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Exercise, Epinephrine and IL-6 Mediated Regulation of Adipose Tissue Metabolism

by

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ABSTRACT

The biochemical and molecular mechanisms underlying the beneficial effects of exercise remain elusive. Growing evidence suggests that white adipose tissue (WAT) is an important organ that exercise exerts beneficial effects on. The role of interleukin 6 (IL-6) in mediating WAT metabolism remains under debate.

The first objective of this thesis is to understand exercise, epinephrine and IL-6 in modulating pyruvate dehydrogenase kinase 4 (PDK4) and phosphoenolpyruvate carboxykinase (PEPCK) gene expression in WAT. Exercise and epinephrine increased PDK4 mRNA levels in WAT from lean rats. The effects of epinephrine on PDK4 were mediated via p38 mitogen-activated protein kinase (MAPK) and peroxisome proliferator-activated receptor gamma (PPARγ). The ability of exercise and epinephrine to induce PDK4 mRNA was maintained in obese rats.

In contrast to epinephrine, IL-6 decreased the gene expression and protein content of PEPCK and PDK4 in cultured WAT. Although an acute bout of treadmill running did not activate IL-6 signalling in adipose tissue, the exercise-induced increases in PEPCK and PDK4 mRNA were attenuated in WAT from IL-$6^{-/-}$ mice.

The second broad objective of this thesis was to understand the role of IL-6 in mediating WAT metabolism by utilizing IL-$6^{-/-}$ mice fed a high fat diet (HFD). IL-$6^{-/-}$ HFD mice were more glucose and insulin intolerant than wild type controls and this was mirrored by reductions in the insulin-stimulated activation of protein kinase B (PKB) and increases in the phosphorylation of extracellular signal regulated kinases (ERK) and c-Jun N-terminal kinases (JNK) in WAT. Epinephrine stimulated lipolysis and hormone

sensitive lipase (HSL) phosphorylation were blunted, adiponectin mRNA expression and secretion, and AMPK phosphorylation were reduced in WAT from IL-$6^{-/-}$ mice. These results suggest a unique role for IL-6 in the maintenance of adipose tissue metabolism during the development of insulin resistance.

In summary, exercise, similar to thiazolidinediones (TZDs), is a potent stimulus that can induce WAT glyceroneogenic enzymes. IL-6 is essential for the metabolic homeostasis in WAT under conditions of metabolic stress: exercise and chronic nutrient excess.

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ACC	acetyl coA carboxylase
AICAR	5-Aminoimidazole-4-carboxyamide ribonucleoside
AMPK	5'AMP activated protein kinase
ANOVA	analysis of variance
aPKCζ/λ	atypical isoforms of PKC
ATF3	activating transcription factor 3
ATGL	adipose triglyceride lipase
ATOC	adipose tissue organ culture
AUC	area under the curve
β2MG	beta 2 microglobulin
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCAC	Canadian Council on Animal Care
cDNA	complimentary deoxyribonucleic acid
C/EBP	CCAAT/enhancer binding protein
Cidea	cell death-inducing DNA fragmentation factor-α-like effecter A
CORE1	ubiquinone:cytochrome c oxidoreductase core I subunit
CPT1	carnitine palmitoyltransferase I
CREB	cAMP response element binding protein
CXCL1	chemokine (C-X-C motif) ligand 1
DCA	dichloroacetate
DIO	diet induced obese

ER	endoplasmic reticulum
ERK1/2	extracellular signal regulated kinases 1/2
eWAT	epididymal white adipose tissue
ES	embryonic stem
ETS	electron transport system
FIRKO	fat-specific disruption of the insulin receptor knockout
FNDC5	fibronectin type III domain 5
G-3-P	glycerol 3-phosphate
GLP-1	glucagon-like peptide-1
GLUT4	glucose transporter type 4
gp130	glycoprotein 130
GTT	glucose tolerance test
HDL-C	high density lipoprotein-cholesterol
HFD	high fat diet
HMW	high-molecular weight
HSL	hormone sensitive lipase
HSP60	heat shock protein 60
IL-6	interleukin-6
IL-6$^{-/-}$	IL-6 deficient
IL-6Rα	IL-6 receptor alpha
iNOS	inducible nitric oxide synthase
IP	intraperitoneal
IR	insulin receptor

IRS	insulin receptor substrate
ITT	insulin tolerance test
JAK	Janus kinase
JNKs	c-Jun N-terminal kinases
KRB	Krebs-Ringer Buffer
LIF	leukemia inhibitory factor
LKB-1	liver kinase B1
MAPK	mitogen-activated protein kinase
MK2	MAPK activated protein kinase 2
mtDNA	mitochondrial DNA
mTOR	mammalian target of rapamycin
mtTFAM	mitochondrial transcription factor A
ND5	NADH dehydrogenase subunit 5
NEFAs	non-esterified fatty acids
NRF-1	nuclear respiratory factor 1
OLETF	Otsuka Long Evans Tokushima Fatty
PDC	pyruvate dehydrogenase complexes
PDK1/2	phosphoinositide dependent kinase-1 and 2
PDK4	pyruvate dehydrogenase kinase 4
PDP	pyruvate dehydrogenase phosphatase
PEPCK	phosphoenolpyruvate carboxykinase
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	phosphatidylinositol 3-kinase

PIP3	phosphatidylinositol 3, 4, 5-trisphosphate
PKA	protein kinase A
PKB	protein kinase B
PP2A	protein phosphatase type 2A
PPARγ	peroxisome proliferator-activated receptor gamma
PPRE	peroxisome proliferator hormone response elements
PRDM16	PRD1-BF1-RIZ1 homologous domain containing 16
rh	recombinant human
S6K1	p70S6 kinase
SAPK/JNK	stress-activated protein kinase/Jun-amino-terminal kinase
SAT	subcutaneous adipose tissue
SEM	standard error of the mean
SHP-2	Src homology2-containing tyrosine phosphatise
siRNA	small interfering RNA
SOCS3	suppressor of cytokine signaling 3
SREBP-1c	sterol regulatory element-binding protein-1c
STAT3	signal transducer and activator of transcription 3
T2DM	type 2 diabetes
TBST	Tris buffered saline/0.1% Tween 20
TGs	triglycerides
TNF-α	tumor necrosis factor-alpha
Tyr	tyrosine
TZDs	thiazolidinediones

UCP1	uncoupling protein 1
VAT	visceral adipose tissue
WAT	white adipose tissue
WT	wild type
ZDF	Zucker Diabetic Fatty

Chapter 1 Study Rationale

1.1 Rationale

There is increasing body of evidence to support that white adipose tissue (WAT) might play an important role in regulating the beneficial effects of exercise on systemic metabolism. For instance, exercise has been reported to induce mitochondrial biogenesis (1-2) and improve insulin sensitivity in WAT (3-5). Catecholamines are central modulators for regulating exercise-induced WAT metabolism such as inducing lipolysis (6) and mitochondrial biogenesis (1). Skeletal muscle has been identified as an endocrine organ that secretes "myokines" with one of the best characterized being interleukin 6 (IL-6) (7). It has been proposed that increases in muscle derived IL-6 may be a potential mediator involved in exercise-induced WAT metabolism (8). For example, IL-6 has been reported to stimulate adipose tissue lipolysis (9) and to activate 5'AMP activated protein kinase (AMPK) (10) in adipocytes.

Thiazolidinediones (TZDs) are a commonly prescribed class of insulin-sensitizing drugs that are thought to mediate a large portion of their beneficial effects through their up-regulation of the glyceroneogenic pathway in WAT (11-12). Given the deleterious side effects of TZDs (13), this thesis initially sought to determine if exercise could mimic TZD's effects on glyceroneogenic enzymes with a special focus on phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate dehydrogenase kinase 4 (PDK4), two key enzymes involved in WAT glyceroneogenesis (11-12). Additionally, this thesis aimed to elucidate the potential involvement of epinephrine and IL-6 in the regulation of exercise-induced

PEPCK and PDK4 mRNA expression and the potential signaling pathways that might be involved.

Currently, the role of IL-6 in the development of systemic insulin resistance is still under debate (14). Owing to the central role of skeletal muscle and liver in the regulation of glucose homeostasis, studies investigating the role of IL-6 in glucose homeostasis have focused primarily on these tissues (15-20). Contradictory results with negative (17-18), neutral (16, 19) and positive (15, 20) effects of IL-6 on liver and/or skeletal muscle insulin sensitivity have been reported.

Interstitial concentrations of IL-6 surrounding adipose tissue are orders of magnitude greater than levels in the circulation (21) suggesting that adipose tissue derived IL-6 could play a pivotal role in the maintenance of adipose tissue metabolism and function in an autocrine and/or paracrine manner. Although evidence from *in vitro* studies suggests that IL-6 causes insulin resistance in adipocytes (22-24), there is also evidence that IL-6 may favorably regulate adipose tissue function presumably through its modulation of AMPK (10). Given the purported role of IL-6 in the control of adipose tissue function and the growing appreciation for adipose tissue in the control of systemic energy homeostasis (25), this thesis further targeted the metabolic action of IL-6 on rodent adipose tissue by studying IL-$6^{-/-}$ mice under both chow and a high fat diet (HFD) conditions.

1.2 Objectives and Hypothesis

1.2.1 Objective 1

To determine the regulation of PDK4 mRNA expression in epididymal adipose tissue (eWAT) from lean rats by exercise (swimming) and epinephrine and the potential signalling pathways involved.

Hypothesis 1 Swimming exercise and epinephrine will increase PDK4 mRNA expression in rats eWAT.

Hypothesis 2 Epinephrine will increase p38 mitogen-activated protein kinase (MAPK) and AMPK signaling and these pathways will be involved in epinephrine induced PDK4 mRNA expression in rats eWAT.

Hypothesis 3 Peroxisome proliferator-activated receptor gamma (PPARγ) will be involved in the pathway through which epinephrine increases PDK4 mRNA expression in rats eWAT.

1.2.2 Objective 2

To determine if 2 hours of swimming exercise and acute increases in epinephrine *in vivo* can induce PDK4 mRNA expression in eWAT from insulin resistant rats and if the reputed signaling pathways mediating this effect, i.e. p38MAPK are intact.

Hypothesis 1 An acute bout of exercise and acute increases in epinephrine *in vivo* will induce PDK4 mRNA expression in eWAT from obese, insulin resistant rats.

Hypothesis 2 p38MAPK activation by epinephrine will be intact in eWAT from obese, insulin resistant rats.

1.2.3 Objective 3

To determine 1) the effects of *ex vivo* IL-6 treatment in mouse eWAT on PEPCK and PDK4 expression; 2) if IL-6 signaling is activated and if PEPCK and PDK4 mRNA expression is increased in mouse eWAT immediately after an acute bout of treadmill running; and 3) through the utilization of IL-$6^{-/-}$ mouse model, to elucidate the role of IL-6 in modulating the effects of treadmill running on PEPCK and PDK4 mRNA expression in mouse eWAT and the reputed signaling pathways therein.

Hypothesis 1 IL-6 will directly attenuate the expression of PEPCK and PDK4 in cultured mouse eWAT.

Hypothesis 2 A single bout of exercise will activate IL-6 signalling and induce PEPCK and PDK4 mRNA expression in mouse eWAT.

Hypothesis 3 The induction of glyceroneogenic enzyme expression will be potentiated in eWAT from IL-$6^{-/-}$ mice.

1.2.4 Objective 4

To investigate the metabolic action of IL-6 on rodent adipose tissue by characterizing the adipose tissue phenotypes from age matched wild type and IL-$6^{-/-}$ mice under both chow and high fat diet (HFD).

Hypothesis 1 eWAT from IL-$6^{-/-}$ mice fed a chow diet will display reductions in insulin action *ex vivo*, have blunted β-adrenergic responsiveness *ex vivo* and decreases in adipose tissue mitochondrial content and respiration compared to age matched WT controls under the same diet.

Hypothesis 2 eWAT from IL-$6^{-/-}$ mice fed a HFD will demonstrate reductions in insulin action *in vivo* and *ex vivo* and have blunted β-adrenergic responsiveness *ex vivo* compared to age matched WT groups under the same diet.

Hypothesis 3 eWAT from IL-$6^{-/-}$ mice fed a HFD will have decreases in AMPK phosphorylation, mitochondrial content and respiration, and reductions in adiponectin expression and secretion in comparison with age matched WT groups under the same diet.

1.3 Chapter Format

1.3.1 Chapter 2

Chapter 2 gives a review of the literature about the study of adipose tissue metabolism related to this thesis research. Firstly, a general introduction to adipose tissue biology and the significant role of adipose tissue mitochondria in mediating adipose tissue metabolism will be outlined. Secondly, insulin signaling pathways, the role of MAPK signaling pathways in adipose tissue insulin resistance and a potential role of adiponectin in regulating adipose tissue proper will be summarized. Thirdly, the effects of exercise on adipose tissue metabolism and the potential mediators (catecholamines, irisin and IL-6) involved in exercise induced adipose tissue metabolism will be provided. Lastly, the role of IL-6 in adipose tissue insulin resistance will be discussed extensively.

1.3.2 Chapter 3

Chapter 3 contains the first objective of this thesis research that has been published in the *American Journal of Physiology - Cell Physiology* (Wan Z, Thrush AB, Legare M, Frier BC, Sutherland LN, Williams DB, Wright DC. 2010). *Epinephrine Mediated Regulation of PDK4 mRNA in Rat Adipose Tissue*. *Am J Physiol Cell Physiol.*

2010, 299(5):C1162-70. Firstly, the effect of exercise and *in vivo* epinephrine treatment on PDK4 mRNA expression in eWAT from lean and insulin sensitive rats will be discussed. Secondly, the effects of *ex vivo* epinephrine treatment on PDK4 mRNA expression in eWAT from lean and insulin sensitive rats and the potential signaling pathways (AMPK, p38MAPK and PPARγ) that might be involved will be presented.

1.3.3 Chapter 4

Chapter 4 contains the second objective of this thesis research that has been published in *Obesity* (Wan Z, Frier BC, Williams DB, Wright DC. 2012). *Epinephrine Induces PDK4 mRNA Expression in Adipose Tissue from Obese, Insulin Resistant Rats*. *Obesity, 2012, 20(2):453-56.* The study of exercise and *in vivo* epinephrine treatment on PDK4 mRNA expression in eWAT from obese and insulin resistant rats will be presented.

1.3.4 Chapter 5

Chapter 5 contains the third objective of this thesis research that has been published in *PLoS One* (Wan Z, Ritchie I, Beaudoin MS, Castellani L, Chan CB, Wright DC. 2012). *IL-6 Indirectly Modulates the Induction of Glyceroneogenic Enzymes in Adipose Tissue during Exercise*. *PLoS One, 2012;7(7):e41719.* First, the effects of *ex vivo* IL-6 treatment in mouse adipose tissue on PEPCK and PDK4 mRNA and protein expression, free fatty acids and glycerol release, and AMPK and p38 MAPK signaling pathways will be presented. Second, the effects of an acute bout of treadmill running on PEPCK and PDK4 mRNA expression and IL-6 signaling in mouse adipose tissue will be shown. Lastly, by utilizing IL-$6^{-/-}$ mice, the role of IL-6 in modulating the effects of

exercise on the expression of PEPCK and PDK4 mRNA expression and the potential signaling pathways that might be involved will be discussed.

1.3.5 Chapter 6

Chapter 6 contains the fourth objective of this thesis research that has been *under preparation for submission*. Firstly, adipose tissue insulin action, beta-adrenergic responsiveness and mitochondrial content/function between age matched WT and IL-$6^{-/-}$ mice under chow diet will be characterized. Secondly, the systemic glucose and insulin tolerance between age matched WT and IL-$6^{-/-}$ mice following 10 weeks of HFD will be characterized by intraperitoneal glucose and insulin tolerance test. Thirdly, adipose tissue insulin action and beta-adrenergic responsiveness between age matched WT and IL-$6^{-/-}$ mice under HFD diet will be presented. Lastly, the potential mechanisms that might be involved in adipose tissue insulin resistance from IL-$6^{-/-}$ HFD mice will also be explored.

1.3.6 Chapter 7

Chapter 7 is a general discussion relating the findings of the thesis research from Chapter 3 to Chapter 6. Firstly, methodological considerations for the utilization of specific models will be discussed. Secondly, the results we have obtained from Chapter 3 to Chapter 6 will be discussed and put into a wider perspective. Thirdly, implications for future research that can be taken from the current thesis will also be outlined.

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Chapter 2 Literature Review

2.1 Overview of White Adipose Tissue

2.1.1 Essential role in metabolic homeostasis

It has now been firmly established that white adipose tissue (WAT) is a multifunctional organ that interacts extensively with other tissues in whole-body metabolic homeostasis through a network of endocrine, paracrine, and autocrine signals (1-2). WAT contains several types of cells: mature adipocytes and other cells usually termed as the stromal vascular fraction including endothelial cells, fibroblasts, macrophages and preadipocytes (2).

The primary physiological functions of WAT can be classified into two aspects. First, it is the largest depot in the body for the triglycerides (TGs) storage under the fed state. In contrast, during fasting and exercise, adipose tissue releases glycerol and fatty acids into the circulation to be used as fuel. Thus, adipose tissue serves as a buffer for regulating lipid flux (3). Second, WAT releases a broad array of endocrine and paracrine factors generally referred to as adipokines (4). These adipokines interact with other organs such as the liver, skeletal muscle and hypothalamus to modulate a wide range of metabolic processes including inflammation, angiogenesis and food intake (4-8).

2.1.2 Adipose tissue as an endocrine organ

WAT is an endocrine organ secreting vast quantities of adipokines with potent metabolic effects (9). The number of identified adipokines has expanded rapidly and includes leptin, adiponectin, apelin, visfatin, resistin, vascular endothelial growth factor,

omentin, insulin-like growth factor 1, retinol binding protein-4, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and monocyte chemotactic protein-1 (9). Two of the best characterized adipokines are leptin and adiponectin.

Leptin was first described in 1994 as the product of the *ob* gene in obese *ob/ob* mice (10). Human obesity is characterized by high circulating leptin levels (11). The central effects of leptin primarily include inhibition of appetite (5). This is exerted mainly through its control on the hypothalamus and other specific regions of the central nervous system (5). The peripheral effects of leptin mainly include stimulating fatty acid oxidation in skeletal muscle (12), modulating the immune system such as increasing cytokine production and macrophage adhesion (13) and inducing angiogenesis in the vascular system (14).

Adiponectin was first described in 1995 and is exclusively secreted by adipocytes (15). Circulating adiponectin is composed of trimer, hexamer, and high-molecular weight (HMW) forms (16). Adiponectin has been attributed to play a number of beneficial roles in the body including improvement of insulin sensitivity (17-18) and vascular function (19), anti-atherogenic, anti-inflammatory actions (20) and cardioprotective effects (21).

2.1.3 Depot-specific phenotype in adipose tissue

Rodent models are commonly used for the investigations of mechanisms involved in adipose tissue metabolism. Generally, WAT in rodent models is classified anatomically as subcutaneous adipose tissue (SAT) or visceral adipose tissue (VAT). SAT is primarily located below the skin in the abdominal, gluteal and femoral areas, whereas VAT includes depots of fat deposited close to or even within the viscera of the abdominal cavity, such as gonadal, mesenteric, omental and retroperitoneal fat (22). It is

well documented that the metabolic features of WAT depend on its anatomical location in the body (23). Compared with SAT, VAT has greater basal and catecholamine-induced lipolysis and is less susceptible to the anti-lipolytic action of insulin (24-25). This is mainly owing to regional variation in the action of catecholamines and insulin, the lipolytic action of catecholamines being more effective and the antilipolytic effect of insulin being weaker in VAT than in SAT (26). VAT expresses higher glucocorticoid receptor levels (27) and 11β-hydroxysteroid dehydrogenase type 1, thus glucocorticoids have a greater impact on VAT than on SAT (28). VAT also has a higher rate of insulin stimulated glucose uptake than SAT (29) and exhibits lower capillary density and angiogenic capacity (30). VAT is richer in mitochondrial content (31-32) and has higher rates of lipid synthesis (33), which is in accordance with higher expression of lipogenesis related genes (34). Finally, there is higher macrophage infiltration into VAT during the progression of obesity (35). These fundamental differences among WAT depots might explain their differential influence on adipose tissue metabolism such as lipolysis, glucose uptake (26, 29, 36) and endocrine function (37).

Omental-mesenteric, gonadal and retroperitoneal adipose tissues are considered to be representative of VAT and are most commonly used in rodent metabolic studies. According to “the portal theory”, an increased delivery of non-esterified fatty acids (NEFAs) from VAT to the liver via the portal vein, can cause liver insulin resistance, therefore visceral fat overaccumulation has a more negative impact on health than subcutaneous fat (38). However, it should be noted that only omental-mesenteric fat drains into the portal vein directly (39). Some findings from rodent studies suggest that SAT might actually have beneficial effects on metabolism. For example, peroxisome

proliferator-activated receptor gamma (PPARγ) agonists improve insulin sensitivity by redistributing VAT to SAT (40-41). Likewise, when SAT in donor mice was transplanted into the VAT of the recipient mice, mice showed improved metabolic characteristics (36). Thus, when studying adipose tissue metabolism, it is important to consider the existence of depot-specific effects.

2.2 WAT Mitochondria

Mitochondria are energy-producing organelles and are the main sites of lipid and glucose oxidation. Mitochondrial biogenesis involves both translation of nuclear and mitochondrial genome-encoded proteins, followed by translocation of the nascent proteins across the mitochondrial membranes and proper assembly of these proteins within mitochondria (42). Although traditionally the role of mitochondria in WAT has been received little attention, accumulating evidence strongly support the importance of mitochondrial function for WAT biology and whole body energy homeostasis. The requirement for a large mitochondrial mass in white fat can be linked to key functions of the adipose tissue.

2.2.1 Mitochondria in WAT glyceroneogenesis

In conditions of increased metabolic demand such as during exercise and fasting, there is a marked rise in lipolytic hormones which stimulate the breakdown of TG molecules within fat cells. The liberated fatty acids are either oxidized within the mitochondria in adipocytes (<0.5% of the total), released into the circulation to be used as fuel source (~60-70%) or re-esterified back to TG (~30-40%) (43). The re-esterification of fatty acids is an energy consuming process and is assumed to be the largest single

consumer of ATP in adipocytes (44). Meanwhile, fatty acid re-esterification requires sufficient provision of glycerol-3-phosphate (G-3-P) to serve as the backbone of the TG molecule and at least in rodent adipose tissue, this occurs primarily through the glyceroneogenesis pathway. Thus, intact mitochondria are required for the provision of not only ATP, but glyceroneogenic enzymes as well. For instance, mitochondrial content has been hypothesized to control the availability of glyceroneogenic substrates (45). The majority of this thesis project focuses on WAT glyceroneogenesis, so it will be reviewed in more detail than other functions of the adipocytes.

2.2.1.1 Glyceroneogenesis pathway

Glyceroneogenesis was first defined by Reshef and colleagues in 1970 as the *de novo* synthesis of G-3-P from sources such as pyruvate, lactate or amino acids (46). It is essentially an abbreviated version of gluconeogenesis. Glyceroneogenesis is the major pathway whereby G-3-P is formed in WAT (47-49) for fatty acid re-esterification when blood glucose levels are low such as under fasting states. Two key regulatory enzymes involved in this process are pyruvate dehydrogenase kinase 4 (PDK4) and cytosolic isoform of phosophoenolpyruvate carboxykinase (PEPCK-C). PDK4 inactivates pyruvate dehydrogenase complexes leading to decreases in the formation of acetyl-CoA and a greater flux of pyruvate towards oxaloacetate (50). PEPCK-C catalyzes decarboxylation of oxaloacetate to form phosphoenolpyruvate. Phosphoenopyruvate is then converted into dihydroxyacetone phosphate, with the reduction of dihydroxyacetone phosphate into G-3-P via G-3-P dehydrogenase (47). The activation of PDK4 and PEPCK-C will favor the glyceroneogenic pathway in adipose tissue (50-52). A general description of glyceroneogenesis pathway in adipose tissue is shown in *Figure 2-1*.

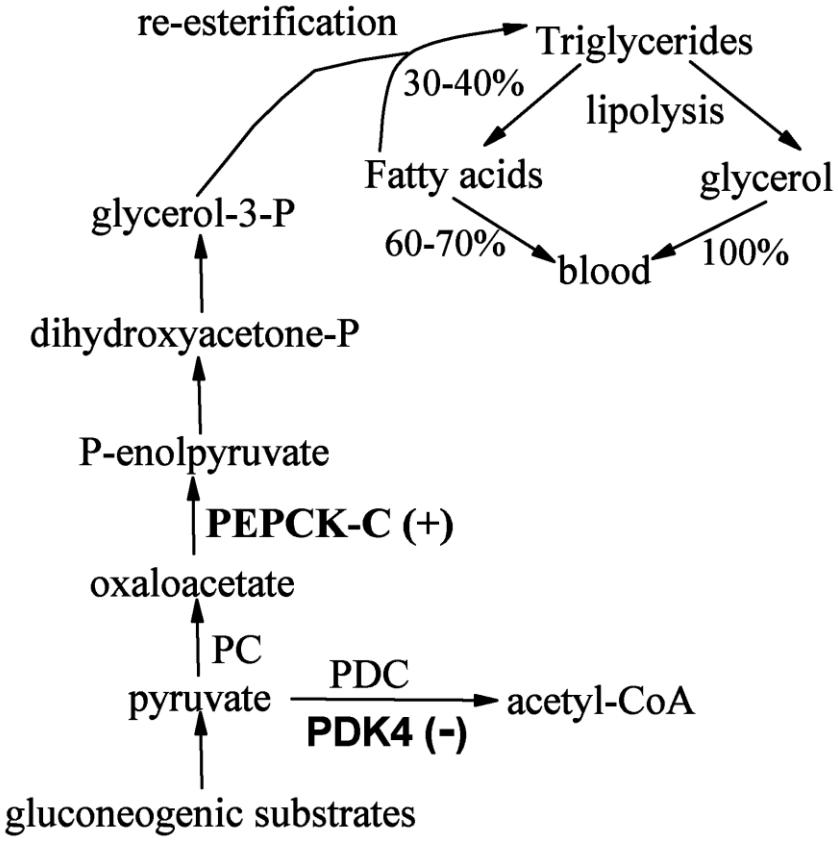

Figure 2-1 General description of glyceroneogenesis and pyruvate routes towards fatty acid re-esterification in adipose tissue.

When blood glucose levels become limiting, lipolysis releases glycerol and fatty acids from triglycerides (TG) stored in fat cells. Almost 100% of the glycerol is released because it is not phosphorylated significantly in adipose per se. In contrast, approximately 40% of released fatty acids are re-esterified back to TGs mainly through the glyceroneogenic pathway. Gluconeogenic substrates other than glucose and glycerol (i.e. pyruvate, lactate, alanine, and citric acid cycle anions) are converted into pyruvate first. Pyruvate can either be used in the tricarboxylic acid cycle after carboxylation by pyruvate dehydrogenase complexes (PDC) into acetyl-CoA or be favored towards glyceroneogenesis if carboxylated to oxaloacetate (OA) by pyruvate carboxylase (PC) in the mitochondria. Pyruvate dehydrogenase kinase 4 (PDK4) inactivates PDC, thereby regulating pyruvate availability for glyceroneogenesis in adipocytes. The cytosolic isoform of phosophoenolpyruvare carboxykinase (PEPCK-C) is involved in the conversion of OA to P-enolpyruvate, the precursor of glycerol-3-phosphate (G-3-P). Thus, the activation of PDK4 and PEPCK-C will favor the glyceroneogenic pathway in adipose tissue. Modified from Cadoudal et al., Diabetes, 2008.

2.2.1.2 PDK4

PDK4 and insulin resistance

On the one hand, PDK4 is postulated to be involved in the etiology of insulin resistance (53). Skeletal muscle PDK4 protein expression is selectively increased in insulin deficient (54-55) and insulin resistant states (56-57). PDK4 knockout (KO) mice are more glucose tolerant compared to wild type (WT) mice (58). In adipocytes, induction of PDK4 by prolactin resulted in decreased insulin-stimulated glucose uptake (59).

On the other hand, PDK4 has been thought to mediate a portion of the beneficial effects of thiazolidinediones (TZDs) on adipose tissue lipid metabolism. TZD treatment of adipocytes leads to increased expression of PDK4 concomitant with an increased rate of glyceroneogenesis (50). PDK4 appears to be essential for the upregulation of glyceroneogenesis by TZDs because the attenuation of PDK4 activity pharmacologically with leelamine or genetically by small interfering RNA (siRNA) both result in decreased fatty acid re-esterification (50).

Regulation of PDK4 expression by exercise and fasting

Pilegaard et al. (60) were the first to report induction of transcription of PDK4 by exercise in human skeletal muscle. They demonstrated that both five consecutive days of one-legged knee extensor exercise (60–90 min) and a single 4h low-intensity cycling bout induced marked increases in *pdk4* mRNA in human skeletal muscle. Likewise, short-term high-intensity exercise can also activate *pdk4* mRNA (61) in skeletal muscle. Similarly, starvation has been shown to induce *pdk4* mRNA expression in both rodent and human skeletal muscle (62-63). Meanwhile, exercise and fasting have also been reported to

induce PDK4 in liver (64-66). Mechanistically, 5'AMP activated protein kinase (AMPK) and p38 mitogen-activated protein kinase (MAPK) signalling cascades have been implicated in the regulation of PDK4. The activation of AMPK has been postulated to either increase (67) or have no effect (68) on *pdk4* mRNA in lean, insulin-sensitive muscle or decrease (68) *pdk4* mRNA in obese and diabetic skeletal muscle. Dexamethasone has been shown to increase PDK4 activity in isolated cardiomyocytes through the phosphorylation and nuclear translocation of p38AMPK (69). The overexpression of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) in rodent skeletal muscle (70) and C2C12 muscle cells (71) results in increases in *pdk4* gene expression, suggesting that PGC-1α might be a key regulator of PDK4. However, considering AMPK is a modulator of PGC-1α (72-73) and the discrepant effects of AMPK activation on PDK4 in skeletal muscle (67-68), it is also possible that the regulation of PDK4 might be through a PGC-1α-independent pathway.

2.2.1.3 PEPCK

PEPCK and insulin resistance

Several lines of evidence suggest that dysregulation of adipocyte PEPCK expression could be an etiological factor in type 2 diabetes (T2DM) (74). First, pharmacologically, TZDs have been reported to induce *pepck* mRNA expression in rodent and human adipocytes and adipose tissue explants concomitant with an increased rate of fatty acid re-esterification and reduced release of fatty acid into the circulation (51-52, 75-76). Second, Olswang et al. (77) genetically engineered mice that have an ablation of PEPCK in WAT. These mice have reduced adipose tissue size and lipid content, and adipose tissue pieces from these mice released higher amounts of NEFAs.

Furthermore, approximately 25% of the mice have mild insulin resistance with notable lipodystrophy. Franckhauser et al. (78) created transgenic mice that overproduced PEPCK specifically in WAT. Although these mice were obese, they never became diabetic and appeared to have modestly increased sensitivity to insulin. However, the following findings from the same research group also demonstrated that PEPCK overexpression in WAT cause insulin resistance when challenged with a high fat diet (HFD) (79). It was suggested that under HFD conditions, the storage capacity of adipose tissue was overwhelmed, consequently resulting in ectopic fat deposition in liver and skeletal muscle, and leading to insulin resistance (79). Millward et al. (80) demonstrated that mice with reduced *pepck* mRNA in WAT develop systemic insulin resistance. Thus, most of the evidence suggests that dysregulation of PEPCK in adipose tissue might cause imbalance of whole body energy homeostasis.

Regulation of PEPCK by exercise and fasting

The mRNA of *pepck* was upregulated by exercise and fasting in mouse liver (81-82). Glyceroneogenesis and *pepck* mRNA have been shown to be increased under fasting condition in rodent adipose tissue (46, 83-84). Further, it was demonstrated that the PEPCK metabolic response to fasting decreased as rats aged (84). Catecholamines might be one mediator for regulating PEPCK in adipose tissue as the infusion of epinephrine in rats resulted in a marked increase in glyceroneogenesis in epididymal adipose tissue (47). Mechanistically, β-2 adrenergic agonists have been reported to stimulate *pepck* mRNA expression through cAMP in adipocytes (85-86).

Regulation of PEPCK by inflammation in WAT

Long-term increases in inflammation (87-88) and in particular IL-6 (89), attenuates PEPCK in fat cells, which might contribute to the increased fatty acid mobilization under inflammatory conditions (89). Furthermore, PEPCK inhibition by inflammation leads to inhibition of glyceroneogenesis and adipogenic deficiency in adipocytes, which might be a mechanism contributing to the adipose dysfunction in obesity (88).

