Characterization of Molecular and Functional Changes Regulating Hypobaric Hypoxia-Induced Right Ventricular Hypertrophy

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DEDICATION

To my parents for instilling work ethic and a sense of purpose in my life

ABSTRACT

Characterization of Molecular and Functional Changes Regulating Hypoxia-Induced Right Ventricular Hypertrophy: Makhosazane Zungu, February 2007

We hypothesized that exposure to chronic hypobaric hypoxia coordinately upregulates mitochondrial regulatory genes as part of an adaptive response to sustain mitochondrial respiratory capacity and contractile function. To test this hypothesis, we initially exposed rats to 2 and 4 weeks of hypobaric hypoxia (11% O_2) and determined cardiac contractile, mitochondrial respiratory function and measured transcript levels of several mitochondrial regulators for the right and left ventricles respectively. For this part of the study, our data demonstrate the coordinate induction of several mitochondrial genes regulating mitochondrial respiratory function and increased mitochondrial DNA content in the hypertrophied RV after 2 weeks of hypoxia. These changes were not observed in the LV. These data suggest a link between the efficiency of mitochondrial oxidative phosphorylation and respiratory function to sustained RV contractile function in response to the increased load. We also found that these adaptations were generally sustained in the RV after 4 weeks of hypoxic exposure. However, the LV displayed diminished contractile and respiratory function at the 4-week time point. We suggest that this mechanism could be part of an adaptive response to conserve energy for contractile purposes.

In parallel, we explored the role of increased fatty acid utilization in the setting of right ventricular hypertrophy by employing a peroxisome proliferator-activated receptor alpha (PPARα) agonist, i.e. Wy-14,643. We found that Wy-14,643 treatment attenuated mitochondrial respiration in the RV and the LV, while promoting uncoupling of mitochondrial oxidative phosphorylation in the LV, suggesting that it may not be an ideal experimental agent to use for in vivo studies aiming to increase myocardial fatty acid oxidation rates.

We next exposed rats to 12 weeks of hypobaric hypoxia to follow up on changes observed at the 2- and 4-week time points. Furthermore, we increased cardiac FA utilization by either: a) cocoa butter, b) fenofibrate or c) coco-butter plus fenofibrate administration. Here we found sustained cardiac contractile function and diminished mitochondrial respiration in both ventricles in response to 12 weeks hypobaric hypoxia. Our data show distinct effects of cocoa butter and fenofibrate on cardiac and respiratory function in the RV and LV following hypoxic exposure. For the RV, the hypoxia-mediated increase in systolic pressure, developed pressure and RPP was reduced following fenofibrate administration under hypoxic conditions. In contrast, RV systolic and developed pressures remained relatively high following cocoa butter treatment. The cocoa butter-fenofibrate combination under hypoxic conditions largely reduced cardiac contractile function in both ventricles. Taken together, these data suggest that chronic hypobaric hypoxia triggers adaptive pathways that may reserve organisms from the potentially damaging effects of high fatty acid supply and support the concept that fuel substrate switch away from fatty acids may be an adaptive mechanism.

We next proceeded to investigate the acute and direct effects of Wy-14,643 and fenofibrate on isolated mitochondria. Our data show that acute exposure to Wy-14,643 and fenofibrate differentially perturb cardiac mitochondrial respiration, i.e. fenofibrate more potently inhibited mitochondrial respiratory function compared to Wy-14,643. We found that both electron transport chain complex I and II are inhibited by fenofibrate, while Wy-14,643 more readily inhibited complex I-dependent respiration.

SUMMARY

An imbalance between tissue oxygen supply and demand results in hypoxia. Hypoxia is caused by many factors which may range from environmental (e.g. high altitude, diving), clinical (e.g. sleep apnea, chronic obstructive pulmonary disease), low oxygen delivery (e.g. heart failure, vascular diseases, stroke, anemia), and low oxygen utilization by tissues (e.g. sepsis, metabolic myopathies). Organisms have evolved adaptive regulatory mechanisms to cope with acute and chronic exposure to hypoxia, and these include 1) pulmonary ventilation, 2) blood hemoglobin, 3) vascularity of the tissues, 4) diffusing capacity of the lungs, 5) metabolic remodeling, and 6) cellular oxygen utilization. Exposure to hypobaric hypoxia also leads to development of right ventricular (RV) hypertrophy, mainly due to an increase in RV mass secondary to pulmonary hypertension.

In the first part of the study (Chapter 2), we hypothesized that exposure to chronic hypobaric hypoxia coordinately upregulates mitochondrial regulatory genes and mitochondrial content in the hypertrophied RV, as part of an adaptive response to sustain mitochondrial respiratory capacity and contractile function. To test our hypothesis, we exposed rats to 2 and 4 weeks of hypobaric hypoxia (11% O_2) and determined cardiac contractile and mitochondrial respiratory function for the right and left ventricles, respectively. Moreover, we performed real-time quantitative RT-PCR analysis to measure transcript levels of several mitochondrial regulators. For this part of the study, our data demonstrate the coordinate induction of several genes (COXII, PGC-1α, NRF1) regulating mitochondrial respiratory function and increased mitochondrial DNA (mtDNA) content in the hypertrophied RV, linking the efficiency of mitochondrial oxidative phosphorylation and respiratory function to sustained contractile function in response to the increased load. Interestingly, these adaptations

were not observed in the LV. Moreover, these changes were generally sustained in the RV after 4 weeks of hypoxic exposure. However, at this time-point the LV began to display diminished contractile and respiratory function.

For the second part of this study, we focused on further exploring the role of increased fatty acid utilization in the setting of right ventricular cardiac hypertrophy. Cardiac hypertrophy is often associated with a substrate switch from fatty acid oxidation to glucose metabolism, due to downregulation of PPARα expression. The hypertrophied and failing heart is associated with a pronounced degree of cardiac metabolic remodeling. These changes appear to manifest in a stage-dependent manner, with earlier stages of heart failure linked to increased fatty acid utilization while end-stage failure is associated with a switch away from fatty acid metabolism (fetal switch). It is also unclear whether these fuel substrate switches are part of an adaptive process or merely an epiphenomenon. Here we proposed that PPARα activation (and subsequent increased fatty acid utilization) in the hypertrophic context would result in decreased efficiency of mitochondrial respiration, thereby leading to impaired contractile function. We devised a series of experiments to activate PPARα (using Wy-14, 643) in the hypertrophied RV (2 weeks hypoxic exposure) and to subsequently assess whether this intervention results in an adaptive or maladaptive phenotype. The major finding of this part of the study is that in vivo Wy-14,643 administration elicited direct effects on mitochondrial respiratory function and cardiac contractility. The data show that Wy-14,643 treatment attenuated mitochondrial respiration (state 3 respiration and rate of ADP phosphorylation) in the RV while promoting uncoupling of mitochondrial oxidative phosphorylation (increased state 4 respiration and basal leak) in the LV.

We then measured the effects of Wy-14,643 administration on the transcript levels of several mitochondrial regulators. The combination of Wy-14,643 and hypoxia resulted in the coordinate induction of PPARα-target genes (PDK-4 and UCP3) in the RV. Wy-14,643 treatment increased PGC-1α, NRF-1, and ANT1 levels in the right ventricle. However, this was associated with impaired respiratory capacity. The Wy-14,643-hypoxia combination increased transcript levels of PDK-4, ANT1 and decreased COXII in the LV. Also, LV, COXIV and UCP3 levels were increased, whereas PGC-1α levels were decreased in response to Wy-14,643 treatment. Reduced PGC-1α was associated with uncoupling and impaired LV function. However, the direct effects of in vivo Wy-14,643 administration make it difficult to draw firm conclusions from these data.

We next exposed rats to 12 weeks of hypobaric hypoxia (11% O_2) (Chapter 3) with two major aims: 1) To investigate whether adaptive cardiac changes we earlier observed (i.e. after 2 weeks) in the hypertrophied RV are sustained at a later time point (12 weeks), and 2) To increase cardiac fatty acid utilization in the hypertrophied heart using two approaches i) enhancing fatty acid delivery by chronic supply of a high fat diet (cocoa butter), and ii) activating PPARα using fenofibrate as a ligand. We demonstrated that cardiac contractile function is sustained (in both RV and LV) at later time points i.e. 12 weeks. Furthermore, we found that LV contractile function is now increased versus the earlier time points (2/4 weeks). Unlike the 2- and 4-week time points (Chapter 2), we observed that exposure to 12 weeks of hypobaric hypoxia resulted in diminished palmitoyl-L-carnitine oxidation and ADP phosphorylation rates (both ventricles).

Since we observed direct effects of PPARα ligands on mitochondrial respiration at longer time point (2 weeks), we investigated the direct and acute effects of two selective PPARα ligands, i.e. fenofibrate and Wy-14,643, on mitochondrial

respiratory function using rat cardiac mitochondria (Chapter 4). We also wanted to ascertain if the uncoupling effects exhibited by PPARα ligands on mitochondrial respiration are substrate-specific. Isolated left ventricular mitochondria were incubated with increasing concentrations of fenofibrate or Wy-14,643 (10, 100, 500 μM) and mitochondrial respiration determined using: malate/glutamate (complex I), succinate (complex II) and palmitoyl-L-carnitine as oxidative substrates. We found that Wy-14,643 and fenofibrate differentially inhibit mitochondrial respiratory function in isolated rat heart mitochondria. We found that acute exposure to fenofibrate markedly inhibited state 3 respiration and rate of ADP phosphorylation. Wy-14,643 elicited dose-dependent effects on respiratory capacity and phosphorylation rate. Our data suggest that both complex I and II are inhibited by fenofibrate while Wy-14,643 more readily inhibited complex I-dependent respiration

In summary, in this study we demonstrated that co-ordinate induction of mitochondrial regulatory genes in the hypertrophied RV is one of the adaptive responses to sustain mitochondrial respiratory capacity and contractile function in response to the hypoxic stimulus at earlier time points (2 and 4 weeks). Interestingly, the later time point (12 weeks) was associated with increased cardiac contractility but reduced mitochondrial respiratory capacity. We propose that this may either be an adaptive measure to conserve energy reserves, or the beginning of a maladaptive phenotype in the RV. These possibilities require further investigation.

We also found that fenofibrate treatment under hypoxia resulted in more severe effects on contractile function, particularly in the LV. Furthermore, we found that adaptive mitochondrial pathways and function of the hypertrophied RV triggered in response to hypobaric hypoxia were not blunted in the presence of high fat supply (cocoa butter). These data therefore indicate that chronic hypobaric hypoxia triggers adaptive pathways that may rescue organisms from the potentially damaging effects

of higher fatty acid supply. My thesis also extends previous work by providing a potential mechanism whereby PPARα activation, and by implication increased fatty acid oxidation, may impair contractile function of the hypertrophied heart i.e. by uncoupling mitochondrial oxidative phosphorylation. Furthermore, the data support the concept that the fuel substrate switch away from fatty acids in the hypertrophied heart may be an adaptive mechanism.

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Chapter 1

General Introduction

An adequate supply of oxygen is vital for the survival of all living organisms. Hypoxia is defined as a relative shortage of oxygen due to lowered partial pressure of oxygen within the cellular environment [Schmedtje and Ji, 1998; Muertz, 1965]. Since a constant oxygen supply is essential to sustain life, organisms have evolved sophisticated molecular sensing mechanisms in order to respond and adapt to lowered oxygen levels (hypoxia). I will now review some of the major adaptive responses to chronic hypoxic exposure, with particular emphasis on the heart.

