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Regulation of PGC-1 alpha in White Adipose Tissue by Exercise

by

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ABSTRACT

This project investigated the effects of exercise and epinephrine on the mRNA expression of peroxisome proliferator activated receptor gamma coactivator-1 alpha (PGC-1α), a master regulator of mitochondrial biogenesis, in rat adipose tissue. Rats that swam 2 hours daily for 4 weeks had increased mitochondrial marker proteins and PGC-1α mRNA expression in epididymal and retroperitoneal adipose tissue ($p<0.05$). Adipose tissue organ culture treatment with epinephrine increased ($p<0.05$) PGC-1α mRNA expression in both depots, but only epididymal adipose responded to a supra-physiological dose. Beta blockade attenuated the effects of an acute bout of exercise on PGC-1α mRNA expression in epididymal, but not in retroperitoneal adipose tissue. This is the first study to demonstrate that rat white adipose tissue PGC-1α mRNA expression is increased by acute and chronic exercise and epinephrine. Increases in circulating catecholamine levels might be one potential mechanism mediating exercise induced increases in PGC-1α mRNA expression in rat abdominal adipose tissue.

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Acetyl CoA, acetyl coenzyme A

AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside

ALAS, δ-aminolevulinate synthase

AMP, adenosine monophosphate

AMPK, 5'-adenosine monophosphate-activated protein kinase

ANOVA, analysis of variance

ATOC, adipose tissue organ culture

ATP, adenosine triphosphate

BCA, bicinchoninic acid

β-GPA, β-guanidinopropionic acid

CAMK, calcium/calmodulin-dependent protein kinase

cAMP, cyclic adenosine monophosphate

CCAC, Canadian Council on Animal Care

CCO, cytochrome c oxidase

cDNA, complimentary deoxyribonucleic acid

CNTF, ciliary neurotrophic factor

CoA-SH, coenzyme A – thiol group

CORE1, ubiquinone-cytochrome c oxidoreductase subunit I

COX, cytochrome c oxidase

COXI, cytochrome c oxidase subunit I

COXIV, cytochrome c oxidase subunit IV

CT, cycle threshold

CV, coefficient of variance

DNA, deoxyribonucleic acid

DN-AMPK, dominant negative 5’-adenosine monophosphate-activated protein kinase

dNTP, deoxyribonucleotide triphosphate

DNTP, 5,5’-dithiobis-(2-nitrobenzoic acid)

ECL, enhanced chemiluminescence

FA, fatty acid

FFA, free fatty acid

GLUT4, glucose transporter 4

HSL, hormone sensitive lipase

IL-6, interleukin-6

I.P., intraperitoneal

IRS, insulin receptor substrate

KO, knock-out

LSD, least significant difference

M199, medium 199

MDH, malate dehydrogenase

MKK3E/6E, mitogen-activated protein kinase kinase 3E or 6E

mRNA, messenger ribonucleic acid

NRF-1, nuclear respiratory factor -1

NRF-2, nuclear respiratory factor -2

OLETF, Otsuka Long-Evans Tokushima Fatty

Oligo(dT), oligodeoxythymidylic acid

p160 mbp, p160 myb binding protein

p38 MAPK, p38 mitogen-activated protein kinase

PBS, phosphate-buffered saline

PDK4, pyruvate dehydrogenase complex kinase 4

PEPCK, phosphoenolpyruvate carboxykinase

PGC-1α, peroxisome proliferator activated receptor-1 alpha

PGC-1β, peroxisome proliferator activated receptor-1 beta

PI3-K, phosphatidylinositol-3-kinase

PPAR, peroxisome proliferator activated receptor

RNA, ribonucleic acid

RT-PCR, reverse transcriptase – polymerase chain reaction

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

SE, standard error

siRNA, small interfering ribonucleic acid

TBST, tris buffered saline/0.01% tween

Tfam, mitochondrial transcription factor A

TFB-1M, mitochondrial transcription factor B -1

TFB-2M, mitochondrial transcription factor B -2

TNB, 5-thio-2-nitrobenzoic acid

TZD, thiazolidinedione

w/w, weight per weight

v/v, volume per volume

Chapter 1. Study Rationale

1.1 RATIONALE

The mechanisms involved in PGC-1α regulation of mitochondrial biogenesis in skeletal muscle and the various activators of this pathway have been studied extensively (Winder *et al.*, 2006; Wright, 2007). Despite the fact that adipose tissue mitochondria are involved in the regulation of whole body metabolism (Wilson-Fritch *et al.*, 2004; Choo *et al.*, 2006; Koh *et al.*, 2007), there is very little known about the regulation of adipose tissue mitochondrial biogenesis and whether PGC-1α, a putative mediator of mitochondrial biogenesis, has a central role in this pathway. One previous report showed that mitochondrial enzyme activity is increased in the white adipose tissue of rats that had undergone a 10 week swim training protocol (Stallknecht *et al.*, 1991), but no work has been done to determine what mechanisms are regulating these changes in markers of mitochondrial biogenesis. Therefore, further research is required to determine what impact exercise has on adipose tissue mitochondria and what pathways are mediating any observed adaptations. To determine the effect of exercise on adipose tissue mitochondrial protein content and gene expression, a two hour per day, four week long swim protocol was used in male Wistar rats and the control group underwent a two minute per day sham swim, as described by Stallknecht et al. (1991). From the exercise study, adipose tissue mitochondrial marker proteins and enzymes were analyzed in addition to the potential regulators of the exercise-induced mitochondrial changes, such as PGC-1α and mitochondrial transcription factor A (Tfam). Furthermore, as circulating catecholamines are elevated during exercise (Gollnick *et al.*, 1970; Galbo *et al.*, 1977; Shah *et al.*, 1984; Stallknecht, 2004), the potential role of epinephrine in activating PGC-1α and mitochondrial biogenesis was of interest. The use of β-blockade during an acute bout of exercise (Miura *et al.*, 2007), in addition to *ex vivo* treatment of adipose tissue organ cultures with epinephrine, allowed for the role of epinephrine to be

determined. Therefore, through the use of both *in vivo* and *ex vivo* approaches, it was determined what the impact of both exercise and epinephrine had on adipose tissue PGC-1α mRNA expression in relation to changes in markers of mitochondrial biogenesis.

1.2 OBJECTIVES AND HYPOTHESES

The objective of this project was to explore the effects of exercise and epinephrine on PGC-1α mRNA expression, a master regulator of mitochondrial biogenesis, in rat abdominal adipose tissue.

1.2.1 Objective 1:

To confirm that exercise training results in an increase in markers of adipose tissue mitochondrial biogenesis and to determine a potential mediator of these mitochondrial adaptations.

Hypothesis 1:

Exercise training would increase PGC-1α mRNA expression, a reputed mediator of mitochondrial biogenesis, in association with increases in mitochondrial marker proteins, ubiquinone-cytochrome c oxidoreductase (CORE) 1 and cytochrome c oxidase (COX) IV, and the activity of citrate synthase.

1.2.2 Objective 2:

To determine whether epinephrine increases PGC-1α mRNA expression. As epinephrine is increased during exercise and is involved in other key pathways of lipid metabolism, we proposed to measure changes in PGC-1α mRNA expression following epinephrine treatment *ex vivo*.

Hypothesis 2:

Treatment of adipose tissue organ cultures with epinephrine would result in an increase in PGC-1α mRNA expression.

1.2.3 Objective 3:

To determine whether epinephrine regulates PGC-1α gene expression during exercise.

Hypothesis 3:

The effect of exercise on PGC-1α mRNA expression in white adipose tissue will be attenuated by treatment with a β-blocker prior to an acute bout of exercise.

1.3 CHAPTER FORMAT

1.3.1 Chapter 2:

Chapter 2 is a general introduction to the study of adipose tissue mitochondrial biogenesis and gives a review of the literature. The regulation of skeletal muscle adaptations to exercise and the impact of exercise and changes in PGC-1α on skeletal muscle mitochondrial biogenesis will be discussed extensively as there is very little known about the effect of exercise on adipose tissue mitochondria. The role of adipose tissue in whole body metabolism and the impact that adipose tissue stores have on health and disease will also be outlined.

1.3.2 Chapter 3:

Chapter 3 contains the bulk of the thesis research that was recently published in *The Journal of Physiology* (Sutherland LN, Bomhof MR, Capozzi LC, Basaraba SA, Wright DC. (2009). Exercise and adrenaline increase PGC-1α mRNA expression in rat adipose tissue. *J Physiol* **587**(7):1607-17). The study of exercise-induced increases in markers of mitochondrial biogenesis and PGC-1α, as well as the effect of epinephrine on PGC-1α gene expression is presented.

1.3.3 Chapter 4:

Chapter 4 is a general discussion relating the findings of the research in Chapter 3 to what was previously known in skeletal muscle and what future directions this area of research should take.

1.3.4 Appendices:

The appendices provide additional information for the methods required to carry out the experiments for Chapter 3. In addition, some of the findings from the studies within Chapter 3 were not published in the paper and are provided in the appendix for future reference and interest to give a more thorough description of the findings.

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Chapter 2. Literature Review

2.1 PGC-1 FUNCTION AND REGULATION

Peroxisome proliferator activated receptor gamma coactivator-1 (PGC-1) is a family of transcriptional coactivators found abundantly in energy demanding tissues such as the heart, skeletal muscle, brown adipose tissue, kidney, liver, and the brain (Larrouy *et al.*, 1999; Eddy & Storey, 2003). PGC-1 was first identified and shown to be elevated in mouse skeletal muscle and brown adipose tissue in response to cold exposure (Puigserver *et al.*, 1998). Since this discovery, PGC-1 has been shown to regulate several transcription factors that are involved in energy metabolism and adaptive thermogenesis through changes in mitochondrial biogenesis, respiration, and fatty acid oxidation (Puigserver *et al.*, 1998; Butow & Bahassi, 1999; Wu *et al.*, 1999; Vega *et al.*, 2000). Changes in PGC-1 mRNA expression have been shown to result from a number of biological and physiological triggers such as cold stress, exercise, increased 5'-AMP-activated protein kinase (AMPK) activation and calcium levels, altered diet, and many others. Interestingly, the regulation and activation of PGC-1 and its targets has been shown to exhibit tissue specificity and as a result, a large body of research surrounds the PGC-1 axis.

PGC-1α functions as the 'master regulator' of mitochondrial biogenesis, as PGC-1α has been shown to induce mitochondrial biogenesis in skeletal (Baar *et al.*, 2002) and heart muscle (Lehman *et al.*, 2000) and brown (Puigserver *et al.*, 1998) and white adipose tissue (Tiraby *et al.*, 2003). Mitochondrial biogenesis involves the coordination of both the nuclear and mitochondrial genomes for the transcription of genes and translation of mRNA into the appropriate mitochondrial proteins to form the multi-subunit respiratory enzyme complexes within the mitochondria. Mitochondrial biogenesis is not only a favourable response to endurance exercise for athletes, but it also has great clinical relevance for the treatment or diagnosis of a variety of human diseases, including Type 2

Diabetes (Chabi *et al.*, 2005; Roden, 2005; Benton *et al.*, 2008). A key component of the ability of PGC-1 overexpression to result in an increase in mitochondrial biogenesis is through the induction and co-activation of nuclear respiratory factors (NRF)-1 and NRF-2. NRF-1 and NRF-2 protein binding sites have been identified on the promoters of several nuclear genes that are required for mitochondrial respiration, such as cytochrome c and most of the nuclear subunits of the cytochrome c oxidase (COX) complexes I-V (Evans & Scarpulla, 1990; Bachman *et al.*, 1996; Scarpulla, 1997). In addition, the NRFs bind and activate the promoters of mitochondrial transcription factor A (Tfam, previously called mtTF-1 or mtTFA), which controls the transcription of the 13 essential mitochondrial encoded mitochondrial genes (Virbasius & Scarpulla, 1994). Tfam works along with the transcription factor B isoforms, TFB1M and TFB2M to lead to increased mRNA expression of key mitochondrial genes. Interestingly, although NRF-1 and NRF-2 are both able to bind to Tfam, rodent Tfam (Choi *et al.*, 2002) does not possess obvious NRF-1 recognition sites. In contrast, both NRF-1 and NRF-2 recognition sites are present in human Tfam. Alternatively, both human and rodent TFB1M and TFB2M genes do not possess NRF-1 binding sites and are controlled mostly through NRF-2 promoter binding (Rantanen *et al.*, 2003). The activation of both nuclear and mitochondrial encoded mitochondrial respiratory genes by PGC-1α highlights the ability of PGC-1α to coordinate and regulate mitochondrial biogenesis in both humans and rodents.

To provide further evidence for the necessity of an increase in PGC-1α content in regulating mitochondrial biogenesis, there have been a number of gain and loss of function studies exploring a number of key points in this pathway. It has been demonstrated that the overexpression of PGC-1 in cardiac myocytes of mice leads to an uncontrolled increase in mitochondrial biogenesis and cellular respiration (Lehman *et al.*, 2000), demonstrating the powerful control that PGC-1 has in cardiac function. Similarly, transgenic mice expressing PGC-

1α from the muscle creatine kinase promoter in the muscle tissues also results in an increased concentration of mitochondrial proteins in the skeletal muscle fibers (St-Pierre *et al.*, 2003). In contrast, the loss of PGC-1α function through the use of PGC-1α knock-out (KO) mice results in reduced cardiac mitochondrial enzyme activity, decreased ATP levels, and a reduction in cardiac output (Arany *et al.*, 2005). In cell lines developed from the brown adipose tissue of PGC-1α KO and wildtype mice, differentiation-induced mitochondrial biogenesis was only slightly reduced in PGC-1α KO or PGC-1β deficient (via siRNA techniques) adipocytes (Uldry *et al.*, 2006). However, if you combine both PGC-1β deficiency and PGC-1α null expression in the brown adipocytes, the normal increase in mitochondrial biogenesis following differentiation is completely ablated in the PGC-1α/β null/deficient adipocytes compared to the wildtype cell lines (Uldry *et al.*, 2006). Although PGC-1α and PGC-1β are often able to compensate for the loss of function of the other PGC-1 isoform, the use of PGC-1α/β null mice in cardiac function and brown adipose tissue mitochondrial density studies have demonstrated that a lack of both PGC-1 coactivators is fatal (Lai *et al.*, 2008). Therefore, although PGC-1α appears to be the master regulator of mitochondrial biogenesis, PGC-1β also plays an important role in some tissues.

Although the majority of studies focus on the role of PGC-1α, it is worth discussing PGC-1β in more detail. PGC-1β was first identified as a homologue of PGC-1α by Lin and colleagues through the use of genomic and expressed sequence tag databases (Lin *et al.*, 2002). Like PGC-1α, PGC-1β mRNA expression is tissue-specific with the majority of expression occurring in the heart and brown adipose tissue and moderate expression found in the liver, skeletal muscle, and white adipose tissue (Lin *et al.*, 2002). Although the two PGC-1 homologues share a large degree of sequence identity in the N-terminus, PGC-1β KOs develop different phenotypes and metabolic responses to biological stressors compared to PGC-1α KO rodent models. For instance, differences

in daily metabolic parameters, such as dark-cycle activity, are observed between PGC-1α KO and PGC-1β KO mice. PGC-1α KO mice have been shown to be hyperactive during the dark cycle (Lin *et al.*, 2004), whereas the PGC-1β KO mice have significantly reduced activity during the dark cycle compared to the wildtype control (Sonoda *et al.*, 2007). Although, PGC-1α and PGC-1β KO mice respond similarly to acute cold exposure, in wildtype mice acute cold stress results in an increase in PGC-1α mRNA expression, with no change in PGC-1β mRNA expression (Meirhaeghe *et al.*, 2003). Therefore, PGC-1β may be capable of mediating the response to the cold stress, such as in the PGC-1α KOs, but when both transcription coactivators are available, PGC-1α regulation appears to be preferred. More studies are required to completely understand this relationship; however, the results of studies involving KO animals or other transgenic models are complicated by the fact that they may not always be representative of what occurs in the wildtype animals.

Of interest to this project, is the response of skeletal muscle to a variety of exercise protocols and the effect of exercise on skeletal muscle PGC-1α and PGC-1β mRNA expression. It is well known that exercise leads to a significant increase in skeletal muscle PGC-1α mRNA expression (Baar *et al.*, 2002; Russell *et al.*, 2003; Wright *et al.*, 2007), which will be discussed further below. However, an acute treadmill exercise in mice (3hr run, 1 hr break, then 3hr run) does not increase skeletal muscle PGC-1β mRNA expression despite significant increases in PGC-1α mRNA (Meirhaeghe *et al.*, 2003) . Similarly, PGC-1β protein content was slightly reduced in human skeletal muscle following an acute cycle exercise protocol (Mathai *et al.*, 2008). The variety of responses of PGC-1β to different biological stimuli across different tissues and species provides further support for future research on the PGC-1α versus PGC-1β response, and this area has not been examined in white adipose tissue.

2.2 REGULATION OF PGC-1α IN SKELETAL MUSCLE

2.2.1 Exercise

Since the first description of exercise-induced increases in skeletal muscle mitochondrial respiratory enzymes in a rodent model (Holloszy, 1967), there has been a tremendous amount of research looking at skeletal muscle adaptations to exercise, with currently almost 15,000 MEDLINE hits for 'skeletal muscle exercise'. With the discovery that PGC-1α increases in response to cold-stress in brown adipose tissue (Puigserver *et al.*, 1998) and its ability to increase markers of mitochondrial biogenesis in skeletal muscle (Wu *et al.*, 1999), the interest in the field of exercise and mitochondrial adaptations has shifted towards the PGC-1α – mitochondria response to exercise. Goto and colleagues demonstrated that after only 3 days of swimming 2 hours per day, PGC-1α mRNA expression in the epitrochlearis muscle of rats was significantly increased compared to sedentary or immersed controls (Goto *et al.*, 2000). In a similar study where rats swam for 6 hours per day for 5 days, it was observed that in addition to increases in muscle PGC-1α mRNA expression, mitochondrial enzymes such as δ-aminolevulinate synthase (ALAS), COX I, cytochrome C, and citrate synthase were also increased in the muscle of the exercised group (Baar *et al.*, 2002). This study supported the previously described increases in muscle mitochondrial enzymes in response to exercise in rats (Holloszy, 1967).

A further question for researchers at this point was determining how the changes in the transcription of nuclear and mitochondrial genes were occurring in response to altered energy demands. The two transcription factors, NRF-1 and NRF-2 were believed to play a role in the control of mitochondrial genes (Evans & Scarpulla, 1990; Virbasius & Scarpulla, 1994) and are also reportedly upregulated by PGC-1α in muscle (Wu *et al.*, 1999). Therefore, to determine whether the exercise-induced increases in mitochondrial genes was associated with increases in the PGC-1α and

NRF-1/NRF-2 pathways, Baar and colleagues measured the mRNA expression and DNA binding of these transcription factors and their coactivator following a single 6 hour swim in rats. They found that triceps mRNA expression of PGC-1α, and the DNA binding of NRF-1 and NRF-2 were all significantly increased in the exercised group (Baar *et al.*, 2002). Although PGC-1α activation of NRF-1 and NRF-2 appear to be responsible for exercise-induced mitochondrial biogenesis, a number of other mediators of mitochondrial protein and enzyme activity have been proposed. A recent study using PGC-1α KO mice, demonstrated that although resting levels of mitochondrial proteins were reduced compared to wild-type controls, the KO mice still responded to 5 weeks of treadmill training with increased muscle mitochondrial protein content (Leick *et al.*, 2008). This study supports the idea that there are multiple regulators of skeletal muscle mitochondrial biogenesis or that compensatory mechanisms are in place.

