGT
  human_adn  CTCTTCAAGA AGGACAAGGC TATGCTCTTC ACCTATGATC AGTACCAGGA AAATAATGTG GACCAGGCCT CCGGCTCTGT
 monkey_adn  CTCTTCAAGA AGGACAAGGC TATGCTCTTC ACCTATGACC AGTACCAGGA AAATAACGTG GACCAGGCCT CCGGCTCTGT
  mouse_adn  CTCTTCAAGA AGGACAAGGC CGTTCTCTTC ACCTACGACC AGTATCAGGA AAAGAATGTG GACCAGGCCT CTGGCTCTGT

                641                                                                             720
porcine_adn  GCTCCTCTAT CTGGAGAAGG GGGACCAAGT CTGGCTCCAG GCATACGGGG ATGAAGAGAA TAATGGGGTC TATGCTGACA
  human_adn  GCTCCTGCAT CTGGAGGTGG GCGACCAAGT CTGGCTCCAG GTGTATGGGG AAGGAGAGCG TAATGGACTC TATGCTGATA
 monkey_adn  GCTCCTGCAT CTGGAGGTGG GCGACCAAGT CTGGCTCCAG GTGTATGGGG AAGGAGAGCG TAATGGACTC TATGCTGATA
  mouse_adn  GCTCCTCCAT CTGGAGGTGG GAGACCAAGT CTGGCTCCAG GTGTATGGGG ATGGGGACCA CAATGGACTC TATGCAGATA

                721                                                                             800
porcine_adn  ATGTCAATGA CTCCATCTTC ACAGGCTTCC TTCTCTACCA CAACATTGAA TGA....... .......... ..........
  human_adn  ATGACAATGA CTCCACCTTC ACAGGCTTTC TTCTCTACCA TGACACCAAC TGATCACCAC TAACTCAGAG CCTCCTC...
 monkey_adn  ATGACAATGA CTCCACCTTC ACAGGCTTTC TTCTCTACCA CGACACCAAC TGATC..... .......... ..........
  mouse_adn  ACGTCAACGA CTCTACATTT ACTGGCTTTC TTCTCTACCA TGATACCAAC TGACTGCAAC TACCCATAGC CCATACA...
```

(B)

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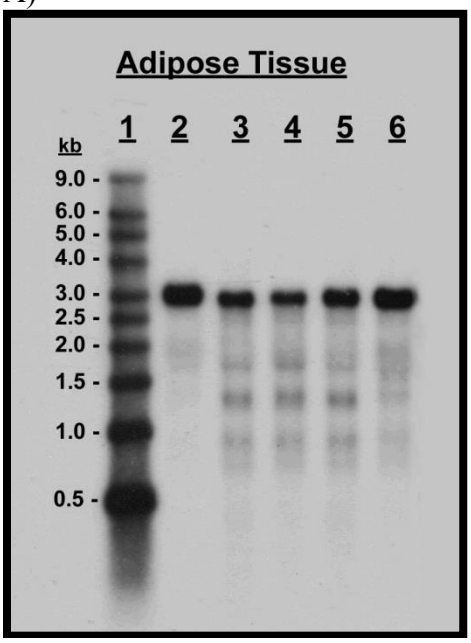
  1 MLLLGAVLLL LALPSLGQET TEKPGALLPM PKGACAGWMA GIPGHPGHNG TPGRDGRDGV
 61 PGEKGEKGDT GLTGPKGDTG ESGVTGVEGP RGFPGIPGRK GEPGESAYVY RSAFSVGLET
121 RVTVPNMPIR FTKIFYNQQN HYDVTTGKFH CNIPGLYYFS FHITVYLKDV KVSLYKKDKA
181 VLFTYDQYQD KNVDQASGSV LLYLEKGDQV WLQAYGDEEN NEVYADNVND SIFTGFLLYH NIE
```

Figure 2.1. Nucleotide sequence and translation of porcine adiponectin.
(A) Comparison of porcine adiponectin (Adn) nucleotide sequence with human (NM_004797 gi4757759), monkey (AF404407 gi15213855), and mouse (BC028770 gi 20381158). The percent identity of porcine nucleotide sequence with that of the species noted above is reported in Table 1. B: The translated amino acid sequence of porcine Adn is compared with the translated human (NM_004797 gi4757759), monkey (AF404407 gi15213855), mouse (BC028770 gi 20381158) and bovine (NP_777167 gi27807433). (B) The amino acid sequence contains the predicted 18 amino acid secretory signal sequence, a series of Gly-X-Y repeats and a 36 amino acid region that is homologous to collagen VIII, collagen X, and the C chain of C1q. The percent amino acid similarity of porcine Adn to other species is also reported in Table 1.

A)



(B)

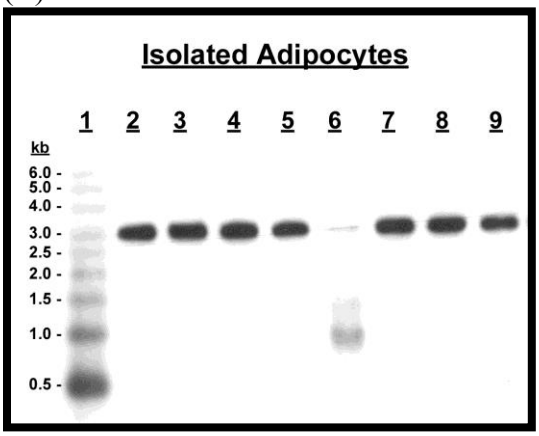


Figure 2.2. Northern blot analysis of adiponectin mRNA in pig adipose tissue (A) and isolated adipocytes (B). Total RNA was extracted from the adipose tissue or adipocytes isolated from the adipose tissue of multiple pigs; 5 µg (adipose tissue) or 2.5 µg (adipocytes) total RNA were probed on the Northern blot with the pig-specific adiponectin riboprobe as described in the methods. Different pigs were used for adipose tissue vs. adipocytes. The results indicate a predominate transcript for pig adiponectin that is approximately 3 kb in size. This transcript was used to establish relative differences among treatments in subsequent experiments. The specificity and origin of the smaller transcripts have not been characterized in any species.

(A) (B)

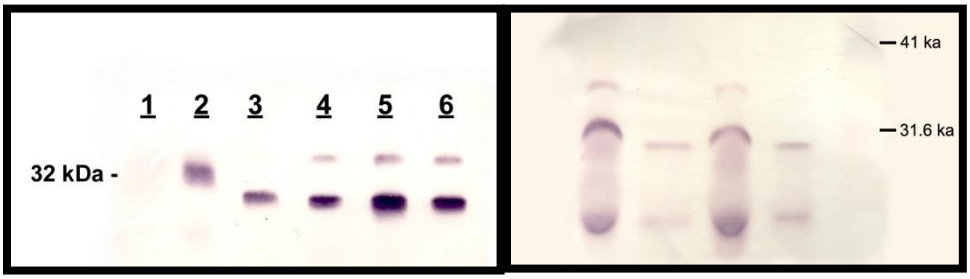


Figure 2.3 Western blot analysis of pig serum using a monoclonal anti-human adiponectin antibody described previously (ANOC 9108; 19, 20).
The serum samples were diluted 1:25. Lane 1 contained the BioRad Rainbow molecular weight markers (not visible in image of blot) and lane 2 is a human serum control. Lanes 3-6 are samples from individual pigs. The antibody detected a single predominant protein in human and pig serum at approximately 32 and 30 kDa, respectively, and also detected a larger protein of approximately 34-35 kDa in the serum of 3 pigs (Lanes 4-6), as was reported previously for mice (Yoda-Murakami et al., 2001). (B) The 30 kDa band was quantified in pig serum samples by western blotting and image analysis as describe in the methods.

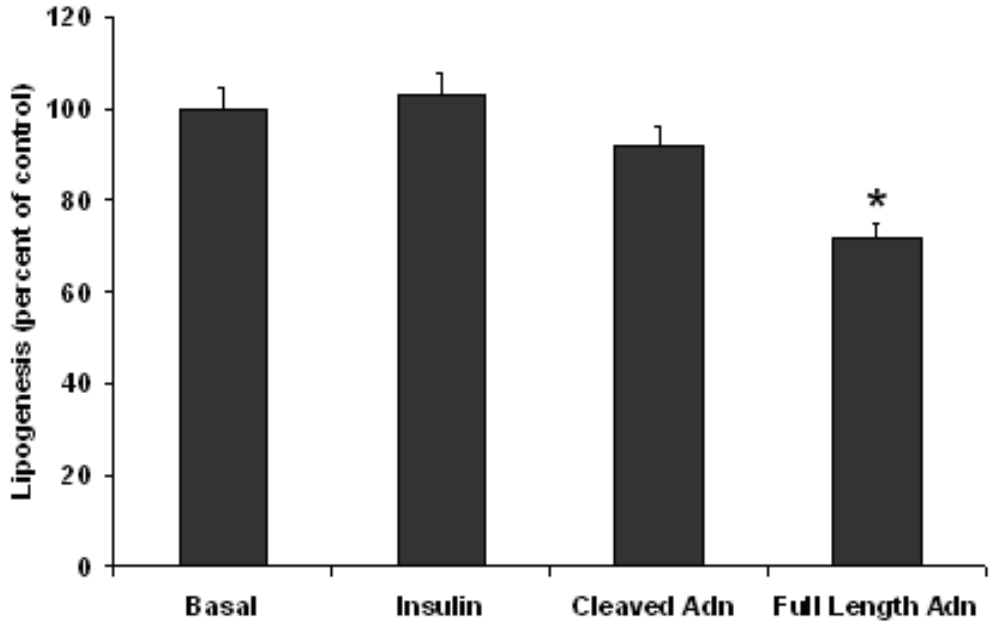


Figure 2.4. Regulation of lipogenesis in isolated pig adipocytes by recombinant pig adiponectin.

Isolated pig adipocytes were incubated with porcine insulin (1 mU/mL), recombinant porcine globular adiponectin (gAdn, 30 μg/mL), or recombinant full-length porcine adiponectin (Intact Adn, 30 μg/mL). Cells were incubated in medium containing 0.75 μCi/2 mL 14C-glucose for 6 h, followed by extraction of total lipids. The data are expressed as a percentage of the basal treatment and represent 5 experiments performed with adipocytes isolated from different pigs. The asterisk indicates significant difference ($P < .04$) vs. the basal control and insulin-stimulated treatments.

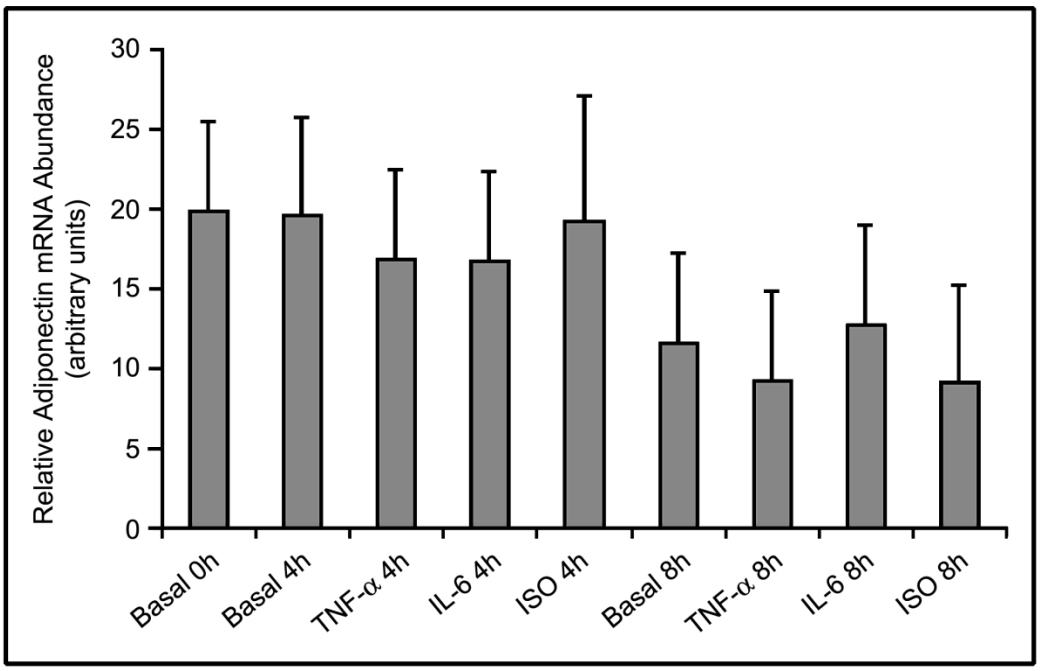


Figure 2.5 Expression of adiponectin mRNA in isolated pig adipocytes treated with selected pro-inflammatory cytokines and isoproterenol
Isolated pig adipocytes were incubated for 4 or 8 hours with 10 ng/ml of TNF-α or IL-6, or 10-6 M isoproterenol. The data are standardized with 18s rRNA and represent 6 experiments performed with adipocytes isolated from different pigs. There was no significant effect of the proinflammatory cytokines or isoproterenol on adiponectin mRNA abundance ($P > .05$).

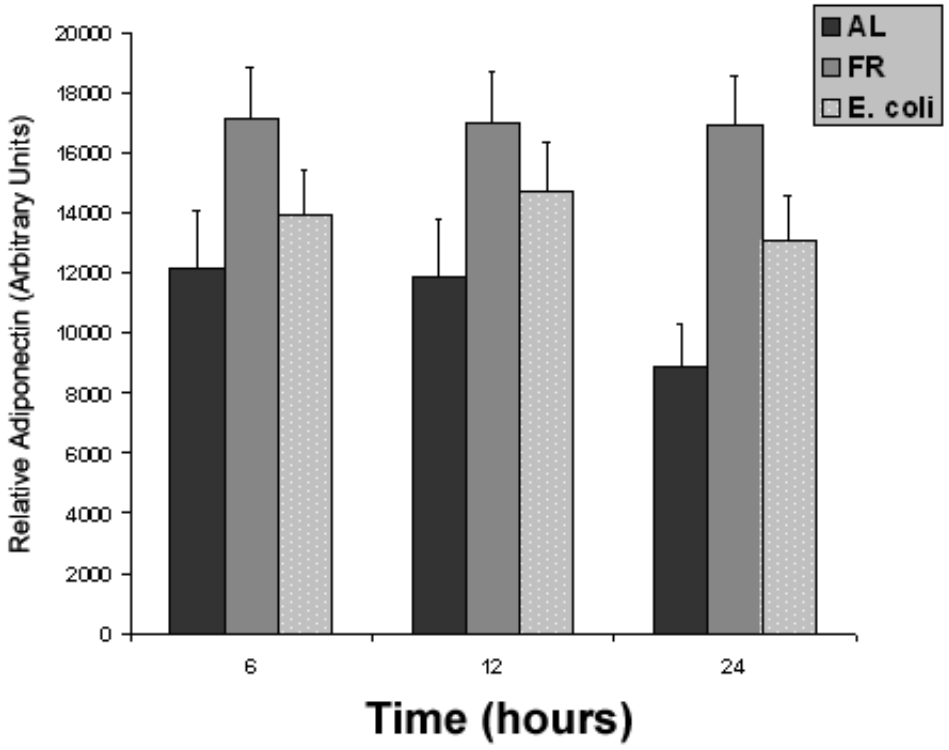
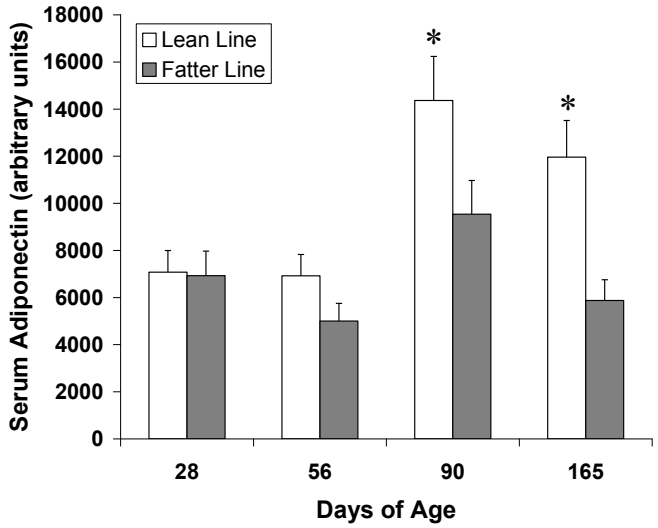


Figure 2.6. Relative serum concentrations of adiponectin in pigs challenged with an i.v. infusion of E. coli

Individually-penned pigs (male castrates weighing approximately 70 kg) were allotted to 3 treatment groups (n = 9) consisting of a control group that was allowed ad libitum feed intake (AL), a feed-deprived group (FR), and a group deprived of feed and infused i.v. with 5 X 109 cfu of a fecal E. coli isolate (E. coli). The AL and FR groups were infused i.v. with the same volume (10 mL) of sterile medium used to deliver the E. coli to the challenged group. Blood samples were obtained by jugular venipuncture from all pigs just prior to infusion and at 6, 12 and 24 hours following infusion. There was no significant effect of treatment, nor was there an interaction ($P > .05$).

(A)



(B)

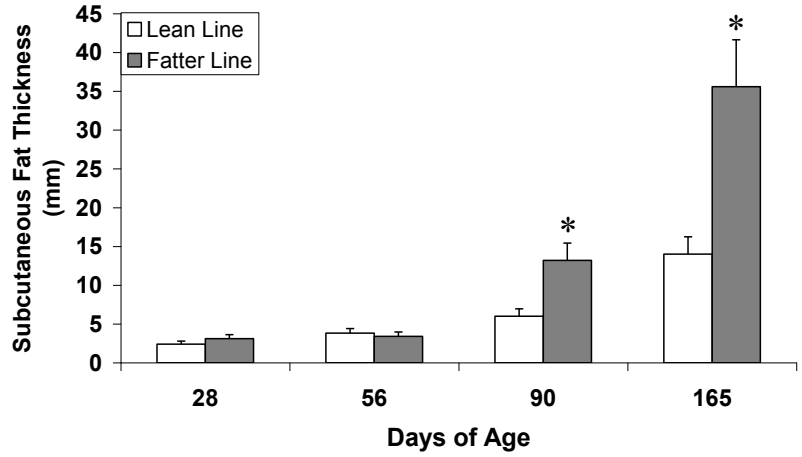


Figure 2.7 Relative serum adiponectin concentrations (A) subcutaneous backfat thickness (B) in a lean vs. a fatter genotype of pigs.
At the ages (days) indicated, serum samples and measures of subcutaneous backfat depth were obtained from pigs (n = 4-5) representing the 2 genetic lines with greatly different potentials for fat accretion. The asterisks indicate significant differences ($P < .01$) with respect to genotype. Additionally, there was an interaction between genotype and age such that the difference in back fat depth between lines increased with age ($P < .01$). Serum leptin was determined using a pig-specific RIA as detailed in the methods. The asterisks indicate significant differences ($P < .01$) with respect to genotype (n = 4-5). There was also an interaction between genotype and time in that the magnitude of the difference in serum leptin between lines increased with time ($P < .01$).

CHAPTER 3. ADIPONECTIN REGULATES THE EXPRESSION OF GENES ASSOCIATED WITH FATTY ACID SYNTHESIS AND OXIDATION IN PRIMARY PIG ADIPOCYTES

3.1 Abstract

Adiponectin has been implicated in the regulation of glucose uptake and fatty acid synthesis in adipocytes, and in the long-term suppression of weight gain when over-expressed in rodents by adenovirus associated virus vectors. The aim of this research was to determine whether adiponectin regulates the expression of genes associated with fatty acid oxidation in porcine adipocytes. Primary adipocytes were isolated from the dorsal subcutaneous adipose tissue of pigs (male castrates, n=5). Cells were incubated in DMEM with 3% BSA, in the presence and absence of recombinant porcine adiponectin (30 μg/mL), and were harvested at 0, 30, 60, 120, 240 and 360 minutes. Total RNA was extracted for cDNA synthesis, and the abundance of selected transcripts determined by real time PCR analysis. Adiponectin transiently increased (2-6.5 fold, $P < .05$) the expression of acetyl Co-A carboxylase (ACC), AMP activated kinase (AMPK), acyl Co-A oxidase (ACO) and peroxisome proliferator activated receptor-α (PPARα). After the initial increase in mRNA abundance, there was either a return to initial levels of expression (PPARα), a significant reduction (AMPK), or a cyclic pattern of expression (ACC, ACO). The abundance of the uncoupling protein-3 (UCP3) transcript was not influenced by adiponectin until the final measure at 360 minutes, at which time it was

increased 3.8-fold ($P < .05$). Collectively, these data indicate that adiponectin acts through autocrine/paracrine signaling pathways to coordinately regulate lipid metabolism in the adipocyte, and may thus be a determinant of adipocyte size and overall lipid accumulation. – Funded by USDA

3.2 Introduction

There are striking similarities in pigs and humans in the development of certain physiological conditions related to cardiovascular disease, hyperglycemia, dyslipidemia, and insulin resistance (Gerrity et al., 2001; Otis et al., 2003). These similarities underscore the potential for the pig as a research model, and also emphasize the need to understand cellular signaling pathways and inter-tissue communications in the pig.