1. Adaptive measures in response to hypoxia

1.1. Oxygen sensing

There are multiple oxygen sensing mechanisms proposed to relay lowered oxygen availability to the intracellular machinery. These include the NAD(P)H oxidase enzyme family, components of the mitochondrial electron transport chain (complex III or IV) [Acker et al., 2006; Murdoch et al., 2006], reactive oxygen species (ROS) [Shah and Sauer, 2006; Chandel and Schumacker, 2000; Michiels et al., 2002], and nitric oxide (NO) [Mateo et al., 2003; Hagen et al., 2003]. An important physiological response to hypoxia requires the coupling of mitochondrial oxygen sensors with downstream signaling molecules in order to activate adaptive functional responses [Goldenthal and Marin-Garcia, 2004]. Hypoxia inducible factors (HIFs) have been identified as key mediators relaying reduced oxygen levels to intracellular signaling and transcriptional pathways. Activated HIF-1 comprises two subunits i.e. HIF-1α and HIF-1β [Wang et al., 1995], with the former identified as a key transcription factor regulating oxygen-dependent gene transcription. The HIF-1α transcriptional system senses oxygen availability to subsequently induce target genes, thereby orchestrating adaptive responses such as increased glycolysis, angiogenesis,

erythropoiesis [Semenza, 2000; Semenza, 2004], and hypoxia-induced pulmonary hypertension [Yu et al., 1999; Shimoda et al., 2001]. HIF-1α is constitutively expressed in the heart and several other tissues [Semenza, 1998]. Under conditions of normal oxygen supply HIF-1α undergoes prolyl-hydroxylation by specific cellular prolyl hydroxylase domain-containing enzymes (PHDs) [Semenza, 2001], and subsequent ubiquitination and degradation by the 26S proteasome (Figure 1) [Huang et al., 1998; Kallio et al., 1999; Salceda and Caro, 1997].

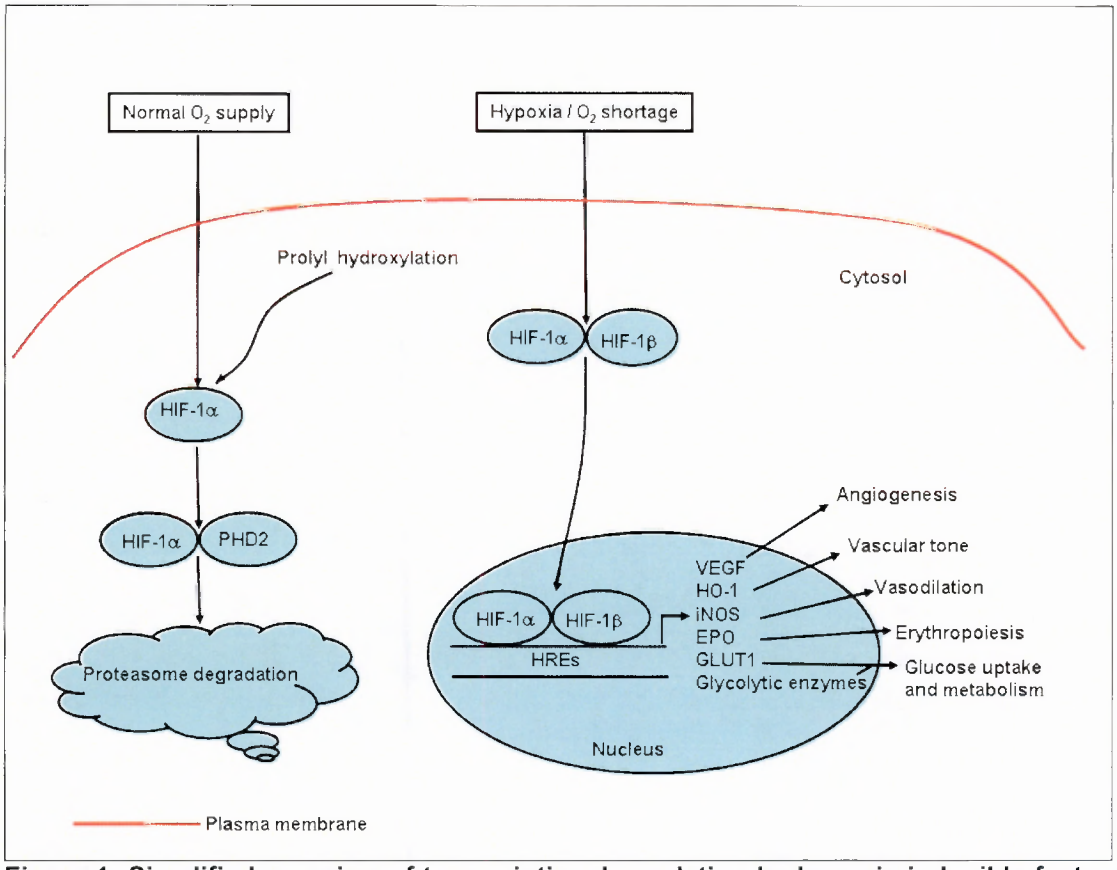

Figure 1: Simplified overview of transcriptional regulation by hypoxia inducible factor-1α (HIF-1α) under normoxic and hypoxic conditions. Abbreviations: hypoxia-inducible factor-1β (HIF-1β), prolyl-4-hydroxylase-2 (PHD2), hypoxia response element (HRE), vascular endothelial growth factor (VEGF), heme oxygenase-1 (HO-1), inducible nitric oxide synthase (iNOS), erythropoietin (EPO), glucose transporter 1 (GLUT1).

Expression of the PHDs varies among cell types and in response to physiologic stimuli [Metzen et al., 2003; D'Angelo et al., 2003]. Prolyl-4-hydroxylase-2 (PHD2) is ubiquitously expressed and exhibits the highest specificity toward HIF-1α [Hirsila et al., 2003]. However, under hypoxic conditions HIF-1α stabilizes thus allowing it to

dimerize with its obligate partner, HIF-1β, to alter the expression of target genes. HIF-1β is also referred to as aryl hydrocarbon nuclear translocase (ARNT). HIF-1α binds to specific hypoxia response elements (HREs) i.e. 5′ -ACCTGC- 3′ that is usually located within, or adjacent to target genes in the 5′-or 3′-flanking sequences [Wang et al., 1995; Tian et al., 1997; Wiesener et al., 1998].

Since ARNT is generally abundant, availability of HIF-1α is the rate limiting step in the formation of HIF-ARNT dimers. As indicated before, HIF-1α plays a crucial role as transcription regulator of several adaptive genes to counteract reduced oxygen supply (Figure 1). For example, vascular endothelial growth factor (VEGF) [Forsythe et al., 1996] is known to be induced by hypoxia in vascular endothelial cells [Namiki et al., 1995]. Moreover, increased VEGF expression leads to angiogenesis in hypoxic tissues [Shweiki et al., 1992] in order to enhance oxygen delivery. There are conflicting reports regarding the development of new myocardial capillaries in animals exposed to hypoxia. While some [Rotta, 1943; Clark and Smith, 1978] have reported decreased ventricular capillary density in chronically hypoxic guinea pigs and rats, others [Miller and Hale; 1970] have found increased capillary density (right and left ventricles) or no changes in the left ventricle [Moravec et al., 1983]. Nevertheless, coronary angiogenesis is considered by many to be a compensatory mechanism to increase oxygen delivery to the heart [Zarain-Herzberg et al., 1996].

HIF-1α also induces the gene expression of erythropoietin (EPO) [Semenza and Wang, 1992], heme oxygenase-1 (HO-1) [Dawn and Bolli, 2005], inducible nitric oxide synthase (iNOS) [Jung et al., 2000], glucose transporter (GLUT1) [Loike et al., 1992], and a panel of glycolytic enzymes such as glyceraldehyde-phosphate dehydrogenase (GAPDH) [Semenza, 1999; Hoppeler et al., 2003], phosphoglycerate kinase 1 (PGK1), aldolase A, hexokinase (HK), and lactate dehydrogenase A [Iyer et

al., 1998; Ryan et al, 1998; Semenza et al., 1994, 1996]. These adaptive measures are not only an attempt to increase oxygen delivery to the heart but also to switch to increased utilization of an alternate metabolic fuel substrate i.e. glucose, that is more efficient in terms of ATP per mole of O_2 (compared to fatty acids) when oxygen supply is limiting. The metabolsim of glucose in mammalian heart is 25-50% more oxygen efficient than the metabolism of free fatty acids [Holden et al., 1995].

Although HIF-1 is a pivotal transcriptional modulator activated in response to diminished oxygen supply, other transcriptional regulators also play important roles in this process. For example, the Sp family (Sp1 and Sp3) of transcription factors have been implicated in hypoxia-sensitive gene expression, acting via GC-rich promoter elements [Boulikas, 1994]. Sp1 is a transcriptional activator while Sp3 may act either as a repressor or activator, depending on the promoter and cellular context [Dennig et al., 1996; Birnbaum et al., 1995; Discher et al., 1998]. Other oxygen-sensitive transcriptional regulators that are directly oxygen-sensitive and not under control of HIF-1 include, for example, the cAMP response element-binding protein-1 (CREB-1) [Safran and Kaelin, 2003], activator protein-1 (AP-1), nuclear factor-κB (NF-κB) [Pahl and Baeurle, 1994; Michiels et al., 2002], peroxisome proliferator-activated receptor α (PPARα), retinoid X receptor α (RXRα) [Sharma et al., 2004; Razeghi et al., 2001b; Huss et al., 2001], and Nkx2.5 [Bar et al., 2003; Pahl and Baeuerle, 1994].

1.2. Hypoxia-mediated cardiac protection

The physiologic responses to chronic hypoxia include polycythemia, pulmonary hypertension, and angiogenesis [Chien et al., 1993; Hasenfuss et al., 1994; Zarain-Herzberg et al., 1996]. Several studies have confirmed such responses in laboratory animals exposed to low oxygen levels [Feldman et al., 1993; O'Rourke et al., 1999;

Studer et al., 1994; Flesch et al., 1996; Litwin et al., 1997; Sipido et al., 2000]. Furthermore, chronic hypoxia also causes an increase in hematocrit [Hunter et al., 1974] as an adaptive response to enhance oxygen delivery. An increase in haemoglobin and red cell count enhances the blood oxygen carrying capacity despite lower than normal arterial oxygen saturation [Norese et al., 2002].

Adaptation to chronic hypoxia is characterized by a variety of functional changes that may protect the heart during episodes of enhanced work. For example, previous studies found that the incidence of myocardial infarction is lower in people naturally acclimatized to high altitude (e.g. Peru, 4000 m) [Hurtado, 1960; Monge et al., 1990]. Moreover, Meerson et al. (1973) showed that rats exposed to chronic intermittent hypoxia and exposed to hypoxia for only a portion of each 24 hr period develop smaller myocardial infarcts. In agreement, Opie et al. (1978) found that exposure to high altitude hypoxia protects against coronary occlusion. Longer term studies show similar results. For example, pre-exposure to chronic hypoxia in adult rat hearts conferred subsequent resistance against an acute hypoxic injury i.e. four months after removal from the hypoxic environment [Ostadal et al., 1995]. This long-lasting effect of hypoxia is in contrast to the protection triggered by ischemic preconditioning, which usually persists for only 3-4 days [Fitzpatrick et al., 2005] and therefore offers the possibility of novel therapeutic intervention. The intracellular signaling mechanisms directing hypoxia-mediated cardiac protection are numerous and the subject of ongoing investigations For instance, adaptation to chronic myocardial hypoxia results in increased resistance to ischemia [Baker et al., 1995] associated with enhanced nitric oxide production from the NOS3 isoform of nitric oxide synthase (NOS) [Shi et al., 2000]. Furthermore, it has been shown that activation of NOS3 activates the sarcolemmal ATP-sensitive potassium (sarcK_{ATP}) channels via a cGMP-dependent mechanism under normoxic and hypoxic conditions [Baker et al., 2001].

