The Effect of Increased Mitochondrial Biogenesis and Activation of PPAR Pathways in a Mouse Model of Aging

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UNIVERSITY OF MIAMI

THE EFFECT OF INCREASED MITOCHONDRIAL BIOGENESIS AND ACTIVATION OF PPAR PATHWAYS IN A MOUSE MODEL OF AGING

By

Lloye M. Dillon

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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THE EFFECT OF INCREASED MITOCHONDRIAL BIOGENESIS AND
ACTIVATION OF PPAR PATHWAYS IN A MOUSE MODEL OF AGING

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Aging is the progressive decline in cellular, tissue and organ function. The mitochondrial theory of aging suggests that the accumulation of mitochondrial DNA (mtDNA) mutations leads to mitochondrial dysfunction, loss of organ function and consequently a decrease in lifespan. This theory is appealing as there is a correlation between age-dependent alterations in mtDNA and an increased risk for developing cardiovascular diseases, neurodegenerative disorders and myopathy. To further investigate the role of mtDNA mutations in aging, the mtDNA mutator mouse, a mouse model with a proof-reading deficient mtDNA polymerase \( \gamma \) (POLG) was created. These mice have a premature aging phenotype and develop hair loss, anemia, kyphosis, sarcopenia, cardiomyopathy and decreased lifespan. This phenotype was associated with an accumulation of mtDNA mutations and mitochondrial dysfunction, suggesting that there is a link between mtDNA mutations, mitochondrial dysfunction and the aging phenotype in mammals.

The work presented in this dissertation demonstrates three strategies employed to compensate for mitochondrial dysfunction in aging using the mutator mouse as a model system. We illustrate that increased mitochondrial biogenesis and activation of peroxisome proliferator-activated receptor (PPAR) pathways can improve some aging phenotypes in the mutator mouse. In chapter 2, we show that increased expression of
PPAR γ coactivator-1α (PGC-1α), a crucial regulator of mitochondrial biogenesis and function, in muscle of mutator mice increased mitochondrial biogenesis and function, and also improved the skeletal muscle and heart phenotypes of the mice. However, deep sequencing analysis of mtDNA showed that the increased mitochondrial biogenesis did not reduce the accumulation of mtDNA mutations in the mutator mouse but rather caused a small increase. Therefore, our results indicate that increased muscle PGC-1α expression is able to improve some premature aging phenotypes in the mutator mice without reverting the accumulation of mtDNA mutations.

Bezafibrate is pharmacological agent that activates peroxisome proliferator-activated receptors (PPARs) and PGC-1α pathways that has been shown to improve mitochondrial function and energy metabolism. In chapter 3 we show that mutator mice treated with bezafibrate for 8-months had delayed hair loss and improved skin and spleen phenotypes. Bezafibrate did not induce global mitochondrial biogenesis/function in mutator mouse; instead it increased mostly markers of fatty acid oxidation. Although we observed positive effects, bezafibrate induced hepatomegaly and did not slow the development of sarcopenia or increased the lifespan of the mutator mice. Our results show that despite its toxic effects, bezafibrate improved some aging phenotypes in the mutator mouse.

Because increased PGC-1α expression in muscle conferred benefits to mutator mice, in chapter 4 we created wild-type and mutator mice that inducibly and ubiquitously express either PGC-1α or its family member PGC-1β. We found that increased systemic expression of PGC-1β was toxic and caused lethality, however, ubiquitous induction of
PGC-1α did not appear to be deleterious. These animals are valuable tools for studying the effects of systemic increases in mitochondrial biogenesis during aging.
DEDICATION

To my husband Philip Dillon, my parents Maurice and Rose Reid

and my brother Troy Reid
ACKNOWLEDGEMENTS

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ABBREVIATIONS

AICAR : 5-Aminoimidazole-4-Carboxamide Ribonucleoside
AMPK : Adenosine Monophosphate Kinase
ATP : Adenosine Triphosphate
ATP5A : ATP synthase subunit 5 alpha
BASO : Basophil
BMC : Bone Mineral Content
BMD : Bone Mineral Density
cAMP : Cyclic Adenosine Monophosphate
COX : Cytochrome c Oxidase
CRMs : Control Region Multimers
CREB : cAMP response element binding protein
CS : Citrate Synthase
D-loop : Displacement loop
Dox : Doxycycline
DRIP : Vitamin D Receptor-Interacting Protein
EOSS : Eosinophil
ERRα : Estrogen Related Receptor alpha
ETC : Electron Transport Chain
GAPDH : Glyceraldehyde 3-Phosphate Dehydrogenase
HAT : Histone Acetyltransferase
HCT : Hematocrit
HGB : Hemoglobin
HIF1α : Hypoxia Inducible Factor 1 alpha
HIF2α : Hypoxia Inducible Factor 2 alpha
IFM : Interfibrillar Mitochondria
LV : Left Ventricle
LVID: d : Left Ventricular Internal Dimension, diastole
Lymph : Lymphocytes
MCH : Mean Corpuscular Hemoglobin
MCHC : Mean Corpuscular Hemoglobin Concentration
MCK : Muscle Creatine Kinase
MCV : Mean Corpuscular Volume
MNC : Monocytes
MTCO1 : Mitochondrial Cytochrome c Oxidase subunit 1
MTCO2 : Mitochondrial Cytochrome c Oxidase subunit 2
MtDNA : Mitochondrial DNA
Mut : Mitochondrial DNA Mutator Mouse
ND1 : NADH Dehydrogenase subunit 1
NDUFB8: NADH Dehydrogenase [Ubiquinone] 1 Beta subcomplex subunit 8
NGS : Next Generation Sequencing
OXPHOS: Oxidative Phosphorylation
PGC-1α : Peroxisome Proliferator-Activated Receptor gamma Coactivator-1alpha
PGC-1β : Peroxisome Proliferator-Activated Receptor gamma Coactivator-1beta
POLG : Mitochondrial DNA Polymerase gamma
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>PPAR</td>
<td>Peroxisome Proliferator-Activated Receptor</td>
</tr>
<tr>
<td>PRC</td>
<td>Peroxisome Proliferator-Activated Receptor gamma Coactivator-related coactivator</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>rtTA</td>
<td>Reverse Tetracycline Transactivator</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate Dehydrogenase</td>
</tr>
<tr>
<td>SDHA</td>
<td>Succinate Dehydrogenase subunit A</td>
</tr>
<tr>
<td>SDHB</td>
<td>Succinate Dehydrogenase subunit B</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>SSM</td>
<td>Subsarcolemmal Mitochondria</td>
</tr>
<tr>
<td>TFAM</td>
<td>Mitochondrial Transcription Factor A</td>
</tr>
<tr>
<td>TFB1M</td>
<td>Mitochondrial Transcription Factor b1</td>
</tr>
<tr>
<td>TFB2M</td>
<td>Mitochondrial Transcription Factor b2</td>
</tr>
<tr>
<td>TRAP</td>
<td>Thyroid Hormone Receptor-Associated Protein</td>
</tr>
<tr>
<td>TRE</td>
<td>Tetracycline Response Element</td>
</tr>
<tr>
<td>Trnd</td>
<td>tRNAd</td>
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<td>Trnk</td>
<td>tRNAk</td>
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<tr>
<td>Trns1</td>
<td>tRNAs1</td>
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<tr>
<td>UQCRC2</td>
<td>Ubiquinol-Cytochrome c Reductase Core protein 2</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>WBC</td>
<td>White Blood Cell</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-Type</td>
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CHAPTER 1
INTRODUCTION

1.1 Overview

Aging is the progressive decline in cellular, tissue and organ function. This complex process often manifests as loss of muscular strength, cardiovascular function and cognitive ability. Mitochondrial dysfunction and decreased mitochondrial biogenesis are believed to participate in metabolic abnormalities and loss of organ function, which will eventually contribute to aging and decreased lifespan. In this chapter, we discuss what is currently known about mitochondrial dysfunction in the aging skeletal muscle and heart. My work is focused on the role of PGC-1 coactivators in the regulation of mitochondrial biogenesis and function and possible therapeutic benefits of increased mitochondrial biogenesis in compensating for mitochondrial dysfunction and circumventing aging and aging-related diseases.

1.2 Mitochondria and Aging

Mitochondria are important for many cellular processes. However, they are primarily responsible for providing cells with energy in the form of adenosine triphosphate (ATP). ATP is produced by oxidative phosphorylation (OXPHOS) using the energy released from electron transfer on the mitochondrial inner membrane. In order to meet the cell’s changing energy requirements, the process of mitochondrial biogenesis needs to regulate mitochondrial protein expression and function in the cell (Handschin and Spiegelman, 2006). The mitochondrial theory of aging has long proposed
mitochondrial dysfunction as a player in the aging process. Recent studies have supported a link between mitochondrial dysfunction associated with aging and decreased mitochondrial biogenesis. A study comparing the transcriptional profile of aging humans to aging mice and flies found that the expression levels of mitochondrial electron transport chain components decrease with age in all three organisms (Zahn et al., 2006). This suggests that across species, mitochondrial biogenesis is decreased during aging.

Over the last few years, the transcriptional coactivators of the peroxisome proliferator-activated receptor (PPAR) gamma coactivator-1 (PGC-1) family have been shown to be crucial regulators of mitochondrial biogenesis and function (Finck and Kelly, 2006; Handschin and Spiegelman, 2006; Mirebeau-Prunier et al., 2010; Philp et al.; Scarpulla, 2008). This family includes PGC-1α and PGC-1β, which are present in many cell types, but more highly expressed in energy demanding tissues such as heart, brain, skeletal muscle and kidney, and PGC-related coactivator (PRC) which is expressed in all tissues (Handschin and Spiegelman, 2006; Lin et al., 2002a; Puigserver et al., 1998). Lately, several independent studies have supported the hypothesis that the PGC-1 protein family is associated with aging. The levels of PGC-1α protein were found to be lower in aged mice than in young mice (Vina et al., 2009) and loss of PGC-1α has been suggested to be an important contributor to mitochondrial dysfunction in aging-associated diseases (Conley et al., 2007b). Besides, a recent study by Sahin et al showed that mitochondrial dysfunction in a mouse model of telomere dysfunction, a phenomenon proposed to be a cause of aging, was associated with repressed levels of PGC-1α and PGC-1β (Sahin et al., 2011). This observation revealed a connection between aging in the
nucleus and the mitochondria (Kelly 2011) and supported the idea that decreased mitochondrial biogenesis and function are key contributors to aging.

1.3 PGC-1 Coactivators and the Regulation of Mitochondrial Biogenesis

Mitochondria are composed of ~ 1500 proteins which are encoded by both the nuclear and mitochondrial DNA (mtDNA). The mtDNA encodes only 13 of these polypeptides, which are subunits of mitochondrial OXPHOS. It also encodes for 2 rRNA genes and 22 tRNA genes required for mitochondrial protein synthesis. All other mitochondrial proteins are encoded by the nuclear DNA. Consequently, the process of mitochondrial biogenesis requires coordination between these two genomes. This coordination is regulated primarily by the PGC-1 family of transcriptional coactivators, which work by activating transcription factors. However, they do not interact directly with DNA. As described below, they bind to several different transcription factors that bind DNA. PGC-1 coactivators also interact with a histone acetyltransferase (HAT) complex that consists of cAMP response element binding protein (CREB)/ p300 that facilitates chromatin remodeling and also with the thyroid hormone receptor-associated protein/vitamin D receptor-interacting protein (TRAP/DRIP) mediator complex in order to activate DNA transcription [reviewed in (Finck and Kelly, 2006; Handschin and Spiegelman, 2006)]. In addition to regulating mitochondrial biogenesis, the PGC-1 coactivators are involved in modulating other metabolic pathways including fatty acid oxidation (FAO), lipogenesis, gluconeogenesis and thermogenesis [reviewed in (Finck and Kelly, 2006; Handschin and Spiegelman, 2006)] (Fig. 1.1). This is in part due to their
ability to coactivate members of the PPAR nuclear receptor family, consisting of PPARα, PPARβ/δ and PPARγ, which are master regulators of lipid and energy metabolism (Wang 2010).

**Figure 1.1. The functions of PGC-1 coactivators in highly oxidative tissues.** PGC-1 coactivators activate a number of transcription factors to regulate mitochondrial biogenesis, fatty acid oxidation (FAO) and other processes in various tissues.

The PGC-1 family initiates mitochondrial biogenesis by activating transcription factors that regulate the expression of nuclear DNA encoded mitochondrial proteins (Anderson and Prolla, 2009). These transcription factors include the nuclear respiratory factor 1 and 2 (NRF-1 and NRF-2) and the estrogen related receptor alpha (ERRα) (Virbasius et al., 1993a; Virbasius et al., 1993b). The activation of these transcription
factors increases the expression of many mitochondrial proteins including the mitochondrial transcription factor A (TFAM) and the mitochondrial transcription factors b1 and b2 (TFB1M and TFB2M respectively) (Rebelo et al., 2011; Wu et al., 1999). TFAM is essential for mtDNA replication, transcription and maintenance (Rebelo et al., 2011; Wu et al., 1999). Therefore, by regulating TFAM levels, the PGC-1 coactivators are able to influence the expression of proteins encoded by the mtDNA. The proteins encoded by the nuclear DNA, are imported into the mitochondria where some will combine with proteins encoded by the mtDNA to form the multi-subunit complexes of the mitochondrial OXPHOS. Therefore, the PGC-1 family regulates mitochondrial biogenesis by coordinating the expression of mitochondrial proteins encoded by both the nuclear and mitochondrial genomes (Fig.1.2).

Although PGC-1α acts mainly in the nucleus; more recently, Aquilano et al suggested that PGC-1α and the NAD⁺-dependent protein deacetylase sirtuin 1 (SIRT1), another pivotal regulator of mitochondrial biogenesis, are also located in the mitochondria (Aquilano et al., 2010). This is an interesting model as prior to this study these proteins were thought to function only in the nucleus. Using fluorescent microscopy, the authors showed that PGC-1α and SIRT1 did not only co-localize with the nuclear Hoechst stain but also with cytochrome c, a mitochondrial protein used to label the mitochondrial network (Aquilano et al., 2010). In addition, they showed that TFAM interacts with PGC-1α and SIRT1 in the mitochondria to form multiprotein complexes (Aquilano et al., 2010). Moreover, they found that the mtDNA D-loop region was present in cross-linked mitochondria immunoprecipitated with anti-PGC-1α antibody.
PGC-1 coactivators are master regulators of mitochondrial biogenesis. PGC-1 coactivators regulate mitochondrial biogenesis by coordinating the expression of mitochondrial proteins encoded by both the nuclear and mitochondrial genome. To accomplish this, they activate transcription factors such as NRF-1/2 and ERRα thereby increasing the expression of nuclear DNA encoded mitochondrial proteins. These mitochondrial proteins are then imported into the mitochondria. One such protein is TFAM, which when unregulated, leads to increased mtDNA replication and increased expression of mtDNA-encoded proteins. These processes initiate an increase in mitochondrial biogenesis.

Figure 1.2. PGC-1 coactivators are master regulators of mitochondrial biogenesis. PGC-1 coactivators regulate mitochondrial biogenesis by coordinating the expression of mitochondrial proteins encoded by both the nuclear and mitochondrial genome. To accomplish this, they activate transcription factors such as NRF-1/2 and ERRα thereby increasing the expression of nuclear DNA encoded mitochondrial proteins. These mitochondrial proteins are then imported into the mitochondria. One such protein is TFAM, which when unregulated, leads to increased mtDNA replication and increased expression of mtDNA-encoded proteins. These processes initiate an increase in mitochondrial biogenesis.

and that PGC-1α and SIRT1 were present in nucleoid (a poorly defined structure containing mtDNA and proteins) enriched mitochondrial fractions (Aquilano et al., 2010). It will be interesting to confirm if PGC-1α has a mitochondrial localization, whether it is directly involved in the regulation of mtDNA transcription/replication and whether PGC-1β and PRC are also present in mitochondria.

Despite sharing similar core functions in the regulation of mitochondrial biogenesis, PGC-1β differs from PGC-1α in its mode of regulation. For example, in contrast to PGC-1α, the expression of PGC-1β in brown adipose tissue and skeletal muscle is not increased by cold exposure and exercise respectively (Arany et al., 2005;
This observation suggests that PGC-1β controls basal mitochondrial biogenesis while PGC-1α controls mitochondrial activity in response to external stimuli (Arany et al., 2005). In addition, PGC-1α and PGC-1β are able to coactivate different transcription factors (Lin et al., 2005). Furthermore, PGC-1α and PGC-1β have some non-redundant roles and both are important to maintain optimal body function since their loss appears to be a key factor in mitochondrial dysfunction, aging and many disease states (Arany et al., 2005; Conley et al., 2007b; Sonoda et al., 2007; Vianna et al., 2006).

Studies performed in mammalian cells and mouse models have highlighted the importance of the PGC-1 family in the regulation of mitochondrial biogenesis. The overexpression of PGC-1α and PGC-1β in C2C12 muscle cells, cardiac myocytes and primary rat neurons was shown to increase mitochondrial density, the expression of nuclear and mitochondrial genes and mitochondrial function (Lehman et al., 2000; St-Pierre et al., 2003; Wareski et al., 2009). Also, increased PGC-1α expression in mouse heart and skeletal muscle triggers an increase in mitochondrial biogenesis (Russell et al., 2004; Wenz et al., 2009b). Accordingly, the ablation of PGC-1α in mouse skeletal muscle and heart leads to decreased expression of most nuclear and mitochondria encoded genes (Arany et al., 2005), decreased mitochondrial enzyme activity and mitochondria content (Adhihetty et al., 2009). Similarly, PGC-1β knockout (KO) mice have reduced expression of nuclear and mitochondrial encoded genes important for mitochondrial function and decreased mitochondrial volume in the heart and muscle (Lelliott et al., 2006; Sonoda et
al., 2007). These studies demonstrate that PGC-1α and PGC-1β play pivotal roles in regulating mitochondrial biogenesis and function.

1.4 Mitochondria and the Aging Skeletal Muscle

In this section, we discuss mitochondrial dysfunction and its implication on aging in skeletal muscle with emphasis on sarcopenia. We also discuss the potential benefits of the PGC-1 coactivators in ameliorating aging-associated metabolic dysfunction in the skeletal muscle.

1.4.1 Mitochondrial Dysfunction in the Aging Skeletal Muscle

The skeletal muscle is essential for movement, body posture and for support of internal organs of the body. It is composed of different fiber types (I, IIA, IIB and IIX) that are classified based on their expression of the myosin heavy chain (MHC) isoforms. Type IIB fibers (‘fast glycolytic’) are composed of few mitochondria and rely primarily on glycolysis for ATP production to perform fast and powerful activity (Arany, 2008a). On the contrary, mitochondria are abundant in type I (‘slow oxidative’) and also in type IIA and IIX (‘fast oxidative/glycolytic’) fibers and produce ATP by OXPHOS making these fibers more fatigue resistant and abundant in glycogen stores (Arany, 2008a).