2.2.2 Mitochondria in WAT *de novo* lipogenesis

De novo lipogenesis in WAT requires acetyl-CoA formed in the mitochondria by the oxidation of pyruvate. However, acetyl-CoA is not able to cross the mitochondrial membrane by itself. Acetyl-CoA and oxaloacetate combine via citrate synthase to form citrate, move to the cytosol and then are simultaneously converted back to acetyl-CoA and oxaloacetate by ATP citrate lyase (90). This shuttle provides the cytosolic acetyl-CoA necessary for *de novo* lipogenesis and requires ATP at several steps (90). To support the significant role played by mitochondria in *de novo* lipogenesis, Kaaman et al. (91) demonstrated that the mitochondrial DNA (mtDNA) number in isolated human subcutaneous adipocytes has a strong positive correlation with basal and insulin stimulated *de novo* lipogenesis. Meanwhile, inhibition of citrate export from the mitochondria at an early differentiation stage of 3T3-L1 adipocytes resulted in decreased accumulation of fat droplets (92). Taken together, the above evidence imply that mitochondrial dysfunction in adipocytes, is associated with a decreased capacity to store fatty acids as TG within adipocytes.

2.2.3 Mitochondria in WAT adipogenesis

Adipogenesis is a biological process involving proliferation of preadipocytes from precursor cells, followed by differentiation into mature adipocytes (93). The acquisition of a mature adipocyte phenotype is characterized by changes in the activity of a number of transcription factors including cAMP response element-binding protein (CREB), CCAAT/enhancer binding protein (C/EBP) family members and PPARγ, that directly influence adipogenesis (94). Mitochondrial biogenesis has been considered as a fundamental aspect of white adipose cell differentiation because mitochondrial proteins are generated at very high levels during the differentiation process (95-96). Adipogenesis is accompanied by changes in mitochondrial structure and biochemical composition, upregulation of components of the mtDNA replication and transcription machinery (93, 97), as well as increased oxygen consumption rate (98). The essential role of citrate export from the mitochondrial matrix to the cytosol for effective differentiation into adipocytes at the early adipogenic stage of 3T3-L1 cells has also been reported (92). Accordingly, chemical inhibition of mitochondrial complex machinery inhibits adipogenic differentiation (99).

2.2.4 Mitochondria in adiponectin synthesis

It has been reported that mitochondrial function is essential for adiponectin synthesis in adipocytes. Enhancement of mitochondrial biogenesis through rosiglitazone and over-expression of nuclear respiratory factor 1 (NRF-1) increased adiponectin synthesis whereas impairment of mitochondrial function through chemical uncouplers and mitochondrial transcription factor A (mtTFAM)-specific siRNA reduced adiponectin synthesis (100). Mechanistically, mitochondrial dysfunction decreases adiponectin

synthesis through pathways involving endoplasmic reticulum (ER) stress, c-Jun N-terminal kinases (JNKs), and activating transcription factor 3 (ATF3) (100).

2.2.5 Adipose tissue mitochondrial dysfunction and insulin resistance

2.2.5.1 Evidence for adipose mitochondrial dysfunction in insulin resistance

Accumulating evidence from clinical observations, and animal and cell culture studies have substantiated a strong relationship between adipose tissue mitochondrial dysfunction and insulin resistance. Activities of mitochondrial respiratory chain complexes and mtDNA are decreased in the adipose tissue or adipocytes from different experimental models of obesity (101-103), subjects with morbid obesity and T2DM (104-108). Impairment of adipose tissue mitochondrial function in insulin resistant states might result from reductions in PGC-1. PGC-1 family members are key components regulating the respiratory chain and the biogenesis of mitochondria (109). For instance, PGC-1α expression is reduced in adipose tissue from several insulin resistant rodent models including Zucker Diabetic Fatty (ZDF) rats, HFD rats, *db/db*, *ob/ob* and diet induced obese (DIO) mice (101, 103, 110-111) and in adipose tissues from individuals with insulin resistance or T2DM (91, 104, 112-113). PGC-1β is suppressed in the inguinal fat in both DIO and *db/db* mice (103). Conversely, reversal of impaired mitochondrial function in adipose tissue could be accomplished through use of PPARγ agonist TZDs treatment (45, 103-104) and exercise (114). These alterations are concomitant with increases in whole body glucose homeostasis.

Whether impairment in adipose tissue mitochondrial function is a cause or consequence of impaired glucose homeostasis is still unclear. On the one hand, there is

evidence suggesting that mitochondrial dysfunction precedes the development of impaired glucose homeostasis. A recent twin study suggests that a decline in mtDNA copy number and mitochondrial activity to catabolise branched chain amino acids may be induced upon development of obesity which precedes development of glucose intolerance (115). The mice with disruption of the insulin receptor knockout (FIRKO) specifically in adipose tissue are protected against obesity, as well as obesity-related glucose intolerance and other metabolic abnormalities. This phenotype could be related to the fact that FIRKO mice from 6 to 36 months of age have sustained high mRNA expression of the nuclear encoded mitochondrial genes involved in glycolysis and tricarboxylic acid cycle, as well as PGC-1α and PGC-1β mRNAs (116). On the other hand, there is evidence to demonstrate that impairment of mitochondrial content may not be an initiating factor for insulin resistance, but appears to play a key role in the worsening of the disease condition. For instance, reduction of WAT mitochondrial content in Otsuka Long Evans Tokushima Fatty (OLETF) rats developed after overt diabetes (114). Similarly, reductions in mitochondrial content and PGC-1α in HFD rats occurred after the onset of glucose intolerance (101).

2.2.5.2 Potential mechanisms by which impaired adipose mitochondrial function could lead to insulin resistance

First, as detailed above, dynamic mitochondrial activity is crucial for adipocyte function. Thus, impairments in adipose tissue mitochondrial function may alter whole body glucose homeostasis through its effects on glyceroneogenic, lipogenic, and adipogenic pathways. Of primary interest to this thesis research, the disorders of glyceroneogenic pathways would lead to increased NEFAs output into the blood.

Increased circulating NEFAs can cause insulin resistance by interfering with the insulin signaling cascade mainly in the liver and skeletal muscle (117). Second, impaired mitochondrial function in adipose tissue can directly lead to insulin resistance in fat cells. For instance, siRNA mediated reduction of TFAM during adipocyte differentiation lead to impaired insulin-stimulated glucose uptake (118). Third, adiponectin provides a physiological link by which the energy status of adipose tissue may influence systemic insulin sensitivity.

2.3 Adipose Tissue Insulin Resistance

2.3.1 Insulin action and signaling pathways in adipose tissue

The insulin signaling cascades in the whole body including adipose tissue comprise a highly integrated and complex network (119). Briefly, under normal physiological conditions, insulin secreted from the pancreas reaches its target tissues and interacts with insulin receptor (IR). The activated IR subsequently phosphorylates insulin receptor substrate (IRS) proteins and then activates two major signaling pathways: (1) phosphatidylinositol 3-kinase (PI3K)-Akt/protein kinase B (PKB) pathway, which is involved in most of the metabolic actions of insulin and (2) the Ras- mitogen-activated protein kinase (MAPK) pathway, which is implicated in most of the transcriptional and mitogenic effects of insulin (120). A schematic diagram of several central points of insulin signaling pathway and its action in WAT is shown below in *figure 2-2*.

There are at least 6 known substrates of IRS proteins expressed in insulin-sensitive tissues with IRS-1 and -2 being the main IRS involved in insulin-mediated metabolic functions (121). IRS proteins are activated mainly through tyrosine (Tyr)

phosphorylation (119). The PI3K cascade is activated by Tyr-phosphorylated IRS proteins and catalyzes the formation of phosphatidylinositol 3, 4, 5-trisphosphate (PIP_3). PIP_3 stimulates phosphoinositide dependent kinase-1 and 2 (PDK1/2) activity, which in turn activates its downstream effectors: Akt/PKB, mammalian target of rapamycin (mTOR), p70S6 kinase (S6K1), as well as the atypical isoforms of PKC (aPKCζ/λ) (122).

Akt/PKB is a key molecular player of insulin-stimulated metabolic pathways. The phosphorylation of Akt/PKB at both serine-473 and threonine-308 is responsible for regulating most of the PI3K-mediated metabolic function of insulin (119). Thus, Akt/PKB phosphorylation states are usually used to demonstrate the insulin stimulation status within a tissue. In adipose tissue, activation of Akt/PKB has the following two major physiological functions: 1) regulates insulin-stimulated glucose uptake via stimulating glucose transporter type 4 (GLUT4) vesicle translocation to the plasma membrane (123); and 2) mediates the phosphorylation and activation of phosphodiesterase 3B (PDE3B), PDE3B then catalyzes the hydrolysis of cyclic adenosine monophosphate (cAMP) to 5'AMP, thus leading to attenuated protein kinase A (PKA) activity and lipolysis (124-125). It should be noted, however, that insulin can also regulate lipolysis through an Akt independent signaling pathway (126).

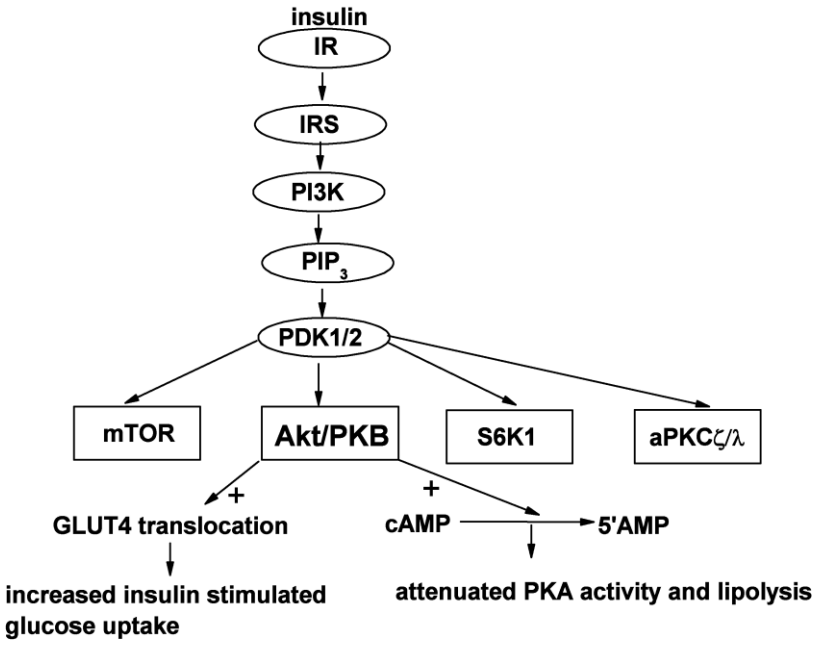

Figure 2-2 A schematic diagram of several central points of insulin signaling pathway and its action in WAT.

Insulin binding to IR leads to the activation of IRS, activating PI3K and its downstream effector PIP_3. PIP_3 stimulates PDK1/2 activity, which result in the activation of its downstream signaling proteins including Akt/PKB, mTOR, S6K1 and aPKCζ/λ. The phosphorylation of Akt/PKB is a key regulator of insulin signal transduction and has two main physiological functions in WAT. First, it can induce the translocation of GLUT4 from the intracellular compartment to the plasma membrane, largely accounting for increased insulin stimulated glucose uptake. Second, it can phosphorylate and activate PDE3B. The activation of PDE3B inactivates cAMP, resulting in attenuated PKA activity and lipolysis. +, stimulation or upregulation.

2.3.2 MAPKs in adipose tissue insulin resistance

A major cause of insulin resistance is the perturbation of insulin signaling system via phosphorylation of IR and IRS on certain serine/threonine residues, as well as the downstream signaling pathways (119). MAPKs are Ser/Thr specific protein kinases that mediate extracellular stimuli into a wide range of cellular responses. There are at least 4 subfamilies of MAPKs: the extracellular signal regulated kinases 1/2 (ERK1/2), ERK5, JNKs and p38MAPK (127). Of primary interest to this thesis, all except ERK5 have been

reported to be involved in the negative regulation of insulin signaling in adipose tissue in response to external stimuli such as NEFAs and inflammation (128-129).

An increased basal activity of ERK1/2, p38MAPK and JNKs has been reported in adipocytes from patients with T2DM (129). Activation of p38 MAPK/JNKs pathways has been proposed to functionally link visceral adiposity with whole body insulin resistance (130). Constitutive activation of ERK1/2, p38 or JNKs in 3T3-L1 adipocytes induces insulin resistance by affecting GLUT4 expression and insulin signaling proteins such as IRS, PI3K and Akt (131).

ERK1/2 is required for early adipocyte differentiation (132). Mice deficient in ERK1 have reduced adiposity and are resistant to diet-induced insulin resistance owing to decreased adipogenesis and elevated energy expenditure (133). Conversely, mice deficient in signaling adaptor p62, an ERK inhibitor, have high basal levels of ERK activity and develop characteristics of metabolic syndrome (134).

JNK1 is a negative modulator of insulin signaling, inhibition of which improves insulin sensitivity in insulin resistant rodent models (135). Mice lacking JNK1 specifically in adipose tissue protect against HFD-induced insulin resistance in the liver through a reduction in IL-6 secretion from adipose tissue (136). The phosphorylation of IRS-1 at serine 307 is an important mechanism by which the activation of JNK can inhibit insulin signaling in adipocytes (137).

In contrast to ERK1/2 and JNK, the role of p38 MAPK in insulin sensitivity *in vivo* has not been established yet. Meanwhile, the role of p38 MAPK in regulating glucose disposal in adipocytes *in vitro* is still in debate. On the one hand, there are studies suggesting that p38 MAPK plays an important role in insulin-stimulated glucose uptake

(138-139). On the other hand, there are also evidence supporting that p38 MAPK is not involved in insulin-stimulated glucose disposal in adipocytes (140-141).

2.3.3 Adiponectin in adipose tissue insulin resistance

2.3.3.1 Adiponectin and adipose tissue biology

Although most studies have been focusing on exploring the endocrine properties of adiponectin in its target tissues such as the skeletal muscle and liver, an increasing body of evidence begin to suggest that adiponectin plays an important role for regulating adipose tissue proper.

Globular adiponectin enhances both basal and insulin-stimulated glucose uptake in isolated rat adipocytes through an AMPK dependent signaling pathway (142). Overexpression of adiponectin in 3T3-L1 fibroblasts has been shown to result in increased adipocyte differentiation and lipid accumulation (143), whereas others reported adiponectin disrupted adipogenesis in 3T3-F442A cells (144). Recently, adiponectin has been reported to inhibit spontaneous and catecholamine-induced lipolysis in human adipocytes from SAT of non-obese individuals, while this effect seems absent in obese subjects (145). Similarly, adiponectin suppresses basal and catecholamine-stimulated lipolysis in mouse adipoytes (146).

Overexpression of adiponectin specifically in WAT of FVB mice resulted in decreased adipose mass and impaired adipocyte differentiation (144). Adiponectin transgenic mice through a dominant mutation in the collagenous domain of adiponectin develop a phenotype that resembles the effects of chronic exposure to β3 adrenergic treatment (147). Specifically, these mice have increased sensitivity to adrenergic stimuli,

increased mitochondrial density in adipocytes, smaller adipocyte size and upregulation of mRNAs involved in lipid storage (147). Thus, it has been proposed by Asterholm et al. (147) that adiponectin plays a crucial role in maintaining the metabolic flexibility of adipose tissue, making adipose tissue more sensitive to metabolic challenges. Meanwhile, the adipose tissues of these transgenic mice have increased expression of genes involved in oxidative phosphorylation, as well as mitochondrial markers including PRD1-BF1-RIZ1 homologous domain containing 16 (PRDM16) and cell death-inducing DNA fragmentation factor-α-like effecter A (Cidea) (148).

Both adipoR1 and adipoR2 are expressed in human and murine adipocytes (149-150), suggesting that adiponectin has autocrine/paracrine effects on adipose tissue. Further studies are required to elucidate the physiological roles of adiponectin in adipose tissue and its potential involvement in adipose tissue insulin resistance under metabolic challenges such as HFD.

2.3.3.2 Regulation of adiponectin in adipose tissue

Adiponectin is initially synthesized as a single polypeptide of 30 kDa and is then assembled in the ER, primarily into 3 forms: trimer, hexamer and HMW multimer (151). The different isoforms of adiponectin have been shown to play distinct biological roles (152-154). The expression, assembly and secretion of adiponectin are regulated at both transcriptional and post-translational level (155). PPARγ is a major player in these processes.

At the transcriptional level, PPARγ may increase adiponectin mRNA directly through its action on adiponectin gene promoter. A functional PPARγ-recognizing peroxisome proliferator hormone response elements (PPRE) site has been identified both

in the human (156) and mouse (157) adiponectin promoters. Point mutations at this site resulted in reduced basal and TZD-induced transactivation of adiponectin promoter (156). At the post-translational level, PPARγ induces the transcription of genes such as Erol-Lα, DsbA-L and ERp44, which encode proteins involved in adiponectin assembly and secretion in the ER (151, 158-159).

2.4 Response of WAT to Exercise

2.4.1 Exercise-induced adipose tissue mitochondrial biogenesis

More than 2 decades ago, Stallknecht et al. (160) demonstrated that exercise training (12 weeks of daily swimming, 6 h per day) increased mitochondrial enzyme activity in the epididymal adipose tissue of rats. The recent results from our research group confirmed this. Sutherland et al. (161) reported that swim training for 4 weeks (2h per day) increased the activity of citrate synthase and mitochondrial respiratory chain enzyme content in epididymal and retroperitoneal fat from rats. Meanwhile, both exercise training and an acute bout of exercise increased PGC-1α mRNA expression. Similarly, Xu et al. (162) reported that treadmill running increased mitochondrial density in mouse epididymal fat by transmission electron microscopy of *in situ* mitochondria. Collectively, the effects of exercise on adipose tissue mitochondrial biogenesis are not exercise type specific because they are observed not only in swim training but also in treadmill running. Exercise induced increases in adipose tissue mitochondrial enzymes and activity further demonstrates the plasticity of adipose tissue in response to exercise.

However, the evidence from human studies is conflicting. Ruschke et al. (163) reported that 4 weeks of training led to increases in PGC-1α mRNA expression only in

human subcutaneous but not omental fat. Camera et al. (164) reported that short-term endurance training did not alter citrate synthase activity, mitochondrial volume or PGC-1α mRNA expression in human subcutaneous adipose tissue although training-induced adaptations in skeletal muscle were observed. As has been discussed in Section1.1.3, subcutaneous fat is less sensitive to adrenergic stimuli than visceral fat. Catecholamines are one of the critical regulators of exercise-induced adipose tissue metabolism (165), thus depot-specific responsiveness to catecholamines might be one contributing factor for the discrepant results from human studies. Meanwhile, the duration and intensity of exercise, as well as the type of exercise might also be important factors for determining exercise-induced mitochondrial biogenesis in WAT.

2.4.2 Exercise-induced adipose tissue lipolysis

During endurance exercise, when carbohydrate reserves are depleted, the mobilization of NEFAs in adipose tissue becomes the main source for energy for the working muscle. The elevated circulating levels of catecholamines are one of the main regulators of adipose tissue lipolysis during exercise. When catecholamines, the hormone adrenaline and the neurotransmitter noradrenaline, bind β-adrenergic receptors, which are seven-transmembrane domain receptors coupled to stimulatory G proteins, there is an activation of adenylyl cyclase leading to a rise in intracellular cAMP levels and the activation of PKA. Activated PKA phosphorylates the lipid droplet protein perilipin and hormone sensitive lipase (HSL), thereby promoting hydrolysis of TG (166-169). HSL is the rate-limiting step for the liberation of fatty acids from adipose tissue into the circulation in this process (170). Reversible phosphorylation at several sites is a hallmark of HSL regulation. It has been demonstrated that Ser-563, Ser-659, and Ser-660 are major

PKA phosphorylation sites responsible for stimulating HSL with Ser-659 and Ser-660 as the major activity controlling sites (171-172).

For a long time, HSL has been viewed as the sole rate-limiting lipase for TG hydrolysis. In 2004, three research groups identified a new enzyme and independently named it adipose triglyceride lipase (ATGL) (173), desnutrin (174) and calcium-independent phospholipase A_2zeta respectively. In 3T3-L1 adipocytes, ATGL can markedly enhance both basal and catecholamine-stimulated lipolysis (173). ATGL-deficient mice have reduced isoproterenol stimulated lipolysis (175). Thus, it seems likely that ATGL is also involved in catecholamine-stimulated lipolysis, however this is still controversial. Two recent findings indicate that ATGL might be a critical regulator of exercise-induced lipolysis. First, Huijsman et al. (176) demonstrated that ATGL deficient mice have impaired running ability and reduced plasma NEFAs availability (176). Second, Wohlers et al. (177) further showed that a single bout of treadmill running significantly increased ATGL content in omental/mesenteric fat of female mice (177). Owing to the insufficient time since its discovery, further studies are required to elucidate the signaling pathways for exercise-induced activation of ATGL in adipose tissue.

2.4.3 Effects of exercise on adipokines

2.4.3.1 Adiponectin

Circulating adiponectin is not affected (178-179) or even decreased (180) in response to exercise in healthy subjects with normal weight; while in obese and overweight subjects, exercise, independently (181-183), or combined with diet-induced weight loss (184), significantly increased plasma adiponectin levels. Generally, it was

considered that the exercise-induced modification of adiponectin is to a large extent determined by changes in body composition (185). Meanwhile, exercise training intensity might also be a factor for influencing plasma adiponectin level, at least in rodent models (186).

2.4.3.2 Leptin

Training has disparate effects concerning leptin concentration with both decreased (187-188) or no alterations (189) having been reported. Generally, plasma leptin concentrations would be decreased if exercise training results in reduced fat mass (190).

2.4.4 Exercise on glucose uptake in adipose tissue

As early as two decades ago, it was reported that 10 weeks of exercise training normalized impaired adipose cell glucose metabolism from streptozocin-treated rats (191). Mechanistically, exercise training increased total GLUT4 protein in adipose cells of rats and adipose tissue of patients with T2DM (192-193). Similarly, exercise training has been reported to increase the response of WAT to insulin through improved activation of IRS/PI3K/Akt/PKB pathway in isolated adipocytes of rats (194). Taken together, the increase in exercise-mediated glucose uptake through increased IRS/PI3K/Akt/PKB/GLUT4 pathway in adipose tissue are at least partially responsible for the improvement in insulin sensitivity of T2DM following exercise training.

2.4.5 Mediators of exercise-induced adipose tissue metabolism

2.4.5.1 Catecholamines

As mentioned above, catecholamines increase during exercise and serve to stimulate adipose tissue lipolysis (168). Epinephrine increases the mRNA expression of

PGC-1α and mitochondrial respiratory chain enzymes, and is considered to mediate at least partially exercise-induced adipose tissue mitochondrial biogenesis (161).

Increases in circulating catecholamine levels have been shown to activate multiple signalling pathways in adipose tissue. Of particular interest to this thesis research is the AMPK and p38 MAPK signalling pathways. β-adrenergic agonists have been reported to increase the activity of AMPK (165, 195-196) and p38 MAPK (197-199) in adipocytes. Studies from both rodent and humans have shown that acute exercise can cause AMPK activation in adipose tissue (165, 196, 200), however this is not a universal finding (201-202). Meanwhile, it has been confirmed that the effects of exercise on AMPK activity are mediated by catecholamines in the adipocytes of female rats (165). The duration and intensity of exercise, as well as the type of exercise might be important factors for influencing the phosphorylation status of AMPK in adipose tissue (201). Furthermore, whether there is gender specific difference in regards to exercise induced AMPK phosphorylation in adipose tissue also needs to be further elucidated.

It seems that the activation of lipolysis plays a major role in the activation of AMPK and p38 MAPK signalling pathways in adipose tissue. The activation of AMPK by catecholamine occurred as a consequence of lipolysis and subsequent increases in FA re-esterification (195). Similarly, β-adrenergic agonists-induced activation of p38 MAPK was attenuated when lipolysis was inhibited (199). Whether exercise and epinephrine can affect *pdk4* and *pepck* mRNA in WAT through AMPK and/or p38 MAPK signaling pathways in adipose tissue remains unknown.

2.4.5.2 Irisin

A recent study from Spiegelman's group has identified a novel peptide as a PGC-

1α dependent myokine that induces the differentiation of mouse subcutaneous white fat cells into a brown adipocytes phenotype during exercise (203). In this study, exercise and overexpression of PGC-1α specifically in muscle cells induced the expression of type I membrane protein fibronectin type III domain 5 (FNDC5) (203). FNDC5 was then cleaved and secreted into the circulation (203). The secreted protein product of FNDC5 was named irisin (after Iris, the Greek messenger goddess) (203). Irisin induced the expression of thermogenic genes in WAT such as uncoupling protein 1 (UCP1) and Cidea, partly through increased PPARα expression (203). It seems irisin is necessary for the exercise-induced thermogenic program in the WAT as injection of anti-FNDC5 reduced the effect of exercise on *ucp1* and *cidea* mRNA expression (203).

2.4.5.3 IL-6

Exercise is a robust perturbation that leads to marked changes in a wide range of hormonal factors. Skeletal muscle has been recently identified as an endocrine organ that secretes "myokines" (reviewed in (204)). One of the most extensively studied is IL-6. IL-6 secretion is increased from contracting skeletal muscle and is thought to be the primary contributor to the increased plasma IL-6 during exercise (205). Pedersen and colleagues have proposed that increases in muscle derived IL-6 plays a role in mediating exercise-associated metabolic changes and may be a potential mediator involved in exercise-induced adipose tissue metabolism (206).

On the one hand, it has been posited that IL-6 stimulates lipolysis in adipose tissue (207-208) and plays a role in regulating exercise-induced increases in adipose tissue AMPK activity (209-210). Conversely, the long-held view that muscle-derived IL-6 signals to adipose tissue during exercise is increasingly being challenged. For instance

IL-6 infusion in healthy humans was shown to selectively stimulate lipolysis in skeletal muscle and have no effect on adipose tissue lipolysis (211). Similarly, IL-6 was found to not play a role in regulating subcutaneous adipose tissue AMPK activity during exercise (201). Recently, exercise in rats has been shown to induce the activation of IL-6 signalling in the liver and that IL-6 could contribute to increase hepatic glucose production by directly upregulating *pepck* mRNA expression (81). Of interest, previous work has demonstrated that long-term increases in inflammation (212-213), and in particular IL-6 (89), attenuate PEPCK expression in fat cells. These findings suggest that increases in IL-6 may serve to dampen the exercise-mediated induction of glyceroneogenic enzymes in adipose tissue. However, it should be noted that interstitial concentrations of IL-6 ranged from 2000 to 3000 pg/ml (214), the circulation concentration of IL-6 is around 35 pg/ml even after extremely large volumes of exercise such as a marathon (215). Therefore, interstitial concentrations of IL-6 are several orders of magnitude higher than levels in the circulation after exercise. Thus, it is of interest to see if IL-6 signalling is activated in adipose tissue during exercise considering the already high concentrations of IL-6 surrounding this tissue at rest. Meanwhile, the role of IL-6 in modulating the effects of exercise on glyceroneogenic enzyme expression in WAT and the reputed signalling pathways therein remains unknown.

2.4.5.4 The interactions between epinephrine and IL-6 during exercise

There are several lines of evidence suggesting that epinephrine increases IL-6 production when administered at supraphysiological concentrations (216-219). β-adrenergic activation via isoproterenol increases IL-6 at both mRNA and protein levels *in vitro* in human breast adipocytes (220). In contrast, there is evidence to suggest that

epinephrine is unlikely to mediate the increased IL-6 mRNA and protein release during exercise. An epinephrine infusion in resting humans, with the circulating epinephrine level comparable to that of exercise-induced increases in plasma epinephrine, only increased plasma IL-6 at a modest levels (about 6-fold), which is much lower than the plasma IL-6 levels observed during exercise (about 30-fold) (221).

2.4.5.5 The interactions between IL-6 and other myokines during exercise

During exercise, skeletal muscle secretes a wide range of myokines other than IL-6, such as myostatin, IL-7, murine chemokine (C-X-C motif) ligand 1 (CXCL1) and leukemia inhibitory factor (LIF) (222). For instance, an acute bout of exercise increased *cxcl1* mRNA expression specifically in skeletal muscle and liver, as well as serum CXCL1 level (223). However the above effects are completely blunted from IL-6-deficient mice (223). Overexpression of IL-6 in muscle cells increased serum CXCL1 and liver *cxcl1* mRNA expression (223). Thus, it is suggested that exercise-induced CXCL1 in the liver is regulated by muscle-derived IL-6 (223). Although exercise-induced CXCL1 in adipose tissue has not been reported at present, we can't exclude this possibility considering the duration and intensity of exercise, as well as the type of exercise might be important factors for determining exercise induced metabolism in WAT. Furthermore, it is tempting to speculate that increases in skeletal muscle-derived IL-6 signal to the liver during exercise and consequently result in increases in CXCL1. CXCL1 signals to regulate adipose tissue metabolism. Clearly, further studies are required to elucidate the crosstalk between muscle/liver/adipose tissue during exercise with a special focus on newly discovered myokines, hepatokines and adipokines.

2.5 IL-6 in WAT Metabolism

2.5.1 IL-6 production and IL-6 signaling in adipose tissue

It has been long known that circulating and adipose tissue IL-6 levels are increased in patients with obesity, insulin resistance and T2DM (224). Chronic low grade elevations of IL-6 in adipose tissue have been posited to correlate with the degree of insulin resistance (225-226). However, it has also been proposed that increased circulating levels of IL-6 are related to increased fat mass instead of insulin resistance (227). Adipocytes and other cells in adipose tissue including stromal cells and macrophages all contribute to IL-6 production (228-230). Meanwhile, fat depot differences in the IL-6 concentration and its metabolic effects on adipocyte metabolism have also been reported, with omental adipose tissue releasing 3 times more IL-6 than subcutaneous adipose tissue (229).

IL-6 transduces cellular signals through receptors that consist of two subunits: glycoprotein 130 (gp130), a non-ligand binding protein responsible for intracellular signal transduction, and the ligand-specific alpha chain, IL-6 receptor alpha (IL-6Rα) (231-232). IL-6Rα can act in both its soluble and membrane bound forms (232). Heterodimers of IL-6, IL-6Rα, and gp130 activate two signaling pathways: 1) Janus kinase (JAK)-signal transducer and activator of transcription 3 (STAT3) signaling pathways (231); and 2) the Src homology2-containing tyrosine phosphatise (SHP-2)/ ERK/ MAPK pathway (232). Phosphorylated STAT3 is translocated to the nucleus to promote the transcription of its target genes, such as suppressor of cytokine signaling 3 (SOCS3), which is highly induced by IL-6 at the transcriptional level (233). SOCS3

serves a negative feedback role on IL-6 signaling (234). SOCS3 may also negatively affect insulin signaling via IRS proteins (235).

2.5.2 IL-6 and insulin resistance

Currently, the role of IL-6 in the determination of insulin sensitivity is still in debate (236-237). This thesis review has attempted to demonstrate that the role of IL-6 in insulin resistance is neither exclusively beneficial nor harmful and appears to be tissue- and context-specific. We primarily focused on three main tissues involved in peripheral insulin resistance and glucose homeostasis: *in vitro* evidence from adipose tissue and *in vivo* evidence in humans and rodents mainly in liver, skeletal muscle and adipose tissue.

2.5.2.1 In vitro effects of IL-6 on adipocyte metabolism

On the one hand, there is evidence pointing to a role of IL-6 negatively regulating insulin signaling in adipocytes. In 3T3-L1 adipocytes, IL-6 decreases adiponectin gene expression and secretion in a dose- and time-dependent manner (238), thus leading to reduced insulin sensitivity. Similarly, IL-6 reduces the mRNA expression of *irs-1*, *glut4*, and PPARγ, IRS-1 protein and tyrosine phosphorylation, decreases insulin-stimulated glucose uptake and lipogenesis, as well as insulin-induced activation of IR-β, Akt/PKB, and ERK1/2 (228, 239). SOCS3 expression, a potential inhibitor of insulin signaling, is increased in adipocyte cell lines exposed to IL-6 *in vitro* (228, 240).

On the other hand, the effects of IL-6 on basal and insulin-stimulated glucose uptake are inconclusive. Stouthard et al. (241) demonstrated that treating 3T3-L1 adipocytes with recombinant murine IL-6 increased basal glucose transport, whereas others found no effect (220, 239, 242). Meanwhile, IL-6 has been reported to either

enhance (241), suppress (228, 239) or have no effect (220) on insulin-stimulated glucose uptake in 3T3-L1 adipocytes. A summary of the controversial reports regarding IL-6 effects on adipocyte metabolism is shown in *table 2-1*.