Since the mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) channel is thought to be a key regulator of cardioprotection [Gross and Fryer, 1999], chronic hypoxia may be a novel way to activate this process.

1.3. Chronic hypoxia induces pulmonary hypertension and right ventricular hypertrophy

It is well documented that chronic exposure to high altitude hypoxia results in pulmonary hypertension [Rabinovitch et al., 1979] via vasoconstriction of the pulmonary arteries. The development of pulmonary hypertension is a highly complex process involving several intracellular pathways [Humbert et al., 2004]. In rats exposed to hypobaric hypoxia, hypoxic pulmonary hypertension develops during the first two weeks of exposure and then stabilizes, and does not increase in severity during the adaptation phase [Herget et al., 1978; Reeves and Herget, 1984]. Hypoxia-induced polycythemia is another factor that contributes to pulmonary hypertension by increasing blood viscosity [Naeye, 1961; Janssens et al., 1991; Barer et al., 1983; Fedde and Wideman, 1996]. This subsequently increases the load on the right ventricle (RV), ultimately leading to the selective development of RV cardiac hypertrophy (RVH) in animals and humans [Ostadal et al., 1995; Kolar and Ostadal, 1991; Favret et al., 2001; Morel et al., 2003; Penaloza et al., 1963; Rabinovitch et al., 1979; Reeves and Herget, 1984]. However, Hislop and Reid (1976) demonstrated that the morphological changes in chronic hypoxia-induced pulmonary hypertension were reversible, requiring ~8 weeks to return to normal.

Several studies have simulated this condition by employing experimental protocols whereby animals are exposed to chronic hypobaric hypoxia. Only a handful of animal models exist that are able to induce selective right ventricular hypertrophy without left

ventricular growth. These include experimental pulmonary artery stenosis [Zierhut et al., 1990; Adachi et al., 1991; Ikeda et al., 1990; Olivetti et al., 1988; Zimmer, 1992], monocrotaline-treatment [Chen et al., 2001; Brown et al., 1998], and pulmonary hypertension by lung irradiation [Zimmer and Zierhut, 1991]. Although the left ventricular mass is not significantly changed by exposure to hypoxia [Rabinovitch et al., 1979; Ostadal et al., 1995; Kolar and Ostadal, 1991], it is recognized that there is a relationship between the functioning of the two ventricles. Therefore, impairment of the RV may eventually influence left ventricular (LV) function (or *vice versa*) [Clyne et al., 1989; Dittrich et al., 1992; Hill et al., 1997; Mizushige et al., 1989; Yu et al., 1996].

1.4. Hypoxia induces mitochondrial biogenesis

Another adaptation to chronic hypoxia is the proliferation of mitochondria. Previous studies in cardiac tissues have reported increased number of smaller mitochondria in rat hearts exposed to hypobaric hypoxia for 21 days [Nouette-Gaulain et al., 2005; Costa et al., 1988]. This is proposed to be an adaptive response to increase mitochondrial surface area, and thus bioenergetic capacity. The basic features of the process of mitochondrial biogenesis will now be summarized.

The ability to modulate mitochondrial number and function is an important adaptive response in all eukaryotes [Wu et al., 1999]. The heart is a highly oxidative tissue that produces more than 90% of its energy from mitochondrial respiration [Ventura-Clapier et al., 2004]. In the heart, mitochondria occupy ~40% of cardiomyocyte space, arranged in rows between myofilaments and under the sarcolemma [Barth et al., 1992]. In addition to energy production, mitochondria are major regulators of apoptosis and therefore play a key role in determining life and death of the mammalian cell [Lee et al., 2000]. Mitochondrial biogenesis increases during cardiac

hypertrophy and exercise and following treatment with xenobiotics, thyroxin, glucocorticoids and electrical stimulation [Attardi et., 1977; Hood, 2001; Totland et al., 2000; Lundgren et al., 1987; Weber et al., 2002; Williams et al., 1987; Nelson, 1990; Xia et al., 1997].

Mitochondria divide during mitosis, providing daughter cells with a normal complement of mitochondria [Bogenhagen and Clayton, 1977]. In agreement, Sanger et al. (2000) showed that mitochondrial mass increases from the onset of S-phase through M-phase. Besides mitosis, there is a suggestion that mitochondria can be either synthesized from submicroscopic precursors present in the cytoplasm, bud from other membranous structures of the cell, or grow and divide from pre-existing mitochondria [Clementi and Nisoli, 2005]. Mitochondrial divisions are not always linked to the cell cycle. For example, muscle mitochondria proliferate during myogenesis and in response to exercise [Brunk, 1981; Moyes et al., 1997]. Mitochondrial biogenesis is accompanied by plasticity of the mitochondrial external shape which is arranged in extended tubular networks [Chen, 1988]. Continuous physical interaction of mitochondria through fusion ensures exchange of both membrane and matrix components to restore local depletions and maintain mitochondrial function [Nakada et al., 2001]. Furthermore, mitochondria have been shown to import most of their phospholipids from the cytoplasm to form and preserve membrane integrity [Moyes and Hood, 2003].

Mitochondrial biogenesis depends on the coordinated expression of both the nuclear and mitochondrial genomes. Animal mitochondria possess their own double-stranded circular DNA (mtDNA) that encodes 13 subunits of the oxidative phosphorylation system (OXPHOS) [Anderson et al., 1991]. Nuclear genes encode all the proteins and enzymes involved in mtDNA replication, transcription, and translation in mitochondria [Shadel and Clayton, 1997]. Two transcription factors play a key role in

facilitating interaction between the nucleus and the mitochondrion. Here nuclear respiratory factors (NRFs) bind and activate the promoters of various nuclear genes that encode for components of mitochondrial oxidative phosphorylation (OXPHOS), and the mitochondrial transcription factor A (mtTFA) that regulate mtDNA replication and transcription [Scarpulla, 2002a]. Upon stimulation, mtTFA translocates to the mitochondrion, where it stimulates mitochondrial biogenesis as indicated by increased mitochondrial DNA replication and mitochondrial gene expression [Garesse and Valejo, 2001; Larsson et al., 1998] (Figure 2).

Peroxisome proliferator-activated receptor–gamma coactivator-1α (PGC-1α) is a key nuclear receptor co-activator for mitochondrial biogenesis and controls mitochondrial number and function in response to energy demands through its interaction with NRFs [Lehman and Kelly, 2002; Scarpulla, 2002a,b; Lehman et al., 2000; Puigserver and Spiegelman, 2003]. In addition, the NRFs (NRF-1 and NRF-2) transcription factors mediate the expression of a number of nuclear genes involved in mitochondrial OXPHOS including subunits of cytochrome c oxidase (COX) and ATP synthase [Vega, 2000; Scarpulla, 2002a,b]. PGC-1α also coactivates estrogen-related receptor α (ERRα), a member of the orphan nuclear receptor family [Giguere et al., 1988; Heard et al., 2000; Hong et al., 1999]. In turn, ERRα interacts with NRF-1 and NRF-2 to regulate mitochondrial biogenesis and expression of OXPHOS enzyme genes [Mootha et al., 2004; Schreiber et al., 2004].

PGC-1α and PGC-1β play key roles in controlling mitochondrial metabolism and oxidative phosphorylation, and in activating expression of nuclear-encoded mitochondrial genes [Scarpulla, 2002b]. In agreement with this, overexpression of PGC-1 α in transgenic mice leads to mitochondrial proliferation in the heart [Lehman et al., 2000], adipocytes [Lowell and Spiegelman, 2000], and myoblasts [Wu et al.,

1999]. Both isoforms are highly expressed in tissues with high oxidative capacity including heart, skeletal muscle, brown adipose tissue, and liver (Puigserver et al., 1998).

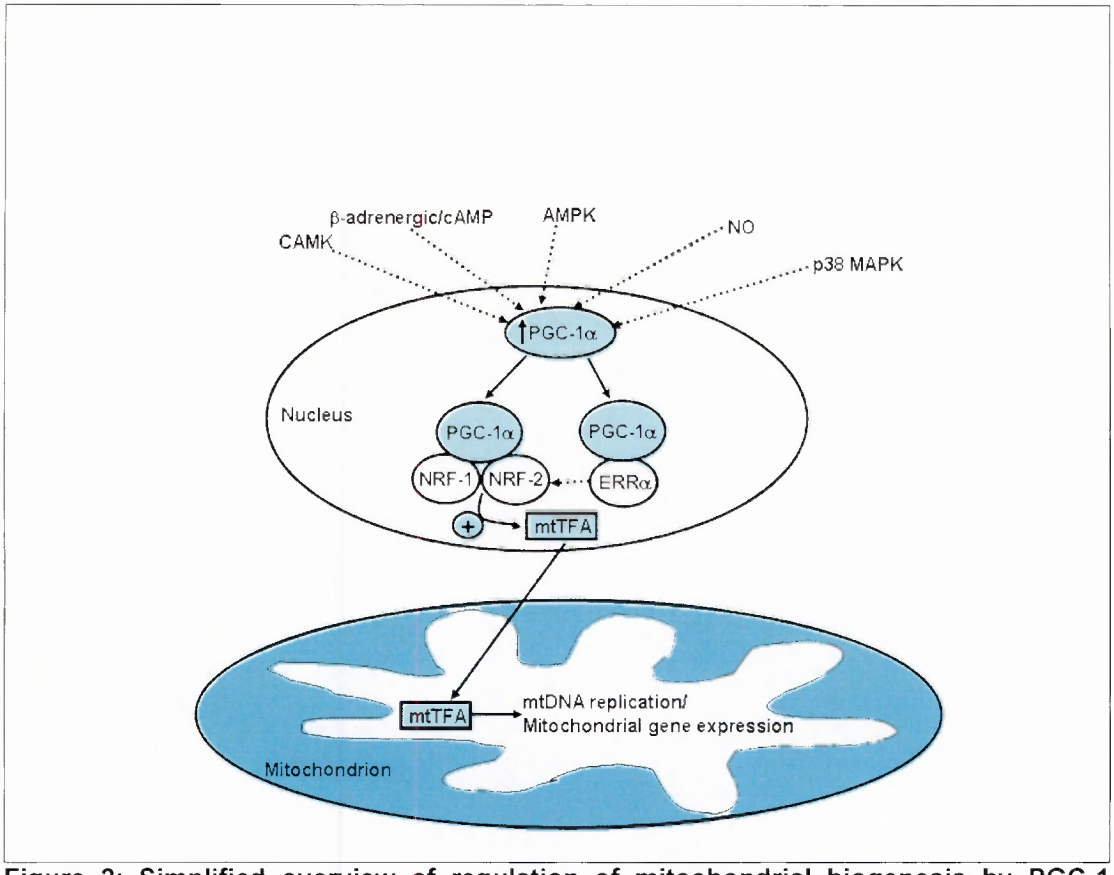

Figure 2: Simplified overview of regulation of mitochondrial biogenesis by PGC-1. Abbreviations: Ca^{+2}-calmodulin kinase (CAMK), cyclic adenosine monophosphate (cAMP), AMP kinase (AMPK), nitric oxide (NO), p38 mitogen-activated protein kinase (p38MAPK), peroxisome proliferator-activated receptor-gamma coactivator-1 α (PGC-1α), nuclear respiratory factor-1 and -2 (NRF-1, NRF-2), estrogen–related receptor α (ERRα), mitochondrial transcription factor (mtTFA), mitochondrial DNA (mtDNA).