As one ages, there is a slow but increasing loss of skeletal muscle mass and function. This condition, known as sarcopenia, is a world-wide public health concern since it affects the entire aging population and leads to dependence on locomotion aids and increases the incidence of falls and bone fractures (Ryall et al., 2008). Because
sarcopenia is such a prevalent condition, many studies have been performed to understand the cellular and molecular mechanisms that are involved in its progression. Over the years, several theories have been proposed to explain sarcopenia. These theories suggest that changes in muscle protein synthesis and degradation, muscle cell apoptosis, denervation/renervation and endocrine changes contribute to the development of sarcopenia (Combaret et al., 2009). Several studies have also proposed a contribution of mitochondrial dysfunction to skeletal muscle aging. The abundance of mtDNA and mitochondrial mRNA transcripts and proteins was shown to decrease with age in the skeletal muscle of healthy men and women (Short et al., 2005). These changes were associated with an increase in DNA oxidation in the skeletal muscle (Short et al., 2005), suggesting that decreased mitochondrial biogenesis leads to mitochondrial dysfunction and increased oxidative DNA damage in the aging skeletal muscle. In another study of aging humans, 20% to 30% of skeletal muscle fibers in individuals between the ages of 67 and 92 were shown to have a defect in the mitochondrial OXPHOS (Bua et al., 2006). This defect was characterized by decreased activity of the nuclear and mitochondrial encoded cytochrome c oxidase (COX) (complex IV) and increased activity of succinate dehydrogenase (complex II) which is entirely nuclear encoded, suggesting that there is a decrease in mitochondrial gene expression (Bua et al., 2006). The fibers with a defective OXPHOS also had high levels of mtDNA deletion mutations (Bua et al., 2006). Additionally, the expression of PGC-1 coactivators and genes involved in mitochondrial OXPHOS were found to be decreased in the skeletal muscle of humans with type 2 diabetes mellitus.
which is an age-associated metabolic disease (Mootha et al., 2003; Patti et al., 2003). Nevertheless, because mitochondrial dysfunction can also be a consequence of the aging process, the contribution of mitochondrial dysfunction to skeletal muscle aging in humans is still a topic of debate (Rasmussen et al., 2003).

Skeletal muscle aging in rodents is also associated with mitochondrial dysfunction similar to that described in aging humans (Bua et al., 2002; Drew et al., 2003; Herbst et al., 2007; Mansouri et al., 2006). Therefore, rodents are a great model system to study how increased mitochondrial dysfunction in the skeletal muscle leads to muscle fiber loss and sarcopenia. In rats, the age dependent increase in mtDNA mutations and OXPHOS defects in individual skeletal muscle fibers contribute to the splitting, atrophy and eventual breakage of muscle fibers during aging (Herbst et al., 2007). Recently, it was shown that mice with a proof-reading deficient mtDNA polymerase gamma (mtDNA mutator mice) that accumulate mtDNA mutations develop sarcopenia (Hiona et al., 2010; Kujoth et al., 2005). These mice have significant skeletal muscle loss, decreased muscle fiber diameter, comprehensive mitochondrial dysfunction and increased apoptosis (Hiona et al., 2010). In addition, another study showed that the levels of PGC-1α and PGC-1β mRNA are dramatically reduced after denervation in rat and mouse skeletal muscle (Sacheck et al., 2007). This is an interesting observation as denervation has been proposed to contribute to sarcopenia and other systemic muscle wasting diseases. These studies demonstrate the marked contribution of mitochondrial dysfunction to skeletal muscle aging.
1.4.2 The Role of PGC-1 Coactivators in Skeletal Muscle

Because mitochondrial dysfunction was suggested to play a role in skeletal muscle aging, the role of regulators of mitochondrial function in skeletal muscle has become a topic of interest. In particular, the involvement of the PGC-1 coactivators in skeletal muscle physiology has been the focus of many recent studies.

PGC-1α was discovered as a nuclear receptor coactivator whose expression is induced in mouse brown fat and skeletal muscle after cold exposure (Puigserver et al., 1998). The expression of PGC-1α in myotubes robustly increases mitochondrial biogenesis and favors uncoupled respiration (Wu et al., 1999). In the mouse skeletal muscle, PGC-1α mRNA is preferentially expressed in slow twitch (type 1) muscle fibers (Lin et al., 2002b). When over-expressed in mouse skeletal muscle, PGC-1α up-regulates mitochondrial biogenesis and induces a change in fiber characteristics to that of oxidative type I and IIA fibers (Lin et al., 2002b). In addition, it was recently shown that increased PGC-1α expression in the mouse skeletal muscle not only increases mitochondrial content but also orchestrates adaptations within each mitochondrion that preferentially enhances fat oxidation favoring the use of fatty acid substrates over carbohydrates for mitochondrial respiration (Hoeks et al., 2012). Conversely, skeletal muscle-specific PGC-1α KO mice have diminished mitochondrial gene expression, a shift from oxidative to glycolytic fiber, myopathy and reduced physical activity along with exercise intolerance (Handschin et al., 2007a). Similar to PGC-1α, increased expression of PGC-1β in the mouse skeletal muscle induces mitochondrial biogenesis, upregulates expression of OXPHOS genes and stimulates the formation of type IIX muscle fibers (Arany et al.,
Interestingly, a recent study by Zechner et al showed that PGC-1β KO in the skeletal muscle of total body PGC-1α KO mice or in wild-type mice, has no effect on the amount or distribution of type II fibers in the skeletal muscle but leads to an increase in type I positive fibers (Zechner et al., 2010). However, they showed that both KO models had a decrease in the expression of genes important for mitochondrial function (Zechner et al., 2010). These findings highlight the importance of PGC-1 coactivators in regulating mitochondrial function in the skeletal muscle but challenge their importance in the regulation of fiber-type switching. Another recent study showed that overexpression of PRC in myotubes increases mitochondrial mass, gene expression, enzyme activity, and respiration though not as robustly as PGC-1α (Philp et al., 2011). PRC was also shown to induce myoblast proliferation and increase the levels of slow MHC protein (Philp et al., 2011).

Mechanistically, PGC-1α regulates fiber type switching through the coactivation of the myocyte enhancing factor 2 (MEF2) transcription factor, which is a master regulator of skeletal muscle determination (Arany et al., 2007; Lin et al., 2002b). Besides, PGC-1α activates its own expression through the coactivation of MEF2 and this effect is increased by calcineurin, a Ca^{2+}-dependent phosphatase (Lin et al., 2002b). PGC-1α also regulates the expression of hypoxia inducible factor 2 alpha (HIF2α), a transcription factor shown to coordinate switch to slow muscle fibers, through its coactivation of ERRα (Rasbach et al., 2010).

The PGC-1 coactivators have also been shown to play an important role in regulating the adaptation of skeletal muscle to exercise. Several studies have
demonstrated that exercise induces upregulation of PGC-1α expression in the skeletal muscle of humans and mice via multiple pathways [reviewed in (Lira et al., 2010)]. These include the phosphorylation and activation of PGC-1α by AMP-activated protein kinase (AMPK) in response to exercise. PGC1-1α can also be phosphorylated and activated by p38γ MAPK (Lira et al., 2010). When phosphorylated, PGC-1α is deacetylated by SIRT1 leading to its sequestration in the nucleus and increased expression of its target genes (Canto et al., 2009; Lira et al., 2010). Subsequently, PGC-1α stimulates mitochondrial biogenesis, fiber-type switching, fatty acid oxidation and other pathways in the skeletal muscle in response to exercise. Recently, it was shown that exercise stimulates the endoplasmic reticulum (ER) unfolded protein response (UPR) in skeletal muscle (Wu et al., 2011). The authors proposed that the UPR is involved in regulating metabolic adaptations and protecting the muscle against ER stress associated with exercise (Wu et al., 2011). Interestingly, they also demonstrated that the UPR response is regulated by PGC-1α through coactivation of ATF6α, a transcription factor known to regulate the UPR (Wu et al., 2011). This finding further emphasizes the role of PGC-1α in coordinating exercise-induced adaptations in the skeletal muscle.

Safdar and colleagues recently demonstrated that acute exercise boosts PGC-1α expression in both the nucleus and the mitochondria of mouse skeletal muscle, leading to increased TFAM levels and increased mitochondrial protein levels (Safdar et al., 2011b). This finding not only confirms the mitochondrial localization of PGC-1α, but also highlights the importance of exercise in promoting the mitochondrial localization of PGC-1α. Furthermore, others have shown that overexpression of PGC-1α in mouse
skeletal muscle increases glucose uptake in the muscle and prevent the depletion of muscle glycogen stores following exercise (Wende et al., 2007). Besides, skeletal muscle specific PGC-1α knockout mice have aberrant glucose homeostasis pointing to the importance of PGC-1α in glucose regulation in skeletal muscle (Handschin et al., 2007b).

In times of nutrient and/or oxygen deprivation, the regulation of angiogenesis is crucial to skeletal muscle as it increases blood supply and nutrient delivery to the muscle. The PGC-1 coactivators are important regulators of angiogenesis. During this process, the expression of PGC-1α in the skeletal muscle increases, leading to an upregulation in the expression of the vascular endothelial growth factor (VEGF) and other angiogenic factors (Arany et al., 2008b). This angiogenic program mediated by PGC-1α occurs independent of HIF-1α and through the coactivation of ERRα (Arany et al., 2008b). Additionally, in response to exercise, PGC-1α is activated by β-adrenergic stimulation and coordinates angiogenesis in the mouse skeletal muscle via this ERRα/VEGF pathway (Chinsomboon et al., 2009). Accordingly, ablation of PGC-1α in the mouse skeletal muscle results in decreased induction of angiogenesis in response to exercise and ischemia (Arany et al., 2008b; Chinsomboon et al., 2009). Lately, Rowe and colleagues showed that increased expression of PGC-1β in mouse skeletal muscle can also induce angiogenesis through ERRα, independently of HIF-1α (Rowe et al., 2011). However, while both PGC-1α and PGC-1β robustly induce increased expression of VEGF, they differ in the induction of other angiogenesis related genes (Rowe et al., 2011). Nevertheless, the PGC-1 coactivators have emerged as crucial regulators of skeletal
muscle angiogenesis and may therefore be critical for the development of strategies aimed at increasing the blood vessel network in the skeletal muscle.

Figure 1.3. The PGC-1 coactivators regulate many adaptations in the skeletal muscle. PGC-1 coactivators interact with many different transcription factors to regulate pathways in the skeletal muscle in response to many types of stimuli.

As described above, PGC-1 coactivators, in particular PGC-1α, are master regulators of skeletal muscle metabolism. They promote increased mitochondrial biogenesis and function, fiber-type switching to enrich oxidative fibers, glucose regulation and angiogenesis in response to exercise and other stimuli (Fig 1.3). However, other factors are also important for the regulation of these processes. Intriguingly, Narkar and colleagues recently showed that fiber-type switch from slow glycolytic to fast oxidative fibers along with increase in mitochondrial respiration and angiogenesis, can occur in mouse skeletal muscle through ERRγ via the AMPK pathway without the
contribution of PGC-1α or stimulation from exercise (Narkar et al., 2011). Nevertheless, the role of PGC-1 coactivators in the regulation of these processes is fundamental to skeletal muscle function.

1.4.3 Benefits of PGC-1 Coactivators and Increased Mitochondrial Biogenesis in the Aging Skeletal Muscle

The stimulation of mitochondrial biogenesis by increased expression of PGC-1α and/or PGC-1β (PGC-1α/β) can compensate for mitochondrial dysfunction in tissues and cells (Bastin et al., 2008; Srivastava et al., 2007; Srivastava et al., 2009; Wenz et al., 2008). Our group showed that increased expression of PGC-1α in the skeletal muscle of aging wild-type mice protects from the development of mitochondrial dysfunction and sarcopenia (Wenz et al., 2009b). Wild-type mice with increased muscle PGC-1α also had improved systemic health at older ages (Wenz et al., 2009b). Increased expression of PGC-1α led to increase in mitochondrial mass, respiration rates and ATP production in COX deficient MLC1F-Cox10−/− mice, a mouse model of mitochondrial muscle myopathy (Wenz et al., 2008). This improvement in mitochondrial function in the skeletal muscle of the mice resulted in a delay in the onset of the myopathy and increase in the animals’ life span (Wenz et al., 2008). Cytochrome c oxidase deficiency is a form of mitochondrial dysfunction previously associated with sarcopenia and skeletal muscle aging (Bua et al., 2002; Safdar et al., 2010). Recently, similar results were obtained by Viscomi and colleagues who showed that increased PGC-1α expression in the skeletal muscle of Surf1 KO mice, which also have a COX deficiency, increased COX expression and activity and
also increased total mitochondrial biogenesis and function in the skeletal muscle (Viscomi et al., 2011).

In addition to improving mitochondrial function in the aging skeletal muscle, increased expression of PGC-1 coactivators can protect skeletal muscle from protein degradation and atrophy, processes proposed to also contribute to skeletal muscle aging and sarcopenia. It was shown that in the skeletal muscle of mice that were either starved or denervated to induce atrophy, increased PGC-1α decreased the expression of genes implicated in protein degradation (Sandri et al., 2006). PGC-1α impeded the activity of FoxO3, a transcription factor involved in regulating the expression of these genes (Sandri et al., 2006). Furthermore, mice with increased PGC-1α were less susceptible to reduction in fiber size, a feature of skeletal muscle atrophy (Sandri et al., 2006). More recently, it was shown that increased expression of PGC-1α and PGC-1β can retard the progression of protein degradation in normal muscle and also in muscle already undergoing atrophy (Brault et al., 2010). Both coactivators were able to reduce the proteosomal and lysosomal protein degradation pathways in starved myotubes without affecting the rate of protein synthesis (Brault et al., 2010). This was proposed to occur by curtailment of the transcription activity of FoxO and also NFkB, a transcription factor known to be important for the induction of atrophy (Brault et al., 2010).

As discussed earlier, exercise promotes increased PGC-1α expression in the skeletal muscle and thereby regulates many subsequent adaptations in skeletal muscle. There is growing evidence that exercise is a physiological method by which the beneficial effects of PGC-1α can be conferred on the aging skeletal muscle. Our
laboratory showed that endurance exercise can increase mitochondrial biogenesis, restore most of the mitochondrial OXPHOS function and delay the onset of mitochondrial myopathy in COX10 KO mice (Wenz et al., 2009a). Intriguingly, Safdar et al recently showed that endurance exercise can reverse the aging phenotype of mtDNA mutator mice (Safdar et al., 2011a). The exercised mtDNA mutator mice had increased PGC-1α and TFAM levels, OXPHOS proteins levels and COX activity in the skeletal muscle compared to sedentary mtDNA mutator mice (Safdar et al., 2011a). This improvement in mitochondrial function in the mouse skeletal muscle was associated with increased endurance (longer time to exhaustion during exercise) and a decrease in the apoptotic index in the skeletal muscle of the mice (Safdar et al., 2011a).

Exercise has also been shown to improve the skeletal muscle phenotype of aging humans. Older adults that engage in the regular resistance exercise training were shown to have a skeletal muscle transcriptional profile similar to that of young adults in comparison to sedentary older adults (Melov et al., 2007). Endurance exercise also increased COX activity and reduced oxidative stress in the older adults (Melov et al., 2007). Recently, it was shown that lifelong endurance exercise not only improves OXPHOS enzyme activity and mitochondrial function in the skeletal muscle of the elderly but it also increases capillarization (angiogenesis) (Iversen et al., 2011). Besides, the skeletal muscle of untrained elderly subjects maintained the ability to respond to exercise by increasing AMPK and p38 mediated phosphorylation events, thus indicating that the elderly skeletal muscle can still respond to exercise induced changes (Iversen et al., 2011).
1.4.4 Effects of Pharmacological Activators of PGC-1 Coactivators on the Aging Skeletal Muscle

Increased expression of PGC-1α and mitochondrial biogenesis can also be activated in skeletal muscle by pharmacological agents. These include bezafibrate (a PPAR pan-agonist used in the clinic to treat hyperlipidemia), 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) (an AMPK agonist) and resveratrol (a naturally occurring compound that may directly or indirectly activate Sirt1) (Puigserver, 2005; Viscomi et al., 2011; Wenz et al., 2008). The effect of bezafibrate on mitochondrial function and biogenesis in skeletal muscle differs between mouse models (Viscomi et al., 2011; Wenz et al., 2008). Our laboratory showed that the administration of bezafibrate to COX deficient MLC1F-Cox10\(^{-/-}\) mice increased PGC-1α expression and improved mitochondrial function in the skeletal muscle of the mice (Wenz et al., 2008). However, the administration of bezafibrate to Surf\(^{-}\)KO mice, which have a mild COX deficiency, did not lead to increased PGC-1α expression or increased mitochondrial biogenesis (Viscomi et al., 2011). In this model bezafibrate was found to only induce fatty acid oxidation through activation of PPARα and PPARβ/δ (Viscomi et al., 2011). In addition, bezafibrate had toxic effects on COX deficient ACTA-Cox15\(^{-/-}\) mice that developed a more severe myopathy and became ill as a result of bezafibrate treatment (Viscomi et al., 2011). Furthermore, it was recently shown that bezafibrate reduced mitochondrial function and protein expression while having no effect on the expression of PGC-1α or the PPARs in mice expressing a mutant Twinkle-helicase (deletor mice) (Yatsuga and Suomalainen 2012). Deletor mice develop COX negative fibers late in adulthood and are used as a model of late-onset mitochondrial myopathy (Yatsuga and Suomalainen 2012).
However, bezafibrate treated deletor mice had reduced percentage of COX negative fibers and reduced deletion load in the skeletal muscle (Yatsuga and Suomalainen 2012). They also had increased lipid oxidation and hepatomegaly (Yatsuga and Suomalainen 2012). In fact, bezafibrate has a well-known rodent-specific hepatoproliferative effect, which can cause health complications in mice (Monk and Todd, 1987). These results indicate that bezafibrate has varied effects on different mouse models of COX deficiency and further studies are needed to determine the effects of this drug. AICAR was shown to increase mitochondrial biogenesis and function in the skeletal muscle of three different mouse models of COX deficiency (Viscomi et al., 2011). Resveratrol has also been shown to have beneficial effects on the skeletal muscle. Recently it was shown to impede age-associated increase in oxidative stress in the skeletal muscle of aged mice by mediating a reduction in H$_2$O$_2$ concentration, lipid peroxidation and protein carbonyls (Jackson et al., 2011). Despite the benefits of resveratrol, it was unable to ameliorate sarcopenia in these aged mice possibly because it did not increase PGC-1α expression or improve mitochondrial function in the skeletal muscle of the aged mice (Jackson et al., 2011). However, in combination with exercise, resveratrol was shown to improve the endurance of senescence-accelerated prone mice (SAMP) compared to senescence-accelerated resistant mice (SAMR) and also increases oxygen consumption and the expression of mitochondrial proteins (Murase et al., 2009).

These findings indicate that PGC-1α activation can confer therapeutic benefits by increasing mitochondrial biogenesis and may be able to circumvent mitochondrial dysfunction and sarcopenia in the aging skeletal muscle.
1.5 Mitochondria and the Aging Heart

In this section, we discuss mitochondrial dysfunction and its implication on aging in heart with emphasis on cardiovascular diseases. We also discuss the potential benefit of the PGC-1 coactivators in reducing aging-associated metabolic dysfunction in the heart.

1.5.1 Mitochondrial Dysfunction in the Aging Heart

Cardiovascular diseases, such as stroke, heart failure and vascular diseases, are the primary cause of death in the elderly, despite the progress being made in preventative and treatment methods (Volkova et al., 2005). These diseases can result from modifications in heart structure and function during aging. They include: impairment of myocardial diastolic relaxation, Ca\(^{2+}\) signaling/flux, myofilament function, cardiac energy metabolism and decrease in cardiomyocytes [reviewed in (Ren et al., 2010; Volkova et al., 2005)]. Many of these aging related changes have been associated with mitochondrial dysfunction in the heart, as mitochondria are the primary energy source in the heart in addition to being involved in several processes such as the regulation of cell death and ROS production (Lesnefsky et al., 2001; Ren et al., 2010). Functioning mitochondria are crucial to the maintenance of optimal heart function and the ability of the heart to adapt to changes in physical activity. This is especially true for cardiomyocytes since mitochondria account for approximately 20-30% of their cell volume (Ren et al., 2010). In fact, mitochondrial dysfunction and reduced mitochondrial biogenesis have been shown to contribute to cardiomyocytes malfunction and the
development of cardiomyopathy (Ren et al., 2010). It has also been shown that in the aging heart there is a greater dependence on carbohydrate metabolism for energy (Lee et al., 2002). This shift in energy source results from an age-associated decrease in the expression of fatty acid metabolism genes (Lee et al., 2002). Furthermore, reduced expression of PGC-1α was shown to be associated with heart failure in humans (Finck and Kelly, 2006; Sihag et al., 2009). In addition, studies have shown that there is an increase in the frequency of mtDNA mutations with age in the heart of humans and also in animal models (Wallace, 2000). These mitochondrial DNA mutations are believed to contribute to the mitochondrial dysfunction associated with the aging. Moreover, mtDNA mutator mice, which accumulate mitochondrial DNA mutations, were shown to develop COX deficiency in cardiomyocytes which most likely contributes to their cardiomyopathy (Trifunovic et al., 2004). Accordingly, mice harboring a mutant human polymerase γ in heart have mitochondrial dysfunction associated with cardiomegaly, biventricular dilation, increased left ventricle mass, cardiac rhythm disturbances and premature death (Lewis et al., 2007). In another study, mtDNA mutator mice were shown to develop age-associated heart dysfunction, cardiomyopathy, which was linked to decrease in PGC-1α expression and decreased mitochondrial biogenesis (Dai et al., 2010).