2.2.2 AMPK

AMPK is a key metabolic regulator of fat metabolism (Merrill *et al.*, 1997) and glucose uptake (Merrill *et al.*, 1997; Mu *et al.*, 2001) in skeletal muscle. AMPK responds to changes in energy through allosteric activation by 5'-AMP and inhibition by ATP. Therefore, during exercise or energy depletion, 5'-AMP is elevated and covalently activated by phosphorylation, and ATP levels are reduced, leading to an increase in AMPK activity. Since AMPK plays such a key role in the regulation of intracellular energy metabolism (Winder *et al.*, 2000; Reznick & Shulman, 2006) and AMPK activation has been shown to increase PGC-1α expression (Terada *et al.*, 2002; Suwa *et al.*, 2003), it was proposed that AMPK could potentially play a role in the adaptation of skeletal muscle mitochondrial content to exercise. Dietary treatment for 6 weeks with 1% w/w β-guanidinopropionic acid (β-GPA), a creatine analogue, depletes muscle creatine levels (Shields & Whitehair, 1973), increases fast-twitch muscle aerobic enzymes (Shoubridge *et*

al., 1985), and increases muscle cytochrome c concentration and citrate synthase activity (Ren *et al.*, 1993) and content (Bergeron *et al.*, 2001). Increases in AMPK activity (Bergeron *et al.*, 2001), and recently in our own lab, increases in p38 MAPK and AMPK signaling, as well as PGC-1α protein and mRNA expression have also been demonstrated in rat skeletal muscle following 6 weeks of β-GPA treatment (Williams *et al.*, 2009). Likewise, treatment with 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), an adenosine analogue, activates AMPK and increases fast-twitch mitochondrial protein and enzyme activity (Winder *et al.*, 2000). Both β-GPA and AICAR treatment studies have been used to mimic the effect of exercise and energy deprivation on skeletal muscle by measuring AMPK activation. AMPK directly phosphorylates PGC-1α protein on Threonine-177 and Serine-538 and this PGC-1α activity is important for the AMPK-induced increases in glucose transporter (GLUT) 4 and mitochondrial biogenesis (Jager *et al.*, 2007). However, similar to the PGC-1α KO studies, transgenic mice with the inactivating D157A mutation in their α2 subunit of AMPK have reduced basal levels of the mitochondrial enzyme, citrate synthase, but they respond to exercise with an equivalent increase in citrate synthase activity when compared to the wild-type exercise group (Rockl *et al.*, 2007). Therefore, exercise-induced increases in mitochondrial enzymes, such as citrate synthase, can occur despite an inactivation of the α2-AMPK subunit. These experiments are unable to rule out any compensation by other AMPK subunits. In contrast, when treating transgenic mice expressing a dominant negative mutant of AMPK (DN-AMPK) in muscle with the energy depleting drug β-GPA, the β-GPA treatment was unable to lead to increases in AMPK activity, mitochondrial content or PGC-1α expression in the DN-AMPK mice (Zong *et al.*, 2002). Therefore, AMPK activation may be responsible for PGC-1α and

mitochondrial responses to energy depletion, but may not be required for, or the only mechanism involved in, exercise-induced increases in PGC-1α expression and mitochondrial biogenesis.

2.2.3 Ca2+

Calcium is an important signaling mediator as it is involved in numerous bodily functions such as contraction, apoptosis, proliferation, ATP synthesis (Berridge *et al.*, 2000), and fuel metabolism (Foot & Leighton, 1994; Sulova *et al.*, 1998). Calcium levels may also be important for upregulating mitochondrial biogenesis in response to exercise. Initial work with muscle cells (L6E9 myotubes) provided evidence of a role for calcium in increasing nuclear genes controlling mitochondrial protein expression (Freyssenet *et al.*, 1999). Exposure of myotubes to a calcium ionophore, ionomycin, led to increases in mitochondrial enzymes (Ojuka *et al.*, 2002a) that were similar to changes reported in rodent skeletal muscle following an exercise protocol (Holloszy, 1967). Calcium is believed to increase mitochondrial biogenesis through the phosphorylation of skeletal muscle calcium/calmodulin-dependent protein kinases (CAMKs) (Ojuka *et al.*, 2002b; Freyssenet *et al.*, 2004; Rose *et al.*, 2006). This was demonstrated by Wu and colleagues who showed that in a transgenic mouse model with constitutively active CAMKIV expression. Skeletal muscle from these mice showed an increase in mitochondrial biogenesis and PGC-1α mRNA expression (Wu *et al.*, 2002). Correspondingly, exposing myotubes in culture to caffeine or ionomycin for 5 days increased cytosolic calcium (Ojuka *et al.*, 2002a) and increased PGC-1α, Tfam, ALAS, COXI, and cytochrome c protein content (Ojuka *et al.*, 2003). Furthermore, Ojuka and colleagues were able to show that by inhibiting either the calcium release from the sarcoplasmic reticulum or by the inhibition of CAMK, all of the increases in PGC-1α, Tfam, and mitochondrial proteins were completely abated (Ojuka *et al.*, 2003). These studies provide strong evidence for the role of calcium in skeletal muscle mitochondrial biogenesis; however, it appears that there is an

important downstream mediator of this pathway known as p38 mitogen-activated protein kinase (p38 MAPK) (Enslen *et al.*, 1996; Chan *et al.*, 2004; Wright, 2007).

2.2.4 p38 MAPK

p38 MAPK is a 38kDa membrane protein that was first described in *Leishmania donovani* promastigotes (Cassel *et al.*, 1991) and it was later discovered that changes in p38 phosphorylation were induced by cyclic AMP (cAMP) in cellular slime mold, *Dictyostelium discoideum* (Schwandner *et al.*, 1993). It was demonstrated very early after its discovery to be involved in the energy metabolism pathways and not surprisingly, the relationship between p38 MAPK and PGC-1α has gained a lot of interest since its identification. Relevant to this project is the fact that p38 MAPK phosphorylates PGC-1, resulting in the stabilization and activation of the transcriptional coactivator. Furthermore, this has been demonstrated in a number of *in vitro* and *in vivo* models showing that cytokines and lipopolysaccharides increase PGC-1 activation in muscle through direct phosphorylation by p38 MAPK (Puigserver *et al.*, 2001; Puigserver & Spiegelman, 2003) and through the removal of a PGC-1α repressor (Knutti *et al.*, 2001), such as p160 myb binding protein (Fan *et al.*, 2004).

Like many other pathways, there are often a number of signals involved and it appears that p38 MAPK is able to both activate and down regulate PGC-1α depending on the environment that the muscle is exposed to. For example, in muscle following high fat feeding or in myotubes incubated with palmitate, PGC-1α mRNA expression is reduced in a p38 MAPK dependent manner, without the involvement of p160 myb binding protein (Crunkhorn *et al.*, 2007). However, in skeletal muscle from rats following an acute bout of exercise (Akimoto *et al.*, 2005; Wright *et al.*, 2007) and in human skeletal muscle following four brief intense exercise bouts (Gibala *et al.*, 2008), PGC-1α mRNA expression is increased concurrently with the activation of the p38 MAPK pathway. This

relationship was supported by *in vitro* work where the overexpression of the p38 upstream kinases, mitogen-activated protein kinase kinase 3E (MKK3E) and MKK6E, in C2C12 myotubes resulted in an increase in PGC-1α mRNA expression (Akimoto *et al.*, 2005). Furthermore, when co-treated with p38 inhibitors, the MKK3E-induced increases in PGC-1α mRNA expression were completely blocked (Akimoto *et al.*, 2005). Many studies on exercise and skeletal muscle support that further upstream, increased muscle calcium levels in response to exercise phosphorylates CAMKII, and that this activated CAMKII is correlated with increased p38 MAPK phosphorylation, which subsequently activates PGC-1α, independent of AMPK (Enslen *et al.*, 1996; Akimoto *et al.*, 2005; Wright, 2007; Gibala *et al.*, 2008).

2.3 OVERVIEW OF ADIPOSE TISSUE

2.3.1 Adipose tissue importance in whole body metabolism

Adipose tissue was once considered to be an inert storage depot for excess fatty acids in the body. However, epidemiological studies in the 1980s highlighted the strong association between increased adipose tissue and cardiovascular disease risk, and that the risks were even greater for individuals with increased abdominal versus lower body adipose tissue (Gillum, 1987; Folsom *et al.*, 1989). These earlier studies helped researchers to recognize that the location of the adipose tissue stores were associated with disease outcome. This resulted in the hypothesis that adipose tissue is interacting with surrounding tissues more than what was once thought. In contrast to an overabundance of adipose stores, individuals who have a scarcity of adipose tissue develop insulin resistance, diabetes mellitus, and hepatic steatosis, as seen in congenital generalized lipodystrophy and other disease or drug-induced lipodystrophies (Agarwal & Garg, 2006). Studies of transgenic and diet-induced lipodystrophic mice show that they develop similar metabolic disorders as humans (Moitra *et al.*, 1998) and that treating the mice with leptin, an important adipokine, can reverse their

insulin resistance and diabetes (Shimomura *et al.*, 1999; Tsuboyama-Kasaoka *et al.*, 2000; Nagao *et al.*, 2008). These studies provided further support for the idea that signals released by adipose tissue, such as adipokines, can have a profound impact on the body as a whole, and that adipose tissue is important for more then just the storage of excess fat.

2.3.2 Differences between adipose tissue depots

When studying adipose tissue *in vivo* or *ex vivo* with humans or animal models, it is important to specify which adipose tissue depot is being described. Although generally merged into two categories of subcutaneous and visceral adipose tissue, there are a number of depots within both of these categories, as they are metabolically different. Subcutaneous adipose tissue can be split into the upper, abdominal, and lower body subcutaneous adipose tissue, as well as superficial and deep adipose tissue with many regional categories within these classifications. Subcutaneous adipose drains into the systemic circulation, whereas visceral adipose tissue is known for its impact on the liver due to the drainage of most visceral adipose directly into the portal vein (Jensen, 2008). However, not all visceral adipose is the same and human visceral adipose tissue is also separated into the omental and mesenteric adipose tissue, which drain into the portal vein and the perinephric/perirenal adipose, which drains into the systemic circulation (Jensen, 2008). The direct drainage of omental and mesenteric adipose tissue into the portal vein may place these depots in a position to have a greater influence on liver physiology and pathology. This also influences how experimental measures should be taken, as the concentration of adipokines and free fatty acids (FFAs) in the portal vein can be significantly different than that found in the systemic circulation (Schaffler *et al.*, 2005). The knowledge that adipose tissue is no longer an inert storage depot and that adipose tissue can influence other tissues has provided support for in depth studies on adipose tissue. Gender-specific distribution of adipose tissue and its impact on disease risk are also important

components to adipose research. For instance, men preferentially store excess fat as visceral and abdominal subcutaneous adipose tissue, whereas women tend to store excess fat in their lower body subcutaneous stores, which are believed to be less of a health risk (Blaak, 2001). Additionally, visceral adipose tissue isolated from males has been reported to have increased FFA mobilization and sensitivity to β3-adrenoreceptor agonists compared to that of females (Lonnqvist *et al.*, 1997). Although this may be partially accounted for by the average increased fat cell volume in the male group, this provides some justification for the increased concern for overweight and obese males. Specifically, an excess of FFAs released from visceral adipose tissue can have a strong impact on liver pathology. For example, increased FAs delivered directly to the liver through the portal vein from visceral adipose can negatively impact the normal insulin-mediated suppression of glucose output from the liver, leading to increased blood glucose levels (Jensen, 2008). Additionally, visceral adipose tissue area has recently been determined to be an independent risk factor for hepatic steatosis (Park *et al.*, 2008).

Rodent models are commonly used to study the detailed mechanisms involved in adipose tissue metabolism and physiology. However, the anatomy of a rat is different from humans and therefore, there has been a great deal of interest in establishing the best comparison between human and rat visceral and subcutaneous adipose depots. Mesenteric, gonadal (perimetrial in females and epididymal in males) and retroperitoneal adipose depots are commonly studied in metabolic and physiology studies, as they are considered to be a representative dissection of visceral adipose tissue (Berthiaume *et al.*, 2007; Laplante *et al.*, 2009). Rat epididymal adipose tissue drains into the systemic circulation (Berthiaume *et al.*, 2007) and is often the most abundant of the visceral adipose tissue depots (Franco-Colin *et al.*, 2000; Laplante *et al.*, 2009), although this may be dependant on the age of the animal (Berthiaume *et al.*, 2007). Some debate surrounds the retroperitoneal adipose

depot as some report retroperitoneal adipose as a non-subcutaneous, non-visceral fat with respect to location and venous drainage (Arner, 1997; Pujol *et al.*, 2005); however, it is generally classified as a good representation of visceral adipose tissue (DiGirolamo *et al.*, 1998; Ishikawa & Koga, 1998; Festuccia *et al.*, 2009). The removal of mesenteric adipose tissue is labour intensive (DiGirolamo *et al.*, 1998), so epididymal or retroperitoneal adipose tissue are often the depot of choice when studying visceral adipose tissue. However, all three of the visceral depots respond differently to metabolic stressors (Berthiaume *et al.*, 2007) and with respect to depot expansion, the mesenteric and epididymal adipose depots increase mostly by hypertrophy, whereas the retroperitoneal and inguinal, an abundant source of subcutaneous adipose, increase by hyperplasia (DiGirolamo *et al.*, 1998). Therefore, the question remains unanswered as to which of the three main visceral adipose depots in male rats is most representative of human visceral adipose tissue and is often study specific, depending on the area of metabolism being examined. For this project, both epididymal and retroperitoneal adipose tissue depots were examined, so that both major sources of visceral adipose tissue are represented.

2.4 RESPONSE OF ADIPOSE TISSUE TO EXERCISE

As previously discussed, the response in muscle to exercise has been studied extensively. However, with the interest expanded to the role of adipose tissue in whole body metabolism, there is now a great deal of literature surrounding the metabolic adaptations in adipose tissue following exercise. The main area of research is focused around exercise-induced changes in adipose tissue lipolysis, glucose uptake, and of great interest to this paper, mitochondrial biogenesis.

2.4.1 Adipose tissue depot-specific changes in lipolysis and FA release following exercise

Endurance exercise requires an abundant source of energy in order for it to be sustained for long periods of time. Fortunately, adipose tissue consists of large storage depots of triglycerides that

are designed to provide a source of energy when required by the working skeletal muscle. In order for the triglycerides to become usable energy, there must be an increase in adipose tissue lipolysis and blood flow. One of the major contributors to mediating adipose tissue lipolysis during exercise is the increase in circulating catecholamines in response to exercise (Gollnick *et al.*, 1970; Galbo *et al.*, 1977). Epinephrine can activate both the β-adrenergic receptors to stimulate lipolysis and the α-adrenergic receptors to inhibit lipolysis (Arner *et al.*, 1990; Galitzky *et al.*, 1993), therefore in the basal state, the α-adrenergic receptors inhibit any unnecessary increases in lipolysis. When the β-adrenergic receptors are activated by increased levels of catecholamines during exercise, this works though G-protein binding, cAMP, and protein kinase A to phosphorylate hormone sensitive lipase (HSL) and perilipin (Londos *et al.*, 1995; Horowitz, 2003; Tansey *et al.*, 2003). Phosphorylated perilipin allows the HSL to gain access to the triglycerides in the lipid droplet, resulting in the release of fatty acids and glycerol. An important application of this research for disease prevention is the fact that intra-abdominal adipose tissue has an increased rate of lipolysis compared to subcutaneous adipose tissue and that training enhances the lipolytic response to epinephrine in adipose tissue even further (Enevoldsen *et al.*, 2000b).

Another important part of the equation is the need for increased adipose tissue blood flow. Exercise-induced increases in adipose tissue blood flow has been demonstrated in female Wistar rats (Enevoldsen *et al.*, 2000a). However, potential limits on adipose blood flow during exercise as a result of epinephrine-stimulated vasoconstriction appears to be responsible for limiting the amount of FFAs delivered to the muscle (Hodgetts *et al.*, 1991; Horowitz, 2003). The regulation of fatty acids during exercise is only one factor, but another aspect is the effect that exercise has on adipose tissue glucose metabolism.

2.4.2 Altered glucose uptake in adipose tissue in response to exercise

An increase in skeletal muscle glucose uptake as a result of contractile activity (Wallberg-Henriksson & Holloszy, 1984) has been studied extensively and a number of mechanisms as a result of acute and chronic adaptations to contraction and exercise are believed to be involved. The acute response to exercise in skeletal muscle is an increase in the translocation of the major glucose transporter, GLUT4, to the plasma membrane to increase glucose transport into the muscle cell. This is believed to be regulated through a number of pathways involving protein kinase C, CAMKs, and AMPK, which are discussed in detail in a review by Rockl and colleagues (Rockl *et al.*, 2008). Of interest to this paper is the effect that chronic contraction or exercise training can have on skeletal muscle glucose uptake. Interestingly, exercise training results in an increase in total GLUT4 protein expression in skeletal muscle, which appears to be regulated by PGC-1α, with some evidence also suggesting direct regulation by AMPK and CAMK (Rockl *et al.*, 2008).

Comparing the research in skeletal muscle to what is known in white adipose tissue, there are some similarities. The discovery that exercise training was able to normalize impaired basal and insulin-stimulated glucose metabolism in isolated adipose cells from streptozocin-treated rats (Goodyear *et al.*, 1991) demonstrated the significant impact that exercise can have on adipose tissue glucose uptake. There is also a small increase in adipose tissue GLUT4 plasma membrane protein expression observed in female rat adipose tissue following 6 weeks of voluntary cage wheel-running exercise (Hirshman *et al.*, 1993). However, there is a much greater increase in total GLUT4 protein content following exercise training (Hirshman *et al.*, 1993; Stallknecht *et al.*, 1993). This chronic effect on GLUT4 is similar to what is reported in skeletal muscle. Exercise has also been shown to increase the response of adipose tissue to insulin through not

only an increase in plasma membrane and total GLUT4 protein, but also through the insulin receptor substrates (IRS), phosphatidylinositol-3-kinase (PI3-K), and Akt pathway (Peres *et al.*, 2005). Taken together, the increase in exercise-mediated glucose uptake through increased total and plasma membrane GLUT4, and the increase in insulin-stimulated glucose uptake are likely contributors to the improvement in insulin sensitivity of Type 2 Diabetic individuals following exercise training. Understanding these mechanisms is important since exercise has been shown to improve insulin sensitivity in Type 2 Diabetics and is viewed as a valuable addition to the treatment plan for these patients (Mourier *et al.*, 1997; Shojaee-Moradie *et al.*, 2007).