PGC-1α activity and expression levels are induced by physiological stimuli such as cold exposure, fasting, and exercise, stimuli known to promote fatty acid metabolism [Lehman et al.; 2000; Goto et al., 2000; Baar et al., 2002]. Signaling pathways involved in the regulation of mitochondrial biogenesis, and therefore activation of PGC-1α, include p38 mitogen-activated protein kinase (p38MAPK) [Puigserver et al., 2001], β-adrenergic/cAMP [Nisoli et al., 2003], nitric oxide [Nisoli et al., 2003], AMP kinase [Zong et al., 2002], and Ca^{+2}-calmodulin kinase (CAMK) [Wu et al., 2002].

1.5. Metabolic adaptation in response to hypoxia

Cardiac metabolic remodeling is another major adaptation during exposure to hypobaric hypoxia. Here, the primary adaptation is a fuel substrate switch from fatty acids to glucose, since the latter is proposed to be a more energetically-efficient fuel substrate when oxygen supply is limiting [Young et al., 2001a; Razeghi et al., 2001a; Barger and Kelly, 1999; Sack et al., 1997]. Likewise, a similar fuel substrate switch has been observed in the hypertrophied heart [Barger and Kelly, 1999; Bishop and Altschuld, 1970; Taegtmeyer and Overturf, 1988; Christe and Rodgers, 1994; Takeyama et al., 1995; Buttrick et al., 1994; Feinendegen et al., 1995]. Since the focus of my thesis relates to metabolic adaptations of the heart in response to hypobaric hypoxia, I will now summarize basic aspects of cardiac metabolism followed by a discussion of conditions where myocardial fuel substrate utilization is altered, particularly in response to chronic oxygen lack.

1.5.1. Cardiac glucose metabolism

Pioneering studies have shown that glucose, lactate and fatty acids are the major fuel substrates of the mammalian heart [Bing et al, 1953, 1954]. Fatty acids are metabolized in mitochondrial matrix by β-oxidation and account for ~60 – 90 % of the total energy production in the normal adult mammalian heart [van der Vusse et al., 1992]. Glucose is catabolized by glycolysis and glucose oxidation, accounting for ~10–40 % of total cardiac energy production [Gertz et al., 1988]. However, despite varying degrees in substrate preference, it is thought that the heart functions optimally when oxidizing both fuel substrates simultaneously.

The heart obtains carbohydrates for metabolism either from the uptake of glucose or lactate, or by the breakdown of glycogen. Glucose uptake into the heart is facilitated by two glucose transporter isoforms, GLUT1 and GLUT4 [Gould and Holman, 1993], a rate-limiting step for exogenous glucose utilization [Depre et al., 1999]. Expression of glucose transporters is developmentally regulated with GLUT1 highly expressed during the fetal stages of development, whereas GLUT4 is postnatally induced and an insulin-sensitive isoform [Santalucia et al., 1992]. GLUT1 is a major mediator of basal cardiac glucose uptake and normally accounts for ~30 % of the total cardiac glucose transporters in the adult heart [Laybutt et al., 1997; Fischer et al., 1997]. The number of glucose transporters and the transmembrane glucose gradient regulate glucose transport into the myocardium [Kodde et al., 2006]. In response to particular stimuli, for example, insulin, ischemia or increased work, there is a higher demand for glucose utilization. In response, GLUTs are mobilized from intracellular vesicular stores to migrate to the sarcolemma, thereby increasing myocardial glucose uptake [Slot et al., 1991; Uphues et al., 1994].

After uptake, glucose is either converted to glycogen or enters the glycolytic pathway where it is ultimately converted to pyruvate with the production of two ATP molecules (Figure 3). Upon entering the cell, free glucose is rapidly phosphorylated by hexokinase to form glucose-6-phosphate (G6P). This ensures that the concentration gradient across the plasma membrane is maintained and that glucose does not leak back out of the cell. G6P can either be converted to glycogen for storage (reaction catalyzed by glycogen synthase) or converted to fructose-6-phosphate (F6P) by the action of phosphohexose isomerase. Glycogen can be mobilized to re-enter the glycolytic pathway via glycogenolysis (reaction catalyzed by glycogen phosphorylase). Phosphofructokinase-1 (PFK-1) is a key regulatory enzyme in the glycolytic pathway and catalyzes the first irreversible step in the breakdown of F6P to

fructose 1,6-bisphosphate (F1,6-BP) (Hue et al., 2002). PFK-1 is inhibited by ATP and activated by ADP, AMP, and P_i [Pogson and Randle, 1966].

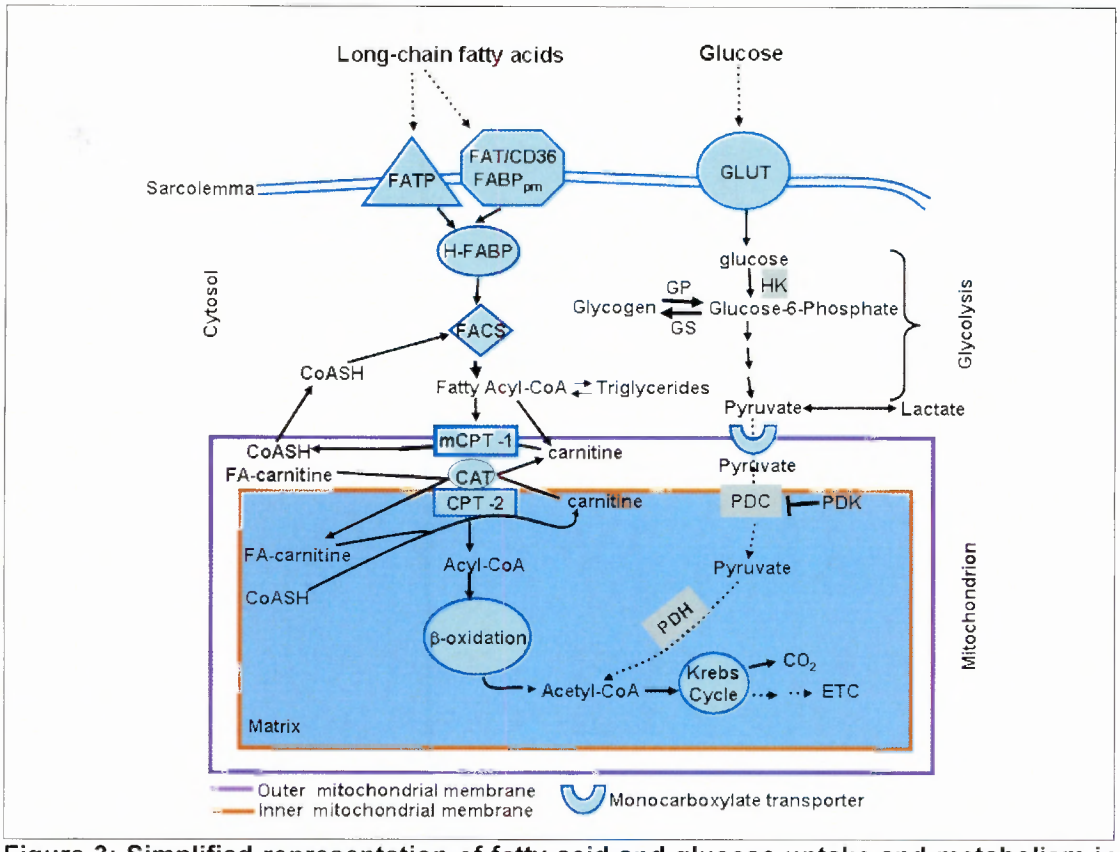

Figure 3: Simplified representation of fatty acid and glucose uptake and metabolism in the normal adult myocardium. Abbreviations: fatty acid transport protein (FATP), fatty acid translocator (FAT/CD36), heart-type fatty acid binding protein (H-FABP), plasma membrane fatty acid binding protein ($FABP_{pm}$), fatty acyl-CoA synthetase (FACS), muscle-type carnitine palmitoyl transferase-1 (mCPT-1), carnitine palmitoyl transferase-2 (CPT-2), carnitine acyl transferase (CAT), coenzyme A (CoASH), GLUT (glucose transporter), hexokinase (HK), pyruvate dehydrogenase kinase (PDK), pyruvate dehydrogenase complex (PDC), pyruvate dehydrogenase (PDH), electron transport chain (ETC).

Fructose 1,6-bisphosphate is subsequently converted to glyceraldehyde 3-phosphate, a reversible reaction catalyzed by fructose 1, 6- bisphosphate aldolase. Further downstream, glyceraldehyde-3-phosphate dehydrogenase catalyzes the conversion of glyceraldehyde 3-phosphate to 1, 3-disphophoglycerate, producing NADH in the process. Sequential reactions catalyzed by phosphoglycerate kinase, phosphoglyceromutase and enolase ultimately result in the production of 2x phosphoenolpyruvate (PEP), which is in turn converted by pyruvate kinase to 2x pyruvate.

The pyruvate formed from glycolysis can be either converted to lactate, decarboxylated to acetyl-CoA, or carboxylated to oxaloacetate or malate. Pyruvate formed from glycolysis may pass the outer mitochondrial membrane via the monocarboxylate transporter. The pyruvate dehydrogenase complex (PDC) on the inner mitochondrial membrane transports pyruvate into the mitochondrial matrix and catalyzes an irreversible step to produce acetyl-CoA [Denyer et al., 1986]. The net reaction: pyruvate + CoA + NAD^+ ⟶ acetyl-CoA +CO_2 + NADH + H^+, is catalyzed by pyruvate dehydrogenase (PDH) which is considered to be the key rate-limiting step of carbohydrate oxidation [Denyer et al., 1986]. Phosphorylation of PDC by members of a family of pyruvate dehydrogenase kinase (PDK) proteins [Bowker-Kinley et al., 1998], inactivates the PDC complex [Harris et al., 2001; Sugden et al., 1998, 2000; Wu et al., 1998, 2000, 2001]. There are currently four known isoforms of PDK (PDK1, PDK2, PDK3, and PDK4), of which PDK1, PDK2 and PDK4 are enriched in the heart [Bowker-Kinley et al., 1998]. The acetyl-CoA produced subsequently enters the Krebs cycle resulting in the production of reducing equivalents, i.e. $NADH^+$ and $FADH_2$. These in turn donate electrons to the mitochondrial electron transport chain to generate ATP, required to sustain the viability of the cell.

1.5.2. Cardiac fatty acid metabolism

Fatty acids are the preferred substrate of the adult myocardium, supplying ~70% of the total ATP [Bing et al., 1954; Shipp et al., 1964; Wisneski et al., 1987]. Fatty acids are stored as triacylglycerols in adipose tissue and are transported by the bloodstream in various forms including: a) a nonesterified form attached to albumin, b) contained within chylomicrons or c) covalently bound in triglycerides. Fatty acids

enter the cardiomyocyte either by passive diffusion or via protein-mediated transport across the sarcolemma. Three myocardial fatty acid transporter proteins have thus far been identified: fatty acid translocase (FAT/CD36), fatty acid transport protein (FATP), and plasma membrane fatty acid binding protein ($FABP_{pm}$) [Sambandam and Lopaschuk, 2003; Hopkins et al., 2003] (Figure 3).

The $FABP_{pm}$ is a peripheral membrane protein [Stump et al., 1993], whereas the heart-type (H-FABP) is often regarded as the cytoplasmic counterpart of plasma albumin [Schaap et al., 1999]. After uptake, long-chain fatty acids are esterified by fatty acyl-CoA synthetase (FACS) and are either converted into triglycerides for intracellular storage or transported into the mitochondria for β-oxidation. Entry of fatty acids into cardiac mitochondria begins with the conversion of fatty-acyl CoA to a fatty acyl-carnitine by the muscle isoform of carnitine palmitoyltransferase-1 (mCPT-1) on the inner side of the outer mitochondrial membrane. This step is considered to be the rate-limiting step for mitochondrial fatty acid oxidation [Kerner and Hoppel, 2002].