Historically, adipocytes were seen as a passive energy storage depot. However, it is now clear that white adipose tissue (WAT) synthesizes and secretes an array of adipocytokines which have been implicated in whole body energy homeostasis (Kahn et al., 2005). Adiponectin, a relatively new adipocytokine, was cloned in the mid-1990s and has since been identified as a significant determinant of insulin sensitivity, glucose homeostasis, and lipid metabolism (Hu et al., 1996; Maeda et al., 1996; Nakano et al., 1996; Scherer et al., 1995).

To date there is limited information as to the mechanisms by which adiponectin regulates energy metabolism in the adipocyte. Most metabolic studies of adiponectin have focused on relationships among adiponectin, insulin resistance and obesity, with the emphasis on skeletal muscle and liver. Fruebis et al. (2001) provided the first evidence that adiponectin regulates lipid metabolism and body composition. Since then, others

have confirmed this and linked adiponectin to improved clearance of circulating free fatty acids, glucose and triglycerides, and suppression of hepatic glucose production (Berg et al., 2001; Combs et al., 2001; Fruebis et al., 2001; Yamauchi et al., 2001).

Yamauchi et al. (2002) and Tomas et al. (2002) independently provided evidence that adiponectin regulates fatty acid oxidation and triglyceride storage in skeletal muscle by activation of 5'AMP-activated protein kinase (AMPK). Activated AMPK phosphorylates acetyl CoA carboxylase (ACC) and deactivates this enzyme to allow depletion of cytosolic malonyl CoA and relieve the allosteric repression of carnitine palmitoyltransferase (CPT-1). Consequently, mitochondrial fatty acid transport and oxidation are increased. We recently published that adiponectin works directly on porcine adipocytes to suppress lipogenesis (Jacobi et al., 2004), and Wu et al. (2003) determined that rat adipocytes treated with globular adiponectin have increased Thr-172 phosphorylation of AMPK and increased Ser-79 phosphorylation of ACC in acute (2 h) cultures. It seems possible that adiponectin could work to modulate hypertrophy of fat cells by regulating lipid metabolism via similar mechanisms.

Predicated on the literature cited above, the first aim of this study was to test the hypothesis that adiponectin acts in autocrine/paracrine fashion in porcine adipocytes to modulate lipogenic activity by activation of AMPK and deactivation of ACC. The second aim was to determine whether adiponectin targets PPARα expression and other genes involved in fatty acid oxidation, and finally, to identify specific regulators of adiponectin expression.

3.3 Methods

Animals and adipocyte isolation

Adipose tissue was obtained from male castrates weighing approximately 90-110 kg, and consuming a nutritionally-complete diet ad libitum. The pigs were maintained according to protocols approved by the Purdue University Animal Care and Use Committee, and esanguinated in the Purdue University abattoir according to USDA regulations. Subcutaneous adipose tissue was removed from the dorsal depot located over the cervical spine, and transported in buffered saline (37^{o}C, 0.15 M NaCl, 10 mM HEPES, pH 7.4) to the laboratory. Adherent skeletal muscle was removed, and the tissue was cubed and sliced with a Stadie-Riggs microtombe. Approximately 6-7 g of sliced tissue was minced, rinsed with saline, and transferred to a flask containing 15 mg collagenase (100 U/mL final concentration; Worthington BioChemical, Lakewood, NJ USA). Following a 30-35 minute digestion period, isolated adipocytes were washed through a 290 μm screen with warmed Krebs Ringer bicarbonate buffer (pH 7.4) containing 10 mM HEPES, 5 mM glucose, and 3% bovine serum albumin (Fraction V, essentially fatty acid free, Invitrogen life technologies, Carlsbad, CA USA) to remove all other cell debris. The cells were resuspended in culture media (DMEM Sigma D6780; St. Louis, MO USA) with 3% BSA and 10 mM HEPES at a final concentration of approximately 2 x 10^5 cells per mL and aliquoted immediately into 15 mL polypropylene scintillation vials for incubation.

Real time quantitative PCR

Total RNA was recovered from cells using Trizol reagent (Invitrogen, Carlsbad, CA) and DNase treated using the Turbo DNase® (Ambion; Houston, TX). RNA concentration was determined using the RiboGreen® assay (Molecular Probes, Eugene, OR USA)

according to the manufacturer's protocol. Total RNA (2 µg) was reverse transcribed using the iScript cDNA synthesis kit (BioRad; Hercules, CA). Primer sequences were: pig adiponectin, 5'-tgctgttgttgggagctgttc -3' and 5'-aggaagcctgtgaagatggag -3'; porcine acetyl CoA carboxylase, 5'- atgtttcggcagtccctgat-3' and 5'-tgtggaccagctgaccttga-3', porcine acetyl CoA oxidase, 5'-ctcgcagacccagatgaaat -3' and 5'-tccaagcctcgaagatgagt-3', porcine AMPK, 5'-acatggctgagaagcagaag-3' and 5'-ggtctcgatgaacaaccata-3', porcine PPARα, 5'-cagcctccagcccctcgtc-3' and 5'-gcggtctcggcatcttctagg-3', porcine UCP3, 5'-tggtgaaggtccgatttcag-3' and 5'-aggcagagacaaagtggcag-3', and porcine 18S, 5'-ttaagccattaccagatata-3' and 5'-agattcattcatgcattaag-3', all sequences are sense and anti-sense respectively. Thermal cycler conditions for PCR reactions were 95 ^{o}C for 3 minutes followed by 36 cycles of 95 ^{o}C for 30 seconds, 64 ^{o}C for 30 seconds, and 72 ^{o}C for 30 seconds. Polymerase chain reaction products were cloned into pGEMT vector (Promega, Madison, WI) and sequenced for verification. Real-time reactions were carried out on an iCycler real-time machine (BioRad, Hercules, CA) using the IQ™ SYBR Green Supermix kit (BioRad, Hercules, CA). Plasmid containing the gene of interest was serially diluted to create a standard curve of gene copy number. The mRNA abundance of each gene product was determined by regression analysis against the standard curve. The housekeeping gene, 18S RNA, did not differ among treatments or pigs.

AMPK activity

Cells and/or tissues were incubated with treatments for six hours in DMEM with 3% BSA. Cell/tissue incubations were stopped by removal of the media and freezing in liquid nitrogen. AMPK activity was assayed in α-AMPK immunoprecipitates of the cells

and/or tissue homogenates similar to Sakoda et al. (2002). Briefly cells were lysed in 10 vol/wt of Buffer A [50 mM Tris-HCl (pH 7.5), 50 mM NaF, 5 mM Sodium Pyrophosphate, 1 mM EDTA, 1 mM DTT, 0.1 mM Phenylmethylsulfonyl fluoride, 10 % glycerol] containing 1% Triton X-100, 5 μM aprotinin, leupeptin, and pepstatin. Following lysis samples were centrifuged at 12,000 x g for 20 minutes at 4^{o}C to remove insoluble material. Supernatants were collected and protein was quantitated using BCA reagents from Pierce (Rockford, IL). Supernatants were aliquoted containing equal amounts of protein (700 μg) and immunoprecipitated overnight at 4^{o}C with 2.5 μg anti-AMPKα (Cell Signaling Technology/New England BioLabs; Beverly, MS). The resultant immuno-complexes were precipitated using protein A agarose beads (Amersham Bioscience Corp; Piscataway, NJ) after which they were washed according to Sakoda et al. (2002). The AMPK activity in the immunoprecipitates was determined as a function of phosphorylation of SAMS peptide as reported by Davies et al. (1989). Assay reagents (Sakoda et al., 2002) were added directly to the immunoprecipitate and incubated for 15 minutes at 30^{o}C while shaking. Aliquots were removed and spotted onto circle filters (Whatman). Filters were washed three times with 1% H_3PO_4, once with acetone and then air dried in a hood. Filters were counted with 5 mL Cytoscint (ICN; Irvine, CA) and activity reported as fold increase over control.

Immunoprecipitation and Western Blot Analyses

Adipocytes were incubated with given treatments for six hours in DMEM with 3% BSA. Cell incubations were stopped by removal of the media and cells were washed twice with PBS. Cells were lysed in 10 vol/wt of Buffer A [50 mM Tris-HCl (pH 7.5), 50 mM NaF, 5 mM Sodium Pyrophosphate, 1 mM EDTA, 1 mM DTT, 0.1 mM Phenylmethylsulfonyl

fluoride, 10 % glycerol] containing 1% Triton X-100, 5 μM aprotinin, leupeptin, and pepstatin. Following lysis samples were centrifuged at 6,000 x g for 10 minutes at 4^{o}C to remove insoluble material. Supernatants were collected and protein was quantified using BCA reagents from Pierce (Rockford, IL). Supernatants were aliquoted containing equal amounts of protein (250 μg) and immunoprecipitated at room temperature for 2 h using the Catch and Release v2.0 Reversible Immunoprecipitation System from Upstate Cell Signaling Solutions (Charlottesville, VA). Phospho-AMPK Thr-172 (Cell Signaling Technologies, Danvers, MA) was immunoprecipitated with 1:100 primary antibody dilutions in 250 μl total volume. Phospho-ACC Ser-79 (Upstate Biotechnology;) was immunoprecipitated with 1:100 primary antibody dilutions in 250 μl total volume. Protein complexes were eluted from the column using 40 μl of 1X denaturing elution buffer containing βmercaptoethanol from the spin columns. Total eluent protein was separated by SDS-PAGE using a 12% resolving gel and 4% stacking gel. Proteins were transferred to a nitrocellulose membrane and probed with the primary antibody for phospho-AMPK or phospho-ACC at a concentration of 1:1000 at room temperature overnight in 5% BSA solution. Blots were probed for 1 h at room temperature with a 1:20,000 dilution of Goat-Anti-rabbit IgG –HRP antibody (Pierce Cat# 31460) and developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Statistical Analysis

All data were checked for normal distribution prior to mixed model analysis. The fixed effects were treatment and time and the random effect was pig. The main effects (treatment and time) were tested using proc mixed analysis blocking for variation across

animals. When protected by a significant F-test, mean separation was accomplished using the least squares means separation (pdiff) procedure (SAS, 1999; Cary, NC).

3.4 Results

Porcine Adiponectin mRNA expression is regulated by adiponectin, TNFα, and isoproterinol

To examine the regulation of porcine adiponectin mRNA abundance in porcine primary adipocytes, cells were isolated and cultured for 6 h in DMEM with 30 μg/mL adiponectin, 20 ng/mL leptin, 10 ng/mL TNFα, 10 ng/mL IL-6, 20 mU/mL insulin, 25 mM glucose, 10 μg/mL LPS and 10^{-6} M isoproterenol. Adiponectin mRNA abundance was up-regulated in porcine adipocytes by treatment with adiponectin itself by 5.7-fold ($P < 0.05$), by 6.6-fold with TNFα treatment ($P < 0.01$), and by 5.2-fold with isoproterenol treatment ($P < 0.05$) (Figure 3.1). In addition, there was a trend for insulin up-regulation of adiponectin message in adipocytes ($P < 0.1$; Figure 3.1). However, leptin, IL-6, 25 mM glucose (high glucose), and LPS treatment did not affect adiponectin mRNA abundance after 6 h of culture ($P > 0.05$; Figure 3.1). Data show differential regulation of adiponectin mRNA abundance by adipocytokines, inflammatory mediators, and regulators of lipid/energy balance in the adipocyte at acute 6 h time points.

Adiponectin regulation of genes involved in cellular energy balance

Regulation of mRNA abundance of the genes, AMPK, ACC, PPARα, ACO and UCP3 in pig primary adipocyte cultures treated with 30 μg/mL recombinant porcine adiponectin for 0, 30, 60, 120, 240, and 360 minutes were analyzed by real time quantitative PCR analysis. Semi-quantitative western blot analysis was used to determine

the regulation of phospho-AMPK or phospho-ACC by adiponectin, and protein levels of PPARα were only evaluated at the 360 minute time point.

Adiponectin regulation of AMPK mRNA, phosphorylation of AMPK, and AMPK activity in porcine adipocytes

Porcine adiponectin transiently regulated mRNA abundance of AMPK over the 360 minutes (Figure3.2). Significant treatment effects were detected between control and adiponectin treated cells at 30, 120, 240, and 360 min ($P < 0.05$; Figure 3.2). The regulation of AMPK mRNA was differentially regulated depending on the time point of measurement. Adiponectin treatment of adipocytes initiated a 4.4-fold increase in AMPK mRNA abundance at 30 minutes compared with control cells at the same time point (trt effect, $P = 0.03$; Figure 3.2). At 60 minutes there is no difference between control and adiponectin treated cells on AMPK mRNA abundance, however by 120 minutes adiponectin down-regulated AMPK by 2.0-fold ($P = 0.03$). Followed by an increase in AMPK mRNA abundance with adiponectin treatment at 240 minute ($P < 0.05$), and a trend for a decrease in AMPK mRNA at 360 minutes stimulated by adiponectin ($P < 0.05$). Interestinly, the regulation of AMPK mRNA was differentially regulated by time in the control verses adiponectin treated cells. While AMPK mRNA abundance declined in adiponectin treated cells from 30 minutes to the 6 h final time point (time effect, $P < 0.05$; Figure 3.2), there was a quadratic pattern of AMPK mRNA expression in the untreated cells ($P < 0.05$; Figure 3.2).

To determine if adiponectin regulation of AMPK mRNA abundance translates into regulation of AMPK protein expression we measure phosph-AMPK levels by semi-quantitative western blots in porcine adipocytes treated with adiponectin and IL-6 over a

6 h time course. Due to large animal to animal variation in phosphorylation of AMPK we did not see any significant treatment effects of adiponectin or IL-6 compared to control cells ($P > 0.05$; Figure 3.3). Interestingly, there was a trend for a significant effect of time seen in control, adiponectin and IL-6 treated cells ($P < 0.1$; Figure 3.3). The effect of time is similar between untreated and treated cells through the first 2 h of culture. We saw a transcient acute increase in phospho-AMPK in the first 30 minutes of incubation followed by decreases through 120 minutes regardless of treatment. Adiponectin and IL-6 caused a sharp increase in phospho-AMPK from 120 minutes to 240 minutes ($P < 0.1$; Figure 3.3), and while the increase in phospho-AMPK was maintained through 360 min with adiponectin treated cells, IL-6 cells at 360 minutes had a decrease in phospho-AMPK from 240 min to 360 min (Figure 3.3). As with phospho-AMPK measurements we found large animal to animal variation in the measurements of AMPK activity and while there were trends for effect of time we did not detect significant difference of treatment or time in AMPK activity ($P > 0.05$; Figure 3.4).

Adiponectin regulation of ACC mRNA and ACC-phosphorylation in porcine adipocytes

Acetyl-CoA Carboxylase mRNA abundance was measure over a 6 hour time course with or without adiponectin treatment (Figure 3.5). There was a trend for a treatment effect of adiponectin on ACC mRNA abundance compared to control cells ($P = 0.1$). This trend can be attributed to an initial 18% increase of ACC mRNA at 30 minutes compared to control cells and small decreases in ACC mRNA at 120 and 240 minute cultures (Figure 3.5). A time effect on ACC mRNA abundance was detected regardless of treatment with ACC mRNA decreasing from 0 to 360 minutes ($P < 0.05$; Figure 3.5).

Acetyl CoA Carboxylase is regulated minute by minute by phosphorylation state. The inactive, phosphorylated, state of ACC is regulated by upstream kinases like AMPK. An experiment studying the effects of the adipocytokines, adiponectin and IL-6, on phospho-ACC protein expression detected large animal to animal variation, therefore, we were unable to detect any significant effects on phopho-ACC protein expression in these studies (Figure 3.6; $P > 0.05$).

Adiponectin regulation of PPARα mRNA and PPARα protein in porcine adipocytes

Peroxisome proliferator-activated receptor-alpha (PPARα) is a transcription factor which plays a major role in lipid metabolism. The oxidative properties of cells have been linked to the activation of PPARα and it's associated with a key gene involved in fatty acid oxidation, acetyl CoA oxidase (ACO). We studied the regulation of PPARα mRNA by adiponectin over 6 h. At 30 minutes adiponectin increased PPARα mRNA abundance by 32% compared with control cells (Figure 3.7; $P < 0.01$). Transcript abundance then declined from 30 minutes to 120 minutes and remained unchanged for the next four hours of measurement ($P < 0.05$; Figure 3.7). A similar trend of PPARα mRNA abundance was seen over the six hours for control cells too. However, measurement of PPARα protein at 6h following adiponectin treatment was up-regulated compared with control cells in porcine adipocytes (Figure 3.8).

Adiponectin regulation of ACO and UCP3 mRNA in porcine adipocytes

Regulation of ACO mRNA abundance by adiponectin in porcine adipocytes depicted similar results to other mRNA measure. Intially after 30 minute incubations ACO mRNA was increased by adiponection compared to control cells ($P < 0.05$; Figure

3.9). However, remaining time points showed no difference in ACO mRNA compared to control cells.

Uncoupling protein 3 (UCP3) expression has been shown to be highly correlated with oxidative fatty acid metabolism in multiple cell types, therefore, determining the UCP3 message expression in adipocytes maybe indicative of the oxidative capacity of the adipocyte. However, we did not detect difference in UCP3 mRNA abundance in adiponectin treated cells compared to the control (Figure 3.10). Again, over the time course there was a significant decrease in UCP3 mRNA from 30 to 360 minutes regardless of treatment ($P < 0.05$; Figure 3.10).

3.5 Discussion

The role of adiponectin in the regulation of energy metabolism could provide significant insight into the underlying mechanisms whole body energy balance in homeostatic states, as well as, provide valuable information regarding the dysfunction during metabolic diseases. The hormonal and biochemical pathways associated with the development of obesity, insulin resistance, diabetes and metabolic syndrome are poorly understood. Adiponectin is inversely correlated with obesity. However, the regulation of adiponectin expression in adipose tissue and if adiponectin plays a role in autocrine/paracrine regulation of lipid metabolism in the adipocyte is not understood. Herein, we report the regulation of porcine adiponectin expression *in vitro* by TNFα and isoproterenol, and show that adiponectin augments its own expression. We also define potentially key roles of adiponectin in the regulation of genes involved in fatty acid synthesis and oxidation.

Acute treatment of porcine adipocytes with adiponectin caused a significant increase in its mRNA abundance. This is the first report of adiponectin regulating its own mRNA abundance. This is particularly exciting, since Ajuwon and Spurlock (2005) reported adiponectin stimulates PPARγ2 protein expression in adipocytes, and that PPARγ induces adiponectin expression. This mechanism may be an auto-regulatory loop by which adiponectin can regulate energy homeostasis in the adipocyte and thereby regulate chronic inflammation known to develop during period of abnormal energy balance.

We also determined the effect of other adipocytokines, insulin, high glucose concentrations (i.e., 5 vs. 25 mM), LPS and β-adrenergic agonist, isoproterenol, on adiponectin mRNA abundance. In contrast to most reports in the literature, we found TNFα significantly increased adiponectin mRNA after 6 hours. While the literature firmly supports the pro-inflammatory cytokine down-regulation of adiponectin mRNA and protein synthesis in 3T3-L1 adipocytes and human adipocytes (Fasshauer et al., 2002; Kappes and Loffler, 2000; Maeda et al., 2001), there could be discrepancies between culture conditions and time which cause these differences. First, Fasshauer et al. (2002) cultured 3T3-L1 adipocytes for a chronic time period of 16 hours in the presence of TNFα, compared to our acute time point of 6h. Second, there could be differences in glucose concentrations used in the culture media. As Lin et al. (2005) demonstrated there are significant differences in the ability of 3T3-L1 adipocytes to respond to insulin stimulated glucose uptake depending if the cultures are maintained in euglycemic vs. hyperglycemic conditions. Therefore, these cultures may have been carried out under euglycemic condition where the regulation of adiponectin expression may be different

from the regulation seen under hyperglycemic conditions, the condition in which our experiments were performed. The same arguments could be used in discussing the difference seen in β-adrenergic agonist regulation of adiponectin in porcine primary adipocytes. While Delporte et al. (2002) report a 30% down-regulation of adiponectin mRNA in human adipose explants by isoproterenol, we show approximately an induction of adiponectin mRNA in 6 hour cultures. The differences between these experiments are not significant in acute and chronic regulation because the explants were cultured only for 8 hours. However, part of the differential regulation may be attributed to the difference in adipose depot. The human explants were extracted from visceral adipose tissue whereas we utilized the subcutaneous adipose depot. In summary, there may be a common thread between TNFα and isoproterenol regulation of adiponectin at acute time point because both stimulate acute FFA release from the adipocyte.