The heart has at least two populations of mitochondria, subsarcolemmal mitochondria (SSM), found below the plasma membrane, and interfibrillar mitochondria (IFM) which are surrounded by the myofibrils in the heart (Lesnefsky et al., 2001). These mitochondria differ in function; studies have shown that IFM have higher mitochondrial
OXPHOS activity than SSM (Lesnefsky et al., 2001). Interestingly, aged rats were shown to have a decrease in mitochondrial OXPHOS in IFM but not in SSM (Fannin et al., 1999). This OXPHOS deficiency was characterized by marked decrease in COX activity and FAO in the heart (Fannin et al., 1999). Others have shown that OXPHOS deficiency in the IFM is specifically associated with decreased activity of complex III and IV since the activity of complexes I and II did not change with age (Lesnefsky et al., 2001). However, a more recent study showed that there is a progressive decrease in the activity of complexes I, II, III and IV of the mitochondrial ETC with age in the heart of rats (Tatarkova et al., 2011). Besides decreased complex activity, there was a gradual increase in lipid peroxidation in the heart of these mice, suggesting a possible contribution of oxidative damaged to their OXPHOS defect and vice versa (Tatarkova et al., 2011). Accordingly, reductions in mitochondrial antioxidant function along with impaired mitochondrial biogenesis are central to the development of metabolic-related myocardial dysfunction (Ren et al., 2010).

1.5.2 The Role of PGC-1 Coactivators in Heart

The heart is a high energy demanding organ that requires a continuous supply of ATP for proper functioning (Rowe et al., 2010). As discussed above, defects in energy metabolism have been associated with heart diseases and aging (Rowe et al., 2010). The PGC-1 coactivators are crucial regulators of heart energy metabolism. They coactivate several transcription factors (NRF-1, NRF-2, ERRα, PPARs) in the coordination of mitochondrial biogenesis and function and fatty acid import and utilization in the heart.
(Finck and Kelly, 2006; Rowe et al., 2010). Both PGC-1α and PGC-1β are highly expressed in heart and recent studies have shed light on their function in the regulation of heart metabolism (Finck and Kelly, 2006; Lin et al., 2002a).

Most of our knowledge on the function of PGC-1 coactivators in the heart came from studies performed in mammalian cells and animal models. Lehman and colleagues showed that the expression of PGC-1α in the heart increases after birth and in response to fasting and conditions that require an increase in mitochondrial biogenesis (Lehman et al., 2000). Increased PGC-1α expression coincided with increased expression of PPARα (a known interacting partner of PGC-1α) and PPARα target genes encoding for mitochondrial and FAO enzymes (Lehman et al., 2000). Activation of the PGC-1α-PPARα pathway in heart requires triglycerides and Atgl, an enzyme that regulates the turnover of triglycerides (TAG) (Haemmerle et al., 2011). These findings indicate that the PPARα/PGC-1α pathway is important in the regulation of heart energy metabolism.

The overexpression of PGC-1α in rat neonatal cardiac myocytes was shown to induce an increase in mitochondrial biogenesis, oxygen consumption and coupled respiration (Lehman et al., 2000). However, the overexpression of PGC-1α in postnatal mouse heart leads to unbridled mitochondrial biogenesis that resulted in severe dilated cardiomyopathy (Lehman et al., 2000). Further studies demonstrated that increased expression of PGC-1α in the neonatal heart is beneficial and robustly increases mitochondrial biogenesis while increased expression of PGC-1α in the adult heart leads to a small increase in mitochondrial biogenesis, aberrant mitochondrial structure and reversible cardiomyopathy (Russell et al., 2004). Therefore, although increased
mitochondrial biogenesis provides benefits to the heart, it needs to be tightly controlled to prevent adverse effects.

Conversely, mice deficient in PGC-1α have decreased expression of FAO, OXPHOS and ATP synthesis genes in the heart (Arany et al., 2005). In addition, lack of PGC-1α caused the heart to become energy deficient and have reduced contractile function in response to external stimuli (Arany et al., 2005). Besides, another PGC-1α deficient mouse model was shown to have a reduction in the maximal capacity for mitochondrial ATP synthesis and FAO and a buildup of triglycerides due to a reduction in their usage (Lehman et al., 2008). These mice were using glucose as a substrate for oxidation/energy production instead of TAG, a metabolic shift believed to contribute to the development of cardiomyopathy (Lehman et al., 2008; Madrazo and Kelly, 2008). These findings point to the importance of PGC-1α in the regulation of mitochondrial function in the heart.

Likewise, mice with global PGC-1β deficiency also have decreased expression of nuclear and mitochondrial encoded genes (Lelliott et al., 2006; Sonoda et al., 2007) in addition to a weakened heart rate response when challenged (Lelliott et al., 2006). In contrast to the relatively mild heart phenotype present in PGC-1α KO and PGC-1β KO mice, ablation of both PGC-1α and PGC-1β (PGC-1αβ-/- mice) resulted in mice with reduced heart size, occasional heart stoppage, low resting heart weight, aberrant mitochondrial structure and density and severely low cardiac output which all contribute to their death (a few days after birth) (Lai et al., 2008). Their phenotype resulted from a cessation of mitochondrial biogenesis in the late fetal stage that prevented the expression
of genes needed for the adult stage development (Lai et al., 2008). These results strongly confirm the importance of the PGC-1 coactivators in the regulation of mitochondrial biogenesis and function in the heart.

1.5.3 Benefits of PGC-1 Coactivators and Increased Mitochondrial Biogenesis in the Aging Heart

Since the PGC-1 coactivators are important for the regulation of mitochondrial biogenesis and energy metabolism in cardiac tissue and reduced expression of these coactivators is a feature of age-associated cardiovascular diseases, they make attractive targets for the treatment of heart disease. As discussed in the previous section, increased expression of PGC-1 coactivators and increase in mitochondrial biogenesis confer many benefits to the aging skeletal muscle and is able to slow the progression of sarcopenia. However, the application of this approach in the aging heart requires careful titration of the levels of PGC-1 coactivators since increased PGC-1α expression in the adult heart leads to cardiomyopathy (Russell et al., 2004). In fact, the therapeutic window lies between high and low levels of PGC-1α in the heart (Handschin, 2009).

Caloric restriction, considered to be the most effective anti-aging intervention thus far, is associated with the activation of PGC-1α (Anderson and Prolla, 2009). This is interesting since it was shown that mice subjected to caloric restriction from middle-age had less decrease in the expression of fatty acid metabolism genes and less increase in carbohydrate metabolism in the heart during aging when compared to non-caloric restricted age-matched mice (Lee et al., 2002). Furthermore, caloric restricted mice had decreased expression of most genes associated with apoptosis in the heart (Lee et al.,
These findings suggest that the decrease in cardiomyocytes observed in the aging heart could be due to increased apoptosis associated with mitochondrial dysfunction. These results also indicate that caloric restriction impedes age-related transcriptional changes in the heart and may be beneficial in preventing age-associated cardiovascular diseases.

Other methods known to increase PGC-1 coactivators and mitochondrial biogenesis have also been employed in an attempt to improve heart function. Studies have shown that exercise, which increases PGC-1α and confers many beneficial effects in the skeletal muscle, can have both positive and negative effects on the human heart depending on its intensity and the health of the subject (Fagard 2011). Nevertheless, exercise therapy is recognized as being important in the prevention and treatment of age-associated cardiovascular diseases (Fagard 2011). However, the effects of exercise on mitochondrial biogenesis and function in the heart are still being unraveled (Eisele et al., 2008; Li et al., 2011).

Bezafibrate, as discussed earlier, is a PPAR (α, β/δ and γ) pan-agonist that has been shown to increase FAO (Viscomi et al., 2011). In addition, it was also shown to increase PGC-1α expression (Wang and Moraes 2011; Wenz et al., 2008) mitochondrial biogenesis and function (Bastin et al., 2008; Wang and Moraes 2011; Wenz et al., 2008). Studies in humans have shown that bezafibrate reduces the risk of developing coronary heart disease and myocardial infarction and also reduced the incidence of cardiac mortality in patients with the metabolic syndrome (Tenenbaum et al., 2005). These findings indicate that activation of the PPAR/PGC-1α pathway confers benefits to the
aging heart. However, further studies are needed to develop other methods to activate this pathway since bezafibrate has been shown to have low potency and possible toxic effects (Tenenbaum et al., 2005).

1.6 Closing Remarks

Mitochondrial dysfunction appears to play a causal role in aging. The PGC-1 coactivators have emerged as crucial regulators of mitochondrial biogenesis and function and decreases in their expression have been linked to age-associated muscle and heart dysfunction (Fig. 1.4). Therefore, increased expression of pathways associated with these coactivators could provide effective improvements of mitochondrial dysfunction during aging.
Figure 1.4. Decreased PGC-1α expression and mitochondrial dysfunction contribute to skeletal muscle and heart aging. During aging, there is a decrease in PGC-1α expression in the heart and skeletal muscle, which contributes to decrease in mitochondrial protein expression, mtDNA levels and OXPHOS. This mitochondrial dysfunction is associated with sarcopenia and may contribute to atrophy, protein degradation, and denervation in the skeletal muscle. The adult heart depends primarily on mitochondrial FAO for ATP production. Therefore, decreased PGC-1α expression and mitochondrial dysfunction are believed to contribute to decrease in FAO and increased reliance on carbohydrate metabolism for ATP. This shift in energy metabolism is associated with cardiomyopathy and other cardiovascular diseases. Recent studies have shown that increased PGC-1α expression, whether by exercise/pharmacological agents, leads to increased mitochondrial biogenesis and OXPHOS and may be able to impede the development of sarcopenia and cardiovascular diseases in the aging skeletal muscle and heart respectively.
CHAPTER 2
INCREASED MITOCHONDRIAL BIOGENESIS IN MUSCLE IMPROVES AGING PHENOTYPES IN THE MTDNA MUTATOR MOUSE

2.1 Overview and Objective

Aging is an intricate process that increases susceptibility to sarcopenia and cardiovascular diseases. The accumulation of mtDNA mutations is believed to contribute to mitochondrial dysfunction, potentially shortening lifespan. The mtDNA mutator mouse, a mouse model with a proofreading deficient mtDNA polymerase γ (POLG), was shown to develop premature aging, including sarcopenia, cardiomyopathy and decreased lifespan. This phenotype was associated with an accumulation of mtDNA mutations and mitochondrial dysfunction. Since the premature aging phenotype of the mutator mouse is characterized by mitochondrial dysfunction in muscle, we hypothesized that increasing mitochondrial biogenesis in the muscle of mutator mice will compensate for mitochondrial defects and slow their premature aging phenotype. Increased mitochondrial biogenesis can be achieved by overexpressing the master regulator of mitochondrial biogenesis, PGC-1α. Therefore, we created mutator mice with increased expression of PGC-1α under the muscle specific muscle creatine kinase (MCK) promoter. We found that increased expression of PGC-1α in muscle of mutator mice increased mitochondrial biogenesis and function, and also improved the skeletal muscle and heart phenotypes of the mice. Deep sequencing analysis of their mtDNA showed that the increased mitochondrial biogenesis did not reduce the accumulation of mtDNA mutations but rather caused a small increase. These results indicate that increased muscle PGC-1α
expression is able to improve some premature aging phenotypes in the mutator mouse without reverting the accumulation of mtDNA mutations.

2.2 Background and Significance

Aging is the progressive decline in cellular, tissue and organ function (Figueiredo et al., 2008; Meissner, 2007; Meissner et al., 2006). This complex process often manifests as loss of muscular strength and cardiovascular function (Meissner et al., 2006). The mitochondrial theory of aging suggests that the accumulation of mtDNA mutations leads to mitochondrial dysfunction, loss of organ function and consequently a decrease in lifespan (Loeb et al., 2005; Taylor and Turnbull, 2005). This theory is appealing as there is a strong correlation between age-dependent alterations in mtDNA and an increased risk for developing cardiovascular diseases, neurodegenerative disorders and myopathy (Figueiredo et al., 2008; Fukui and Moraes, 2008; Mohamed et al., 2006; Polisecki et al., 2004). Accordingly, there is an association between the accumulation of mtDNA point mutations and deficiencies in mitochondrial OXPHOS in aging muscle fibers and hippocampal neurons (Trifunovic, 2006).

The mtDNA mutator mouse, a mouse model with a proofreading deficient POLG, is a valuable model system to study the contribution of mitochondrial dysfunction to aging (Kujoth et al., 2005; Trifunovic et al., 2004; Wiesner et al., 2006). Animals homozygous for this mutant POLG (mtDNA mutator mice, designated Mut mice) age prematurely, have reduced lifespan and show increased accumulation of mtDNA point mutations (Kujoth et al., 2005; Trifunovic et al., 2004). The premature aging phenotype
of the mutator mice begins at about 6 months of age with hair loss and graying, presbycusis, weight loss and kyphosis become apparent at approximately 10 months of age (Kujoth et al., 2005; Trifunovic et al., 2004). Reminiscent of normal human aging, mutator mice have left ventricular hypertrophy, cardiomyopathy, sarcopenia (loss of skeletal muscle mass) and high levels of mtDNA mutation in the brain and heart (Kujoth et al., 2005; Trifunovic et al., 2004). Mitochondrial dysfunction is also evident in the mutator mice as they have mitochondrial respiratory chain dysfunction along with increased apoptosis in different tissues (Kujoth et al., 2005; Trifunovic et al., 2004).

As previously discussed in chapter 1, PGC-1α is a master regulator of mitochondrial biogenesis. Over-expression of PGC-1α in mouse skeletal muscle and heart has been shown to increase mitochondrial biogenesis and function (Lehman et al., 2000; Wenz et al., 2008). In addition, PGC-1α induces fiber type switch from glycolytic to oxidative fibers (Lin et al., 2002b), angiogenesis (Arany et al., 2008) and retards protein degradation and atrophy in the skeletal muscle (Brault et al., 2010; Sandri et al., 2006). Furthermore, PGC-1α has been shown to positively regulate cardiac function (Arany et al., 2005). Because Mut mice have mitochondrial dysfunction, sarcopenia and cardiomyopathy and PGC-1α has been shown to increase mitochondrial biogenesis and function, we tested whether increased PGC-1α expression could compensate for the mitochondrial dysfunction in Mut mice and improve their aging phenotype. Here we show that Mut mice with increased expression of PGC-1α under the MCK promoter, have increased mitochondrial biogenesis and function, and improved skeletal muscle and heart function.
2.3 Results

2.3.1 MCKPGC-1αMut mice have increased PGC-1α in the skeletal muscle

We created Mut mice transgenically expressing PGC-1α under the MCK promoter (hereetofore referred to as MCKPGC-1αMut mice). In MCKPGC-1αMut mice, PGC-1α overexpression in skeletal and cardiac muscle begins at birth.

We allowed MCKPGC-1αMut mice to age until they were 10 month-old, the age when most of the phenotypes reported in the Mut mice become evident (Kujoth et al., 2005; Trifunovic et al., 2004). To ensure that 10 month-old MCKPGC-1αMut mice had increased PGC-1α levels in the skeletal muscle, RNA and total homogenates were prepared from the quadriceps of male MCKPGC-1αMut, MCKPGC-1αWT (wild-type mice transgenically expressing PGC-1α under the MCK promoter), Mut and WT mice and used for qRT-PCR and western blot analysis respectively. We found that 10 month-old MCKPGC-1αMut mice had a four-fold increase in PGC-1α mRNA levels compared to Mut and our positive controls, MCKPGC-1αWT mice, had a twenty fold increase in PGC-1α mRNA compared to WT mice (Fig. 2.1A). MCKPGC-1αMut mice also had a 2 fold increase in PGC-1α protein in the quadriceps compared to Mut, and MCKPGC-1αWT mice had ~ 3 fold increase compared to WT mice (Fig. 2.1B and 2.1C). These results indicate that 10 month-old MCKPGC-1αMut mice have increased PGC-1α in the skeletal muscle; however, the levels are lower than that of MCKPGC-1αWT mice. Unless otherwise stated, we performed all our experiments with 10 month-old male MCKPGC-1αMut mice and age matched controls.
Figure 2.1. MCKPGC-1αMut mice have increased PGC-1α levels in the skeletal muscle. (A) Gene expression of PGC-1α relative to WT in the quadriceps of 10 month-old male mice. mRNA levels are normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (B) Western blot showing PGC-1α protein levels in the quadriceps of 10 month-old male mice with loading control actin. (C) Quantification of western blot in (B) showing PGC-1α protein levels normalized to actin. n = 4/group. * P< 0.05, *** P< 0.001 Student’s t-test. Error bars represent S.E.M.

2.3.2 Increased PGC-1α expression increases mitochondrial biogenesis and function in the skeletal muscle of Mut mice

To assess changes in mitochondrial biogenesis, we measured mitochondrial protein levels and mtDNA levels in the quadricep of MCKPGC-1αMut mice and
controls. We found that MCKPGC-1αMut mice had increased levels of several subunits of the mitochondrial oxidative phosphorylation system (OXPHOS) (Fig. 2.2A and 2.2B)

Figure 2.2. Increased PGC-1α expression increases mitochondrial biogenesis and function in the quadricep of Mut mice. (A) Western blot showing the levels of mitochondrial proteins in the quadriceps of mice and loading control actin. ATP5A (subunit of complex V), UQCRC2 (subunit of complex III), MTCO1 (subunit of complex IV), SDHA and SDHB (subunits of complex II) and NDUFB8 (subunit of complex I) (n = 4/group). (B) Quantification of western blot in (A) showing protein levels normalized to actin. (C) Histology of the quadriceps showing COX (complex IV) and SDH (complex II) activity staining (n = 3/group). (D) Complex I and III activity, (E) Complex IV activity and (F) citrate synthase activity in total quadricep homogenate (n = 4/group). (G) MtDNA levels relative to WT in the quadriceps based on ND1 copy number (subunit of complex I) normalized to GAPDH (n = 4/group). Analyzed 10 month-old male mice. *P<0.05, **P<0.01, ***P<0.001 Student’s t-test. Error bars represent S.E.M.
and increased mtDNA levels in the quadriceps compared to Mut mice (Fig. 2.2G). MCKPGC-1αMut mice also had increased levels of these mitochondrial proteins in the gastrocnemius compared to Mut mice (Fig. 2.3A and 2.3B).