Acute regulation of mitochondrial fatty acid uptake occurs via allosteric control of CPT-1 by malonyl-CoA within the cytosol. Malonyl-CoA levels depend on opposing reactions i.e. it is synthesized by acetyl-CoA carboxylase (ACC) from acetyl-CoA, and degraded by malonyl-CoA decarboxylase (MCD) [Dyck and Lopaschuk, 2002]. ACC activity is controlled by 5'-AMP activated protein kinase (AMPK), which phosphorylates and inhibits it, thereby reducing malonyl-CoA levels [Hardie, 1992; Winder, 2001]. AMPK activators include 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) [Henin et al., 1996; Russell et al., 1999; Holmes et al., 1999], low insulin [Makinde et al., 1998], cardiac hypertrophy [Tian et al., 2001], hypoxia [Marsin et al., 2002], exercise [Hardie, 2004], ischemia [Russell et al., 1999], high AMP/ATP ratio [Hardie and Hawley, 2001], low ratio of phosphocreatine to creatine

(PCr/Cr) [Hardie and Carling, 1997; Ponticos et al., 1998; Stapleton et al., 1996], and fasting [Munday et al., 1991; Witters et al., 1991].

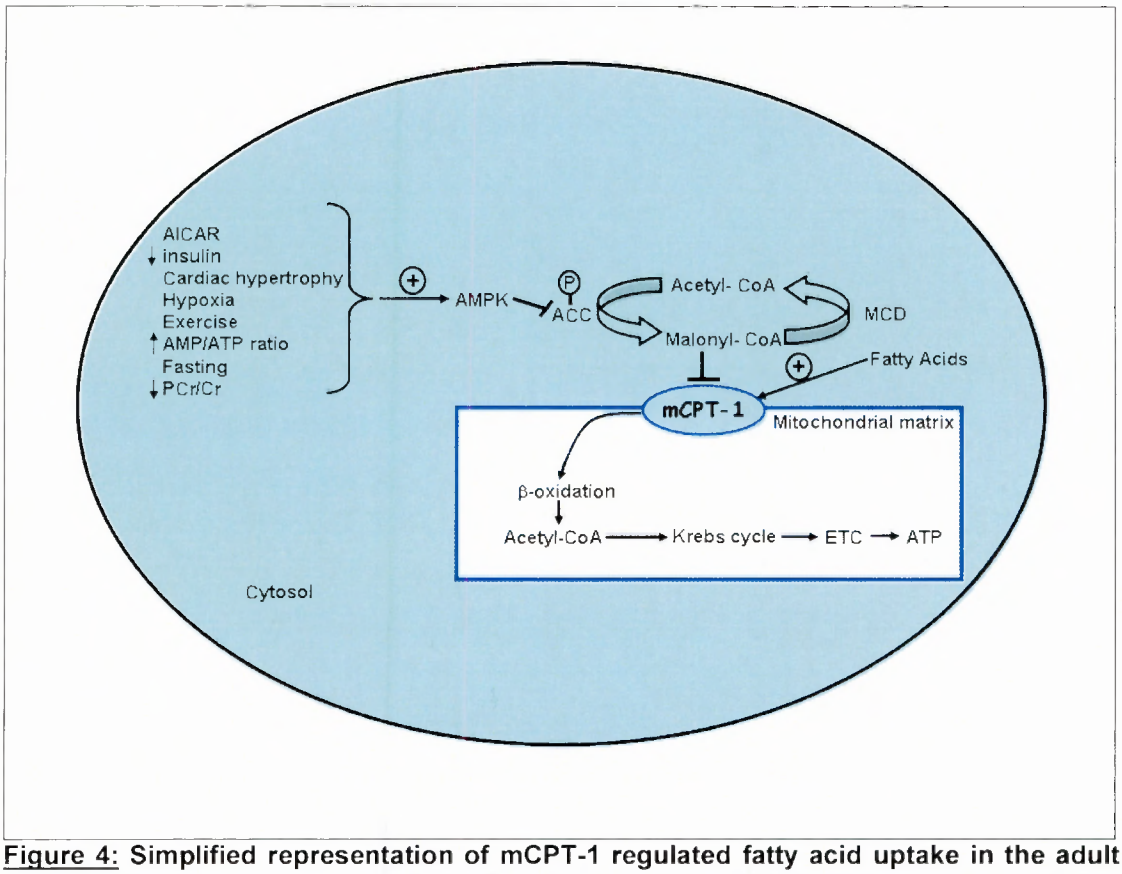

Figure 4: Simplified representation of mCPT-1 regulated fatty acid uptake in the adult heart. Abbreviations: 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR), adenosine monophosphate (AMP), phosphocreatine (PCr), creatine (Cr), 5'-AMP activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC), malonyl-CoA decarboxylase (MCD), muscle-type carnitine palmitoyltransferase-1 (mCPT-1), electron transport chain (ETC), adenine triphosphate (ATP).

Reduction of malonyl-CoA levels increases fatty acid uptake into mitochondria by relieving inhibition of CPT-1 [Lopaschuk, 1997; Muoio et al., 1999] (Figure 4). AMPK activity also increases glucose uptake by increasing GLUT4 translocation to the sarcolemma [Holmes et al., 1999; Russell et al., 1999]. Carnitine acyl-transferase (CAT) transfers the fatty acyl-carnitine into the mitochondrial matrix. CPT-2 on the inner surface of the inner mitochondrial membrane converts the fatty acyl-carnitine back to the fatty acyl-CoA. The fatty acyl-CoA in the mitochondrial matrix then enters the β-oxidation spiral catalyzed by either: (1) long- or medium-chain acyl-CoA

dehydrogenase i.e. LCAD or MCAD. LCAD is a mitochondrial matrix flavoprotein which catalyzes the first reaction in the β-oxidation of straight-chain fatty acids [Beinert, 1963] and forms an enzyme family with the short, medium, and very long-chain acyl-CoA dehydrogenase (MCAD and VLCAD) [Zhang et al., 1997]; (2) enoyl-CoA hydratase; (3) β- hydroxyacyl-CoA dehydrogenase (3-HAD), or (4) acyl-CoA acetyltransferase (ketothiolase). Each cycle of the mitochondrial β-oxidation spiral shortens fatty acids by 2 carbons and produces acetyl-CoA, 1x NADH and 1x $FADH_2$ with each successive turn of the cycle. The acetyl-CoA produced condenses with oxaloacetate to form citrate in the first reaction of the Krebs cycle. NADH and $FADH_2$ formed from β-oxidation and the Krebs cycle donate reducing equivalents to the mitochondrial electron transport chain to drive ATP production.

1.5.2.1. PPARs and fatty acid metabolism

There are several regulatory processes that control fatty acid utilization by the heart. These include substrate availability, uptake, expression levels of fatty acid enzymes and control of enzymes regulating cardiac fatty acid metabolism. Since a strong emphasis of my thesis relates to transcription factors driving fatty acid utilization, I will now focus on the regulation of a family of transcriptional modulators that play a key role to modulate cardiac fatty acid metabolism. Several fatty acid metabolism genes are regulated by a group of transcription factors referred to as the peroxisome proliferator-activated receptors (PPARs) [Desvergne and Wahli, 1999; Kliewer et al., 2001]. Three PPAR isoforms have thus far been identified namely, PPARα, PPAR β/δ and PPARγ. PPARs are ligand-activated nuclear receptors which bind as obligate heterodimers with retinoid X receptor α (RXRα) to consensus peroxisome proliferator response elements (PPRE) located within the regulatory regions of target genes (Figure 5). PPRE consists of a direct repeat (DR) of a hexameric 5'-AGGTCA-

3′ separated by one (DR-1) or two nucleotides (DR-2) [Ijpenberg et al., 1997; Gervois et al., 1999]. PPARs are also able to recruit coactivators, for example PGC-1α, or corerepressors, such as chicken ovalbumin upstream promoter-transcription factor (COUP-TF), thereby inducing or inhibiting transcriptional activity [Miyata et al., 1993].

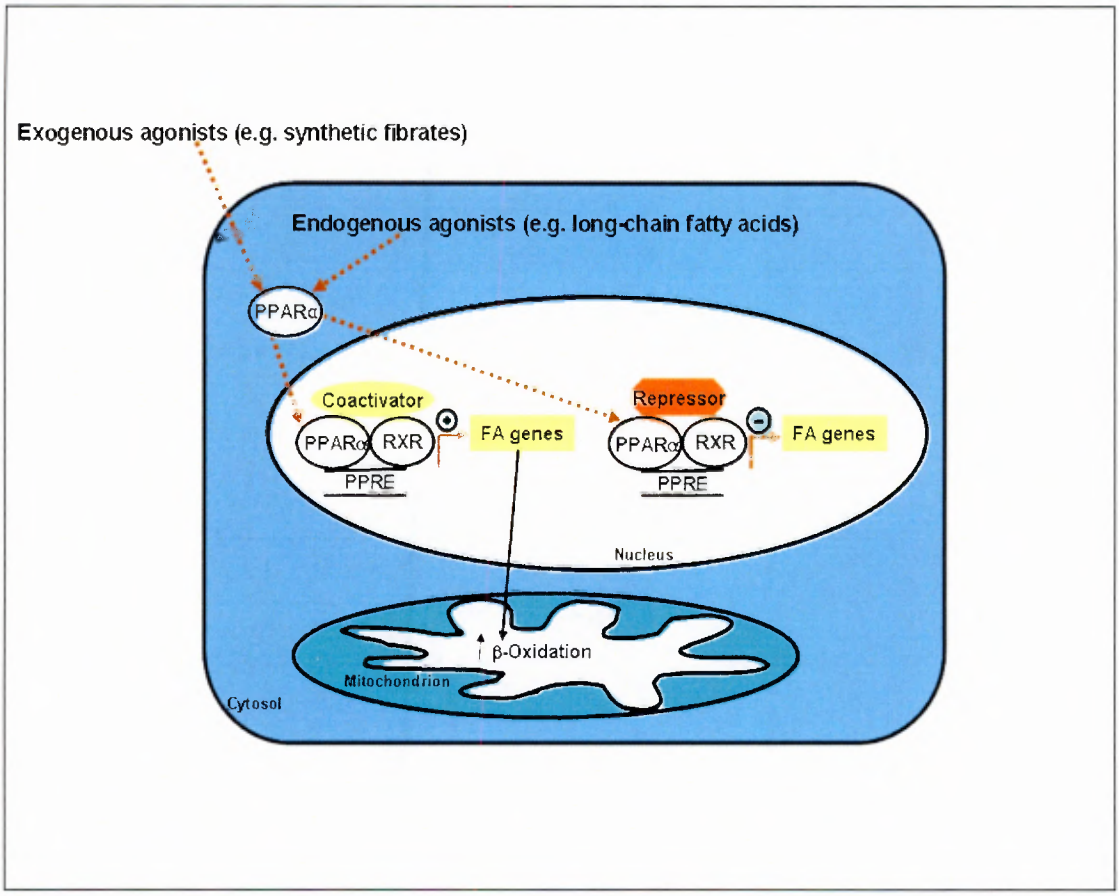

Figure 5: Simplified overview of PPARα-mediated transcription of genes encoding fatty acid regulators. Abbreviations: peroxisome proliferator-activated receptor (PPAR), retinoid X receptor (RXR), peroxisome proliferator response element (PPRE), fatty acid (FA).