The literature is very much divided on insulin regulation of adiponectin. One of the most interesting findings we report here is adiponectin can significantly induce the transcription of its own transcript. This is an intriguing concept that needs to be investigated further to determine if adiponectin plays a role in modulating its protein expression.

The autocrine regulation of adiponectin on lipid metabolism in the adipocyte has been reported by Jacobi et al. (2004) showing a down-regulation of lipogenesis by full length porcine adiponectin. A potential mechanism for this was also demonstrated by Wu et al. (2003) who determined that globular adiponectin induces phosphorylation of AMPK and ACC in rat primary adipocytes. In pig adipocytes, adiponectin transiently increased the expression of ACC and AMPK at the mRNA level at 30 minutes. The

regulation at the mRNA level over a 6 h period was cyclical for both enzymes, which perhaps reflects a tightly regulated coupling of these enzymes. In the current work we also determine phosphorylation state of AMPK and ACC in pig adipocytes. In contrast to Wu et al. (2003), although we saw numerical increases in phospho-AMPK and phospho-ACC they were not statistically significant. These differences may be attributed to adipocytes derived from different species, different isoforms of adiponectin, or the fact that our culture system was under hyperglycemic conditions compared with their euglycemic conditions. Altogether, these differences between culture systems could have a significant impact on the ability of adiponectin to regulate phosphorylation of AMPK and ACC.

While there is little information on the oxidative capacity of the adipocyte, it has been well documented adiponectin induces oxidation in skeletal muscle and liver (Hu et al., 1996; Maeda et al., 1996; Nakano et al., 1996; Scherer et al., 1995). Therefore, we hypothesized that adiponectin may impact the oxidative capacity of the adipocyte and convert the adipocyte from an energy storage cell to an energy consuming cell. In fact at 30 min time point, adiponectin stimulates PPARα and ACO mRNA indicating there is a potential for increased oxidative capacity of adipocytes. Adiponectin also increases PPARα protein in porcine adipocytes at 6 h of treatment, indicating there is an increased potential for oxidative capacity of the adipocyte given that PPARα is a key transcription factor in regulating genes of fatty acid oxidation. Additionally, adiponectin also significantly enhanced UCP3 mRNA at 6 h post treatment. Altogether, these data suggest adiponectin can modulate glucose and lipid metabolism in the adipocyte potentially be regulating cell size through modulation of fatty acid synthesis and

oxidation. This hypothesis is corroborated by Claret et al. (2005) who demonstrated tungstate fed to diet-induced obese rats increases fatty acid oxidation in adipose tissue and limits excessive production of ROS by induction of uncoupling proteins. As well as, Li et al. (2005) demonstrated chronic stimulation of β-adrenegic signaling in adipocytes remodels WAT in two manners. First, β-adrenergic stimulation induces an immediate pro-inflammatory response which is correlated with suppressed adipocyte-specific genes. Second, β-adrenergic activation stimulated mitochondrial biogenesis and up-regulated genes involved in fatty acid transport, activation and oxidation. These data and the data presented here suggest the adipocyte has metabolic machinery and the plasticity to increase its catabolic activity to protect against excess nutrient induced insulin resistance and adiponectin may be a key regulator of the system.

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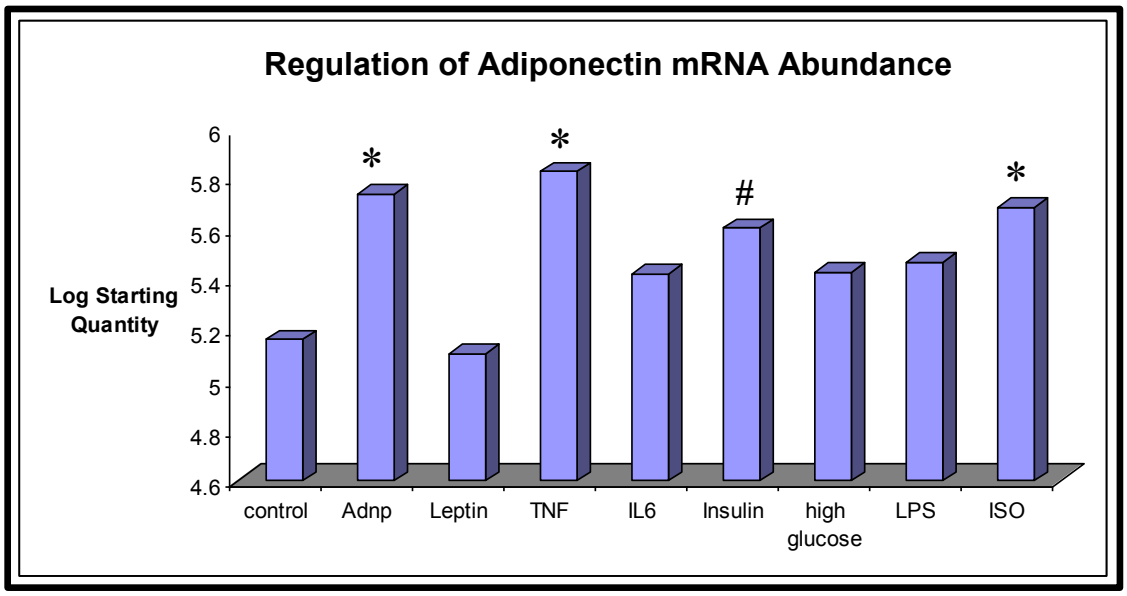

Figure 3.1 Regulation of adiponectin mRNA abundance in porcine adipocytes. Adipocytes from five pigs (n=5; 6 replicates within pig) were isolated and cultured for 6 hours the presence of indicated treatments (30 µg/mL pAdn, 20 ng/mL leptin, 10 ng/mL TNFα, 10 ng/mL IL-6, 20 mU/mL insulin, 25 mM glucose, 10 µg/mL LPS and 10^{-6} M isoproterenol). *indicates treatment different from control at $P < 0.05$; #indicates treatment different from control at $P < 0.1$; standard error = 0.12.

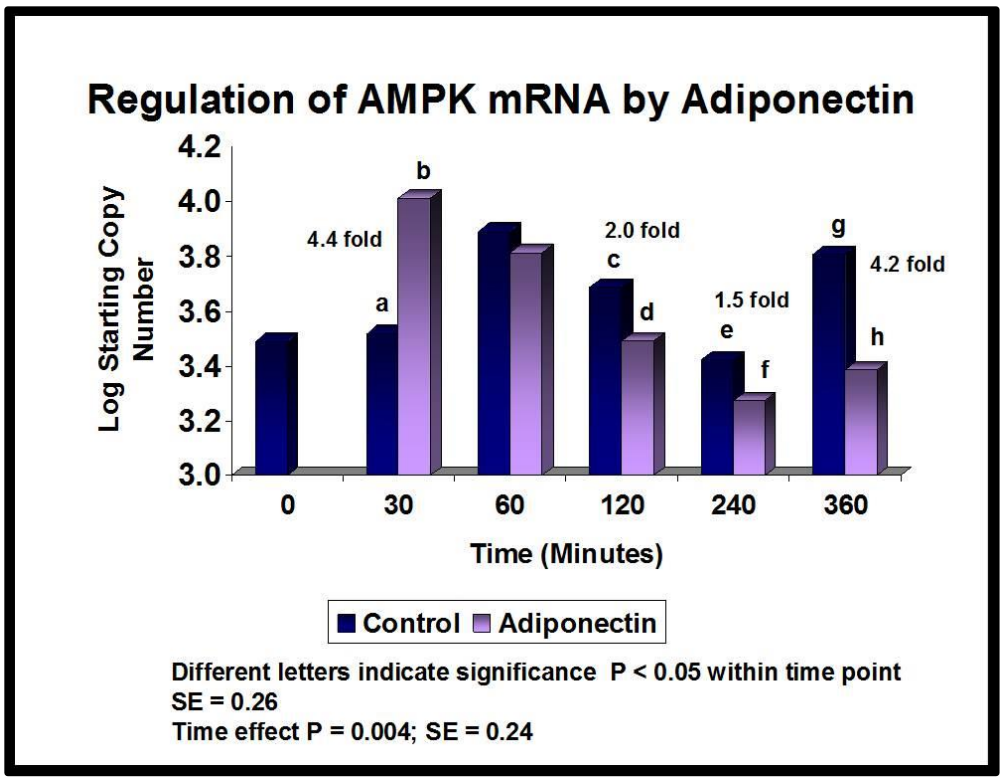

Figure 3.2Effect of adiponectin on AMPK mRNAabundance pig adipocytes from 0 to 6 hours.
Adipocytes were isolated and cultured in the presence or absence of adiponectin (30 µg/mL adiponectin) (n=5 pigs; 6 replicates within pig). Different letters indicate significant difference between control and adiponectin treated cells within a time point; $P < 0.05$; standard error = 0.26. The main effect of time was significant at $P = 0.04$; standard error = 0.24.

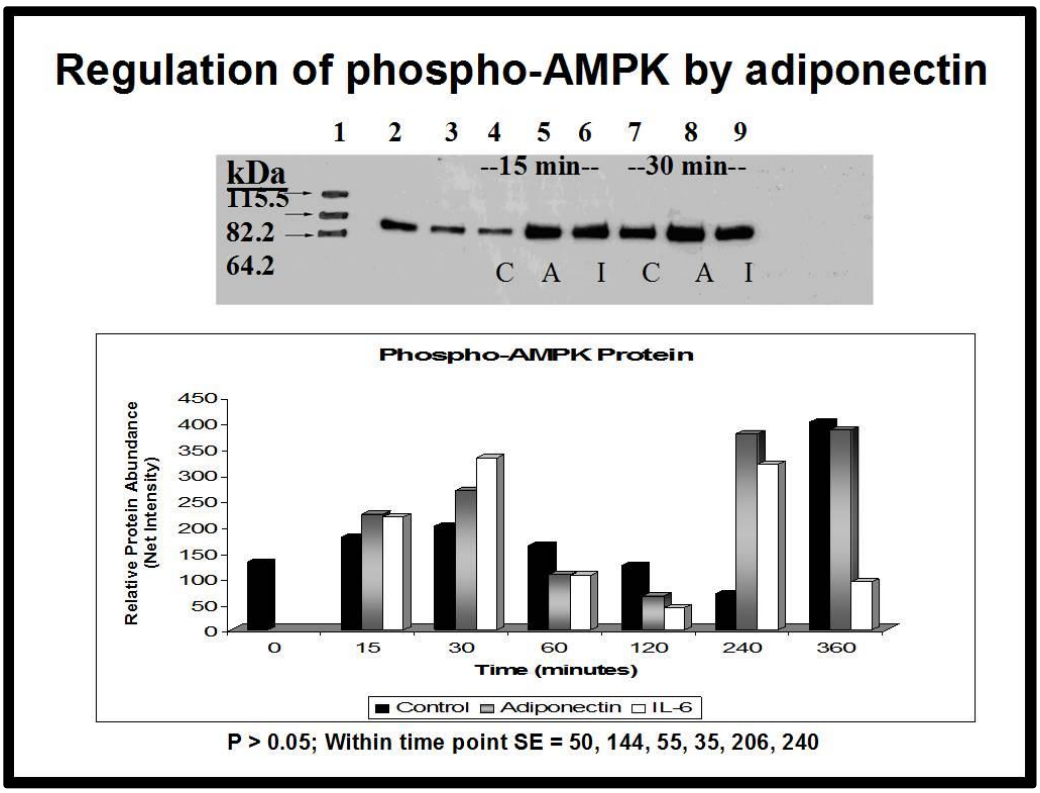

Figure 3.3Effect of adiponectin and IL-6 on phosphorylation of AMPK in pig adipocytes. Adipocytes were cultured in the presence or absence of adiponectin (30 µg/mL) or IL-6 (20 ng/mL). (n=5 pigs; 6 replicates within pig) Standard error = 50, 144, 55, 35, 206, and 240, respectively for increasing time.

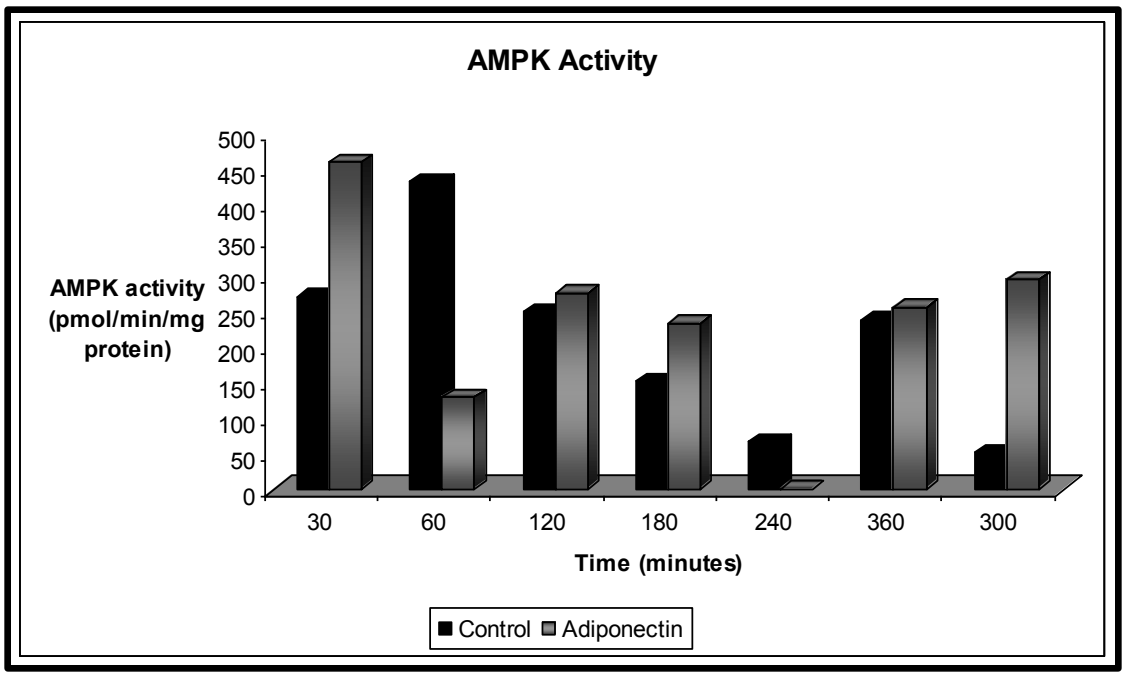

Figure 3.4.Effect of adiponectin on AMPK activity in pig adipocytes
Adipocytes were cultured for given time points in the presence or absence of adiponectin (30 μg/mL adiponectin). (n=5 pigs; 6 replicates within pig); standard error = 199

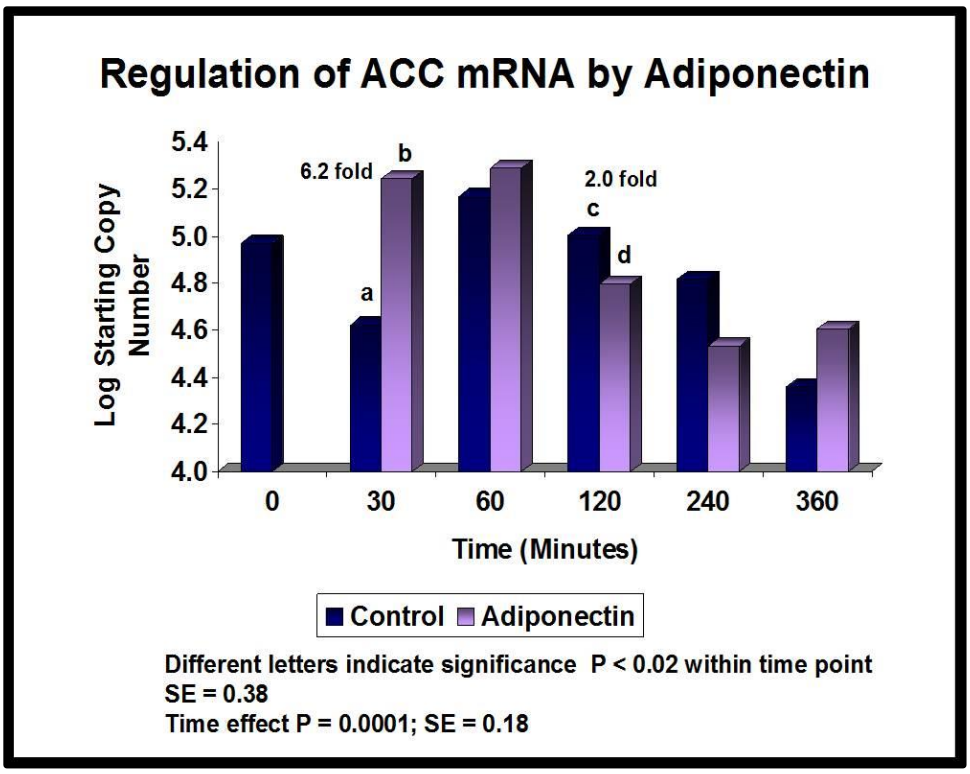

Figure 3.5Effect of adiponectin on ACC mRNA abundance in pig adipocytes. Adipocytes were cultured for given time points in the presence or absence of adiponectin (30 µg/mL adiponectin). (n=5 pigs; 6 replicates within pig); different letters indicate significance difference within a time point at P< 0.05. standard error = 0.18.

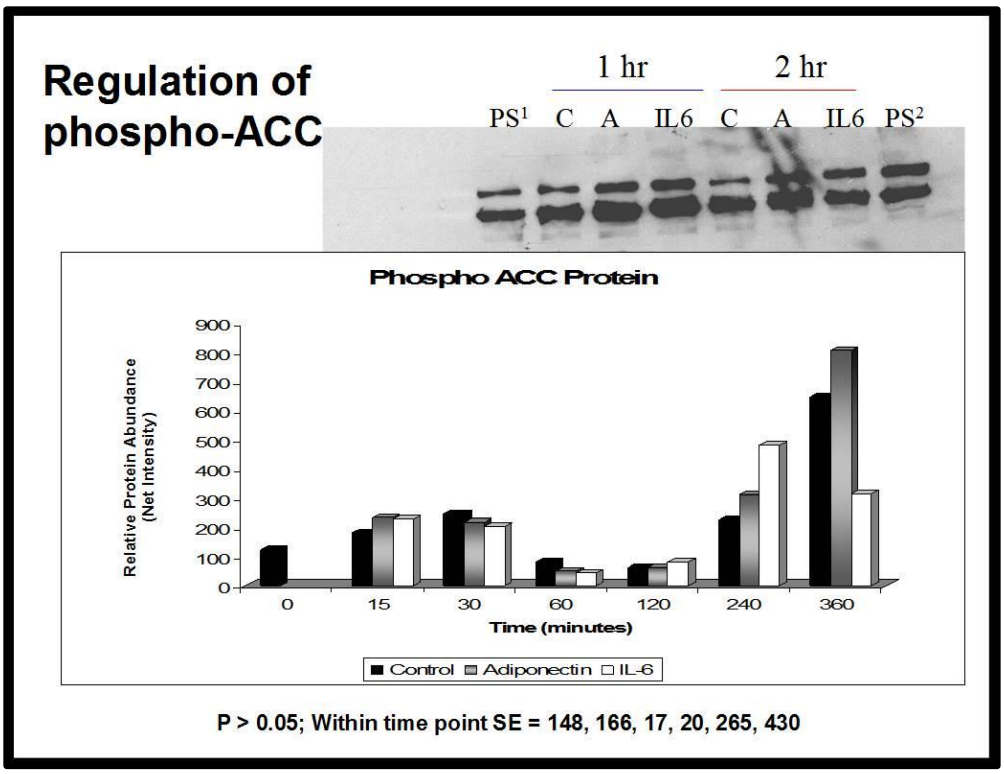

Figure 3.6 Effect of adiponectin and IL-6 on phosphorylation of ACC in pig adipocytes. Adipocytes were cultured for given time points in the presence or absence of adiponectin or IL-6 (30 µg/mL adiponectin and 20 ng/mL IL-6). (n=5 pigs; 6 replicates within pig) Standard error = 148, 165, 17, 20, 265, and 430 respectively.