Table 2-1 *In Vitro* Effects of IL-6 on Adipocyte Metabolism

Reference	***In vitro* effects of IL-6 on adipocyte metabolism**
Fasshauer et al., 2003 (238)	Decreases adiponectin gene expression and secretion in 3T3-L1 adipocytes
Lagathu et al., 2003 (228); Rotter et al., 2003 (239)	Negatively affects insulin signaling pathways in 3T3-L1 adipocytes and human fat cells
Stouthard et al., 1996 (241)	Increases basal and insulin stimulated glucose uptake in 3T3-L1 adipocytes
Path et al., 2001 (220); Huppertz, 1996 (242)	No effects on basal and insulin stimulated glucose uptake in 3T3-L1 adipocytes
Rotter et al., 2003 (239)	No effects on basal glucose uptake while suppresses insulin stimulated glucose uptake in 3T3-L1 adipocytes

In summary, studies investigating the role of IL-6 in insulin action in adipocytes *in vitro* have mainly looked at short-term effects of high IL-6 levels and the results are conflicting. In order to mimic the situation seen in individuals with obesity or T2DM, studies of the effect of long-term low-grade IL-6 challenge of primary cultured adipocytes and/or cultured adipose tissue on insulin sensitivity are warranted.

2.5.2.2 In vivo effects of IL-6 on whole body glucose homeostasis and lipid metabolism

The effects of IL-6 on whole body glucose homeostasis and lipid metabolism have been studied both in humans and rodents. The results are contradictory, with improvement, no effect and deterioration having been reported. Most of the data available *in vivo* in humans are related to the acute effects of IL-6. An acute increase in plasma IL-6 levels through recombinant human (rh) IL-6 infusion has been reported to increase (243-245), decrease (246-247) or not affect (207, 211, 248) plasma glucose levels. Of

note, the increased plasma glucose levels may be caused by concomitant increases in other hormones such as epinephrine and glucagon owing to the supra-physiological concentration of plasma IL-6 (243-244). Infusion of IL-6 at a physiological concentration inhibits endotoxin-induced TNF-α production (249), increases SAT glucose uptake (246) and enhances the glucose infusion rate (248), indicating that IL-6 might improve insulin sensitivity *in vivo* in humans. Meanwhile, functional lack of IL-6 in patients with rheumatoid arthritis through IL-6 receptor-inhibiting monoclonal antibody leads to increased plasma lipid and glucose levels, arguing against IL-6 as an insulin resistance agent in humans *in vivo* (250-251). Infusion of IL-6 has also been reported to increase fatty acid rate of appearance and oxidation (243, 252), systemic lipolysis (211, 252) and *hsl* mRNA in adipose tissue of T2DM (247). It was recently confirmed that rhIL-6 infusion stimulated lipolysis in skeletal muscle instead of adipose tissue could account for the systemic changes in fatty acid metabolism (211). However, there is also a report suggesting that infusion of rhIL-6 has no effect on lipolysis during low intensity exercise (253).

In vivo studies in rodents suggest that IL-6 has tissue-specific effects, promoting (254-258) or having no effect (259) on insulin resistance in liver. Similarly, contradictory results with negative (256-257), neutral (255, 259) and positive (260-261) effects of IL-6 on skeletal muscle insulin sensitivity have been reported. Intracerebroventricular administration of IL-6 decreases adipose tissue mass in rats (262), indicating that IL-6 might be beneficial for adipose tissue metabolism *in vivo*.

2.5.3 Effects of IL-6 on metabolism in genetically modified mouse model

Mice lacking the gene encoding IL-6 (IL-$6^{-/-}$) develop characteristic features of the metabolic syndrome such as mature-onset obesity and insulin resistance (263-265), but this is not a universal finding (266). The increased adiposity and insulin resistance were partly reversed following chronic central administration of IL-6, highlighting the importance of centrally mediated effects of IL-6 (262). IL-$6^{-/-}$ mice also have reduced fat utilization at room temperature (264), and impaired increases in energy expenditure when challenged with cold exposure or new cage stress (267). Meanwhile, IL-$6^{-/-}$ mice have decreased endurance and energy expenditure during exercise (264). Hepatocyte-specific IL-6Rα deficient mice have hepatic inflammation and systemic insulin resistance (268). Transgenic *ob/ob*$^{IL\text{-}6}$ mice generated by mating *ob*$^{+/-}$/IL6^{tg} mice with *ob*$^{+/-}$ mice were more insulin sensitive than *ob/ob* mice under chow and HFD, and responsive to central leptin action, suggesting that rhIL-6 can promote systemic insulin sensitivity in mice (269). Whereas, overexpression of IL-6 in skeletal muscle by an electro-transfer procedure resulted in hypoadiponectaemia, liver inflammation, hyperinsulinaemia, hypoglycemia and reduced body fat (257).

To sum up, the effects of IL-6 on whole body energy homeostasis have been mainly focusing on the liver and skeletal muscle, with a striking paucity of data available in adipose tissue. Genetically-modified mouse models have shed new light on the metabolic action of IL-6 in the whole body with contradictory results being reported. Studies of the effect of IL-6 action on adipose tissue metabolism by utilizing IL-6 infusion *in vivo* and genetic mouse models are further required.

2.6 Thesis Overview

Thiazolidinediones (TZDs) are a commonly prescribed class of insulin sensitizing drugs that are thought to mediate a large portion of their beneficial effects through their up-regulation of the glyceroneogenic pathway in WAT (50, 270). Although there is an increasing body of evidence that WAT might play an important role in regulating the beneficial effects of exercise on systemic metabolism, it is not known if exercise can mimic TZD effects on PEPCK and PDK4 mRNA expression in adipose tissue. Furthermore, catecholamines are central modulators for regulating exercise-induced effects on WAT metabolism such as inducing lipolysis (168) and mitochondrial biogenesis (161). Skeletal muscle has been identified as an endocrine organ that secretes "myokines" with one of the best characterized being interleukin 6 (IL-6) (204). It has been proposed that increases in muscle derived IL-6 may be a potential mediator involved in exercise-induced WAT metabolism (271). It is not known if epinephrine and/or IL-6 play roles in the regulation of exercise-induced PEPCK and PDK4 mRNA expression and the signaling pathways that might be involved.

Currently, the role of IL-6 in the development of systemic insulin resistance is still under debate (272). Interstitial concentrations of IL-6 surrounding adipose tissue are orders of magnitude greater than levels in the circulation (214) suggesting that adipose tissue derived IL-6 could play a pivotal role in the maintenance of adipose tissue metabolism and function in an autocrine and/or paracrine manner. Although evidence from *in vitro* studies suggests that IL-6 causes insulin resistance in adipocytes (228, 238-239), there is also evidence that IL-6 may favorably regulate adipose tissue function presumably through its modulation of AMPK (209). Although not a universal finding

(266), IL-6$^{-/-}$ mice have been reported to have impaired glucose homeostasis when fed a chow diet (263). Likewise, IL-6$^{-/-}$ mice developed hepatic inflammation and insulin resistance under HFD feeding conditions (273). It is not known if there is phenotypic differences in adipose tissue between age-matched WT and IL-6$^{-/-}$ mice under chow and HFD fed conditions.

Consequently, this thesis has been designed to:

1) determine the regulation of PDK4 mRNA expression in epididymal adipose tissue (eWAT) from lean rats by swimming and epinephrine and the potential signalling pathways involved.

2) determine if 2 hours of swimming exercise and acute increases in epinephrine *in vivo* can induce PDK4 mRNA expression in eWAT from insulin resistant rats and if the reputed signaling pathways mediating this effect, i.e. p38MAPK are intact.

3) determine a) the effects of *ex vivo* IL-6 treatment in mouse eWAT on PEPCK and PDK4 expression; b) if IL-6 signaling is activated and if PEPCK and PDK4 mRNA expression is increased in mouse eWAT immediately after an acute bout of treadmill running; and c) through the utilization of IL-6$^{-/-}$ mouse model, to elucidate the role of IL-6 in modulating the effects of treadmill running on PEPCK and PDK4 mRNA expression in mouse eWAT and the putative signaling pathways therein.

4) investigate the metabolic action of IL-6 on rodent adipose tissue by characterizing the adipose tissue phenotypes from age matched wild type and IL-6$^{-/-}$ mice under both chow and HFD conditions.

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Chapter 3 Epinephrine Mediated Regulation of PDK4 mRNA in Rat Adipose Tissue[1]

3.1 Introduction

Adipose is a vibrant metabolic tissue secreting a wide range of hormones and metabolites that influence whole body fuel metabolism (1). The primary metabolic function of adipose tissue is to serve as a storage depot for lipids. In conditions of nutrient excess, glucose and fatty acids are stored in adipose tissue as triglycerides (TGs). When blood glucose levels become limiting, such as during fasting or prolonged exercise, fatty acids are liberated from TGs via lipolysis. While a large percentage of these fatty acids are released into the circulation, a significant amount are re-esterified back to TG (2). Re-esterification requires the provision of glycerol 3-phosphate (G-3-P) and, at least in rodent adipose tissue, the generation of G-3-P occurs primarily through *de novo* synthesis from sources such as lactate and pyruvate, in a process termed glyceroneogenesis (3-4). Since fluctuations in fatty acid levels have been linked to the development of systemic insulin resistance (5), an understanding of the factors which regulate glyceroneogenesis is vital in understanding the pathogenesis of type 2 diabetes.

Accumulating evidence suggests that pyruvate dehydrogenase kinase 4 (PDK4) is involved in the regulation of glyceroneogenesis in white adipose tissue (6). PDK4, along with the related enzymes PDK1, -2 and -3, phosphorylates and inhibits pyruvate dehydrogenase complexes (PDC) leading to decreases in the formation of acetyl CoA and

[1] A version of this chapter has been published. Wan et al. Am J Physiol Cell Physiol. 2010. Nov;299(5):C1162-70.

a greater flux of pyruvate towards oxaloacetate and the glyceroneogenic pathway (6). Thiazolidinediones (TZDs), a commonly prescribed class of anti-diabetic drugs and potent peroxisome proliferator-activated receptor gamma (PPARγ) agonists, increase PDK4 expression, lower fasting fatty acid levels and improve insulin sensitivity in insulin resistant Zucker *fa/fa* rats (6). Moreover, TZD treatment of adipocytes leads to increases in the expression of PDK4 concomitant with increased rates of glyceroneogenesis (6). PDK4 would appear to be essential for the up-regulation of glyceroneogenesis by TZDs as the attenuation of PDK4 activity pharmacologically with leelamine or reducing PDK4 content by short interfering RNA both result in decreased fatty acid re-esterification (6). Given the effects of TZDs on PDK4 expression and glyceroneogenesis (6-7) and owing to the unwanted side effects of these drugs (8) the identification of alternative approaches to induce PDK4 in white adipose tissue is warranted.

The regulation of PDK4 expression has been extensively studied in skeletal muscle and has been shown to be robustly induced by fasting (9) and exercise (10-12). The over-expression of the transcriptional coactivator 1-α (PGC-1α) in rodent skeletal muscle (13) and C2C12 muscle cells (14) results in increases in PDK4 expression, thus lending credence to the supposition that this molecule is a key regulator of PDK4. Interestingly, we have recently made the novel discovery that exercise and epinephrine increase PGC-1α mRNA expression in rat epididymal adipose tissue (15). Moreover, β-adrenergic agonists have been reported to increase the activity of the 5'AMP activated protein kinase (AMPK) (16-18) and p38 mitogen-activated protein kinase (MAPK) pathways in adipocytes (19-20), signaling cascades which have been implicated in the regulation of PDK4 (10, 21-22). Taken in concert, these findings suggest a role for β-

adrenergic agonists in the regulation of PDK4 in white adipose tissue. In this context, we hypothesized that fasting, exercise and epinephrine would increase PDK4 mRNA expression in rat epididymal adipose tissue. We further surmised that epinephrine would increase p38 and AMPK signaling and these pathways would be involved in the regulation of PDK4 in rat adipose tissue. Lastly, since PPARγ agonists have been implicated in regulating PDK4 mRNA expression, we postulated that this transcription factor would also be involved in the pathway through which epinephrine increases PDK4 in adipose tissue.

3.2 Methods and Materials

3.2.1 Materials

RNeasy kit was purchased from Qiagen (Toronto, ON). SuperScript II Reverse Transcriptase, oligo(dT) and dNTP were products from Invitrogen (Burlington, ON). Taqman Gene Expression Assays for beta 2 microglobulin (β2MG), beta actin, PDK1, PDK2, PDK3, PDK4, pyruvate dehydrogenase phosphatase 1 (PDP1) and PDP2 were from Applied Biosystems (Foster City, CA). 5-Aminoimidazole-4-carboxyamide ribonucleoside (AICAR) was purchased from Toronto Research Chemicals (Toronto, ON) while rosiglitazone was obtained from Cayman (Ann Arbor, MI). Total and phosphorylation specific antibodies for the detection of AMPK, ACC acetyl coA carboxylase (ACC), p38 MAPK, MAPK activated protein kinase 2 (MK2) and hormone sensitive lipase (HSL) were from Cell Signaling (Danvers, MA). Phosphorylated pyruvate dehydrogenase **(**PDH) antibodies and SB202190/SB203580 were products of

EMD Biosciences (San Diego, CA). All other chemicals were purchased from Sigma (Oakville, ON).

3.2.2 Treatment of Rodents

All protocols followed Canadian Council on Animal Care (CCAC) guidelines and were approved by the Animal Use and Welfare Committee at the University of Alberta. Male Wistar rats (Charles River, Wilmington MA) weighing ~200g were housed 2 per cage, with a 12/12-hour light/dark cycle, and were provided with water and standard rat chow *ad libitum*. The 12-hour light cycle was from 6am to 6pm and all experimental protocols were performed between 7am and 10am. Swim exercise and epinephrine injections were performed as described previously by our laboratory (15). Briefly, rats were acclimatized to the animal housing unit for 1 week prior to experiments. For the exercise studies, half of the animals swam for 2 hours while the remaining animals served as controls, which swam for 10 min to control for exposure to water. For the epinephrine experiment, rats were injected intraperitoneally (IP) with a weight adjusted bolus of epinephrine (20 μg/100 gram body weight) or an equivalent volume of sterile saline. To determine the effects of fasting on PDK4 mRNA levels in adipose tissue, food was removed at ~ 5:00 pm the evening before the experiment. Immediately following exercise, 2 hours after epinephrine injections or ~18 hours following the removal of food animals were anesthesized with sodium pentabaritol (5mg/100g body weight), epididymal adipose tissue was dissected free of the testes, immediately weighed and then clamp frozen in tongs cooled to the temperature of liquid nitrogen and stored at -80 ℃ until further analysis.

3.2.3 Adipose Tissue Organ Culture

Adipose tissue organ culture (ATOC) is a well characterized technique that has been used to determine changes in adipose tissue metabolism and gene expression (23). Epididymal adipose tissue was cultured as we have described in detail previously (15). Briefly, 250 mg of epididymal adipose tissue was minced into ~ 5-10 mg pieces and placed into culture dishes containing 7.5 ml of M199 medium supplemented with 1% antibiotic/antimycotic, 50 μU insulin and 2.5 nM dexamethasone. The epididymal adipose tissue was then kept in an incubator at 37 ℃ to equilibrate for 24 h. To assess the effects of epinephrine (1 μM), AICAR (1 mM) and metformin (1mM) on the expression of PDK4 mRNA, adipose tissue was cultured for 24 hours and then treated with these agents for 2 hours. To determine the effects of p38 inhibition on PDK4 expression, cultures were treated with 1 μM SB202190 or SB203580 for 30 minutes prior to and during the 2 hour epinephrine treatment. These compounds are cell permeable inhibitors of p38 MAPK that binds to the ATP pocket of the active kinase. We chose this concentration of SB202190/SB203580 since previous studies have demonstrated that higher concentrations of the pyridinyl imidazole compounds may possess some non-specific effects (24). For the determination of changes in the phosphorylation of AMPK, ACC, p38, MK2 and HSL experiments were conducted as described above except cultures were treated with epinephrine for 30 minutes. At the end of the experiments adipose tissue cultures were rinsed in ice cold sterile PBS, strained and adipose tissue fragments snap frozen and stored at -80 ° C for further analysis.

3.2.4 Western Blot Analysis

Protein was extracted from adipose tissue and changes in the phosphorylation status of AMPK, ACC, p38, MK2 and HSL determined by Western blotting, as described in detail by our laboratory previously (25). Briefly, adipose tissue samples were homogenized in 1.5 volumes of ice-cold cell lysis buffer supplemented with Protease Inhibitor Cocktail (Sigma) and phenylmethylsulfonyl fluoride. Homogenized samples were centrifuged for 10 min at 1500 X G at 4 ℃. The fat cake was removed and the infranatant was collected and protein concentration determined using the BCA method (26). Equal amounts of protein were separated on 7.5% gels. Proteins were wet transferred to nitrocellulose membranes at 200mA/tank and subsequently blocked in Tris buffered saline/0.1% Tween 20 (TBST) supplemented with 5% (W/V) non-fat dry milk for 1 hour at room temperature with gentle agitation. Membranes were incubated in TBST/5% non-fat dry milk supplemented with appropriate primary antibodies overnight at 4°C with gentle agitation. The following morning, membranes were briefly washed in TBST and then incubated in TBST/1% non-fat dry milk supplemented with HRP conjugated secondary antibodies for 1 hour at room temperature. Bands were visualized using ECL plus and captured using a Typhoon Imaging system (GE Health Care, Baie d'Urfe Quebec). ImageQuant (GE Healthcare), software was used to quantify relative band intensities. Beta actin or tubulin were used as internal controls.

3.2.5 Lipolysis Assays

Adipose tissue was cultured as described above in *section 3.2.3*. On the morning of the experiment media were removed and replaced with fresh M199 supplemented with 3% (W/V) fatty acid-free BSA. Epinephrine, SB202190 or dichloroacetate (DCA) were

added as described above. Media was collected at the end of the 2 hour epinephrine treatment and adipose tissue fragments weighed and frozen in liquid nitrogen. Glycerol release into the media was determined using a Free Glycerol Kit (Sigma, St. Louis, MO). The coefficient of variation for this assays in our laboratory is <10%.

3.2.6 Real Time RT-PCR

RNA was isolated from epididymal adipose tissue, complementary DNA (cDNA) synthesized and PCR analysis performed as we have described in detail previously (15, 25). Briefly, RNA was isolated from epididymal adipose tissue using an RNeasy kit according to the manufacturer's instructions. One microgram of RNA was used for the synthesis of cDNA using SuperScript II Reverse Transcriptase, oligo(dT) and dNTP. Real-time PCR was performed using a 7900HT Fast Real-Time PCR system (Applied Biosystems). A Relative difference in gene expression between groups was determined using the $2^{-\Delta\Delta CT}$ method (27). The amplification efficiencies of the gene of interest and the housekeeping gene were equivalent and there was no effect of the experimental manipulation on the expression of housekeeping genes.

3.2.7 Statistical Analysis

Data are presented as means ± standard error of the mean (SEM). Comparisons between control and exercised rats were made using a one-way analysis of variance (ANOVA) followed by a post-hoc comparison using Fisher's LSD test. Comparisons between fed and fasted rats, vehicle and epinephrine, AICAR or metformin treated cultures were made using a Student's T-test. The effects of SB202190 on cell signaling and lipolysis were analyzed using a 2 X 2 ANOVA with LSD post-hoc comparisons. The

effects of SB202190 or SB203580 on PDK4 mRNA expression were analyzed using a one way ANOVA on ranks followed by Dunn's test. Statistical significance was set at $p<0.05$.

3.3 Results

3.3.1 Exercise, Fasting and Epinephrine Increase PDK4 mRNA Expression in Epididymal Adipose Tissue

PDK4 mRNA expression was increased ~ 6 fold immediately following 2 hours of swim exercise (*Figure 3-1A*) and remained elevated 2 (3.83 ± 0.32 fold increase versus control, $p < 0.05$) and 4 hours (3.34 ± 0.70 fold increase versus control, $p < 0.05$) following exercise cessation. Similarly, an overnight fast led to a ~ 3 fold increase in PDK4 mRNA levels. PDK4 mRNA levels were increased in epididymal adipose tissue 2 hours following a bolus injection of epinephrine (20 μg/100 gram body weight) and were also increased in cultured epididymal adipose tissue following a 2 hour epinephrine (1 μM epinephrine) treatment. Exercise, fasting and epinephrine did not increase mRNA levels of PDK1, 2 or 3 (*Figure 3-1A*). Similarly the mRNA expression of PDP1 and 2, enzymes which dephosphorylate PDH complexes, were not altered by any of our experimental manipulations (data not shown). Increases in PDK4 mRNA levels after an overnight fast or 4 hours following exercise cessation were associated with increases in the phosphorylation of PDH on serine 293 and serine 300, PDK4 phosphorylation sites (28) (*Figure 3-1B and C*). Confirming the importance of PDK4 in regulating fatty acid release (6), we found that DCA, a PDK4 inhibitor (28), increased the ratio (μmol/g tissue

fatty acid to μmol/g tissue glycerol) of fatty acid to glycerol release in cultured adipose tissue (1.76 ± 0.10 control, 2.67 ± 0.45 DCA, p =0.05).

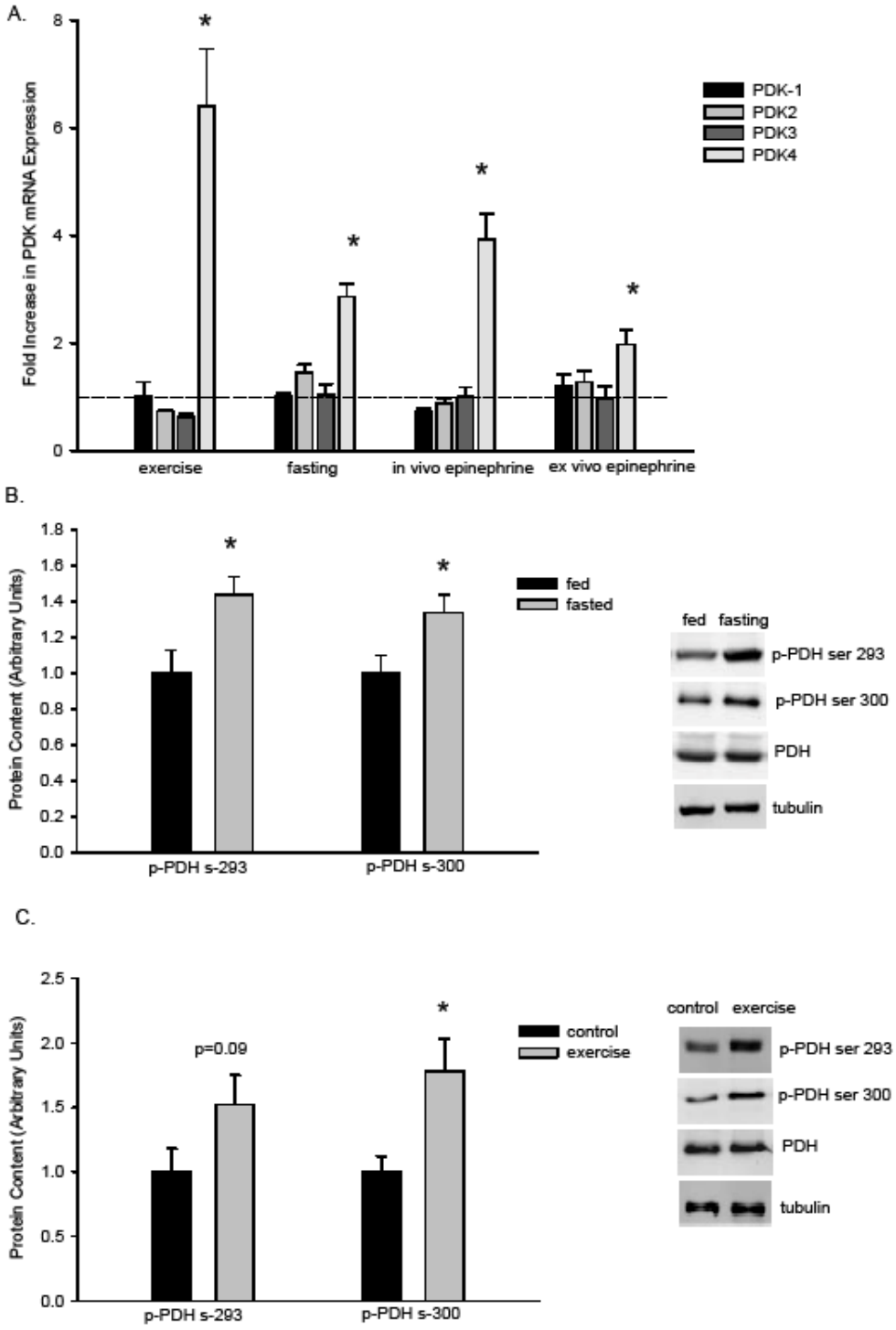

Figure 3-1 Effects of exercise, fasting and epinephrine on PDK4 mRNA and the phosphorylation of PDH protein expression. A) Exercise, fasting and epinephrine increase the mRNA expression of PDK4, but not PDK1, PDK2 or PDK3 in rat epididymal adipose tissue. Increases in PDK4 mRNA levels are associated with increases in the phosphorylation of PDH on serine 293 and 300 B) after an overnight fast and C) 4 hours following exercise cessation. Data are presented as means + SEM for 5-11 animals or tissue cultures per group. The mRNA data is normalized to beta actin and expressed as fold differences compared to sedentary controls, fed controls, saline injected animals or vehicle treated tissue cultures. Representative Western blot images are given to the right of the quantified data in B and C. * p <0.05 versus control.

3.3.2 Epinephrine Increases AMPK and P38 MAPK Signaling in Cultured Epididymal Adipose Tissue

As illustrated in *Figure 3-2*, epinephrine treatment (1 μM, 30 minutes) led to increases in the phosphorylation of AMPK and its downstream substrate ACC (29). Similarly, epinephrine also increased the activity of the p38 MAPK signaling pathway as evidenced by increases in the phosphorylation of p38 and MK2. p38 MAPK phosphorylates MK2 on threonine-222 and thus MK2 phosphorylation can be used as a marker of p38 MAPK activity (30).

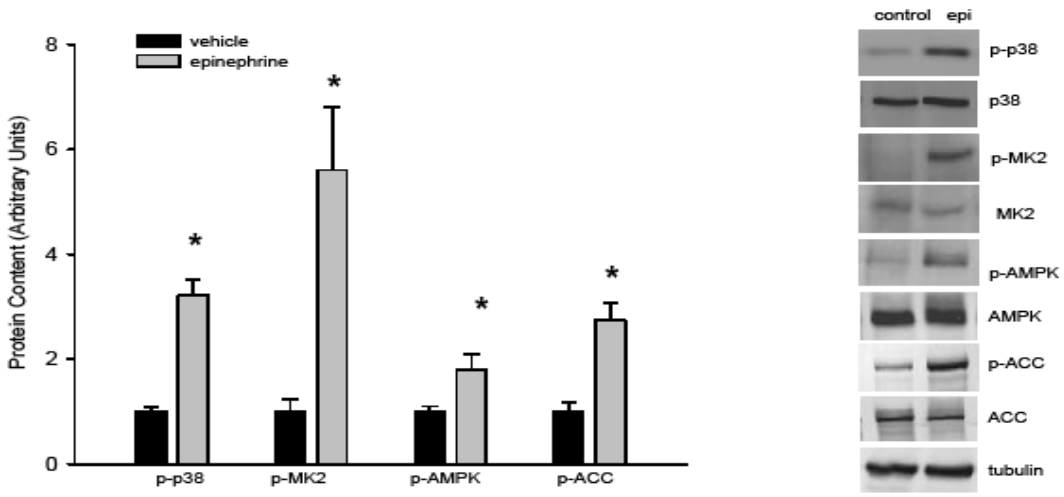

Figure 3-2 Effects of *ex vivo* epinephrine on p38 and AMPK signalling pathways. *Ex vivo* epinephrine treatment (30 minutes, 1μM) increases the phosphorylation of p38, MK2, AMPK and ACC in cultured rat epididymal adipose tissue. Data are presented as means + SEM for 5-8 tissue cultures per group. Representative Western blot images are given to the right of the quantified data. **p* <0.05 versus control.

3.3.3 AMPK Agonists Do Not Increase PDK4 mRNA Expression in Rat Epididymal Adipose Tissue

As illustrated in *Figure 3-3A and B*, AICAR (1 mM, 1 hour) and metformin (1 mM, 1 hour) both increased AMPK and ACC phosphorylation while having no effect on p38 MAPK phosphorylation. These compounds decreased PDK4 mRNA expression (*Figure 3-3C*).

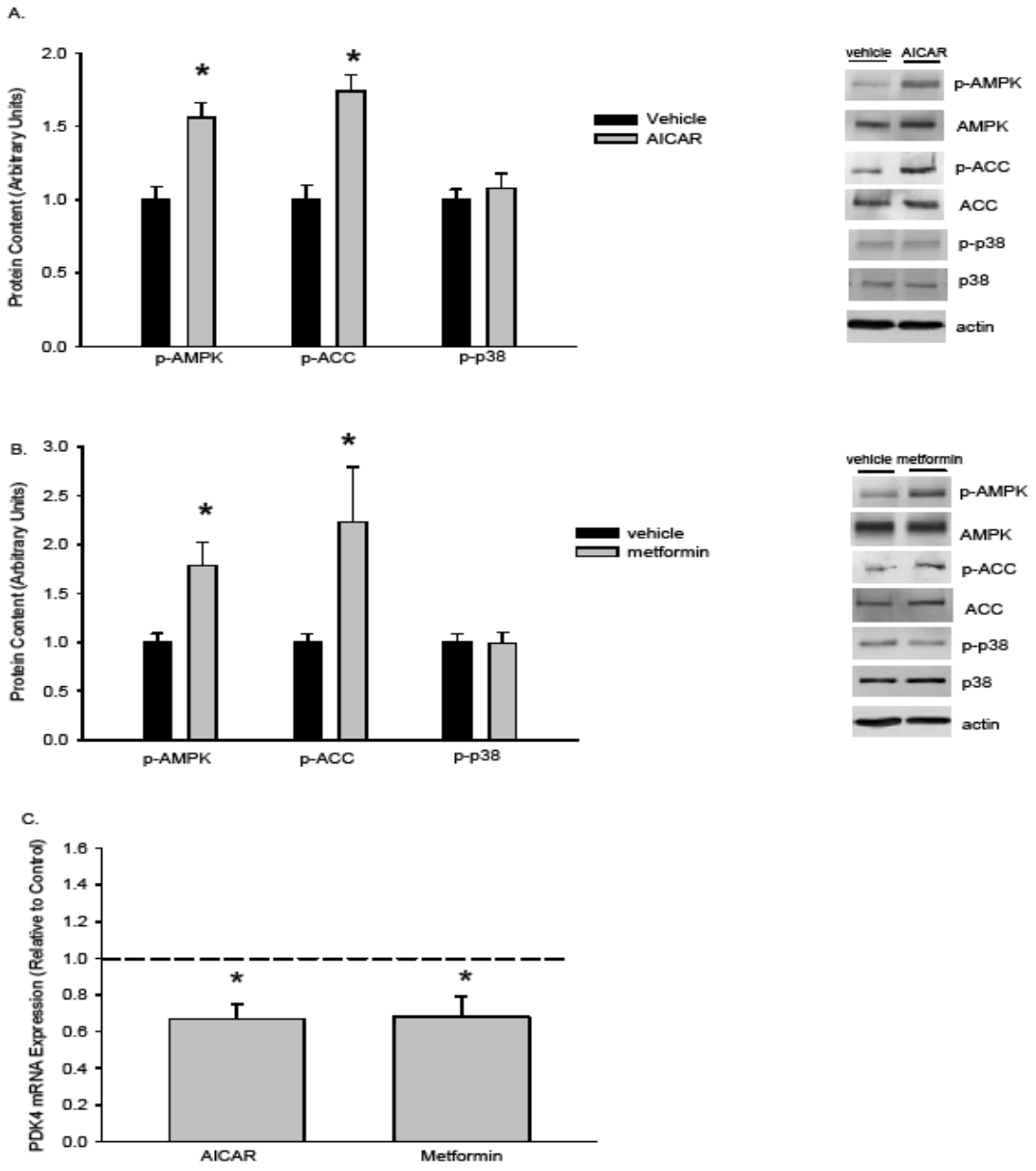

Figure 3-3 Effects of *ex vivo* AICAR and metformin on AMPK and p38 signalling pathways and PDK4 mRNA. The AMPK agonists A) AICAR (1 mM) and B) metformin (1 mM) increase AMPK signaling (1 hour treatment) in cultured epididymal adipose tissue but do not increase the expression of C) PDK4 mRNA levels (2 hour treatment). Data are presented as means + SEM for 5-11 samples per group. RT-PCR data was normalized to beta actin. Representative Western blot images are given to the right of the quantified Western blot data. **p* <0.05 versus control. (For figure 3-3C, the relative PDK4 mRNA expression in vehicle treated group for the *ex vivo* AICAR and metformin treatment experiment is 1.10 ± 0.49.)