2.4.3 What is known about exercise-induced adipose tissue mitochondrial biogenesis?

It is now well known that adipose tissue mitochondria play a role in the regulation of whole-body fuel metabolism (Bogacka *et al.*, 2005a; Frayn *et al.*, 2008; Maasen, 2008). For example, treatment of male Zucker fa/fa rats for 4 days with the insulin-sensitizing drug thiazolidinedione (TZD), increased adipose tissue phosphoenolpyruvate carboxykinase (PEPCK), as well as the mitochondrial pyruvate dehydrogenase complex kinase 4 (PDK4), which together lead to an increase in adipose tissue glyceroneogenesis (Tordjman *et al.*, 2003; Leroyer *et al.*, 2006; Cadoudal *et al.*, 2007; Cadoudal *et al.*, 2008). Increased adipose tissue glyceroneogenesis reduces fatty acid release from adipocytes, lowering plasma NEFA concentrations, and altering peripheral insulin sensitivity (Oakes *et al.*, 2001; Ye *et al.*, 2004; Cadoudal *et al.*, 2008). The effect of adipose tissue fatty acid oxidation on whole-body fuel metabolism is controversial, but some researchers hypothesize that it is a major contributor (Maassen *et al.*, 2007), while others feel that fatty acid re-esterification is more important (Frayn *et al.*, 2008). Either way, both of these pathways are affected by the function and amount of mitochondria in the adipocytes. Therefore, recent studies have focused on the

regulation of adipose tissue mitochondrial biogenesis and how this could influence whole-body fuel metabolism (Wilson-Fritch *et al.*, 2004; Choo *et al.*, 2006; Koh *et al.*, 2007a).

Rodent models of insulin resistance (ob/ob) and Type 2 Diabetes (db/db) exhibit a lower concentration of adipose tissue mitochondria (Wilson-Fritch *et al.*, 2004; Choo *et al.*, 2006; Valerio *et al.*, 2006), suggesting that either the lower number of mitochondria is a result of, or an initiating factor in, Type 2 Diabetes. These genetic models of insulin resistance and Type 2 Diabetes were also used to test the effect of TZDs on PGC-1α and mitochondrial protein and gene expression. The ability of the TZDs to increase PGC-1α and mitochondria helped support the argument for the importance of adipose tissue in whole-body metabolism, but still was unable to conclude whether these reductions in mitochondria were the cause or result of insulin resistance (Wilson-Fritch *et al.*, 2004; Choo *et al.*, 2006). However, we recently explored this phenomenon in our laboratory and found that reductions in mitochondrial biogenesis and PGC-1α occurred after the initiation of impaired glucose homeostasis, and suggested that the reduction in mitochondrial proteins is not an initiator of impaired glucose tolerance in high fat fed rats (Sutherland *et al.*, 2008). This study does not rule out that the reduction in mitochondrial proteins and expression could assist in the further development of severe insulin resistance and Type 2 Diabetes, and therefore increasing mitochondrial biogenesis is still likely to be of therapeutic benefit for improving insulin resistance.

Similar studies have also been conducted in insulin-resistant and Type 2 Diabetic humans, where reductions in adipose tissue mitochondria and PGC-1α gene expression have been observed (Bogacka *et al.*, 2005b; Heilbronn *et al.*, 2007). However, treatment with the PPAR-γ agonist drugs, TZDs, results in an increase in PGC-1α mRNA expression, mitochondrial DNA copy number, and markers of mitochondrial aerobic capacity in adipose tissue of Type 2 Diabetics (Bogacka *et al.*, 2005b). Of clinical relevance, these increases in adipose tissue mitochondrial markers and PGC-1α

gene expression occur in concert with decreased HbA_{1C} and plasma triglyercide levels (Einhorn *et al.*, 2000; Rosenstock *et al.*, 2002; Bogacka *et al.*, 2005b). Although it is evident that the major action of TZDs occurs through changes in adipose tissue, which leads to improvements in whole-body glucose and lipid regulation in Type 2 Diabetics, these drugs come with many side effects (Chiarelli & Di Marzio, 2008; Li *et al.*, 2008).

Therefore, we were interested in examining how adipose tissue mitochondrial biogenesis is regulated, so that potential nutritional, lifestyle, or pharmacological interventions could be used to lead to increased mitochondrial biogenesis. There is very little known about the impact of exercise on adipose tissue mitochondria, despite the wealth of research surrounding exercise-induced mitochondrial biogenesis in muscle (Holloszy, 1967; Irrcher *et al.*, 2003; Mahoney & Tarnopolsky, 2005; Lumini *et al.*, 2008). A study by Stallknecht et al. (1991), showed that the mitochondrial respiratory chain enzyme, cytochrome c oxidase (CCO) and the Krebs cycle enzyme, malate dehydrogenase (MDH), were significantly increased in the adipose tissue of male and female Wistar rats following a 10 week swimming protocol of 6 hours per day (Stallknecht *et al.*, 1991). However, since this study was published, no one has looked at the potential mechanisms responsible for the increased mitochondrial enzymes, so this became a major objective of this project. As reductions in adipose tissue mitochondrial proteins and gene expression are associated with decreased PGC-1α expression in rodent models of insulin resistance and Type 2 Diabetes (Wilson-Fritch *et al.*, 2004; Rong *et al.*, 2007; Sutherland *et al.*, 2008), this suggests that increases in mitochondrial biogenesis observed with exercise (Stallknecht *et al.*, 1991) may be regulated by PGC-1α. Additionally, although there are often many tissue-specific regulatory pathways, PGC-1α is known to regulate mitochondrial biogenesis in skeletal muscle (Wu *et al.*, 1999). Additionally, it appears to be by the activation of

PGC-1α in skeletal muscle following exercise that mitochondrial biogenesis is increased in skeletal muscle (Wright *et al.*, 2007). Furthermore, the over-expression of PGC-1α in white adipocytes has been shown to result in increased mitochondrial respiratory chain proteins and enzymes (Tiraby *et al.*, 2003). Therefore, the research presented in this thesis investigates whether exercise is able to increase adipose tissue PGC-1α mRNA expression in concert with increases in markers of mitochondrial protein and enzyme activity. The mitochondrial transcription factor, Tfam, is controlled by PGC-1α and is responsible for the mitochondrial encoded mitochondrial genes (Virbasius & Scarpulla, 1994; Scarpulla, 2008). Therefore measuring changes in adipose tissue Tfam mRNA expression as a result of exercise is important in answering the thesis question.

2.5 BETA-ADRENERGIC REGULATION

During exercise, the sympathetic nervous system is activated (Hartley *et al.*, 1972b, a) and plasma epinephrine is increased (Gollnick *et al.*, 1970; Galbo *et al.*, 1977; Shah *et al.*, 1984; Stallknecht, 2004). Epinephrine is able to activate both α- and β-adrenergic receptors, which are expressed throughout the body in a tissue-specific manner (Arner *et al.*, 1990; Galitzky *et al.*, 1993). For example, although α-, β_1-, and β_2-adrenergic receptors are found abundantly in skeletal muscle, only β_2-adrenergic receptor agonist treatment in muscle (*in vivo* or *ex vivo*) led to increases in PGC-1α mRNA expression, whereas the α-, β_1-, and β_3-adrenergic receptor agonists had no effect (Miura *et al.*, 2007). This can be compared to white adipose tissue, where the majority of β-adrenergic receptors are the β_3-isoform, with only a minor regulatory contribution from the small proportion of β_1-adrenergic receptors (Farias-Silva *et al.*, 2004). Therefore, the majority of sympathetic nervous system-related studies in adipose tissue are focused on catecholamines and the β_3-adrenergic

receptors and how they affect adipose tissue metabolism in health and disease (Esler *et al.*, 2001; Esler *et al.*, 2003).

Although the major β-adrenergic receptor in white adipose tissue is the β3-form (Farias-Silva *et al.*, 2004), there are a total of five adrenergic receptor types found on the plasma membranes of white adipocytes in rats, all of which are activated by catecholamines: α1, α2, β1, β2, and β3 (Kobatake *et al.*, 1991; Farias-Silva *et al.*, 2004). There is a low abundance of the α1-adrenergic receptors in white adipose tissue (Kobatake *et al.*, 1991; Torres-Marquez *et al.*, 1992; Lafontan & Berlan, 1993) and the α1-adrenergic receptor has no action on cAMP, as instead, its functions are mediated through the phosphatidylinositolbisphosphate cycle and phospholipase C (Zhong & Minneman, 1999; Cheng *et al.*, 2000). Its activation results in an increase in intracellular calcium and protein kinase C, and results in the activation of glycogen phosphorylase and an inactivation of the glycogen synthase pathways (Fain & Garcija-Sainz, 1983). Due to the fact that there is a very low abundance of the α1-receptors in white adipose tissue and that they are not involved in the cAMP-dependent pathways, these receptors were not considered further in this study. However, the α2-adrenergic receptors have a major role in white adipose tissue as they are coupled to adenylate cyclase by the Gi-proteins in the plasma membrane. Activation of the Gi proteins by α2-adrenergic receptor stimulation results in the inhibition of adenylate cyclase and a reduction in cAMP production and lipolysis. Alternatively, the β-adrenergic receptors are coupled to the Gs-proteins and when stimulated by catecholamines, this leads to an increase in adenylate cyclase activity and cAMP production. cAMP activates cAMP-dependent protein kinase A (Levitzki, 1988; Birnbaumer *et al.*, 1990; Spiegel *et al.*, 1992), which is responsible for the phosphorylation of hormone sensitive lipase, driving an increase in lipolysis (Horowitz, 2003; Tansey *et al.*, 2003). Protein kinase A also phosphorylates cAMP response binding protein (CREB), which activates a number of transcription

factors and regulators (Shepherd & Bah, 1988), such as PGC-1α (Puigserver *et al.*, 1999; Xue *et al.*, 2005). Therefore, the activation of the α2- and β-adrenergic receptors has an important impact on white adipose tissue metabolism. Although the α2-adrenergic receptors are important for the negative regulation of cAMP, it is believed that catecholamines are preferentially bound to the α2-adrenergic receptors at low concentrations during rest, but to the β-adrenergic receptors at higher concentrations, such as during exercise (Arner *et al.*, 1990). The topic of this project is on the regulation of white adipose tissue during a state of elevated epinephrine levels and exercise, therefore the β-adrenergic receptors are the main focus.

Norepinephrine is also increased in response to exercise (Galbo *et al.*, 1977). It has also been demonstrated that norepinephrine stimulation in epididymal and inguinal adipocytes increases cellular respiration in both depots *in vitro* (Deveaud *et al.*, 2004). However, in a spontaneously active rat model, basal plasma epinephrine, but not plasma norepinephrine levels are increased along with increases in epididymal adipose tissue AMPK phosphorylation (Hattori *et al.*, 2009). It has previously been shown that epinephrine and exercise increase epididymal adipose tissue AMPK activity (Koh *et al.*, 2007b) and AMPK is also proposed to increase PGC-1α expression in adipose tissue (Pirinen *et al.*, 2007; Gaidhu *et al.*, 2009). Therefore, we focused on measuring the impact of epinephrine, rather than norepinephrine, on adipose tissue PGC-1α mRNA expression and changes in adipose tissue mitochondrial biogenesis.

An actively researched area of Type 2 Diabetes research is the area of adipose tissue sympathetic nervous system response to diet, exercise, or other physiological stimuli. For instance, in the obesity-prone mouse model (AKR/J) fed a high fat diet, there was significantly decreased PGC-1α mRNA expression in concert with decreased β_3-adrenergic receptor mRNA in the brown adipose tissue compared to that of the obesity resistant mice (SWR/J) (Prpic *et al.*, 2002).

Additionally, it has been noted that the use of β-adrenergic receptor agonists are therapeutic in rodent models of insulin resistance and Type 2 Diabetes, as the drugs result in improvements in glucose tolerance (Yoshida *et al.*, 1991; Koza *et al.*, 2004). Since β-adrenergic receptor activation occurs during exercise and it has been shown to play a role in exercise-induced increases in adipose tissue lipolysis (Shepherd & Bah, 1988), then perhaps increased epinephrine is also partially responsible for the hypothesized exercise-induced increases in adipose tissue PGC-1α mRNA expression.

Further evidence for this hypothesis is provided by work in skeletal muscle that has demonstrated that β-adrenergic receptor activation leads to increases in PGC-1α gene expression in skeletal muscle (Miura *et al.*, 2007). Miura et al. (2007) also used an acute exercise protocol with or without the injection of propranolol, a non-specific β-blocker, which was able to attenuate the increases in PGC-1α in the skeletal muscle by up to 70%. β_3-adrenergic receptor agonists also increase PGC-1α mRNA expression in brown adipose tissue (Gomez-Ambrosi *et al.*, 2001), providing potential for epinephrine to increase PGC-1α mRNA expression in white adipose tissue by the stimulation of β-adrenergic receptors.

However, catecholamine stimulation and β-adrenergic receptor activity can be different even within adipose tissue depots. Since differences between retroperitoneal and epididymal adipose tissue depots are commonly identified in metabolic studies (Blennemann *et al.*, 1992; Shi *et al.*, 2007; Bueno *et al.*, 2008), it is believed that the study of the sympathetic nervous system in these adipose depots is not exempt from this phenomenon. For example, sympathetic noradrenergic nerves are involved in lipid mobilization within white adipose tissue and therefore the sympathetic nervous system responds to fasting (Giordano *et al.*, 2005). Interestingly, the retroperitoneal adipose tissue response is very different from the epididymal adipose tissue response and this is believed to be due

to an acute increase in sympathetic nerve density in the retroperitoneal adipose tissue when in the fasted state, compared to an increased level of hormonal regulation in the epididymal adipose tissue (Giordano *et al.*, 2005). Furthermore, there is evidence for an increased sensitivity to β-adrenergic agonists in epididymal adipose tissue resulting in greater increases in β-adrenergic stimulated lipolysis in the epididymal versus retroperitoneal adipose tissue (Tavernier *et al.*, 1995). Similar results have been demonstrated when comparing catecholamine stimulated lipolysis in human adipose tissue, where a greater response to catecholamines was reported in omental adipose tissue compared to subcutaneous adipose tissue (Lafontan & Berlan, 1993). Therefore, when studying the effect of epinephrine in white adipose tissue, it is important to look at both the retroperitoneal and epididymal adipose tissue depots, as more work is needed to understand the depot-specific responses to β-adrenergic stimulation.

2.6 THESIS OVERVIEW

It is clear that there is a wealth of knowledge on the changes in skeletal muscle mitochondrial biogenesis in response to acute and chronic exercise. However, the impact that exercise has on adipose tissue mitochondrial biogenesis and the regulatory pathway leading to the increased mitochondrial proteins and enzyme activity has not be determined. Evidence from the study of the impact of exercise on skeletal muscle (Holloszy, 1967; Wu *et al.*, 1999) and pharmacological treatment with insulin-sensitizing drugs (TZDs) affecting white adipose tissue (Wilson-Fritch *et al.*, 2004; Choo *et al.*, 2006) would suggest a role of PGC-1α in regulating exercise-induced adipose tissue mitochondrial biogenesis. The role of PGC-1β in heart (Sonoda *et al.*, 2007; Lai *et al.*, 2008) and skeletal muscle (Meirhaeghe *et al.*, 2003) mitochondrial biogenesis has been studied, but very little is known about the role of PGC-1β in white adipose tissue in resting and with exercise. In addition, β-adrenergic receptor stimulation in brown adipose tissue has been shown to increase PGC-

1α mRNA (Gomez-Ambrosi *et al.*, 2001), suggesting that the increased epinephrine during exercise may also be partially responsible for the changes in adipose tissue mitochondria. Consequently, the following series of studies have been designed to: 1) determine if exercise training will increase adipose tissue PGC-1α mRNA expression and mitochondrial marker enzymes, 2) determine whether epinephrine can increase adipose tissue PGC-1α mRNA expression and 3) establish whether the effect of exercise on PGC-1α mRNA expression is attenuated by blocked adrenergic stimulation using a beta blocker.

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Chapter 3. Exercise and Epinephrine Increase PGC-1α mRNA Expression in Rat Adipose Tissue[1]

3.1 INTRODUCTION

In recent years a growing number of studies have focused on the regulation of adipose tissue mitochondrial biogenesis, in large part due to the purported role of adipose tissue mitochondria in the regulation of whole body fuel metabolism (Koh *et al.*, 2007;Wilson-Fritch *et al.*, 2004;Choo *et al.*, 2006). For instance in rodent models of insulin resistance and Type 2 Diabetes, adipose tissue mitochondrial content is reduced (Choo *et al.*, 2006;Wilson-Fritch *et al.*, 2004;Valerio *et al.*, 2006). Interestingly, peroxisome proliferator activated receptor (PPAR) gamma agonists (e.g. thiazolidinediones (TZDs)) (Choo *et al.*, 2006;Wilson-Fritch *et al.*, 2004;Rong *et al.*, 2007) and ciliary neurotrophic factor (CNTF) (Crowe *et al.*, 2008) have been shown to induce mitochondrial biogenesis in adipose tissue from insulin resistant animals. These changes are associated with increases in the mRNA expression of PPAR gamma co-activator 1 alpha (PGC-1α) (Crowe *et al.*, 2008;Wilson-Fritch *et al.*, 2004) and the related co-activator PGC-1β (Rong *et al.*, 2007). PGC-1α and β are key regulators of mitochondrial biogenesis that co-activate and induce the expression of transcription factors such as nuclear respiratory factors (NRF)-1 and 2 and mitochondrial transcription factor A (Tfam), molecules involved in the coordinated regulation of nuclear and mitochondrial encoded genes, respectively (Scarpulla, 2008). When over-expressed in white adipocytes, PGC-1α leads to increases in the expression of mitochondrial respiratory chain proteins and enzymes involved in fatty acid oxidation (Tiraby *et al.*, 2003), resulting in a phenotype similar to that of brown adipose tissue. Of interest from a clinical perspective, work from Smith's laboratory has shown a correlation between adipose

[1] A version of this paper has been published. Sutherland et al. (2009). *J Physiol* **587**(7):1607-1617.

tissue PGC-1α mRNA expression and whole body insulin sensitivity (Hammarstedt *et al.*, 2003). Collectively these findings highlight the importance of PGC-1α in white adipose tissue.

Given the increasing interest surrounding adipose tissue mitochondrial biogenesis it is surprising that only one isolated report has examined the effects of exercise on the regulation of this process in adipose tissue (Stallknecht *et al.*, 1991). Stallknecht and colleagues (1991) found that 6 hours of daily swimming for 10 weeks led to increases in cytochrome c oxidase and malate dehydrogenase activities in rat epididymal adipose tissue. Given the close association between increases in PGC-1α mRNA expression and the induction of mitochondrial biogenesis it seems likely that exercise training could increase the mRNA expression of this key transcriptional co-activator in adipose tissue. While PGC-1α and β regulate similar genes (Scarpulla, 2008), it would appear that they respond differently to external stimuli. For instance, in skeletal muscle, PGC-1β expression is not increased by exercise. It is yet to be determined if exercise induced increases in mitochondrial enzymes are paralleled by similar changes in PGC-1β mRNA expression in white adipose tissue.