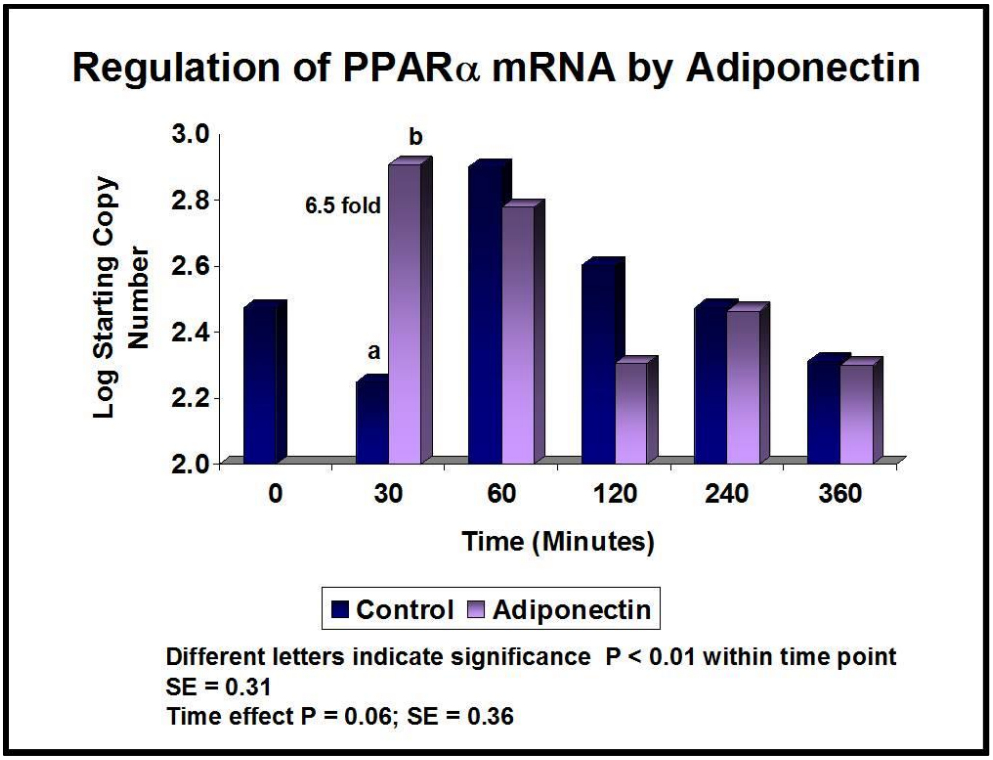

Figure 3.7.Effect of adiponectin on PPARα mRNA abundance in pig adipocytes. Adipocytes were cultured for given time points in the presence or absence of adiponectin (30 µg/mL adiponectin). (n=5 pigs; 6 replicates within pig) Different letters indicate significance of P< 0.05; standard error = 0.31.

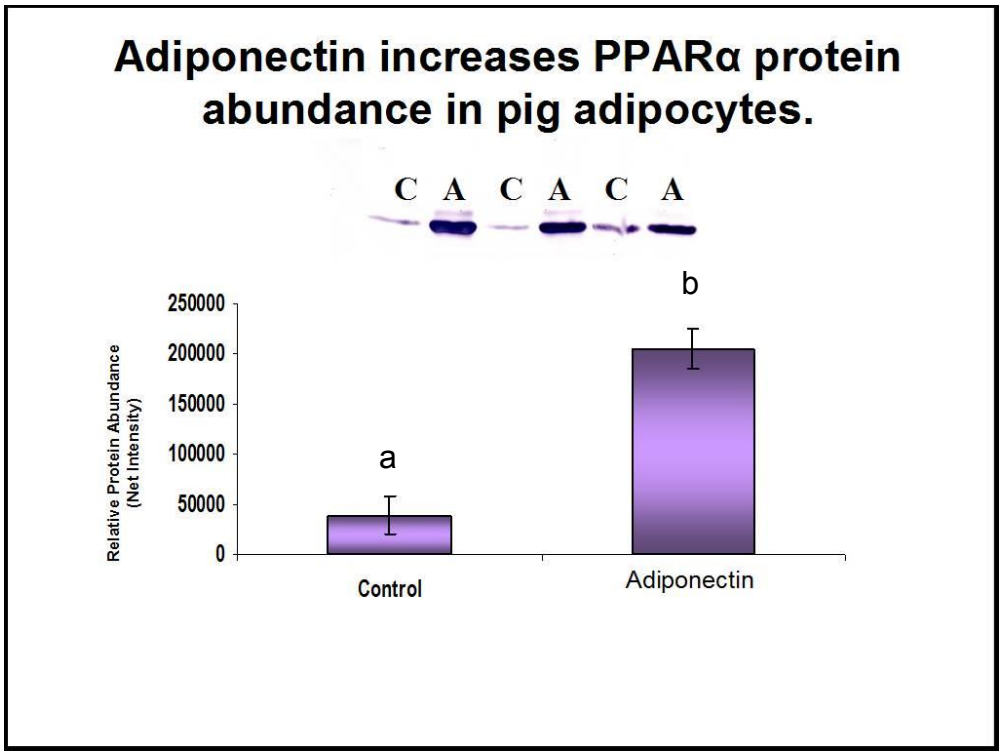

Figure 3.8Effect of adiponectin on abundance of PPARα protein pig adipocytes. Adipocytes were cultured for 6 h in the presence (-) or absence (+) of adiponectin (30 µg/mL adiponectin). (n=3 pigs; 6 replicates within pig) Different letters indicate significant treatment difference $P < 0.05$.

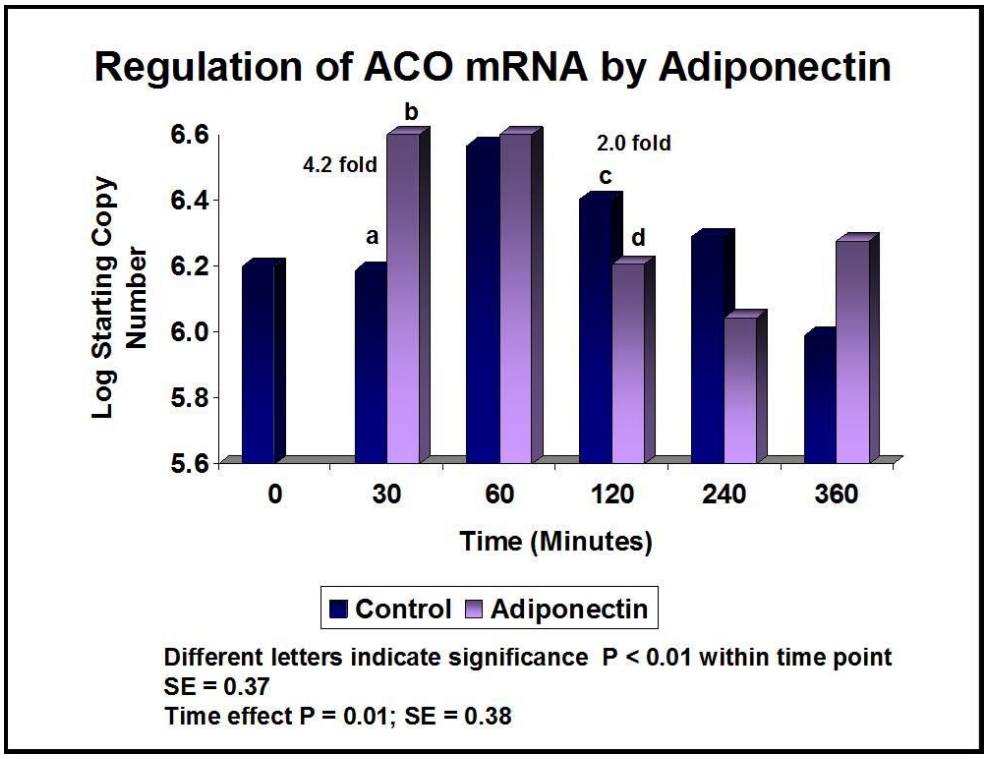

Figure 3.9. Effect of adiponectin on ACO mRNA abundance in pig adipocytes. Adipocytes were cultured for given time points in the presence or absence of adiponectin (30 µg/mL adiponectin). (n=5 pigs; 6 replicates within pig) Different letters indicate significant difference $P < 0.01$. Standard error = 0.35, 0.29, 0.37, 0.45, and 0.47.

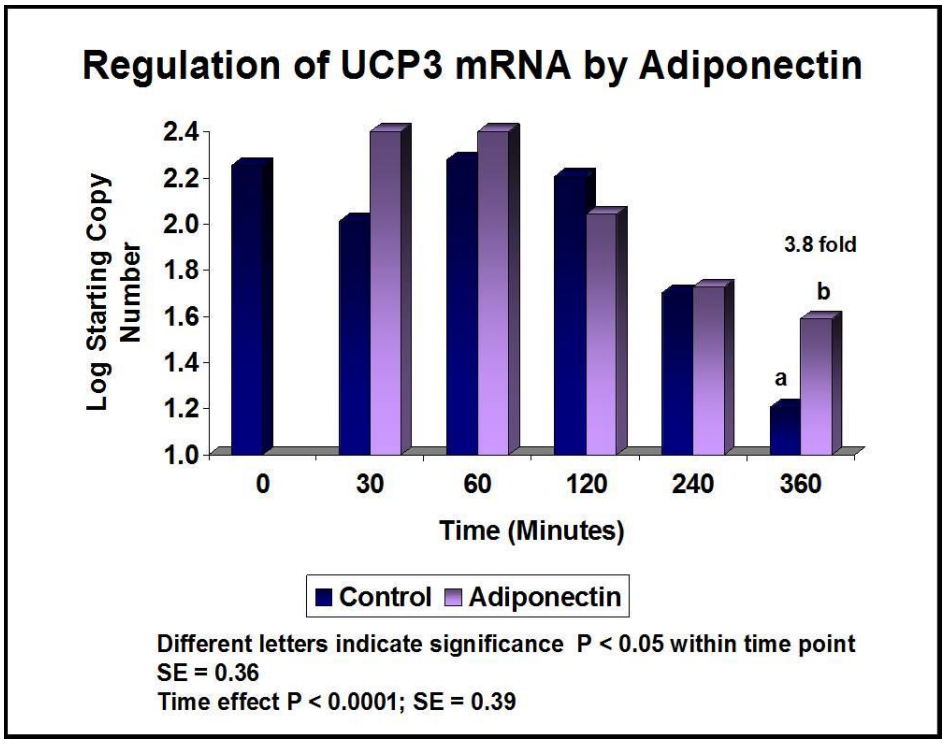

Figure 3.10 Effect of adiponectin on UCP3 mRNA abundance in pig adipocytes. Adipocytes were cultured for given time points in the presence or absence of adiponectin (30 µg/mL adiponectin). (n=5 pigs; 6 replicates within pig). Different letters indicate significance of $P < 0.05$. standard error = 0.36.

CHAPTER 4. PRO-INFLAMMATORY CYTOKINES AND GLUCOSE REGULATE EXPRESSION OF ADIPONECTIN RECEPTORS IN PORCINE ADIPOCYTES

4.1 Abstract

The anti-inflammatory activity of adiponectin may contribute to its insulin sensitizing effects. Two adiponectin receptors have been identified as adipoR1 (R1) and adipoR2 (R2), and down regulation of these receptors may promote insulin resistance. The aim of this research was to determine if tumor necrosis factor α (TNFα), interleukin-6 (IL-6), or hyper-glycemic conditions regulate receptor expression in adipocytes, and whether inhibition of the JAK-STAT pathway by AG490 alters this regulation. Primary adipocytes were prepared from subcutaneous adipose tissue of pigs (n=3). Cells were incubated ± TNFα or IL-6 (30 ng/mL), with or without pretreatment of 10 μM AG490, in hyper-glycemic or normo-glycemic conditions for 6 h. Total RNA was extracted and gene expression determined by real time PCR. There was no effect of cytokines at 5.5 mM glucose for R1 expression. However, there was a trend for a down-regulation of R2 by AG490-TNFα ($P < 0.07$). At 25 mM glucose, there was an increase in R1 by AG490-TNFα treatment ($P = 0.06$). R2 was marginally reduced by IL-6, but there was a reduction ($P < .01$) with AG490 plus IL-6. High glucose caused a reduction of both receptors, whereas TNFα increased both in the high glucose media. R1 ($P < 0.1$) and R2 ($P < 0.05$) were further elevated by AG490 plus TNFα in high glucose. Interestingly,

IL-6 and AG490 plus IL-6 ($P < 0.05$) reduced R1 and R2 expression, but only in the high glucose media. Collectively, these data indicate that the effects of TNFα and IL-6 on adiponectin receptor expression are influenced by glucose concentration, and that the JAK-STAT pathway may be a determinant of adiponectin receptor expression.

4.2 Introduction

Adipose tissue is a functional endocrine organ involved in regulating its own metabolism, as well as whole body energy homeostasis. Adiponectin is an adipokine which moderates whole body glucose and energy metabolism. Additionally, adiponectin is involved as a paracrine and autocrine regulator of white adipose tissue (WAT) metabolism (Wu et al., 2003; Aujwon et al, 2004, 2005; Jacobi et al., 2004; Kadowaki and Yamauchi, 2005). The mechanisms of adiponectin action have been investigated by multiple researchers, who demonstrated different forms of adiponectin have potentially different biological functions (Pajvani et al., 2004). The pathways which regulate the synthesis of different forms of adiponectin have been elusive; however, there are thoughts that different forms may be involved in regulating glucose metabolism versus regulating inflammation.

While the form of adiponectin may have a critical role in determining physiological responses, receptor mediated cell-signaling will be integral in understanding adiponectin's physiological responses. Adiponectin signaling appears to be predominantly through the recently cloned adipoR1 and adipoR2 receptors (Yamauchi et al., 2003). AdipoR1 is ubiquitously expressed and most abundantly expressed in skeletal muscle, whereas AdipoR2 is most abundantly expressed in liver.

Hyperinsulinemia induces a two-fold increase of adipoR1 in skeletal muscle (Debard et al., 2004). Additionally, Chinetti et al. (2004) demonstrated pharmacological regulation of adipoR2 by synthetic PPAR ligands, thiazolidinediones (TZD) and fibrates in human macrophages. Bluher et al. (2005) demonstrated both receptors are expressed in adipose tissue, and adipoR2 seems to be more abundant in porcine subcutaneous adipose tissue than adipoR1 (Ding et al., 2004). Therefore, it seems plausible that the regulation of adiponectin receptors in white adipose tissue could be important in regulation of glucose metabolism and whole energy balance. In fact, Tuschida et al. (2005) provided the first evidence demonstrating activation of PPARs may play a critical role in adiponectin and adiponectin receptor expression. This information led them to hypothesize that activation of PPARα suppresses inflammation in white adipocytes in part by suppressing adipocyte hypertrophy. Secondly, dual activation of PPARα and PPARγ may lead to induction of adiponectin receptors, as well as regulation of different forms of adiponectin present. Previously we reported adiponectin suppress lipogenesis in porcine adipocytes (Jacobi et al., 2004) and induces PPARα, both findings supporting Tuschida et al. (2005) report that adiponectin induces PPARs and reduces inflammation. In addition, reports from our lab have also shown adiponectin activates PPARγ2 and inhibits TNFα and IL-6 secretion (Ajuwon et al., 2005). Therefore, the hypothesis of the current study was that down-regulation of adiponectin receptors by pro-inflammatory cytokines antagonizes the anti-inflammatory action of adiponectin to potentiate the innate immune response in adipocytes, and this differentially regulated under euglycemic and hyperglycemic conditions.

4.3 Methods

Animals and adipocyte isolation

Adipose tissue was obtained from five male castrates weighing approximately 90-110 kg consuming a nutritionally-complete diet ad libitum. The pigs were maintained according to protocols approved by the Purdue University Animal Care and Use Committee, and killed in the Purdue University abattoir according to USDA regulations. Subcutaneous adipose tissue was removed from the dorsal depot located over the cervical spine, and transported in buffered saline (37^{o}C, 0.15 M NaCl, 10 mM HEPES, pH 7.4) to the laboratory. Adherent skeletal muscle was removed, and the tissue was cubed and sliced with a Stadie-Riggs microtombe. Approximately 6-7 g of sliced tissue was minced, rinsed with saline, and transferred to a flask containing 15 mg collagenase (100 U/mL final concentration; Worthington BioChemical, Lakewood, NJ USA). Following a 30-35 minute digestion period, isolated adipocytes were washed through a 290 μm screen with warmed Krebs Ringer bicarbonate buffer (pH 7.4) containing 10 mM HEPES, 5 mM glucose, and 3% bovine serum albumin (Fraction V, essentially fatty acid free, Invitrogen Life Technologies, Carlsbad, CA USA) to remove all other cell debris.

The isolated adipocytes were resuspended in culture media (DMEM Sigma D6780; St. Louis, MO USA) containing 3% BSA and 10 mM HEPES at a final concentration of approximately 2 x 10^5 cells per mL and aliquoted immediately into 15 mL polypropylene scintillation vials for incubation. Porcine adipocytes were then treated with either recombinant porcine TNFα (30 ng/mL), recombinant porcine IL-6 (30 ng/mL), 10 μM AG490, 10 μM darglitazone, and/or differing levels of glucose concentration as indicated under actual experiments.

Real time quantitative PCR

Total RNA was recovered from cells using Trizol reagent (Invitrogen, Carlsbad, CA) and DNase treated using the Turbo DNase® (Ambion; Houston, TX). RNA concentration of sample was determined using the RiboGreen® assay (Molecular Probes, Eugene, OR USA) according to the manufacturer's protocol. Total RNA (2 μg) was reverse transcribed using the iScript cDNA synthesis kit (BioRad; Hercules, CA). Primer sequences were: pig adipoR1, 5'-attgctacaggactcattcg-3' and 5'-tagccattacggattact-3'; pig adipoR2, 5'-attacggttaacgttact -3', 5'-aggcttatcgactatggtta -3'and pig 18S, 5'-ttaagccattaccagatata-3' and 5'-agattcattcatgcattaag-3', all sequences are sense and anti-sense, respectively. Thermal cycler conditions for PCR reactions were 95 ^{o}C for 3 minutes followed by 36 cycles of 95 ^{o}C for 30 seconds, 62 ^{o}C for 30 seconds, and 72 ^{o}C for 30 seconds. Polymerase chain reaction products were cloned into pGEMT vector (Promega, Madison, WI) and sequenced for verification of identity. Real-time reactions were carried out on an iCycler real-time machine (BioRad, Hercules, CA) using the IQ™ SYBR Green Supermix kit (BioRad, Hercules, CA). Plasmid containing the gene of interest was serially diluted to create a standard curve of gene copy number. The mRNA abundance of each gene product was determined by regression analysis against the standard curve. The housekeeping gene, 18S RNA, did not differ among treatments or pigs.

Immunoprecipitation of Phospho-Proteins and Western Blots

Adipocytes were incubated with given treatments for two to six hours in DMEM containing 3% BSA. Cell incubations were stopped by removal of the media and cells were washed three times with PBS. Cells were lysed in 10 vol/wt of Buffer A [50 mM

Tris-HCl (pH 7.5), 50 mM NaF, 5 mM Sodium Pyrophosphate, 1 mM EDTA, 1 mM DTT, 0.1 mM Phenylmethylsulfonyl fluoride, 10 % glycerol] containing 1% Triton X-100, 5 μM aprotinin, leupeptin, and pepstatin. Cell lysates were then centrifuged at 6,000 x g for 10 minutes at 4^{o}C to remove any insoluble material. Supernatants were collected and protein was quantified using BCA reagents from Pierce (Rockford, IL). Supernatants protein (250 μg) was then immunoprecipitated at room temperature for 2 h using the Catch and Release v2.0 Reversible Immunoprecipitation System from Upstate Cell Signaling Solutions (Charlottesville, VA). The immunoprecipitate reaction was in a total volume of 250 μl and in the presence of AdipoR1 (G-001-44; Phoenix Pharmaceuticals; Belmont,CA) or AdipoR2 (G-001-23;Pheonix Pharmaceuticals; Belmont, CA) antibodies at 1:100 or 1:50 dilutions, respectively. Protein complexes were eluted from the column using 40 μl of 1X denaturing elution buffer containing βmercaptoethanol from the spin columns. The protein was then separated by SDS-PAGE using a 12% resolving gel and 4% stacking gel. Proteins were transferred to a nitrocellulose membrane and probed with the primary antibody for AdipoR1 or AdipoR2 at a concentration of 1:1000 at room temperature overnight in 5% BSA solution. Blots were probed for 1 h at room temperature with a 1:20,000 dilution of Goat-Anti-rabbit IgG – HRP antibody (Pierce Cat# 31460) and developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Cat #34080).

Statistical Analysis

All data were checked for normal distribution prior to mixed model analysis. The fixed effects were treatment and time, and the random effect was replicate. The main effects (treatment and time) were tested using prox mixed analysis. When protected by a

significant F-test, mean separation was accomplished using the least squares means separation (pdiff) procedure (SAS, 1999; Cary, NC).

4.4 Results

Adiponectin receptors are differentially regulated over time and inflammatory cytokines, TNFα and IL-6 modulate

To access adipoR1 and adipoR2 mRNA abundance in primary porcine adipocytes, cells were cultured for 0, 2, 4 and 6 hours in the euglycemic conditions and then collected to total RNA isolation. Porcine primary adipocytes express adipoR1 and adipoR2 mRNA. Interestingly, the mRNA abundance was differentially regulated over time. AdipoR1 mRNA abundance was significantly up-regulated over time in culture from 0 to 6 hours (Figure 4.1; $P < 0.05$), while adipoR2 mRNA abundance was significantly decreased from 0 to 6 hours of culture ($P < 0.05$; Figures 4.2).