**Figure 2.3 Increased PGC-1α expression increases mitochondrial protein levels in the gastrocnemius of Mut mice.** (A) Western blot showing the levels of mitochondrial proteins in the gastrocnemius of 10 month-old male mice showing loading control actin. ATP5A (subunit of complex V), UQCRC2 (subunit of complex III), MTCO1 (subunit of complex IV), SDHB (subunits of complex II) and NDUFB8 (subunit of complex I) (n = 4/group). (B) Quantification of western blot in (A) showing protein levels normalized to actin. * $P<0.05$, **$P<0.01$ Student’s $t$-test. Error bars represent S.E.M.
To determine if this increase in mitochondrial biogenesis increased mitochondrial function in the quadricep of MCKPGC-1αMut mice, we measured the activity of mitochondrial electron transport chain (ETC) and citric acid cycle-associated enzymes. Frozen transverse sections were prepared from the quadricep of the mice and activity stainings were performed to detect the activity of cytochrome c oxidase (COX), complex IV and succinate dehydrogenase (SDH), complex II of the mitochondrial ETC. We found that MCKPGC-1α-Mut mice had more fibers positively stained for COX activity (dark brown stain) and SDH activity (blue stain) compared to Mut mice (Fig. 2.2C). These findings indicate that PGC-1α overexpression increased mitochondrial function in the quadriceps of Mut mice. Total homogenate from quadriceps also showed increased activity of complex I and III (Fig. 2.2D), complex IV (Fig. 2.2E), and citrate synthase (a marker of mitochondrial mass, Fig. 2.2F) measured spectrophotometrically. These results indicate that, as expected, MCKPGC-1αMut mice have increased mitochondrial biogenesis and function in the skeletal muscle.

### 2.3.3 Increased PGC-1α expression induces fiber type switch and improves skeletal muscle function of Mut mice

It was previously shown that Mut mice develop decreased quadricep and gastrocnemius weight (Kujoth et al., 2005). Therefore, we compared the quadricep and gastrocnemius weight of MCKPGC-1αMut to Mut mice to test if increased PGC-1α expression could protect the mice from loss of skeletal muscle mass. Surprisingly, we found that increased PGC-1α expression had no effect on the skeletal muscle weight of
the mice (Fig. 2.4A). However, Mut mice fell more often when running on a treadmill than MCKPGC-1α-Mut mice (Fig. 2.4B). This was observed not only at 3 and 5 months,

![Graph A](image1.png)

**Figure 2.4.** Increased PGC-1α expression has no effect on skeletal muscle weight but improves skeletal muscle function of Mut mice. (A) Weight of quadriceps and gastrocnemius of 10 month-old male mice (n = 4-5/group). *P < 0.05, **P < 0.01 Student’s t-test. Error bars represent S.E.M. (B) Number of falls of mice when put to run on a treadmill for 3 minutes at 9 meters/minute (n = 5-10/group). *P < 0.05 represents difference between Mut and MCKPGC-1αMut; ##P < 0.01 and ###P < 0.001 represents difference between WT and Mut; ++P < 0.01 and +++P < 0.001 represents difference between MCKPGC-1αWT and Mut; φP < 0.05 represents difference between WT and MCKPGC-1αMut; Difference between MCKPGC-1αWT and MCKPGC-1αMut is significant at the 9 month time point only and the difference between WT and MCKPGC-1α is not significant at any time point. Statistics represent Two-way ANOVA followed by Bonferroni post tests. Error bars represent S.E.M.
but also at 9-10 months of age when Mut mice develop sarcopenia. This suggests that increased expression of PGC-1α in the skeletal muscle of the Mut mice is able to improve mitochondrial function, boost endurance and skeletal muscle function.

PGC-1α has been shown to regulate the conversion of muscle fibers from glycolytic (type IIB) to oxidative (type I and IIA) fiber types (Lin et al., 2002b). Oxidative muscle fibers are characterized by high mitochondrial density and resistance to fatigue (Handschin et al., 2007a). Therefore, we sought to determine if the improved treadmill performance of MCKPGC-1αMut mice was associated with the formation of more oxidative fiber types. We performed immunohistochemistry for myosin heavy chain (MHC) I and MHC IIA (markers of type I and type IIA oxidative fibers respectively) in

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**Figure 2.5. Increased PGC-1α expression increases oxidative fibers in the quadriceps of Mut mice.** Immunohistochemistry results showing MHC I (type I) and MHC IIA (type IIA) (green) positive fibers in frozen transverse sections from the quadriceps of 10 month-old male mice (n = 3/group).
frozen sections from the quadriceps of 10 month-old male mice. We found that MCKPGC-1αMut mice had more type I and IIA positive skeletal muscle fibers than Mut mice (Fig. 2.5), which explains their improved treadmill performance despite no increase in their skeletal muscle weight.

2.3.4 Increased PGC-1α expression improves heart function of Mut mice

We found that similar to WT controls, MCKPGC-1αMut mice showed a trend towards increased PGC-1α mRNA levels in the heart compared to Mut mice (Fig. 2.6A). In addition, they showed a trend towards increased heart mtDNA levels (Fig. 2.6D). To determine the effect of this mild increase in PGC-1α expression and mtDNA levels on mitochondrial protein levels in the heart, we performed western blot to detect the levels of subunits of mitochondrial OXPHOS enzymes. We found that both MCKPGC-1αMut and MCKPGC-1αWT mice had no detectable increase in the levels of mitochondrial markers in the heart which suggests that there is no increase in heart mitochondrial biogenesis (Fig. 2.7A and 2.7B). Next we measured the activity of COX and citrate synthase to determine if the function of heart mitochondria was improved. Our results showed that MCKPGC-1αMut mice had increased COX activity in total heart homogenate (Fig. 2.6C) and frozen transverse sections from the heart (Fig. 2.7C) when compared to Mut mice. However, they had no increase in heart citrate synthase activity (Fig. 2.6B). These results indicate that mild PGC-1α overexpression in the myocardium of MCKPGC-1αMut mice along with systemic effects of increased PGC-1α expression in
Figure 2.6. Increased PGC-1α expression stabilizes mtDNA levels and improves mitochondrial function and ejection fraction in the heart of Mut mice. (A) Gene expression of PGC-1α in the heart relative to WT (n = 4/group). mRNA levels are normalized to GAPDH. (B) Citrate synthase activity and (C) COX (complex IV) activity in total heart homogenate (n = 4/group). (D) MtDNA levels in the heart relative to WT based on ND1 copy number (subunit of complex I) normalized to GAPDH (n = 4/group). We analyzed 10 month-old male mice. (E) Percent heart ejection fraction based on echocardiogram of mouse heart (n = 11-12 10 month-old male and female mice per group). * P< 0.05, *** P< 0.001 Student’s t-test. Error bars represent S.E.M.

the skeletal muscle, improved heart mitochondrial function without causing a major increase in mitochondrial biogenesis.

We next examined if improved mitochondrial function in MCKPGC-1αMut mice had any effect on overall cardiac function. To measure heart function, we performed
Figure 2.7. Increased PGC-1α expression has no effect on mitochondrial protein levels but increases COX activity in the heart of Mut mice. (A) Western blot showing the levels of mitochondrial proteins in the heart of 10 month-old male mice showing loading control actin. ATP5A (subunit of complex V), UQCRC2 (subunit of complex III), MTCO1 (subunit of complex IV), SDHB (subunits of complex II) and NDUFB8 (subunit of complex I) (n = 4/group). (B) Quantification of western blot in (A) showing protein levels normalized to actin. (C) Histology of the heart showing transverse heart sections from 10 month-old mice stained for COX (complex IV) activity (n = 3/group). Error bars represent S.E.M.

echocardiograms on 10 month-old male and female mice and determined ejection fraction as a quantitative measure of overall cardiac function. We found that compared to WT mice, Mut mice had decreased ejection fraction (Fig. 2.6E); however, this was
restored in MCKPGC-1αMut mice to levels comparable to WT animals (Fig. 2.6E). Interestingly, MCKPGC-1αMut mice had no change in heart size and ventricle lumen diameter compared to Mut mice (not shown).

Many studies have linked cardiac fibrosis, increase in heart collagen content, to heart aging (Biernacka and Frangogiannis 2011). Therefore, we asked if the improved heart function in MCKPGC-1αMut mice was associated with a reduction in heart collagen levels. We performed immunohistochemistry to detect collagen I in frozen sections from the heart of 10 month-old male mice. Surprisingly, we found collagen I was reduced along muscle fibers and around blood vessels in the heart of Mut mice and was restored in MCKPGC-1αMut mice (Fig. 2.8). Since collagen I is needed for proper ventricular diastolic function in the heart (de Souza, 2002), its increase likely contributes to improved heart ejection fraction in MCKPGC-1αMut mice. It is possible that increased mitochondrial function or systemic effects resulting from increased PGC-1α expression in skeletal muscle affects heart collagen I levels.

2.3.5 Increased PGC-1α expression increases the abundance of mtDNA point mutations in the skeletal muscle of Mut mice

The premature aging phenotype of Mut mice is believed to be caused by the accumulation of mtDNA mutations resulting from their error prone POLG (Edgar et al., 2009; Trifunovic et al., 2004). Therefore, we determined the effect of increased PGC-1α expression on the abundance of mtDNA point mutations in the skeletal muscle of Mut mice. Total DNA was prepared from the quadriceps of the mice and we amplified two
amplicons spanning the entire mtDNA. We then sequenced this mtDNA using next generation sequencing (NGS) to determine sequence variations in MCKPGC-1αMut mice compared to Mut, MCKPGC-1αWT and WT mice.

As previously shown (Williams et al., 2010), we found that mice with a Mut background had more mtDNA sequence variations than WT mice (Fig. 2.9A). NGS demonstrated that the increased levels of point mutations were evenly distributed
throughout the whole mitochondrial genome of the Mut mouse. An interesting exception was the D-loop, which showed a cold spot for point mutations (position \~16,000 in Fig. 2.9A; (Ameur et al., 2011). Mut and MCKPGC-1αMut mice had similar overall abundance of mtDNA point mutations (Fig. 2.9B). Further analysis showed that all the mice tested (WT, MCKPGC-1αWT, Mut and MCKPGC-1αMut mice) shared similar abundance of their most frequent mtDNA point mutations. Highly abundant point mutations (ranked as #1 to #100 in abundance; 1-40% heteroplasmy) are likely inherited mutations (Fig. 2.9C), as these mice were littermates born to parents heterozygous for the D257A mutation in POLG, which have increased levels of mtDNA mutations in their germ line (Vermulst et al., 2007). Interestingly, WT and MCKPGC-1αWT had much lower levels of the less abundant mtDNA point mutations (ranked as #100 to #10,000 in abundance) compared to Mut and MCKPGC-1αMut mice (Fig. 2.9C). These rare mutations likely represent somatic mutations accumulated during life. In Mut mice, they are caused by the error-prone POLG function in muscle mtDNA. Although individually these mutations are less abundant, there are many more of them, explaining the increased total load of mutations in the Mut mice (Fig. 2.9A and 2.9B). When we took a closer look at low frequency mutations (ranked from #100 to #10,000), we found that MCKPGC-1αMut mice had a higher average abundance of these mutations compared to Mut mice (Fig. 2.9D). These results indicate that increased PGC-1α expression increases the abundance of somatically-generated mtDNA point mutations in the skeletal muscle of Mut mice. This increase is likely a result of increases in mtDNA replication and mitochondrial biogenesis orchestrated in the skeletal muscle by PGC-1α.
Figure 2.9. Increased PGC-1α expression increases the average abundance of somatic mtDNA point mutations in the skeletal muscle of Mut mice. (A) Representative plot showing the first minor allele frequency of variants throughout the entire mtDNA from the quadriceps of WT and Mut mice. (B) Data as in (A) for variants between positions 5,300 to 7,800 of the mtDNA (which spans MTCO1, Trns1, Trnd, MTCO2 and Trnk genes) for three different mice (numbered 1-3) for each group. (C) Graph showing abundance versus rank abundance for first minor allele variants in the quadriceps. (D) Average abundance of rare (rank #100 – #10,000) variants in the quadriceps. n = 3/group 10 month-old male mice. *P < 0.05, **P < 0.01 Student’s t-test. Error bars represent S.E.M.

2.3.6 Increased PGC-1α expression does not increase CRMs in the heart of Mut mice

The mtDNA control region, which includes the D-loop, regulates mtDNA replication and transcription (Rebelo et al., 2011). We recently showed that Mut mice have a signature genetic abnormality, namely, high levels of control region multimers (CRMs), in heart and brain mtDNA (Williams et al., 2010). CRMs contain novel
recombination sites that allow them to be detected and quantified by PCR. We were able to amplify CRMs in the heart of Mut and MCKPGC-1αMut mice but not in WT mice (Fig. 2.10A). QPCR showed that increased PGC-1α expression in the heart had no effect on the relative CRMs levels in MCKPGC-1αMut mice compared to Mut mice (Fig. 2.10B). These results indicate that increased PGC-1α levels in the heart of Mut mice did not affect mtDNA structure in this tissue. They also suggest that CRMs do not contribute to the heart phenotype of the Mut mice.

**Figure 2.10. Increased PGC-1α expression has no effect on CRMs in the heart of Mut mice.** (A) Agarose gel showing the amplification of CRMs in total DNA from the heart of 10 month-old male Mut and MCKPGC-1αMut mice. Positive control (PC) Mut mouse brain sample is used as a marker for CRMs. The “wild-type” D-loop is shown in all 10 month-old male mice analyzed. (n = 4/group numbered 1-4). (B) QPCR result showing mtDNA D-loop expression in the heart of 10 month-old mice relative to WT. Expression is normalized to COX1 (subunit of complex IV) (n = 4/group). * P< 0.05 Student’s t-test. Error bars represent S.E.M.
2.3.7 Increased PGC-1α expression has mild systemic effects on Mut mice

We also assessed the effect of muscle PGC-1α overexpression on non-muscle phenotypes in the Mut mouse. We found that MCKPGC-1αMut mice and Mut mice have very similar appearance (not shown) and weights at each age (Fig. 2.11A). In addition, we found no change in bone mineral density and content (Fig. 2.11D and 2.11E), body area, lean mass, total fat and body fat percentage of 10 month-old male MCKPGC-1αMut and Mut mice (Fig. 2.11F). This indicates that overexpression of PGC-1α in the skeletal muscle of Mut mice did not have a major influence on these parameters.

We also compared the blood cell count and blood chemistry of the mice at 10-months to search for systemic effects. Our results showed that there was no change in liver or kidney function markers (not shown). Although there was no change in blood cell count in MCKPGC-1αMut compared to Mut mice (Fig. 2.11B), MCKPGC-1αMut mice had decreased mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) compared to Mut mice (Fig. 2.11C). Because MCV and MCH are increased in Mut mice due to severe anemia, these findings suggest that increased muscle PGC-1α expression ameliorates these blood parameters in Mut mice.

We also compared the survival of MCKPGC-1αMut mice to Mut mice. We found that while MCKPGC-1αMut mice did not live longer than Mut mice, they started dying six weeks after Mut mice (Fig. 2.11G). This indicates that increased PGC-1α levels in the skeletal muscle and heart may delay onset of death in the MCKPGC-1αMut group of mice.
Figure 2.11. Increased PGC-1α expression has mild systemic effects on Mut mice. (A) Changes in the body weight of male mice from 3 to 10 months of age (n = 11/group). (B) Data from complete blood cell count in 10 month-old male mice showing RBC (red
blood cells, x10^6µl), WBC (white blood cells, x10^6µl), HGB (hemoglobin, g/dL), MNC (monocytes, %), BASO (basophils, %) and EOSS (eosinophils, %) (n = 5-7/group). (C) Results of complete blood cell count in 10 month-old male mice showing HCT (hematocrit, %), MCV (mean corpuscular volume, fl), MCH (mean corpuscular hemoglobin, pg), MCHC (mean corpuscular hemoglobin concentration, %) and Lymph (lymphocytes, %) (n = 5-7/group). (D) BMD (bone mineral density) and (E) BMC (bone mineral content) measurement in 10 month-old male mice (n = 5-7/group). (F) Measurement of total body area (cm^2), lean mass (g), total body fat (g) and percent fat (%) (n = 5-7/group). (G) Percent survival of MCKPGC-1αMut (n = 17) compared to Mut (n = 13), MCKPGC-1αWT (n = 17) and WT mice (n = 17). * P< 0.05, **P< 0.01, ***, P< 0.001 Student’s t-test. Error bars represent S.E.M

2.4 Discussion

2.4.1 Phenotypic changes triggered by increased muscle PGC-1α in the Mut mouse.

Recent studies have highlighted an association between decreased mitochondrial biogenesis and function, decreased PGC-1α expression and aging (Conley et al., 2007a; Vina et al., 2009; Zahn et al., 2006). Consequently, methods of improving mitochondrial biogenesis and function have become topics of interest. Our laboratory previously showed that in a mouse model with mitochondrial muscle myopathy, increased expression of PGC-1α leads to an increase in mitochondrial mass, respiration and ATP production (Wenz et al., 2008). We also showed that increased expression of PGC-1α in the skeletal muscle of aging WT mice protects from mitochondrial dysfunction and sarcopenia associated with normal aging (Wenz et al., 2009b). In the study described in this chapter, we tested whether increased PGC-1α expression in the Mut mice could compensate for mitochondrial dysfunction and confer protection against premature aging phenotypes.
We found that although MCKPGC-1αMut mice had increased PGC-1α levels in the skeletal muscle, this increase was blunted when compared to their WT counterparts. We do not have an explanation for this difference, but it may be related to feedback mechanisms controlling PGC-1α stability in different bioenergetics states. In any case, the increase in mitochondrial biogenesis and associated increase in oxidative skeletal muscle fibers in the Mut muscle was sufficient to promote protection against some of the aging phenotypes.

Similar increases in mtDNA levels, mitochondrial protein levels and COX activity were observed in the skeletal muscle of endurance trained Mut mice (Safdar et al., 2011a). This is not surprising as it is known that exercise induces PGC-1α expression in skeletal muscle and PGC-1α has been shown regulate the adaptation of skeletal muscle to exercise (Lira et al., 2010; Safdar et al., 2011b). However, while endurance exercise rescued the decreased quadriceps and gastrocnemius weight in the Mut mice (Safdar et al., 2011a), we found that increased PGC-1α expression had no effect on skeletal muscle weight. The increase in skeletal muscle weight observed in endurance exercised Mut mice may result from effects of exercise that are parallel to the PGC-1α pathway.

Furthermore, we found that MCKPGC-1αMut mice trended to have increased COX activity and mtDNA levels in the heart despite having only a small increase in PGC-1α expression in this tissue. This small increase may be optimal since high levels of PGC-1α in the heart can lead to cardiomyopathy (Russell et al., 2004). We also observed that MCKPGC-1αMut mice had increased percent heart ejection fraction, suggesting that increased PGC-1α expression and mitochondrial function in the heart improved heart
function. This finding supports the role of PGC-1α in regulating heart energy metabolism and function (Arany et al., 2005). Besides its role in increasing mitochondrial biogenesis, PGC-1α can also increase the levels of specific factors that improve mitochondrial activity, anti-oxidant function and microvascularity (Rowe et al., 2010). Moreover, increased PGC-1α expression in muscle can lead to the release of myokines that can impact heart function (Arnold et al., 2011).

Despite having improvements in skeletal muscle and heart, MCKPGC-1αMut mice did not display changes in physical appearance, body weight, bone mineral density or fat/lean mass that was observed in endurance exercised Mut mice (Safdar et al., 2011a). However, similar to endurance exercised Mut mice, MCKPGC-1αMut mice had decreased MCV and MCH suggesting that increased PGC-1α expression improved some blood parameters in the Mut mice (Safdar et al., 2011a). Although we did not observe an extension in the lifespan of MCKPGC-1αMut mice as was observed in exercised Mut mice (Safdar et al., 2011a), the increased longevity in that study was determined by analyzing very few animals, and confirmation in larger groups would be required. In any case, the overall health of MCKPGC-1αMut mice appeared improved, as the early deaths in this group were observed 6 weeks after the early deaths in the Mut mice.