PPARα is highly expressed in tissues with high fatty acid oxidation capacity such as heart, liver, kidney, and skeletal muscle [Barger and Kelly, 2000]. Well-described PPARα ligands include lipid-derived molecules such as long-chain fatty acids, eicosanoids, and leukotriene B4, as well as synthetic ligands such as fenofibrate, gemfibrozil and the experimental hypolipidemic compound, Wy-14,643 [Krey et al., 1997; Forman et al., 1997; Devchand et al., 1996]. PPARα regulates basal and fatty acid-induced transcription of genes encoding multiple enzymes for fatty acid

oxidation (including fatty acid uptake and oxidation) [Barger and Kelly, 2000]. For example, PPARα-target genes in the heart include FATP, FABP, acyl-CoA synthetase (ACS) [van Bilsen et al., 1997, van der Lee et al., 2000], mCPT-1 [Brandt et al., 1998; Djouadi et al., 1998], MCAD, LCAD, VLCAD [Djouadi et al., 1999; Gulick et al., 1994; van der Lee et al., 2000], PDK4 [Finck et al., 2002]; uncoupling protein 3 (UCP3) [Young et al., 2001b], and mitochondrial acyl-CoA thioesterase 1(MTE1) [Stavinoha et al., 2004]. In agreement, PPARα knockout mice exhibit low expression of fatty acid oxidation enzymes and reduced cardiac fatty acid oxidation rates [Campbell et al., 2002]. Finck et al. (2002) demonstrated that FAT/CD36 is under the control of PPARα in the heart. However, a time delay in PPAR-mediated regulation of FAT/CD36 has been demonstrated, suggesting that FAT/CD36 activation by PPARα is indirect [Sato et al., 2002].

PPARβ/δ is ubiquitously expressed and at relatively high levels in cardiomyocytes [Gilde et al., 2003]. PPARβ/δ is suggested to play multiple functional roles, including wound healing [Di Poi et al., 2002], cholesterol metabolism [Leibowitz et al., 2000], and fatty acid oxidation (similar to PPARα) [Wang et al., 2003; Gilde et al., 2003; Cheng et al., 2004]. In agreement, mice with cardiac-specific deletion of PPARβ/δ exhibit severe defects in myocardial fatty acid oxidation gene expression, reduced FAO rates, increased lipid accumulation, and lipotoxicity [Cheng et al., 2004]. However, more recently it has also been suggested that PPARβ/δ may also induce glucose metabolism genes [Lee et al., 2006]. PPARβ/δ also protects isolated cardiomyocytes from oxidative stress-induced apoptosis by upregulating the expression of catalase, a known scavenger of reactive oxygen species (ROS) such as hydrogen peroxide [Pesant et al., 2006]. Compared to PPARα, fewer PPARβ/δ selective agonists have been identified. PPARβ/δ agonists include the fibrate

derivatives, GW 2433 [Brown et al., 1997], L16504 and GW501516 [Oliver et al., 2001; Berger et al., 1999].

PPARγ regulates lipid storage, controlling the expression of genes involved in fatty acid storage and adipogenesis [Desvergne and Wahli, 1999]. Synthetic compounds, collectively termed thiazolidinediones (TZDs) are true PPARγ ligands and display antidiabetic properties [Lehmann et al., 1995]. Other PPARγ ligands include the prostanoid 15-deoxy-Delta (12,14)-PGJ2 (15d-PGJ2) [Forman et al., 1995; Kliewer et al., 1995; Krey et al., 1997] and polyunsaturated fatty acids [Krey et al., 1997; Willson et al., 1996]. TZDs (e.g. troglitazone, pioglitazone and rosiglitazone/BRL 49653), have positive effects on insulin action while negatively regulating plasma glucose levels [Hihi et al., 2002], therefore reducing insulin resistance. It is suggested that TZDs act by directing fatty acids away from skeletal muscle to adipose tissue, thereby increasing glucose utilization and insulin sensitivity [Hihi et al., 2002]. In addition, PPARγ signaling suppresses the production of proinflammatory cytokines in monocytes [Jiang et al., 1998], and inhibits proliferation and migration in vascular smooth muscles [Marx et al., 1998; Law et al., 2000]. Moreover, Asakawa et al. (2002) identified PPARγ as a transducer of anti-hypertrophic signaling in the heart. In this study, they found that heterozygous PPARγ-deficient mice displayed a robust hypertrophic response to pressure overload which was not blunted following treatment with pioglitazone. In agreement, both troglitazone and the endogenous PPARγ-agonist, 15d-PGJ2, were able to prevent hypertrophy and hypertrophic marker expression in isolated cardiomyocytes [Yamamoto et al., 2001].

1.5.3. Mitochondrial oxidative phosphorylation

Acetyl-CoA derived from glucose and fatty acid metabolism is oxidized by the Krebs cycle, generating NADH. Reduced flavin adenine dinucleotide ($FADH_2$) is generated via substrate flux through the β-oxidation spiral. NADH or $FADH_2$ can initiate electron transfer through the electron transport chain (Figure 6). The reducing equivalents (NADH and $FADH_2$) from glucose and fatty acid metabolism, are oxidized by complex I (NADH dehydrogenase) and complex II (succinate dehydrogenase) of the electron transport chain, respectively. Both complex I and complex II pass electrons to the membrane-bound electron carrier, coenzyme Q (CoQ). Reduced CoQ from either complex I or complex II then passes electrons to complex III (ubiquinol cytochrome c reductase), which in turn transfers it to oxidized cytochrome c. Reduced cytochrome c passes electrons to complex IV (cytochrome c oxidase), which reduces molecular oxygen to water in the final step. As electrons are transferred between complex I and complex IV, protons are translocated across the inner mitochondrial membrane and a proton gradient is established. ATP is generated from ADP and P_i as protons are shuffled down an electrochemical gradient through complex V (ATP synthase) into the matrix. Therefore, ATP synthase couples ATP production to proton translocation and is completely dependent on the generation of an electrochemical proton gradient across the inner mitochondrial membrane. Finally, ATP is transported from the mitochondrial matrix to the cytoplasm through the adenine nucleotide transporter (ANT), thereby supplying mitochondrially generated energy to help meet intracellular requirements.

ANT is an integral inner mitochondrial membrane protein that catalyzes the rapid and unlimited exchange of cytosolic ADP for mitochondrial ATP. ANT regulation is stringently coupled to the mitochondrial F_1-F_0-ATPase that phosphorylates ADP to ATP in conjunction with proton flux from the inter-mitochondrial membrane space into

the mitochondrial matrix [Heineman and Balaban, 1990]. Three isoforms of ANT have been described in mammals namely, ANT1, ANT2 and ANT3 [Neckelmann et al., 1987; Houldsworth and Attardi, 1988; Cozen et al., 1989], expressed in a tissue-specific pattern [Cozen et al., 1989; Ku et al., 1989]. Only ANT1 and ANT2 have thus far been identified in rodents [Levy et al., 2000; Li et al., 1989]. Expression of ANT1 is postnatally induced and it is highly expressed in skeletal muscle and heart [Li et al., 1989; Stepien et al., 1992; Portman et al., 1997].

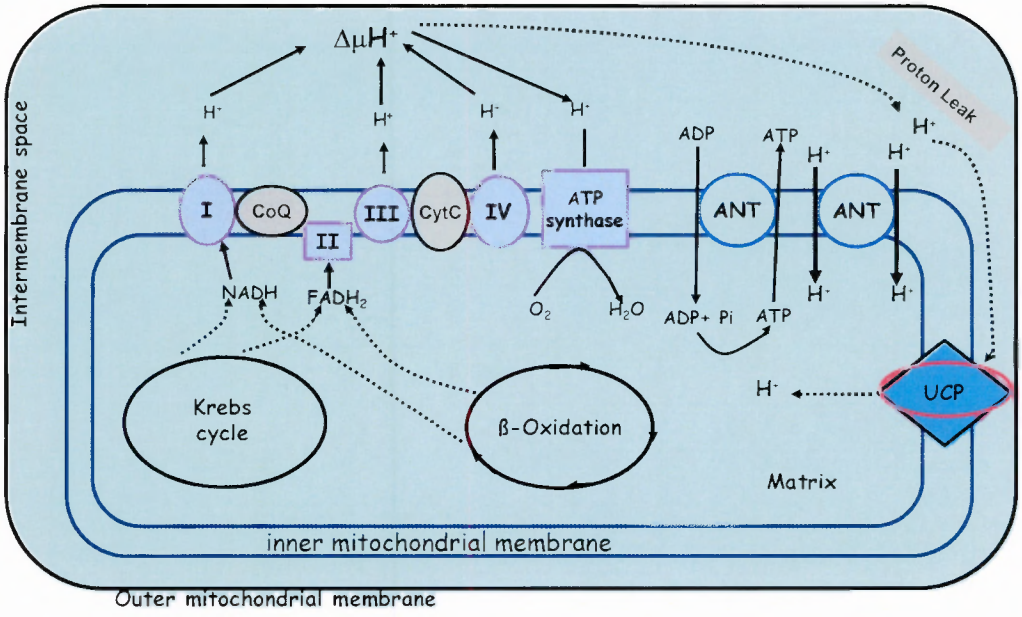

Figure 6: Simplified representation of mitochondrial oxidative phosphorylation. Abbreviations: reduced nicotine adenine dinucleotide (NADH), reduced flavin adenine dinucleotide ($FADH_2$), complex I (I), coenzyme Q (CoQ), complex II (II), complex III (III), cytochrome c (CytC), complex IV (IV), adenine nucleotide translocator (ANT), uncoupling protein (UCP), electrochemical proton gradient ($\Delta\mu H^+$).

The role of ANT in the heart was recently highlighted following its genetic depletion [Graham et al., 1997; Esposito et al., 1999]. Here ANT1 depletion was maladaptive, resulting in the development of diminished mitochondrial oxidative phosphorylation,

excessive mitochondrial ROS generation, mitochondrial genomic mutations, and progressive development of cardiomyopathy [Ning et al., 2000; Graham et al., 1997; Esposito et al., 1999]. On the contrary, ANT2 is a fetal isoform, thus weakly expressed in most tissues after birth. Stepien et al. (1992) found that ANT2 expression was increased in cells with high glycolytic capacity for example C2C12 myoblasts, leading to the suggestion that it may mediate nucleotide transport during glycolytic conditions (e.g. hypoxia). ANT3 expression in humans is ubiquitous and it has been proposed to form ANT1/ANT3 heterodimers [Stepien et al., 1992].

In summary, reducing equivalents generated from cardiac metabolic pathways trigger electron transfer across the inner mitochondrial membrane to generate an electrochemical gradient that is utilized to generate mitochondrial ATP. However, these processes are not always tightly linked and in some instances may result in the 'uncoupling' of the electrochemical gradient and actual mitochondrial ATP synthesis.

1.5.3.1. Mechanisms regulating uncoupling of mitochondrial oxidative phosphorylation

ATP synthase is not the only route by which the mitochondrial electrochemical proton gradient is dissipated [Nicholls, 1974]. Uncoupled oxidative phosphorylation or 'proton leak' results from electron transfer and proton translocation across the inner mitochondrial membrane into the matrix without phosphorylation of ADP to ATP via ATP synthase, resulting in reduced ATP production (Figure 6). Studies have shown that uncoupling can be facilitated by inner mitochondrial membrane proteins such as the uncoupling proteins (UCPs) [Krauss et al., 2005], ANTs [Samartsev et al., 1997; Speakman et al., 2004] and cytochrome oxidase [Kadenbach et al., 2000]. It is suggested that proton leak accounts for up to 20% of mitochondrial oxygen

thermogenesis [Nicholls and Locke, 1984; Cannon and Nedergaard, 1985; Klingenberg, 1990]. However, UCP2 is expressed in most cells [Fleury et al., 1997; Gimeno et al., 1997], while UCP3 is found mainly in skeletal muscle [Boss et al., 1997; Vidal-Puig et al., 1997; Yoshitomi et al., 1998] and expressed at lower levels in the heart [Yoshitomi et al., 1998], UCP4 [Mao et al., 1999] and UCP5 [Sanchis et al., 1998] are mainly expressed in the brain. Expression of UCP2 gene in the heart is species-specific, with relatively higher levels in mice compared to humans [Fleury et al., 1997].