In contrast to skeletal muscle (Wright, 2007;Winder *et al.*, 2006) little is known regarding the specific mechanisms which are responsible for exercise-induced increases in PGC-1α mRNA expression and mitochondrial biogenesis in white adipose tissue. Given the recent findings that beta adrenergic agonists can increase PGC-1α mRNA expression in hepatocytes (Ding *et al.*, 2006) and brown fat pre-adipocytes (Puigserver *et al.*, 1998) it seems likely that increases in circulating catecholamine levels, that occur during exercise, could initiate exercise-induced increases in adipose tissue PGC-1α mRNA expression. Within this context the purpose of the present investigation was to explore the regulation of adipose tissue PGC-1α mRNA expression by exercise. We hypothesized that both exercise training and an acute bout of exercise would lead to increases in PGC-1α mRNA

expression. We further surmised that epinephrine, a key catecholamine released during exercise, would lead to increases in PGC-1α mRNA expression. Lastly, we postulated that the acute effects of exercise on PGC-1α mRNA expression would be attenuated in the presence of beta blockade. To achieve these objectives we studied PGC-1α mRNA expression and markers of mitochondrial biogenesis in two rat abdominal adipose tissue depots.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Reagents, molecular weight markers, and nitrocellulose membranes for SDS-PAGE were purchased from Bio-Rad (Mississauga, Ontario). ECL Plus was a product of Amersham Pharmacia Biotech (Arlington Heights, IL). Antibodies against COXIV, and CORE I were purchased from Molecular Probes (Eugene, Oregon). An antibody against beta-actin was a product of Sigma (St. Louis, Missouri). Horseradish peroxidase-conjugated donkey anti-rabbit and goat anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, Pennyslvania). SuperScript II Reverse Transcriptase, oligo(dT) and dNTP were purchased from Invitrogen (Carlsbad, CA). Citrate Synthase activity kits were obtained from Sigma (St. Louis, MO). All other chemicals were purchased from Sigma.

3.2.2 Treatment of rats

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. As we hypothesized that epinephrine would increase PGC-1α expression and since epinephrine levels would increase following an overnight fast, all animals were studied in the fed condition. After 1 week acclimatization, rats were randomly divided into 2 groups. Half of the animals were subjected to exercise training, starting at 15 minutes per day of swimming for two days and then 2 hours per day, 7 days per week for 4 weeks. As described previously by Stallknecht et al. (1991), the remaining rats swam for 2 minutes per day and served as a sham control. Approximately 20 hours

following the last bout of exercise, rats were anesthesized with sodium pentobarbital (5mg per 100 gram body weight). Epididymal and retroperitoneal adipose tissue was dissected free of the testes and kidneys, respectively, immediately weighed and then clamp frozen in tongs cooled to the temperature of liquid nitrogen and stored at -80 ºC 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 (Trujillo *et al.*, 2006;Fried *et al.*, 1993;Lee *et al.*, 2007). The major strength of this method is the ability to maintain gene expression over extended periods of time (Fried & Moustaid-Moussa, 2001). Epididymal and retroperitoneal fat pads were removed from male Wistar rats (~200 grams), weighed, and immediately placed in 50 ml conical tubes containing sterile PBS with 1% v/v antibiotic/antimycotic. Under sterile conditions, 500mg of tissue was placed into culture dishes containing 15 ml of M199 supplemented with 1% v/v antibiotic/antimycotic, 50μU insulin and 2.5 nM dexamethasone added to the media. The tissue was then minced into ~5-10 mg pieces and kept in an incubator at 37ºC to equilibrate for 24 hours. Approximately 3-4g of adipose tissue was collected from each rat, which provided adequate adipose tissue to use a 500mg sample from each individual rat in all treatment groups. For the dose-response experiment epinephrine (1, 5, 10 or 50 μM) or vehicle (sterile dH_2O) was added to the media and the plates were returned to the incubator for 6 hours. After 6 hours, the culture media containing the adipose tissue minces was poured into ice-cold PBS and then cell strainers were used to collect the adipose tissue minces from the media/PBS. The adipose tissue minces were then snap frozen in liquid nitrogen and stored at -80ºC until further analysis. For the time course study adipose tissue was allowed to equilibrate for 24 hours and then 1μM of epinephrine was added to the media and plates were returned to the incubator

for 2, 4, 6 or 12 hours. In an additional experiment the effects of a 2 hour treatment with 100 nM epinephrine was examined. After each time point, the adipose tissue minces were collected as described above and stored at -80ºC until further analysis.

3.2.4 Acute exercise and beta blockade

Rats were acclimated to swim exercise as described above. Seventy and 10 minutes prior to the start of exercise rats were injected (I.P.) with a weight adjusted bolus of propranolol hydrochloride (0.2 mg per 100 gram body weight) or an equivalent volume of sterile saline. This protocol has previously been used to inhibit beta adrenergic signaling in rats during swim exercise (Nolte *et al.*, 1994). Immediately following 2 hours of swimming rats were anesthetized and adipose tissue harvested. There were 6 rats in the control group and 9 rats each, in the swim and swim + propranolol groups.

3.2.5 Western blotting

Clamp-frozen epididymal and retroperitoneal fat was homogenized in a 2:1 volume-to-weight ratio of ice cold cell lysis buffer supplemented with Protease Inhibitor Cocktail and phenylmethylsulfonyl fluoride using a motor driven glass on glass mortar and pestle. Homogenized samples were sonicated for 5 seconds and centrifuged for 15 minutes at 2500 X G at 4° C. The protein concentration of the supernatant was determined using the BCA method (Smith *et al.*, 1985). The CV for this assay is <5% in our laboratory. Relative levels of the protein content of CORE 1 and COXIV were determined by Western blot analysis as described previously (Wright *et al.*, 2007;Sutherland *et al.*, 2008). Briefly, equal amounts of protein were separated on either 10% (CORE 1) or 15% (COXIV) gels that were prepared in lab. Proteins were wet electrophoretically transferred to nitrocellulose membranes for 90 minutes at 200 mA per tank. Membranes were blocked in Tris-buffered saline/0.01% Tween (TBST) supplemented with 5% non-fat dry milk at

room temperature for 1 hour 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 blots were briefly washed in TBST and then incubated in TBST/1% non-fat dry milk supplemented with HRP conjugated goat-anti-mouse secondary antibody for 1 hour at room temperature. Bands were visualized using ECL (enhanced chemiluminescence) plus and captured using a Typhoon Imaging system (General Electric, Piscataway, NJ). Imagequant software was used to quantify relative band intensities (General Electric, Piscataway, NJ). To control for equal loading and transfer of proteins beta actin was used as an internal control. In preliminary experiments, we found that 4 weeks of swimming had no effect on the protein content of beta actin in epididymal and retroperitoneal adipose tissue (3.22 ± 0.09 control, 3.27 ± 0.12 trained in epididymal adipose tissue and 4.72 ± 0.33 control, 4.77 ± 0.39 trained in retroperitoneal adipose tissue, arbitrary densitometric units n=5-6).

3.2.6 Citrate synthase activity

Frozen adipose tissue samples were homogenized and protein extracted as described above, in the description of Western blotting. Citrate synthase activity was determined by measuring the formation of 5-thio-2-nitrobenzoic acid spectrophotometrically (412 nm) in a microplate reader (Molecular Devices, Sunnyvale, CA, USA) as described in the manufacturer's instructions.

3.2.7 Real time RT-PCR

RNA was isolated from epididymal and retroperitoneal adipose tissue and adipose tissue minces using an RNeasy lipid kit (Qiagen, Mississauga, ON) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1μg of RNA using SuperScript II Reverse Transcriptase, oligo(dT) and dNTP. Real time PCR was performed using a 7900 HT Fast Real-Time PCR system (Applied Biosystems). Taqman Gene Expression Assays (Applied

Biosystems, Streetsville, ON) were used to determine the mRNA expression of beta actin and Tfam. Primers and probes for PGC-1α, and PGC-1β were designed using Primer Express 3.0 software (Table 3.1). Samples were run in duplicate on a 96 well plate. Results were normalized to the mRNA expression of beta actin as we had found in preliminary experiments that this gene did not change with any of our experimental manipulations. Relative differences in gene expression between groups were determined using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Standard curve assays were performed for beta actin and PGC-1α, PGC-1β, and Tfam. The amplification efficiencies of the gene of interest and beta actin were equivalent as determined using the equation $10^{(-1/slope)}$ -1. Similarly, when plotting log cDNA dilution versus delta CT (ΔCT, $CT_{gene\ of\ interest}$-$CT_{beta\ actin}$) the slope of this relationship was <0.1, indicating that the genes of interest were amplified with equal efficiency.

3.2.8 Statistical analysis

Data are presented as means ± SE. Comparisons between the sham control and trained groups were made using an unpaired Student's t-test. Comparisons between the vehicle and treated groups during ATOC experiments were made using a one-way ANOVA followed by a post-hoc comparison using Fishers LSD test. Similarly, differences between control, swim and swim plus propranolol groups were made using a one-way ANOVA and Fisher's LSD test. Statistical significance was set at $P < 0.05$.

3.3 RESULTS

3.3.1 Effect of exercise on body weight, fat pad mass and food intake

Body weight gain in the swim-trained group was significantly less than the control group (Table 3.2). Food intake did not differ between groups. Fat pads from the swim-trained rats weighed less than those in the control group.

3.3.2 Exercise-induced increases in mitochondrial protein content and enzyme activity

Four weeks of swim training led to increases in the protein content of COXIV and CORE 1, proteins of complex IV and complex III of the respiratory chain, respectively. Additionally, citrate synthase activity was increased in epididymal and retroperitoneal adipose tissue from trained rats (Figure 3.1). The measurement of these enzymes have previously been used as markers of mitochondrial content in skeletal muscle and adipose tissue (Garcia-Roves *et al.*, 2006;Sutherland *et al.*, 2008;Rong *et al.*, 2007).

3.3.3 Exercise training increases the mRNA expression of PGC-1α and Tfam

PGC-1α and Tfam mRNA expression were increased in epididymal and retroperitoneal adipose tissue following training (Figure 3.2). On the other hand, the mRNA expression of PGC-1β was not significantly increased in either fat pad following swim training.

3.3.4 Acute Exercise increases the mRNA expression of PGC-1α

Immediately following an acute, 2 hour bout of exercise, PGC-1α mRNA expression was increased in both fat pads. Four hours following exercise cessation, PGC-1α mRNA expression was not different than control values (Figure 3.3). Tfam mRNA expression was not significantly increased in either fat pad immediately, or 4 hours following exercise cessation.

3.3.5 Epinephrine causes a dose and time dependent increase in PGC-1α mRNA expression

To observe the initial, direct effects of epinephrine on the induction of PGC-1α mRNA expression independent of systemic changes in other metabolites and hormones we utilized adipose tissue organ culture. Adipose tissue minces were cultured and exposed to various concentrations of epinephrine over a number of time points. The initial epinephrine concentrations that we utilized were based on previous findings in hepatocytes (Ding *et al.*, 2006). Treatment with pharmacological (1-50μM) doses of epinephrine for 6 hours resulted in a dose dependent increase in PGC-1α mRNA expression in cultured epididymal and retroperitoneal adipose tissue (Figure 3.4). Although 10 μM epinephrine resulted in the highest induction of PGC-1α mRNA expression, we used a 1 μM dose of epinephrine for the time course experiments to avoid any potential issues with toxicity at the high concentrations we were using. The highest measured increase in PGC-1α mRNA expression was observed following a 2 hour exposure to 1 μM epinephrine with progressive decreases thereafter (Figure 3.5). The increases in PGC-1α mRNA expression preceded the epinephrine-mediated rise in Tfam mRNA expression in epididymal adipose tissue. Treatment of adipose tissue cultures for 2 hours with 100 nM epinephrine, a concentration more representative of the levels of circulating catecholamines during exercise (epinephrine levels during swimming in rats are ~ 15 nM (Higashida *et al.*, 2008)), led to significant increases in PGC-1α mRNA expression in epididymal adipose tissue (2.3±0.6, $p=0.02$), but not in retroperitoneal adipose tissue (1.4±0.1, $p>0.05$).

3.3.6 Beta blockade attenuates exercise induced increases in adipose tissue PGC-1α mRNA expression

Rats were treated with the beta blocker propranolol (200 μg per 100 gram body weight) 70 and 10 minutes prior to 2 hours of swim exercise. In preliminary experiments we found that this dosing protocol almost completely blocked the *in vivo* effects of epinephrine (20 μg per 100 gram

body) on PGC-1α mRNA expression in adipose tissue (epinephrine 3.69 ± 1.26 fold increase above control, epinephrine + propranolol 1.44 ± 0.36 fold increase above control). This epinephrine treatment results in increases in epinephrine (~190 nM) (Fell *et al.*, 1981) to levels much higher than seen during exercise (~15 nM) (Higashida *et al.*, 2008). The same propranolol treatment has previously been used in rat swim models and does not effect the ability of rats to complete the swim exercise (Nolte *et al.*, 1994). As seen in figure 3.6, propranolol reduced the exercise induced rise in PGC-1α mRNA expression in epididymal but not retroperitoneal adipose tissue.

3.4 DISCUSSION

Adipose tissue mitochondria are increasingly being recognized as key players in the regulation of whole body metabolism. Surprisingly, and in sharp contrast to skeletal muscle, few studies have explored the effects of exercise on mitochondrial biogenesis in adipose tissue. Consistent with one previous report (Stallknecht *et al.*, 1991), we found that 2 hours of daily swim exercise for 28 consecutive days led to increases in markers of adipose tissue mitochondrial biogenesis, such as CORE1 and COXIV protein content and citrate synthase activity.

Mitochondrial biogenesis is a complex process involving the coordinated regulation of both nuclear and mitochondrial encoded genes. A central cog in this process would appear to be PGC-1α. The over-expression of PGC-1α in skeletal muscle (Lin *et al.*, 2002) or white adipocytes (Tiraby *et al.*, 2003) induces mitochondrial biogenesis, whereas the deletion of this gene leads to reductions in mitochondria (Leone *et al.*, 2005). Exercise has been shown to have both an acute (Baar *et al.*, 2002;Mathai *et al.*, 2008;Miura *et al.*, 2007) and chronic, training related effect (Goto *et al.*, 2000) on PGC-1α mRNA expression in skeletal muscle. Similar to these aforementioned findings, we made the novel observation that 4 weeks of daily swim training led to increases in the mRNA expression of PGC-1α, in white adipose tissue. On the other hand training did not significantly increase the expression of PGC-1β in either fat depot. These findings are consistent with a previous report which demonstrated that cold exposure, fasting and exercise increased PGC-1α but not PGC-1β mRNA levels in brown adipose tissue, liver and skeletal muscle respectively (Meirhaeghe *et al.*, 2003). PGC-1α mRNA expression in white adipose tissue was increased immediately after exercise and returned to control levels four hours following exercise cessation. Since tissue was harvested well after this time point, our results suggest that the long term increases in PGC-1α mRNA

expression were the result of a training effect and not related to the residual effects of the last bout of exercise. The rise in PGC-1α was associated with increases in the mRNA expression of Tfam, a transcription factor involved in the regulation of mitochondrial encoded genes whose expression is controlled, at least in part, by PGC-1α (Gleyzer *et al.*, 2005;Wu *et al.*, 1999). While our findings are consistent with the notion that PGC-1α could be involved in mediating exercise-induced mitochondrial biogenesis in adipose tissue, future studies are needed to determine if the changes in PGC-1α mRNA expression are paralleled by increases in the protein content of this transcriptional co-activator.

Biochemical changes within the contracting muscle itself, such as perturbations in high energy phosphates (Bergeron *et al.*, 2001;Zong *et al.*, 2002;Baar *et al.*, 2002), and increases in cytosolic calcium concentration (Ojuka *et al.*, 2003;Ojuka *et al.*, 2002), are believed to initiate, to a large extent, exercise-induced mitochondrial biogenesis in skeletal muscle. On the other hand, the triggering mechanisms mediating this process in adipose tissue have not been established. Hormonal factors such as epinephrine are intimately involved in the acute regulation of adipose tissue metabolism during exercise (McMurray & Hackney, 2005). Moreover, micromolar concentrations of epinephrine have been shown to induce PGC-1α mRNA expression in hepatocytes (Ding *et al.*, 2006). Given these findings it seems likely that epinephrine may be a mediator of PGC-1α mRNA expression in white adipose tissue and perhaps serve as an extracellular signal in the exercise mediated induction of PGC-1α.

As an initial approach to test this hypothesis we used adipose tissue organ culture and determined the dose-response and time-course of epinephrine induced increases in PGC-1α mRNA expression. Across a range of concentrations, we found that epinephrine markedly increased PGC-1α mRNA expression and that these changes preceded increases in Tfam mRNA expression.

Interestingly, epididymal adipose tissue appeared much more responsive to the effects of pharmacological doses of epinephrine as witnessed by larger increases in PGC-1α mRNA expression in epididymal adipose tissue and the absence of epinephrine-induced increases in Tfam mRNA expression in retroperitoneal adipose tissue. Consistent with these findings, PGC-1α mRNA expression in retroperitoneal adipose tissue organ cultures did not significantly increase when treated with supra-physiological (100 nM) concentrations of epinephrine. Our findings in epididymal adipose tissue are consistent with recent results from Miura and colleagues (2007) who reported that beta adrenergic stimulation leads to increases in PGC-1α mRNA expression in murine skeletal muscle.

Having shown that both exercise and epinephrine treatment induce PGC-1α mRNA expression in white adipose tissue we wanted to gain insight into a potential role of epinephrine in mediating the acute effects of exercise on the induction of PGC-1α mRNA expression in white adipose tissue. Rats were treated with the non-specific beta blocker propranolol prior to, and tissue harvested immediately following, 2 hours of swimming. In epididymal adipose tissue, beta blockade led to a ~40% reduction in the exercise-induced increase in PGC-1α mRNA expression. It should be noted that the same propranolol treatment blocked the epinephrine-induced rise in PGC-1α mRNA expression suggesting that this treatment was sufficient to block the effects of epinephrine on adipose tissue gene expression. The partial attenuation of the exercise-induced increase in PGC-1α mRNA expression in combination with the results of our epinephrine experiments, are consistent with the hypothesis that elevations in catecholamines may mediate a portion of the exercise-induced increase in PGC-1α mRNA expression in epididymal adipose tissue.

In contrast to epididymal adipose tissue, beta blockade did not significantly attenuate the exercise-induced increase in PGC-1α gene expression in the retroperitoneal fat pad. Given the greater effect of epinephrine on the induction of PGC-1α expression in epididymal compared to retroperitoneal adipose tissue, these results are not entirely surprising. Taken in combination with previous results showing enhanced lipolysis in epididymal versus retroperitoneal adipocytes (Tavernier *et al.*, 1995), our findings would suggest the existence of depot-specific differences in responsiveness to beta adrenergic stimulation. While the mechanisms underlying these apparent depot-specific differences are not clear, our results suggest that multiple extracellular signals are likely involved in the exercise induced up-regulation of PGC-1α expression in white adipose tissue. For example, exercise has been shown to increase circulating levels of thyroid hormone (Wirth *et al.*, 1981;Fortunato *et al.*, 2008;Limanova *et al.*, 1983), a hormone that has been shown, at least in skeletal muscle, to increase PGC-1α protein content (Branvold *et al.*, 2008). Along a similar line, the expression and secretion of interleukin 6 (IL-6) from skeletal muscle has been shown to increase dramatically during exercise (Pedersen & Febbraio, 2008). Interestingly, IL-6 has been shown to activate 5'AMP activated protein kinase (Kelly *et al.*, 2004), a reputed mediator of PGC-1α mRNA expression (Jager *et al.*, 2007). The potential roles of IL-6 and/or thyroid hormone in the regulation of PGC-1α in white adipose tissue are questions that need to be explored in further detail.