Therefore, increased expression of PGC-1α in muscle conferred protection mostly to muscle and heart, likely by increasing the mitochondrial pool, which minimizes the partial defects associated with increased levels of mtDNA mutations. There were also improvements in some blood parameters, which may be associated with the release of myokines (Arnold et al., 2011).
2.4.2 Genotypic changes triggered by increased muscle PGC-1α in the Mut mouse

When we analyzed mtDNA point mutations in the skeletal muscle, we found that increased PGC-1α expression in Mut mice did not decrease the abundance of somatic point mutations, but instead led to a small but significant increase. This finding suggests that increased PGC-1α expression induces mtDNA replication in Mut mice, leading not only to increased mtDNA levels but further accumulation of mtDNA point mutations with each round of replication since these mice have a proof-reading deficient POLG. In contrast, endurance exercised Mut mice were reported to have decreased frequency of mtDNA point mutations in the skeletal muscle (Safdar et al., 2011a). We do not have an explanation for this difference, but they may be due to either different methodology or to systemic effects of exercise that possibly mediate an increase in mtDNA repair or removal of dysfunctional mitochondria in the skeletal muscle of the Mut mice. It has been shown that skeletal muscle of the Mut mice have a lower abundance of mtDNA point mutations in the D-loop compared to the rest of the mtDNA (Ameur et al., 2011). This pattern was preserved in MCKPGC-1αMut mice. The reason for the existence of this “cold spot” remains unexplained.

We previously showed that CRMs are present in the heart of Mut and not WT mice and can be used as markers of mtDNA damage (Williams et al., 2010). Here we observed that MCKPGC-1αMut mice had similar levels of CRMs in the heart as Mut mice. This finding suggests that increased PGC-1α expression did not increase mtDNA damage in the heart, although we observed a trend towards increased mtDNA levels.
Our findings highlight the potential of PGC-1α in circumventing mitochondrial dysfunction in aging and age-related conditions. We showed that increased PGC-1α expression under the MCK promoter increases mitochondrial biogenesis and function and improves skeletal muscle and heart function of Mut mice despite not improving their mtDNA mutation load.

2.5 Material and Methods

Generation of MCKPGC-1αMut mice

Transgenic mice expressing PGC-1α driven by the muscle/heart specific MCK promoter (MCKPGC-1α) were previously described (Lin et al., 2002b). MtDNA mutator mice (Polg\(^{D257A/D257A}\)) were previously characterized (Kujoth et al., 2005; Trifunovic et al., 2004). We crossed MCKPGC-1α\(^{+/-}\) with Polg\(^{D257A/+}\) mice to obtain MCKPGC-1α\(^{+/-}\)-Polg\(^{D257A/+}\) mice. The MCKPGC-1α\(^{+/-}\)-Polg\(^{D257A/+}\) mice were then crossed with Polg\(^{D257A/+}\) to obtain mutator mice expressing PGC-1α (MCKPGC-1αMut). The resulting littermates of WT, MCKPGC-1α\(^{+/-}\) in a wild-type background (MCKPGC-1αWT) and Polg\(^{D257A/D257A}\) (Mut) were used as controls.

Animal Husbandry

Mice were housed in a virus-antigen-free facility of the University Of Miami Division Of Veterinary Resources in a 12-h light/dark cycle at 22°C and fed ad libitum with irradiated standard mouse diet.
Quantitative PCR

Total RNA was isolated from snap frozen quadriceps and heart using the RNeasy Fibrous Tissue Mini kit (Qiagen). cDNA was synthesized from 1µg of RNA using the Superscript III reverse transcriptase kit (Invitrogen). Quantitative real-time PCR, with specific primers for PGC-1α (5’-CTGCGGGATGATGGAGACA, 5’-AGCAGCGAAAGCGTCACA) and GAPDH (5’-GCAGTGCAAGTGAGATT, 5’-GAATTTGCCGTGGAGTGAGT) was performed using Maxima SYBR Green/ROX PCR Master Mix (Fermentas).

Total DNA was extracted from frozen quadriceps and heart with phenol: chloroform and mtDNA copy number was quantified using ND1 (5’-CAGCCTGACCCATAGCCATA, 5’-ATTCTCTTCTGTCAGGTCGA), and GAPDH primers. MtDNA D-loop (control region) levels were quantified using control region primers (CRMF, CCCCTCCCCCATTTGGTCTATT; CRMB, TTGATGGCCCTGAAGTAAGAACC) and COX1 primers (CO1F, AGGCTTCACCCTAGATGACACA; CO1B, GTAGCGTCGTGGATTTCTGAA). ΔΔCt method was used to analyze abundance of PGC-1α, mtDNA and D-loop.

PCR

For detection of CRMs, total DNA was isolated from frozen heart by phenol: chloroform extraction. CRMs were amplified with Expand Long Template PCR kit (Roche) using primers 15720F (CACCAATGCCCCTTCTTC) and 16022B (TTGAGTTTTGCGGACTAATGAT). Reaction was performed with Buffer 2 using an extension time of 2 minutes.
Western Blot

Western blot analysis was performed as previously described (Pickrell et al., 2011). Primary antibodies used were PGC-1α (Santa Cruz H-300), SDHA (Mitosciences), Total OXPHOS Rodent Cocktail (Mitosciences) and Actin (Sigma). All primary antibodies were used at 1:1000 dilution, except for PGC-1α antibody which was used at 1:200 dilution, and incubated overnight at 4°C. Secondary antibodies used were infrared-conjugated anti-rabbit 700 (1:3000) and anti-mouse 800 (1:5000) (Rockland). Blots were visualized with Odyssey Infrared Imaging System (LI-COR Biosciences) and band intensity/optical density was quantified with default software supplied by LI-COR.

Histochemistry and Immunohistochemistry

Deeply anesthetized mice were perfused with cold PBS and quadriceps and heart were removed and immediately frozen in isopentane cooled in liquid nitrogen. For histochemistry, triplicate 10 µm thick cross-sections of these frozen tissues were stained for SDH and COX activities as previously described (Sciacco and Bonilla, 1996). The stained sections were analyzed using a light microscope. For immunohistochemistry, frozen sections were blocked in PBS 5% BSA for 30 minutes. For heart sections, primary antibody for collagen I (1:200) (Abcam) was incubated overnight at 4°C and wheat germ agglutinin conjugated to Texas Red-X (1:100) (Molecular Probes) was added for 10 minutes. Secondary antibody conjugated to Alexa 488 (1:400) (Molecular Probes) was added for 1 hour in the dark. Sections were mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories) and then analyzed with a LSM710 confocal microscope (Zeiss). Alexa 488 intensity was quantified using the default confocal
microscope software (Zeiss). For fiber typing in the quadriceps, primary antibodies recognizing myosin heavy chain (MHC) IIA (SC-71-c) and MHC I (A4.951-c) (DSHB) were incubated overnight at 4°C. Secondary antibody conjugated to Alexa 488 (1:400) (Molecular Probes) was added and incubated for 2 hours in the dark. Sections were analyzed with a fluorescent microscope.

**Spectrophotometric Assays**

The activity of complex IV and citrate synthase (CS) was determined spectrophotometrically in total homogenate from the quadriceps and heart of mice as described previously (Barrientos, 2002). The activity of complex I and III was determined spectrophotometrically in total homogenate from the quadriceps of mice as described previously (Martinez et al., 2001). However, complex I reaction was started with coenzyme Q1 and inhibited with rotenone and complex III reaction was started with coenzyme Q2 and inhibited with stigmatelin. Assay results were normalized to protein concentration obtained by the Bradford method.

**Treadmill Test**

Mice were acclimated to the treadmill (Columbus Instruments, Columbus, OH) prior to testing; they were put to run for about 1-2 minutes until they knew how to perform the test. For the test, mice were put to run on the treadmill at 9 meters per minute for 3 minutes. Mice performance was measured by the number of times a mouse falls off the running belt onto the grid during the test.
**Echocardiogram**

Transthoracic echocardiography was performed on 10 month-old mice anesthetized with 1% isoflurane as previously described (Peacock et al., 2010). The VisualSonics 770 system (Toronto, Canada) was used for this procedure and percent ejection fraction was calculated from the obtained images.

**Next Generation Sequencing**

Sequencing libraries were prepared from two amplicons covering the entire mtDNA using the Kapa high fidelity PCR system. Fragments were amplified using 5’-blocked (C6-TFA) primers 12728F (CTGTACCCACGCATTCTTCA) and 4200B (GGATAGGCCTATTAATGTTATGT) and 4075F (AGCAGCAACAAAATACTTCGTCACAC) and 12886B (GTGAGGCGAGGCTTCGATTAC). Products were cleaned using the Sigma Genelute PCR cleanup kit, quantified with an Invitrogen Qubit fluorometric quantitation system and mixed in equimolar amounts. Sequencing libraries were prepared using Illumina TruSeq sample prep and 2x 100 bp paired-end sequencing was done on an Illumina HiSeq 2000 system. Read files were created using CASAVA 1.8 without duplicate removal. CLCBio 4.7.2 was used for assembly and variant detection. Reads were imported and quality trimmed at default settings with a length cutoff of 60 bp to produce working files which were assembled with a cutoff of 0.9 similarities and 0.95 lengths with paired-end distance optimized for each sample. Variants were detected using the CLCBio single nucleotide polymorphism (SNP) detection algorithm with default quality scoring and no significance scoring. Thus values reported are sum variance
(biological and technical) at each position and differences in mutation frequency between WT and other mice is assumed to reflect differences in actual mutation relative to WT variance. SNP tables were analyzed and graphics produced in MS Excel. Only the first minor allele frequencies were considered.

**Blood Analysis**

Mice were deprived of food overnight and the following day anesthetized and blood was collected by cardiac puncture. Blood was sent to the University of Miami Comparative Pathology Laboratory for complete blood cell count and liver/kidney panel analysis.

**DEXA Scan**

The body composition of mice was determined as previously described (Cheung et al., 2009). In brief, mice were anesthetized and weighed and bone mineral density and content along with fat and lean body mass was measured using a Lunar PIXImus II Densitometer (GE Medical Systems, Waukesha, WI).
CHAPTER 3

LONG-TERM BEZAFIBRATE TREATMENT IMPROVES SKIN AND SPLEEN PHENOTYPE OF THE MTDNA MUTATOR MOUSE

3.1 Overview and Objective

Pharmacological agents, such as bezafibrate, that activate PPAR and PGC-1α pathways have been shown to improve mitochondrial function and energy metabolism. The mtDNA mutator mouse (Mut) is a mouse model of aging that harbors a proofreading-deficient mtDNA polymerase γ (POLG). As mentioned in chapter 2, these mice develop many features of premature aging including hair loss, anemia, osteoporosis, sarcopenia and decreased lifespan. They also have increased mtDNA mutations and marked mitochondrial dysfunction. In this chapter we tried a pharmacological approach to improve mitochondrial function in all tissues of the Mut mice in an attempt to slow their premature aging. We placed Mut mice on a diet containing bezafibrate for 8-months. We found that bezafibrate treated Mut mice had delayed hair loss and improved skin and spleen phenotype. Although we observed an increase in markers of fatty acid oxidation, we did not detect a generalized increase in mitochondrial markers. There were no improvements in muscle function or lifespan of the Mut mouse, which we attribute to the rodent-specific hepatomegaly associated with fibrate treatment. These results show that despite its secondary effects in rodent’s liver, bezafibrate was able to improve some of the aging phenotypes in the Mut mouse. Because the associated hepatomegaly is not observed in primates, long-term bezafibrate treatment in humans can have beneficial effects on tissues undergoing chronic bioenergetic-related degeneration.
3.2 Background and Significance

Many recent studies have linked mitochondrial dysfunction with aging and aging-associated diseases (Dillon et al., 2012). The Mut mouse is a mouse model of premature aging that supports this connection. This mouse has a mutant POLG that lacks its proofreading ability (Kujoth et al., 2005; Trifunovic et al., 2004). Mut mice were shown to develop many features of premature aging such as early hair loss, anemia, osteoporosis, sarcopenia, cardiomyopathy and decreased lifespan (Kujoth et al., 2005; Trifunovic et al., 2004). These aging-like phenotypes were associated with increased mtDNA mutation and mitochondrial dysfunction (Kujoth et al., 2005; Trifunovic et al., 2004). We showed in chapter 2 that increasing mitochondrial biogenesis and function in muscle, by transgenic overexpression of PGC-1α, improved skeletal muscle and heart phenotypes of Mut mice (Dillon et al., 2012).

Bezafibrate is a pharmacological agent, used in clinical practice to treat hyperlipidemia, which activates the PGC-1α/PPAR pathway (Tenenbaum et al., 2005). It functions as a pan-agonist of the PPAR (α, δ and γ) nuclear receptor family (Tenenbaum et al., 2005). The PPARs regulate a variety of metabolic pathways, primarily lipid metabolism; however they are also involved in regulating cell differentiation and proliferation and immune/inflammatory responses of the cell (Kuenzli and Saurat, 2003; Wang 2010). Recent studies using bezafibrate to activate the PGC-1α/PPAR pathway in mouse models with mitochondrial dysfunction have yielded varied results. Bezafibrate increased mitochondrial biogenesis and function and increased the lifespan of mice with a muscle-specific defect in cytochrome c oxidase (COX) (Wenz et al., 2008). Bezafibrate
was also shown to increase PGC-1α expression and mitochondrial biogenesis/function in several tissues of a mouse model of Huntington’s disease associated with reduced PGC-1α levels (Johri et al., 2012). In mice expressing a mutant Twinkle-helicase (deletor mice), bezafibrate reduced the number of COX negative muscle fibers, however, it did not increase mitochondrial biogenesis and function (Yatsuga and Suomalainen 2012). Instead, bezafibrate induced fatty acid oxidation and hepatomegaly in the deletor mice (Yatsuga and Suomalainen 2012). In Surf1 knock-out (KO) mice, bezafibrate also induced fatty acid oxidation and hepatomegaly while increasing the expression of PPARs (Viscomi et al., 2011). However, it did not induce mitochondrial biogenesis or improve the myopathy in this mouse model (Viscomi et al., 2011).

In an attempt to compensate for mitochondrial dysfunction in the Mut mouse, we placed them on a bezafibrate diet (BD) for 8-months. Here we show that bezafibrate did not induce several tested markers of mitochondrial biogenesis/function; however, it delayed hair loss and drastically improved the skin and spleen phenotype of Mut mice. These results suggest that activation of PPARs can have beneficial effects in certain tissues undergoing mitochondrial stress.

3.3 Results

3.3.1 Systemic effects of bezafibrate in the Mut mouse

To improve mitochondrial function in all tissues, 2 month-old male Mut mice were placed on a standard mouse diet containing 0.5% bezafibrate (BD). This mouse group is heretofore referred to as MutBD. As a positive control for the effects of bezafibrate, same aged male WT mice (heretofore referred to as WTBD) were also placed
on BD. Mice on the BD were compared to same aged male Mut and WT mice that were fed a standard mouse diet (standard diet, SD). These mice will be referred to as MutSD and WTSD respectively. The animals were fed their respective diets for 8 months and then analyzed at 10 month-old to uncover the effects of long-term bezafibrate administration on physical phenotype, organ structure/function and mitochondrial function in a model of mitochondrial aging.

The Mut mouse was previously shown to have decreased body weight compared with WT mice (Kujoth et al., 2005; Trifunovic et al., 2004). Therefore, we monitored body weight changes in both mice on BD and SD monthly beginning at 2 months of age. Similar to previous reports, we found that beginning at 3 months MutSD mice had a lower body weight compared with WT mice (Fig. 3.1A). We observed that mice in all groups had a similar body weight at 2 month-old but only WTSD mice experienced a steady increase in body weight between 4 and 11 month-old (Fig. 3.1A). We also found that MutBD and WTBD mice weighed less than MutSD and WTSD mice respectively beginning at 3 months of age (Fig. 3.1A). This difference in body weight was not due to a decrease in the food intake of MutBD mice (not shown). These results indicate that bezafibrate reduced the body weight of both Mut and WT mice.

In addition, we monitored changes in BMD and BMC, body area and body fat and lean mass of Mut and WT mice on BD and SD by DEXA scanning. Mut were previously shown to have decreased BMD, BMC and body fat at 10 month-old (Kujoth et al., 2005; Trifunovic et al., 2004). We found that 10 month-old MutBD and WTBD mice had decreased body BMD, BMC and area compared with MutSD and WTSD mice (Fig.
3.1B-3.1D). The less dense bones may be related to lower locomotor activity or muscle function. Surprisingly, bezafibrate had no effect on fat levels in these mice (Fig. 3.1D).

**Figure 3.1. Systemic effects of bezafibrate on Mut and WT mice.** (A) Body weight of mice from 2 to 14 month-old (n = 11-26/group for 2 to 11-month; 2-6/group for 12 to 14-month). The difference in body weight between WTSD and MutSD mice is statistically significant from 3 to 14 months, between WTSD and WTBD mice is significant at every time point and the difference between MutSD and MutBD is significant from 3 to 14 months. Measurement of total (B) bone mineral density (BMD), (C) bone mineral content (BMC) (D), body area (cm$^2$), lean mass (g), total body fat (g) and percent (%) fat of 10 month-old mice (n = 5-7/group). (E) Percent survival of mice (n = 11-15/group). *, $P<0.05$; **, $P<0.01$, Student’s t-test. Error bars represent the SEM.
Because the Mut has decreased lifespan compared to WT mice (Kujoth et al., 2005; Trifunovic et al., 2004), we compared the lifespan of bezafibrate treated and SD mice. Our survival studies showed that bezafibrate did not increase the percent survival of MutSD mice (Fig. 3.1E).

3.3.2 Bezafibrate delayed hair loss and restored the skin structure of the Mut mouse

As part of their premature aging phenotype, Mut mice develop grey hairs and start showing signs of hair loss at about 6 to 7 months of age (Kujoth et al., 2005; Trifunovic et al., 2004). Therefore, we compared the coat phenotype of MutBD with MutSD mice. We observed that at ~ 7 months of age, MutSD mice started showing signs of hair loss on their back and under their neck while age matched MutBD mice that had been on the BD for 5 months did not (Fig. 3.2A). The hair loss in MutSD mice advanced with age, whereas MutBD mice only began showing signs of hair loss on their back at 10 month-old (Fig. 3.2A). In addition, we observed that 10 month-old MutBD mice had fewer gray hairs than age matched MutSD mice (not shown). These findings indicate that bezafibrate is able to delay loss and graying of the hair, two phenotypes of the Mut mice.

To further characterize the effects of bezafibrate on hair loss and graying, dorsal and ventral skin were collected from 10 month-old WTSD, MutBD and MutSD mice. Paraffin embedded skin sections were stained with hematoxylin and eosin (H&E) to determine structural changes. We observed that dorsal skin sections from MutSD mice were thinner and lacked the distinct layers that were present in WTSD mice, while MutBD mice had a skin structure more similar to WTSD mice (Fig. 3.2B, H&E).
Specifically, the dorsal skin sections from MutSD mice were primarily composed of the dermis/connective tissue and had a non-continuous epidermis layer (Fig. 3.2B, H&E). In addition, the skin sections from MutSD mice had smaller hair follicles compared with WTSD and MutBD mice (not shown). No distinct structural differences were observed in ventral skin sections from these latter mice (not shown).

Because collagen and elastin fibers are the primary functional components of the dermis/connective tissue layer of the skin (Oikarinen, 1994), we wanted to determine if bezafibrate had an effect on the expression of these components. To detect elastin fibers and collagen, dorsal skin sections were stained with the Verhoeff’s Van Geison (EVG) and Masson’s trichrome stain respectively. We found that elastin fibers (black/dark brown stain) were present in the connective tissue of both MutSD and MutBD mice at levels similar to WTSD mice (Fig. 3.2B, EVG). However, compared with WTSD mice, MutSD mice had essentially no collagen (blue stain) in the connective tissue of the skin (Fig. 3.2B, Masson’s trichrome). Surprisingly, the collagen levels in the skin were restored in MutBD mice (Fig. 3.2B, Masson’s trichrome).