3.3.4 SB202190 Does Not Attenuate AMPK or PKA Signaling in Epididymal Adipose Tissue Cultures

Treating cultured epididymal adipose tissue with 1 μM SB202190 for 30 minutes prior to and during epinephrine treatment led to reductions in MK2 phosphorylation (*Figure 3-4A*). SB202190 did not inhibit the phosphorylation of HSL on serine 563 and 660 (*Figure 3-4B*) which are protein kinase A (PKA) phosphorylation sites (31). Similarly AMPK phosphorylation was not inhibited by SB202190 (*Figure 3-4C*). Epinephrine-induced increases in lipolysis, as determined by glycerol release into the culture media, was not reduced by prior treatment with SB202190 (*Figure 3-4D*).

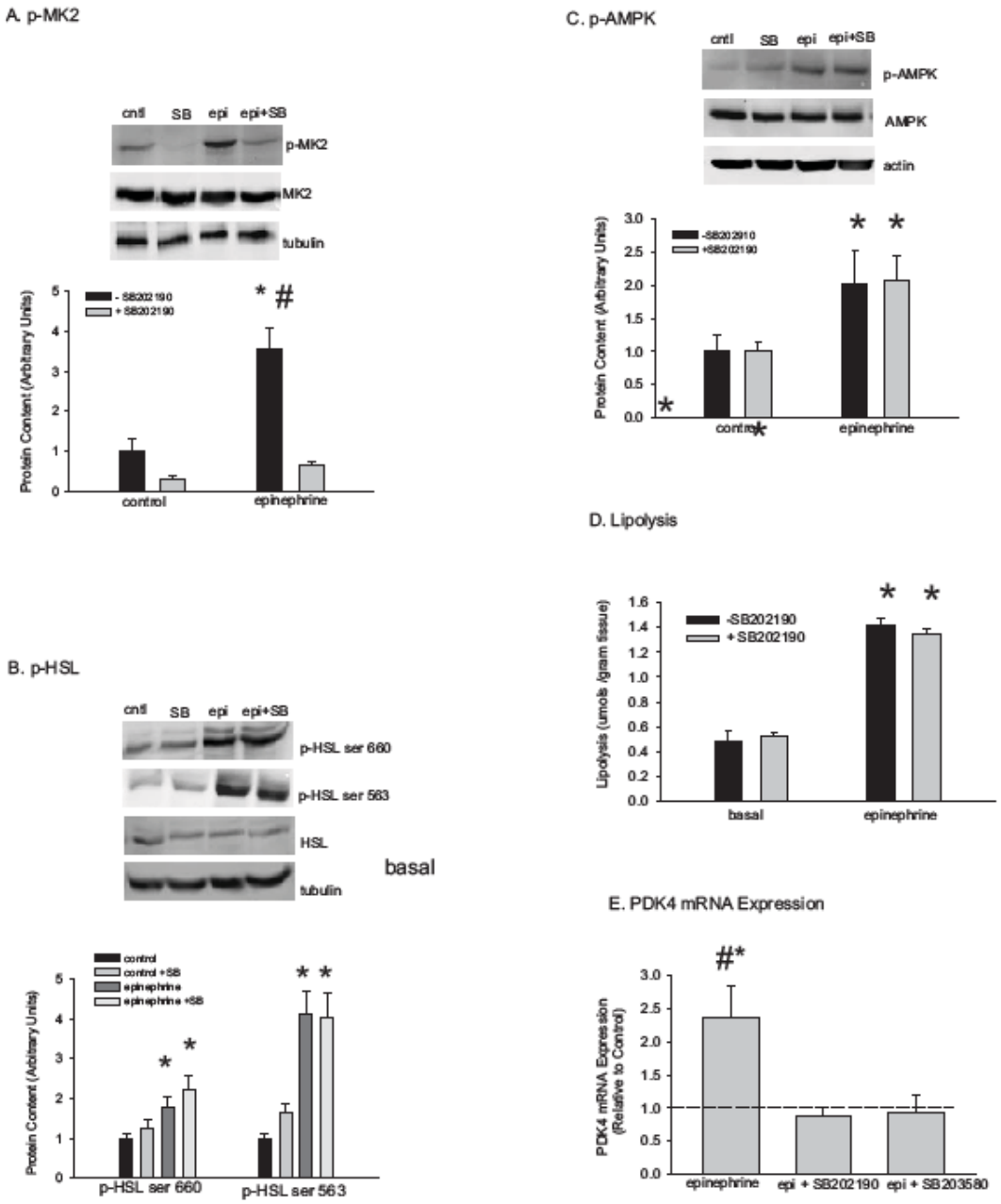

Figure 3-4 Effects of *ex vivo* SB on the phosphorylation of MK2, AMPK, HSL, lipolysis and PDK4 mRNA. SB202190 inhibits epinephrine induced increases in A) MK2 phosphorylation but not B) HSL phosphorylation (serine 563 and 660) C) AMPK phosphorylation or D) lipolysis. E) Epinephrine mediated increases in PDK4 mRNA expression are abrogated by SB202190 and SB203580. Data are presented as means + SEM for 5-15 tissue cultures per group. The RT-PCR data is normalized to beta actin and expressed as fold differences compared to controls. Representative Western blot images are given above

the quantified data. * p <0.05 versus vehicle treated control, $^{\#}$ p <0.05 versus SB202190 treated epinephrine condition. (For figure 3-4E, the relative PDK4 mRNA expression in vehicle treated group is 1.31 ±0.64.)

3.3.5 SB202190 Attenuates Epinephrine Induced Increases in PDK4 mRNA

Epinephrine-induced increases in PDK4 mRNA were blunted by SB202190 (*Figure 3-4E*, as shown above). Similarly the related compound SB203580 (1 μM) also blocked the effects of epinephrine on PDK4 mRNA levels. In the absence of epinephrine these compounds had no effect on PDK4 mRNA (data not shown).

3.3.6 Epinephrine Mediated Increases in PDK4 Are Inhibited by PPARγ Antagonist

The PPARγ agonist rosiglitazone (2 μM) increased PDK4 mRNA levels in cultured adipose tissue and this effect was abrogated by GW9662 (20 μM), a PPARγ antagonist (*Figure 3-5A*). These reductions did not appear to be secondary to decreases in β adrenergic signaling as GW9662 did not affect epinephrine mediated increases in the phosphorylation of p38 MAPK, AMPK or HSL (*Figure 3-5B*).

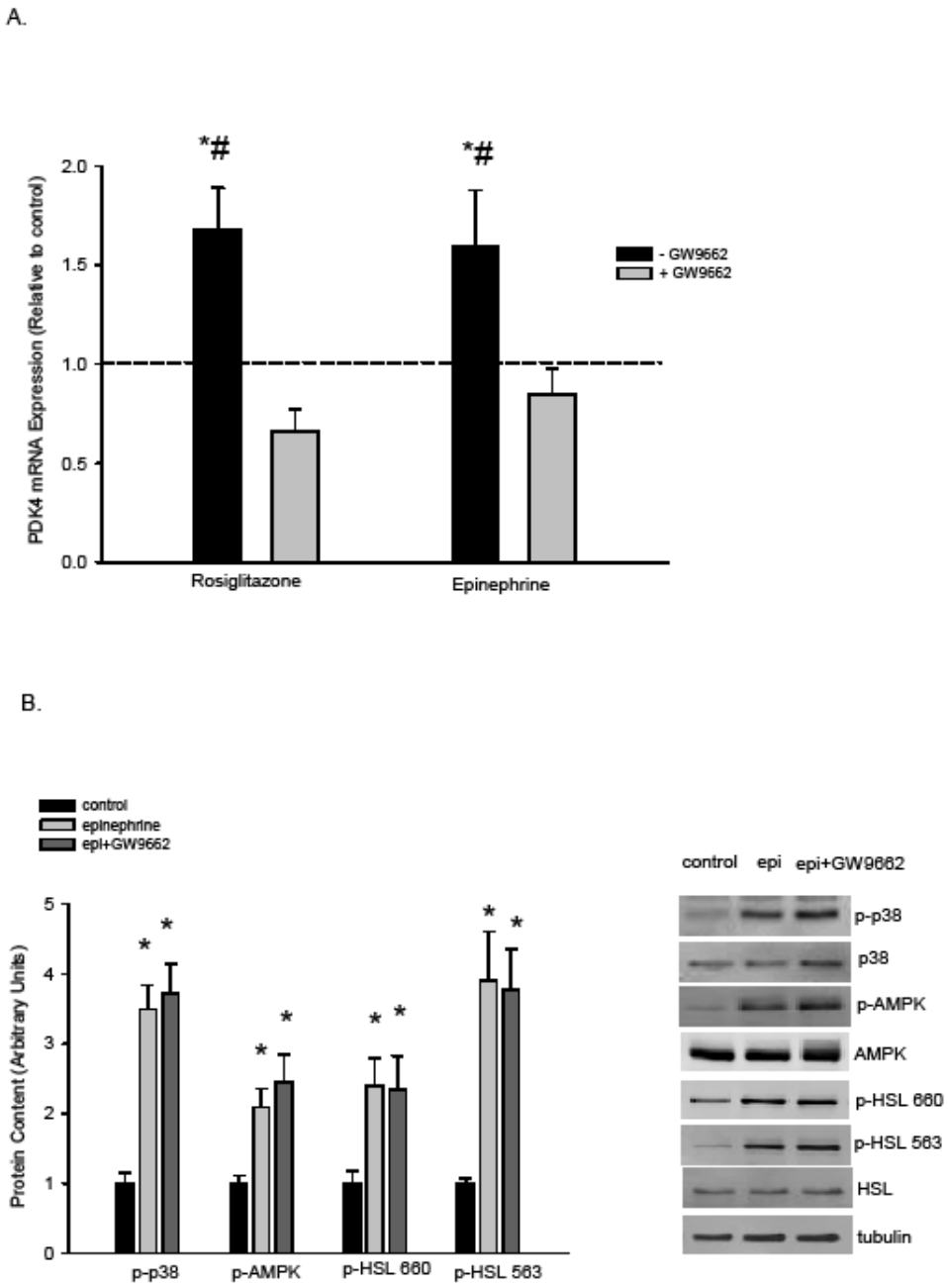

Figure 3-5 Effects of *ex vivo* GW9662 on rosiglitazone and epinephrine mediated increases in PDK4 mRNA and signalling. GW9662 (20μM), a PPARγ antagonist, inhibits A) rosiglitazone (2μM) and epinephrine (1 μM) mediated increases in PDK4 mRNA levels while having no effect on epinephrine mediated increases in the phosphorylation of B) p38, AMPK, or HSL. Data are presented as means + SEM for 6-8 cultures per group. The RT-PCR data is normalized to beta actin and expressed as fold differences compared to control. Representative Western blot images are given to the right of the quantified data. * *p*

<0.05 versus control, $^{\#}p$ <0.05 versus epinephrine + GW9662. (For figure 3-5A, the relative PDK4 mRNA expression in vehicle treated group for *ex vivo* rosiglitazone and epinephrine treatment is 1.01 ± 0.07 and 1.09 ± 0.09 respectively.)

3.4 Discussion

Adipose tissue is an active metabolic organ that is intimately involved in the regulation of systemic carbohydrate and lipid metabolism (1). Accumulating evidence suggests that the modulation of white adipose tissue metabolism is a beneficial approach in the treatment of type 2 diabetes. In this regard it has been demonstrated that TZDs increase fatty acid re-esterification and limit fatty acid release from white adipocytes (6). Cadoudal and collegues (6) have reported that the inhibition or knockdown of PDK4 attenuates the effects of TZDs on glyceroneogenesis thus demonstrating the essential role of this enzyme in fatty acid recycling. Given the key role of PDK4 in the regulation of white adipose tissue metabolism, we sought to explore the regulation of this kinase by physiological stimuli such as exercise, fasting and epinephrine. We have made the novel discovery that exercise leads to rapid increases in PDK4 mRNA levels that persist for upwards of 4 hours following exercise cessation. These findings are similar in magnitude and time course to the reported effects of exercise on PDK4 mRNA levels in skeletal muscle (10-12) and further demonstrate (15) the plasticity of adipose tissue in response to exercise. In addition to exercise, and consistent with one previous report (32), we found that PDK4 mRNA levels were also increased by fasting. In contrast to PDK4, PDK1, 2 and 3, were not responsive to these perturbations. These findings are congruent with previous studies which demonstrated negligible changes in PDK2 levels in skeletal muscle following fasting (33). In addition to increasing PDK4 mRNA levels, we found that the phosphorylation of PDH on serine 293 and 300 was increased after an overnight

fast or 4 hours following exercise cessation. Since PDK4 phosphorylates these residues (28), these findings suggest an increase in PDK4 activity.

Swim exercise (34) and fasting (35) increase circulating epinephrine levels. To determine if epinephrine could be a signal which initiates increases in PDK4 mRNA levels, we examined PDK4 in adipose tissue harvested from rats two hours following epinephrine injections or in adipose tissue cultures treated with epinephrine. Similar to what has previously been reported in mouse skeletal muscle following treatment with the β_2-adrenergic agonist formoterol (36), we found that epinephrine increased PDK4 mRNA levels both *in vivo* and *ex vivo.*

In isolated adipocytes β-adrenergic agonists increase the activity of AMPK (16-17, 37). Since AMPK has been postulated to regulate PDK4 mRNA expression in skeletal muscle (10, 21), we reasoned that this kinase may serve a similar role in white adipose tissue. Consistent with this line of thinking we found that *ex vivo* epinephrine treatment increased the phosphorylation of AMPK and ACC concomitant with increases in PDK4 mRNA expression. To determine if the direct activation of AMPK could increase PDK4 mRNA levels we treated adipose tissue cultures with AICAR or metformin. AICAR is an adenosine analogue which is converted to ZMP and mimics the effects of AMP on AMPK activation (38) while metformin activates AMPK independent of changes in the AMP:ATP ratio (38). Despite increasing AMPK and ACC phosphorylation we demonstrate that these mechanistically distinct AMPK agonists did not increase, and in fact reduced, PDK4 mRNA expression in cultured epididymal adipose tissue. In adipocytes the activation of AMPK results in the phosphorylation of HSL (39-41) on serine 565, leading to decreases in HSL activity and reductions in lipolysis (42-44). Since

rates of fatty acid re-esterification change in proportion to lipolysis (39, 45) it is not entirely surprising that AMPK agonists would down regulate the expression of PDK4 given reductions in lipolysis, and presumably by extension, decreases in the absolute rate of fatty acid re-esterification. Regardless of the mechanisms involved these findings clearly demonstrate tissue specific differences in the mechanisms regulating PDK4.

In addition to AMPK, catecholamines have also been shown to activate p38 MAPK signaling in adipocytes (19-20). Since this pathway has been implicated in modulating the expression of PDK4 in cardiomyocytes (22), we postulated that p38 MAPK would regulate PDK4 in white adipose tissue. Consistent with this hypothesis we found that the p38 MAPK inhibitor SB202190 attenuated the epinephrine-induced increases in MK2 phosphorylation and PDK4 mRNA expression. While the pyridinyl imidazole compounds are potent inhibitors of p38 MAPK (46), these agents have also been shown to possess non-specific effects (24, 47). In this light we thought it vital to determine if SB202190 impaired epinephrine signaling, independent of reductions in p38 MAPK activity. Under the conditions utilized we found that SB202190 did not reduce epinephrine-induced increases in the phosphorylation of HSL on serine 660 and 563 which are specific PKA phosphorylation sites (31) nor did the compound inhibit epinephrine-induced increases in lipolysis. Similarly, SB202190 did not attenuate epinephrine-induced increases in AMPK phosphorylation. Collectively, these results strongly suggest that inhibition of p38 MAPK, and not a general abrogation of β adrenergic signaling mediates the effects of SB202190 on epinephrine-induced increases in PDK4 mRNA levels in white adipose tissue.

In addition to providing novel information regarding the processes which regulate PDK4 mRNA expression in white adipose tissue our findings also lend insight into the relationship between AMPK and p38 MAPK signaling in this tissue. It has been suggested by some (48) but not all (49) that AMPK may lie upstream of p38 MAPK. Conversely, it has also been demonstrated that SB202190, at levels ~10 fold higher than used in the current study, attenuates AMPK activity in heart muscle (50). Contrary to these studies we did not find an interaction between p38 and AMPK signaling in cultured white adipose tissue. For instance, AICAR and metformin did not increase the phosphorylation of p38 MAPK while the inhibition of p38 MAPK signaling was not associated with reductions in AMPK phosphorylation. Collectively these results imply that epinephrine increases the activation of AMPK and p38 MAPK through parallel, not serial pathways in white adipose tissue.

Previous work has demonstrated that rosiglitazone causes a rapid increase in PDK4 mRNA levels in rodent adipose tissue (6). Since the time-course and magnitude of change in PDK4 mRNA with rosiglitizone are similar to what we see following epinephrine treatment we reasoned that PPARγ may be involved in the pathway through which epinephrine-induces PDK4 in white adipose tissue. Consistent with this hypothesis, and in line with work from Caudoudal and associates (6), we found that rosiglitazone, a potent PPARγ agonist, increased the expression of PDK4. Conversely, GW9662, a PPARγ antagonist prevented rosiglitazone and epinephrine-mediated increases in PDK4. The reduction in epinephrine-induced PDK4 was not secondary to attenuated β-adrenergic signaling as epinephrine-induced increases in p38, AMPK and HSL phosphorylation were unaffected by GW9662. In combination with the p38

inhibition experiments, these findings provide evidence suggesting the involvement of p38 MAPK and PPARγ in regulating epinephrine mediated increases in PDK4. What is not clear at this juncture is whether there is a direct link between p38 activation, PPARγ and increases in PDK4 or whether both p38 MAPK and PPARγ are required for the induction of PDK4 by epinephrine, but do so through distinct pathways. While speculative, the activation of p38 MAPK may activate PGC-1α (51), which in turn may bind to and co-activate PPARγ thus leading to the induction of PDK4 in white adipose tissue. Clearly, this is a question that needs to be explored in greater detail in the future.

In summary we have made the novel observation that exercise, fasting and epinephrine increase the mRNA expression of PDK4 in rat epididymal adipose tissue. We have further demonstrated a disconnect between the activation of AMPK and the induction of PDK4 mRNA and provide data suggesting that p38 MAPK and PPARγ are involved in the regulation of PDK4 mRNA levels in adipose tissue. These findings provide novel information regarding the biochemical processes regulating PDK4 and importantly highlight alternative, non-pharmaceutical based approaches that can be used to induce PDK4 mRNA expression.

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Chapter 4 Epinephrine Induces PDK4 mRNA Expression in Adipose Tissue from Obese, Insulin Resistant Rats[2]

4.1 Introduction

Thiazolidinediones (TZDs) are a commonly prescribed medication used in the treatment of type 2 diabetes (T2DM). These PPARγ agonists mediate a large portion of their beneficial effects on insulin sensitivity through increasing fatty acid re-esterification in adipose tissue, reducing fatty acid efflux and limiting ectopic lipid deposition (1). Increases in fatty acid re-esterification require the formation of glycerol-3-phosphate (G3P) and this occurs primarily through the conversion of non-glucose molecules such as pyruvate in a process termed glyceroneogenesis (2).

Pyruvate dehydrogenase kinase 4 (PDK4) is up-regulated in skeletal muscle in insulin-resistant states and is postulated to be involved in the etiology of insulin resistance (3-4). In adipocytes, increases in PDK4 are associated with an inhibition of glucose uptake (4), yet despite this, this enzyme is thought to mediate a portion of the beneficial effects of TZDs on adipose tissue lipid metabolism. PDK4 inactivates pyruvate dehydrogenase complexes resulting in a shunting of pyruvate to oxaloacetate and ultimately the generation of glycerol-3-phosphate (G3P) (1). TZDs increase the expression of PDK4, concomitant with improvements in insulin sensitivity in Zucker *fa/fa* rats (1). The inhibition of this enzyme attenuates TZD mediated increases in glyceroneogenesis and leads to increases in fatty acid release (1).

[2] A version of this chapter has been published. Wan et al. Obesity (Silver Spring). 2012 Feb;20(2):453-6.

While effective, TZDs possess many deleterious side effects (5) and thus the identification of alternative approaches to mimic the beneficial effects of these compounds is needed. In this regard we have previously demonstrated that epinephrine increases the expression of PDK4 through PPARγ and p38 MAPK (p38) dependent pathways (6). While these findings would suggest that the activation of beta adrenergic signaling may serve as an effective means with which to mimic one of the beneficial effects of TZDs, it should be noted that this study was completed using adipose tissue from lean, insulin-sensitive animals. Adipocytes from high fat fed rodents display a blunted response to the effects of catecholamines (7) and thus it is not clear if acute elevations in beta adrenergic signaling would be an effective means with which to induce the expression of PDK4 in adipose tissue from obese, insulin resistant animals. Therefore, the purpose of the present investigation was to determine if acute increases in epinephrine could induce PDK4 in adipose tissue from insulin resistant rats and if the signaling pathways mediating this effect were intact. As exercise increases plasma catecholamine levels, we further wanted to determine if this perturbation would have similar beneficial effects on PDK4 expression in adipose tissue from obese rodents.

4.2 Methods and Materials

4.2.1 Methods and Procedures

All protocols followed Canadian Council on Animal Care (CCAC) guidelines and were approved by the Animal Use and Welfare Committee at the University of Alberta. Male Wistar rats (Charles River, Wilmington, MA) were housed 2 per cage, with a 12/12-hour light/dark cycle and were provided with water and either standard rat chow a high

fat diet *ad libitum* for 6 weeks. The chow diet contained 28.5% protein, 13.5% fat, and 58.0% carbohydrates, expressed as percentage of total energy. The high fat diet contained 18.4% protein, 21.3% carbohydrate and 60.3% fat (Harlan, Madison, WI). Rats were injected intraperitoneally (IP) with a weight-adjusted bolus of epinephrine (20 μg/100 gram body weight) or an equivalent volume of sterile saline (8) or swam for 2 hours with the remaining animals serving as controls (6). Immediately following exercise, or 30 minutes or 2 hours after epinephrine injections, animals were anesthesized with sodium pentobarbitol (5mg/100g body weight) and epididymal adipose tissue was harvested and stored at -80 ℃ until further analysis.

As we have described previously (6) and detailed in chapter 3, the protein content and/or phosphorylation of AMPK (5'AMP-activated protein kinase), p38, MK-2 (MAPK-activated protein kinase 2) and ACC (acetyl CoA carboxylase) were determined by Western blot analysis, while changes in PDK4 mRNA expression were determined using real time RT-PCR with beta actin as a housekeeping gene (6). Relative differences in gene expression between groups were determined using the $2^{-\Delta\Delta CT}$ method.

4.2.2 Statistical analysis

Data are presented as means ± standard error of the mean (SEM). Data were compared with two-way analysis of variance (ANOVA) followed by a post-hoc comparison using Fisher's LSD test. Statistical significance was set at $p < 0.05$.

4.3 Results and Discussion

Although TZDs increase the expression of PDK4 in adipose tissue from obese, insulin resistant rodents (1), these compounds possesses many unwanted side effects (5)

and thus it was of great interest to determine if acute increases in epinephrine induce PDK4 mRNA expression in adipose tissue from obese, insulin resistant animals. Rats fed a HFD weighed more (chow 448 ± 6, HFD 519 ± 8 g, $p<0.05$), had heavier epididymal fat pads (chow 5.7 ± 0.3, HFD 15.4 ± 1.2 g, $p<0.05$) and were hyperglycemic (chow 98.6 ± 3.3, HFD 115.9 ± 5.7 mg/dl, $p<0.05$). As seen in *Figure 4-1A below*, we made the novel observation that epinephrine-induced PDK4 mRNA levels to a similar extent in adipose tissue from chow and HFD rats. It should be stressed that these were mRNA measurements and longer duration studies are needed to determine if equivalent changes occur in PDK4 protein.

We have previously shown that p38 plays a role in the pathway(s) through which epinephrine induces PDK4 mRNA in adipose tissue from lean rats (6). The activation of p38 has been reported to be impaired in skeletal muscle from insulin resistant mice (9) and thus it was of interest to determine if p38 signaling was intact in adipose tissue from HFD rats. As the activation of p38 would occur prior to the induction of PDK4 gene expression, we harvested tissue 30 minutes following a bolus injection of epinephrine. To our surprise we found that the phosphorylation of p38, and its downstream substrate, MK2, were markedly blunted in adipose tissue from HFD compared to chow fed rats (*Figure 4-1 B, C*). This is particularly surprising given the robust induction of PDK4 in epididymal adipose tissue from these animals. To determine if the blunted effect of epinephrine on intracellular signaling was specific to p38, or a more generalized effect of the HFD, we measured changes in AMPK signaling. Consistent with previous reports we found that epinephrine increased the phosphorylation of AMPK and its substrate ACC in chow fed rats (10). In contrast to p38, the phosphorylation of AMPK and ACC were not

blunted in adipose tissue from HFD rats (*Figure 4-1 D, E*). These findings are in contrast to a recent report from Ceddia's laboratory (7) demonstrating a marked reduction in AMPK protein content and signaling in isolated adipocytes from HFD fed mice. This discrepancy could be explained by species-related differences, the mode of epinephrine administration or the use of intact fat pads vs. isolated adipocytes.

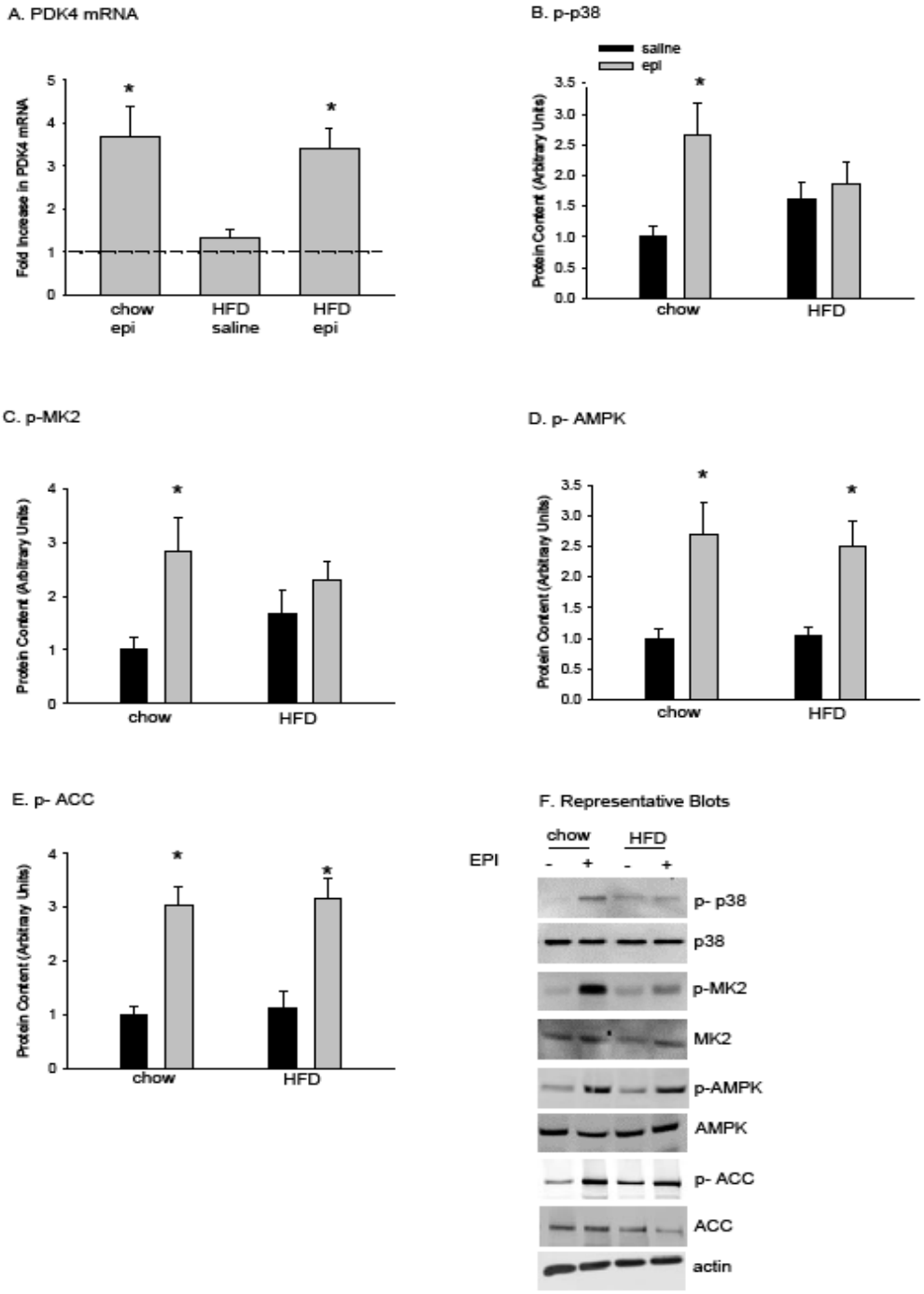

Figure 4-1 Effects of epinephrine injection on PDK4, p38 and AMPK signalling pathways from chow and HFD rats. A) Epinephrine (epi) increases PDK4 mRNA levels in adipose tissue from HFD rats. p38 MAPK signaling is attenuated in HFD rats (B and C) while AMPK signaling is intact (D and E). Data are presented as means + SEM for 8-10 animals per group. Real time RT-PCR data is presented as a fold

change compared to saline injected chow fed rats. Representative Western blots are displayed in panel F. * $p<0.05$ compared to saline treated condition within the same diet group. (For figure 4-1A, the relative PDK4 gene expression in chow saline injection group is 1.07±0.14.)

The induction of PDK4, despite the blunted activation of p38 in adipose tissue from HFD rats suggests that alternative mechanisms mediate the effects of epinephrine on the induction of PDK4. In skeletal muscle AMPK has been implicated in the control of this enzyme (11). However, in cultured adipose tissue we have shown that AMPK agonists such as metformin and AICAR decrease the expression of PDK4 (6). As absolute rates of fatty-acid re-esterification parallel lipolysis (12), and given the effect of AMPK on inhibiting lipolysis (13) this finding is not entirely unexpected. Collectively these findings suggest that AMPK- and p38- independent pathways are involved in the epinephrine-mediated induction of PDK4 *in vivo* in adipose tissue from HFD rats. TZDs have been shown to induce the expression of PPARγ dependent genes, in part, through the inhibition of cyclin dependent kinase 5-mediated phosphorylation of this transcription factor (14). While speculative, it is possible epinephrine may mediate the induction of PDK4 in adipose tissue from HFD rats through a similar mechanism.

Regardless of the specific pathways(s) involved our data clearly highlights the beneficial effect of acute increases in epinephrine on the induction of PDK4 in adipose tissue from HFD rats. The procedure utilized in our experiments raises plasma epinephrine levels to ~190 nmol/l, a concentration much higher than what is seen under normal physiological circumstances (8). Given this we wanted to determine if exercise, a condition that robustly increases plasma epinephrine levels, but to levels lower than what is seen with epinephrine injections (~15 nmol/l (15)), would be a beneficial approach to induce PDK4 in adipose tissue. Two hours of swim exercise increased PDK4 mRNA

levels to an equivalent or even greater extent in adipose tissue from HFD rats (chow exercise 5.49 ± 0.73, HFD sedentary 1.36 ± 0.17, HFD exercise 9.36 ± 1.05 fold increase versus chow sedentary).

In summary our data is the very first to demonstrate that epinephrine and exercise, similar to TZDs, are potent stimuli that can be used to induce PDK4 in adipose tissue from obese, insulin resistant rodents. We have made the further novel observation that the pathways regulating the effects of epinephrine on PDK4 in adipose tissue from HFD rats may be distinct from those in lean animals. Our findings suggest that interventions that acutely increase epinephrine levels, such as exercise, may serve as an effective TZD independent approach with which to modulate adipose tissue metabolism.

4.4 Literature Cited

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Chapter 5 IL-6 Indirectly Modulates the Induction of Glyceroneogenic Enzymes in Adipose Tissue during Exercise[3]

5.1 Introduction

When blood glucose levels are limiting such as during exercise, the breakdown of triglyceride (TG) molecules within fat cells is accelerated. While the majority (~65-75%) of liberated fatty acids following lipolysis are released into the circulation to be used as a fuel source, a significant amount are retained in the fat cell and are re-esterified back to TG (1). The re-esterification of fatty acids requires the provision of glycerol 3-phosphate (G-3-P), and in rodent adipose tissue, the generation of G-3-P occurs primarily through *de novo* synthesis from sources such as lactate and pyruvate, in a process termed glyceroneogenesis (2-3). Phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate dehydrogenase kinase 4 (PDK4) have been identified as essential components of the glyceroneogenic enzymatic machinery (4-5).

Similar to what has been reported in skeletal muscle (6), we have found that exercise leads to a robust induction of PDK4 in white adipose tissue (7). We demonstrated that this effect was recapitulated by epinephrine and could involve p38 mitogen activated protein kinase (MAPK) (7). On the other hand, 5-aminoimidazole-4-carboxyamide ribonucleoside (AICAR) and metformin, which are 5' AMP activated protein kinase (AMPK) agonists, decreased PDK4 mRNA expression in rat adipose tissue (7). AMPK is an energy-sensing enzyme that inhibits energy-consuming processes (8).