The purpose of the present study was to examine the effects of exercise and epinephrine on PGC-1α mRNA expression in white adipose tissue. Although swimming may not be representative of all exercise models, given the relatively large increases in catecholamines (Higashida *et al.*, 2008), we have made the novel observations that both acute swim exercise and long term training lead to increases in PGC-1α mRNA expression in white adipose tissue. Interestingly, it does not appear that beta adrenergic agonists are the sole regulators of exercise-induced increases in PGC-1α

mRNA expression. A further elucidation of the specific extracellular signals which regulate exercise induced increases in PGC-1α mRNA expression is an area ripe for investigation and will lend much insight into the regulation of adipose tissue gene expression by exercise.

Table 3.1. Sequences for the primers and probes used for real-time PCR procedures.

Gene of Interest	Forward	Reverse	Probe
PGC-1α	5′-GTGCAGCCAAGACTCTGTATGG-3′	5′-GTCCAGGTCATTCACATCAAGTTC-3′	5′-AGTGACATAGAGTGTGCTGCC-3′
PGC-1β	5′-CCGATCCCGGCAAACC-3′	5′-CAGAAGTTCCCTTAGGATGGAGAA-3′	5′-CCAAAGCCTTCTGGACTG-3′

Table 3.2. The effect of exercise training on body weight, epididymal and retroperitoneal fat pad weight, and food intake in male Wistar rats.

	Sedentary	Trained
Initial body weight (g)	248.5 ± 2.2	251.6 ± 2.7
Final body weight (g)	410.9 ± 4.8	389.4 ± 14.0
Weight gain (g)	162.4 ± 3.8	137.8 ± 13.8*
Epididymal fat pad (g)	4.4 ± 0.2	3.4 ± 0.3*
Retroperitoneal fat pad (g)	4.3 ± 0.4	2.0 ± 0.3*
Food intake (g (100 g body weight)$^{-1}$)	5.9 ± 0.2	6.4 ± 0.3

Data are presented as means ± S.E.M. for 8 per group. *$P < 0.05$ compared to sedentary values.

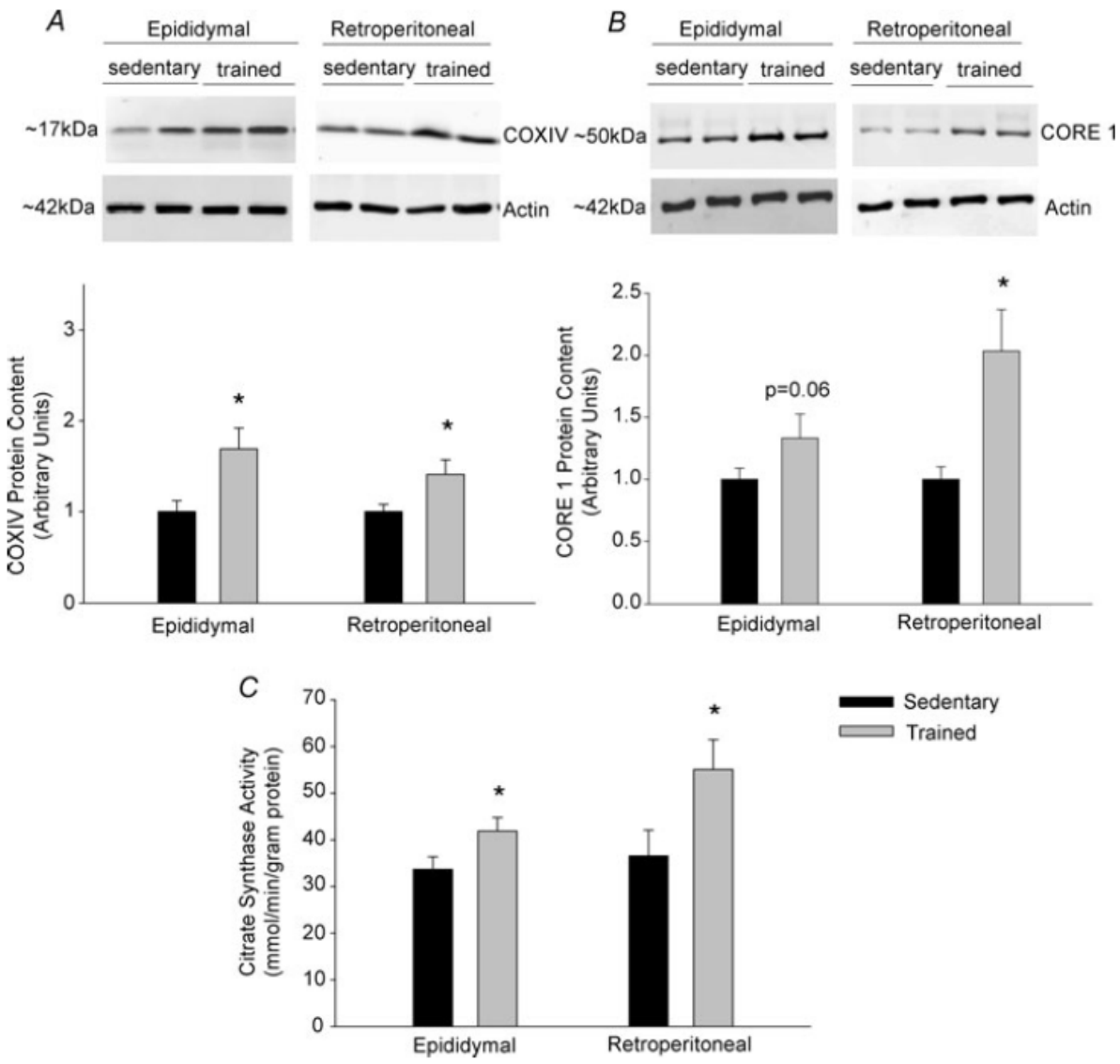

Figure 3.1. The effects of exercise training on COXIV protein content (A), CORE1 protein content (B) and citrate synthase activity (C) in rat epididymal and retroperitoneal adipose tissue. Data are presented as means + SE for 10-14 samples per group. Representative western blots for CORE1, COXIV and β-actin are shown above the quantified data and results were normalized to actin protein content; * $P<0.05$.

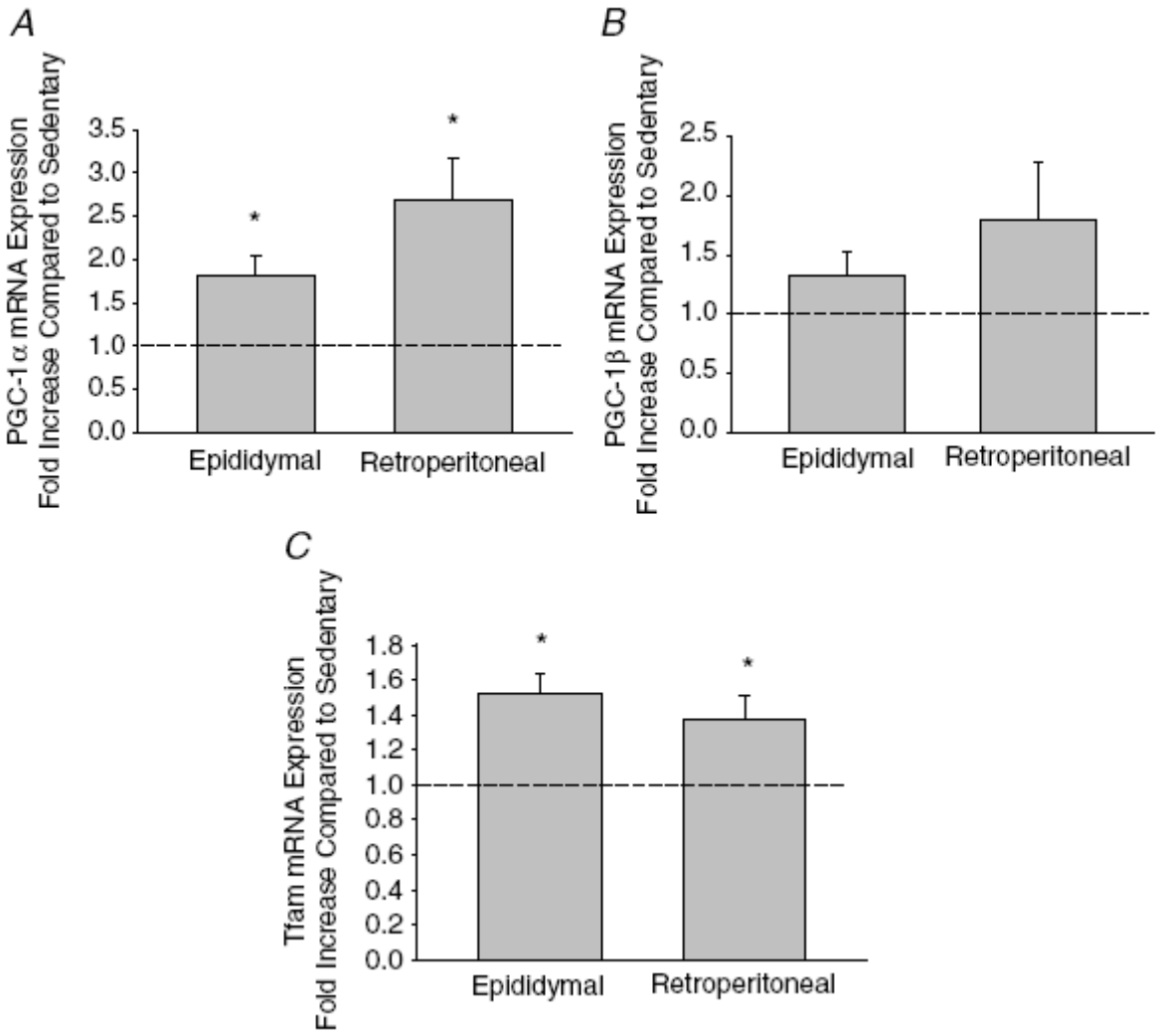

Figure 3.2. The effects of exercise training on the mRNA expression of PGC-1α (A), PGC-1β (B) and Tfam mRNA expression (C) in epididymal and retroperitoneal adipose tissue. Data are presented as means + SE for 10-14 samples per group, normalized to actin mRNA expression, and expressed as fold differences compared to sedentary controls; * $P<0.05$.

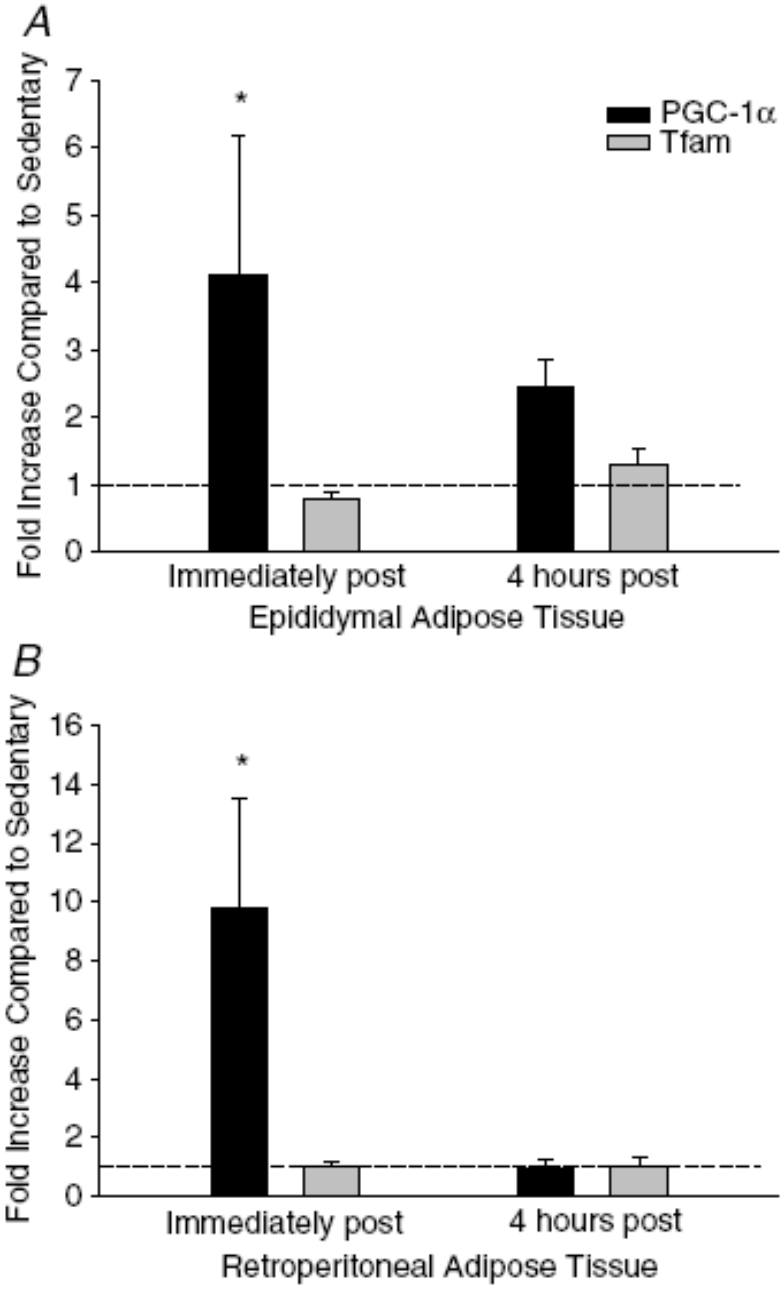

Figure 3.3. The time course of exercise induced increases in PGC-1α and Tfam mRNA expression in epididymal (A) and retroperitoneal (B) adipose tissue. Data are presented as means + SE for 5-6 samples per group, normalized to actin mRNA expression, and expressed as fold differences compared to sedentary controls; * $P<0.05$.

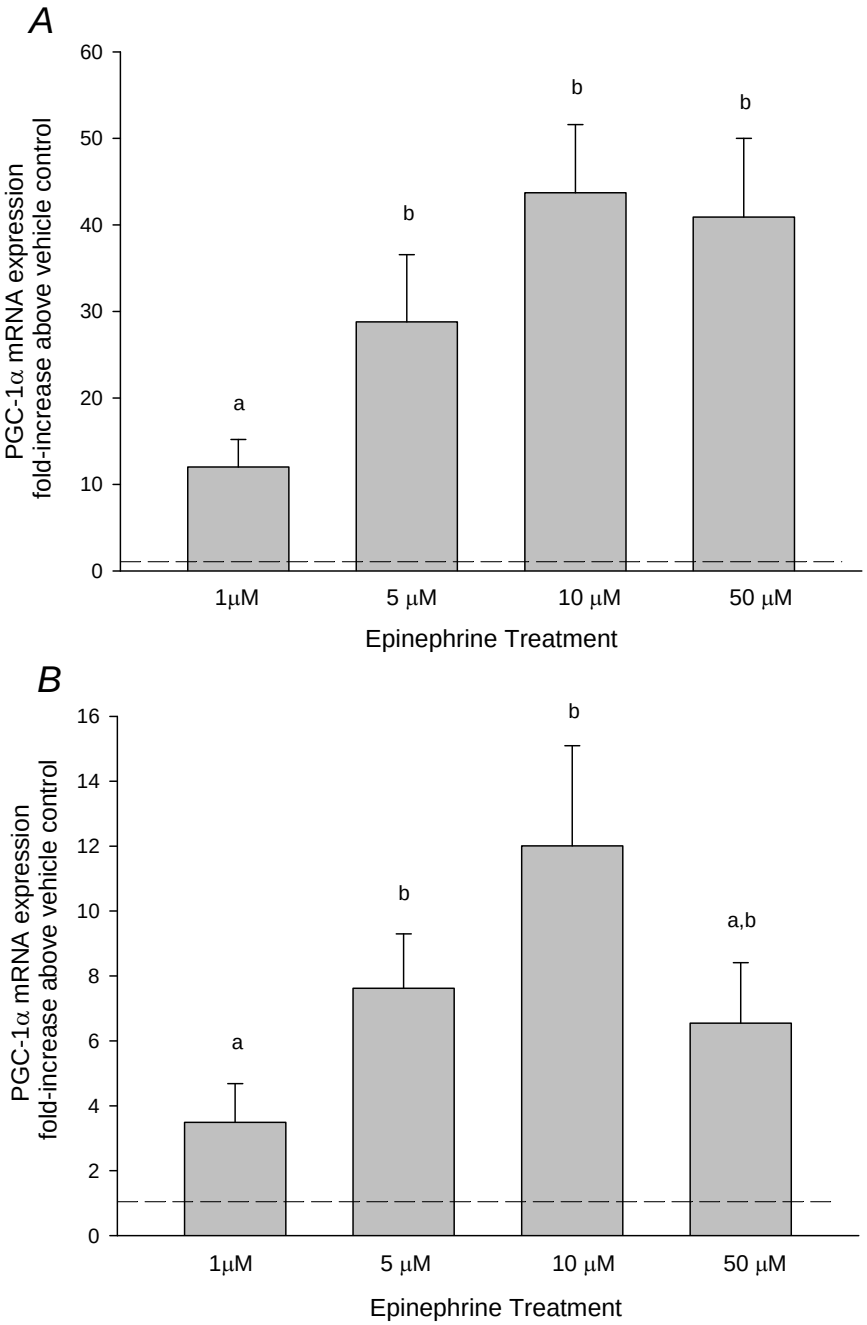

Figure 3.4. The dose-response of epinephrine on PGC-1α mRNA expression in epididymal (A) and retroperitoneal (B) adipose tissue organ cultures. Data are presented as means + SE for 4-6 samples per group, normalized to actin mRNA expression, and expressed as fold differences compared to vehicle treated controls; letters represent differences from vehicle control, different letters represent differences between treatment groups; $P<0.05$.