Glycemic conditions may modulate adiponectin receptor expression by pro-inflammatory cytokines

It has been well documented hyperglycemia is highly correlated with the development of insulin resistance and adiponectin has insulin-sensitizing properties. However, the understanding of how adiponectin exerts these effects is in the early stages of development, and insulin resistance is correlated with pro-inflammatory mediators. To address the relationship between adiponectin and pro-inflammatory mediators we investigated how the cytokines, TNFα and IL-6 modulate adiponectin receptor mRNA abundance under normal glycemic and hyperglycemic conditions. Porcine adipocytes were cultured for 6 hours in the presence or absence of recombinant porcine TNFα or IL-

6 (30 ng/mL) with or without 10 μM AG490 (JAK-STAT inhibitor) in both 5.5 mM and 25 mM glucose. Under normal glycemic (5.5 mM glucose) conditions, TNFα and IL-6 had no effect on adipoR1 mRNA abundance (Figure 4.3). However in the presence of 25 mM glucose, adipoR1 mRNA abundance was up-regulated compared to control when treated with AG490 + TNFα (Figure 4.3; $P = 0.06$). There was a trend for down-regulation of adipoR2 mRNA abundance when adipocytes were treated with the combination of AG490 + TNFα in 5.5 mM glucose (Figure 4.4; $P = 0.8$). In 25 mM glucose media, AdipoR2 mRNA abundance was marginally reduced by IL-6, but significantly reduced when treated with AG490 + IL-6 (Figure 4.4; $P < 0.05$). To determine if there was a correlation between adipoR1 and adipoR2 message and protein expression preliminary semi-quantitative western blots were ran and analyzed for receptor protein abundance basal media condition, 30 ng/mL TNFα, and 30 ng/mL IL-6 at three different levels of glucose supplementation (5.5, 25, and 40 mM). Preliminary results showed no differences in adipoR1 and adipoR2 protein expression in the presence or absence of TNFα or IL-6 at normo- or hyper-glycemic conditions (Figures 4.5 and 4.6; $P > 0.05$). Collectively, these data indicate that the effects of TNFα and IL6 on adiponectin receptor expression are influenced by glucose concentration, and that the JAK-STAT pathway may be a determinant of adiponectin receptor mRNA expression, however, acutely protein levels were not regulated by these factors.

<u>AdipoR1 and R2 were not acutely regulated at the protein level by glycemic condition or PPARγ agonist</u>

While we found marginal regulation of adipoR1and adipoR2 mRNA abundance by the pro-inflammatory cytokines, TNFα and IL-6, and no regulation of protein

abundance of the adiponection receptors by these pro-inflammatory cytokines in a small sample size, we want to investigate if PPAR signaling hormones or pathways regulate receptor expression. To address this question porcine adipocytes were cultured for 6 hours in basal media alone, plus 30 ug/mL porcine adiponectin, plus 10 μM darglitazone, or the combination of adiponectin and darglitazone in normal or high glucose media. AdipoR1 protein expression was not affected by treatment with adiponectin, darglitazone, or glucose levels (Figure 4.7). Similar to adipoR1 protein expression, there was no significant change in adipoR2 proteins expression by any of the treatments at 5.5 mM glucose concentrations (Figure 4.8). However, adiponectin, darglitazone and adiponectin plus darglitazone increased adipoR2 protein expression in porcine adipocytes cultured in 25 mM glucose containing media (Figure 4.8; $P < 0.05$). These data suggest differential regulation of adipoR2 by adiponectin and darglitazone under differing glycemic conditions.

4.5 Discussion

Regulation of whole body energy homeostasis is regulated by complex biochemical reactions that are modulated by substrate and product abundance, as well as, hormonal regulation. The complexity in maintenance of energy homeostasis is correlated with the abundance of adipose tissue. Adipose tissue once thought to be a passive energy storage depot is now known to produce adipokines needed to maintain whole body energy homeostasis. An imbalance of adipose tissue mass (too little or too much) is associated with diseases metabolic imbalance. The aim our study was to investigate changes in adiponectin receptors expression in cell culture that mimic physiological associated with metabolic disease.

Herein, we provide evidence that pro-inflammatory cytokines, glycemic conditions, adiponectin and PPARγ agonist play a role in the differential regulation of adipoR1 and adipoR2. These findings are of considerable significance for several reasons. Firstly, in addition to obesity and insulin resistance, chronic inflammation accompanies the pre-diabetic condition known as the metabolic syndrome (Yudkin, 2003). Secondly, obesity is accompanied with an increase in macrophage infiltration into white adipose tissue (Weisberg et al., 2003; Xu et al., 2003a) and both adipocyte and macrophage gene profiles reflect inflammation. Furthermore, our lab has shown adiponectin to suppress TNFα and IL-6 production in macrophages and adipocytes (Ajuwon and Spurlock, 2005; Wulster-Radcliffe et al., 2004). Now we found TNFα plus a JAK/STAT inhibitors tends to down-regulate adipoR2 mRNA abundance in adipocytes culture in euglycemic conditions, and under hyperglycemic conditons IL-6 with inhibition of JAK/STAT signaling leads to a 29% reduction in adipoR2 mRNA abundance compared to control

hyperglycemic culture adipocytes. In addition, adipoR2 protein abundance was increased in porcine adipocytes culture in hyperglycemic conditions with the addition of PPARγ agonist, darglitazone and porcine adiponectin compared to control cells. This regulation could indicate differing physiological functions for adipoR1 and adipoR2.

Under conditions of hyperinsulinemia, adipoR1 is induced two-fold in skeletal muscle (Debard et al., 2004). Hyperinsulinemic conditions are reflective of an insulin resistant state, suggesting there is regulation of adiponectin receptors in glucose sensitive tissues during altered metabolic conditions. Consistent with these findings, we demonstrated under acute inflammatory conditions that TNFα down-regulates adipoR2 expression in primary adipocytes. This down-regulation could lead to inability of adiponectin to exert anti-inflammatory effects on the adipocyte and lead to the increased infiltration of macrophages associated with chronic inflammation and obesity. This hypothesis is reasonable considering we have shown that adiponectin up-regulates its mRNA in WAT (data unpublished) and adiponectin and darglitazone increase adipoR2 protein expression in adipocytes under hyperglycemic conditions. Moreover, this regulation of adipoR2 is supported by Chinetti et al. (2004), who demonstrated pharmacological regulation of adipoR2 by synthetic PPAR ligands, thiazolidinediones (TZD) and fibrates in human macrophages. Bluher et al. (2005) demonstrated both receptors to be expressed in adipose tissue, and adipoR2 seems to be more abundant in porcine subcutaneous adipose tissue verses adipoR1 (Ding et al., 2004). Furthermore, Tuschida et al. (2005) have provided the first evidence demonstrating activation of PPARs may play a critical role in adiponectin and adiponectin receptor expression in WAT of KKAy mice. These authors also extended the link between PPARs, adiponectin

and inflammation by demonstrating the PPARα agonist, WY-14643 directly increased expression of both adiponectin receptors in WAT and also decreased monocyte chemoattractant protein-1 in adipocytes and macrophages.

Taken together, these data along with evidence we provide here linking pro-inflammatory cytokines, hyperglycemic state, adiponectin and PPARγ agonist to the regulation of adipoR2 in adipocytes, makes a very strong argument for the hypothesis that the activation of PPARα suppresses inflammation in white adipocytes in part by suppressing adipocyte hypertrophy. Secondly, dual activation of PPARα and PPARγ may lead to induction of adiponectin receptors, as well as, regulation of different forms of adiponectin present compared to total adiponectin. Additionally, this is also supported by the fact we show adiponectin can stimulate PPARα mRNA and protein in isolated porcine adipocytes.

Collectively, these data indicate that the effects of TNFα and IL6 on adiponectin receptor expression are influenced by glucose concentration, and that the JAK-STAT pathway may be a determinant of adiponectin receptor mRNA expression, and acutely protein levels of adipoR2 may be regulated by adiponectin and PPARγ agonist. Interestingly, the adipocyte plays a crucial role in regulating its own metabolism and by maintaining the cell size in a "normal" metabolic state. This in turn will allow for the production of adipokines which maintain normal glucose homeostatsis that is lost during the development of obesity and insulin resistance.

4.6 References

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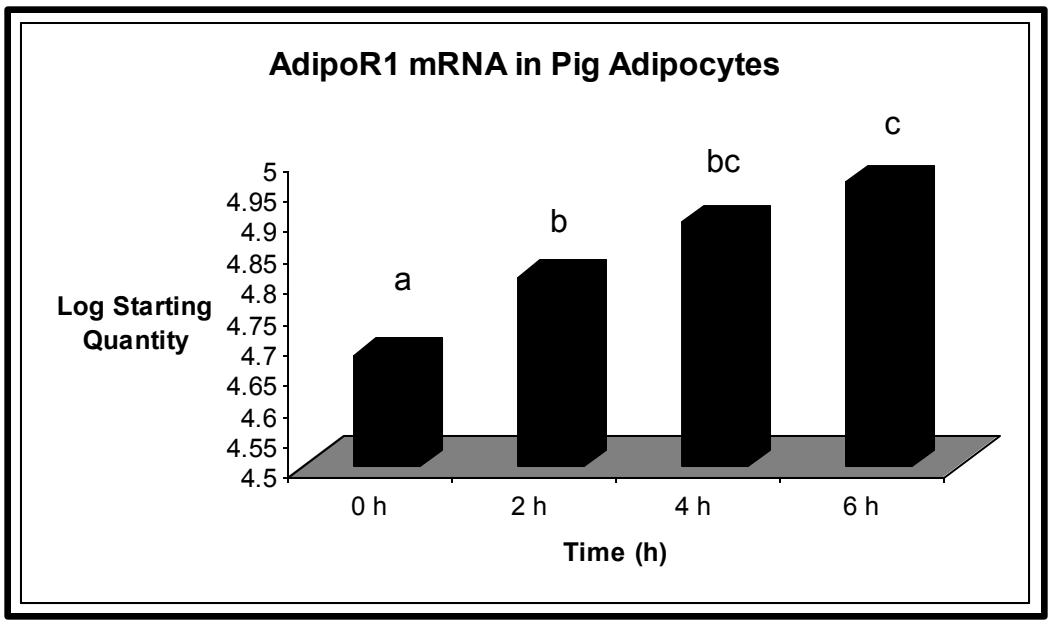

Figure 4.1 Regulation of AdipoR1 mRNA overtime in pig adipocytes.
Adipocytes were cultured for given time points in media with 5.5 mM glucose. (n=5 pigs; 6 replicates within pig) Time effect $P < 0.05$. Means without common letter differ ; standard error = 0.09).

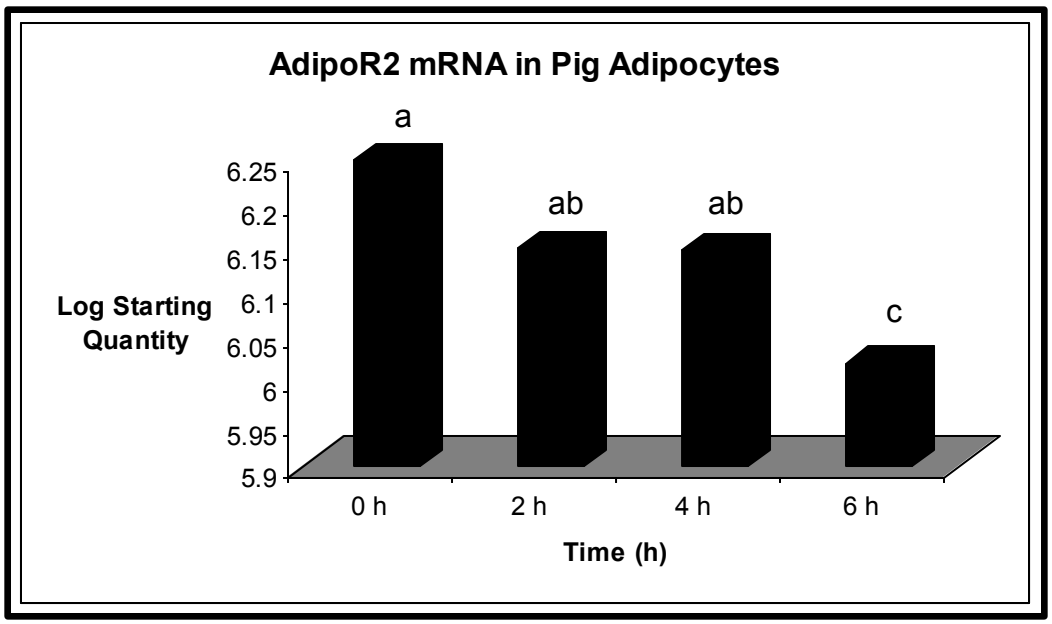

Figure 4.2Regulation of AdipoR2 mRNA overtime in pig adipocytes.
Adipocytes were cultured for given time points in media with 5.5 mM glucose. (n=5 pigs; 6 replicates within pig) Time effect $P < 0.05$. Means without common letter differ ; standard error = = 0.06).

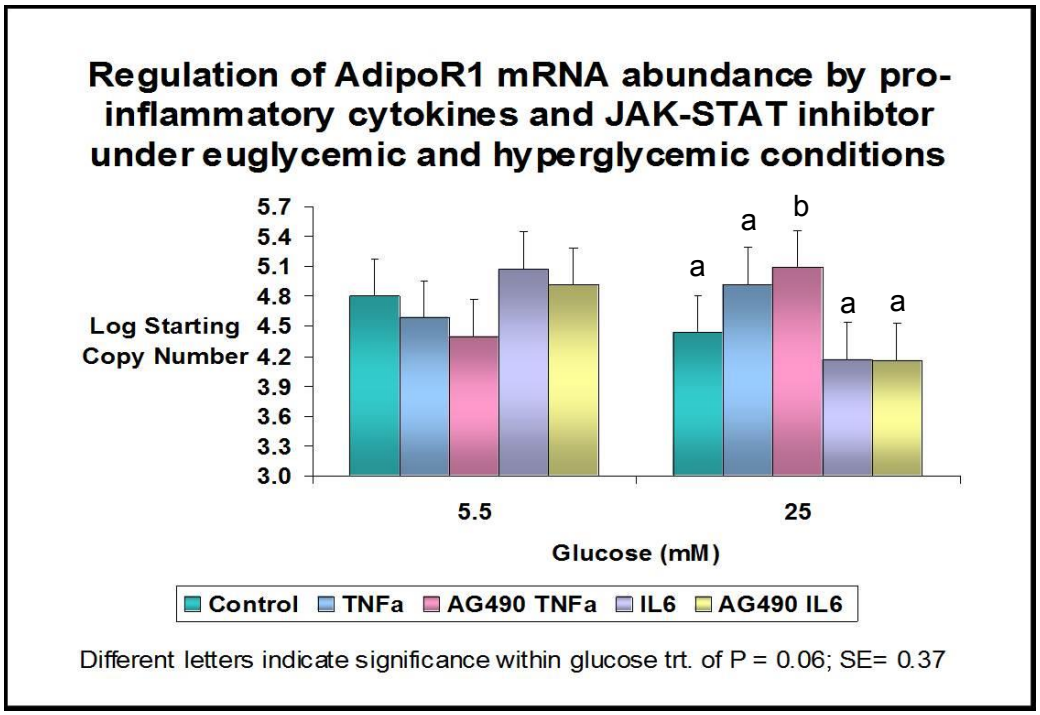

Figure 4.3Effect of TNFα and IL-6 on AdipoR1 mRNA abundance in pig adipocytes under euglycemic and hyperglycemic conditions.
Adipocytes were cultured for 6 h in the presence or absence of TNFα (30 ng/mL), AG490 (10uM), and/or IL-6 (30 ng/mL). (n=3 pigs; 6 replicates within pig). Different letters indicate significance of $P < 0.1$, standard error = 0.37.

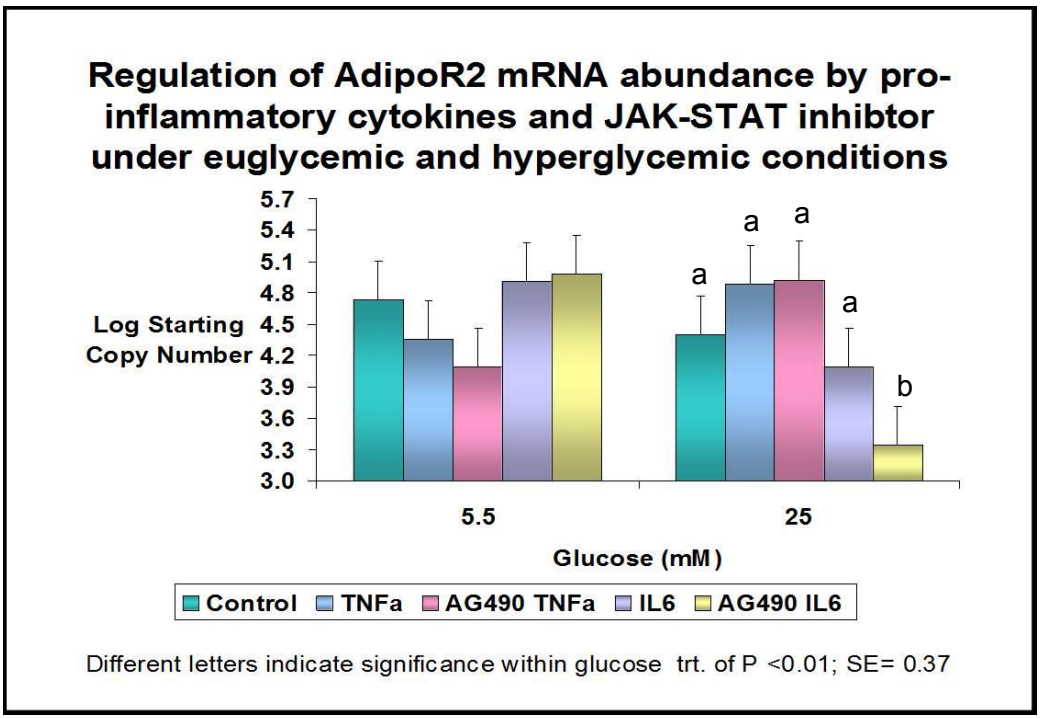

Figure 4.4 Effect of TNFα and IL-6 on AdipoR2 mRNA abundance in pig adipocytes under euglycemic and hyperglycemic conditions.
Adipocytes were cultured for 6 h in the presence or absence of TNFα (30 ng/mL), AG490 (10uM), and/or IL-6 (30 ng/mL). (n=3 pigs; 6 replicates within pig). Different letters indicate significance of $P < 0.01$, standard error = 0.37

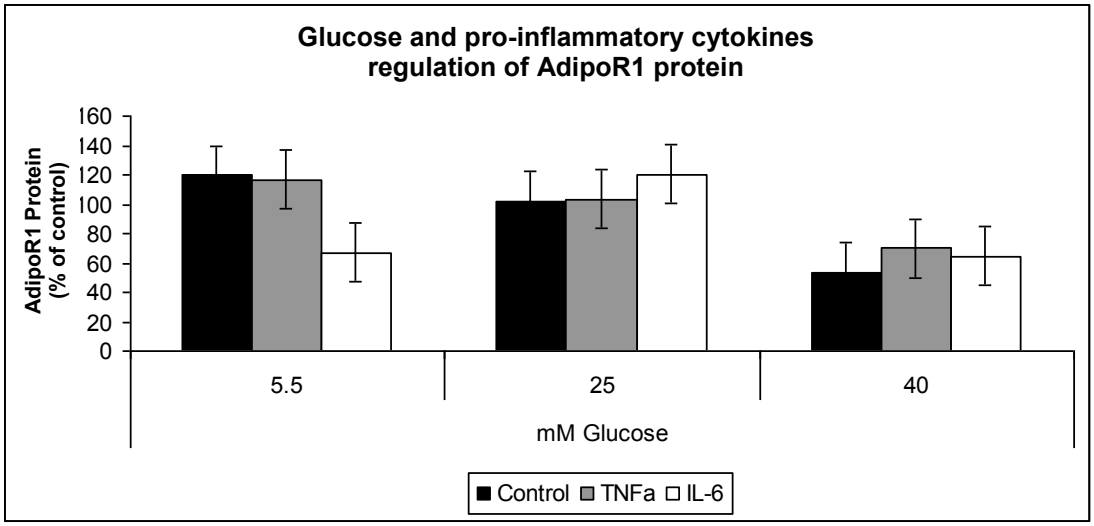

Figure 4.5 Regulation of AdipoR1 protein by pro-inflammatory cytokines under euglycemic and hyperglycemic conditions.
Adipocytes were cultured for 6 h in the presence or absence of TNFα (30 ng/mL) or IL-6 (30 ng/mL) under varying concentrations of glucose. (n=2 pigs; 6 replicates within pig) standard error = 20.5