[3] A version of this chapter has been published. Wan et al. PLoS One. 2012;7(7):e41719. Epub 2012 Jul 23.

As fatty acid re-esterification is one of the primary drains on ATP levels in adipocytes (9) it is not entirely surprising that AMPK agonists would decrease the expression of enzymes involved in this process.

Given the vital function of adipose tissue in the provision of fatty acids, it is likely that there are multiple systemic factors involved in the regulation of genes involved in fatty acid handling. In this light recent work has highlighted the potential involvement of skeletal muscle derived interleukin 6 (IL-6) as a mediator of adipose tissue metabolism during exercise (10). For example, IL-6 has been reported to stimulate adipose tissue lipolysis (11) and to activate AMPK (12) in adipocytes. Moreover, the activation of AMPK during exercise is blunted in adipose tissue from IL-6 knockout mice (12). Of interest, previous work has demonstrated that long-term increases in inflammation (13-14), and in particular IL-6 (15), attenuate PEPCK expression in fat cells. These findings suggest that increases in IL-6 may serve to dampen the exercise-mediated induction of glyceroneogenic enzymes in adipose tissue.

The long-held view that muscle derived IL-6 signals to adipose tissue during exercise is increasingly being challenged. For instance, it has been reported that IL-6 infusions do not increase lipolysis or activate IL-6 signalling in adipose tissue from healthy humans (16). Likewise, *in vivo* AMPK activity was recently shown to be similar in subcutaneous adipose tissue from wild type and IL-6 deficient mice (17). Moreover, and somewhat surprisingly, to the best of our knowledge it is not known if IL-6 signalling is activated in adipose tissue during exercise.

With the aforementioned points in mind the purpose of the present investigation was to determine the role of IL-6 in the induction of glyceroneogenic enzymes during

exercise in white adipose tissue. Using a combination of *ex vivo* adipose tissue culture approaches and whole body IL-6 deficient mice (IL-$6^{-/-}$) we hypothesized that a) IL-6 would directly and rapidly attenuate PEPCK and PDK4 mRNA expression in mouse adipose tissue, b) IL-6 signalling would be activated in adipose tissue during exercise and c) the induction of glyceroneogenic enzymes following exercise would be enhanced in adipose tissue from IL-$6^{-/-}$ mice.

5.2 Methods and Materials

5.2.1 Materials

Reagents, molecular weight marker and nitrocellulose membranes for SDS-PAGE were purchased from Bio-Rad (Mississauga, ON). ECL Plus was a product of Amersham Pharmacia Biotech (Arlington Heights, IL). Antibodies against p-cAMP response element binding protein (p-CREB) (CAT# 4276), p-p38 MAPK (CAT #9211), p38 MAPK (CAT#9212), p-MAP kinase-activated protein kinase 2 (p-MK-2) (CAT#3044), MK2 (CAT#3042), p-AMPK (CAT#2531), AMPKα (CAT#2793), p-acetyl-CoA carboxylase (p-ACC) (CAT#3661), ACC (CAT#3662), p- signal transducer and activator of transcription 3 (STAT3) tyrosine 705 (CAT#4113), STAT3 (CAT#9132), p-hormone sensitive lipase (p-HSL) (Ser563) (CAT#4139), HSL (CAT#4107), protein phosphatase type 2A (PP2A) (CAT# 2041), liver kinase B1 (LKB-1) (CAT#3050) and PPARγ (CAT#2430) were from Cell Signaling (Danvers, MA). Antibodies against tubulin (CAT#ab7291), the beta 3 adrenergic receptor (β3-AR) (CAT#ab94506), PDK4 (CAT#ab38242), and PP2C (CAT# ab27267) were purchased from Abcam (Cambridge, MA) Anti PEPCK antibody (CAT# 10004943) was obtained from Cayman Chemicals

(Ann Arbor, MI). Horseradish peroxidase-conjugated donkey anti-rabbit and goat anti-mouse IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Fatty acid-free bovine serum albumin (FA-free BSA) (CAT# 152401) was from MP Biomedical (Solon, OH). Free glycerol reagent (CAT#F6428) was from Sigma (Oakville, ON). Non-esterified fatty acids assay kits (NEFA-HR kit) were purchased from Wako Chemicals (Richmond, VA). Recombinant mouse IL-6 (RMIL6I) was obtained from Thermo Scientific (Rockford, IL) while a mouse IL-6 ELISA was from Biolegend (San Diego, CA). AICAR was purchased from Toronto Research Chemicals (Toronto, ON). SuperScript II Reverse Transcriptase, oligo(dT) and dNTP were products from Invitrogen (Burlington, ON). Taqman Gene Expression Assays for mouse β actin (4352933E), eukaryotic 18S rRNA (4352930E), PDK4 (Mm01166879_m1), PEPCK (Mm01247058_m1) and suppressor of cytokine signaling3 (SOCS3) (Mm00545913_s1) were from Applied Biosystems (Foster City, CA). All other chemicals were purchased from Sigma (Oakville, ON).

5.2.2 Treatment of Animals

All protocols followed Canadian Council on Animal Care (CCAC) guidelines and were approved by the University of Guelph Animal Care Committee. 3 month old male IL-$6^{-/-}$ mice (Jackson Laboratories B6.12952-$IL6^{tmlkopf/}$J) on a C57BL/6J background and age-matched C57BL/6J wild-type (WT) mice were housed 3 per cage, with a 12/12-hour light/dark cycle, and were provided with water and standard rodent chow *ad libitum*. Mice were acclimated to the animal housing facility for 1 month prior to the start of the exercise experiment. Before the experiment, all mice were acclimated to running on a motor-driven treadmill during a 3-day period with 15 min of running at 15

m/min, 5% grade per day. On the day of the experiment, one-half of the mice in each group (IL-$6^{-/-}$ and WT) were sedentary (KOSED and WTSED), and the other half subjected to running on a motorized treadmill at 15 m/min, with an incline of 5% for 90 min (KOEX and WTEX). This intensity of exercise is well tolerated by IL-6 deficient mice (18). Immediately following exercise cessation, mice were killed by cervical dislocation and epididymal white adipose tissue (eWAT) removed, immediately weighed and then clamp-frozen in tongs cooled to the temperature of liquid nitrogen and stored at -80 ℃ until further analysis. In a separate set of experiments blood was collected from the saphenous vein prior to, and immediately following 90 minutes of treadmill exercise. Blood was spun down and the plasma collected for the determination of glucose (YSI Glucose Analyzer), fatty acids, TGs and IL-6.

5.2.3 Adipose Tissue Organ Culture

Adipose tissue organ culture is a well characterized technique that has been used to determine changes in adipose tissue metabolism and gene expression (19). Epididymal adipose tissue was cultured as we have described in detail previously (7, 20) and detailed in chapter 3. To assess the effects of IL-6 or AICAR on the mRNA expression of PEPCK and PDK4, adipose tissue was cultured for 24 h and then treated with mouse IL-6 (150 ng/ml) or AICAR (1 mM) for 2, 6 or 12 h. To determine the effects of IL-6 on the activation of the AMPK and p38MAPK signalling pathways, experiments were conducted as described above except cultures were treated with mouse IL-6 (150 ng/ml) for 30 min. To determine the effects of IL-6 on lipolysis, adipose tissue was cultured as described above. On the morning of the experiment, media was replaced with fresh M199 supplemented with 2.5% FA-free BSA. IL-6 (150 ng/ml) or epinephrine (1 μM) was

added and media were collected after a 2h treatment. To determine the effects of IL-6 on fatty acid re-esterification, adipose tissue was cultured as described above and treated with mouse IL-6 (150 ng/ml) for 24h. Thereafter, the media was replaced by fresh M199 supplemented with 2.5% FA-free BSA and pyruvate (4 mM), media collected 4h later. At the end of all the experiments adipose tissue cultures were rinsed in ice-cold sterile PBS, strained and adipose tissue fragments snap frozen and stored at -80 ° C for further analysis.

5.2.4 Lipolysis in Adipose Tissue Explants

To determine the effects of *ex vivo* treatment of epinephrine on lipolysis, epididymal adipose tissue minces (~50 mg cut into 10-20 mg fragments) from 4 month old male WT and IL-$6^{-/-}$ mice were incubated in 1.0 ml of oxygenated Krebs-Ringer Buffer (KRB) (118 mM NaCl, 4.8 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 5 mM glucose, pH 7.4) with 2.5% FA-free BSA supplemented with or without 1 μM epinephrine at 37° C in a shaking water bath. Buffer samples were collected after 2 hours. NEFA and glycerol were analyzed by colormetric assays according to the manufacturer's instructions. NEFA and glycerol concentrations were corrected for tissue weight and reported as μmol released per g tissue.

5.2.5 Western Blot Analysis

Protein was extracted from adipose tissue and changes in the phosphorylation status or content of AMPK, p38, MK2, STAT3, CREB, HSL, β3-AR, LKB1, PP2A, PP2C and PPARγ were determined by Western blotting, as described in detail by our laboratory previously (7, 20). Briefly, adipose tissue samples were homogenized in 2

volumes of ice-cold cell lysis buffer supplemented with Protease Inhibitor Cocktail (Sigma) and phenylmethylsulfonyl fluoride. Homogenized samples were centrifuged for 10 min at 1500 X G at 4 ℃. The fat cake was removed and the infranatant was collected and protein concentration determined using the BCA method. Equal amounts of protein were separated on 7.5% or 10% SDS-PAGE gels. Proteins were wet transferred to nitrocellulose membranes at 200mA/tank and subsequently blocked in Tris buffered saline/0.1% Tween 20 (TBST) supplemented with 5% non-fat dry milk for 1 h at room temperature with gentle agitation. Membranes were incubated in appropriate primary antibodies diluted in TBST/5% non-fat dry milk overnight at 4° C with gentle agitation. The following morning, membranes were briefly washed in TBST and then incubated in HRP-conjugated secondary antibodies diluted in TBST/1% non-fat dry milk for 1 h at room temperature. Signals were detected using enhanced chemiluminesence and were subsequently quantified by densitometry by Gene Tool according to the manufacturer's instructions (SynGene, ChemiGenius2, PerkinElmer).

5.2.6 Real Time PCR

RNA was isolated from adipose tissue using an RNeasy kit according to the manufacturer's directions. Total RNA (1 µg) was used for the synthesis of complementary DNA (cDNA) using SuperScript II Reverse Transcriptase, oligo(dT) and dNTP. Real time PCR was performed using a 7500 Fast Real-Time PCR system (Applied Biosystems). Samples were run in duplicate in a 96-well plate format. Each assay (20 µl total volume) contained 1 µl gene expression assay, 1 µl cDNA template, 10 µl Taqman Fast Universal PCR Master Mix and 8 µl RNase-free water. For β-actin or 18S, each 50 µl reaction contained 25 µl PCR Master mix, 2.5 µl each of gene expression assay, 1 µl

cDNA template, and 21.5 µl RNase-free water. Results were normalized to the mRNA expression of β-actin or 18S. Relative differences in gene expression between groups were determined using the $2^{-\Delta\Delta CT}$ method (21). The amplification efficiencies of the gene of interest and the housekeeping gene were equivalent.

5.2.7 Statistical Analysis

Data are presented as means ± standard error of the mean (SEM). Comparisons between vehicle and IL-6 signalling, and differences between the exercise-induced increases in PEPCK and PDK4 mRNA expression in WT and IL-$6^{-/-}$ were made using a Student's T-Test. Comparisons between vehicle and IL-6 or AICAR treated cultures on gene expression, and the effects of IL-6 and epinephrine on lipolysis in cultured adipose tissue were made using a one-way ANOVA followed by a post hoc comparison using Fisher's LSD test. Comparisons between WT and IL-6 KO mice in regards to signalling, *ex vivo* lipolysis and plasma glucose and fatty acid levels were completed using a 2 X 2 ANOVA with LSD post hoc analysis. Statistical significance was set at $p<0.05$.

5.3 Results

5.3.1 Effects of *ex vivo* IL-6 Treatment on Signalling and Gene expression in eWAT

IL-6 treatment (150 ng/ml, 30 min) of cultured eWAT led to an ~ 3-fold increase in the tyrosine 705 phosphorylation of STAT3. Similarly, the phosphorylation of AMPK and its downstream substrate ACC were also increased by IL-6. The phosphorylation of p38MAPK and its substrate MK-2, was not increased by IL-6 (*Figure 5-1*). IL-6 led to a rapid induction of SOCS3 (2 hours 4.76 ± 1.26 fold increase, 6 hours 5.48 ± 1.49 fold increase, 12 hours 4.24 ± 1.33 fold increase, $p<0.05$ vs control) a transcriptional target of

IL-6 while decreasing PEPCK and PDK4 mRNA expression (*Figure 5-2A*). Reductions in PEPCK and PDK4 mRNA expression were mirrored by decreases in the protein content of these enzymes (*Figure 5-2B*). To determine if decreases in PEPCK and PDK4 resulted in a functional impairment in fatty acid handling we treated cultured adipose tissue with IL-6 (24 hours, 150 ng/ml) and then measured the ratio of fatty acid to glycerol released into the media 4 hours following the removal of IL-6. As seen in *figure 5-2C*, IL-6 treatment resulted in increases in the FFA/glycerol which is indicative of reductions in fatty acid re-esterification. As seen in *Table 5-1*, AICAR (1 mM) treatment reduced the mRNA expression of PEPCK and PDK4.

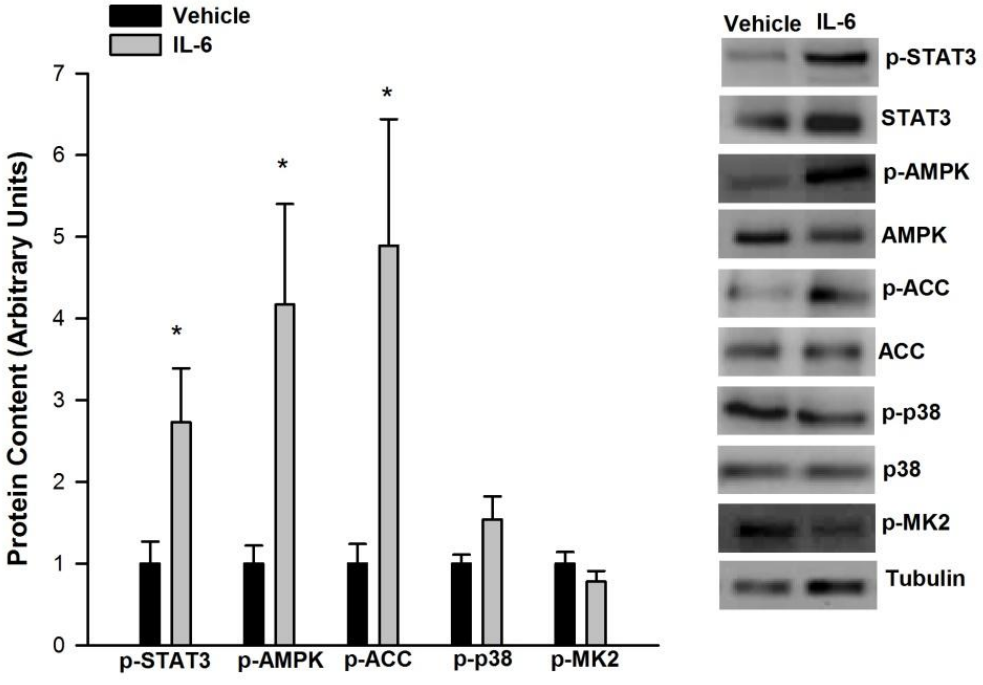
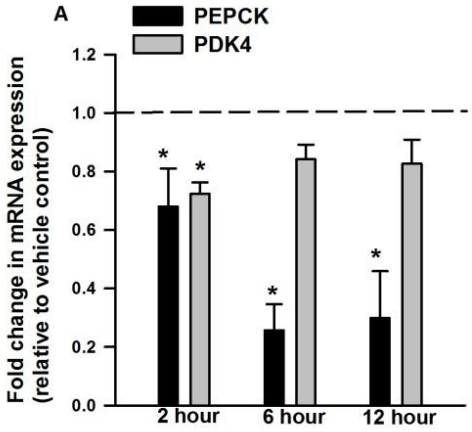


Figure 5-1 IL-6 signalling in cultured adipose tissue. *Ex vivo* IL-6 treatment (150 ng/ml, 30mins) increases the phosphorylation of STAT3, AMPK and ACC but not the p38MAPK signalling pathways in cultured eWAT. Data are presented as means + SEM for 7 cultures per group. Representative Western blot images are given to the right of the quantified data. * $p<0.05$ versus vehicle control.


A
PEPCK
PDK4
Fold change in mRNA expression (relative to vehicle control)
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1.0
0.8
0.6
0.4
0.2
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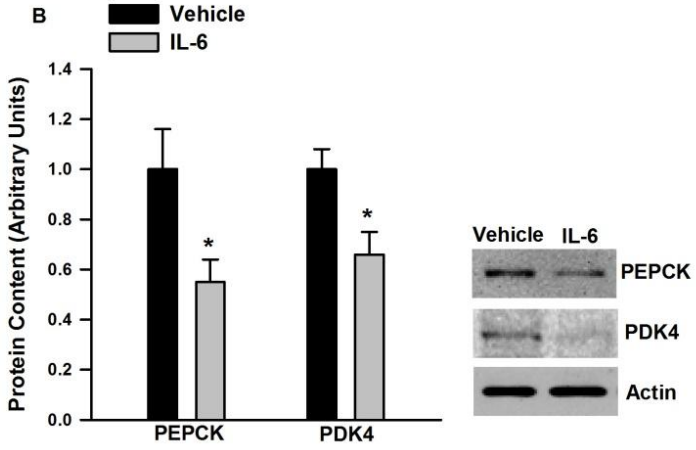
2 hour
6 hour
12 hour


B
Vehicle
IL-6
Protein Content (Arbitrary Units)
1.4
1.2
1.0
0.8
0.6
0.4
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*
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PEPCK
PDK4
Vehicle
IL-6
PEPCK
PDK4
Actin

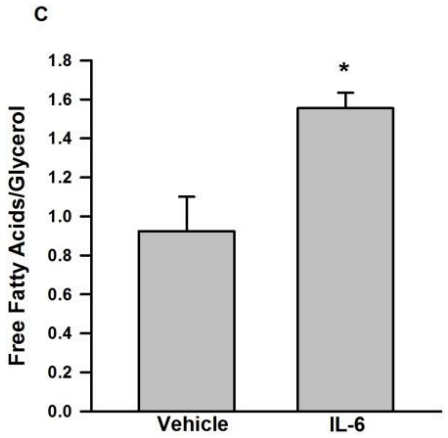
C
Free Fatty Acids/Glycerol
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Vehicle
IL-6

Figure 5-2 Effects of *ex vivo* IL-6 on PEPCK and PDK4. *Ex vivo* IL-6 treatment (150 ng/ml) decreases A) PEPCK and PDK4 mRNA expression and B) protein content in cultured eWAT (150 ng/ml, 12h). These changes are associated with increases C) in fatty acids to glycerol ratio following long-term (150 ng/ml, 24 h) IL-6 treatment. Data are presented as means + SEM for 7 cultures per group. The mRNA data are normalized to beta actin mRNA and expressed as fold differences compared to vehicle controls in A. Representative Western blot images are given to the right of the quantified data in B. * $p<0.05$ versus vehicle control. (For figure 5-2A, the relative PEPCK and PDK4 mRNA expression in vehicle treated groups is1.45 ±0.34 and 1.39 ±0.52, respectively.)

Table 5-1 Effects of *ex vivo* AICAR Treatment on PEPCK, PDK4 mRNA Expression in eWAT.

Group	PEPCK	PDK4
AICAR 6h	0.49 ± 0.13 [a]	0.90 ±0.21
AICAR 12h	0.44 ± 0.12 [a]	0.64 ± 0.09 [a]

Data are presented as means ± SEM for 6 cultures per group. The mRNA data are normalized to 18S mRNA and expressed as relative mRNA differences compared to vehicle controls (vehicle =1.0). [a] $p<0.05$ versus vehicle control.

5.3.2 The IL-6 Mediated Activation of AMPK Occurs Independent of Lipolysis

As presented in *Figure 5-3*, short-term IL-6 treatment (150 ng/ml, 2 h) did not stimulate lipolysis as determined by the release of glycerol (*Figure 5-3A*) and fatty acids (*Figure 5-3B*) into the culture media. Epinephrine (1 μM) was included in these experiments as a positive control, and resulted in marked increases in glycerol and fatty acid release. In contrast to short term incubations, IL-6 treatment for 12 hours moderately enhanced glycerol release (control 5.1 ± 0.7, IL-6 6.8 ± 0.8 μmol/gram tissue/12 hours, n=6 per group, $p=0.053$).

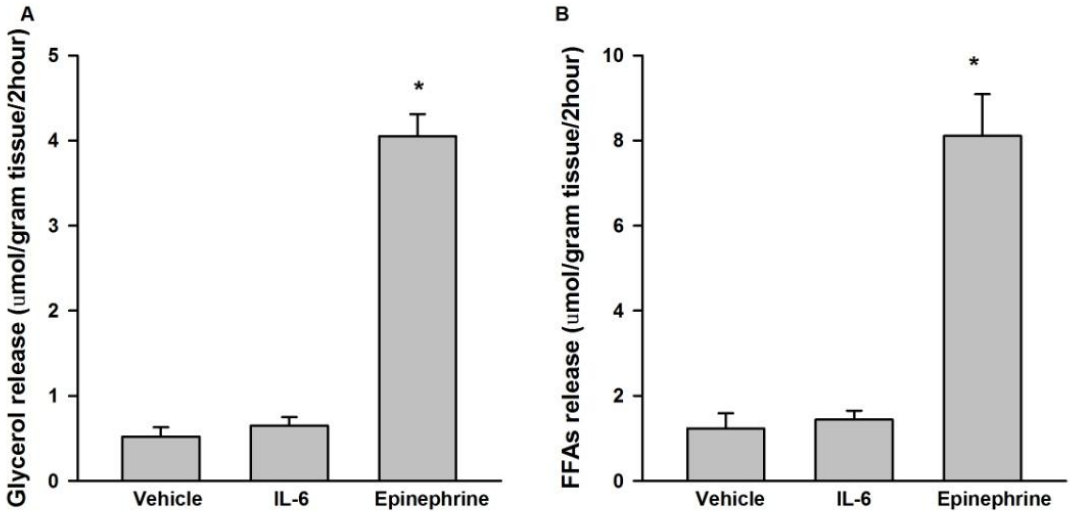

Figure 5-3 Effects of *ex vivo* IL-6 on adipose tissue lipolysis. Short term (2 hours) IL-6 (150 ng/ml) treatment does not increase A) glycerol or B) fatty acid release from cultured eWAT. Epinephrine (2 hours, 1 μM) treated cultures were included as a positive control. Data are presented as means + SEM for 7 cultures per group. * $p<0.05$ compared to vehicle and IL-6 groups.

5.3.3 Markers of IL-6 Signalling Are Not Activated in Adipose Tissue during Exercise

SOCS3 mRNA expression in eWAT did not increase in response to the exercise protocol we utilized (*Figure 5-4A*). Meanwhile, a significant decrease in the phosphorylation status of STAT3 (Tyr705) was observed immediately after exercise (*Figure 5-4B*). Plasma IL-6 levels tended to be higher following exercise (13.4 ± 6.1 sedentary, 25.3 ± 5.4 exercise pg/ml, $p=0.085$).

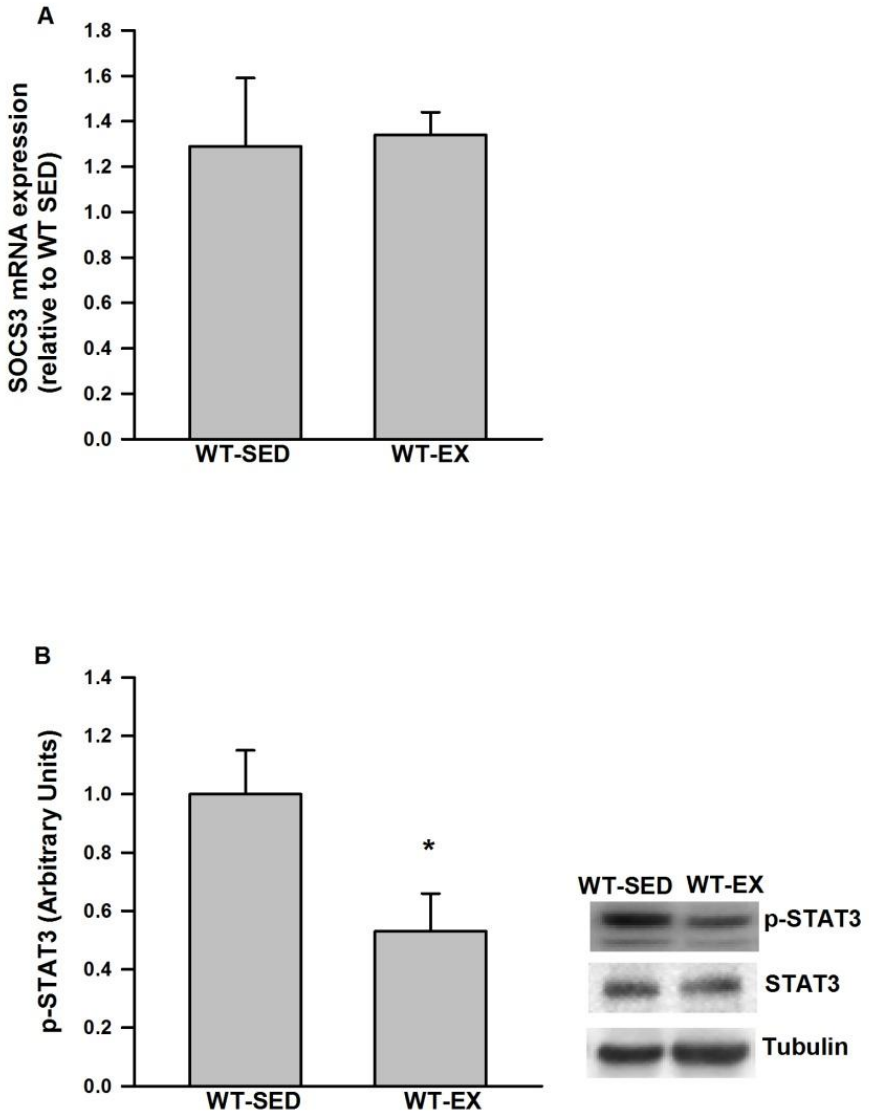

Figure 5-4 Markers of IL-6 signalling in adipose tissue following exercise. An acute bout of exercise does not activate IL-6 signalling in eWAT as demonstrated by A) unchanged SOCS3 mRNA levels and B) decreases in the phosphorylation of STAT3 (Tyr705). Data are presented as means + SEM for 10 animals per group. The mRNA data is normalized to 18S and expressed as fold differences compared to the WT sedentary (SED) group in A. Representative Western blot images are given to the right of the quantified data in B. * $p<0.05$ versus WT SED.

5.3.4 Exercise-Mediated Increases in PEPCK and PDK4 Expression Are Attenuated in IL-6$^{-/-}$ Mice

The expression of PEPCK and PDK4 in eWAT from WT and IL-6$^{-/-}$ sedentary mice were similar. Consequently, we compared the exercise-mediated induction of PEPCK and PDK4 between WT and IL-6$^{-/-}$ mice. As seen in *Figure 5.5*, the induction of glyceroneogenic enzymes, especially PDK4, was blunted in adipose tissue from IL-6 deficient mice. There were no differences in PPARγ protein content in adipose tissue from WT and KO mice (WT 1.00 ± 0.11, IL-6$^{-/-}$ 1.03 ± 0.08, arbitrary units N= 8-10/group, p>0.05).

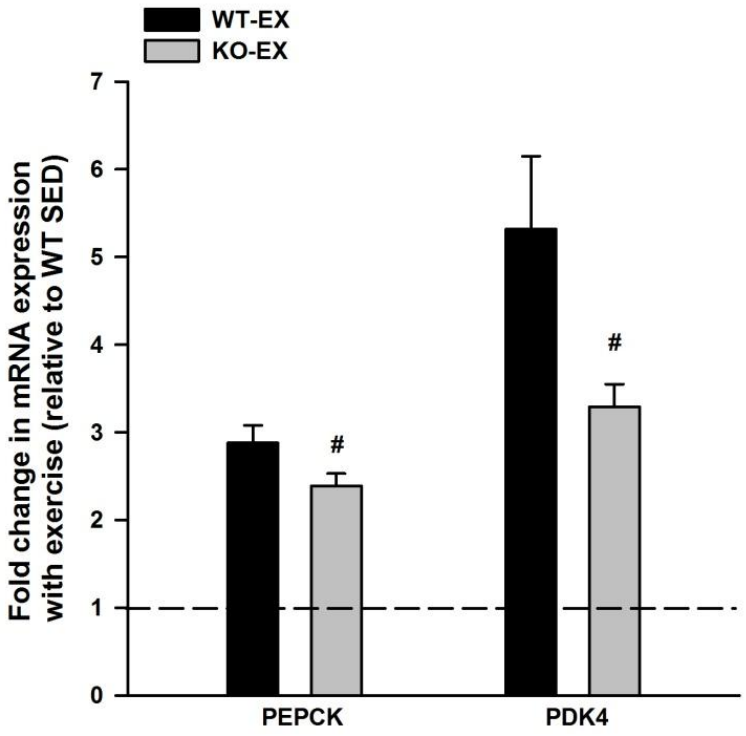

Figure 5-5 Exercise-mediated induction of PEPCK and PDK4 from WT and IL-6$^{-/-}$ mice. The exercise-mediated induction of PEPCK and PDK4 is attenuated in eWAT from IL-6$^{-/-}$ deficient mice. Data are presented as means + SEM for 10-12 animals per group and are expressed as fold increases in gene expression compared to the sedentary control group of the same genotype. $^{\#}p$ <0.05 compared to WT EX.

5.3.5 AMPK Phosphorylation Is Altered in eWAT from IL-6$^{-/-}$ Mice

In sedentary IL-6$^{-/-}$ mice, phosphorylated AMPK (p-AMPK) in eWAT was diminished by ~40% compared to WT sedentary mice. A single bout of treadmill running did not lead to an increase in p-AMPK from WT mice while exercise significantly increased p-AMPK in IL-6$^{-/-}$ mice (*Figure 5-6A*). The phosphorylation of p38 MAPK was not changed following exercise in either genotype (*Figure 5-6B*). Total AMPK and p38 MAPK protein were similar in both genotypes (data not shown). Alterations in AMPK phosphorylation were not associated with changes in the protein content of LKB-1, PP2A or PP2C (*Figure 5-6C*).