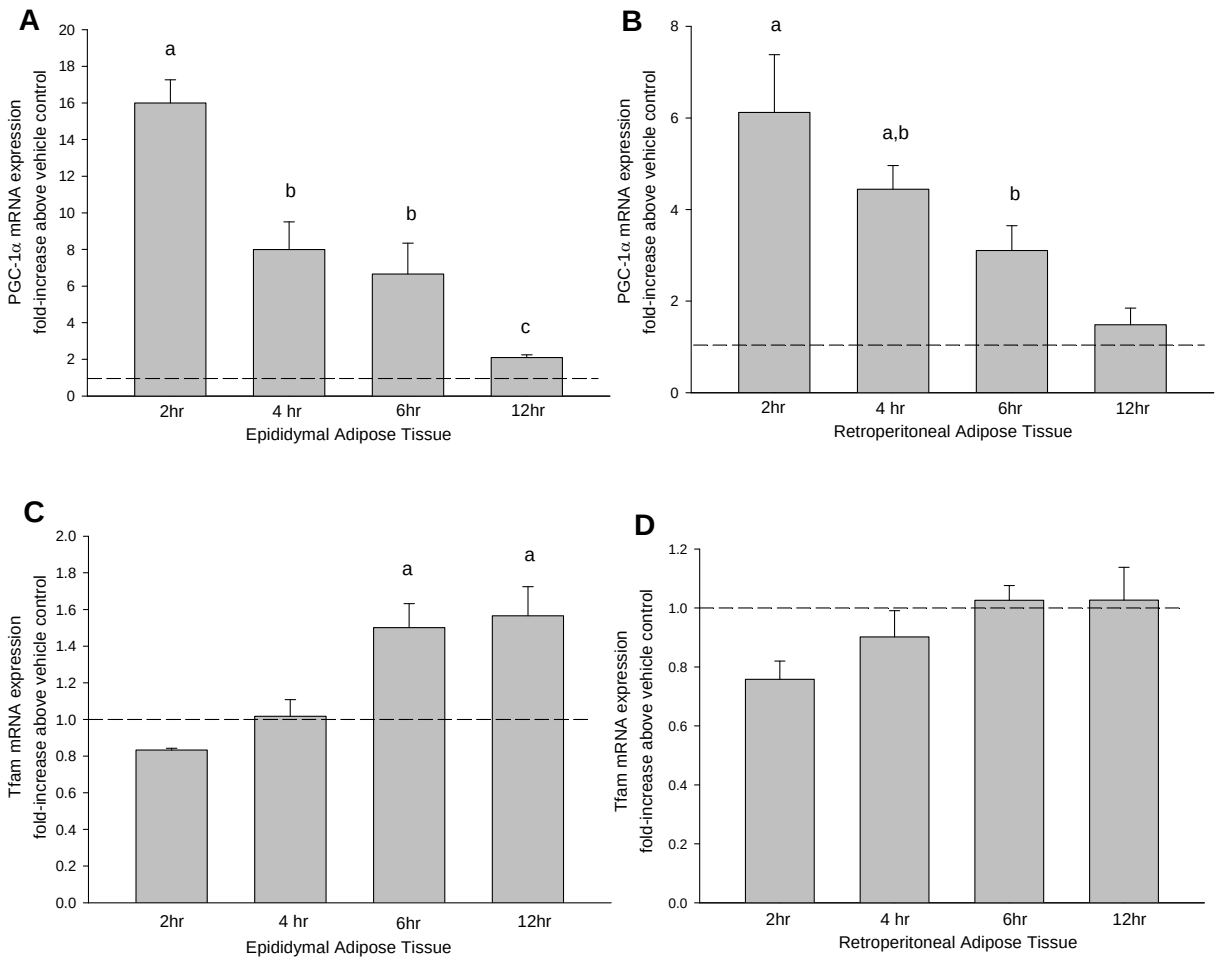

Figure 3.5. The time course of epinephrine (1 μM) induced increases in PGC-1α and Tfam mRNA expression in rat epididymal (A and C) and retroperitoneal (B and D) organ cultures. Data are presented as means + SE for 4-6 samples per group, normalized to actin mRNA expression, and expressed as fold differences compared to vehicle treated controls; letters represent differences from vehicle control, different letters represent differences between treatment groups; $P<0.05$.

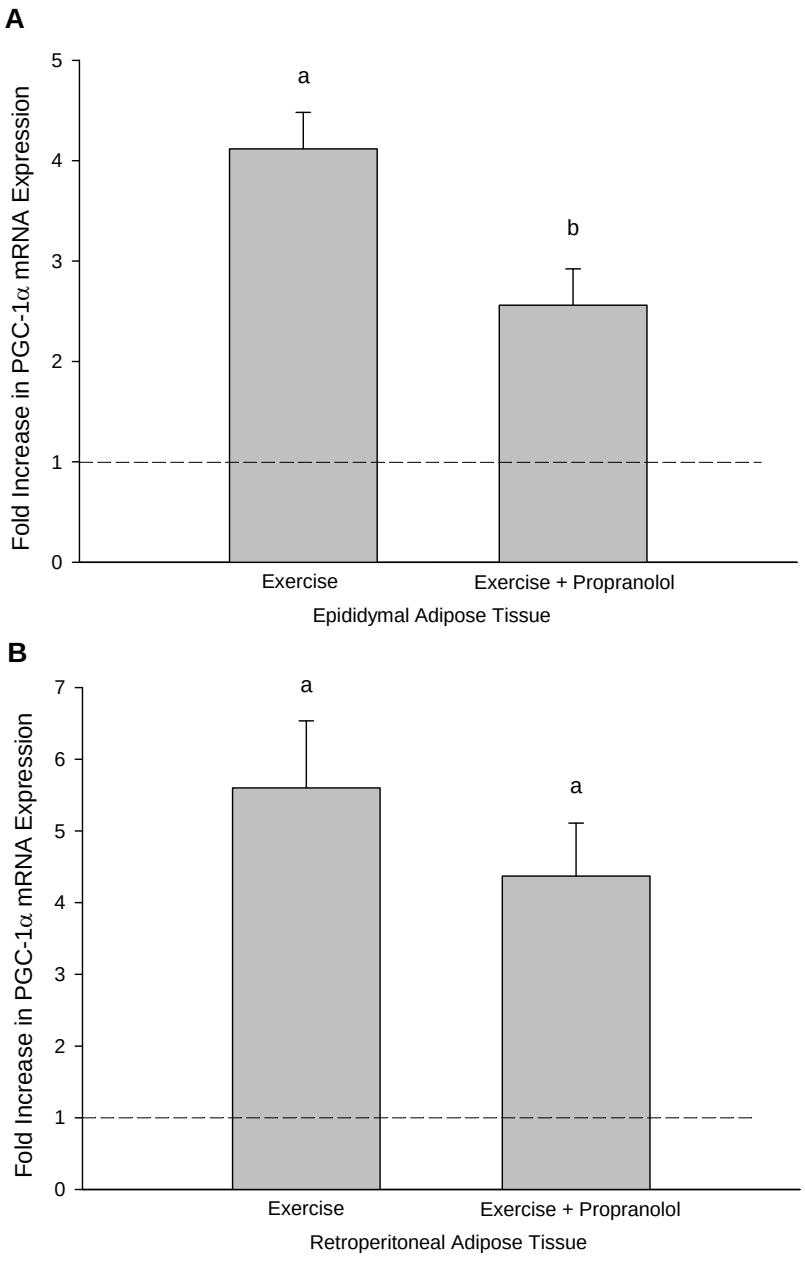

Figure 3.6. The effects of propranolol on the exercise induced increases in PGC-1α mRNA expression in rat epididymal (A) and retroperitoneal (B) adipose tissue. Data are presented as means + SE for 6-9 samples per group, normalized to actin mRNA expression, and are expressed as fold differences compared to non-exercised rats; letters represent differences from non-exercised rats, different letters represent differences between treatment groups; $P<0.05$.

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Chapter 4. General Summary and Discussion

4.1 SUMMARY OF RESULTS

The overall objective of this study was to explore the effects of exercise and epinephrine on adipose tissue PGC-1α mRNA expression in relation to changes in markers of adipose tissue mitochondrial biogenesis.

1. **The first objective of this thesis research was to confirm that exercise training results in an increase in markers of adipose tissue mitochondrial biogenesis and to determine a potential mediator of these mitochondrial adaptations.**

It was hypothesized that exercise training would increase PGC-1α mRNA expression, a reputed mediator of mitochondrial biogenesis, in association with increases in mitochondrial marker proteins, CORE1 and COXIV, and citrate synthase enzyme activity.

This hypothesis was supported, as it was shown that four weeks of swim training in male Wistar rats resulted in a significant increase in epididymal and retroperitoneal adipose tissue PGC-1α mRNA expression in concert with increases in CORE1 and COXIV protein expression and citrate synthase enzyme activity. An acute, two hour swim also increased PGC-1α mRNA expression in both adipose tissue depots.

2. **To determine whether epinephrine increases PGC-1α mRNA expression, since epinephrine is increased during exercise and is involved in other key pathways of lipid metabolism.**

It was hypothesized that treatment of adipose tissue organ cultures with epinephrine would result in an increase in adipose tissue PGC-1α mRNA expression.

This hypothesis was supported by the findings that incubation of adipose tissue organ cultures with epinephrine resulted in an increase in PGC-1α mRNA expression; however, this was most prominent in epididymal adipose tissue. Additionally, as shown in Figure D.1 of the Appendix

D, there is data to support that markers of mitochondrial gene expression, COXIV and COXI mRNA, were also increased in concert with PGC-1α.

3. **To determine if epinephrine is a key regulator of adipose tissue PGC-1α gene expression during exercise.**

It was hypothesized that the effect of exercise on PGC-1α mRNA expression in epididymal and retroperitoneal adipose tissue would be attenuated by treatment with a β-blocker prior to an acute bout of exercise. This would demonstrate that epinephrine plays a role in the regulation of exercise-induced PGC-1α mRNA expression in adipose tissue.

In Chapter 3 it was shown that the use of propranolol, a non-specific β-adrenergic antagonist, was able to partially attenuate the exercise-induced increases in PGC-1α mRNA expression in epididymal adipose tissue; however, there was no significant effect of propranolol on the retroperitoneal adipose tissue. These results coincided with the decreased response of retroperitoneal adipose tissue to *ex vivo* and *in vivo* epinephrine treatment. Collectively, these findings demonstrate a depot-specific response to epinephrine treatment and that PGC-1α regulation during exercise may differ between epididymal and retroperitoneal adipose tissue depots.

4.2 GENERAL DISCUSSION

4.2.1 Exercise Increases Adipose Tissue PGC-1α Gene Expression

Adipose tissue is now recognized as an active endocrine organ containing mitochondria which play a key role in the regulation of whole body metabolism. Similar to skeletal muscle, adipose tissue mitochondria have previously been shown to respond to exercise, which was demonstrated by increases in mitochondrial enzyme activity following 10 weeks of swimming (Stallknecht *et al.*, 1991). Although there has been a great deal of research focused on the regulation of skeletal muscle mitochondrial adaptations to exercise (Wu *et al.*, 1999; Goto *et al.*, 2000; Baar *et al.*, 2002), few studies have explored the regulation of adipose tissue mitochondrial biogenesis following exercise training. This study was able to show that 2 hours of daily swimming for 4 weeks leads to an increase in markers of adipose tissue mitochondrial biogenesis, such as CORE1 and COXIV protein and citrate synthase enzyme activity, which was consistent with the previous report by Stallknecht et al. (1991). It also demonstrated that adipose tissue PGC-1α mRNA expression increased in concert with the increased markers of mitochondrial biogenesis, providing support for PGC-1α playing a role in the regulation of mitochondrial biogenesis in adipose tissue. However, it should be noted that one caveat to these studies was that the PGC-1α protein was not measured. Changes in mRNA expression do not necessarily represent absolute changes in protein content and it is ideal to measure both mRNA and protein expression for the gene of interest. Our laboratory has previously tested all commercially available antibodies for PGC-1α, but we are not confident that the results obtained were specific to the PGC-1α protein using any of the antibodies tested. Therefore, our findings are specific to changes in PGC-1α mRNA expression. As this was the first known study to show an increase in PGC-1α mRNA expression in adipose tissue following exercise, we were then interested in looking at potential mediators of the increased PGC-1α gene expression.

4.2.2 Epinephrine Increases Adipose Tissue PGC-1α Gene Expression

Epinephrine is a potential regulator of exercise-induced increases in PGC-1α mRNA expression, as epinephrine is increased during exercise (Gollnick *et al.*, 1970; Galbo *et al.*, 1977), and catecholamines are known to play a role in the acute regulation of the metabolic response to exercise (McMurray & Hackney, 2005). Additionally, epinephrine and β-agonists have previously been shown to increase PGC-1α in hepatocytes (Ding *et al.*, 2006) and brown fat pre-adipocytes (Puigserver *et al.*, 1998), therefore there was a rationale for why it was thought that epinephrine would increase PGC-1α expression in white adipose tissue.

The treatment of adipose tissue organ cultures with epinephrine led to an increase in PGC-1α mRNA expression in both epididymal and retroperitoneal adipose tissue. However, an important observation was that the increase in epididymal adipose PGC-1α gene expression was over twice that of the retroperitoneal adipose organ cultures. Additionally, in epididymal adipose organ cultures the increase in PGC-1α was then followed by an increase in Tfam, a known regulator of mitochondrial-encoded mitochondrial genes (Chapter 3), as well as markers of mitochondrial gene expression, COXI and COXIV (Appendix D). In contrast, the much smaller increase in PGC-1α gene expression in retroperitoneal adipose organ cultures did not lead to an increase in Tfam or COXIV, and resulted in only a slight increase in COXI after 12 hours. This suggests that *ex vivo*, this level of increase in PGC-1α mRNA was not sufficient to result in changes in the expression of Tfam or markers of mitochondrial gene expression, at least at the time points examined. These results also highlight the likely involvement of other extra-cellular signals in the regulation of PGC-1α expression that may not be activated by epinephrine in retroperitoneal adipose tissue. Collectively, this provides further evidence for the role of increases in PGC-1α mRNA regulating mitochondrial

gene expression in adipose tissue, but that epinephrine regulation appears to be depot-specific with respect to PGC-1α gene expression.

Following the *ex vivo* treatment of adipose tissue with epinephrine, it was important to determine the effects of epinephrine *in vivo* and whether the increases in epinephrine during exercise were responsible for the observed changes in adipose tissue. Preliminary experiments were completed to determine the appropriate dose of propranolol, a non-specific β-adrenergic antagonist, required to block the effects of epinephrine. This dosage of propranolol was then administered to rats prior to a 2 hour swim. Propranolol has previously been used to study the effects of β-adrenergic stimulation on skeletal muscle PGC-1α mRNA expression (Miura *et al.*, 2007). The propranolol treatment resulted in an approximate 40% attenuation of the increase in PGC-1α mRNA expression compared to saline injected swim controls in epididymal adipose. However, the observation that β-blockade had no effect on the exercise-induced increases in retroperitoneal PGC-1α gene expression provided further support for the *ex vivo* data, suggesting that epinephrine does not play a significant role in the exercise induced regulation of PGC-1α in retroperitoneal adipose tissue.

The use of the non-specific β-adrenergic antagonist, propranolol, is just one way of studying the effects of β-blockade. There are also specific β_1-, β_2-, and β_3-aderengic receptor antagonists that could be used to study β-blockade (Carpene *et al.*, 1999) during exercise treatments, with the β_3-adrenergic receptor antagonist of greatest interest to adipose tissue studies. Future studies could also use adrenalectomy to study the adaptations to exercise in the absence of glucocorticoids (Berthiaume *et al.*, 2004). These surgeries are much more involved and an alternative exercise protocol may need to be used to avoid getting the surgical site wet, as would be the case with swimming. A third additional way of studying the effects of the absence of β-adrenergic stimulation would be to study

β-less mice, which have previously been used to measure the effect of exercise on skeletal muscle PGC-1α mRNA expression in this genetic model (Miura *et al.*, 2007).

4.2.3 Adipose Tissue Depot Specific Regulation

The depot-specific differences responsible for the diverse response to epinephrine in adipose tissue remain to be determined; however, there are a number of hypotheses that could explain these findings. It has been suggested that the differences between epididymal and retroperitoneal adipose tissue lies in their differences in sensitivity to β-adrenergic agonists. Tavernier and colleagues have shown that there is a decreased sensitivity to β-adrenergic agonists in retroperitoneal compared to epididymal adipose tissue in male Wistar rats (Tavernier *et al.*, 1995). The *ex vivo* epinephrine dose-response presented in Chapter 3 is consistent with the reduced β-adrenergic sensitivity hypothesis, as the increases in PGC-1α mRNA in retroperitoneal adipose tissue organ cultures were much smaller than that observed in epididymal adipose, even at very high concentrations of epinephrine (50μM). Furthermore, the PGC-1α mRNA expression in retroperitoneal adipose tissue organ cultures at the highest tested concentration of epinephrine began to decrease compared to the lower epinephrine concentrations. This suggested that increases in the concentration of epinephrine alone, are not sufficient to result in further increases in PGC-1α in retroperitoneal adipose tissue organ cultures. Since the increases in PGC-1α gene expression following exercise training were similar between adipose tissue depots, this suggests that retroperitoneal adipose may not only be less responsive to β-adrenergic agonists, but it appears that this depot also relies heavily on other extracellular signals to increase PGC-1α gene expression. Furthermore, the slightly greater response in retroperitoneal adipose tissue to an acute bout of swimming provides further support for depot-specific extracellular signals involved in regulating PGC-1α gene expression in retroperitoneal versus epididymal adipose tissue.

Another explanation for the depot-specific differences between fat pads could be linked to sympathetic nerve density. It has been shown that in response to fasting, retroperitoneal adipose tissue has an increase in sympathetic nerve density, whereas the epididymal adipose tissue appears to respond more heavily to hormonal regulators during fasting (Giordano *et al.*, 2005). However, with the propranolol injection and the acute swim study in Chapter 3, there would not be sufficient time for an increase in sympathetic nerve density to occur. This could potentially account for the lack of response to the β-blocker in retroperitoneal adipose tissue, however more studies would be required to examine this hypothesis.

Another potential reason for the differences in response to epinephrine in epididymal and retroperitoneal adipose tissue could be due to differences in the amount of β-adrenergic receptors in each depot. A number of studies have characterized the differences in β_1-, β_2-, β_3-, and α- adrenergic receptors in either human omental adipocytes (Richelsen *et al.*, 1991; Hellmer *et al.*, 1992) or pooled adipocytes from both retroperitoneal and epididymal adipose tissue in rats (Yang *et al.*, 2004) compared to subcutaneous. However, there is one study that measured the concentration of $\beta_{1/2}$- and α_2-adrenergic receptors in epididymal, retroperitoneal, and subcutaneous adipocyte membranes. It was found that there were no differences between epididymal and retroperitoneal adipose tissue, but that both of these visceral depots had significantly more $\beta_{1/2}$-adrenergic receptors and significantly less α_2-adrenergic receptors than the subcutaneous depot (Tavernier *et al.*, 1995). Studying the amount of β_3-adrenergic receptors would be of interest, since this is the main β-adrenergic receptor isoform in adipose tissue (Farias-Silva *et al.*, 2004). A more detailed characterization of the differences in the amount of α- and β-adrenergic receptors in these depots could provide a better understanding of the observed differences in epididymal and retroperitoneal response to β-adrenergic stimulation. It should be noted that 7-11 weeks of swim training of male Wistar rats resulted in an

increase in responsiveness of epididymal adipose tissue to β-adrenergic stimulation without an increase in the amount of β-adrenergic receptors (Bukowiecki *et al.*, 1980), but retroperitoneal adipose tissue was not studied.