The role of UCPs in the heart is unclear at this stage with some studies suggesting it may play a classic uncoupling role, while others argue against it. For example, it was shown that elevated expression of UCPs in skeletal and cardiac muscle results in increased uncoupling of oxidative phosphorylation and decreased myocardial efficiency and mitochondrial membrane potential [Boehm et al., 2001]. However, others have found that overexpression of UCP2 in cardiac myocytes protected against damaging ROS [Teshima et al., 2003]. Furthermore, it is also proposed that UCP3 may play a physiological role in the export of fatty acids out the mitochondrial matrix, thus preventing the build up of damaging lipid intermediate species [Himms-Hagen and Harper, 2001; Schrauwen et al., 2003]. Thus at this stage there is no precisely defined role for the cardiac-enriched UCP isoforms (UCP2 and UCP3). However, a recent study [Murray et al., 2005] reported that UCP2 and UCP3 protein expression levels in the heart are controlled by different regulatory mechanisms, suggesting distinct functional roles. Ongoing and future studies should more clearly define its role in the heart.

1.6. Metabolic fuel substrate switches in relatively hypoxic environments

1.6.1. Developmental

The fetal heart exists in a relatively hypoxic state. In the past, opinions about which energy source is preferable for cardiac metabolism during early development have varied [Girard et al., 1992; Lopaschuk et al., 1991; Sack et al., 2000]. It has now emerged, however, that the transition from the fetal to postnatal phenotype is characterized by the shift of substrate preference from glucose to fatty acid metabolism [Lopaschuk et al., 1992]. This substrate switch is accordingly matched by transcriptional and posttranslational remodeling [Sack et al., 2000]. Mitochondrial function is also altered during the course of cardiac development. For instance, during the transition from fetal to the postnatal period, mitochondrial number and activity increase [Goodwin et al., 1976], associated with higher activity of citrate synthase, a key enzyme in the Krebs cycle [Razeghi et al., 2001a].

The fetal heart largely relies on glycolysis as a source of energy [Lopaschuk et al., 1992; Fisher et al., 1980]. This is matched by increased activity of glycolytic enzymes [Fisher et al., 1980; Hoerter and Opie, 1978; Makinde et al., 1997; Rolph and Jones, 1983]. In particular, lactate utilization is increased in the fetal heart [Werner and Sicard, 1987] due to high availability of circulating lactate [Fisher et al., 1980]. Moreover, mitochondrial oxidative capacity is high in fetal and newborn hearts despite high glycolysis rates [Lopaschuk and Spafford, 1990; Lopaschuk et al., 1991; Warshaw, 1969, 1972]. The hypoxic environment of the fetal myocardium favors reliance on a more efficient fuel substrate under these conditions i.e. utilization of carbohydrates compared to fatty acids [Fisher et al., 1980; Kostreva and Wood, 1991].

After birth, there is a marked increase (~10-fold) in fatty acid oxidation that is paralleled by decreased glycolytic rates [Itoi and Lopaschuk, 1993; Lopaschuk et al., 1991]. The multiple mechanisms responsible for increased fatty acid metabolism in the postnatal heart are not yet completely understood at present. However, increased CPT-1 expression is thought to play a role [Bartelds et al., 2004; Cook et al., 2001; Onay-Besikci et al., 2003]. The heart is the only known organ that expresses both liver (CPT-Iα) and muscle (CPT-1β) isoforms in the same cell [Cook et al., 2001]. CPT-1α predominates as the fetal isoform in the rat [Brown et al., 1995; Cook et al., 2001], lamb [Bartelds et al., 2004] and human [Razeghi et al., 2001a] heart. CPT-1β is also expressed during the fetal stages [Razeghi et al., 2001a; Marin-Garcia and Goldenthal, 2002]. Furthermore, CPT-1α activity decreases whilst CPT-1β increases after birth in rats [Park and Cook, 1998].

Control of fatty acid oxidation in the newborn period also occurs via malonyl-CoA at the level of mitochondrial fatty acid uptake [Lopaschuk et al., 1994; Makinde et al., 1997]. Previous studies have shown that myocardial malonyl-CoA content decreases within a few days after birth [Dyck et al., 1998; Lopaschuk et al., 1994; Makinde et al., 1997]. Decreased malonyl-CoA levels may be due to reduced activity of ACC, leading to increased CPT-1 activity and higher fatty acid oxidation rates [Lopaschuk et al., 1994]. In agreement, activity of AMPK, an inhibitor of ACC, is increased postnatally [Dyck et al., 1998]. Consistent with this switch to higher myocardial fatty acid oxidation, MCAD and LCAD gene expression are also robustly induced during the transition from the fetal to the postnatal state [Hainline et al., 1993; Nagao et al., 1993], thus matching higher myocardial energy requirements [Kelly et al., 1987, 1989].

In terms of the regulation of glucose metabolism, GLUT1 protein levels decline in the rat heart after birth, whereas insulin-responsive GLUT4 protein levels are increased ~3-fold during the transition from the fetal to the postnatal phase [Santalucia et al., 1992]. This induction of GLUT4 is thought to be due to increased levels of postnatal insulin or thyroid hormones [Weinstein and Harber, 1992; Studelska et al., 1992; Sugden et al., 2000]. Moreover, cardiac PDK4 protein was found to be more robustly expressed than PDK1 during early development [Sugden and Holness, 1994, 2000], thereby diminishing glucose oxidation [Sugden et al., 2000].

Cardiac PGC-1α expression increases sharply at birth coincident with a perinatal shift from glucose to fatty acid metabolism [Lehman et al., 2000]. Furthermore, overexpression of PGC-1α in cardiomyocytes activates mitochondrial biogenesis and coupled respiration [Lehman et al., 2000]. In the developing mouse heart, there is increased mitochondrial biogenesis and oxidative metabolism that is preceded by higher expression of PGC-1α, concomitant with the increased reliance on fatty acid oxidation [Lehman et al., 2000]. PGC-1α is also thought to be the key regulator of fatty acid oxidation since it interacts with PPARα to promote transcription of nuclear genes encoding mitochondrial fatty acid oxidation enzymes [Vega et al., 2000; Lehman et al., 2000]. The PGC-1α [Kodde et al., 2007] and PGC-1β regulatory circuit coactivates ERRs leading to increased MCAD expression, a key enzyme of the β-oxidation spiral [Kamei et al., 2003]. ERRα also cooperates with NRF-1 and NRF-2 to regulate mitochondrial biogenesis and expression of oxidative phosphorylation enzyme genes [Mootha et al., 2004; Schreiber et al, 2004]. Therefore, PGC-1 activation increases fatty acid utilization by promoting the expression of PPARα and ERRα [Kodde et al., 2007].

Several metabolic changes have also been reported in the hypertrophied/failing heart. It is proposed that some degree of hypoxia also exists in the hypertrophied/failing heart due to reduced capillary volumes. Since my thesis deals with hypoxia-induced hypertrophy, I will now discuss some of these pertubations in more detail.

1.6.2. Physiologic/ pathophysiologic cardiac hypertrophy and heart failure

1.6.2.1. Physiologic hypertrophy

The hypertrophic response in the heart is usually associated with myocyte hypertrophy (increased mass) rather than hyperplasia (increased number), an observation first made in 1860 by German pathologist Rudolph Virchow [Virchow, 1860]. Therefore, cardiac hypertrophy is defined as the enlargement of the heart associated with increases in cardiomyocyte size and changes in the organization of the sarcomeric structure [Lorell and Carabello, 2000; Frey et al., 2004]. Physiologic hypertrophy is a favourable, adaptive response of the heart to increases in physical demand, and does not culminate in heart failure [Buttrick and Scheuer, 1987]. This is, for example, observed during developmental growth, exposure to hypobaric hypoxia and exercise training [Izumo et al., 1988; Buttrick and Scheuer, 1987]. Cardiomyocytes also respond to hypertrophic stimuli with characteristic changes in gene expression as part of a compensatory process for increased hemodynamic load [Katz, 1990]. Early genes to be expressed include the immediate early genes known as proto-oncogenes i.e. *c-jun, c-fos* and *c-myc*. Expression of these genes may be mechanistically linked to activation of other target genes that are later upregulated during the hypertrophic response [Chien et al., 1991]. Here it has been found that

transcription takes place within 30 minutes and peaks within the first hour of mechanical stress [Komuro et al., 1990]. In agreement, aortic constriction in rats results in cardiac expression of *c-fos* and *c-myc* at 30 minutes and 2 hours, respectively [Komuro et al., 1988]. Furthermore, Diez et al. (2001) showed that the expression levels of the early genes are high initially and normalize after a few hours, even though the hypertrophic stimulus persists.

Physiologic hypertrophy is usually associated with modest wall thickening, normal ventricular collagen content, maintained coronary blood flow [Medugorac, 1980; Di Bello et al., 2003] and a relatively normal pattern of cardiac gene expression [Fagard, 1997; Kaplan et al., 1994]. It has been shown that the right ventricular mass is linearly related to pulmonary arterial pressure in hypoxic rats [Rabinovitch et al., 1979]. The initial rise in pulmonary artery pressure is due to vasoconstriction [Reeves and Herget, 1984], which is a physiologic response that serves to improve alveolar ventilation and perfusion [Von Euler and Liljestrand, 1946]. In response to moderate hypobaric hypoxia (2-12 week exposure period), Sharma et al. (2004) and Adrogue et al. (2005) found RV cardiac hypertrophy without any visible fibrosis. On the contrary, most models of pathological hypertrophy are characterized by fibrosis and sarcomere disarrangement [Izumo et al., 1988; Swynghedauw, 1999].

Cardiac hypertrophy was initially described as a beneficial response to maintain cardiac output under conditions of overload [Meerson, 1972]. However, it is now recognized as a risk factor for increased mortality [Levy et al., 1990; Casale et al., 1986; Koren et al., 1991; Vakili et al., 2001; Bolognese et al., 1994; Carreno et al., 2006]. The clear differences between physiologic and pathophysiologic cardiac hypertrophy are still evolving. For instance, it has been proposed that a major underlying difference between physiologic and pathologic hypertrophy is the duration of stress i.e. transient (physiologic) versus chronic (pathophysiologic) [Lorell and

Carabello, 2000]. However, a recent study suggested that the nature of the stress, rather than its duration may indeed be a key determinant of a maladaptive cardiac phenotype [Perrino et al., 2006].

Signaling pathways may also determine whether hemodynamic overload manifests in an adaptive or maladaptive phenotype. For example, the phosphoinositide 3-kinase (PI3K) pathway has been implicated in the development of physiologic hypertrophy [McMullen et al., 2003; McMullen et al., 2007]. Conversely, G-protein-coupled-receptor-associated ($G\alpha_q$) protein signaling has been linked to the onset of pathophysiologic hypertrophy [Kempf and Wollert, 2004]. For example, transgenic mice overexpressing cardiac-specific $G\alpha_q$ develop hypertrophy that rapidly progresses to heart failure [Sakata et al., 1998]. Moreover, inhibition of $G\alpha_q$ in transgenic mice attenuates cardiac hypertrophy in response to aortic stenosis [Akther et al., 1998]. Angiotensin II and norepinephrine are potent activators of $G\alpha_q$-dependent signaling pathways in cardiomyocytes. Thus, attenuated signaling via these pathways for e.g. angiotensin-converting enzyme (ACE) inhibitors or adrenoreceptor blockers, prevents the hypertrophic response [Wollert and Dexler, 1999; Flather et al., 2000; Bristow, 2000]. Therefore, distinct molecular signaling pathways underlie the development of physiologic versus pathologic hypertrophy and the subsequent development of heart failure [Kang et al., 2004].