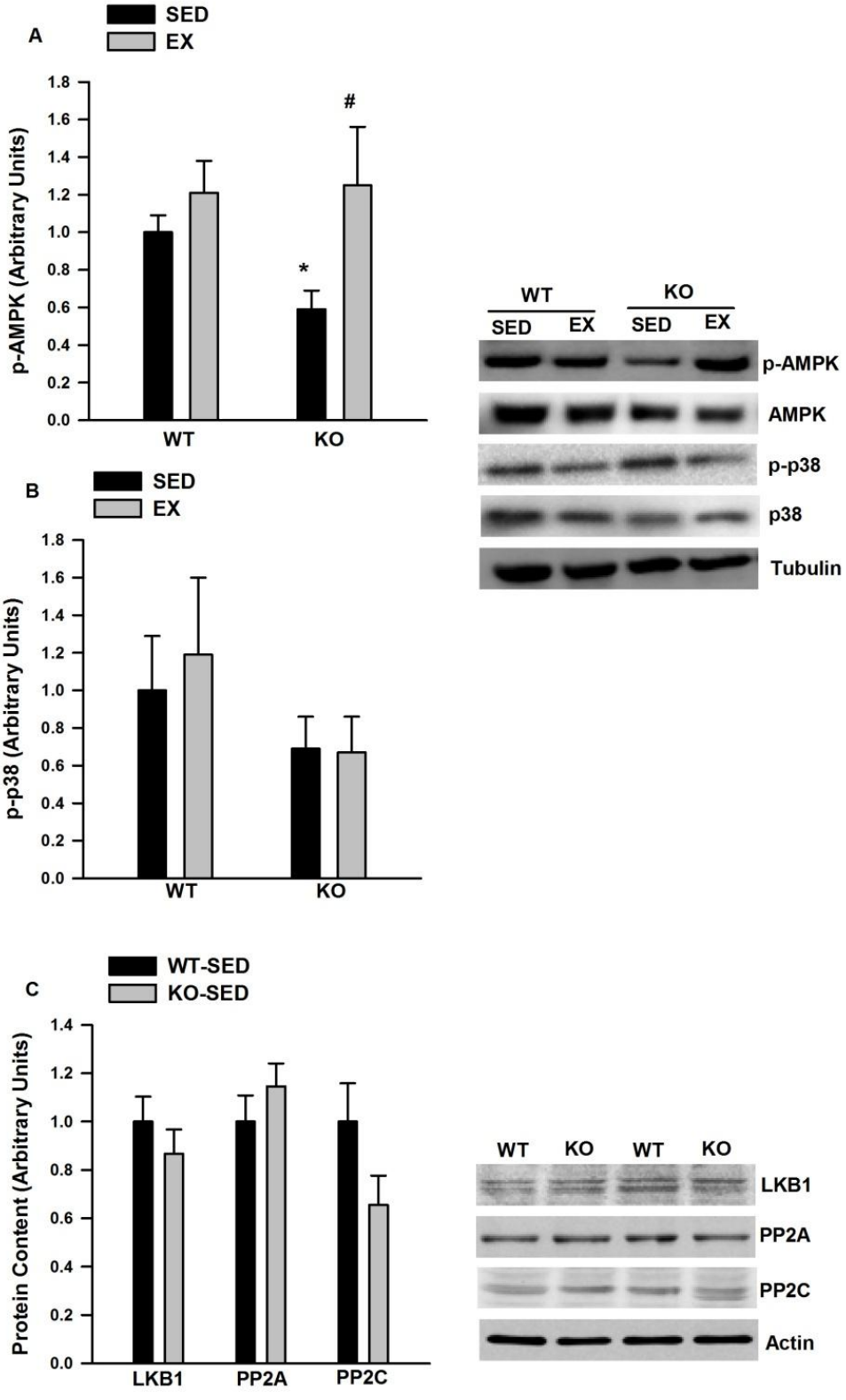
A
SED
EX
p-AMPK (Arbitrary Units)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
#
WT
KO
WT
KO
SED
EX
SED
EX
p-AMPK
AMPK
p-p38
p38
Tubulin
B
SED
EX
p-p38 (Arbitrary Units)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
WT
KO
C
WT-SED
KO-SED
Protein Content (Arbitrary Units)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
LKB1
PP2A
PP2C
WT
KO
WT
KO
LKB1
PP2A
PP2C
Actin

Figure 5-6 Exercise mediated AMPK signaling from WT and IL-6$^{-/-}$ mice. A) P-AMPK is reduced in eWAT from sedentary IL-6$^{-/-}$ compared to WT mice and increased following an acute bout of exercise in IL-6$^{-/-}$ mice only. B) Exercise did not change the phosphorylation of p38 in either genotype. C) There is no differences for the protein expression of LKB1, PP2A and PP2C between WT and KO SED group. Data are presented as means + SEM for 10-12 animals per group. Representative Western blot images are shown to the right of the quantified Western blot data. * $p<0.05$ versus WT SED. $^{\#}$ versus IL-6$^{-/-}$ KOSED in A.

5.3.6 There Are No Apparent Reductions in Beta Adrenergic Signalling in eWAT from IL-6$^{-/-}$ Mice

As beta-adrenergic agonists have been shown to induce glyceroneogenic enzymes we wanted to determine if the attenuation of PDK4 and PEPCK during exercise was secondary to reductions in adrenergic signalling. In this regard we found that the phosphorylation of CREB (*Figure 5-7A*) and HSL (*Figure 5-7B*), proteins that are phosphorylated via beta adrenergic dependent pathways (22-24) were similar in adipose tissue from WT and IL-6 deficient mice following exercise. Likewise, the ability of epinephrine to stimulate lipolysis *ex vivo* was similar in adipose tissue from WT and IL-6$^{-/-}$ mice (*Figure 5-7C, D*). There were no differences in the protein content of the beta 3 adrenergic receptor in eWAT from WT and IL-6$^{-/-}$ mice (WT 1.00 ± 0.27, IL-6$^{-/-}$ 1.03 ± 0.36 arbitrary units N= 8-10/group, $p>0.05$). Plasma fatty acid levels were similar at rest between genotypes and increased to a greater extent in IL-6$^{-/-}$ mice. Exercise decreased plasma TG levels in both WT and KO mice. There was no effect of genotype or exercise on plasma glucose or glycerol levels (*Table 5-2*).

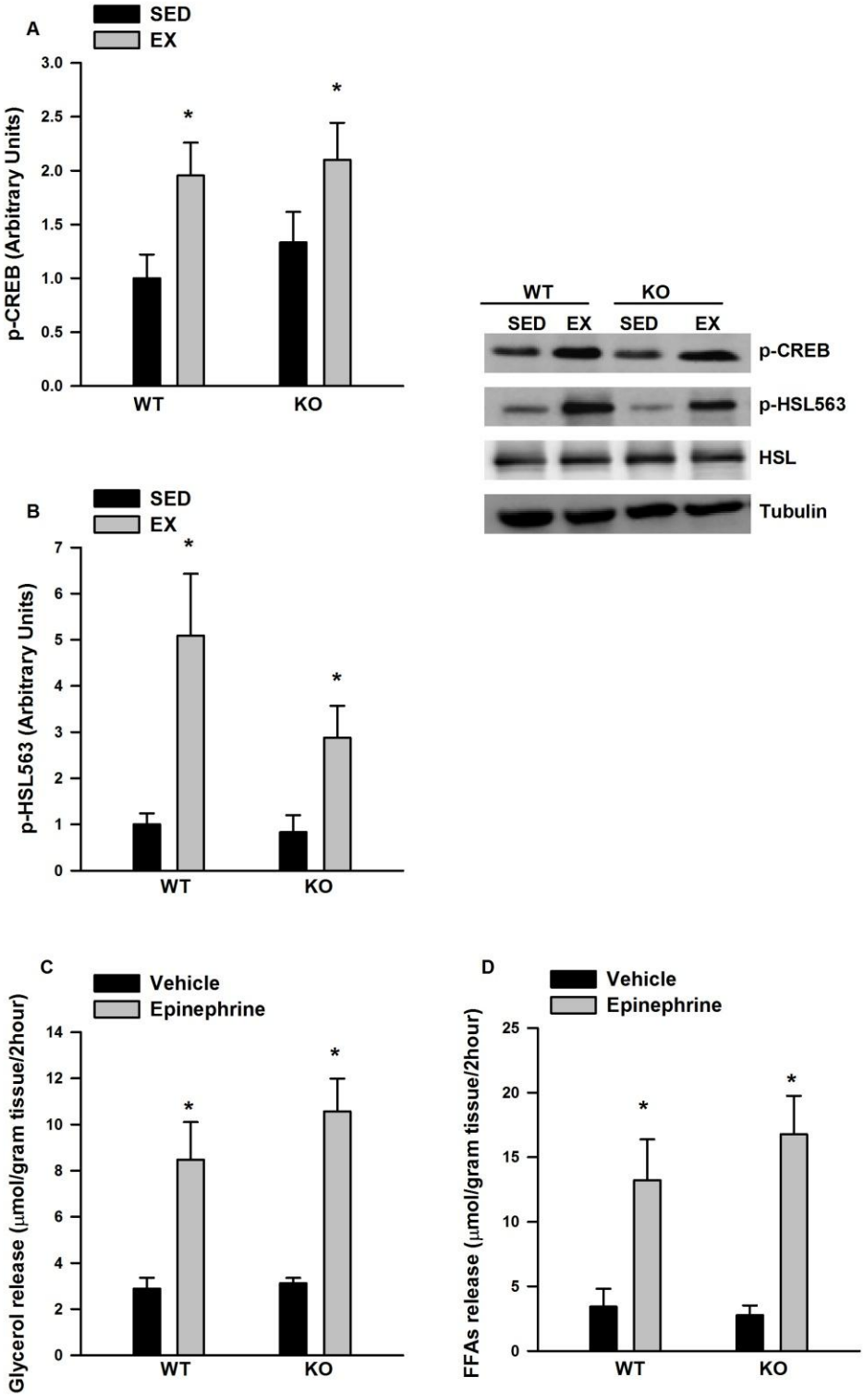
A
SED
EX
p-CREB (Arbitrary Units)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
WT
KO
WT
KO
SED
EX
SED
EX
p-CREB
p-HSL563
HSL
Tubulin
B
SED
EX
p-HSL563 (Arbitrary Units)
7
6
5
4
3
2
1
0
*
*
WT
KO
C
Vehicle
Epinephrine
Glycerol release (μmol/gram tissue/2hour)
14
12
10
8
6
4
2
0
*
*
WT
KO
D
Vehicle
Epinephrine
FFAs release (μmol/gram tissue/2hour)
25
20
15
10
5
0
*
*
WT
KO

Figure 5-7 Beta adrenergic signalling in adipose tissue from WT and IL-6$^{-/-}$ mice. Exercise-induced increases in A) CREB and B) HSL phosphorylation are not different in eWAT from WT and IL-6$^{-/-}$ mice. Epinephrine stimulated C) glycerol and D) fatty acid release are not different in eWAT from either WT or IL-6$^{-/-}$ mice. Data are presented as means + SEM for 6-12 samples/animals per group. * $p<0.05$ compared to sedentary control group in A and B or vehicle treated control group in C and D.

Table 5-2 Effects of Exercise on Plasma Glucose, NEFA, Glycerol and Triglyceride levels from WT and IL-6$^{-/-}$ Mice.

Group	Glucose (mmol/L)	NEFA (mmol/L)	Glycerol	TG (mmol/L)
WT-SED	9.50 ±0.82	0.51 ±0.03	0.27 ±0.02	0.73 ±0.11
WT-EX	12.59 ±0.74	0.74 ±0.05 [a]	0.32 ±0.01	0.44 ±0.06 [a]
KO-SED	9.86 ±1.49	0.49 ±0.05	0.30 ±0.02	0.73 ±0.07
KO-EX	13.86 ±0.46	0.92 ±0.06 [a,b]	0.28 ±0.01	0.45 ±0.02 [a]

Values are means ±SEM for 4–7 animals per group. NEFA, nonesterified fatty acid; Glucose and NEFA in sedentary group were measured in the fed state [a] P <0.05 vs. sedentary group within genotype. [b] $p<0.05$ compared to WT-EX group.

5.4 Discussion

In previous work we have shown that exercise increases the expression of PDK4 in rat adipose tissue (7). While this effect is likely mediated, at least in part, by catecholamines, it is not clear if additional systemic factors modulate the expression of enzymes involved in fatty acid re-esterification. As it has been proposed that exercise increases IL-6 secretion from skeletal muscle and stimulates adipose tissue lipolysis (11), it was of interest to determine if this myokine modulated the expression of PDK4 and PEPCK in mouse white adipose tissue during exercise. As a first step in addressing this question we treated cultured epididymal mouse adipose tissue with IL-6 and found a rapid activation of IL-6 signalling and reductions in the expression of glyceroneogenic enzymes.

The activation of AMPK in fat cells has been suggested to occur as a consequence of lipolysis and subsequent increases in fatty acid re-esterification (25). To determine if this was the case in our model we measured the ability of IL-6 to stimulate fatty acid and

glycerol release in cultured eWAT. In contrast to a previous report demonstrating small increases (~5-10%) in glycerol release in 3T3 adipocytes with a similar IL-6 treatment (11), we were unable to detect an effect of IL-6. We included epinephrine-treated cultures as a positive control and clearly showed a robust increase in glycerol and fatty acid release with this hormone, demonstrating the suitability of our preparation for the measurement of lipolysis. The discrepancy between previous work done in cultured adipocytes (26-27), and the current results are likely due to the duration of IL-6 exposure. In this regard we found that longer incubations (i.e. 12 hours) increased lipolysis in cultured adipose tissue. As increases in lipolysis occurred at a much later time point than the activation of AMPK this suggests that the modulation of AMPK signalling by IL-6 occurred independent of lipolysis. Moreover, given the prolonged duration of treatment required to increase lipolysis, our findings would question the role of IL-6 as a stimulator of lipolysis in adipose tissue during exercise.

Having shown that IL-6 directly and rapidly reduces the expression of enzymes involved in fatty acid re-esterification we next sought to determine if the deletion of IL-6 would potentiate the effects of exercise on the induction of PDK4 and PEPCK in mouse adipose tissue. As an initial approach we analyzed the effects of exercise on the activation of reputed markers of IL-6 signalling in adipose tissue, i.e. STAT3 phosphorylation (28) and the induction of SOCS3 mRNA (29). While we did not detect changes in these parameters, perhaps these findings are not entirely unexpected. In this regard it has been reported that interstitial concentrations of IL-6 (30) are several orders of magnitude higher than levels in the circulation, even after extremely large volumes of exercise such as a marathon (31). Thus, it seems unlikely that an increase in plasma IL-6 in the low

pg/ml range would be a sufficient enough stimulus to activate IL-6 signalling in a tissue that is bathed by much higher concentrations at rest. With these points in mind we can not rule out the possibility that longer durations, and/or a greater intensity of exercise could lead to increases in IL-6 signalling in adipose tissue.

Despite the fact that IL-6 signalling did not appear to be activated in adipose tissue during exercise, we found that the exercised-mediated induction of PEPCK and PDK4 was attenuated in adipose tissue from IL-$6^{-/-}$ mice. We interpret these findings as suggesting that the attenuated effect of exercise on these genes was indirect. In an effort to elucidate the mechanisms that could, at least in part, explain the attenuation in glyceroneogenic enzymes we assessed changes in AMPK signalling. Consistent with previous work from Kelly *et al.* (12) we found that the phosphorylation of AMPK was decreased in adipose tissue from IL-6 deficient mice at rest. Although exercise did not appreciably increase AMPK phosphorylation in adipose tissue from WT mice, a finding similar to recent work from Pilegaard's group (17), exercise lead to an ~ 2 fold increase in AMPK phosphorylation in adipose from IL-6 deficient mice. Alterations in AMPK phsophorylation could not be explained by differences in the total protein content of LKB-1, an upstream AMPK kinase (32), or PP2C/PP2A, protein phosphatises which de-phosphorylate AMPK (33-34). As we have shown that AICAR treatment reduces PEPCK and PDK4 mRNA expression, these results provide evidence suggesting that the greater relative increase in AMPK activation in adipose tissue from IL-$6^{-/-}$ mice may be associated with the attenuated induction of PEPCK and PDK4. At this point it is not clear why exercise increased AMPK phosphorylation to a greater extent in eWAT from IL-$6^{-/-}$ versus WT mice.

In addition to AMPK, we have previously demonstrated a role for p38 MAPK and PPARγ in the regulation of PDK4 mRNA in rat adipose tissue (7). Thus, we thought it plausible that genotypic differences in these parameters could explain, at least in part, the blunted induction of PDK4 in adipose tissue from IL-6 deficient following exercise. As the protein content of PPARγ was not different between groups and p38 MAPK phosphorylation not increased in either genotype post-exercise, it seems unlikely that differences in these pathways account for the attenuated induction of PDK4.

We (7, 35), and others (36), have demonstrated a role for beta-adrenergic agonists in the control of glyceroneogenic enzymes in adipose tissue. Therefore, it is plausible that alterations in beta-adrenergic signalling could account for the observed changes in the exercise-mediated induction of PEPCK and PDK4 in adipose tissue from IL-$6^{-/-}$ mice. We found that the exercise-induced phosphorylation of HSL and CREB, proteins that are phosphorylated via beta-adrenergic dependent pathways (22-24), were similar between genotypes. Likewise, the ability of epinephrine to stimulate lipolysis in adipose tissue *ex vivo* was nearly identical in adipose tissue from WT and IL-6 deficient mice. Lastly, the exercise-mediated increase in plasma fatty acid levels was, if anything, slightly elevated in IL-6 deficient mice. Collectively these results provide evidence that reductions in the exercise-induced expression of glyceroneogenic enzymes in adipose from IL-$6^{-/-}$ mice occurred independent of decreases in beta adrenergic signalling.

If IL-6 modulates the expression of glyceroneogenic enzymes indirectly and apparently independent of alterations in beta adrenergic signalling, the question arises as to the specific mechanisms which could be mediating this effect. In this regard, a growing body of literature has demonstrated the existence of complex tissue-to-tissue

communication during exercise. For instance it has recently been shown that exercise stimulates the secretion of chemokine (C-X-C motif) ligand 1 (CXCL1) from the liver through an IL-6 dependent mechanism (37). Increases in circulating CXCL1 levels via the over-expression of this cytokine in skeletal muscle leads to an up-regulation of enzymes involved in fatty acid oxidation (38). Thus, while speculative, it could be argued that increases in skeletal muscle-derived IL-6 signal to the liver during exercise resulting in increases in CXCL1, which in turn acts as a signal involved in the regulation of PEPCK and PDK4 in adipose tissue. Clearly, this is an area of research that requires further attention.

In summary we have found that IL-6 signalling is not activated in adipose tissue during exercise, nor does IL-6, at least in the short-term, increase adipose tissue lipolysis. Despite these results we have evidence to suggest that IL-6 plays a role, albeit most likely indirect, in mediating the effects of exercise on the induction of glyceroneogenic enzymes. These intriguing results shed insight into the complex regulatory pathways governing the expression of genes involved in fatty acid re-esterification and challenge the long held paradigm that IL-6 is a direct mediator of exercise-induced changes in adipose tissue metabolism and gene expression.

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Chapter 6 IL-6 Deficient Mice Fed a High Fat Diet Display Alterations in Adipose Tissue Metabolism

6.1 Introduction

Interleukin 6 (IL-6) has traditionally been viewed as a pro-inflammatory cytokine that is linked to the etiology of insulin resistance (1-3). For instance IL-6 in both *in vivo* and *in vitro* models has been shown to cause hepatocyte insulin resistance (4-5). However, accumulating evidence has begun to challenge this long held view. Carey et al. (6) reported that the infusion of IL-6 in humans increased basal and insulin stimulated glucose uptake in skeletal muscle. These results are particularly interesting given the recent work by Matthews et al. (7) demonstrating that IL-6 deficient mice develop insulin resistance and, when fed a high fat diet (HFD), display signs of hepatic steatosis and liver inflammation. Collectively these findings imply that IL-6 possesses insulin-sensitizing properties in liver and muscle.

Owing to the central role of skeletal muscle and liver in the regulation of carbohydrate metabolism, studies investigating the role of IL-6 in glucose homeostasis have focused primarily on these tissues (7-8). However interstitial concentrations of IL-6 surrounding adipose tissue are orders of magnitude greater than levels in the circulation (9) suggesting that adipose tissue-derived IL-6 could play a pivotal role in the maintenance of adipose tissue metabolism and function in an autocrine and/or paracrine manner. Given the growing appreciation for adipose tissue in the control of systemic carbohydrate and lipid metabolism (10) it is somewhat surprising that effects of IL-6 ablation on adipose tissue metabolism have not been examined.

While some studies have reported that IL-6 causes adipose tissue insulin resistance (3), others have provided evidence that IL-6 favorably regulates adipose tissue function. For instance, IL-6 activates 5'AMP activated protein kinase (AMPK) in adipocytes (11). AMPK is a master regulator of cellular metabolism and recent work suggests that AMPK regulates mitochondrial biogenesis in fat cells (12). The induction of mitochondrial biogenesis in adipocytes is mirrored by improvements in insulin action (13), whereas reductions in mitochondrial content/function are associated with adipocyte insulin resistance (14). In addition to mitochondrial content, it has been suggested that AMPK controls the expression of the insulin sensitizing adipokine, adiponectin either directly (15) or secondary to increases in adipose tissue mitochondrial biogenesis (16). Increased expression of adiponectin has been shown to have favorable effects on adipose tissue metabolism such as increasing beta-adrenergic responsiveness and mitochondrial density (17).

Given the increasingly well-recognized role of adipose tissue in regulating glucose homeostasis (10) and the apparent controversy regarding the role of IL-6 in the pathogenesis of insulin resistance, the purpose of the present study was to examine the effects of IL-6 deficiency on adipose tissue metabolism. Given the possibility that a clear manifestation of IL-6 deficiency might not be evident under the condition of a standard chow diet we examined the metabolic outcomes of IL-6 deficiency under both chow and HFD conditions. We hypothesized that IL-$6^{-/-}$ mice would display reductions in adipose tissue insulin action, would have blunted beta adrenergic responsiveness and decreases in adipose tissue mitochondrial content and respiration, and adiponectin expression and secretion.

6.2 Methods and Materials

6.2.1 Materials

Reagents, molecular weight marker and nitrocellulose membranes for SDS-PAGE were purchased from Bio-Rad (Mississauga, ON). Antibodies against ubiquinone: cytochrome c oxidoreductase core I subunit (CORE1) (CAT# MS303) were obtained from Mitosciences (Eugene, OR) while heat shock protein 60 (HSP60) antibodies (CAT# SPA-807) were from Enzo Life Sciences (Ann Arbor, MI). Antibodies against p-threonine 308 protein kinase B (PKB) (CAT#9275), p-serine 473 PKB (CAT#9271), PKB (CAT#4685), p-p38 mitogen-activated protein kinase (MAPK) (CAT #9211), p38 MAPK (CAT#9212), p-stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK) (CAT#4671), p-extracellular regulated kinase (ERK) 1/2 (CAT#9101), ERK1/2 (CAT#4695), p-AMPK (CAT#2531), AMPKα (CAT#2793), p-serine563 hormone sensitive lipase (HSL) (CAT#4139), HSL (CAT#4107) and peroxisome proliferator-activated receptor gamma (PPARγ) (CAT#4685) were from Cell Signaling (Danvers, MA). Antibodies against tubulin (CAT#ab7291) were from Abcam (Cambridge, MA). Horseradish peroxidase-conjugated donkey anti-rabbit and goat anti-mouse IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Fatty acid-free bovine serum albumin (BSA) (CAT# 152401) was from MP Biomedicals (Solon, OH). Free glycerol reagent (CAT#F6428) was from Sigma (Oakville, ON). A kit for the determination of non-esterified fatty acids (NEFA-HR kit) was purchased from Wako Chemicals (Richmond, VA). An ELISA for the detection of mouse adiponectin (CAT# EZMADP-60K) was from Millipore (Billerica, MA). SuperScript II Reverse Transcriptase, oligo(dT),dNTP and SYBR Green qPCR

SuperMix was products of Invitrogen (Burlington, ON). Taqman Gene Expression Assays for mouse β actin (4352933E), eukaryotic 18S rRNA, TNFalpha (CAT# Mm99999068_m1), adiponectin (CAT# Mm00456425_m1), F4/80 (Emr1) (CAT# Mm00802529_m1), IL-1-beta (CAT# Mm00434228_m1), CxC11 (CAT#Mm00433859_m1), Arg1 (CAT# Mm00475988_m1), iNOS (CAT# Mm00440502_m1), Cc12 (CAT#Mm00441242), CD206 (Mm00440502_m1) and CD11c (CAT# Mm00498698_m1) were from Applied Biosystem (Foster City, CA). All other chemicals were purchased from Sigma (Oakville, ON).

6.2.2 Treatment of Animals

All protocols followed Canadian Council on Animal Care (CCAC) guidelines and were approved by the University of Guelph Animal Care Committee. Twelve week old male IL-$6^{-/-}$ mice (Jackson Laboratories B6.12952-$IL6^{tmlkopf}$/J) and age-matched C57BL/6J wild-type (WT) mice were housed 1 per cage, with a 12/12-hour light/dark cycle. Mice were acclimated to the animal housing facility for 1 week prior to the start of the diet manipulation. At 13 weeks of age, mice were fed a high-fat diet (HFD) *ad libitum* for 10 week. The high-fat diet contained 18.4% protein (casein), 60.3% fat, and 21.3% carbohydrate, and was purchased from Harlan Laboratories Inc. (CAT# TD06414, Madison, WI). Body weight and food intake were determined weekly for the duration of the study. Pre-weighed food was placed in the food hoppers and measured on a per-cage basis. Food intake was determined as the average grams consumed per week over the course of the study.

6.2.3 Glucose and Insulin Tolerance Tests

Intraperitoneal glucose (GTT) and insulin tolerance tests (ITT) were performed at the end of the 10 weeks of HFD. For the GTT, mice were fasted for 6h prior to an intraperitoneal (I.P.) injection of glucose (2g/kg body weight). For the ITT, mice had free access to HFD prior to an I.P. injection of insulin (0.75U/kg body weight). Blood glucose levels were determined by tail vein sampling at the indicated intervals using a glucometer. Changes in glucose over time were plotted, and the area under the curve (AUC) was calculated for each.

6.2.4 *In Vivo* Insulin Signaling

Mice were anesthetized with sodium pentobarbitol (5mg/100g body weight) and a small incision (~1-2 cm) was made in the abdominal cavity allowing for the removal of the distal tip of one epididymal fat pad. Minimal blood loss occurred during the removal of the pre-insulin sample. Following this, mice were then injected I.P with a weight adjusted (3.75 U/kg body weight) bolus injection of insulin. 15 minutes post injection the contra lateral epididymal fat depot was removed. Adipose tissue was immediately clamp frozen in tongs cooled to the temperature of liquid nitrogen and then stored at -80°C until further analysis. The insulin injection protocol in these experiments allowed for the comparison of the effects of insulin on PKB phosphorylation within the same animal.

6.2.5 Adipose Tissue Explants

Adipose tissue minces (~50 mg cut into 10-20 mg fragments) from WT or IL-$6^{-/-}$ mice were incubated in 2.0 ml of oxygenated Krebs-Ringer buffer (KRB) (118 mM NaCl, 4.8 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 4

mM pyruvate, pH 7.4) with 2.5% fatty acid-free BSA at 37° C in a shaking water bath. Explants were treated with epinephrine (1 μM) and IL-6 (150 ng/ml) for either 30 minutes (signaling) or 2 hours (lipolysis). For the determination of insulin signaling explants were incubated in KRB supplemented with vehicle, 0.25 or 1 mU/ml of insulin for 30 minutes. Lipolytic rates are linear during this time period. Media and tissue minces were collected for the determination of fatty acid and glycerol release and the activation of the lipolytic and insulin-signaling pathway respectively. At the end of the experiments adipose tissue minces were rinsed in ice-cold sterile PBS, strained and adipose tissue fragments snap frozen and stored at -80 ℃ until further analysis.

6.2.6 Plasma Adiponectin and Adiponectin Secretion

The adiponectin levels in plasma and adiponectin secretion in KRB from vehicle treated adipose tissue explants were measured using a mouse specific adiponectin ELISA. Samples were run in duplicate.

6.2.7 Mitochondrial Respiration in Adipose Tissue

High-resolution O_2 consumption measurements were conducted in 2 mL of Miro5 using the OROBOROS Oxygraph-2k (OROBOROS INSTRUMENTS, Corp., Innsbruck, AT) with stirring at 750 rpm at 37 °C. Epididymal adipose tissue was removed and ~ 40-50 mg were minced with scissors (~30 seconds) and placed in an Oxygraph-2k reaction chamber. In agreement with a previous report (18), preliminary experiments demonstrated no additional effect of standard membrane permeabilization detergents (digitonin, saponin) on respirometric assessments. This suggests the surgical and mincing procedures are sufficient for maximal respiration in adipose tissue.

Adipose samples were allowed to incubate for ~10 min before initiating the respirometric protocols. 10 mM glutamate (G) and 5 mM malate (M) were then added as complex I substrates (State IV respiration) followed by 5mM ADP (D; State III respiration). Additional electron entry into complex I and II were achieved with 10mM pyruvate (P) and 20mM succinate (S) respectively. Electron transport system (ETS) capacity was determined via titration of FCCP to induce maximal uncoupled respiration (~typically occurred with 0.5 μM FCCP). All experiments were completed before oxygraph chamber [O_2] reached 100 μM, which is well above the threshold of O_2 limitation we have observed in adipose tissue (~60-80 μM). Polarographic oxygen measurements were acquired in 2-second intervals, with the rate of respiration derived from 40 data points, and expressed as pmol • s^{-1} • μg^{-1} protein at 37 ˚C. Cytochrome *c* was added to test for mitochondrial membrane integrity and respiration was never increased by >10%.

6.2.8 Western Blot Analysis

Protein was extracted from adipose tissue and the content and/or phosophorylation of PKB, CORE1, HSP60, HSL, PPARγ, ERK1/2, JNK, p38MAPK, AMPK and the beta 3 adrenergic receptor determined by Western blotting, as described in detail by our laboratory previously (19-20) and detailed in chapter 3. Signals were detected using enhanced chemiluminesence (PerkinElmer Life Science) and were subsequently quantified by densitometry by Gene Tool according to the manufacturer's instructions (SynGene, ChemiGenius2, PerkinElmer).

6.2.9 Mitochondrial DNA

Total DNA was isolated using DNeasy blood and tissue kit (Qiagen) and relative mitochondrial DNA copy number was determined as described in detail previously by Smith et al (21). Briefly, mtDNA primers were designed using the mouse mitochondrial genome sequence (Genbank accession number NC_001665) within the NADH dehydrogenase subunit 5 (ND5) gene: forward, 5'-GCAGCCACAGGAAAATCCG-3'; and reverse, 5'-GTAGGGCAGAGACGGGAGTTG-3'. Primers measuring genomic content were designed within the solute carrier family 16, member1 gene sequence on chromosome 2 (Genbank accession number NC_005101): forward, 5'-TAGCTGGATCCCTGATGCGA-3'; and reverse, 5'-GCATCAGACTTCCCAGCTTCC-3'. Each assay (25 µl total volume) contained 2.5 µl forward and reverse primers, 5 µl of DNA template, 10 µl of SYBR Green qPCR SuperMix and 5 µl of RNase free water. Relative mtDNA content was calculated by the $2^{-\Delta\Delta CT}$ method using genomic DNA content as an internal standard with 7500 System SDS Software Version 1.2.1.22 (Applied Biosystems).

6.2.10 Real Time PCR

RNA was isolated from adipose tissue using an RNeasy kit according to the manufacturer's instructions. 1 µg of RNA was used for the synthesis of complementary DNA (cDNA) using SuperScript II Reverse Transcriptase, oligo(dT) and dNTP. Real time PCR was performed using a 7500 Fast Real-Time PCR system (Applied Biosytems) or Rotorgene 6000 (Qiagen), as described previously (22). Each assay (20 µl total volume) contained 1 µl gene expression assay, 1 µl of cDNA template, 10 µl of Taqman Fast Universal PCR Master Mix and 8 µl of RNase free water. For β-actin or 18S, each

50µl reaction contained 25 µl of PCR Master mix, 2.5 µl each of gene expression assay, 1 µl of cDNA template, and 21.5 µl of RNase free water. Results were normalized to the mRNA expression of β-actin or 18S. Relative differences in gene expression between groups were determined using the $2^{-\Delta\Delta CT}$ method (23). The amplification efficiencies of the gene of interest and the housekeeping gene were equivalent.

6.2.11 Statistical Analysis

Data are presented as means ± standard error of the mean (SEM). The effects of epinephrine and insulin *ex vivo* on lipolysis and insulin signaling pathways in chow groups between WT and IL-$6^{-/-}$ mice were made using a two way ANOVA followed by a LSD post hoc analysis. Comparisons between two groups were made using a Student's T-Test. Statistical significance was established at a p level <0.05.

6.3 Results

6.3.1 No Phenotypic Differences in Adipose Tissue Metabolism in Chow Fed IL-$6^{-/-}$ Mice

Ex vivo insulin-induced PKB phosphorylation on both threonine-308 and serine-473 phosphorylation sites were similar in epididymal adipose tissue explants from WT and IL-6 deficient mice (*Figure 6-1A*). Similarly the ability of epinephrine to stimulate lipolysis was not different between genotypes (*Figure 6-1B*). Markers of mitochondrial content such as CORE1 and HSP60 (*Figure 6-1C*) and mitochondrial respiration in response to successive additions of electrons into complex I (GM, GMD, GMDP) and complex II (GMDP-S) and mitochondrial uncoupler (FCCP) were not different between groups (*Figure 6-1D*). There was no difference in adiponectin expression in epididymal adipose tissue between genotypes (1.33 ± 0.18 fold increase in IL-$6^{-/-}$ mice versus WT).