Differences in the pathways downstream of the β-adrenergic stimulation and activation of cAMP may also be responsible for the depot-specific responses in epididymal and retroperitoneal adipose tissue. A recent study compared the effect of incubating epididymal, retroperitoneal, and subcutaneous adipocytes with AICAR, prior to epinephrine-stimulation. Although the basal levels of lipolysis were higher in the retroperitoneal compared to the other depots, the effect of the AMPK-activation and epinephrine treatment produced similar results between all adipocytes studied (Anthony *et al.*, 2009). However, this study was conducted in an opposite fashion to what normally occurs *in vivo*, where AMPK is normally activated as a result of epinephrine-stimulation, rather than occurring prior to increased epinephrine (Koh *et al.*, 2007; Anthony *et al.*, 2009). Studies have also examined increases in AMPK activity in epididymal adipose tissue (Moule & Denton, 1998) and 3T3-L1 adipocytes (Fu *et al.*, 2007) following treatment with a β-adrenergic agonist, as well as in epididymal adipose tissue following *in vivo* or *ex vivo* treatment with epinephrine (Koh *et al.*, 2007). However, a comparison between epididymal and retroperitoneal adipose tissue AMPK activation following β-adrenergic stimulation would be useful to determine whether this pathway is different between the two visceral adipose depots. Similarly, there may be differences between epididymal and retroperitoneal adipose tissue in the activation of the p38 MAPK pathway following β-adrenergic stimulation. This pathway has been examined in epididymal adipose tissue (Moule & Denton, 1998) and white adipocytes (C3H10T1/2 cell line) (Cao *et al.*, 2001), showing the ability of β-adrenergic stimulation to activate p38 MAPK. Nonetheless, a study comparing the activation of p38 MAPK and downstream increases in PGC-1α gene expression in both epididymal and

retroperitoneal adipose tissue could lead to a better understanding of the differences that were observed in response to exercise and epinephrine in this project.

4.2.4 Differences in Male and Female Rat Responses to Exercise and Epinephrine

A lot of emphasis has been placed on the importance of studying both epididymal and retroperitoneal adipose tissue, but it is also important to realize that there may be differences between male and female adipose tissue. It should be noted that although there is a similar increase in β-adrenergic agonist sensitivity (Bukowiecki *et al.*, 1980) and a similar increase in mitochondrial enzyme activity (Stallknecht *et al.*, 1991) between white adipose tissue of male and female Wistar rats, it is also known that exercise training in females results in very different changes in adipocyte morphology compared to males (Bukowiecki *et al.*, 1980). For example, in a 7-11 week swim training study, exercise trained female rats weighed ~5% less than their sedentary controls and the decrease in parametrial adipose tissue was due to both a reduction in adipocyte size and number (Bukowiecki *et al.*, 1980). Conversely, exercise trained male rats weighed ~20% less than sedentary controls following the 7-11 week swim protocol and the decrease in epididymal adipose tissue was due only to reductions in the size of the adipocytes (Bukowiecki *et al.*, 1980). It has also been demonstrated in female rats that an increased androgen to estrogen ratio, as occurs in post-menopausal females, promotes the accumulation of visceral adipose tissue by inhibiting AMPK activation and increasing lipogenesis (McInnes *et al.*, 2006). Since AMPK activation is known to increase PGC-1α mRNA expression in epididymal adipocytes of male rats (Gaidhu *et al.*, 2009), changes in female AMPK activity could potentially alter mitochondrial biogenesis due to changes in PGC-1α regulation. The impact of changes in androgen and estrogen levels in female rats on AMPK activation and lipogenesis further demonstrates the importance of studying specific age-groups and genders when investigating the regulatory pathways involved in lipid metabolism. Therefore, the

results from this study are potentially specific to male Wistar rats and further studies in female rats are required to explore whether or not the observed changes in PGC-1α and markers of mitochondrial biogenesis in response to exercise and epinephrine are sex-specific.

4.2.5 Comparison Between Adipose Tissue and Skeletal Muscle PGC-1α Regulation

It appears as though there are a number of other signaling mechanisms that could account for the differences in response to epinephrine between epididymal and retroperitoneal adipose tissue. The increases in PGC-1α mRNA expression following acute or chronic exercise (Goto *et al.*, 2000; Baar *et al.*, 2002) and epinephrine stimulation (Miura *et al.*, 2007) are similar to what has been demonstrated in skeletal muscle. Therefore, there may be extracellular signals, such as increased circulating epinephrine and downstream cellular effectors, such as p38 MAPK and AMPK, involved in adipose tissue that would be similar to what has been demonstrated in skeletal muscle.

One of the potential mediators of PGC-1α expression in adipose tissue may be AMPK, which has been demonstrated to increase PGC-1α expression in skeletal muscle (Terada *et al.*, 2002; Suwa *et al.*, 2003). AMPK activity is increased in adipose tissue with exercise (Ruderman *et al.*, 2003; Koh *et al.*, 2007) and AMPK is also proposed to increase PGC-1α expression in adipose tissue (Pirinen *et al.*, 2007; Gaidhu *et al.*, 2009). We have recently demonstrated in our lab that the treatment of adipose tissue organ cultures with AMPK agonists for 2 hours does increase PGC-1α mRNA expression (unpublished data). Furthermore, epinephrine treatment *in vivo* has been shown to increase AMPK activation in adipocytes and the use of the β-blocker propranolol prevented the exercise-induced increase in AMPK activity (Koh *et al.*, 2007). Treatment of 3T3-L1 adipocytes with β-adrenergic agonists also leads to an increase in lipolysis, the AMP:ATP ratio, and AMPK activity (Gauthier *et al.*, 2008). This increase in the AMP:ATP ratio and AMPK activity is subsequently blocked when co-treated with the general lipase inhibitor, orlistat (Gauthier *et al.*,

2008). Further research is required to determine exactly how the increase in the AMP:ATP ratio occurs. However, it does not appear to be due to a direct regulation from changes in cAMP or PKA activity, but may instead be partially due to the acylation of intracellular fatty acids released from lipolysis (Gauthier *et al.*, 2008). Although the data from this project suggests that, at least in epididymal adipose tissue, epinephrine plays a role in exercise-induced increases in PGC-1α mRNA expression, it is not known whether this is occurring through the activation of AMPK. It is also not known whether AMPK is responsible for the observed exercise-induced increases in adipose tissue mitochondrial biogenesis. One method to study this pathway would be to exercise an AMPK KO rodent, with and without treatment with a β-blocker, with the caveat that when using this transgenic rodent model, alternative pathways could overcompensate for the lack of AMPK and may complicate the interpretation of the data. Together, these studies have contributed to the proposed model of activation shown in figure 4.1.

Calcium is also believed to play a role in the regulation of skeletal muscle PGC-1α expression. Although the exact process has not been completely confirmed, there are a few suggested modes of action that have been explored. Increased calcium activates the calcium/calmodulin-dependent protein kinases (CAMKs). The activation of CAMK appears to play a role in the up-regulation of skeletal muscle PGC-1α during exercise (Wu *et al.*, 2002; Ojuka *et al.*, 2003). However, the regulation of PGC-1α by increases in calcium and CAMK activation appears to act, at least partially, through the phosphorylation and subsequent activation of p38 MAPK, a downstream mediator of CAMK (Enslen *et al.*, 1996; Chan *et al.*, 2004). p38 MAPK has been shown to phosphorylate PGC-1α and remove a repressor of PGC-1α transcriptional activity (Knutti *et al.*, 2001; Puigserver *et al.*, 2001), such as p160 mbp (Fan *et al.*, 2004), as well as increase the expression of PGC-1α through increasing promoter activity (Akimoto *et al.*, 2005). This is what has been demonstrated in skeletal

muscle, but has not been fully explored in adipose tissue. The treatment of adipocytes with epinephrine has been demonstrated to increase calcium binding to the plasma membrane fractions of the isolated adipocytes (McDonald & Jarett, 1980). For the calcium-activated CAMK pathway to be involved in the regulation of PGC-1α in adipose tissue, adipose tissue calcium levels would need to be increased, but this is unlikely and has not been shown. A more probable pathway would be through the coupling of cAMP by epinephrine. Stimulation of the β-adrenergic receptor by increased levels of epinephrine during exercise, activates the G-protein receptor mechanism that by activation of adenylate cyclase and resulting cAMP production, leads to the protein kinase A-dependent phosphorylation of cAMP response binding protein (CREB) and p38 MAPK (Shepherd & Bah, 1988; Xue *et al.*, 2005). As discussed above, p38 MAPK increases PGC-1α transcriptional activity by increasing promoter activity, such as CREB (Puigserver *et al.*, 1999; Xue *et al.*, 2005) (as depicted in figure 4.1). Therefore, these pathways may be another potential area of interest for future research in the area of adipose tissue PGC-1α regulation. In order to study the role of cAMP and p38 MAPK in the control of exercise-induced increases in adipose tissue PGC-1α mRNA expression, techniques similar to those used to explore the role of epinephrine in this project could be employed.

It has also been hypothesized that lactate may play a role in the regulation of PGC-1α expression and mitochondrial biogenesis in skeletal muscle. The incubation of L6 cells with lactate leads to a coordinated increase in PGC-1α mRNA expression, NRF-2 DNA binding, COX protein expression, and COXIV mRNA expression (Hashimoto *et al.*, 2007). Although this suggests an ability of lactate to increase mitochondrial biogenesis in coordination with increases in PGC-1α gene expression, it remains unknown whether lactate is responsible for changes in these pathways during exercise. Whether lactate is partially responsible for mitochondrial biogenesis and PGC-1α regulation in adipose tissue has not been explored. However, it has been demonstrated that exercise leads to

increases in adipose tissue lactate levels and that a large portion of this increase may be due to endogenous production in adipose tissue, rather than an increase in plasma lactate circulation (Ardevol *et al.*, 1997). The ability of adipose tissue to produce more lactate during exercise would support the possibility that lactate could play a role in PGC-1α and mitochondrial biogenesis regulation during and after exercise.

4.2.6 Discussion of Exercise Protocol

There are a number of rodent models of endurance exercise protocols that are commonly used, including treadmill training, voluntary wheel running, and swimming. Limitations to the swim protocol are that there is no quantitative measurement of the amount of exercise performed by each individual rat and that swimming results in a relatively large increase in circulating catecholamines in the rat (Higashida *et al.*, 2008). In contrast, a benefit to the treadmill and wheel running protocols is the ability to quantitatively measure the amount of exercise being performed by setting the speed and angle of the treadmill (Miura *et al.*, 2007; Leick *et al.*, 2008) and collecting measurements of the duration and running speed using the cage wheel (Rodnick *et al.*, 1989; Halseth *et al.*, 1995). Disadvantages to the treadmill protocol are that shocking devices are often required to keep the animals running (Brown *et al.*, 2007) and the treadmills are costly and require a designated room. Disadvantages to the wheel running protocol are that there may be large differences in the amount of exercise performed by each rat (Rodnick *et al.*, 1989) and these sophisticated computers and cage wheels are also expensive. The swim training protocol was chosen for this experiment as it has been used extensively for many similar studies looking at changes in skeletal muscle (Arias *et al.*, 2007; Wright *et al.*, 2007) and adipose tissue (Stallknecht *et al.*, 1991) mitochondria, in addition to the fact that swim training is cost-effective, does not require a large work area, and many rats can be exercised at one time.

An important methodological aspect of swim training experiments is the control group. Stallknecht et al. (1991) demonstrated that there was no significant difference in the adipose tissue mitochondrial enzymes between a sedentary control, a sham control (swam 2 minutes per day), or a cold-stressed group (kept at 4°C). The reason for the lack of response in the sham and cold-stressed groups has not been determined. However, it is known that cold stress increases plasma norepinephrine, but not epinephrine concentrations in Wistar rats (Storm *et al.*, 1981; Picotti *et al.*, 1982). It has also been shown that adrenal gland weight and adrenal medulla volume per body weight is increased in swim-trained rats, but not in sedentary control, sham swim, or cold-stressed groups (Stallknecht *et al.*, 1990). If future research is able to provide further support for the role of epinephrine as a key extracellular signaling mechanism of exercise-induced increases in PGC-1α mRNA expression and mitochondrial proteins, then the lack of increase in circulating plasma epinephrine may account for the differences between swim trained and the sham or cold-stressed groups.

Similarly, the water temperature is also very important. It has been demonstrated that swimming at 35-37°C does not alter the rectal temperature of the trained rats (Baker & Horvath, 1964; Stallknecht *et al.*, 1991), whereas swimming at 20°C decreases and 42°C increases the rectal temperature of the rats and leads to exhaustion prior to 45 minutes in both the 20°C and 42°C water (Baker & Horvath, 1964). Therefore, a 2 minute per day sham swim and a water temperature of 35-37°C was chosen for the swim experiments for this project.

Future studies could include the use of other exercise techniques, such as voluntary wheel-running or treadmill running to compare results between different methods of exercise in rats.

4.2.7 Relation to Type 2 Diabetes Research

Other future studies could include studying the effects of exercise in rodent models of obesity, insulin resistance, or Type 2 Diabetes. These studies could be used to determine whether exercise is able to prevent or reverse the decreases in PGC-1α mRNA expression and mitochondrial biogenesis observed in high fat feeding (Sutherland *et al.*, 2008) and genetic models of obesity, insulin resistance and Type 2 Diabetes (Choo *et al.*, 2006; Rong *et al.*, 2007). Thiazolidinediones (TZDs) are a class of drugs used to improve insulin-sensitivity and glucose and lipid metabolism in Type 2 Diabetics. TZDs are potent PPARγ agonists that increase adipose tissue PGC-1α mRNA expression and markers of mitochondrial protein content, similar to the effects observed from exercise training (Stallknecht *et al.*, 1991; Wilson-Fritch *et al.*, 2004; Rong *et al.*, 2007). Currently, there are reservations about the use of TZDs as they are associated with a long list of serious side effects, such as fluid retention, weight (fat) gain, and increased incidence of heart attack (Li *et al.*, 2008). However, these side effects are likely due to the non-specific activation of other transcription factors and nuclear receptors (Li *et al.*, 2008). Therefore, a better understanding of the mechanisms involved in TZD and exercise-induced increases in adipose tissue PGC-1α expression are needed to help improve Type 2 Diabetes drug therapy. The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is a model of hyperphagic obesity, yet the rats continue to run voluntarily on cage wheels when provided. This model has been used to study skeletal muscle adaptations in obesity with or without exercise (Morris *et al.*, 2008), and future research could measure changes in adipose tissue mitochondrial and PGC-1α regulation from these obese animals with or without exercise. Understanding the pathways involved in the prevention and reversal of obesity and Type 2 Diabetes induced decreases in mitochondrial and PGC-1α expression could be very beneficial to the field of diabetes research.

4.2.8 Conclusion

This project was designed to study the effects of exercise and epinephrine on PGC-1α mRNA expression in white adipose tissue. It was confirmed that chronic exercise leads to increases in markers of adipose tissue mitochondrial biogenesis, as previously described in male Wistar rats (Stallknecht *et al.*, 1991). The novel observations were made that acute and long term swim training leads to increases in PGC-1α mRNA expression in both epididymal and retroperitoneal white adipose tissue. Due to the fact that β-blockade only partially attenuated the exercise-induced increases in PGC-1α mRNA expression in epididymal adipose, and not at all in the retroperitoneal depot, it appears that β-adrenergic agonists do not exclusively control the regulation of PGC-1α mRNA expression during exercise. There does appear to be very different regulatory mechanisms between epididymal and retroperitoneal adipose tissue depots that need to be further explored with a broadening of research to study other extracellular signals and effectors that may be involved.

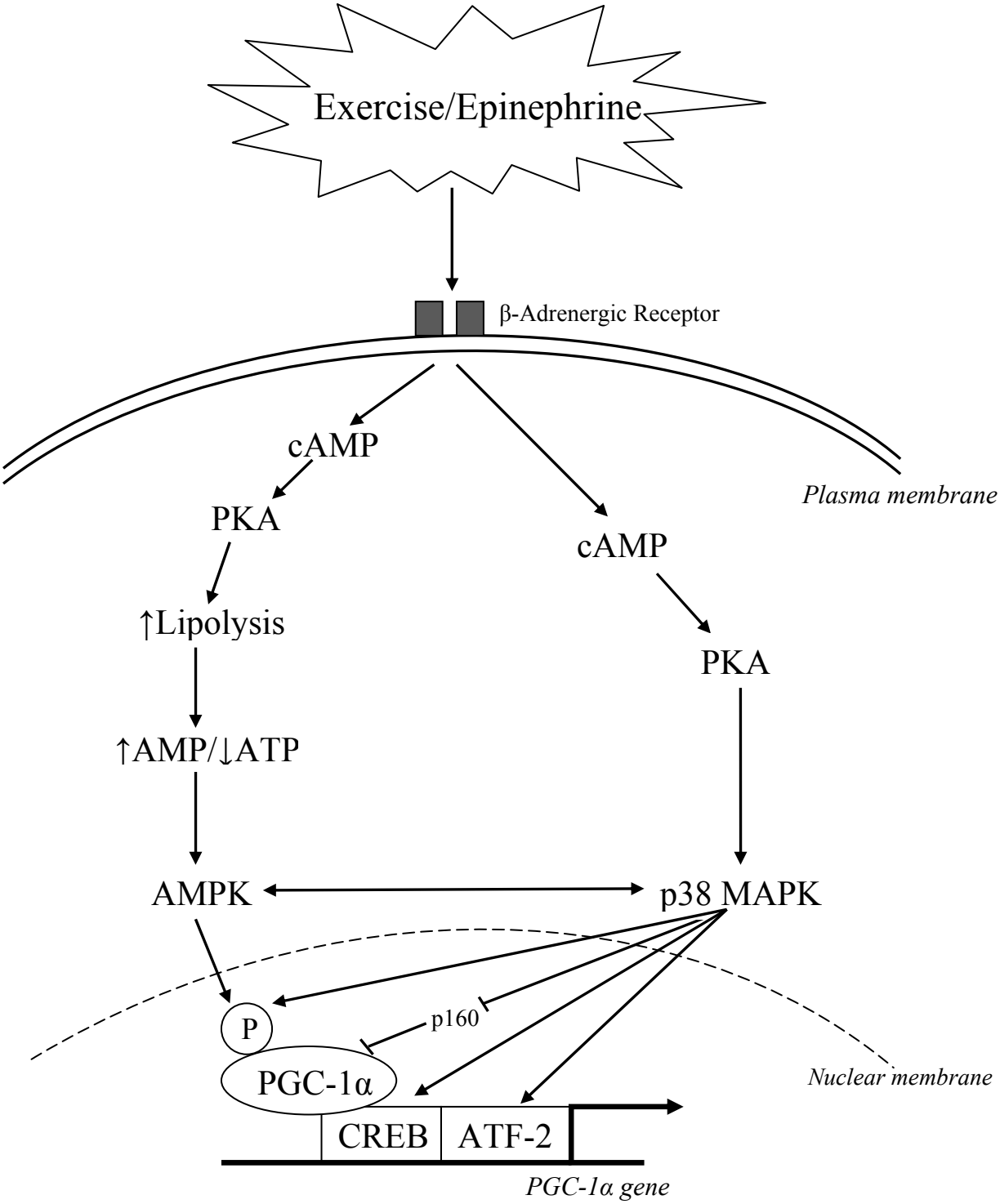
Exercise/Epinephrine
β-Adrenergic Receptor
cAMP
PKA
↑Lipolysis
↑AMP/↓ATP
AMPK
Plasma membrane
cAMP
PKA
p38 MAPK
P
p160
PGC-1α
Nuclear membrane
CREB
ATF-2
PGC-1α gene

Figure 4.1. Proposed mechanisms leading to exercise-induced increases in PGC-1α mRNA expression in adipose tissue. Exercise and epinephrine activate the β-adrenergic receptors which results in increased lipolysis and downstream effects on AMPK and PGC-1α phosphorylation. Additionally, cAMP activates p38 MAPK, which has been shown to phosphorylate PGC-1α, inhibit the p160 repressor of PGC-1α, and activate the transcription factors CREB and ATF-2 on the PGC-1α promoter. Overall, this leads to an increase in PGC-1α transcription. Interaction between AMPK and p38 MAPK may also be an important part of this pathway.