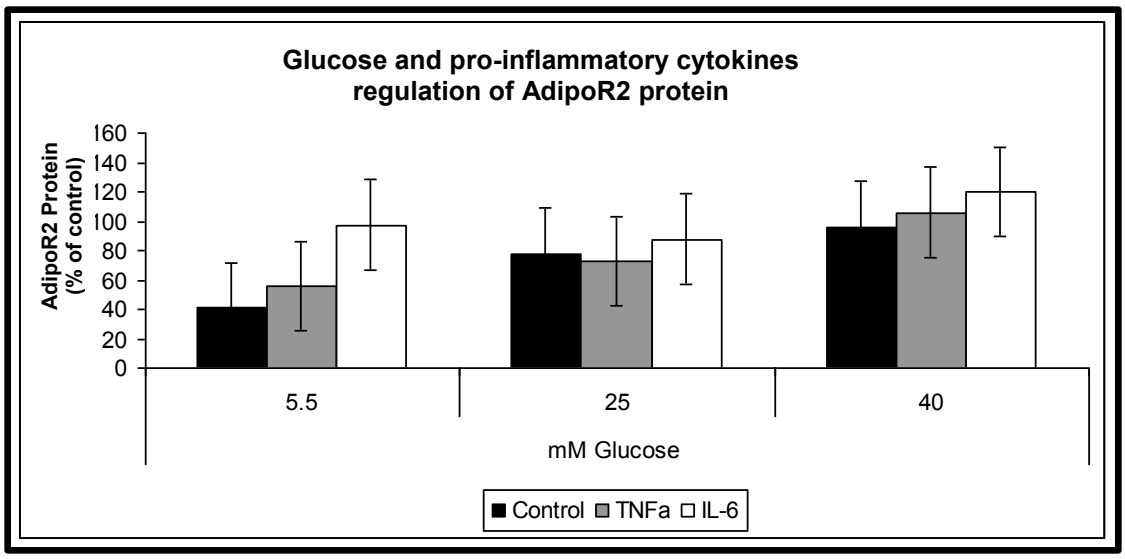

Figure 4.6 Regulation of AdipoR2 protein by pro-inflammatory cytokines under euglycemic and hyperglycemic conditions
Adipocytes were cultured for 6 h in the presence or absence of TNFα (30 ng/mL) or IL-6 (30 ng/mL) under varying concentrations of glucose. (n=2 pigs; 6 replicates within pig) standard error = 30.6

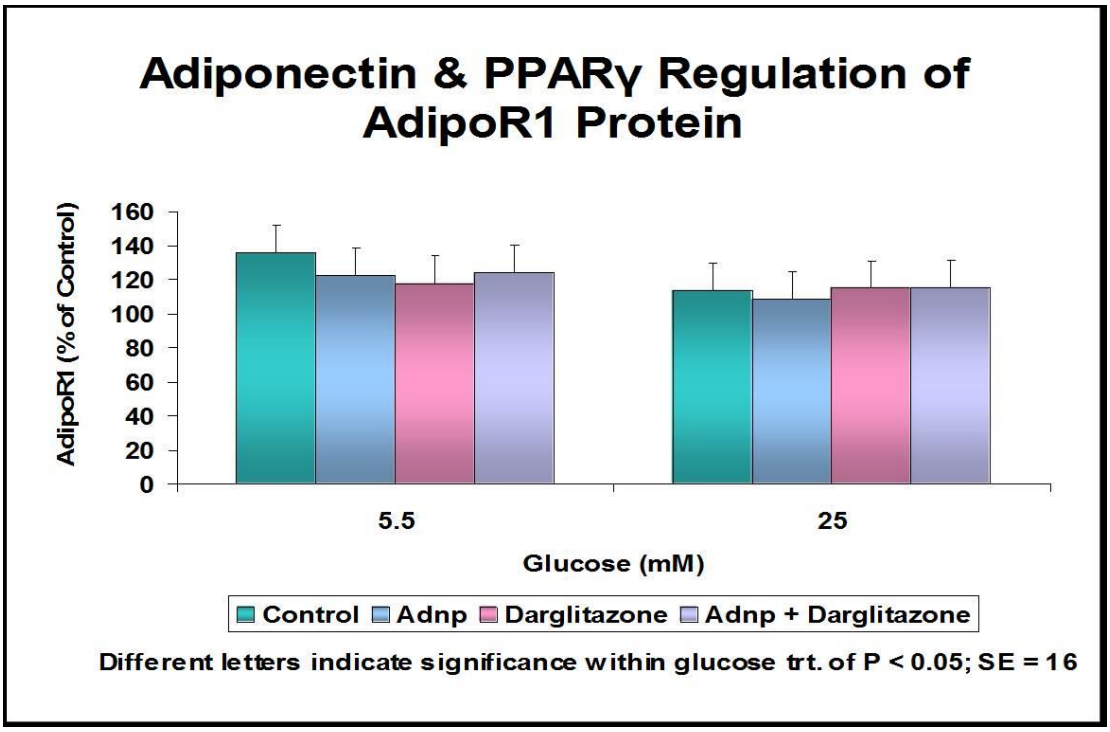

Figure 4.7 Regulation of AdipoR1 protein by adiponectin and darglitazone under euglycemic and hyperglycemic conditions.
Adipocytes were cultured for 6 h in the presence or absence of adiponectin (30 μg/mL) or darglitazone (10 μM) under varying levels of glucose. (n=2 pigs; 6 replicates within pig) standard error = 16.01

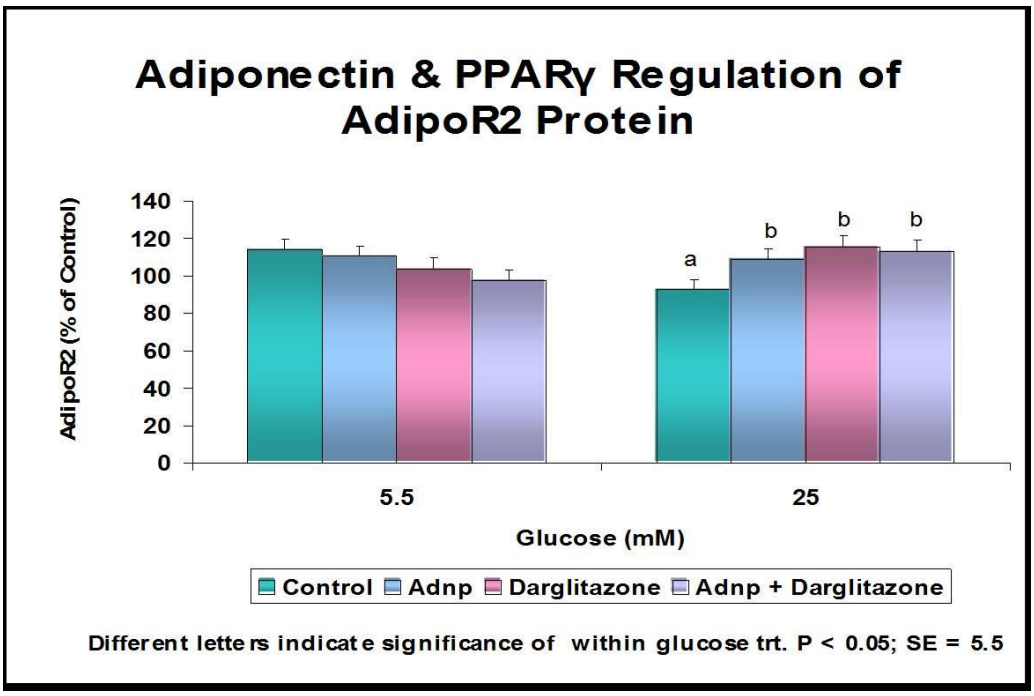

Figure 4.8 Regulation of AdipoR2 protein by adiponectin and darglitazone under euglycemic and hyperglycemic conditions.
Adipocytes were cultured for 6 h in the presence or absence of adiponectin (30 μg/mL) or darglitazone (10 μM) under varying levels of glucose. (n=2 pigs; 6 replicates within pig) Means without common letter differ $P < 0.05$. Standard error = 5.5

CHAPTER 5. HYPERGLYCEMIA-INDUCED IMPAIRMENT OF ADIPOCYTE FUNCTION

5.1 Abstract

Induction of insulin resistance is a major complication associated with many metabolic abnormalities linked to obesity. It has recently been established that hyperglycemic conditions can induce insulin resistance in adipocytes and perpetuate the inflammatory response associated with development of metabolic syndrome. However, it has not been investigated as to whether adiponectin, the insulin sensitizing and anti-inflammatory adipokine, functions to combat glucose intolerance by inhibiting or reversing the effects seen by hyperglycemic treatments. Here, we analyzed the effects of hyperglycemia on insulin sensitivity in pig adipocytes, as well as, determining the effect of adiponectin on hyperglycemic treated 3T3-L1 adipocytes. In pig adipocytes, we found 25 and 40 mM glucose in the culture media is enough to induce hyperglycemic inhibition of insulin-stimulated glucose uptake ($P < 0.05$). Glucose induced insulin resistance in primary pig adipocytes could not be attributed to changes in phosphosphorlation of the insulin receptor or its down-stream target Akt ($P > 0.05$). To test a more chronic (2 days) glycemic regulation of insulin-induced glucose uptake in adipocytes we used the 3T3-L1

mouse derived adipocyte culture system. Similar to the effects seen in acute porcine adipocyte cultures hyperglycemia inhibited insulin stimulated 2-deoxyglucose uptake in 3T3-L1 adipocytes too ($P < 0.05$), and 24 hour treatment with adiponectin could not reverse the insulin resistance. Again, difference in phosphorylation of the insulin receptor and Akt could not be detected in hyperglycemic culture cells compared with control cells. Interestingly, although adiponectin did not appear to impact insulin signaling pathways in these cells to combat hyperglycemic conditions it did have an effect on inflammatory pathways in the adipocyte. Adiponectin reduced the production of intracellular ROS under hyperglycemic conditions in 3T3-L1 adipocytes ($P < 0.05$). Taken together these data implicate adiponectin suppressing intracellular inflammation during hyperglycemia, but was not able to reverse hyperglycemia induced insulin resistance effect on glucose uptake.

5.2 Introduction

The development of insulin resistance is a pleiotropic condition which is impacted by many genetic and environmental factors. Insulin resistance is a condition which develops over time and is highly associated with obesity and type II diabetes. The degree of insulin resistance is directly linked to glucose tolerance. Once a certain level of insulin resistance is met, there is a severe deterioration of glucose homeostasis in multiple tissues throughout the body.

Adipocytes have come to the forefront of being major regulators of whole body energy metabolism, and in beginning to understand the role of the adipocyte it has become apparent that excessive hypertrophy of adipocytes/adipose tissue leads to

abnormal metabolic states associated with disease and morbidity. The mechanisms behind these phenotypes are beginning to be unraveled. In fact, hyperglycemic and hyperinsulinemic states result in impaired insulin receptor signaling (Tang et al., 2001). Others have demonstrated 3T3-L1 cells under hyperglycemic conditions have impaired IRS-1 phosphorylation and down-stream insulin signaling (Lin et al., 2005). Hyperglycemia has also been shown to induce reactive oxygen species (ROS) in rat primary adipocytes and 3T3-L1 adipocytes (Lu et al., 2001; Lin et al. 2005). Increased ROS is associated with increased inflammation, and an increased inflammatory state is highly associated with obesity.

The adipocytokine adiponectin has been shown to have anti-inflammatory and insulin-sensitizing properties (ref). Our lab has previously demonstrated that adiponectin can inhibit lipopolysacharide induced cytokine production in treated adipocytes and macrophages (Ajuwon et al., 2004; Ajuwon and Spurlock, 2005; Wulster-Radcliffe et al., 2004). Given this information it seemed logical adiponectin, an insulin-sensitizing adipocytokine, mechanistically may regulate insulin stimulated glucose uptake in adipocytes and the production of ROS, thereby alleviating the effects of hyperglycemia induced insulin-resistance.

Therefore, the first aim of this research was to determine if hyperglycemia could impair glucose uptake in porcine adipocytes, and if so, does it occur through impairment of insulin signaling pathways. Secondly, to investigate using the 3T3-L1 adipocytes model of hyperglycemia induced insulin resistance if adiponectin could reverse the inflammatory state induced by high glucose restore insulin sensitivity.

5.3 Methods

Animals and adipocyte isolation

Adipose tissue was obtained from male castrates weighing approximately 90-110 kg, and consuming a nutritionally-complete diet ad libitum. The pigs were maintained according to protocols approved by the Purdue University Animal Care and Use Committee, and killed in the Purdue University abattoir according to USDA regulations. Subcutaneous adipose tissue was removed from the dorsal depot located over the cervical spine, and transported in buffered saline (37^{o}C, 0.15 M NaCl, 10 mM HEPES, pH 7.4) to the laboratory. Adherent skeletal muscle was removed, and the tissue was cubed and sliced with a Stadie-Riggs microtombe. Approximately 6-7 g of sliced tissue was minced, rinsed with saline, and transferred to a flask containing 15 mg collagenase (100 U/mL final concentration; Worthington BioChemical, Lakewood, NJ USA). Following a 30-35 minute digestion period, isolated adipocytes were washed through a 290 μm screen with warmed Krebs Ringer bicarbonate buffer (pH 7.4) containing 10 mM HEPES, 5 mM glucose, and 3% bovine serum albumin (Fraction V, essentially fatty acid free, Invitrogen life technologies, Carlsbad, CA USA) to remove all other cell debris. The cells were resuspended in culture media (DMEM Sigma D6780; St. Louis, MO USA) with 3% BSA and 10 mM HEPES at a final concentration of approximately 2 x 10^5 cells per mL and aliquoted immediately into 15 mL polypropylene scintillation vials for incubation.

Cell Culture

3T3-L1 murine fibroblast (ATCC; Manassas, VA) were seeded in 12-well plates or 3.5 cm dishes and cultured according to standard conditions. Briefly, cells were grown in 5%

CO_2 in low glucose DMEM containing 10% calf serum (GIBCO, Carlsbad, CA). Two-days post-confluence (Day 0), cells were induced to differentiate with low glucose medium containing 10% fetal bovine serum (GIBCO), 150 nM insulin, 250 μM dexamethasone, and 0.5 mM isobutylmethylxanthine (IBMX) for two days (Day 2). Thereafter, cells were maintained in fresh medium containing only insulin for another 8 to 10 days. Cells were used between days 10 and 14 post-differentiation. Glucose treatments (5.5 or 25 mM) were applied to cells 48 hours prior to harvest and 30 μg/mL adiponectin treatments were applied 24 hours prior to harvest.

Measurement of [1-^{3}H]2-Deoxyglucose Uptake

Pig adipocytes were assayed for [1-^{3}H]2-Deoxyglucose (Amersham) uptake under the following conditions. Pig adipocytes were cultured in 5.5, 25, or 40 mM glucose for three hours and then media was spiked with 0.33 μCi of [1-^{3}H]2-deoxyglucose/mL for 5 minutes followed by media being spiked with increasing concentrations of insulin (0, 1, 5, 20, and 100 nM) for 15 minutes. Transport assay was terminated by removal of media and two washes with cold PBS. A hundred microliter alliqout of cells was added to scintillation cocktail and counted for glucose uptake. All samples were standardized to cells which were spiked with labeled glucose and washed immediately for a background count. 3T3-L1 glucose uptake was performed similarly to Lin et al. (2005) except cells were not glucose starved for 30 minutes prior to the assay.

Immunoprecipitation of Phospho-Proteins and Western Blots

Adipocytes were incubated with treatments for six hours in DMEM with 3% BSA. Cell incubations were stopped by removal of the media and cells were washed twice with PBS.

Cells were lysed in 10 vol/wt of Buffer A [50 mM Tris-HCl (pH 7.5), 50 mM NaF, 5 mM Sodium Pyrophosphate, 1 mM EDTA, 1 mM DTT, 0.1 mM Phenylmethylsulfonyl fluoride, 10 % glycerol] containing 1% Triton X-100, 5 μM aprotinin, leupeptin, and pepstatin. Following lyses samples were centrifuged at 6,000 x g for 10 minutes at 4^{o}C to remove insoluble material. Supernatants were collected and protein was quantified using BCA reagents from Pierce (Rockford, IL). Supernatants were aliquoted containing equal amounts of protein (100 μg) and immunoprecipitated at room temperature for 2 h using the Catch and Release v2.0 Reversible Immunoprecipitation System from Upstate Cell Signaling Solutions (Charlottesville, VA). Phospho-Akt (Thr 308) antibody (Cat # 9275; Upstate Cell Signaling) was immunoprecipitated with 1:100 primary antibody dilutions in 250 μl total volume. Akt antibody (Cat # 9272; Upstate Cell Signaling) was immunoprecipitated with 1:500 primary antibody dilutions in 250 μl total volume. Phospho-insulin receptor (Tyr) (BioSource) was immunoprecipitated with 100 ug total protein and a 1:50 primary antibody dilution in 250 ul total volume. Protein complexes were eluted from the column using 40 μl of 1X denaturing elution buffer containing β-mercaptoethanol. Total eluent protein was separated by SDS-PAGE using a 12% resolving gel and 4% stacking gel. Proteins were transferred to a nitrocellulose membrane and probed with the primary antibody. All antibodies were used in a 1:500 dilution to probe blots at room temperature overnight in 5% BSA solution or 5% milk powder in TTBS. Blots were probed for 1 h at room temperature with a 1:20,000 dilution of Goat-Anti-rabbit IgG –HRP antibody (Pierce Cat# 31460) and developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Cat #34080).

Measurement of Intracellular ROS Generation

Cells were washed in Krebs-Ringer biocarbonate buffer and then incubated in the dark with fluorescent probe 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) *mixed isomers (10 uM; Invitrogen CAT# C400) for 45 minutes at 37^{o}C. The flourensence of CM-DCF was analyzed using a fluorescent plate reader at an excitation wavelength of 435 nm and emission at 550 nm. ROS production was determined by an H_2O_2 standard curve (10-200 nmol/ml).

Statistical Analysis

All data were checked for normality before being analyzed using the mixed model analysis. The fixed effect was the treatment and the random effect was the replicate. When protected by a significant F test, mean separation was accomplished using the least squares means separation (pdiff) procedure (SAS, 1999; Cary, NC).

5.4 Results

Hyperglycemia inhibits insulin-stimulated glucose uptake in porcine adipocytes and 3T3-L1 adipocytes

Insulin resistance is highly correlated with hyperglycemia, but the mechanisms for this induction of insulin resistance are not well understood. However, recently obesity and the development of insulin resistance have been linked to chronic inflammation, and adiponectin has been implicated as an insulin-sensitizing hormone. First, we wanted to determine if hyperglycemia could induce insulin resistance in pig adipocytes, and if so does it occur through direct inhibition of insulin signaling. As presented in Figure 5.1, hyperglycemic (25 mM and 40 mM glucose) conditions can

inhibit insulin stimulated 2-deoxyglucose uptake in pig adipocytes ($P < 0.01$). However, pre-treatment with adiponectin for 2 hours could not reverse this inhibition on glucose uptake (data not shown) during this acute time frame. To examine a more chronic model of insulin resistance used 3T3-L1 adipocyte cultures. At 10 day post-differentiated adipocytes were changed to either 5.5 or 25 mM glucose media for 24 hours. The following day either vehicle or adiponectin was added to the cell media for 24h. Twenty-four hours after adiponectin treatment all cells were given 2-deoxyglucose followed by corresponding insulin treatment. Similar to results in acute porcine adipocyte cultures, even with more chronic adiponectin treatment the hormone could not reverse hyperglycemic inhibition of insulin stimulated glucose uptake (Figure 5.2; $P < 0.01$).

Effect of hyperglycemia on insulin signaling pathway in pig adipocytes and 3T3-L1 adipocytes

To further extend these results we wanted to see if the mechanism for hyperglycemic induced insulin resistance is related to insulin signaling pathway. Therefore, we measured phospho-Akt at Thr 308, total Akt, and phospho-insulin receptor at Tyr 92. Conversely, to what we expected there was no significant change across treatments for phospho-Akt, Akt and phosphor-IR in pig adipocytes which had glycemic inhibition of insulin stimulated glucose uptake (Figure 5.3, 5.4, and 5.5). Interestingly, we also measured these same proteins in the 3T3-L1 experiments and again found no difference between treatments on phosphorylation state of Akt or the insulin receptor ($P > 0.05$; Figures 5.6, 5.7, 5.8).

The role of hyperglycemic conditions and adiponectin on development of intracellular ROS in 3T3-L1 adipocytes

To investigate whether adiponectin could reverse the increase in intracellular ROS species associated with hyperglycemic conditions in 3T3-L1 cells we used a 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA)*mixed isomers assay to measure intracellular ROS. In contrast to the insulin signaling data, we measured significantly elevated intracellular ROS under hyperglycemic conditions which were abrogated with adiponectin treatment (Figure 5.9; $P < 0.05$).