The transforming growth factor beta 1 (TGF-β1)/Smad3 signaling pathway was shown to regulate a number of collagen gene promoters in human dermal fibroblasts (Verrecchia et al., 2001). Also, it was shown that activated PPARδ can regulate collagen levels in skin via the TGF-β1/Smad3 pathway (Ham et al., 2010). Since bezafibrate is a PPARδ agonist, we wanted to determine whether this pathway was activated in the skin of 10 month-old MutBD mice. We performed western blot for Smad3 in total homogenate from the skin of 10 month-old mice. Our results showed an accumulation of
total Smad3 protein in the skin of WTBD and MutBD mice compared with WTSD and MutSD mice (Fig. 3.2C). The accumulation of total Smad3 protein was previously shown to be associated with increased Smad2/3 signaling in human dermal fibroblasts.

Figure 3.2. Bezafibrate delayed hair loss and restored the skin structure of Mut mice. (A) Pictures of mice at 7 month-old and 10 month-old showing their coat phenotype (n = 6/group). Squares highlight area of coat being described. (B) Dorsal skin sections from 10 month-old mice showing hematoxylin and eosin (H&E) staining to depict structural changes (black arrow indicates break in the epidermis layer of MutSD mice), Verhoeff's Van Geison (EVG) staining for elastic fibers shown in black/dark brown (yellow arrow) and Masson’s trichrome staining showing collagen in blue (n = 2/group). (C) Western blot showing total Smad3 and glyceraldehyde 3-phosphate (GAPDH) protein levels in total skin homogenate from 10 month-old mice and quantification of total Smad3 band intensity normalized to GAPDH (n = 4/group). Error bars represent the SEM.
Therefore, our results suggest that bezafibrate activates the TGF-β1/Smad3 pathway thereby causing an increase in collagen in the skin of MutBD mice.

### 3.3.3 Bezafibrate decreased spleen size and restored the spleen structure of the Mut mouse

Mut mice were previously shown to have enlargement of some organs, one of which was the spleen (Trifunovic et al., 2004). We found that bezafibrate reduced the spleen weight and size of 10 month-old MutBD and WTBD mice (Fig. 3.3A and 3.3B). The spleen size of MutBD mice was restored to that of WTSD mice (Fig. 3.3A). To further investigate this effect of bezafibrate on the spleen of Mut and WT mice, we performed H&E staining of paraffin embedded spleen sections. We found that bezafibrate improved the spleen structure of MutBD mice (Fig. 3.3D). Our results showed that MutSD mice had an aberrant spleen structure characterized by marked atrophy and decreased white pulp (purple areas in Fig. 3.3D) and an increase in red pulp of the spleen (Fig. 3.3D). As the white pulp of the spleen synthesizes antibodies, the atrophy of this area in MutSD mice can impair their immune response (Cesta, 2006). Interestingly, the spleen of MutBD mice had a white and red pulp distribution and organization that were similar to WTSD mice (Fig. 3.3D). These findings indicate that bezafibrate protected the spleen in the Mut mouse.

Because MutSD mice had pronounced atrophy in the spleen, we performed immunohistochemistry for cleaved caspase-3 in paraffin embedded spleen sections from 10 month-old mice. After quantifying the number of positive cells, we found that compared with WT mice, MutSD mice had increased cleaved caspase-3 (Fig. 3.3C).
However, there was a trend towards a reduction in the number of cleaved caspase-3 positive cells in MutBD mice (Fig. 3.3C), indicating that bezafibrate could reduce apoptosis in the spleen of Mut mice. This attenuation of apoptosis could explain the decreased atrophy and restoration of white pulp observed in the spleen of MutBD mice (Fig. 3.3D).

It was reported that Mut mice develop anemia, decreased red blood cells (RBC), which is a clinical feature in aging humans (Kujoth et al., 2005; Trifunovic et al., 2004). Because our results showed that bezafibrate improved the spleen structure of Mut mice, we wanted to determine if bezafibrate had an effect on RBC count. We performed a complete blood cell panel (CBC) in 10 month-old mice and found that while bezafibrate had no effect on white blood cell (WBC) (not shown), MutBD and WTBD mice had a moderate but significant increase in RBC count compared with MutSD and WTSD mice (Fig. 3.3E). However, the RBC count in MutBD mice was not restored to that of WTSD mice. These results suggest that bezafibrate increased RBC in both WT and Mut mice and we speculate that this improvement may be associated with other beneficial effects of bezafibrate in the spleen.

To further investigate the effects of bezafibrate in the spleen, we performed quantitative reverse transcriptase – polymerase chain reaction (qRT-PCR) to determine the expression level of PPARγ and PGC-1α. Both genes are known targets of bezafibrate, and PPARγ has been shown to be involved in regulating anti-inflammatory responses (Harries and Paus, 2009), which can influence spleen size. We found that PPARγ mRNA levels were elevated in the spleen of MutSD compared with WTSD mice.
Figure 3.3. Bezafibrate improved spleen size and structure of Mut mice. (A) Spleen weight of 10 month-old mice and (B) picture of spleen from Mut mice (n = 4-6/group). (C) Quantification of cleaved caspase-3 immunostaining in paraffin sections from the spleen of 10 month-old mice (n = 4/group) (D) H&E staining of the spleen of 10 month-old mice showing the organization of white pulp (purple) and red pulp (pink) (n = 3/group). (E) Results from complete blood cell count in 10 month-old mice showing RBC (red blood cells, x10⁶/µl ) (n = 5-6/group) (F) PGC-1α and PPARγ mRNA levels in the spleen of 10 month-old mice normalized to actin. (G) Quantification of western blot showing mitochondrial protein levels in total homogenate from the spleen of 10 month-old mice normalized to actin. NADH dehydrogenase (ubiquinone) 1β subcomplex subunit 8 (NDUFB8; subunit of complex I), succinate dehydrogenase subunit B (SDHB; subunits of complex II), ubiquinol-cytochrome c reductase core protein 2 (UQRC2; subunit of complex III), and ATP synthase subunit 5α (ATP5A; subunit of complex V). *, P<0.05; **, P<0.01, Student’s t-test. Error bars represent the SEM.
and its levels were restored in MutBD mice (Fig. 3.3F). \textit{PPAR}\textgamma levels were also reduced in WTBD mice indicating that this effect was due to bezafibrate (Fig. 3.3F). These findings suggest that bezafibrate may reduce inflammation and thus also the inflammatory response regulated by PPAR\textgamma in the spleen. We also found that PGC-1\textalpha levels were reduced in the spleen of MutSD mice compared with WTSD and there was a trend towards an increase in PGC-1\textalpha levels in MutBD mice (Fig. 3.3F). To determine if this mild increase in PGC-1\textalpha levels caused an increase in mitochondrial biogenesis in the spleen of MutBD mice, we performed western blot to assess mitochondrial protein levels in total spleen homogenate. We found that there was no increase in mitochondrial protein levels in the spleen (Fig. 3.3G). These results indicate that beneficial effects of the bezafibrate in the spleen are not due to an increase in mitochondrial biogenesis but perhaps due to reduced apoptosis and anti-inflammatory responses regulated by PPAR\textgamma.

### 3.3.4 Bezafibrate induced fatty acid oxidation and not mitochondrial biogenesis in the skeletal muscle phenotype of Mut mice

As part of their premature aging phenotype, Mut mice develop sarcopenia and mitochondrial dysfunction in the skeletal muscle (Hiona et al., 2010). To determine if bezafibrate was able to induce mitochondrial biogenesis/function in the skeletal muscle of Mut mice, we performed western blot to detect the levels of PGC-1\textalpha and mitochondrial proteins in total quadricep homogenate from 10 month-old mice. We found that MutSD mice had reduced PGC-1\textalpha protein levels compared with WTSD mice and the levels were not restored in MutBD mice (Fig. 3.4A). Similarly, MutSD mice had reduced \textit{PGC-1}\beta mRNA levels compared with WTSD mice and these levels were also not
increased in MutBD mice (Fig. 3.4B). Also, bezafibrate did not increase mitochondrial protein levels or citrate synthase activity (CS) in the quadriceps of MutBD and WTBD mice (Fig. 3.4C and 3.4D respectively). We also did not detect an increase in cytochrome c oxidase (COX) activity in total quadriceps homogenate of MutBD when compared to MutSD mice (not shown). To further characterize the effects of bezafibrate on mitochondria in the skeletal muscle, we quantified mtDNA levels in the quadriceps by qPCR. Paradoxically, we found that MutBD and WTBD mice had decreased mtDNA levels compared with MutSD and WTBD (Fig. 3.4E). These results indicate this bezafibrate regimen did not induce mitochondrial biogenesis in the skeletal muscle of Mut and WT mice.

To determine the effect of bezafibrate on the expression of PPARs in the skeletal muscle, we performed qRT-PCR and found that bezafibrate had no effect on the mRNA levels of PPARs in the skeletal muscle of Mut mice (Fig. 3.4B). However, we found that the expression of some fatty acid oxidation-related genes, known to be regulated by the PPARs, was increased in the skeletal muscle (Fig. 3.4F). The mRNA levels of acyl-coenzymeA oxidase 1 (ACOX), an enzyme that oxidizes long chain and branched fatty acids, and carnitine palmitoyl transferase (CPT1), enzyme involved in fatty acid transport into mitochondria, were elevated in the quadriceps of MutBD mice (Fig. 3.4F). Interestingly, these increases were not found in WTBD mice (Fig. 3.4F). In addition, there was no change in the mRNA levels of cluster of differentiation 36 (CD36), an integral membrane protein that internalizes lipoproteins and fatty acids, and short-chain-acyl-coenzymeA dehydrogenase (SCAD), an enzyme that metabolizes short-chain fatty
Figure 3.4. Beazafibrate induces fatty acid oxidation and not mitochondrial biogenesis in the skeletal muscle of Mut and WT mice. (A) Western blot of PGC-1α and loading control actin in total quadricep homogenate from 10 month-old mice and quantification of PGC-1α band intensity normalized to actin (n = 4/group). (B) Gene expression of PGC-1β and PPARs in the quadriceps of 10 month-old mice normalized to actin (n = 4/group). (C) Citrate synthase activity in the total quadricep homogenate from 10 month-old mice (n = 4/group). (D) Quantification of western blot of mitochondrial proteins in the total homogenate from the quadriceps of 10 month-old mice (n = 4/group). NADH dehydrogenase (ubiquinone) 1β subcomplex subunit 8 (NDUFB8; subunit of complex I), succinate dehydrogenase subunit B (SDHB; subunits of complex II), ubiquinol-cytochrome c reductase core protein 2 (UQRC2; subunit of complex III), mitochondrial cytochrome c oxidase subunit 1 (MTO1; subunit of complex IV) and ATP synthase subunit 5α (ATP5A; subunit of complex V) (E) Quantification of mtDNA levels in the quadriceps of 10 month-old mice based on ND1 (subunit of complex I) levels normalized to glyceraldehyde 3-phosphate (GAPDH) (n = 4/group). (F) Gene
expression of markers of fatty acid oxidation, acyl-coenzymeA oxidase 1 (*ACOX*), cluster of differentiation 36 (*CD36*), carnitine palmitoyl transferase (*CPT1*) and short-chain-acyl-coenzymeA dehydrogenase (*SCAD*) in the quadriceps of 10 month-old mice normalized to actin (n = 4/group). *, *P* < 0.05; **, *P* < 0.01, ***, *P* < 0.001, Student’s *t*-test. Error bars represent the SEM.

acids (Fig. 3.4F). These results indicate that bezafibrate increased mitochondrial fatty acid oxidation in the skeletal muscle of MutBD mice.

### 3.3.5 Bezafibrate did not improve the skeletal muscle weight or function of Mut mice

Because Mut mice were shown to have loss of skeletal muscle mass (Hiona et al., 2010), we also monitored the effect of bezafibrate on skeletal muscle weight. We found that while WTBD mice had reduced quadriceps and gastrocnemius weight compared with WTSD mice, bezafibrate had no effect on the weight of these muscles in MutBD mice (Fig. 3.5A). Nevertheless, we went on to compare the motor function/skeletal muscle function of MutBD and WTBD mice to that MutSD and WTSD mice by assessing their performance on a treadmill test. We began testing mice at 3 months of age and monitored their performance monthly. Our results showed that between 4-6 months of age both MutBD and WTBD mice fell more than MutSD and WTSD mice (Fig. 3.5B). This trend continued between 7-10 months of age for MutBD (Fig. 3.5B). These results indicate that bezafibrate did not prevent the development of sarcopenia or improved the skeletal muscle function of Mut mice.
Figure 3.5. The effect of bezafibrate on the skeletal muscle phenotype and function of Mut and WT mice. (A) Skeletal muscle weight of 10 month-old mice (n = 4-6/group). (B) The number of falls of mice when put to run on a treadmill for 3 minutes at 9 meters/minute (n = 5-10/group). *, $P<0.05$; **, $P<0.01$, ***$P<0.001$, Student’s $t$-test. Error bars represent the SEM.

3.3.6 Bezafibrate induced hepatomegaly and fatty acid oxidation in the liver of Mut and WT mice

Bezafibrate was previously shown to have hepatoproliferative effects on rodent liver (De Souza et al., 2006; Yatsuga and Suomalainen 2012). Therefore, we monitored the liver phenotype of our mice. We found that bezafibrate doubled the liver weight of 10 month-old MutBD and WTBD mice compared to MutSD and WTSD mice (Fig. 3.6A). To determine whether there was a liver pathology, the liver of MutBD and WTBD and standard controls were subjected to histological analysis. H&E staining of the liver showed that MutBD and WTBD mice seemed to have enlarged hepatocytes primarily around the central vein of the liver, consistent with the observed hepatomegaly (Fig. 3.6B). Furthermore, we performed a hepatic panel and found that 10 month-old MutBD and WTBD had significantly increased levels of liver enzymes aspartate aminotransferase
(AST) and alanine aminotransferase (ALT) in their blood compared to MutSD and WTSD mice, a sign of liver damage (Fig. 3.6C). These results indicate that bezafibrate induced hepatomegaly and liver damage in Mut and WT mice.

To determine the effect of bezafibrate on the RNA levels of PGC-1α and β, as well as PPARs in the liver of Mut and WT mice, we performed qRT-PCR. We found that bezafibrate had no effect on the mRNA levels of PPARδ, PPARγ and PGC-1β in liver of 10 month-old mice (Fig. 3.6D). However, bezafibrate reduced the mRNA levels of PPARα in both MutBD and WTBD mice and we found a trend towards increased mRNA for PGC-1α levels in the liver of MutBD mice (Fig. 3.6D). To determine if this increase in PGC-1α RNA resulted in increased mitochondrial biogenesis in the liver, we performed western blot to detect the levels of mitochondrial protein. We found that bezafibrate did not increase the levels of mitochondrial proteins in the liver of MutBD and WTBD mice (not shown). Finally, it was recently shown that bezafibrate induces fatty acid oxidation in the liver of mice (Yatsuga and Suomalainen 2012). We confirmed this by performing qRT-PCR. Our results show that bezafibrate increased the mRNA levels of ACOX and CD36 in the liver of both MutBD and WTBD mice (Fig 3.6E). However, there was no change in the gene expression of CPT1 and SCAD (Fig 3.6E). These results indicate that bezafibrate induced fatty acid oxidation in the liver of Mut and WT mice.
Figure 3.6. Bezafibrate induces hepatomegaly and fatty acid oxidation in Mut and WT mice. (A) Liver weight of 10 month-old mice (n = 4–6/group). (B) H&E staining of the liver of 10 month-old mice showing hepatocytes and central vein (n = 4/group). (C) The level of liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the blood of 10 month-old mice (n = 6/group). (D) Gene expression of PGC-1 coactivators and PPARs in the liver of 10 month-old mice normalized to actin (n = 4/group). (E) The mRNA level of markers of fatty acid oxidation in the liver of 10 month-old mice normalized to actin (n = 4/group). Acyl-coenzymeA oxidase 1 (ACOX), cluster of differentiation 36 (CD36), carnitine palmitoyl transferase (CPT1) and short-chain-acyl-coenzymeA dehydrogenase (SCAD). *, P < 0.05; **, P < 0.01, ***, P < 0.001, Student’s t-test. Error bars represent the SEM.
3.4 Discussion

We hypothesized that bezafibrate would induce mitochondrial biogenesis in the Mut mouse and the latter would ameliorate some of the premature aging phenotypes observed in these mice. Our results showed a more complex picture, where beneficial effects were observed for some tissues, but not others. Moreover, the beneficial effects were not clearly linked to increased mitochondrial biogenesis.

In the skin, the most striking finding was the restoration of collagen protein in the dermis of MutBD mice. Since decreased collagen synthesis is a feature of skin aging (Oikarinen, 1994), bezafibrate seemed to have protected the skin in Mut mice from premature aging-like phenotypes. Furthermore, because collagen is an important structural component of the dermis skin layer, restoration of collagen levels may have contributed to the preservation of hair follicles and the delay in hair loss we observed in MutBD mice.

Although we were surprised by the beneficial effects of bezafibrate in the skin; several studies have highlighted the importance of PPARs in skin biology (Kuenzli and Saurat, 2003). PPARs have been shown to play a role in skin permeability, epidermal cell growth/differentiation, skin inflammatory response and wound healing (Kuenzli and Saurat, 2003). In fact, activation of PPARδ was shown to be able to influence collagen expression and promote wound healing in the skin via TGF-β1/Smad3 signaling (Ham et al., 2010). We showed that bezafibrate treated mice had an accumulation of total Smad3 protein in the skin, an indicator of increased Smad2/3 signaling (Smaldone et al., 2011),
suggesting that this pathway may have been activated by bezafibrate to promoting an increase in collagen levels.

We also showed that mice on the BD had reduced spleen weight and restoration of the red and white pulp organization in the spleen. The increased cleaved caspase-3 positive staining detected in the spleen of MutSD mice was not surprising since Mut mice were shown to have increased apoptosis in many tissues (Kujoth et al., 2005). However, we found that MutBD mice had a trend towards reduction in the number of cleaved caspase-3 positive staining; suggesting that bezafibrate was reducing apoptosis in the spleen. We also found that bezafibrate treated mice had increased red blood cell count which may have resulted from the restoration of spleen structure. In addition, PPARγ mRNA levels were lower in the spleen of MutBD mice. Studies have shown that PPARγ controls inflammation by inhibiting the expression of inflammatory cytokines (Kuenzli and Saurat, 2003; Wang 2010). Therefore, we speculate that PPARγ levels were increased in MutSD mice to promote an anti-inflammatory response and this response was no longer needed in MutBD mice and so PPARγ levels were reduced.

Similar to previous findings in deletor (Yatsuga and Suomalainen 2012) and Surf1 KO mice (Viscomi et al., 2011), bezafibrate did not induce increased generalized mitochondrial biogenesis/function in skeletal muscle of Mut mice. Interestingly, we found that PGC-1α protein levels were reduced in the skeletal muscle of Mut mice and they were not restored by bezafibrate. This raises the question of whether reduction in PGC-1α contributes to the skeletal muscle phenotype of Mut mice. Nevertheless, we found that bezafibrate increased markers of fatty acid oxidation in the skeletal muscle,
similar to what was shown in Surf1 KO mice (Viscomi et al., 2011). However, while bezafibrate increased the expression of PPARs in the skeletal muscle of Surf1 KO mice (Viscomi et al., 2011), PPAR expression was either decreased or unaffected in all the tissues we analyzed in bezafibrate treated Mut mice. We cannot eliminate the possibility that bezafibrate increased PPAR expression in Mut mice, but this was not observed when we analyzed 10-month-old mice on BD for 8-months.

Our laboratory previously showed that bezafibrate increased PGC-1α expression and mitochondrial biogenesis in MLC1F-Cox10⁻/⁻ mice thereby delaying the onset of the myopathy (Wenz et al., 2008). However, we could not demonstrate a generalized increase in mitochondrial content in the Mut mice, although the expression of some genes coding for mitochondrial proteins associated with β-oxidation were increased. We showed in chapter 2 that transgenic expression of PGC-1α in the skeletal muscle of Mut mice increased mitochondrial biogenesis and function and improved mouse treadmill performance (Dillon et al., 2012). Taken together, these findings showed that overexpression of PGC-1α confers more benefits to the skeletal muscle of Mut mice than bezafibrate administration.