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APPENDIX A

Additional Methods for Real Time RT-PCR

RNA was extracted and cDNA was prepared as described in section 3.3.7 of Chapter 3. An additional Taqman Gene Expression Assay (Applied Biosystems, Streetsville, ON) was used to determine the mRNA expression of NRF-1. Primers and probes for COXIV and COXI were designed using Primers Express 3.0 software (Table A.1).

Table A.1. Sequences for the primers and probes used for real-time PCR procedures**.**

Gene of Interest	Forward	Reverse	Probe
COX IV	5'-CTACCAGGGCACTTAGCCTAAT-3'	5'-TTGACGTGGGCCACATC-3'	5'-TAGTCTTCACTCTTC ACAACACTCCCATGT-3'
COXI	5′-TGCTGAAGGAGGAGCATCCT-3′	5′-TGAGGCGAGTGGTCTGGAA-3′	5′-TGGGATGACGAGCAG C-3′

Equal efficiency of amplification was determined for the PGC-1α, PGC-1β, COXI, and COXIV probes. Figure A.1 shows the slopes of cycle thresholds (CT) versus the log of the dilution of the cDNA for PGC-1α and β-actin to determine if these primers/probes were amplified with equal efficiency. If the primer/probe set are amplified with 100% efficiency, the slope would be -3.33; therefore, slopes of -3.5964 and -3.4922 are within an acceptable range (Nicklas *et al.*, 2004). The efficiency is determined by $E = -1 + [10^{(-1/slope)}]$, with 1.0 being a perfect efficiency relationship. For the slopes obtained from PGC-1α and β-actin, efficiency is close to 1.0 and so they are considered to be acceptable for use. Similar slopes were found for the other primer/probes used.

Data was analyzed using the $2^{-\Delta\Delta CT}$ method as previously determined to be a reliable method for analyzing quantitative RT-PCR data (Livak & Schmittgen, 2001). Briefly, $\Delta\Delta C_T$ is determined by $(C_{T,Target} - C_{T,Actin})_{Time\ x} - (C_{T,Target} - C_{T,Actin})_{Time\ 0}$ (Livak & Schmittgen, 2001). Time *x* is any time point, where Time 0 is determined by the averages of the target and β-actin CT values, therefore normalizing the values to the β-actin.

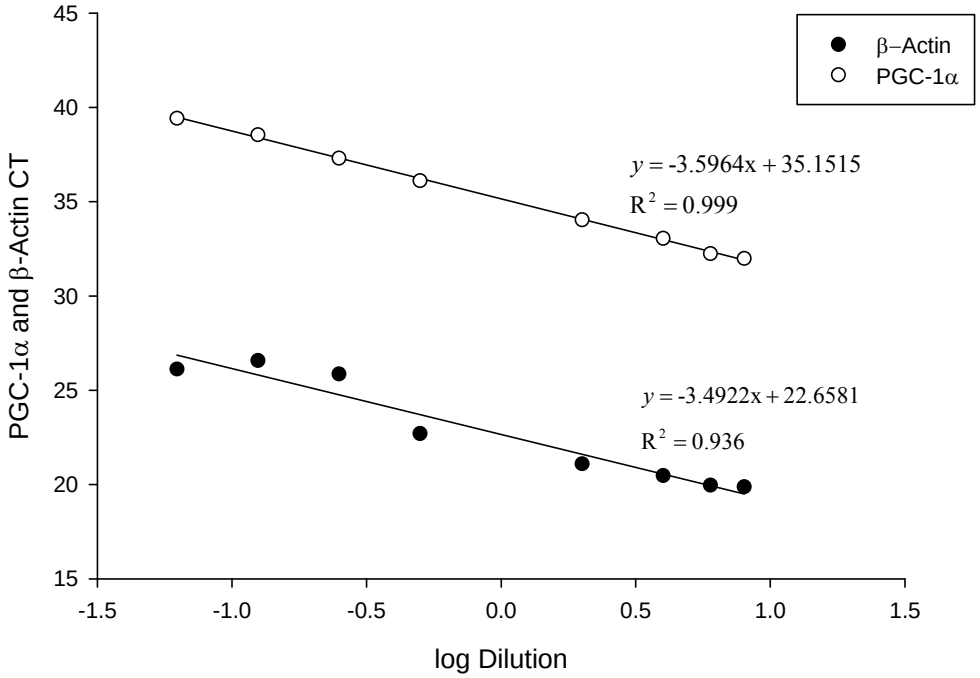

Figure A.1. Slopes of CT versus the log dilution of the cDNA to determine whether the PGC-1α and β-actin are amplified with equal efficiency.

For the assessment of beta actin, Tfam, and NRF-1 mRNA expression, 1µl of gene expression assay, 1µl of cDNA, 10µl PCR Master Mix, and 8µl of nuclease free water were added to each well. For PGC-1α and PGC-1β, 12.5µl of PCR Master Mix, 0.225µl each of forward and reverse primers, and 0.05µl of probe were added to each well. For COXI and COXIV, 10µl of PCR Master Mix, 0.225µl each of forward and reverse primers, and 0.05µl of probe were added to each well. Additionally, an optimal ratio of cDNA to reagents was determined for each of the designed probes by a trial test and the amplification curves were assessed for the most desirable efficiency. The following ratios were determined: for PGC-1α, 4µl of cDNA with 3µl of nuclease free water, for PGC-1β, 2µl of cDNA with 5µl of nuclease free water and for COXIV and COXI, 1µl of cDNA with 8.5µl of nuclease free water was added to each well in addition to the above reagents. Together, the reagents, cDNA, and nuclease free water added up to a total of 20µl per well.

APPENDIX B

Additional Methods for Citrate Synthase Activity

Frozen adipose tissue samples were homogenized and protein extracted as described in section 3.3.5 on Western Blotting from Chapter 3. Citrate synthase activity was determined by measuring the formation of 5-thio-2-nitrobenzoic acid (TNB) spectrophotometrically (412 nm) in a microplate reader as described in the manufacturer's instructions (Sigma, MO, USA).

The assay works due to the following series of reactions that occur in the tissue extract. The citrate synthase in the adipose tissue protein extractions catalyzes the reaction between acetyl coenzyme A (acetyl CoA) and oxaloacetic acid, resulting in the formation of citric acid. The thioester of acetyl CoA is hydrolyzed to form a thiol group, CoA-SH. This thiol group then reacts with the 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in the reaction forming a yellow product, TNB acid, that is measured spectrophotometrically at an absorbance of 412nm.

The following is the reaction catalyzed by citrate synthase:

$$\text{Acetyl CoA} + \text{Oxaloacetate} \rightarrow \text{Citrate} + \text{CoA-SH} + H^{+} + H_2O$$

Below is the colourimetric reaction that produces the TNB used to represent the relative amount of citrate synthase activity in the sample:

$$\text{CoA-SH} + \text{DTNB} \rightarrow \text{TNB} + \text{CoA-S-S-5-TNB}$$

By comparing the amount of TNB in each of the samples, a relative difference in the amount TNB will represent the relative amount of citrate synthase present in the samples.

APPENDIX C

Exercise Training Increases NRF-1 mRNA Expression in Epididymal Adipose Tissue

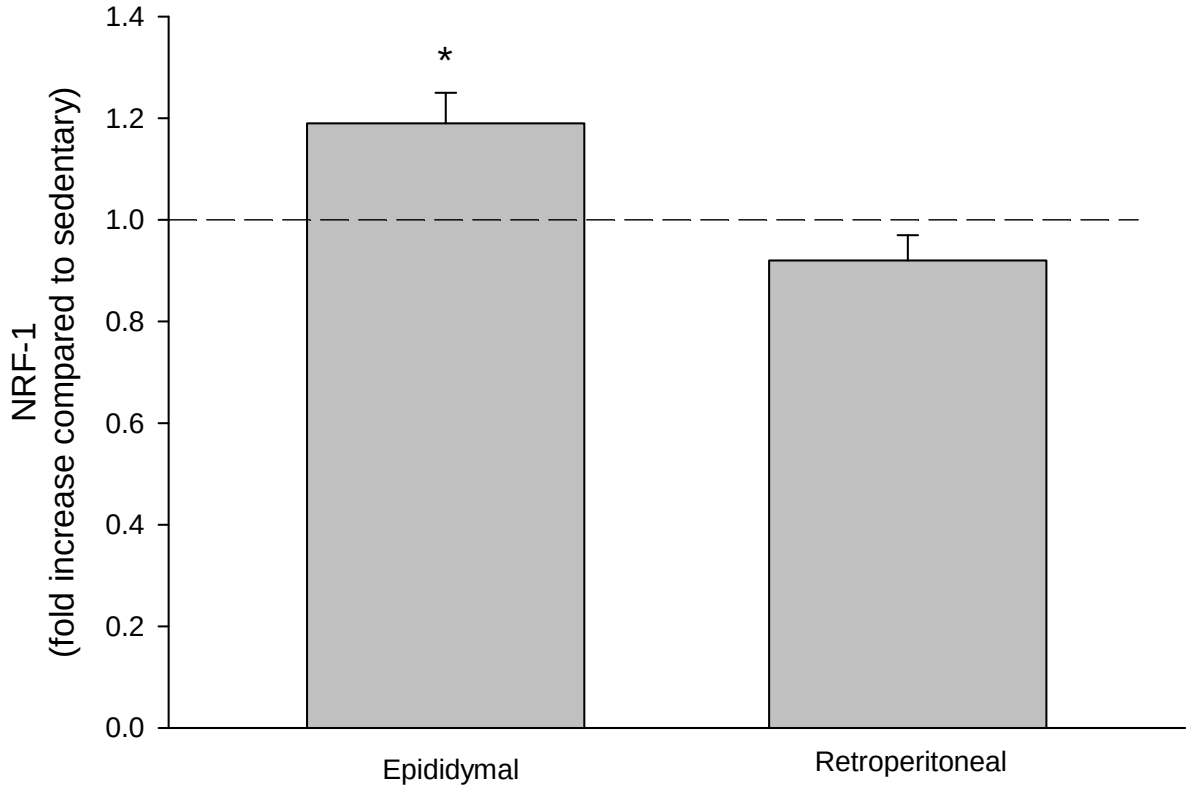

Figure C.1. The effects of exercise training on the mRNA expression of nuclear respiratory factor (NRF)-1 mRNA expression in epididymal and retroperitoneal adipose tissue. NRF-1 is a transcription factor known to regulate the expression of nuclear encoded mitochondrial genes and is controlled by PGC-1α. There was a significant increase in the epididymal adipose tissue, but no change in retroperitoneal adipose tissue. This data was not included in the accepted version of the Sutherland et al. (2009) *J Physiol* **587**(7):1607-1617. Data are presented as means + SE for 10-14 samples per group, normalized to actin mRNA expression, and expressed as fold differences compared to sedentary controls * $P<0.05$.

APPENDIX D

Time Course of Increases in COXI and COXIV mRNA Expression in Adipose Tissue Organ Culture

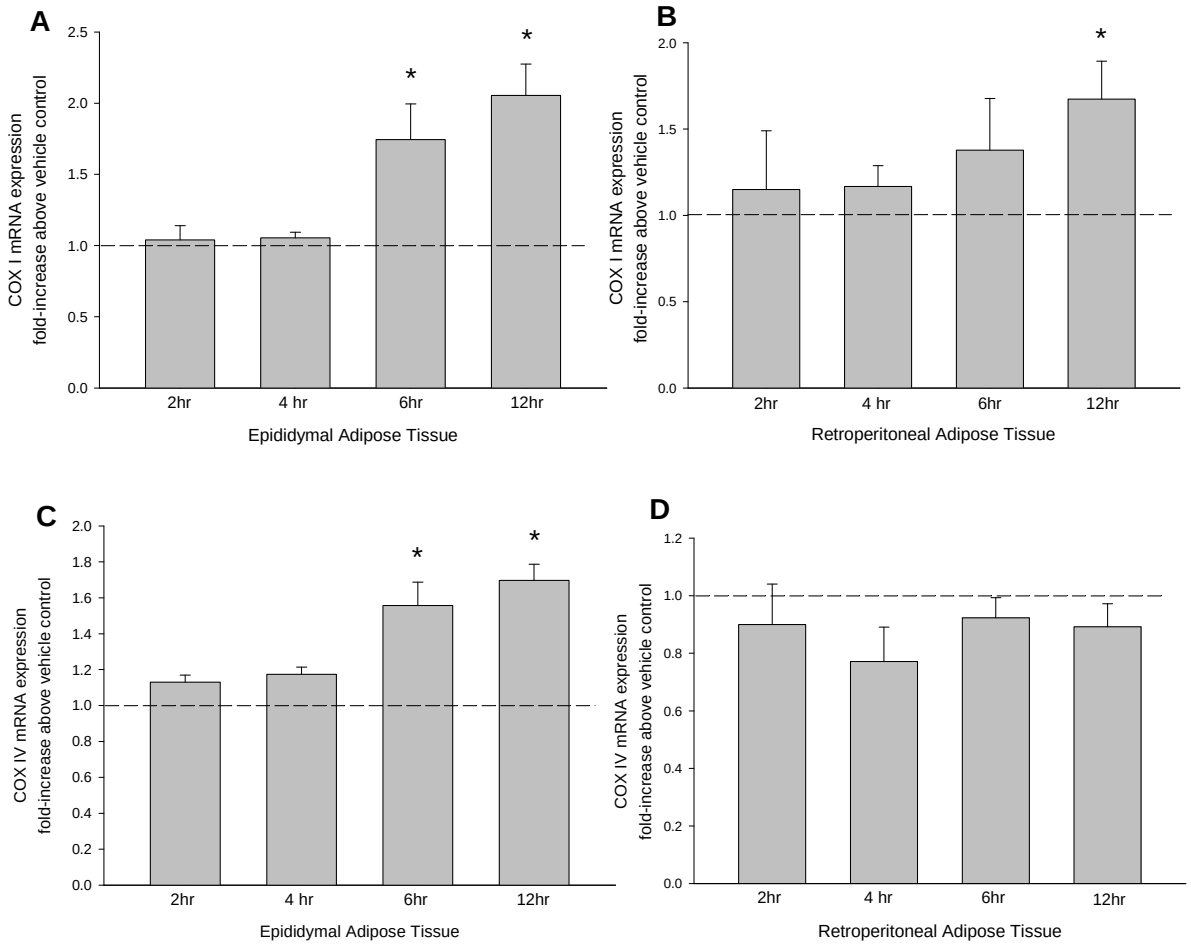

Figure D.1. The time course of epinephrine (1 μM) induced increases in cytochrome c oxidase subunit (COX) I and IV mRNA expression in rat epididymal (A and C) and retroperitoneal (B and D) organ cultures. COXI is a mitochondrial-encoded mitochondrial respiratory chain enzyme, whereas COXIV is a nuclear-encoded mitochondrial respiratory chain enzyme. Therefore, they were both examined to confirm upstream control by Tfam (controls mitochondrial-encoded mitochondrial genes) and NRF-1 (controls nuclear-encoded mitochondrial genes). Interestingly, as shown in Figure C.1, NRF-1 was not increased in the

retroperitoneal adipose tissue following swim training, and here, COXIV mRNA was not increased in the retroperitoneal adipose tissue following treatment with epinephrine. Although it is not completely clear whether these two findings are related, they do present an interesting finding for future research. This data was not included in the accepted version of the Sutherland et al. (2009) *J Physiol* **587**(7):1607-1617. Data are presented as means + SE for 4-6 samples per group, normalized to actin mRNA expression, and expressed as fold differences compared to vehicle treated controls, * $P<0.05$.

APPENDIX E

Adipose Tissue Organ Culture Treatment with a Supra-physiological Dose of Epinephrine

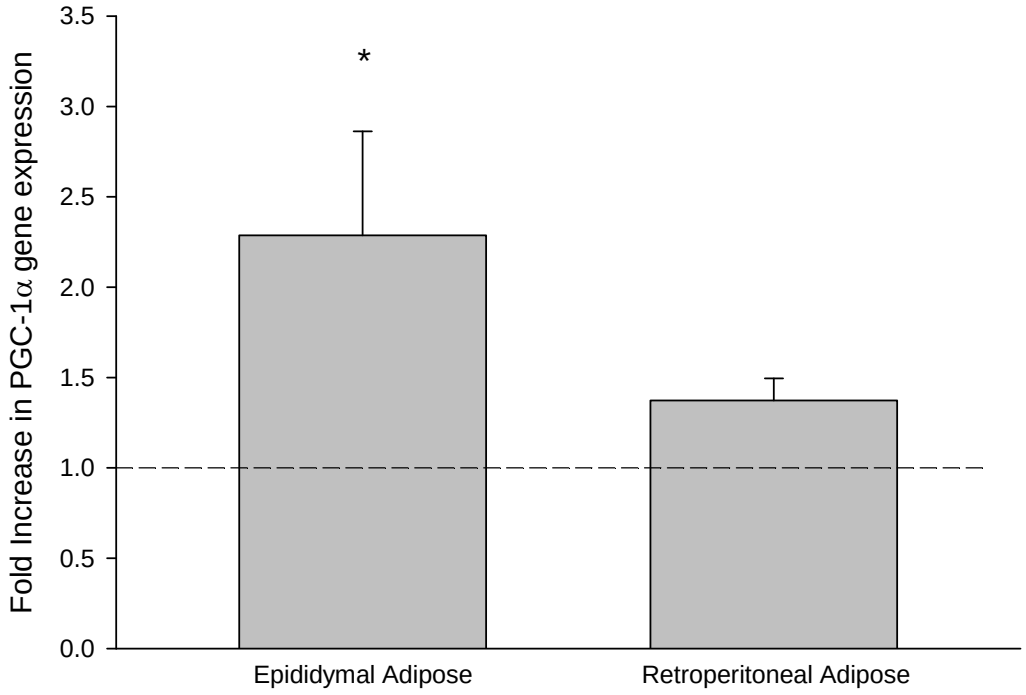

Figure E.1. The effect of a supra-physiological dose of epinephrine on PGC-1α mRNA expression in epididymal and retroperitoneal adipose tissue organ cultures. Treatment of epididymal and retroperitoneal adipose tissue with 100nm of epinephrine for 2 hours led to a significant increase in epididymal adipose tissue PGC-1α mRNA expression, but no change in the retroperitoneal adipose tissue. This is not entirely surprising since at the higher concentration of epinephrine treatment (1μm) as shown in Chapter 3, Figures 3.4 and 3.5, the increase in PGC-1α mRNA expression in the retroperitoneal adipose tissue was 2.5-fold less than the increase in the epididymal adipose tissue. *$P<0.05$ significant increase compared to vehicle control.

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