5.5 Discussion

These experiments provide a limited view of hyperglycemic induced mechanisms involved in insulin resistance. Our data show hyperglycemic cell culture conditions induce insulin resistance in primary porcine adipocytes, as it relates to insulin stimulated glucose uptake. This is a significant contribution to the literature which consistently reports pig adipocytes are only mildly sensitive to insulin stimulation (Mills et al., 1999). However, conventional primary adipocyte methodologies supplement culture media with 25 mM glucose (Walton et al., 1996; Mills et al., 1999), possibly inducing an insulin resistant phenotype. Although adiponectin is known to be an insulin sensitizing hormone, we were not able to reverse the effect of hyperglycemic conditions by pre-treatment with adiponectin. There are a few plausible reasons why adiponectin was not able to reverse insulin-resistance. First, in pig primary adipocyte cultures the length of time primary cells can be cultured is extremely acute (6-8 hours). This very small duration of treatment with adiponectin may not be enough time for reversal of the effects of high

glucose. Furthermore, the form of adiponectin involved in glucose and insulin tolerance signaling pathways could be the high molecular weight or the cleaved protein and not the full length protein. In fact, Tuschida et al. (2005) reported the high molecular weight isoforms of adiponectin may be the form of the protein involved in modulating insulin resistance (Tuschida et al., 2005). If this is the case, then there is the possibility the recombinant expressed full length adiponectin, which we used in our cultures, is not mediating insulin sensitivity pathways but functions to regulate inflammatory mediators in the adipocyte which indirectly affect insulin sensitivity.

Similar to the pig adipocyte experiments, we also determined recombinant expressed full length adiponectin did not reverse hyperglycemic induced inhibition of glucose uptake in 3T3-L1 adipocytes. Given that this system allowed for more chronic exposure for adiponectin treatment the same arguments from above still apply to the reasons we may not have seen a reversal of insulin resistance in these cells. In contrast to Lin et al (2005) we could not show hyperglycemic conditions inhibited phosphorylation of key components of the insulin signaling pathway. These results may be attributable to inclusion of 10% serum in culture media if 3T3-L1 cells throughout the assay system. Serum components may have interfered with ability to pick up reduction in phosphorylation of the insulin receptor and its down-stream effector Akt.

As depicted by the increase of intracellular ROS, inflammation does appear to be induced in 3T3-L1 adipocytes by hyperglycemic conditions. Interestingly, under low glucose conditions ROS are also present within the cells. This may be due to an acute increase of intracellular fatty acids within the cell stimulating an acute inflammatory response. Although, it does not appear adiponectin has a conventional effect on lipolysis

in adipocytes we do know that it can stimulate PPARα in these cells thereby eliciting an increase in oxidative capacity of the adipocyte. This initial increase in intracellular fatty acid could lead to an acute inflammatory response from these cells. It will be interesting to determine if this is a prolonged effect of adiponectin treatment at more chronic time points. An alternative point could be IL-6 is actually acting in an anti-inflammatory capacity in these short time frames. This would not be completely unwarranted because Bruunsgard et al. (2005) have shown IL-6 release from muscle tissue during exercise has anti-inflammatory properties.

Researchers have found hyperglycemia has a significant effect on the development of intracellular ROS in other cell types (ref), and Lin et al. (2005) reported hyperglycemia induces intracellular ROS in 3T3-L1 adipocytes. If adiponectin is insulin-sensitizing it seems plausible adiponectin could reverse the induction of ROS in 3T3-L1 cells. Our data demonstrated adiponectin does reduce intracellular ROS compared to high glucose treated cells alone. Recombinant adiponectin does protect the adipocyte against increases in ROS in adipocytes, but did not impact insulin sensitivity pathways. These findings lend support to the hypothesis that different forms of adiponectin are impacting glucose metabolism pathways and inflammatory pathways in the adipocyte in different ways. This theory adds more complexity to understanding how adiponectin works as an insulin sensitizing compound and an anti-inflammatory compound in the adipocyte to protect against metabolic abnormalities associated with obesity and type II diabetes.

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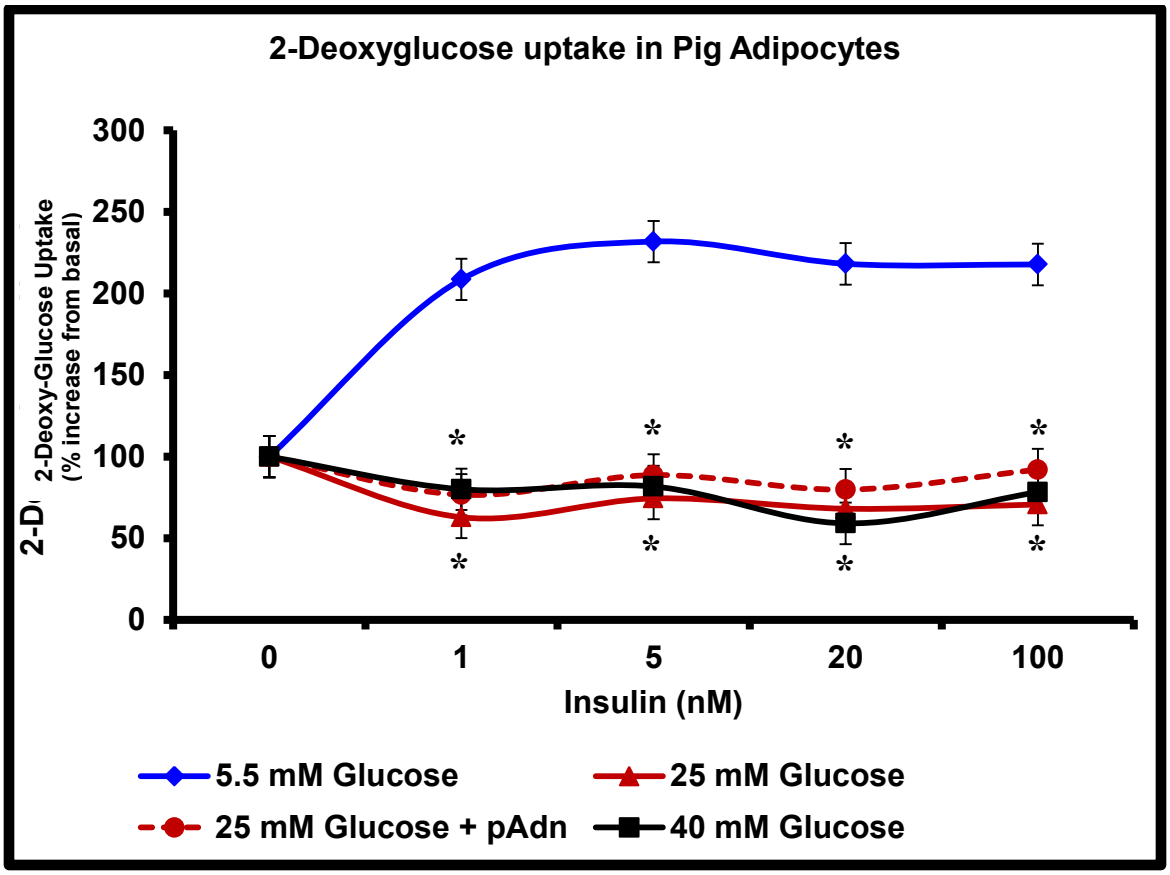

Figure 5.1 Hyperglycemic inhibition of insulin stimulated glucose uptake in pig adipocytes
Adipocytes were and cultured with increasing concentrations of glucose for three hours followed by 2h insulin treatment with 2-deoxyglucose in the media. n = 3 pigs; error bars are stardard error = 12.77; *mean significantly different from 5.5 mM glucose treatment; $P < 0.05$.

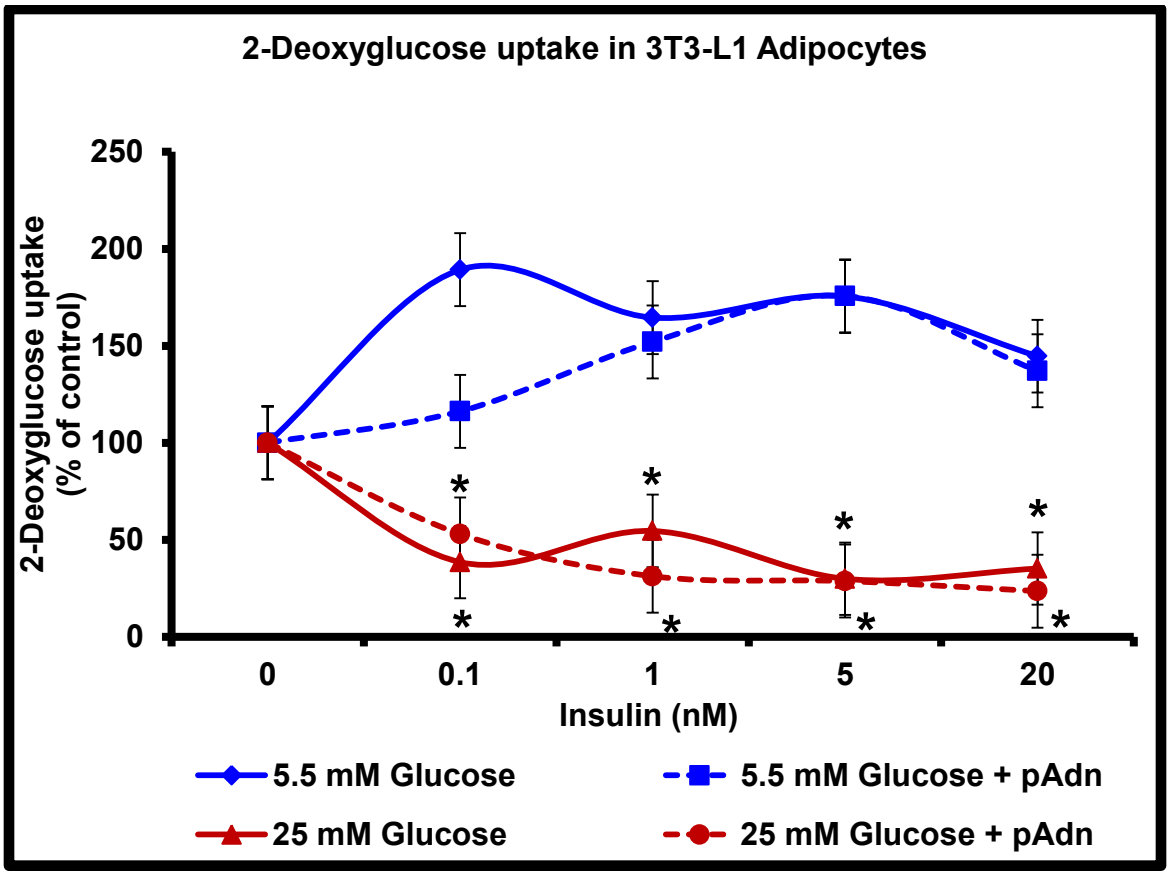

Figure 5.2 Hyperglycemic inhibition of insulin stimulated glucose uptake in 3T3-L1 adipocytes
is not attenuated by 24h treatment with adiponectin. Adipocytes were cultured for 24 h in euglycemic or hyperglycemic media followed by 24 h treatment with 30 ug/ml adiponectin. At 48 h post glucose treatments cells were treated with insulin in the presence of 2-deoxyglucose for 2 h. n=6 wells/treatment; *Indicate significant difference from 5.5 mM glucose treated cell, $P < 0.05$; error bars represent standard error = 18.87.

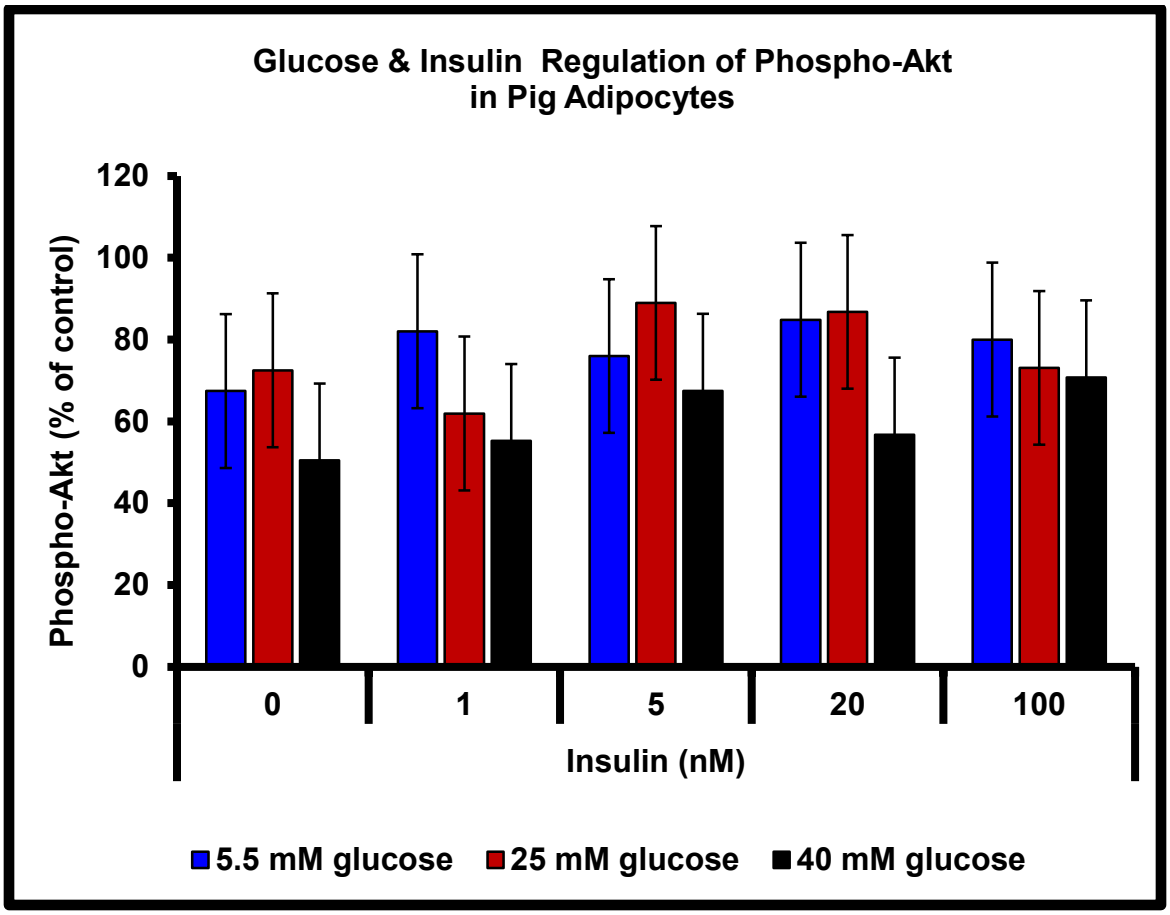

Figure 5.3 Hyperglycemic regulation of Phospho-Akt in pig adipocytes.
Adipocytes were cultured for 6 h in different glucose containing media with increasing doses of insulin. n= 2 pigs; standard error = 18.81

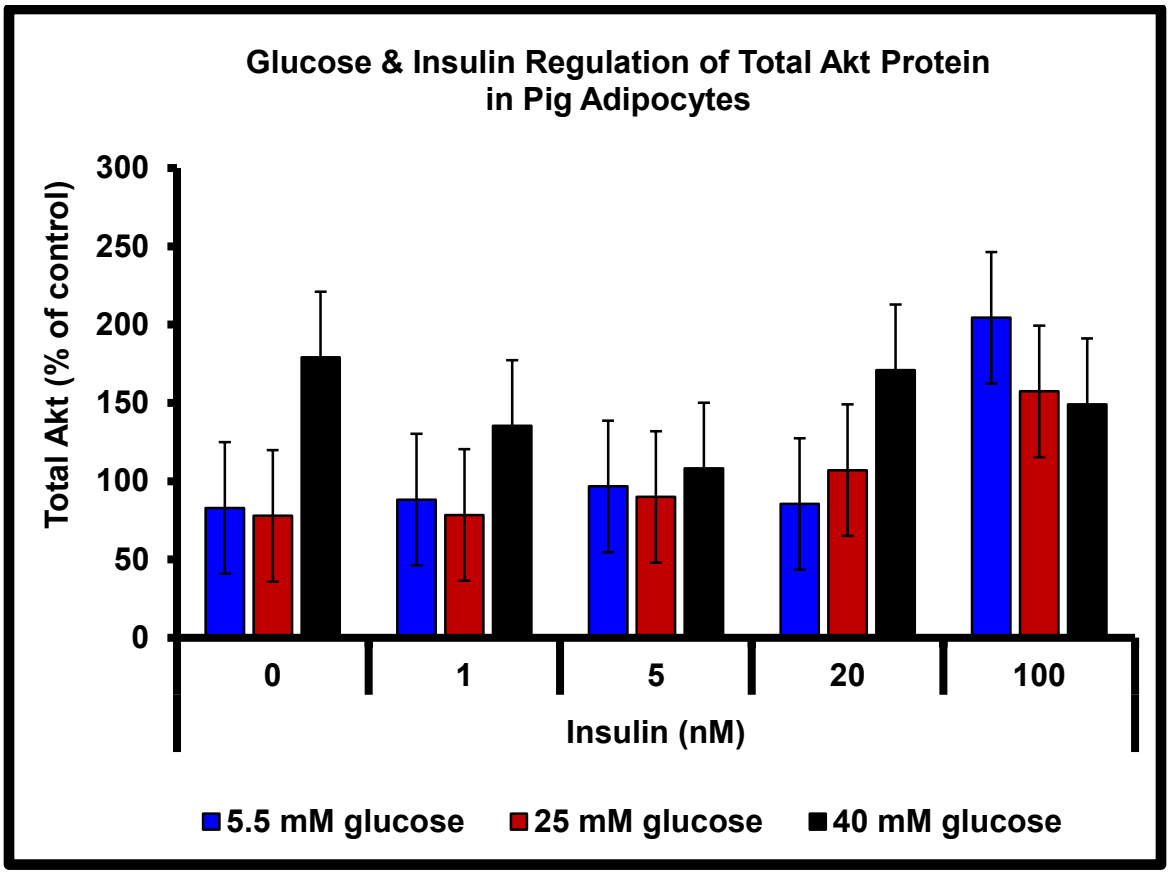

Figure 5.4 Hyperglycemic regulation of Total Akt protein in pig adipocytes. Adipocytes were cultured in euglycemic and hyperglycemic media for 6 h and stimulated for 2 h with insulin. n= 2 pigs; standard error = 42.60.