In this chapter, we confirmed that bezafibrate has a toxic effect on the liver; it induced hepatomegaly in both WT and Mut mice. This is a rodent specific effect of bezafibrate that was also observed in the Surf1 KO and deletor mouse (Viscomi et al., 2011; Yatsuga and Suomalainen 2012). In addition, bezafibrate induced fatty acid oxidation in the liver of both WT and Mut mice, a pathway known to be regulated by PPARs (Wang 2010). Furthermore, we found that bezafibrate treated WT and Mut mice
had decreased body weight, BMD, BMC and body area. The decreases in body weight and body area were probably the result of increased fatty acid oxidation induced by bezafibrate. We suspect that decreased locomotion is related to the effects of bezafibrate on BMD and BMC. We speculate that these non-beneficial effects of bezafibrate on the overall well-being of the mice may have obscured its pharmacological beneficial effects. The early death of MutBD mice may be the result of the liver damage they sustained in combination with the phenotypes that were not ameliorated by bezafibrate.

Here we showed that bezafibrate improved some premature aging-like phenotypes of Mut mice, most clearly observed in skin and spleen. Recently, it was shown that mitochondrial dysfunction in somatic stem cells may be contributing to the progeroid phenotypes present in Mut mice (Ahlqvist et al., 2012). Therefore, our findings suggest that bezafibrate may help maintain the stem cell population in replicating tissues like skin and spleen in Mut mice. Interestingly, the benefits observed did not correlate with generalized increased mitochondrial biogenesis or function, and may also result from the effects of PPARs in other related regulatory pathways involved in the control of inflammation, fatty acid metabolism and apoptosis.

3.5 Material and Methods

Mouse Model

MtDNA mutator mice (Polg^{D257A/D257A}) (Mut) were previously characterized (Kujoth et al., 2005; Trifunovic et al., 2004). We placed 2 month-old male Mut and WT mice (MutBD and WTBD respectively) on a standard mouse diet containing 0.5%
bezafibrate (bezafibrate diet, BD) (Bio-Serv). As a control, we placed same age male Mut and WT mice on the standard mouse diet (standard diet, SD) (MutSD and WTSD respectively). Mice were kept on their respective diets for 8 months and analyzed at 10 month-old.

**Animal Husbandry**

Mice were housed in a virus-antigen-free facility of the University Of Miami Division Of Veterinary Resources in a 12-h light/dark cycle at 22°C and fed *ad libitum* with irradiated standard mouse diet or bezafibrate diet.

**DEXA Scan**

This procedure was performed as previously described (Cheung et al., 2009). In summary, mice were anesthetized then weighed and bone mineral density and content along with fat and lean body mass was measured using a Lunar PIXImus II Densitometer (GE Medical Systems, Waukesha, WI).

**Histology**

The dorsal and ventral skin of euthanized mice was shaved, and skin biopsies were collected and stored in formalin for at least 24 hours before being sent to the University of Miami Lois Pope Life Centre histology core for paraffin embedding. Paraffin skin sections were stained with hematoxylin and eosin (H&E), Verhoeff’s Van Geison (EVG) and Masson’s trichrome stain at the University of Miami Dermatology Pathology Core. The stained sections were analyzed using a light microscope. For spleen and liver analysis, deeply anesthetized mice were perfused with cold PBS and the liver
and spleen were collected and stored in formalin for at least 24 hours. Fixed tissues were then paraffin embedded, sectioned, stained for H&E and analyzed for structural changes at the University of Miami Comparative Pathology Laboratory.

**Immunostaining**

Paraffin embedded spleen sections were warmed then deparaffinized and rehydrated. Sections were incubated in 70% formic acid to retrieve antigen before being permeabilized with 0.2% Triton. Endogenous peroxidases were quenched then sections were blocked for 1 hour with normal goat serum (KPL). Sections were incubated overnight in 1:250 dilution of primary antibody for cleaved caspase-3 (Cell Signaling). Secondary antibody, biotinylated goat anti-rabbit (KPL), was used then sections were treated with streptavidin peroxidases (KPL) for 30 minutes before being incubated in Diaminobenzidine (DAB). Slides were dehydrated in alcohol then mounted in xylene with aqueous mounting medium and viewed with a light microscope. The number of positive (brown) staining was counted in 4 field of view from each section and averaged.

**Western Blot**

Western blot was performed as previously described (Pickrell et al., 2011). Primary antibodies used were Smad3 (Cell Signaling), GAPDH (GeneTex), Total OXPHOS Rodent Cocktail (Mitosciences), PGC-1α (Santa Cruz H-300), and Actin (Sigma). Primary antibodies were used at 1:1000 dilutions, except PGC-1α which was used at 1:200 dilution, and incubated overnight at 4°C. Secondary antibodies used were infrared-conjugated anti-rabbit 700 (1:3000) and anti-mouse 800 (1:5000) (Rockland)
and goat anti-rabbit HRP-linked (1:1000) (Cell Signaling). Blots were either visualized with Odyssey Infrared Imaging System (LI-COR Biosciences) and band intensity quantified with default software supplied by LI-COR or they were visualized with X-ray film developer and band intensity quantified with ImageJ software.

**Quantitative PCR**

Total RNA was isolated from snap frozen tissue using the RNeasy Fibrous Tissue Mini kit (for quadriceps) and RNeasy Mini kit (for liver and spleen) (Qiagen). cDNA was synthesized from 1µg of RNA using the iScript cDNA synthesis kit (BIO-RAD). Quantitative real-time PCR, with specific primers for PGC-1α (5′-CTGCGGGATGATGGAGACA, 5′-AGCAGCGAAAGCGTCACA), PGC-1β (5′-TGGCCCAGATACACTGACTATG, 5′-TGGGCTTCTTTCAGTAAGCT), PPARα (5′-TTCCCTGTGTGTGGCTGCTAT, 5′-CCCTCCTGCAACTTCTCAATGTAG), PPARγ (5′-CGGAAGCCTTTTGGTGACTTTA, 5′-GCGGTCTCCACTGAGAATAATGAC), PPARδ (5′-ACCGCAACAAAGTGTCAGTAC, 5′-CTCCGGCATCCGTCCAAAG), ACOX (5′-ACCGCCTATGCCCTCCACTTTTC, 5′-GCAAGCCATCCGACATTTCTTCG), CD36 (5′-CCAAATGAAGATGAGCATAGGACAT, 5′-GTTGACCTGCAGTCGTTTGC), CPT1 (5′-TTTCGACAGGTTGTTTGAC, 5′-TCTGCGTTTATGCCTATCTTG), SCAD (5′-CCTGGATTGTGCTGTGAA, 5′-TGTCTGGCCAGCTTTGAAGC) and β-Actin (5′-TGACAGGATGCAGAAGGAG, 5′-GCAGTCAGGAGGAGCAAT) was performed using SsoAdvanced SYBR Green (BIO-RAD). MtDNA copy number was
quantified as previously described (Dillon et al., 2012). ΔΔCt method was used to
determine the relative abundance of each gene.

**Spectrophotometric Assays**

Citrate synthase (CS) activity was determined spectrophotometrically in total
homogenate from the quadriceps of mice as previously described (Dillon et al., 2012).
Assay results were normalized to protein concentration obtained by the Bradford method.

**Treadmill Test**

The treadmill test was performed as previously described (Dillon et al., 2012). Mice were first acclimated to the treadmill (Columbus Instruments, Columbus, OH) then
they were put to run at 9 meters per minute for 3 minutes. Mice performance was
determined by the number of times a mouse falls off the running belt onto the grid during
the test.

**Blood Analysis**

Mice were fasted overnight and the following day anesthetized and blood was
collected by cardiac puncture. Blood was sent to the University of Miami Comparative
Pathology Laboratory for analysis of complete blood cell count (CBC) and for a hepatic
blood panel to measure markers of liver damage/inflammation.
4.1 Overview and Objective

PGC-1α and PGC-1β are master regulators of mitochondrial biogenesis and function. Since decreased mitochondrial biogenesis has been associated with aging, we wanted to test if ubiquitous overexpression of PGC-1α or PGC-1β could ameliorate aging phenotypes in mouse models. The mtDNA mutator mouse (Mut) is a mouse model of aging with a mutant mtDNA polymerase γ (POLG) that has profound mitochondrial dysfunction. Our aim was to increase the expression of PGC-1α/β ubiquitously in Mut and WT mice using an inducible system and characterize the immediate effects of this induction on mouse phenotype to establish a model that could be used to access long-term effects during aging. We found that increased expression of PGC-1β systemically in either WT or Mut mice was toxic and caused lethality after 2-3 weeks of PGC-1β induction. On the other hand, ubiquitous induction of PGC-1α in WT mice did not appear to be deleterious. These results indicate that our mouse model of ubiquitous expression of PGC-1α may be a useful model to study the effects of overexpression of PGC-1α on aging phenotypes in all tissues of Mut mice.

4.2 Background and Significance

Studies have shown that stimulation of mitochondrial biogenesis by increasing the expression of PGC-1α and/or PGC-1β (PGC-1α/β) compensates for mitochondrial...
dysfunction in tissues and cells (Lehman et al., 2000; Srivastava et al., 2007; Srivastava et al., 2009; Wenz et al., 2008). Our laboratory showed that overexpressing PGC-1α/β in cells with a OXPHOS defect caused by nonsense mutations in the mtDNA or in OXPHOS deficient cells from patients with mitochondrial disorders leads to increased mitochondrial respiration and expression of OXPHOS genes in the cells (Srivastava et al., 2007; Srivastava et al., 2009). Also we showed that in a mouse model with mitochondrial muscle myopathy, increased expression of PGC-1α leads to increase in mitochondrial mass and an increase in respiration and ATP production (Wenz et al., 2008). This improvement in mitochondrial function resulted in a delay in the onset of the myopathy and importantly, an increase in the animals’ life span (Wenz et al., 2008). Interestingly, our lab also showed that increased expression of PGC-1α in the skeletal muscle of aging wild-type (WT) mice protects from mitochondrial dysfunction and sarcopenia associated with normal aging (Wenz et al., 2009b). In addition, the mice with increased muscle PGC-1α had improved whole-body health and increased lifespan compared to Wt mice (Wenz et al., 2009b).

Since mitochondrial dysfunction and decreased mitochondrial biogenesis have been associated with aging, we hypothesized that increasing mitochondrial biogenesis in all tissues of WT and Mut mice (mouse model of aging) will compensate or prevent the development of mitochondrial defects and slow aging. In chapter 2, we showed that muscle specific overexpression of PGC-1α in Mut mice increased mitochondrial biogenesis in heart and skeletal muscle and ameliorated the aging phenotype in these tissues (Dillon et al., 2012). However, this muscle specific expression did not confer
much systemic benefits or improvements to non-muscle tissues in Mut mice (Dillon et al., 2012). In chapter 3, we tried to increase mitochondrial biogenesis in all tissues of the Mut mice using bezafibrate (a pharmacological drug). We showed that bezafibrate did not promote generalized increase in mitochondrial biogenesis; instead it increased fatty acid oxidation and improved the aging phenotype in the skin and spleen of Mut mice.

In a second attempt to increase mitochondrial biogenesis in all tissues of the Mut and WT mice, we created two mouse models, one with inducible ubiquitous expression of PGC-1β and the other with PGC-1α. Using doxycycline (an analog of tetracycline) to induce expression of the transgene in our mice, we determined the conditions necessary for induction. We found that 7 days induction with standard mouse chow containing 1.5 g/Kg doxycycline was enough to induce expression of both PGC-1β and PGC-1α in tissues of the mice. Our results showed that increased expression of PGC-1β systemically in either WT or Mut mice was lethal after 2-3 weeks of PGC-1β induction. However, ubiquitous induction of PGC-1α in WT mice did not cause deleterious effects in the mice and so this model may be used to assess the effects of increased PGC-1α expression in all tissues of Mut mice.

4.3 Results and Discussion

4.3.1 Generation of iROSAPGC-1α/βMut and iROSAPGC-1α/βWT mice

We wanted to test whether we could induce increased expression of PGC-1α/β, regulators of mitochondrial biogenesis, throughout the body of the Mut and WT mice.
Therefore, we have created iROSAPGC-1α/βMut and iROSAPGC-1α/βWT mice, mice that are inducibly and ubiquitously expressing PGC-1α/β.

In the iROSAPGC-1α/βMut and WT mice, the ROSA promoter directs the expression of PGC-1α/β in all tissues of the mice only in the presence of tetracycline or a tetracycline analog such as doxycycline (Dox). In all our experiments we used Dox to induce the expression of PGC-1α/β in the iROSAPGC-1α/βMut and WT mice. The animals were fed Dox-containing food (Bio-Serv) only during periods of induction; otherwise they were fed the standard diet.

We used several mouse models to create iROSAPGC-1α/βMut mice. The first is the Mut mouse. The second is inducible PGC-1α mouse and inducible PGC-1β mouse. These are separate mice, one expressing PGC-1α and the other expressing PGC-1β from a tetracycline response element (TRE) (TRE-PGC-1α/β mice) (Russell et al., 2004). We obtained these TRE-PGC-1α/β mice in a FVB/N background so we backcrossed them to WT C57BL/6 mice so that all our animals would have the same genetic background. We used F6 pups from these backcrossings for matings. Thirdly, we used a mouse expressing Reverse Tetracycline Transactivator (rtTA) mouse under the ubiquitous ROSA26 locus (ROSA26-rtTA-IRES-EGFP) (Belteki et al., 2005).

We performed several crosses to obtain iROSAPGC-1α/βMut mice (Fig. 4.1). We generated mice homozygous for the TRE-PGC-1α/β transgene and mice homozygous for the ROSA-rtTA-IRES-EGFP transgene that were both heterozygous for POLG. These mice were crossed to obtain mice heterozygous for the TRE-PGC-1α/β and ROSA-rtTA-
IRES-EGFP and homozygous for POLG (iROSAPGC-1α/βMut mice). This mating plan increased the yield of iROSAPGC-1α/βMut mice to 25 percent of each litter.

**Figure 4.1. Creation of iROSAPGC-1α/βMut mice.** Diagrammatic representation of crosses performed to generate iROSAPGC-1α/βMut mice.

### 4.3.2. Assessment of the inducibility of PGC-1β expression in mutator and WT mice

**Effects of 500mg/kg (low) and 10g/kg (high) doxycycline diet on PGC-1β expression**

We performed our initial experiments with iROSAPGC-1βWT (inducible mice - mice heterozygous for both ROSA-rtTA-IRES-EGFP and TRE-PGC-1β) and un-inducible controls (mice not heterozygous for both ROSA-rtTA-IRES-EGFP and TRE-PGC-1β) to determine the inducibility of the expressed PGC-1β in a WT background. Six
weeks old male mice were induced for 7 days with 200 mg/kg (low) Dox diet and for 7 days with 10 g/kg (high) Dox diet. For this pilot study mice were placed in the following groups: iROSAPGC-1βWT mice on the standard diet, iROSAPGC-1βWT mice on Dox diet and un-inducible mouse on Dox diet. The mice were weighed and transthoracic echocardiogram was performed before and after induction. All mice were sacrificed immediately after induction was halted and tissues were collected and subjected to RNA analysis.

Our results showed that after 7 days of induction with the high Dox diet, iROSAPGC-1βWT mice had an unhealthy appearance (not shown) and a decrease in body weight (Fig. 4.2A). These mice also had a decrease in heart left ventricular internal dimension, a sign of left ventricle hypertrophy, and an increase in percent heart ejection fraction, suspected to result from the heart’s attempt to compensate for left ventricular malfunction (Fig. 4.2A and 4.2B respectively). No change in these parameters was observed in un-inducible mice on the high Dox diet or iROSAPGC-1βWT mice on the standard diet (Fig. 4.2). In addition, iROSAPGC-1βWT and un-inducible mice on the low Dox diet for 7 days also had no changes in these parameters (Fig. 4.2). These results suggest that inducing PGC-1β expression with a high Dox diet may have negative effects on the heart.

To determine if we were able to induce PGC-1β expression in iROSAPGC-1βWT mice with low and high Dox diets, and if the changes seen in body weight and heart function of the mice were due to an increase in PGC-1β expression or side effects of
Figure 4.2. The effects of low and high concentrations of Dox on body weight and heart phenotype of iROSAPGC-1βWT. (A) Body weight of 6 week-old iROSAPGC-1βWT mice on a high Dox diet (10g/kg doxycycline), low Dox (200mg/kg) diet or standard mouse diet (No Dox.) for 7 days. (B) Representative M-mode echocardiographic images showing a section through the left ventricle of the heart of mice. Yellow arrows indicate the left ventricular internal dimension at diastole (LVID:d). Graphs show quantification of LVID:d. (C) Percent heart ejection fraction of iROSAPGC-1βWT mice on a high, low or No Dox diet for 7 days. (n=1-2 male mice/group)

doxycycline, RNA was prepared from the heart and liver of mice from all experimental groups. QRT-PCR was done to detect changes in PGC-1β mRNA levels of both tissues and the results were normalized to beta actin. Our results show that only iROSAPGC-
1βWT mice on the high Dox diet had increased PGC-1β levels in the heart and liver following the 7 day induction (Fig 4.3A and 4.3B). No increase in PGC-1β level was seen in un-induced mice on the high Dox diet for 7 days or iROSAPGC-1βWT mice on a no Dox diet (Fig. 4.3A and 4.3B). Similarly, no increase in PGC-1β level was observed in iROSAPGC-1βWT mice on the low Dox diet for 7 days (Fig. 4.3A and 4.3B). These results indicate the high Dox diet is able to induce PGC-1β expression in 7 days whereas the low Dox diet is not.

Figure 4.3. High concentration Dox diet induces increased PGC-1β expression in heart and liver of iROSAPGC-1βWT mice. (A) PGC-1β mRNA levels in the liver and (B) PGC-1β mRNA levels in the heart of 6 week-old iROSAPGC-1βWT (inducible, Ind) and un-inducible mice (un-ind) on the high Dox diet or low dox diet (200mg/Kg) for 7 days (n=1-2 mice/group)

Furthermore, we observed that iROSAPGC-1βWT mice on the high Dox diet for 7 days had a 12-14 fold increase in PGC-1β mRNA levels in the heart compared to 2-6 fold increase in the liver (Fig 4.3A and 4.3B). This extremely high expression of PGC-1β in the heart seems to underly the signs of cardiac hypertrophy and the weight loss observed in induced iROSAPGC-1βWT mice on the high Dox diet. Therefore, although we were able to increase PGC-1β levels with the high Dox diet and prove the inducibility
of iROSA-PGC-1βWT mice, this concentration of Dox induces too high expression of PGC-1β and so is not suitable to be used in future studies.

Effects of 1.5g/Kg doxycycline (medium Dox) diet on PGC-1β expression

In an attempt to find a concentration of Dox that induces increased PGC-1β expression without conferring negative effects, we obtained a diet containing 1.5g/Kg doxycycline (medium Dox diet). 4 month-old male iROSA-PGC-1βWT and age matched un-inducible controls (mice not heterozygous for both ROSA-rta-IRES-EGFP and TRE-PGC-1β) were placed on the medium Dox diet for 7 days. The mice were weighed and transthoracic echocardiogram was performed before and after induction.

After 7 days induction with the medium Dox diet, inducible mice (iROSA-PGC-1βWT) had a marked decrease in body weight while there was no change in the body weight of the un-inducible controls (Fig. 4.4A). The transthoracic echocardiogram showed no change in the left ventricular internal dimension at diastole or left ventricle mass after the induction of both inducible and un-inducible mice (Fig. 4.4B and 4.4C). However, there was a trend towards a decrease in percent heart ejection fraction in both induced and un-induced mice (Fig. 4.4D).