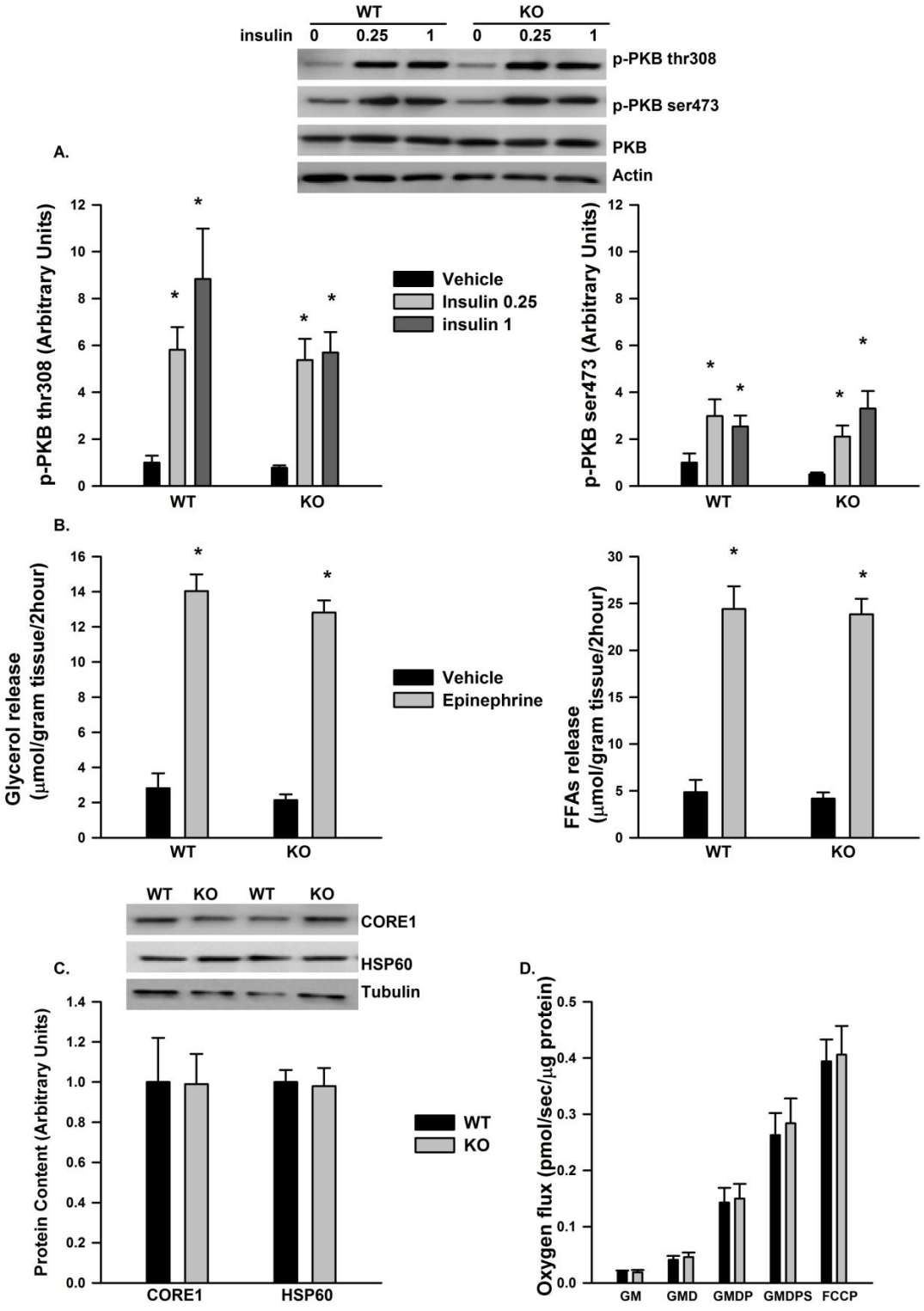
A.
WT
KO
insulin 0 0.25 1 0 0.25 1
p-PKB thr308
p-PKB ser473
PKB
Actin
p-PKB thr308 (Arbitrary Units)
Vehicle
Insulin 0.25
insulin 1
WT
KO
p-PKB ser473 (Arbitrary Units)
B.
Glycerol release (μmol/gram tissue/2hour)
Vehicle
Epinephrine
FFAs release (μmol/gram tissue/2hour)
C.
WT KO WT KO
CORE1
HSP60
Tubulin
Protein Content (Arbitrary Units)
D.
Oxygen flux (pmol/sec/μg protein)
GM
GMD
GMDP
GMDPS
FCCP

Figure 6-1 Adipose tissue metabolism in chow fed WT and IL-6$^{-/-}$ mice. A) *Ex vivo* insulin (0.25 and 1 mU) signaling, B) *ex vivo* epinephrine (1μM, 2hour) induced glycerol and FFA release, C) mitochondrial proteins and D) mitochondrial respiration in epididymal adipose tissue from WT and IL-6$^{-/-}$ mice. Data are presented as means + SEM for 6-10 samples per group. Representative Western blots are shown above in A and C. Mitochondrial respiration was measured by high-resolution O_2 consumption. GM (glutamate, malate), GMD (glutamate, malate, ADP), GMDP (glutamate, malate, ADP, pyruvate), GMDPS (glutamate, malate, ADP, pyruvate, succinate).*p<0.05 compared to vehicle treated condition.

6.3.2 Food Intake, Body Weight and Fat Pad Mass in WT and IL-6$^{-/-}$ Fed a HFD

There were no differences in body weight, cumulative food intake or epididymal fat pad mass in WT or IL-6$^{-/-}$ mice fed a HFD for 10 weeks (*Table 6-1*).

Table 6-1 Descriptive Characteristics of WT and IL-6$^{-/-}$ Mice Fed a High Fat Diet

	WT	**KO**	***p***
Initial body weight (g)	27.6 ± 0.5	27.4 ± 0.4	0.80
Final body weight (g)	41.8 ± 1.4	44.2 ± 1.0	0.09
Epididymal fat pad weight (g)	1.85 ± 0.41	2.14 ± 0.15	0.44
Average food intake (g per week)	18.90 ± 0.4	18.7 ± 0.7	0.78

Data are presented as means ± SEM for 9-10 animals per group.

6.3.3 IL-6$^{-/-}$ Mice Have Impaired Glucose Homeostasis Compared to WT Fed a HFD

Following 10 weeks of a HFD WT and IL-6 deficient mice underwent an I.P. GTT. As seen in *Figure 6-2A and B*, IL-6$^{-/-}$ mice had impaired glucose tolerance compared to WT controls. Similarly, IL-6$^{-/-}$ mice were insulin intolerant compared to WT as shown by a blunted reduction in blood glucose levels (*Figure 6-2C and D*).

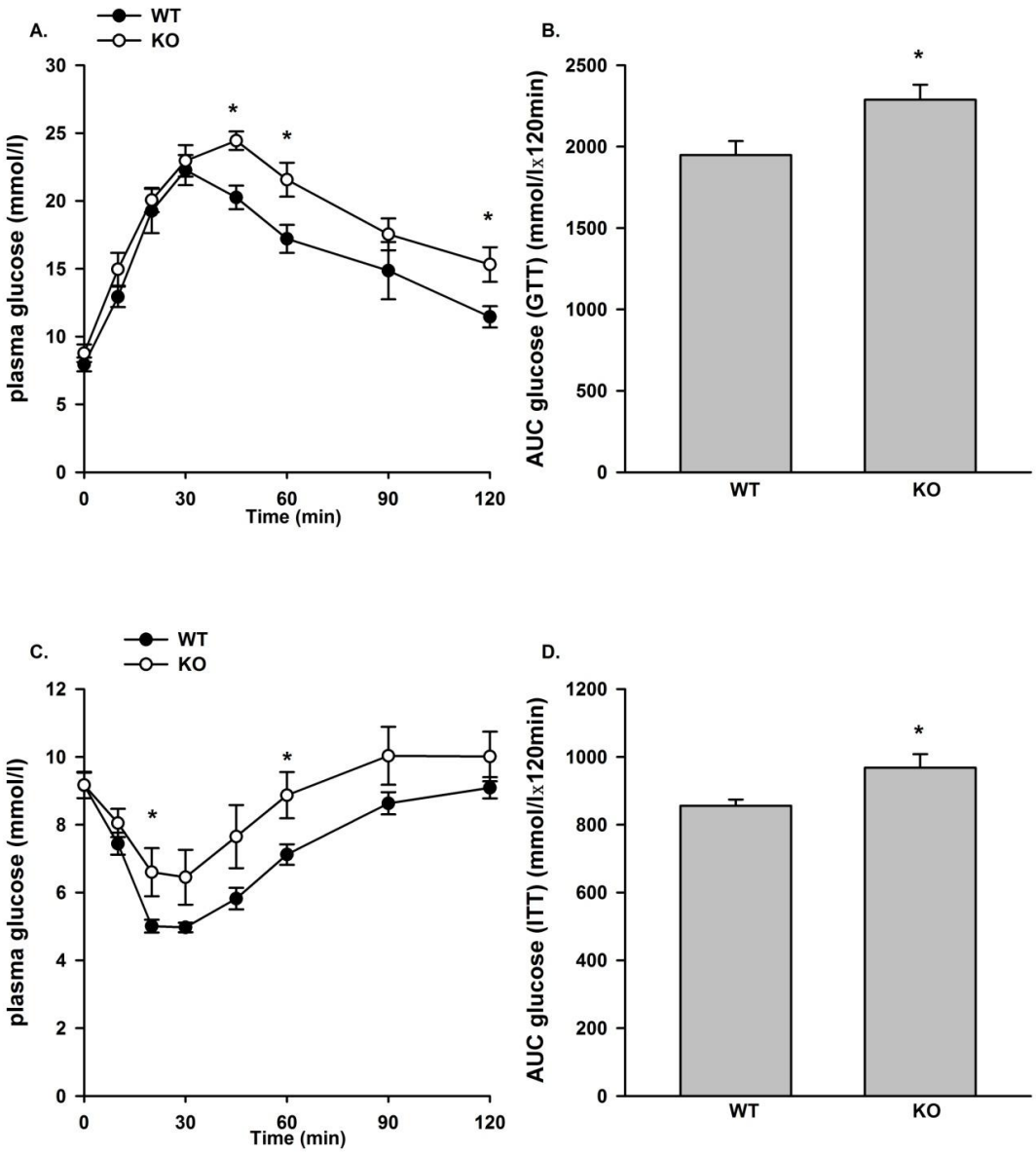

Figure 6-2 Glucose and insulin tolerance in WT and IL-6$^{-/-}$ mice fed a HFD. Glucose curves and the quantified area under the curves for glucose (A, B) and insulin (C, D) tolerance tests. Data are presented as means +SEM for 8-10 mice per group. * p<0.05 versus WT group at the same time point in A and C. * p<0.05 versus WT group in B and D.

6.3.4 Insulin Signaling Is Attenuated in Adipose Tissue from IL-6$^{-/-}$ Mice Fed a HFD

To ascertain if systemic glucose and insulin intolerance were reflected in defects in insulin signaling in adipose tissue we injected mice *in vivo* with insulin and assessed changes in the serine and threonine phosphorylation of PKB. As adipose tissue was

procured from the same animal prior to, and following insulin injection the data is expressed as a fold increase by insulin within the same animal. There were no differences in PKB phosphorylation between genotypes in samples taken prior to the injection of insulin (data not shown). As shown in *Figure 6-3A*, the insulin-induced phosphorylation of PKB was markedly attenuated in adipose tissue from IL-6 deficient compared to WT mice. Similar effects were found when insulin signaling was measured in adipose tissue explants *ex vivo* (data not shown). The reductions in PKB phosphorylation were associated with increases in the phosphorylation of ERK and JNK, but not p38 (*Figure 6-3B*).

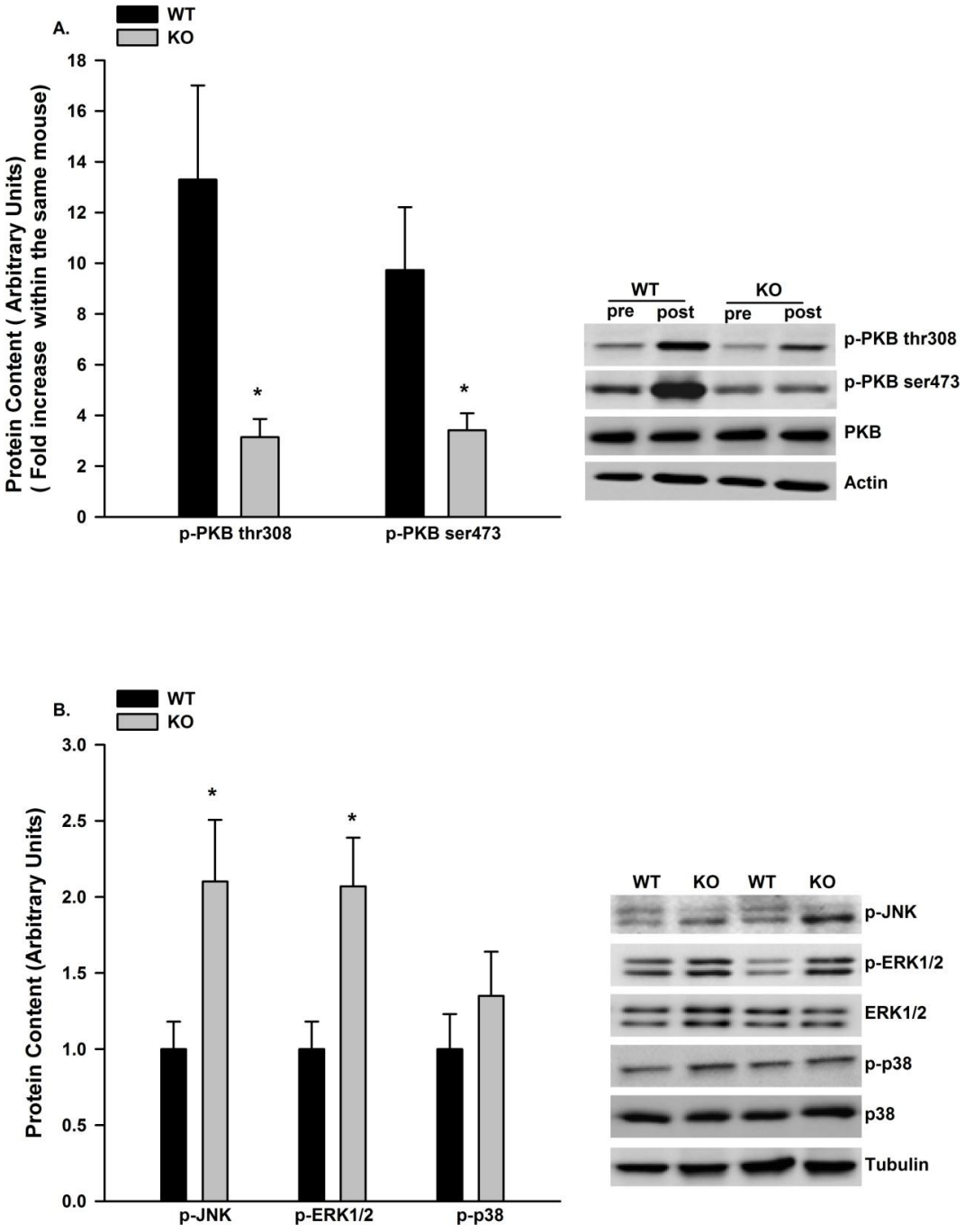

Figure 6-3 *In vivo* insulin signaling in adipose tissue from WT and IL-6$^{-/-}$ mice fed a HFD. A) *In vivo* insulin-mediated (3.75U/kg BW, 15 mins) phosphorylation of PKB on threonine 308 and serine 473. B) The attenuated phosphorylation of PKB is associated with increases in the phosphorylation of ERK and JNK in adipose tissue from IL-6$^{-/-}$ mice. Data are presented as means + SEM for 8-10 animals per group. In A) data are presented as fold increases in PKB phosphorylation relative to the control sample from the same animal. Representative Western blot images are given to the right of the quantified data. * $p<0.05$ versus WT group.

6.3.5 Beta-Adrenergic Signaling Is Attenuated in Adipose Tissue from IL-6$^{-/-}$ Mice

To determine if catecholamine signaling is altered in adipose tissue from IL-6 deficient mice fed a HFD we treated epididymal adipose tissue explants with epinephrine (1μM) and measured changes in glycerol and fatty acid release *ex vivo*. Epinephrine significantly increased glycerol and fatty acid release in eWAT from WT (*Figure 6-4A*) but not IL-6$^{-/-}$ (*Figure 6-4B*) mice. There were no differences in vehicle treated HSL phosphorylation between groups (data not shown). The attenuation of lipolysis in explants from IL-6$^{-/-}$ mice was associated with reductions in the epinephrine-induced phosphorylation of HSL (*Figure 6-4C*). Reductions in beta-adrenergic signaling were not explained by decreases in the protein content of the beta 3 adrenergic receptor (WT 1.00 ± 0.23, KO 1.20 ± 0.18, arbitrary units, N=10 per groups).

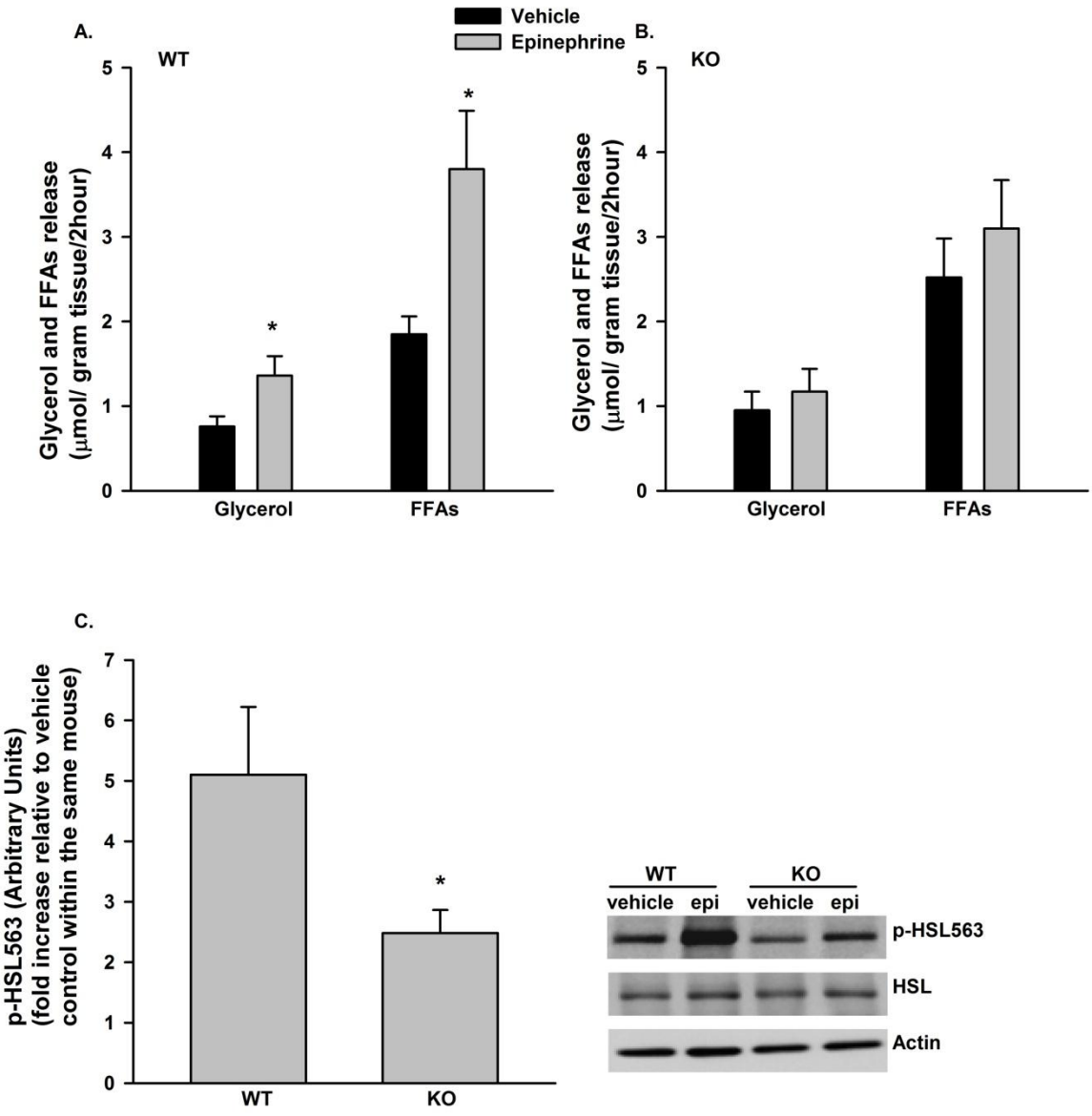

Figure 6-4 *Ex vivo* epinephrine-stimulated lipolysis in adipose tissue from WT and IL-6$^{-/-}$ mice fed a HFD. Epinephrine-induced (1μM, 2h) glycerol and FFA release in epididymal adipose tissue from A) WT and B) IL-6$^{-/-}$ mice fed a HFD. C) Epinephrine-induced (1μM, 30 minutes) increases in the phosphorylation of HSL at ser563. Increases in phosphorylation are expressed relative to the vehicle control condition from the same animal. Data are presented as means + SEM for 8-10 animals per group. Representative Western blot images are given to the right of the quantified data in C. * $p<0.05$ versus vehicle control within the same genotype in A and versus WT in C.

6.3.6 IL-6 Does Not Potentiate Epinephrine-Stimulated Lipolysis

IL-6 (150 ng/ml) did not increase or potentiate the effects of epinephrine (1 μM) on glycerol release in adipose tissue explants from WT mice (*Table 6-2*).

Table 6-2 Effects of IL-6 alone, or in Combination with Epinephrine, on Glycerol Release in Epididymal Adipose Tissue Explants from Male C57BL/6J Mice Fed Chow.

Experimental Condition	Glycerol Release (μmol/gram tissue/2hours)
vehicle	1.31 ± 0.24
IL-6 (150 ng/ml)	1.45 ± 0.35
epinephrine (1 μM)	2.89 ± 0.54*
IL-6 + epinephrine	2.88 ± 0.4*

Data are means ± SEM for 5-8 explants per group. * $p<0.05$, significant effects compared to vehicle or IL-6 alone determined by 2-way ANOVA.

6.3.7 Reductions in Adiponectin mRNA Expression and Secretion in Adipose Tissue from IL-6$^{-/-}$ Mice

As seen in *Figure 6-5A*, adiponectin mRNA expression was reduced ~ 50% in adipose tissue from HFD-fed IL-6$^{-/-}$ mice concomitant with reductions in the protein content of PPARγ (*Figure 6-5B*). Despite plasma adiponectin levels being similar between genotypes (*Figure 6-5C*), adiponectin secretion *ex vivo* was reduced in eWAT from IL-6 deficient mice (*Figure 6-5D*).

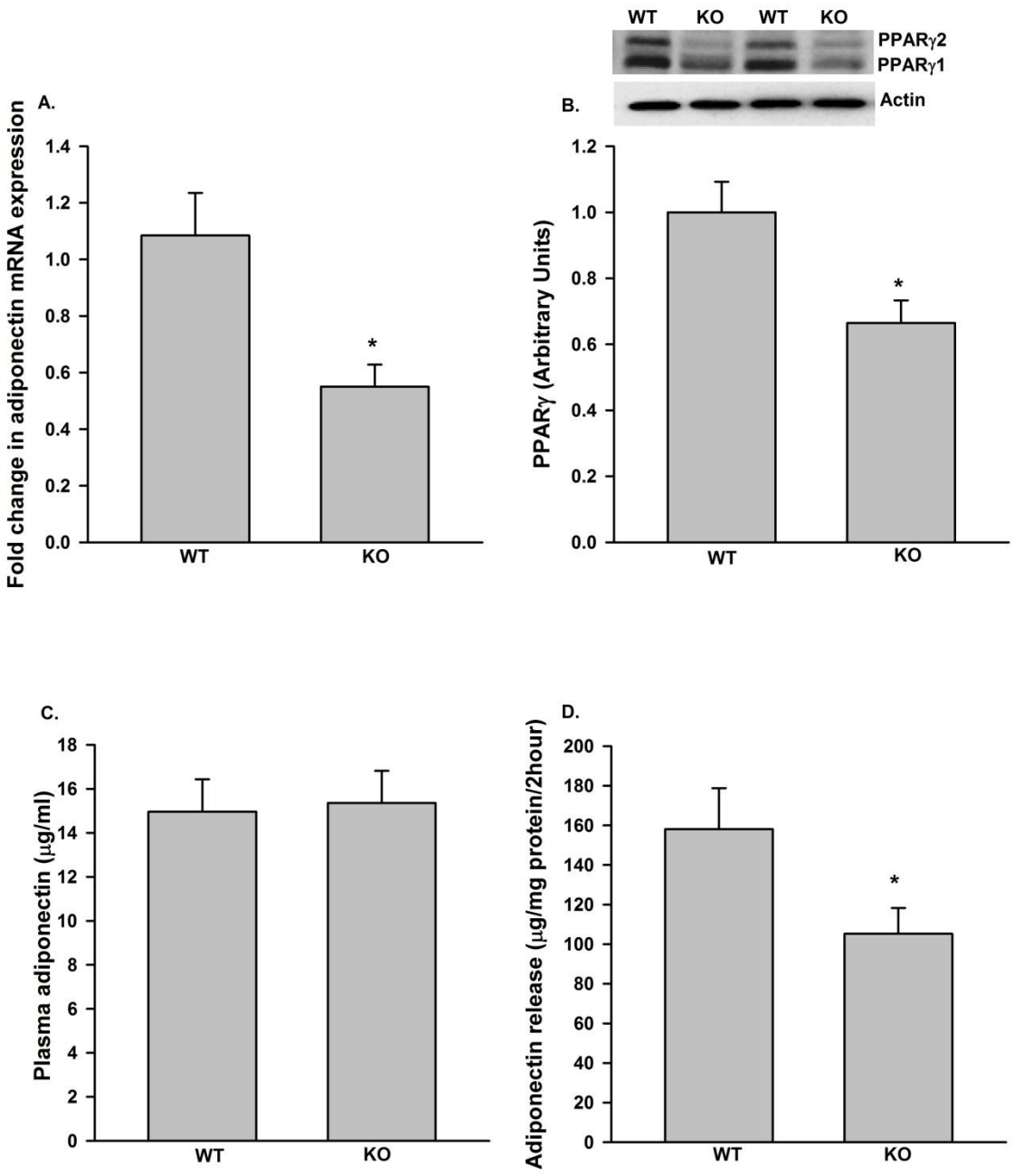

Figure 6-5 Adiponectin expression, secretion and plasma levels in WT and IL-6$^{-/-}$ mice fed a HFD. A) Adiponectin mRNA expression and B) PPARγ protein expression in epididymal adipose tissue from WT IL-6$^{-/-}$mice. C) Plasma adiponectin levels and D) adiponectin secretion from epididymal adipose tissue from WT and IL-6$^{-/-}$ mice fed a HFD. Data are presented as means + SEM for 8-10 animals per group. The mRNA data is normalized to 18S and expressed as fold differences compared to the WT group in A. Western blot images are given above the quantified data in B. * p<0.05 versus WT group.

6.3.8 Markers of Inflammation and Macrophage Infiltration in Adipose Tissue from IL-6$^{-/-}$ Mice

As shown in *Figure 6-6*, there were modest increases in markers of macrophage infiltration such as EmR1 (F4/80) in eWAT. Interestingly the expression of markers of pro-inflammatory macrophages such as CD11c was not increased, while CD206, a marker of macrophages in the M2 state was significantly increased. The inducible nitric oxide synthase (iNOS) to arginase ratio was reduced in adipose tissue from IL-6$^{-/-}$ mice.

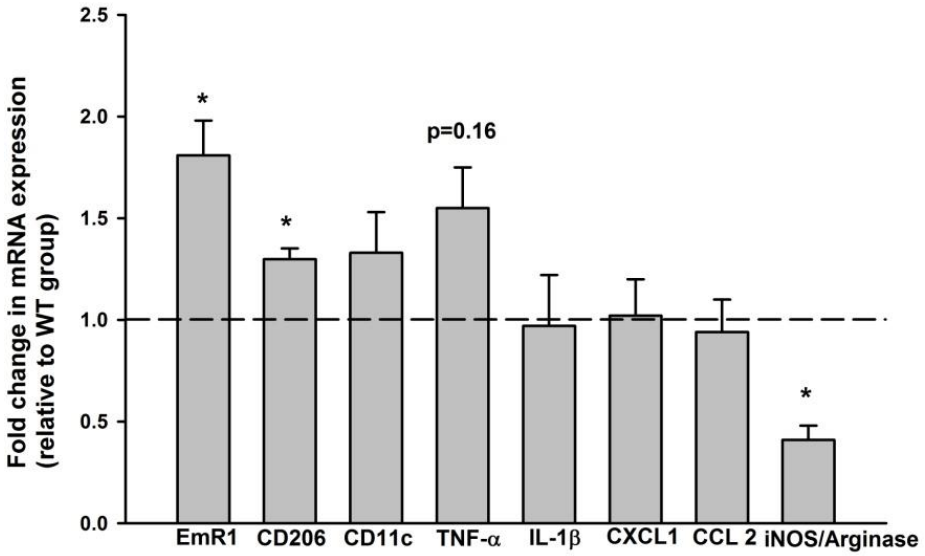

Figure 6-6 mRNA markers of inflammation in eWAT from WT and IL-6$^{-/-}$ fed a HFD. Markers of macrophage infiltration are increased while the iNOS/arginase is reduced in adipose tissue from IL-6 deficient fed a high fat diet. Data are presented as means + SEM for 8-10 animals per group. The mRNA data is normalized to beta actin and expressed as fold differences compared to the WT group. * $p<0.05$ versus WT group. (For figure 6-6, the relative mRNA expression of EmR1, CD206, CD11c, TNF-α, IL-1β, CXCL1, CCL2, iNOS and arginase in WT HFD group is 1.19 ± 0.21, 1.03 ± 0.08, 1.15 ± 0.20, 1.14 ± 0.20, 1.16 ± 0.23, 1.24 ± 0.31, 1.10 ± 0.15, 1.13 ± 0.18 and 1.31 ± 0.52, respectively.)

6.3.9 Mitochondrial Content Is Not Different in Adipose Tissue from WT and IL-6$^{-/-}$ Mice fed a HFD

The phosphorylation of AMPK, a reputed mediator of mitochondrial biogenesis in adipose tissue, was reduced in adipose tissue from IL-6$^{-/-}$ mice (*Figure 6-7A*). Despite the

reduction in AMPK, mitochondrial respiration was not altered in adipose tissue from IL-$6^{-/-}$ compared to WT mice fed a HFD as demonstrated by similar levels of respiration in response to successive additions of electrons into complex I (GM, GMD) and complex II (GMDP-S) and FCCP (*Figure 6-7B*). Data were similar whether expressed relative to tissue weight (data not shown) or total protein content. The content of mitochondrial marker proteins (*Figure 6-7C*) and mitochondrial DNA copy number (*Figure 6-7D*) were not different between genotypes.

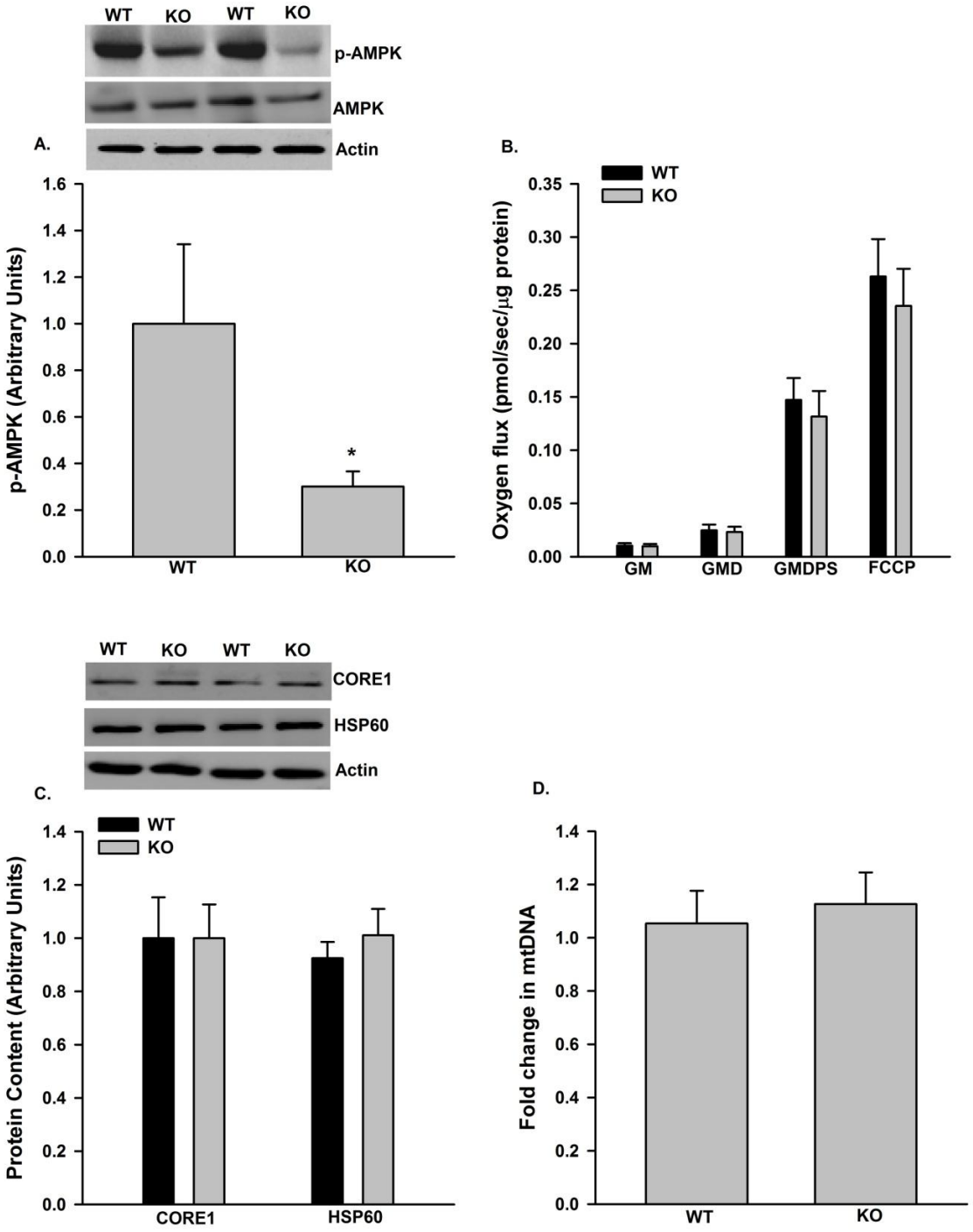

Figure 6-7 AMPK and mitochondrial content and respiration in adipose tissue from WT and IL-6$^{-/-}$ fed a HFD. A) AMPK phosphorylation, B) mitochondrial respiration, C) mitochondrial marker proteins and D) mtDNA content in epididymal adipose tissue from WT and IL-6$^{-/-}$ mice fed a HFD. Data are presented as means + SEM for 8-10 animals per group. Western blot images are given above the quantified data in A and C. Mitochondrial respiration was measured by high-resolution O_2 consumption. GM (glutamate, malate), GMD (glutamate, malate, ADP), GMDPS (glutamate, malate, ADP, pyruvate, succinate). Relative mtDNA content is normalized to genomic MCT1 DNA content and expressed as fold differences compared to the WT group in D. * $p<0.05$ versus WT group in A.