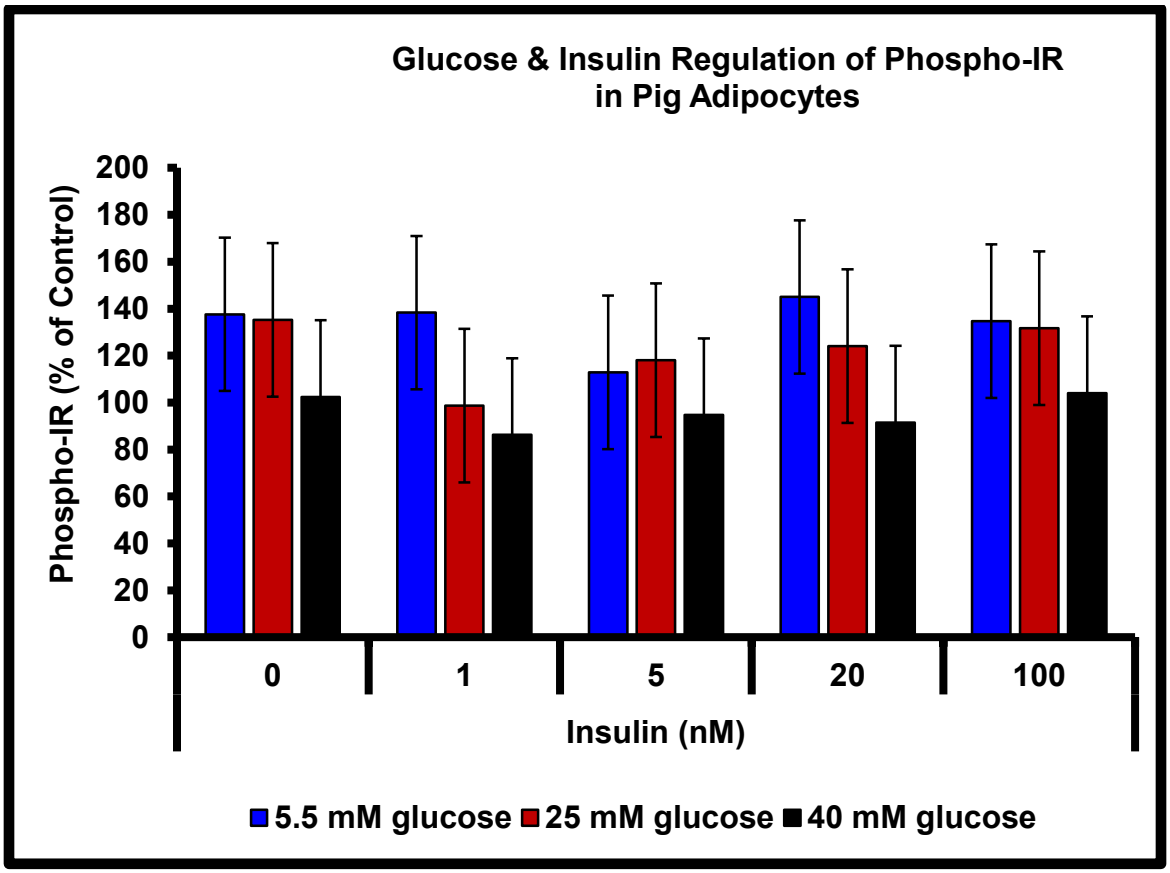

Figure 5.5 Hyperglycemic regulation of phosphorylation of the insulin receptor in pig adipocytes.
Adipocytes were cultured in euglycemic and hyperglycemic media for 6 h and stimulated for 2 h with insulin. n= 2 pigs; standard error = 32.68

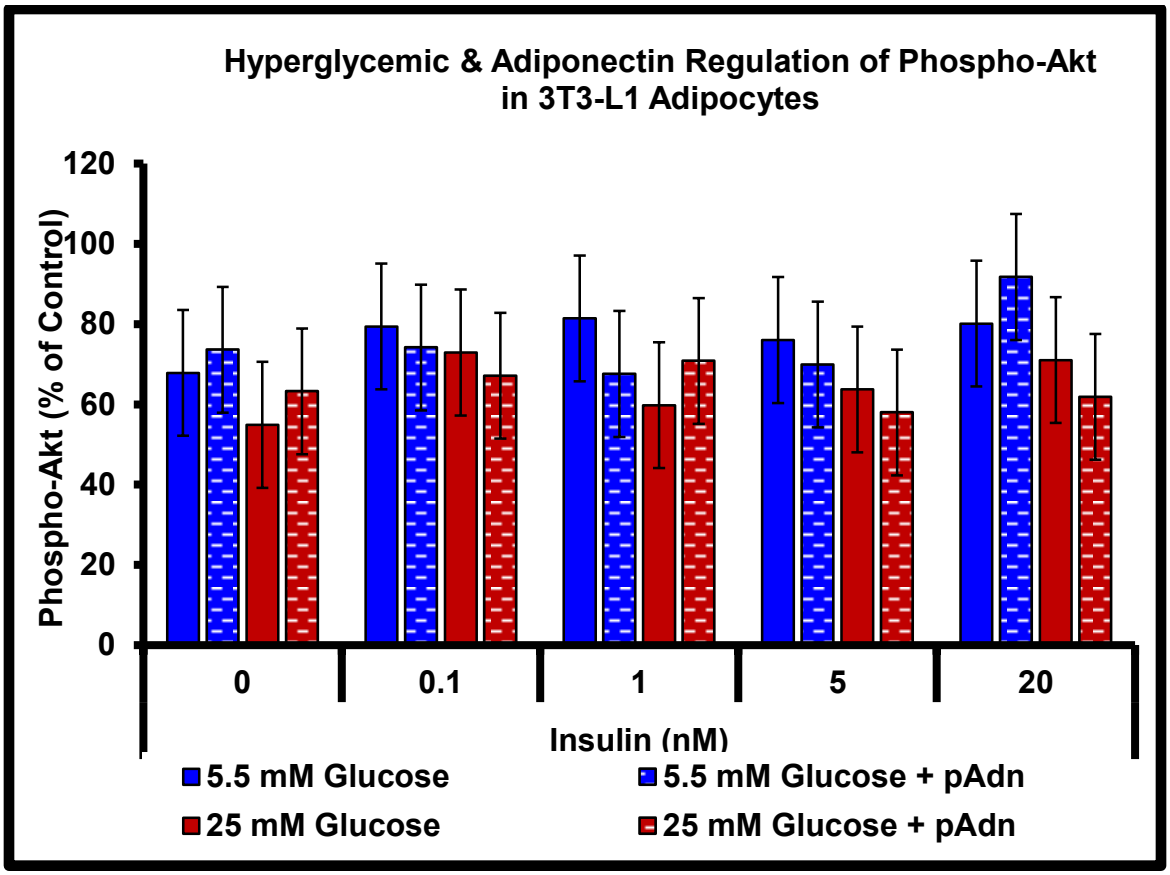

Figure 5.6 Hyperglycemic regulation of phosphorylation of Akt in 3T3-L1 adipocytes Adipocytes were cultured in euglycemic and hyperglycemic media for 48 h and stimulated for 2 h with insulin. n= 6 wells/treatment; standard error = 15.69.

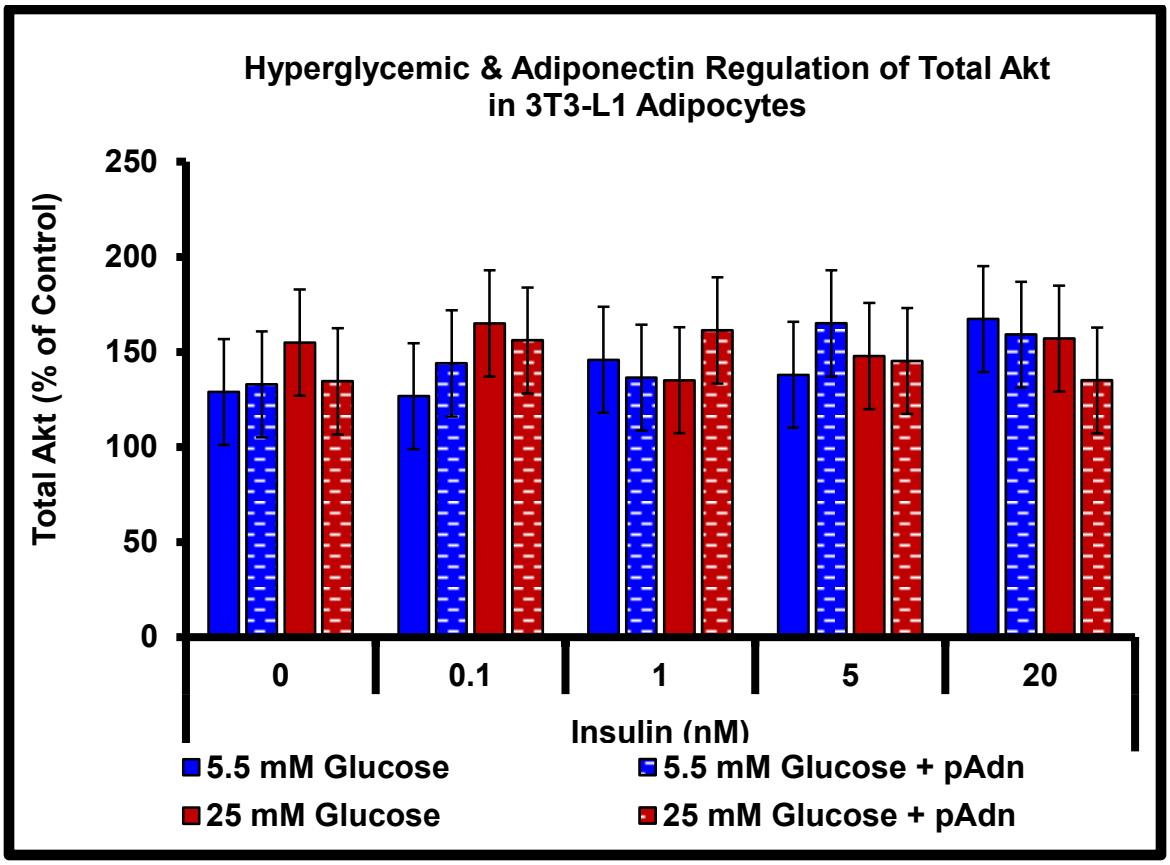

Figure 5.7 Hyperglycemic regulation of total Akt protein in 3T3-L1 adipocytes .Adipocytes were cultured in euglycemic and hyperglycemic media for 48 h and stimulated for 2 h with insulin. n= 6 wells/treatment; standard error = 27.89.

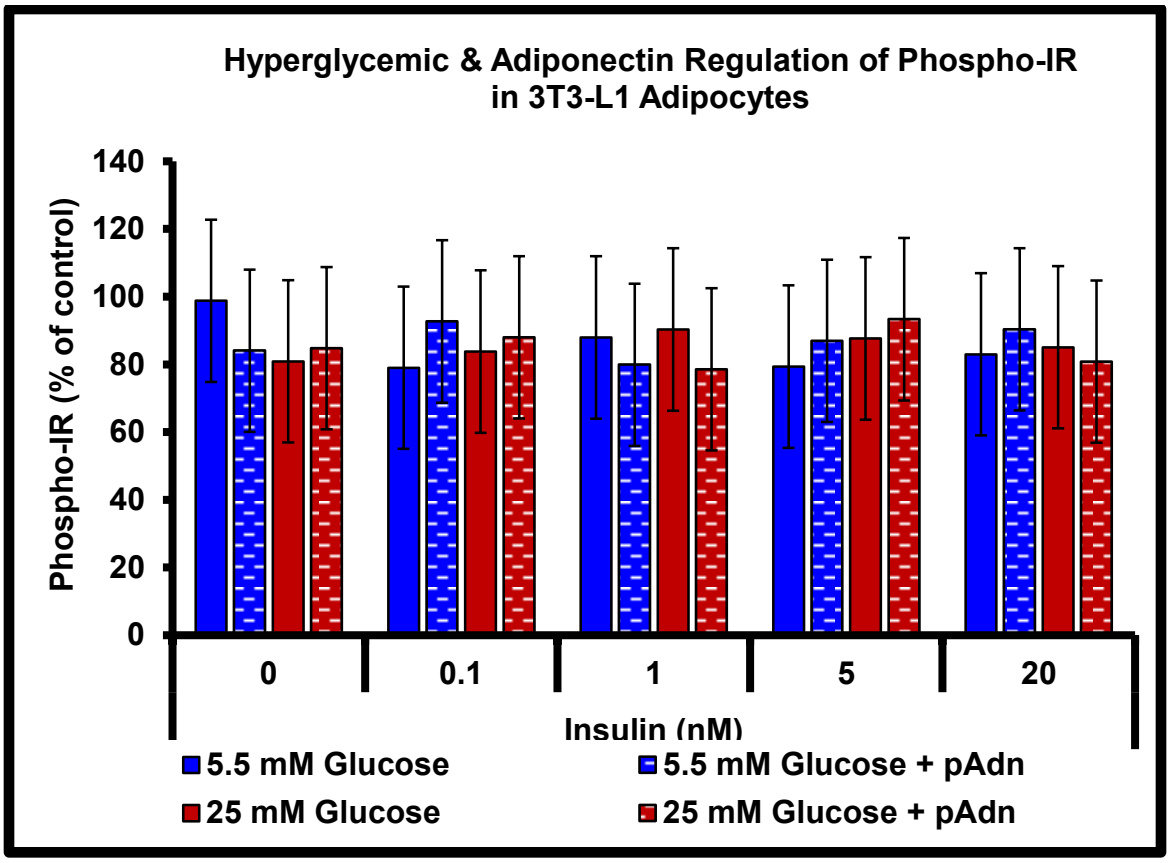

Figure 5.8.Hyperglycemic regulation of phosphorylation of the insulin receptor in 3T3-L1 adipocytes
Adipocytes were cultured in euglycemic and hyperglycemic media for 48 h and stimulated for 2 h with insulin. n= 6 wells/treatment; standard error = 23.98

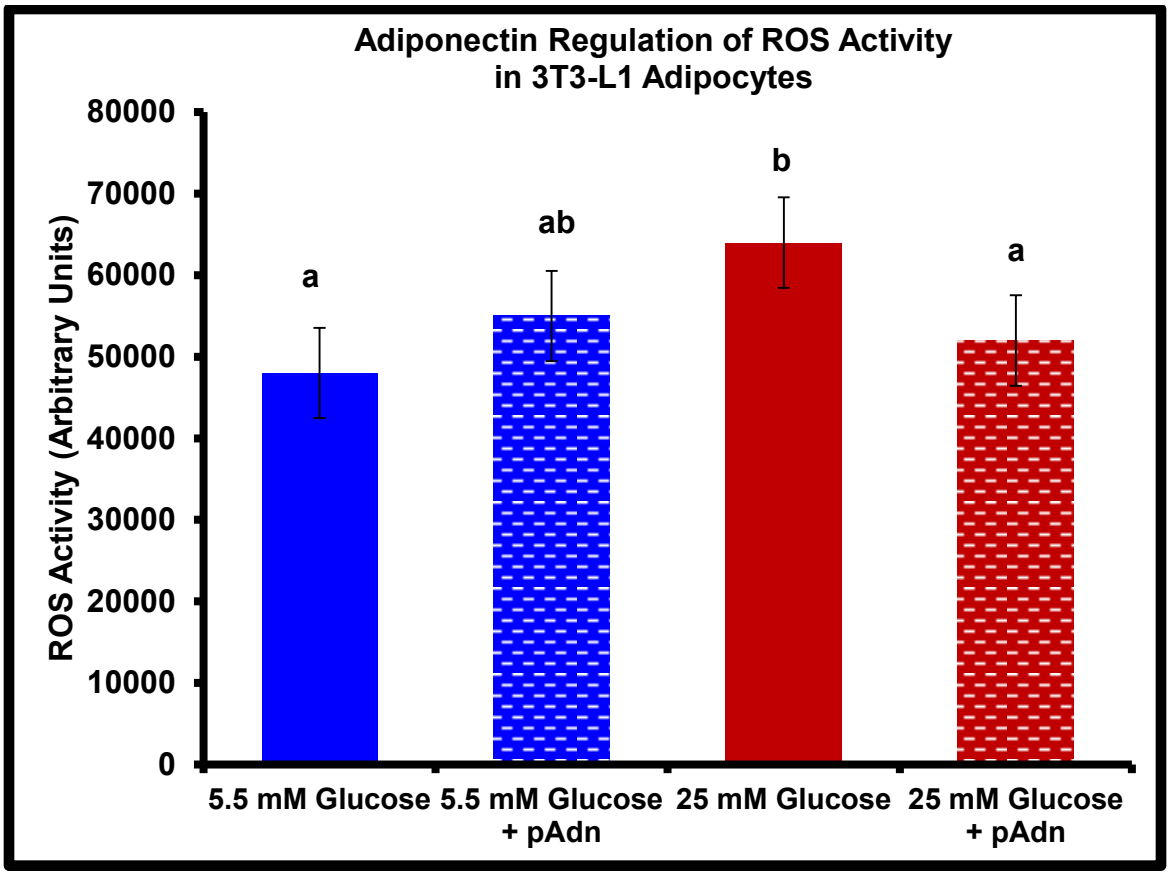

Figure 5.9Adiponectin regulation of intracellular ROS activity in 3T3-L1 adipocytes cultured under euglycemic and hyperglycemic conditions.
n=3 per treatment. Main effect of treatment was significant at P = 0.034; different letters indicate significant treatment differences at $P < 0.05$; error bars represent standard error = 5540

CHAPTER 6. SUMMARY

The adipocyte has come to the forefront of having pleitropic functions in energy metabolism and a role in immune function. Although the role of energy metabolism is thought to be fairly well characterized, it appears the intricate balance between energy homeostasis in the adipocyte and abnormal metabolic state is tightly linked. We provide evidence that the regulation of energy balance in the adipocyte is a critical component of maintaining a normal glycemic and insulinemic state in the pig and 3T3-L1 adipocytes, and that the adipocytokine, adiponectin, plays a critical role in this balance. First, we provided evidence of adiponectin impacting lipid metabolism in the adipocyte by directly decreasing lipogenesis. Additionally, we provide evidence of the mechanism behind this reduction in lipogeneic capacity may be partially mediated by induction of AMPK activation which impacts the phosphorylation of ACC, thereby, inhibiting a key regulatory enzyme in lipid synthesis. This theory has also been demonstrated by Wu et al., (2003) in rat adipocytes using the globular form of adiponectin. The inhibition of ACC leads to a reduction in the conversion of acetyl CoA to malonyl CoA. Malonyl CoA functions acutely as an allosteric regulator of the rate limiting enzyme, CPTI, which tightly controls oxidative capacity of the cell. By decreasing the flux of malonyl CoA to the cell one has increased the capacity for activity of CPTI and thereby, increasing the capacity of oxidation within the cell. We have further data to support this hypothesis,

which shows adiponectin acutely stimulates AMPK, PPARα, ACO, and UCP3 mRNA in pig primary adipocytes, as well as, up-regulates PPARα protein in these cells. The last three genes of interest are all known to play important roles in increased oxidative capacity of cells.

The regulation of lipid metabolism and storage of excess energy in the adipocyte are traditionally linked to adipose tissue. More recently it appears this physiology is the component of adipocytes which couple it to the inflammatory tone of the adipose tissue, as well as the body. Research has shown 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), a synthetic compound, which activates the energy sensing kinase, AMPK, can dose-dependently increase adiponectin gene expression in human adipose tissue (Lihn et al., 2004). In addition, AICAR attenuates the release of TNFα and IL-6 from human adipose tissue. Thus, reduced concentrations of these cytokines, increased levels of adiponectin, and AMPK activity appear to play a role in the insulin-sensitizing effects of AICAR and adiponectin. This is consistent with the findings that adiponectin attenuates the translocation of nuclear factor kappa B (NFκB) to the nucleus of adipocytes stimulated with lipopolysacharide (LPS) (Ajuwon and Spurlock 2005). Attenuation of NFκB translocation is coupled with an expected decrease in production of pro-inflammatory cytokines. These date support a tight link between adipocyte energy metabolism and immune function of the adipocyte.

Additionally, we demonstrate adiponectin induces its own expression in pig adipocytes. Data suggesting adiponectin has tight control over energy metabolism of the cell and regulation of adipocytokines involved in whole body energy homeostasis and inflammatory tone. Adiponectin expression is known to be regulated by PPARγ and

research from our lab confirms adiponectin can increase PPARγ2 expression in the adipocyte. Therefore, these data suggest a strong theory that adiponectin may be a master regulator of energy metabolism, glucose homeostasis and inflammation in the adipocyte. In addition, we have shown adiponectin regulates PPARα mRNA and protein abundance in the adipocyte and Tsuchida et al. (2005) report PPARα increases adiponectin receptor expression. These researchers also demonstrated that dual activation of PPARα and PPARγ had additive effects in reversing insulin resistance in KKAy obese mice, which provides supporting evidence adiponectin regulates the tight control energy metabolism in the adipocyte.

Furthermore, we also provide evidence that pro-inflammatory cytokines, glucose concentrations, adiponectin and PPARγ agonist are involved in differential regulation of adipoR1 and adipoR2. These data suggest potential mechanisms for regulation of adiponectin and adiponectin signaling and the inverse correlation of the adipokine the development of obesity and insulin resistance. The chronic inflammation known to accompany metabolic syndrome (Yudkin, 2003) antagonizes the ability of adiponectin to regulate the adipocyte microenvironment which plays a critical role in modulating whole body insulin resistance.

The regulation of adiponectin and the adiponectin receptors have key roles in the regulation of insulin resistance. Although our data did not find that recombinant adiponectin could reverse hyperglycemic inhibition of insulin stimulated glucose uptake in adipocytes, we did find evidence adipocytes cultured in high glucose media that are insulin resistant have increased intracellular ROS and treatment with adiponectin attenuates production of ROS. It will be interesting to see how the dual regulation of

adiponectin, its receptors, glucose homeostasis and inflammation unfolds. The research reported here provides key pieces to the mechanisms of adiponectin physiology suggesting adiponectin functions in an autocrine and paracrine mechanism to regulate its synthesis and its receptor synthesis. The complexity of the involvement of adipocyte physiology in regulation of whole body energy homeostasis is a marvel to how well regulated the human body is at modifying signaling pathways under complex metabolic stress; and to how excess or depletion of adipose tissue can negatively impact the ability of all tissue in the body to maintain energy homeostasis.

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Sheila Kay Jacobi was born in Floyds Knobs, Indiana, where she attended Floyd Central High School. She entered college at Purdue University where she graduated with a B.S. in Animal Sciences in December 1996. She continued her education at the University of Nebraska-Lincoln by obtaining an M.S. in nutritional biochemistry fall of 1999. She then worked for The Ohio State University for a year working with faculty to develop undergraduate internship opportunities and course development. She then moved back to Purdue to pursue her PhD. After defending her dissertation, Sheila has accepted a position at North Carolina State University.