To determine if 7 days of medium Dox diet was able to induce PGC-1β expression in iROSA-PGC-1βWT and iROSA-PGC-1βMut mice, we prepared RNA from the heart and liver of 3 month-old male mice. QRT-PCR was performed to detect changes in PGC-1β mRNA levels of both tissues and the results were normalized to actin. Our
results showed that 7 days of medium Dox diet induced a 15 fold increase in PGC-1β expression in the heart of iROSAPGC-1βWT and a 10 fold increase in iROSAPGC-1β Mut mice (Fig. 4.5). It also increased PGC-1β expression ~8 fold in the heart of iROSAPGC-1βMut mice and there was a trend towards increased PGC-1β in the heart of iROSAPGC-1βWT mice (Fig. 4.5).

Since 7 days induction with medium Dox diet was able to induce PGC-1β expression in the heart of iROSAPGC-1βWT and iROSAPGC-1βMut mice without
causing a detectable heart defect; we placed 7 month-old male iROSAPGC-1βMut, Mut, iROSAPGC-1βWT and WT mice on the medium Dox diet and monitored their physical phenotype. All mice were weighed before induction and weekly following the start of

![Graph](image)

**Figure 4.5. Medium concentration Dox diet induced increased PGC-1β expression in liver and heart.** PGC-1β mRNA levels in liver and heart of 4 month-old male mice on the medium doxycycline diet for 7 days normalized to actin (n = 3-4/group). *, P< 0.05; **, P<0.01, Student’s t-test. Error bars represent the SEM.

the induction. After one week induction, iROSAPGC-1βMut mice had on average a 7 gram decrease in body weight while iROSAPGC-1βWT mice had a 10 grams decrease (Fig. 4.6). The decrease in body weight continued after two weeks of induction but was much less, only between 2-3 grams for both groups (Fig. 4.6). We also observed that un-induced Mut and WT mice had a small decrease in body weight each week after the start of induction (Fig. 4.6). This small decrease in body weight in the un-inducible mice could be due to adjustment to the new diet while the marked decrease in body weight of the inducible mice is probably due to a combination of the induced expression of PGC-1β and adjustment to the new diet.
After two weeks of continuous induction with the medium Dox diet, some of the iROSAPGC-1βMut mice died. All mice with this genotype were dead before the third week of continuous induction while all the un-induced Mut mice were still alive. Blood and tissues were collected from one iROSAPGC-1β-Mut mouse on the verge of death and used for a hepatic blood panel. Our results showed that this mouse had high alanine aminotransferase (ALT) levels, low albumin and low blood urea to creatine ratio (not shown). Histological studies were also performed on the heart, lung, liver, spleen, kidney and small and large intestines of this mouse. The heart had moderate myofibril degeneration and necrosis and the liver had moderate hepatocellular atrophy (not shown). All other tissues were within normal limits. These results suggest that continuous induction of PGC-1β expression in 7 month-old iROSAPGC-1βMut mice with the medium Dox diet, leads to heart and liver damage which may have contributed to their death. We confirmed these results with a second group of mice. Based on these results we

Figure 4.6. Medium concentration Dox diet induced decreased body weight of iROSAPGC-1βMut and iROSAPGC-1βWT mice. Body weight of 7 month-old mice on the medium Dox diet continuously for 2-3 weeks (Mut groups, n = 4 mice and WT groups, n = 6 mice). Error bars represent the SEM.
conclude that continuous induction of 7 month-old iROSAPGC-1βMut and iROSAPGC-1βWT mice with the medium Dox diet for two or more weeks is detrimental to their health.

To determine if the non-beneficial effects of induction of PGC-1β expression in 7 month-old mutator and wild-type mice was due to the age of the mice, we placed 3 month-old iROSAPGC-1βMut and iROSAPGC-1βWT mice on the medium Dox diet. Similar to 7 month-old mice, we observed that all 3 month-old iROSAPGC-1βMut mice died after two weeks while iROSAPGC-1βWT mice died after three weeks of continuous induction. These results indicate that death of continuously induced iROSAPGC-1βMut and iROSAPGC-1βWT is most likely due to effects of PGC-1β induction and not related to the mouse age at the start of induction.

Next, we wanted to determine the cause of death in continuously induced iROSAPGC-1βMut and iROSAPGC-1βWT mice. Since we observed that continuous induction with the medium Dox diet for two weeks caused heart and liver damage in a 7 month-old iROSAPGC-1βMut mouse, we tried to confirm these findings by repeating this experiment in 3 month-old mice. Our results showed that 3 month-old iROSAPGC-1βMut and iROSAPGC-1βWT mice also had a decrease in body weight and are smaller in size after one week of induction with medium Dox diet when compared with un-induced mutator and wild-type mice respectively (Fig. 4.7A and 4.7B). In addition, iROSAPGC-1βMut mice had decreased heart, liver and spleen, kidney and gastrocnemius weight after one week of induction compared with un-induced mutator mice (Fig. 4.7C and 4.7D). This corresponds to their decrease in body weight. However, the 3 month-old
iROSAPGC-1βWT mice only had a decrease in kidney weight, while their thymus weight increased (Fig. 4.7D and 4.7C respectively). The results of the pathology report showed that all iROSAPGC-1βMut and iROSAPGC-1βWT mice had mild hepatocellular atrophy while only one iROSAPGC-1βMut mouse had mild myocardial degeneration

![Graphs and images](image)

**Figure 4.7.** 3 month-old ROSAPGC-1βMut and ROSAPGC-1βWT mice have decreased body and organ weight after 7 days induction with medium concentration Dox diet. (A) Body weight of mice before and after 7 days induction with medium Dox diet. (B) Picture of mice after 7 days induction with the medium Dox diet. (C) and (D) Organ weight of mice after 7 days induction with the medium Dox diet. n=3-4/group. *, P< 0.05; **, P<0.01, Student’s t-test. Error bars represent the SEM.

(not shown). This indicates that induction of PGC-1β expression in iROSAPGC-1βMut and iROSAPGC-1βWT leads to liver atrophy. The atrophy was mild possibly because
these mice were analyzed only after one week of induction and death was observed after 2 to 3 weeks of induction, nevertheless it is unlikely that liver atrophy is causing the death of induced mice.

We also analyzed the blood of 3-month-old iROSAPGC-1βMut, iROSAPGC-1βWT and un-inducible Mut and WT mice on the medium Dox diet for 7 days to determine the systemic effects of PGC-1β induction. We found that while Mut mice had decreased red blood cell count, hemoglobin and hematocrit compared with WT mice, iROSAPGC-1βMut mice had restored levels of these parameters after 7 days induction with the medium Dox diet (Fig. 4.8A and 4.8B). iROSAPGC-1βWT mice had increased white blood cell and neutrophils compared with WT mice after 7 days induction with the medium Dox diet (Fig. 4.8A and 4.8B). We also observed that both iROSAPGC-1βMut and iROSAPGC-1βWT mice had either increased or a trend towards increased blood creatine and blood urea nitrogen (BUN) levels. This may be an indicator of kidney damage (Fig. 4.8C and 4.8D).

In conclusion, although 7 days induction of iROSAPGC-1αMut and iROSAPGC-1αWt mice with the medium Dox diet induced PGC-1β expression and seems to ameliorate the anemia in mutator mice, it lead to weight loss, hepatocellular atrophy and signs of kidney damage in both WT and mutator mice. To conclude this aim, we decided to focus on inducing PGC-1α expression in iROSAPGC-1αMut and iROSAPGC-1αWT mice as we have shown in other mouse models that increased PGC-1α expression in muscle confers benefit to both mutator and wild-type mice.
Figure 4.8. Systemic effects of 7 days PGC-1β induction with medium concentration Dox diet. Complete blood cell count results of 3 month-old mice (A) RBC (Red Blood Cells, $\times 10^6$µl), HGB (Hemoglobin, g/dL), WBC (White Blood Cells, $\times 10^6$µl), MNC (Monocytes, %), BASO (Basophils, %) and EOSS (Eosinophils, %) and (B) HCT (Hematocrit, %), MCV (Mean Corpuscular Volume, fL), MCH (Mean Corpuscular Hemoglobin, pg), MCHC (Mean Corpuscular Hemoglobin Concentration, %), Lymph (Lymphocytes, %) and NEU (Neutrophils, %) (C) and (D) hepatic panel results showing blood creatine, BUN (Blood Urea Nitrogen) and total bilirubin levels all in mg/dL (n=3-4/group). *, $P < 0.05$; **, $P < 0.01$, Student’s t-test. Error bars represent the SEM.

4.3.3 Assessment of the inducibility of PGC-1α expression in Mut and WT mice

In an initial experiment to determine the inducibility of PGC-1α expression, we placed 3 month-old male and female iROSAPGC-1αWT mice and un-inducible WT controls continuously on the medium Dox. After 2-3 months, the mice looked well and were steadily increasing their body weight (Fig 4.9A and 4.9B). Therefore, to test of the
medium Dox diet induces PGC-1α expression; we placed 4-6 week-old iROSAPGC-1αWT and WT mice on the medium Dox for 7 days. All mice were weighed before and after 7 days of induction. Immediately after induction, mice were sacrificed and the liver, spleen, thymus, quadriceps, gastrocnemius, kidney, testes, heart and soleus were removed. RNA was prepared from these tissues and used for qRTPCR. Our results showed that after 7 days induction with the medium Dox diet, iROSAPGC-1αWT and WT mice had a trend towards increased body weight (Fig. 4.10A). We also found that all iROSAPGC-1αWT mice had increased PGC-1α expression in the quadriiceps and gastrocnemius but not in heart after 7 days induction when compared with WT mice (Fig. 4.10B). They also had increased PGC-1α expression in testes, kidney, thymus and spleen (Fig. 4.10C and 4.10D). However, the highest level of PGC-1α expression was found in the thymus. These results indicate that we are able to induce PGC-1α expression in
iROSAPGC-1αWT mice with the medium Dox diet without causing a decrease in body weight or detrimental effects in the mice, which contrasts with PGC-1β.

Since Mut mice have premature aging phenotypes in the skeletal muscle, thymus, and spleen (tissues that had high expression of PGC-1α in iROSAPGC-1αWT mice), our inducible ubiquitous PGC-1α mouse model is a good model system with which to test the effects of ubiquitous expression of PGC-1α on aging phenotypes in the Mut mouse.

Figure 4.10. iROSAPGC-1αWT mice on medium concentration Dox diet have increased PGC-1α expression (A) Body weight of 4-6 week-old mice before and after 7 days induction with medium Dox diet. (B), (C) and (D) PGC-1α mRNA expression in the different tissues of 4-6 week-old iROSAPGC-1αWT and un-inducible WT mice after 7 days induction with medium Dox diet normalized to glyceraldehyde 3-phosphate (GAPDH). *, P < 0.05; **, P < 0.01, Student’s t-test. Error bars represent the SEM.
4.4 Materials and Methods

Animal Husbandry

Mice were housed in a virus-antigen-free facility of the University Of Miami Division Of Veterinary Resources in a 12-h light/dark cycle at 22°C and fed ad libitum with irradiated standard mouse diet or standard mouse diet containing doxycycline. Doxycycline diet used was low concentration (200 mg/Kg), medium concentration (1.5 g/Kg) or high concentration (10 g/Kg).

Echocardiogram

Transthoracic echocardiography was performed on mice anesthetized with 1% isoflurane as previously described (Peacock et al., 2010). The VisualSonics 770 system (Toronto, Canada) was used for this procedure and percent ejection fraction, left ventricular internal dimension during diastole and left ventricle mass was calculated from the obtained images.

Blood Analysis

Mice were deprived of food overnight and the following day anesthetized and blood was collected by cardiac puncture. Blood was sent to the University of Miami Comparative Pathology Laboratory for complete blood cell count, blood biochemistry and hepatic panel analysis.

Quantitative RT-PCR

Total RNA was isolated from snap frozen tissue using the RNeasy Fibrous Tissue Mini kit (for heart and skeletal muscle) and RNeasy Mini kit (for all other tissues)
cDNA was synthesized from 1µg of RNA using the iScript cDNA synthesis kit (BIO-RAD). Quantitative real-time PCR, with specific primers for PGC-1α (5’-CTGC GGATGATGGAGACA, 5’-AGCAGCGAAAGCGTCACA), PGC-1β (5’-TGGCC AGATACACTGACTATG, 5’-TGGGCCTCTTTTCAGTAAGCT), β-Actin (5’-TGACAGGATGCAGAAGGAGAT, 5’-GCGCTCAGGAGGAGCAAT) and GAPDH (5’-GCAGTGGCAAAGTGAGGAGATT, 5’-GAATTTGCCGTGAGTGGAGT) was performed using SsoAdvanced SYBR Green (BIO-RAD). ΔΔCt method was used to determine the relative abundance of each gene.
CHAPTER 5
CONCLUDING REMARKS

Aging is a major risk factor for developing neurological disorders, cardiovascular diseases and sarcopenia. Over the years, many studies have focused on identifying and targeting molecular pathways that contribute to aging in an attempt to slow the aging process. Recently, a unified theory of aging was proposed and it suggests that the major molecular pathways thought to promote aging all contribute to mitochondrial dysfunction in aging (Sahin and Depinho 2012). This emphasizes the importance of proper regulation of mitochondrial biogenesis and function during the aging process. However, there is a need for effective methods to compensate for mitochondrial dysfunction and decreased bioenergetic potential during aging. The work presented in this dissertation demonstrates that increasing mitochondrial biogenesis is a promising method to compensate for decreased mitochondrial biogenesis and function during aging. It also shows that activation of PPAR regulated pathways can confer beneficial effects during aging.

The results presented in this dissertation showed that overexpression of PGC-1α, a master regulator of mitochondrial biogenesis and function, is an effective way to improve mitochondrial function in aging. We studied the mtDNA mutator mouse, a model of aging that has a proofreading deficient mtDNA polymerase γ that causes the accumulation of mtDNA mutations and mitochondrial dysfunction in different tissues. We found that increased PGC-1α expression in the muscle of the mtDNA mutator mouse, increased mitochondrial biogenesis and function in heart and skeletal muscle. This improvement in mitochondrial function did not prevent skeletal muscle wasting but it
maintained the skeletal muscle function of the mice. As a result of the improvements in skeletal muscle and improved mitochondrial function in heart, Mut mice with increased PGC-1α expression also had improved heart function. This finding highlights the fact that skeletal muscle health or phenotype has a strong influence on the function of other tissues. Our results also showed that increased PGC-1α expression in muscle slightly increased the abundance of somatic mtDNA mutations in the skeletal muscle of the Mut mouse. This was not surprising since PGC-1α increased mitochondrial biogenesis and mtDNA replication. However, these results demonstrate that increased mitochondrial biogenesis can confer benefits without repairing DNA damage. Therefore, this approach is not only applicable to aging but also to conditions caused by DNA mutations that are characterized by mitochondrial dysfunction.

Our results also showed that increased mitochondrial biogenesis in muscle did not confer many systemic benefits or alleviate the aging phenotype in non-muscle tissues in the Mut mice. However, it was recently shown that endurance exercise rescued all the aging phenotypes in the Mut mouse (Safdar et al., 2011a). Because exercise increases PGC-1α expression, this highlights the benefits of increased PGC-1α expression, but it also indicates that exercise regulates many other pathways that likely conferred systemic benefits to Mut mice. It would be interesting to determine the non-PGC-1α related pathways that are activated by exercise and perhaps these pathways could be activated simultaneously with pathways regulating mitochondrial biogenesis to provide an effective approach to slow aging in cases where exercise therapy is not a feasible option.
The work presented in this dissertation also showed that pharmacological activation of PPARs can confer benefits during aging. We found that Mut mice treated with bezafibrate, a PPAR panagonist, for 8 months had improved skin and spleen phenotype. Bezafibrate delayed hair loss, improved skin structure and restored collagen levels in the skin. This was a surprising finding that highlights the important role of PPARs in cutaneous biology and suggests that these proteins may be therapeutic targets to combat hair loss during aging. We also found that bezafibrate reduced spleen size and restored spleen structure of Mut mice. It also increased the red blood cell count of Mut mice, demonstrating that it conferred systemic benefits to the mice. However, contrary to our initial hypothesis, bezafibrate did not induce generalized mitochondrial biogenesis or function. Instead it induced fatty acid oxidation similar to what was observed in other mouse models (Viscomi et al., 2011; Yatsuga and Suomalainen 2012). We also found that bezafibrate had some non-benefical effects. It induced hepatomegaly, decreased body weight and bone mineral density/content. These findings confirm that bezafibrate has a toxic effect on rodents. This toxic effect may have overshadowed some potential beneficial effects of bezafibrate and also poses a challenge in testing the effects of this drug on rodent models. Our results also demonstrate that overexpression of PGC-1α is a more effective method to improve mitochondrial biogenesis and function than administration of bezafibrate. Consequently, there is a need for the development of more effective pharmacological agents that can be used to induce mitochondrial biogenesis.

AICAR, a pharmacological agent that activates AMPK, was recently shown to increase mitochondrial biogenesis and function in the skeletal muscle of mice with a
deficiency in COX activity (Viscomi et al., 2011). In addition, AICAR did not have a toxic effect on the mice (Viscomi et al., 2011). Based on these findings, it would be interesting to test if this agent can increase mitochondrial biogenesis and function and improve the skeletal muscle phenotype of Mut mice. Because AICAR specifically targets AMPK pathways, its effects can be easily interpreted unlike bezafibrate which activates all the members of the PPAR family. Therefore, AICAR may be a promising alternative to bezafibrate that can be used to compensate for mitochondrial dysfunction in aging.

Finally, in this dissertation we developed mouse models with inducible ubiquitous expression of PGC-1α and PGC-1β that can be used to induce systemic mitochondrial biogenesis. We showed that ubiquitous expression of PGC-1β had a deleterious effect on WT and Mut mice. Although we still do not know the reason for this effect, it is possible that overstimulation of metabolic pathways regulated by PGC-1β may have contributed to this phenotype. The expression of PGC-1β was strongly induced in the heart and liver of both WT and Mut mice, tissues that were found to have an abnormal pathology after 7 days induction of PGC-1β expression. We speculate that perhaps high levels of PGC-1β expression in the heart and liver induced huge increases in mitochondrial biogenesis and lipid metabolism that eventually lead to aberrant heart and liver function. In fact, it was previously shown that high expression of PGC-1α in the adult heart leads to cardiomyopathy and early death due to increases in mitochondrial biogenesis (Russell et al., 2004). Perhaps this is also true for high expression of PGC-1β in the adult heart. However, further studies are needed to determine the expression level of PGC-1β in other tissues of these mice and also to identify the pathways activated by ubiquitous expression
of PGC-1β. These studies will be crucial in determining the cause of death in mice with ubiquitous PGC-1β expression.

On the contrary, we found that ubiquitous expression of PGC-1α in WT mice did not have a deleterious effect although a similar induction paradigm was used as in the PGC-1β mouse model. This difference in effect may be due to the fact that expression of PGC-1α in the heart and liver of induced WT mice was not as high as that of PGC-1β; perhaps due to differences in gene copy number. Since mouse health was not affected by ubiquitous PGC-1α expression, further studies are needed to determine if the expression of PGC-1α observed is sufficient to induce systemic increase in mitochondrial biogenesis and function. This unique mouse model will help to expand our knowledge on the effects of PGC-1α in different tissues in wild-type mice. Furthermore, the findings presented in this dissertation suggest that ubiquitous expression of PGC-1α in the Mut mouse will be a good system in which to study the effects of systemic increase in mitochondrial biogenesis and function on aging phenotypes. This inducible mouse model is a valuable tool as it will allow one to test whether increased mitochondrial biogenesis confers more protection against aging if induced in early or late adulthood.
WORKS CITED