6.4 Discussion

Elevations in IL-6 have traditionally been thought of as a causal event in the development of insulin resistance. For instance IL-6 administration both *in vivo* and *in vitro* has been shown to cause insulin resistance in hepatocytes (4-5). In contrast to these studies, previous work has clearly shown that IL-6 deficient mice develop impaired glucose tolerance and insulin resistance when fed a chow diet (7). When challenged with a HFD, IL-6 deficient mice display signs of liver damage, hepatocyte inflammation and reductions in mitochondrial content in the liver (7). While these are not universal findings (24) they would suggest that a re-evaluation of the tissue specific functions of IL-6 is needed.

Adipose tissue is a key contributor to the regulation of systemic fuel metabolism through the release of fatty acids and various adipokines, such as adiponectin (10). Increases in fatty acid release and reductions in adiponectin secretion are hallmark features of insulin resistance (10). Given the surprising phenotype of IL-6 deficient mice we sought to explore the effects of systemic IL-6 deficiency on adipose tissue metabolism. In our initial experiments we examined insulin signaling, lipolysis and mitochondrial content in adipose tissue from WT and IL-$6^{-/-}$ mice fed a low fat chow diet. Surprisingly, despite the reported impaired glucose homeostasis in chow fed IL-$6^{-/-}$ mice (7-8), we found that there were no phenotypic differences in epididymal adipose between WT and IL-6 deficient mice. Given these unexpected results we reasoned that the provision of a metabolic challenge to IL-6 deficient mice, in the form of a HFD, could uncover genotypic differences in adipose tissue metabolism.

Similar to some (7), but not all (24) previous studies, we found that IL-6$^{-/-}$ mice fed a HFD were more insulin and glucose intolerant than WT mice given a HFD. The attenuation in glucose homeostasis was associated with reductions in insulin-stimulated PKB phosphorylation in eWAT *in vivo*. The activation of stress kinases such as ERK (25) and JNK (26) have been shown to inhibit insulin signaling and thus the greater activation of these kinases in adipose tissue from IL-6 deficient mice could explain the abrogated activation of PKB. These results are similar to what has been reported in liver from IL-6$^{-/-}$ mice (7).

Elevations in fatty acid levels have been causally linked to the pathogenesis of insulin resistance (27-28) and thus it was of interest to determine if fatty acid release was perturbed in adipose tissue from IL-6$^{-/-}$ mice. Similar to the attenuated insulin-mediated activation of PKB, we found that the ability of epinephrine to stimulate glycerol and FFA release and to increase the phosphorylation of HSL was blunted in epididymal adipose tissue from IL-6 deficient mice. These results suggest that increases in lipolysis do not account for the observed differences in systemic glucose homeostasis between genotypes, a finding that is consistent with previous work reporting similar plasma fatty acid levels in WT and IL-6$^{-/-}$ mice (7).

The role of IL-6 in stimulating lipolysis is controversial. While some studies have demonstrated a direct effect of IL-6 on lipolysis in adipocytes (29-30), others have shown that IL-6 infusions do not stimulate adipose tissue lipolysis in humans (31). Based on the attenuated catecholamine-induced lipolysis in IL-6$^{-/-}$ mice, it is tempting to speculate that IL-6 may play a synergistic role with epinephrine in mediating lipolysis. However, arguing against this premise we found that IL-6 treatment did not potentiate the effects of

epinephrine on lipolysis *ex vivo*. Moreover, as catecholamine mediated lipolysis was similar in adipose tissue from chow fed WT and IL-$6^{-/-}$ mice it would suggest that the blunted effects of epinephrine in adipose from IL-6 deficient mice were not the direct result of the absence of IL-6. Rather our findings would point towards an indirect role of IL-6 in mediating lipolysis under conditions of chronic nutrient excess.

Adiponectin is an adipose tissue-derived hormone, or adipokine, that possesses noted insulin sensitizing effects in a wide range of tissues including adipose (32). Moreover, recent work from Scherer's group has provided evidence that adiponectin plays a role in sensitizing adipocytes to beta-adrenergic stimulation (17). Given these findings it would seem reasonable to assume that alterations in adiponectin may account for some of the observed phenotypic differences between WT and IL-6 deficient mice. Despite reductions in adiponectin mRNA levels, PPARγ protein content and *ex vivo* adiponectin secretion in adipose tissue from IL-$6^{-/-}$ mice, plasma adiponectin levels were not different between groups. These results suggest that there are differences in the clearance/degradation of adiponectin, or an upregulation of adiponectin secretion from another fat depot in IL-$6^{-/-}$ mice. Regardless, our results would imply that reductions in adiponectin could be modulating adipose tissue beta-adrenergic responsiveness through an autocrine/paracrine mechanism, a concept that has previously been put forth by others (33).

Adiponectin increases the activity of AMPK (34), a reputed mediator of mitochondrial biogenesis in adipose tissue (12). Moreover, AICAR, which activates AMPK, can induce adiponectin mRNA expression in adipocytes (15). Similarly, it has been suggested that a disruption of mitochondrial function or decreases in mitochondrial

content lead to reductions in adiponectin synthesis (16). Collectively these findings point towards a complex interplay between adiponectin, AMPK and mitochondrial biogenesis. In our model, despite seeing reductions in the phosphorylation of AMPK, markers of mitochondrial content and respiration were not different in adipose tissue from WT and IL-6 deficient mice. These findings are somewhat surprising as they demonstrate a lack of association between not only AMPK and mitochondrial biogenesis, but mitochondrial content/function and adiponectin as well. As AICAR has been shown to induce adiponectin expression (15), it is tempting to speculate that reductions in AMPK phosphorylation could be linked to decreases in adiponectin secretion/expression. However, in adipose tissue from chow fed IL-$6^{-/-}$ mice, despite reductions in AMPK activity (35), the expression of adiponectin mRNA was not reduced. These findings demonstrate that decreases in AMPK alone are not sufficient to lead to reductions in adiponectin expression and suggest the involvement of additional, yet to be resolved mechanisms.

Emerging evidence has suggested that inflammation can decrease the activity of AMPK (36). Given this we were surprised to find only marginal alterations in markers of inflammation in adipose tissue from IL-$6^{-/-}$ mice. If anything, our results, particularly the reduction in the iNOS to arginase ratio, demonstrates a reduction in specific markers of inflammation in adipose tissue from IL-6 deficient mice. Although these findings do not fit with previous work from Febbraio's group (7) who elegantly demonstrated increases in hepatic inflammation in IL-6 knockout mice, our results are not entirely unexpected given the pronounced effect of IL-6 on the induction of pro-inflammatory processes in adipose tissue.

The current study, for the first time, clearly demonstrates phenotypic differences in adipose tissue from WT and IL-6$^{-/-}$ mice when fed a HFD. Although we cannot ascertain with certainty if these effects are directly due to a loss of IL-6 signaling, or secondary to changes in other circulating factors, they point towards a unique role for IL-6 in the regulation of adipose tissue metabolic flexibility. In contrast to mice fed a HFD we did not see differences in adipose tissue insulin signaling or beta-adrenergic responsiveness in WT or IL-6 deficient mice fed a low fat diet. As chow fed IL-6$^{-/-}$ mice from the same commercial distributor as used in the current study are insulin resistant compared to WT controls (8), this would suggest that perturbations in adipose tissue metabolism are not a causal event in the etiology of impaired glucose homeostasis in IL-6 knockout mice and likely implicates skeletal muscle and/or liver as the primary site of impairment. Although speculative, our results would further suggest that the increase in IL-6 during the development of obesity and insulin resistance (37-38) might serve as a protective mechanism in the maintenance of adipose tissue metabolism.

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Chapter 7 General Discussion

7.1 Summary of Main Findings

The aim of this thesis is 1) to understand exercise and the role of hormonal factors (epinephrine and IL-6) that mediate the regulation of pyruvate dehydrogenase kinase 4 (PDK4) and phosphoenolpyruvate carboxykinase (PEPCK) gene expression in rodent white adipose tissue (WAT) and the potential signalling pathways therein; and 2) to understand the metabolic action of IL-6 on rodent WAT metabolism by utilizing IL-$6^{-/-}$ mice under both chow and a high fat diet (HFD) conditions.

First of all, we determined if an acute bout of exercise and epinephrine treatment can induce PDK4 mRNA expression in epididymal adipose tissue (eWAT) from lean rats and explored the potential signalling pathways that might be involved in. We found that exercise, *in* or *ex vivo* epinephrine treatment increased PDK4 mRNA and p38 mitogen-activated protein kinase (MAPK) and peroxisome proliferator-activated receptor gamma (PPARγ) could be involved in epinephrine-mediated regulation of PDK4 mRNA in eWAT from lean and insulin sensitive rats. Whereas, 5' AMP activated protein kinase (AMPK) agonists including 5-aminoimidazole-4-carboxyamide ribonucleoside (AICAR) and metformin decreased PDK4 mRNA expression in cultured eWAT.

Second, we determined the regulation of PDK4 mRNA expression in eWAT from obese and insulin resistant rats by an acute bout of exercise and acute increases in epinephrine *in vivo*. We demonstrated that the abilities of exercise and epinephrine to induce PDK4 mRNA were maintained in eWAT from obese and insulin resistant rats,

while the pathways mediating the effects of epinephrine on PDK4 mRNA in obese rats may be distinct from those in lean animals.

Third, we determined the effects of *ex vivo* IL-6 treatment on PEPCK and PDK4 expression in cultured mouse eWAT, and the effects of an acute bout of treadmill running on IL-6 signaling and PEPCK and PDK4 mRNA expression in mouse eWAT. We also elucidated the role of IL-6 in modulating the effects of exercise on PEPCK and PDK4 mRNA expression and the putative signaling pathways therein by utilizing an IL-$6^{-/-}$ mouse model. We found that *ex vivo* IL-6 treatment decreased PEPCK and PDK4 mRNA and protein expression, and increased the phosphorylation of AMPK, acetyl coA carboxylase (ACC) and signal transducer and activator of transcription 3 (STAT3) and induced suppressor of cytokine signaling 3 (SOCS3) mRNA expression. The activation of AMPK by IL-6 was independent of increases in lipolysis. IL-6 signaling was not activated immediately following an acute bout of treadmill running (15 meters/min, 5% incline, 90 mins). Exercise-induced increases in PEPCK and PDK4 mRNA were attenuated in mouse eWAT from IL-$6^{-/-}$ mice.

Finally, we characterized the adipose tissue phenotypes between age matched wild type (WT) and IL-$6^{-/-}$ mice under both chow and HFD (10 weeks) conditions. In IL-$6^{-/-}$ mice fed a normal chow diet, there were no differences in eWAT insulin action, beta-adrenergic responsiveness or mitochondrial content/function compared to WT controls. IL-$6^{-/-}$ mice fed a HFD, were more glucose and insulin intolerant than WT controls fed the same diet and this was mirrored by reductions in the insulin-stimulated activation of protein kinase B (PKB) and increases in the phosphorylation of extracellular signal regulated kinases (ERK) and c-Jun N-terminal kinases (JNK) in eWAT. Epinephrine

stimulated lipolysis and hormone sensitive lipase (HSL) phosphorylation were blunted in eWAT from IL-6$^{-/-}$ mice. These changes were associated with decreased mRNA expression and secretion of adiponectin and AMPK phosphorylation. However, mitochondrial content and respiration in eWAT were similar between genotypes under HFD challenge.

In this chapter, the results we have obtained will be discussed and put into a broader perspective and important issues for future research will be addressed. First, methodological considerations for the specific models that I used will be discussed.

7.2 Methodological Considerations

7.2.1 Adipose tissue organ culture

One of the main techniques used in this thesis was adipose tissue organ culture (ATOC). Compared with primary adipocyte culture or *in vitro* cell lines, it has advantages and disadvantages. The major advantages of ATOC is 1) the paracrine and/or autocrine interactions between adipocytes and other cell types in adipose tissue remain intact and more representative of what is seen *in vivo* compared to isolated adipocyte preparations; and 2) depot-specific differences in adipose tissue remain during the culture procedure (1). The primary disadvantage of using ATOC is that it can't be determined whether the observed effects are owing to direct effects on adipocytes *per se* or on other types of cells resident within the adipose tissue.

Primary culture of adipocytes could be a complementary approach to differentiate whether the effect is on adipocytes themsleves. However, it has been reported that the isolation of adipocytes could result in the induction of the inflammatory response and

decrease key adipose-specific genes such as glucose transporter type 4 (GLUT4) and PPARγ (2). Isolation of adipocytes is labor intensive (1), and the crosstalk between adipocytes and nonadipose cells within adipose tissue is lost. Meanwhile, the primary culture of adipocytes is unable to completely recapitulate the whole adipocyte population since it is limited by the specific selected adipocytes that are recovered. For instance, owing to the fragile nature of adipocytes, the larger fat cells are particularly easy to be disrupted and lost during the isolation procedure (3).

7.2.2 Selection of adipose depots

As has been detailed in chapter 1, there are fundamental differences among different adipose depots with regards to their influence on adipose tissue metabolism. An increased delivery of non-esterified fatty acids (NEFAs) from visceral adipose tissue (VAT) to the liver via the portal vein, can cause liver insulin resistance, therefore visceral fat overaccumulation has a more negative impact on health than subcutaneous fat (4). Omental-mesenteric, gonadal and retroperitoneal adipose tissues are considered to be representative of VAT and are most commonly used in rodent models of metabolic studies. Omental-mesenteric fat drains into the portal vein directly (5), however, the isolation and acquisition of mesenteric adipose tissue is difficult (6). Although epididymal adipose tissue drains into the systemic circulation (7), it has been demonstrated that the removal of gonadal, but not mesenteric or retroperitoneal fat, improves insulin action and glucose tolerance in mice (8). Consequently, this thesis focused on epididymal adipose tissue.

7.2.3 Exercise models

7.2.3.1 Swimming

One of the major differences between swimming and running is their distinct differences in the sympathoadrenal function (9) with swimming inducing greater increase in circulating catecholamine levels (9-10). The major advantages of swimming include 1) less mechanical stress and/or damage (11); and 2) cost-effective as swimming requires less expensive apparatus than treadmill running. The main disadvantages of swimming are that the exercise intensity can't be quantitatively measured and accordingly, the applicability of the findings to exercise prescription in a clinical setting is limited. Meanwhile, researchers must be extremely alert to make sure animals swim continuously.

The water temperature is also an important factor for influencing swimming performance. If the water temperature is greater than 42 ℃ or lower than 20 ℃, the exercise performance will be greatly affected (12). Most swimming studies are conducted at a water temperature between 32 and 36 ℃ (13-16).

7.2.3.2 Treadmill running

The main advantage of treadmill running is that researchers can precisely manipulate the duration and the intensity of exercise and thus can keep all the mice running at the same level in the study. The major disadvantage of using treadmill running is the use of external stimuli to keep animals running, such as continuous tail shock. It has been reported that shock can elicit significant increases in both plasma norepinephrine and epinephrine levels (9). Meanwhile, treadmill training requires a specialized device, which is more expensive than that required for swimming. Like

swimming, researchers must also be vigilant to make sure all the animals finish the designed exercise protocol.

7.2.3.3 Gender differences in response to exercise

There are gender differences in response to exercise in rodent models. For instance, in contrast to male rats, female rats lose little or no weight during intensive training (17). Female mice of C57BL/6J strain have increased treadmill running ability and greater capacity to increase their cardiac mass when challenged with similar amounts of exercise compared with their male counterparts (18). From a mechanistic perspective, in skeletal muscle, estrogen has been shown to activate AMPK (19) and affect genes involved in glucose and lipid metabolism such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), PPARα, PPARβ/δ, carnitine palmitoyltransferase I (CPT1), sterol regulatory element-binding protein-1c (SREBP-1c) and GLUT4 (20). In adipose tissue, estradiol supplementation has been reported to affect adipose triglyceride lipase (ATGL) and perilipin expression in ovariectomized mice (21). Since AMPK, PGC-1α and lipolytic signaling pathways are, or are closely related, to the endpoints of this thesis research, we focused on male rodents.

7.2.4 IL-6$^{-/-}$ mouse model

To elucidate the role of IL-6 in modulating the effects of exercise on PEPCK and PDK4 mRNA expression and in regulating adipose tissue metabolism under HFD, we utilized mice with a global deletion of IL-6. Both IL-6$^{-/-}$ mice (B6.12952-IL6$^{tmlkopf/}$J) and their age matched WT mice (C57BL/6J) were purchased from the Jackson Laboratory. The IL-6 targeted mutation was originally created by Kopf et al (22). The Jackson

Laboratory mice database provides the following information for the IL-$6^{-/-}$ mice used in this thesis:

"……a targeting vector was designed to place a neomycin resistance cassette into the first coding exon (exon 2) of the targeted gene. This construct was electroporated into 129S2/SvPas-derived D3 embryonic stem (ES) cells. Correctly targeted ES cells were injected into C57BL/6J blastocysts and the resulting mice were sent to The Jackson Laboratory where the mouse line was bred to C57BL/6J (Stock No. 00664) mice for 11 generations".

It has been reported that 129 background genes can be reduced to 0.1% of all genes if back-crossing 10 generations (23). Thus, IL-$6^{-/-}$ and WT mice utilized in the current thesis have an almost identical strain background, i.e. C57BL/6J.

It should be mentioned that there are also some limitations for using an IL-$6^{-/-}$ mouse model. Like other transgenic or knockout mice, the interpretation of results generated from IL-$6^{-/-}$ mouse model should be viewed cautiously. The total absence of any factor may result in compensatory adaptations and alterations during development (24).

This thesis aimed to investigate the effects of IL-6 on adipose tissue metabolism. A systemic deletion of IL-6 might be unable to differentiate whether the observed effects are on adipose tissue *per se* or compensatory effects in other organs. Future studies via adipose-tissue-specific IL-6 knockout or transgenic mice may shed more light on the effects of IL-6 on adipose tissue metabolism.

7.2.5 HFD

Over the past several decades, multiple rodent models of insulin resistance have been utilized in type 2 diabetes (T2DM) related research. These mainly include 1) genetic rodent models such as Zucker Diabetic Fatty (ZDF) rats, Otsuka Long Evans Tokushima Fatty (OLETF) rats, *ob/ob* and *db/db* mice; and 2) diet induced obese (DIO) rats and mice. Diabetes develops spontaneously in male ZDF rats at ages 8-10 weeks owing to leptin receptor mutation (25). OLETF rats develop impaired glucose regulation at ~ 8 weeks of age and progress to diabetes by ~24 weeks of age (26) due to a spontaneous lack of cholecystokinin-1 receptor (27). Mice lacking the functional leptin (*ob/ob*) or leptin receptors (*db/db*) develop diabetes at a relatively early age ~10 weeks (28-29). Although these genetic rodent models are useful in exploring the molecular mechanisms in the pathogenesis of T2DM, the primary limitation is that they are unable to accurately model human situations as diabetes and T2DM in modern society is induced mainly by environmental manipulation (30). Alternatively, high fat feeding-induced obese rodent models may more closely mirror the development of obesity and insulin resistance in humans.

To determine if an acute bout of exercise and acute increases in epinephrine could induce PDK4 mRNA expression in adipose tissue from insulin resistant rats, male Wistar rats weighing 200 g were fed a chow or a high fat diet *ad libitum* for 6 weeks. Previous findings from our lab had shown that 6 weeks of HFD in rats resulted in abdominal obesity, fasting hyperinsulinemia, adipose tissue insulin resistance, and decreased adipose tissue mitochondrial content (31), thus it served as a suitable diet induced insulin resistant model for *objective 2*.

The C57Bl/6J mouse strain is an obesity prone mouse line (32). HFD induced obesity in C57BL/6J mouse strain was first described by Surwit et al. (33) in 1988. When challenged with a HFD, C57BL/6J mice exhibit severe obesity, hyperglycemia, hyperinsulinemia, hypertension, progressive peripheral and then central leptin resistance (33-34). In contrast, C57BL/6J mice remain euglycemic and lean on low fat, chow diets (35). DIO in the C57BL/6J genetic background has been widely used for studying the mechanisms underlying the progression or resistance to T2DM (36-37). Meanwhile, HFD is commonly used in transgenic mouse models to uncover the importance of specific genes in the etiology of insulin resistance (38).

It should be noted, however, that there are some limitations to HFD-induced obesity in the current study: First, the high percentage of calories from fat (60% calories from fat) and the fatty acid profile in the diet (fat mainly from lard) is rarely seen in most populations. Second, not all of the phenotypes induced by HFD in C57BL/6J mice mimic the situations in humans. For instance, HFD resulted in an increase in plasma high density lipoprotein-cholesterol (HDL-C) in almost all mouse strains (39), whereas in humans one of the hallmarks of the metabolic syndrome is reduced levels in HDL-C (40). Thus, further studies are required to develop diets that will more closely represent most western diet: a diet with less energy from fat (20-35% from fat) (41), as well as a diet enriched in mono-unsaturated fatty acids and poly-unsaturated fatty acids (42).

7.3 Exercise on Adipose Tissue Metabolism

Exercise is a fundamental tool in the management of T2DM. However, the biochemical and molecular mechanisms underlying the beneficial effects of exercise have remained elusive. Thiazolidinediones (TZDs) are a commonly prescribed medication

used in the treatment of T2DM. A large portion of TZD's beneficial effects on insulin sensitivity is through its upregulation on PEPCK and PDK4 activity in adipose tissue, resulting in increased fatty acid re-esterification through the glyceroneogenic pathway. This will consequently prevent ectopic lipid deposition (43-44). While effective, these drugs cause a wide range of side effects (45) and thus the identification of alternative approaches that mimic the beneficial effects of these compounds on adipose tissue function is needed. Within this broad context, a central effort of this thesis has been exploring the hormonal factors, underlying biochemical/cellular mechanisms, mediating the effects of exercise on glyceroneogenic enzymes in adipose tissue.

In this thesis, both swimming exercise in rats and treadmill running in mice led to rapid increase in PDK4 mRNA. Similarly, we also observed increased PEPCK mRNA expression in mice immediately after exercise. The exercise-induced PDK4 and PEPCK mRNA expression in rodent white adipose tissue not only further demonstrates the plasticity of adipose tissue in response to exercise (16), but also demonstrates that exercise, similar to TZDs, is a potent stimuli which can be used to induce adipose tissue glyceroneogenic enzymes.

The rate of fatty acid re-esterification is proportional to the rate of lipolysis (46-47). Exercise is a robust activator of lipolysis in adipose tissue (48-49). Thus, it is reasonable to assume that exercise would induce PDK4 and PEPCK mRNA expression, presumably favoring the glyceroneogenic pathway for fatty acid re-esterification. It should be mentioned that, during exercise, although the relative rate of fatty acid re-esterification remains constant (~30-40%), the absolute amount of fatty acids that are re-esterified back to triglycerides (TG) is greatly enhanced owing to exercise-induced

lipolysis. For instance, Wolfe et al. (50) quantified the TG-fatty acid cycling in humans during 4 hour walking. The TG-fatty acid cycling in adipocytes increased from 1.3 μmol/kg/min at rest to 3 μmol/kg/min throughout the exercise (50). Similarly, the whole body TG-fatty acid cycling is about 4 times higher in athletes than untrained volunteers (51). This was also supported by *ex vivo* and *in vivo* evidence from rodent studies. Treatment of cultured epididymal adipose tissue with norepinephrine *ex vivo* led to about 2 fold increase in TG-fatty acid cycling (47). Likewise, epinephrine infusion in rats resulted in about 2 fold increase in the glyceroneogenic pathways for fatty acid re-esterification in adipose tissue (52).

7.4 IL-6 and Adipose Tissue Metabolism

To date the role of IL-6 in mediating adipose tissue metabolism is still under debate (53). Although evidence from *in vitro* studies suggest that IL-6 causes insulin resistance via decreasing adiponectin mRNA expression and negatively affecting insulin signaling pathways (54-56), there is also evidence that IL-6 may favorably regulate adipose tissue function. For instance, IL-6 activates AMPK in adipocytes (57), a central player in remodeling adipose tissue metabolism (58). This thesis was interested in the regulation of IL-6 on adipose tissue metabolism under two conditions of metabolic stress: an acute bout of exercise and chronic nutrient excess.

We found that IL-6 treatment in adipose tissue *ex vivo* activated AMPK signaling pathways independently of lipolysis. AMPK has been reported to regulate mitochondrial biogenesis in fat cells (59). It has also been suggested that AMPK controls the expression of adiponectin either directly (60) or secondary to increases in adipose tissue mitochondrial biogenesis (61). Adiponectin is intimately involved in mediating adipose

tissue metabolism through its auto/paracrine actions on adipose tissue (62-63). Interstitial concentrations of IL-6 surrounding adipose tissue are orders of magnitude greater than levels in the circulation (64). Collectively, our findings suggested that IL-6 plays a pivotal role in maintaining adipose tissue function.

We also found that IL-6 plays an indirect role in mediating the effects of exercise on the induction of glyceroneogenic enzymes. During exercise, a wide range of myokines, hepatokines and adipokines are secreted from skeletal muscle, liver and adipose tissue, such as myostatin, IL-7, leukemia inhibitory factor, follistatin and irisin (65-67). A growing body of evidence has also demonstrated the existence of complex tissue to tissue communication during exercise at least partly through myokines, adipokines and hepatokines (66, 68). For instance it has recently been shown that exercise stimulates the secretion of chemokine (C-X-C motif) ligand 1 (CXCL1) from the liver through a muscle derived-IL-6 dependent mechanism (68). The current findings argue a potential role of IL-6 in mediating adipose tissue metabolism during exercise indirectly through its interactions with other organs or myokines, adipokines and hepatokines such as CXCL-1.

We further demonstrated that under conditions of calorie excess, the ablation of endogenous IL-6 leads to marked alterations in adipose tissue metabolism. The dysregulated phenotypes mainly include attenuated sensitivity to both insulin and catecholamines, as well as decreased adiponectin mRNA expression and secretion in adipose tissue. These findings further point towards a unique role of IL-6 in modulating adipose tissue insulin signaling, beta adrenergic responsiveness and adiponectin secretion. Thus, it is reasonable to assume that the increased secretion of IL-6 from adipose tissue during the development of obesity is a protective mechanism for the maintenance of

adipose tissue function. We propose that at least part of the salutary effects of IL-6 on adipose tissue metabolism is through its action on the AMPK signaling pathway. Meanwhile, similar to the role that IL-6 is playing in exercise induced glyceroneogenic enzymes, it is likely that the effects of IL-6 on adipose tissue action is through its interactions with other organs or other hormonal factors such as glucagon-like peptide-1 (GLP-1) (69). For instance, it has been reported that elevated systemic IL-6 could stimulate GLP-1 secretion from intestinal L cells and pancreatic alpha cells, resulting in improved insulin secretion and glycemia (69).

In summary, this thesis proposes that IL-6 is essential for the metabolic homeostasis in adipose tissue under conditions of metabolic stress such as exercise and chronic nutrient excess. Future studies are required to further elucidate the mechanisms for the beneficial effects of IL-6 on adipose tissue metabolism.

7.5 Relevance of the Current Findings to Human Health Research

A large portion of TZD's beneficial effects on insulin sensitivity is through their up-regulation of PEPCK and PDK4 activity in adipose tissue. Currently, there are reservations about the use of TZDs because they are associated with multiple unwanted side effects, such as worsening of the coronary heart diseases, increasing body weight and fluid retention (45). In the current thesis, we found that an acute bout of exercise can induce PEPCK and PDK4 mRNA in epididymal adipose tissue from rodents, consequently resulting in increased fatty acid re-esterification in adipose tissue. This is in consistent with the findings by Wolfe et al. (50) who reported increased TG-fatty acid cycling in humans during 4 hours of walking and suggests that rodent models recapitulate

human physiology to the extent that they are useful models for future studies in this area of research. Future studies are required to elucidate 1) if exercise can also induce PEPCK and PDK4 mRNA in adipose tissue from lean and obese human subjects and 2) if depot-specific and gender-specific responses exist for exercise induced glyceroneogenic enzymes. It is noteworthy that gender-specific responses to exercise have been reported in human population studies. For instance, in regards to the normal cardiac response to exercise, men had a higher systolic blood pressure compared to women (70). Females oxidize more fat and less carbohydrate and amino acids during endurance exercise compared to males, and this was at least partially owing to the higher levels of 17-β-estradiol in females (71). Of relevance to this thesis, women have a higher percentage of body fat in the gluteal-femoral region, in contrast, men have more body fat in the abdominal region (72). Using stable isotope methods, it has been reported that the rate of glycerol appearance was higher in females compared to male during submaximal cycling exercise (73). A gender difference in sympathetic nervous system activity during exercise has also been reported with males having a greater increase in plasma epinephrine and norepinephrine levels (74). Through the utilization of in-situ microdialysis it has been shown that women have a decreased α2-mediated antilipolysis (75) and increased β-adrenergic mediated lipolysis (76) in abdominal subcutaneous tissue compared to men.

The current thesis also suggested that, in mice, IL-6 is essential for the maintenance of adipose tissue function (mainly the responses to both catecholamines and insulin, as well as adiponectin mRNA expression and secretion) under chronic nutrient excess. Similarly, the lack of functional IL-6 in patients with rheumatoid arthritis through IL-6 receptor-inhibiting monoclonal antibody leads to increased plasma lipid and glucose

levels, arguing against IL-6 as an insulin resistance causing agent in humans *in vivo* (77-78). Further studies are required to re-evaluate the effects of IL-6 on adipose tissue metabolism in human population studies during the development of insulin resistance.

7.6 Future Directions

The studies in this thesis support 1) an increasingly recognized role that adipose tissue is playing in mediating exercise-induced health benefits and 2) a unique role of IL-6 in modulating adipose tissue metabolism under conditions of metabolic stress. Although this thesis has contributed significantly to our understanding of exercise-mediated adipose tissue metabolism and in elucidating the role of IL-6 in the etiology of metabolic derangements in adipose tissue, some important questions are still unanswered:

1) Whether IL-6 signaling is activated post-exercise and how IL-6 is involved in regulating glucose and lipid metabolism in adipose tissue and interacts with other tissues during the post-exercise period?

2) How exercise-induced myokines, hepatokines and adipokines communicate with each other and what are their physiological roles in exercise mediated whole body energy metabolism?

3) Besides epinephrine and IL-6, how are the other hormonal factors such as glucagon involved in mediating exercise induced adipose tissue metabolism?

4) For the observed phenotype in adipose tissue of IL-$6^{-/-}$ mice under HFD from this thesis, what is the causal event that leads to metabolic derangements in adipose tissue? What is the role of IL-6 in the etiology of adipose tissue and systemic insulin resistance? If increased IL-6 in adipose tissue is protective, does it act on adipose tissue metabolism directly or through its interactions with other organs or other hormonal factors?

5) Broadly, what messages can be obtained from the first three studies for the personalized exercise prescriptions in the prevention and/or treatment of obesity and T2DM over the long term? What information can be taken from the last study for the therapeutic treatment of obesity related disorders considering the enigmatic roles that IL-6 is playing systematically?

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