1.6.2.2. Altered expression of contractile proteins in the hypertrophied heart

Several contractile proteins have been identified that show a shift to a fetal gene program during the onset of cardiac hypertrophy. These include atrial natriuretic peptide (ANP), β-myosin heavy chain [Izumo et al., 1987] and α-skeletal actin [Chien

et al., 1991; Komuro et al., 1988; Chien et al., 1993]. The fetal gene program also includes increased expression of sarcoplasmic/endoplasmic reticulum Ca^{+2} ATPase 2a protein (SERCA2a) [de la Bastie et al., 1990] and sarcolemmal Na^{+}, K+ -ATPase [Charlemagne et al., 1986].

Myosin heavy chain (MHC) is the main component of myosin and exists in two distinct isoforms, MHC-α and MHC-β [Pope et al., 1980]. MHC-β isoform is characterized by decreased ATPase activity compared to MHC-α. The latter isoform is associated with reduced contractile velocity but greater economy in force generation [Holubarsch et al., 1985]. Currently, data on MHC-β expression patterns in the heart is conflicting. For example, it has been demonstrated that MHC-β expression is the predominant isoform in the heart regardless of the stage of development or disease [Razeghi, 2001a] and that it predominates throughout all developmental stages in the human heart [Reiser et al., 2001]. However, others showed that both MHC-α and MHC-β are downregulated in fetal and failing hearts [Isaacs et al., 1992; Kostin et al., 2000]. Moreover, myosin heavy chain isoform switching in response to hypoxia is thought to be an adaptive mechanism to conserve ATP per contraction. Razeghi et al. (2003) showed increased myocardial gene expression of MHC-β (fetal isoform) and reduced MHC-α (adult isoform) levels in response to hypoxia. In agreement, Hashimoto et al. (2003) showed that increased expression of MHC-β is an adaptive mechanism necessary to sustain cardiac contractile efficiency in response to impaired oxidative metabolism of hypoxia-induced hypertrophied ventricles. Also, Sharma et al. (2004) exposed rats to hypobaric hypoxia and found that MHC-β mRNA expression was upregulated while MHC-α was downregulated after 2 days, independent of hemodynamic load.

Intracellular Ca^{+2} cycling is largely determined by the activity of SERCA2a in the sarcoplasmic reticulum [Bers et al., 1993]. Previous work showed that intracellular calcium released from the sarcoplasmic reticulum is the major source of intracellular calcium during excitation-contraction coupling [Razeghi et al., 2003]. Therefore, SERCA2a activity is crucial in the regulation of cardiac contractility [Razeghi et al., 2003]. An earlier study reported diminished SERCA2a mRNA levels during the transition from LV hypertrophy to failure after aortic banding [Feldman et al., 1993]. Consistent with these findings, Sharma et al. (2004), found reduced SERCA2a mRNA expression levels in the hypertrophied heart in response to chronic hypobaric hypoxia while SERCA2a transcript levels were downregulated in fetal [Sack et al., 1996] and failing hearts [Gwathmey et al., 1987; Movsesian and Schwinger, 1998]. Downregulation of SERCA2a is thought to contribute to contractile dysfunction of the failing heart [Pieske et al., 1999].

Myocardial tissues also exhibit markedly reduced activities of myofibrillar and sarcoplasmic reticulum Ca^{2+} ATPase in a canine model of pacing-induced heart failure [O'Brien et al., 1990]. Moreover, SERCA2a expression is downregulated in pressure-overload induced right ventricular hypertrophy in parallel with impaired calcium handling and contractile function [Kogler et al., 2003]. Possible mediators of decreased SERCA2a expression in pressure overload-induced myocardial hypertrophy include calcium-dependent signaling pathways involving calcineurin and CAMK [Frey and Olson, 2003], as well as calcium-independent signaling pathway that is associated with mechanical stress [Ruwhof and van der Laarse, 2000]. Ion pumps, including SERCA2a, exhibit preference for glycolytic ATP as opposed to ATP derived from β-oxidation, as an adaptive response that helps minimize disturbances in Ca^{+2} homeostasis associated with cardiac hypertrophy [van Bilsen et al., 1998].

In most animal models of chronic hemodynamic overload, decreased SERCA2a mRNA levels are paralleled with increased atrial natriuretic peptide (ANP)-mRNA levels, a marker of cardiac hypertrophy [Mercadier et al., 1989]. ANP is a peptide hormone which exhibits diuretic, natriuretic, and vasorelaxant properties [Rosenzweig and Seidman, 1991; de Bold et al., 1996]. Under physiological conditions in the mammalian heart, atrial levels of ANP are ~100-fold greater than ventricular levels [Gardner et al., 1986]. Increases in ventricular expression of ANP have been documented in numerous experimental models of cardiac hypertrophy and failure [Calderone et al., 1995; Fukui et al., 1989; Mercadier et al., 1989; Matsubara et al., 1990; Feldman et al., 1993; Boluyt et al., 1994; Yokata et al., 1995; Yoshimine et al., 1997], as well as in human heart failure [Feldman et al., 1991; Takahashi et al., 1992; Pasternac and Cantin, 1990]. However, Vikstrom et al. (1998) failed to show correlation between cardiac hypertrophy and ventricular expression of ANP in a transgenic mouse model of hypertrophic cardiomyopathy, leading to the alternate suggestion that ANP should not be considered a stable marker of cardiac hypertrophy.

1.6.2.3. Mitochondrial perturbations

Cardiac hypertrophy is associated with increased mitochondrial number and size [Maron et al., 1975], and a marked increase in mtDNA synthesis [Zak et al., 1980]. Chronic heart failure is also linked with mitochondrial morphological abnormalities such as increased number, reduced size and compromised structural integrity [Schaper et al., 1991]. Impairment of mitochondrial function in the human failing heart is accompanied by reduced activity of the respiratory chain [Buchwald et al., 1990]. In agreement, mitochondrial oxygen utilization is diminished in rat [Sanbe et al., 1993],

and dog [Sharov et al., 1998] models of heart failure. The reduced expression of mitochondrial proteins is associated with limited ATP generating capacity and abnormalities in high-energy phosphate kinetics in heart failure [Ning et al., 2000]. Large-scale deletions in heart mtDNA have also been reported in patients with cardiomyopathy [Suomalainen et al., 1992; Marin-Garcia et al., 1996; Li et al., 1995]. Furthermore, Sanbe et al. (1995) found reduced rates of mitochondrial oxygen consumption in an experimental model of post-infarction cardiac failure and in explanted hearts from patients with ischemic and idiopathic dilated cardiomyopathy [Sharov et al., 2000]. In agreement, several studies have shown that intracellular ATP concentrations are ~25% to 30% lower in failing human hearts [Nascimben et al., 1996; Baars et al., 2002].

Experimental and clinical studies of heart failure show reduced activity of several electron transport chain complexes. For example, myocardial tissues from a canine model of pacing-induced cardiomyopathy exhibit reduced activities of key ATP-utilizing and ATP-generating enzymes, including mitochondrial ATP synthase (complex V) [O'Brien et al., 1990; Niknahad et al., 1995]. Furthermore, Marin-Garcia et al. (2001) showed diminished complex III and complex V activities with no change in citrate synthase in an experimental model of decompensated pacing-induced heart failure. However, Jarreta et al. (2000) showed selective reduction of complex III activity in myocardial biopsies from heart failure patients. Patients with dilated cardiomyopathy also displayed reduced cytochrome content and activities of complexes III and IV with no change in complex II and complex V [Buchwald et al., 1990]. Moreover, Scheubel et al. (2002) demonstrated a 28% decrease in complex I activity, but no changes in complexes II, III, IV or citrate synthase in heart failure patients. A ~50% decline in complex I activity was reported in a pacing-induced canine model of heart failure, and this was associated with greater production of ROS [Ide et al.,1999].

ROS have been suggested to play a causative role in the development of mitochondrial-induced metabolic cardiomyopathy [Russell et al., 2005]. Mitochondria are a major source of ROS and therefore a prime target of ROS-induced damage. The mitochondrial respiratory chain generates ROS especially in state 4 respiration, when the electrochemical gradient between the inner mitochondrial membrane is high and the rate of electron transport is limited [Teshima et al., 2003]. In this context, it has been suggested that uncoupling protein 2 (UCP2) affect ROS production by dissipating the electrochemical gradient [Teshima et al., 2003]. ROS compromise mitochondrial aerobic capacity by causing damage to mtDNA and mitochondrial proteins [van Bilsen, 2004]. mtDNA damage by ROS exarcebates mitochondrial dysfunction and reduces mitochondrial energy production [Duchen, 2004; Fariss et al., 2005]. In addition to impairing mitochondrial coupling [Echtay et al., 2002], ROS can also oxidize lipids and proteins and other intracellular molecules such as nitric oxide (NO) to produce highly reactive products, which are damaging to the cell [Ceylan-Isik et al., 2006; Shen et al., 2006;Ye et al., 2004]. However, it has been demonstrated that superoxide activates UCPs in various tissues, indicating that UCPs may be recruited endogenously as a compensatory mechanism to counteract oxidative stress [Echtay et al., 2002]. Teshima et al. (2003) demonstrated that overexpression of UCP2 attenuates ROS generation and prevent Ca^{2+} overload in isolated cardiomyoctes.

Lastly, in both volume- and pressure-overloaded rat hearts, phosphocreatine levels are reduced in left ventricular hypertrophy [Kapelko et al., 1988]. Likewise, heart failure patients exhibit marked reductions in levels of phosphocreatine [Kalsi et al., 1999]. It has been demonstrated that reduced phosphocreatine-to-ATP (PCr/ATP) ratio levels in human hearts predict motality from cardiovascular causes [Neubauer et al., 1997]. Furthermore, it was shown that mitochondrial creatine kinase activity and

content, which transfers mitochondrial ATP to the myofibril, were reduced in a rat model of pressure-overload hypertrophy [De Sousa et al., 1999]. Together these studies therefore suggest that reduced ATP production, caused by defects in the mitochondrial oxidative mechanism, may lead to "energy starvation" of the heart, thereby contributing to contractile dysfunction.

1.6.2.4. Metabolic remodeling

Cardiac energy metabolism may also be altered in hypertrophied and failing hearts [Sambandam et al., 2002]. Pressure overload [Allard et al., 1994; Christe and Rodgers, 1994], volume-overload [El Alaoui-Talibi et al., 1992, 1997], and myocardial infarction [Remondino et al., 2000] have all been associated with increased reliance on glucose utilization versus fatty acid oxidation, resembling a fetal-type fuel substrate utilization. Increased reliance on glucose utilization is associated with more efficient ATP production per mole of oxygen consumed [Barger and Kelly, 1999]. The fetal gene program is regulated in response to developmental, hormonal, and hemodynamic stimuli [Parker et al., 1990]. Reactivation of fetal gene program in the hypertrophied heart may serve to reduce ATP and oxygen consumption requirements [Sack et al., 1997] since a relative degree of hypoxia may exist. However, unlike the rapid early induction of hypertrophic genes initiation of the fetal gene program usually occurs within 6 to 12 hours [Carreno et al., 2006]. Conversely, other evidence suggest that such a switch may be a key contributor to the transition from compensated hypertrophy to heart failure. Thus, the latter argues, metabolic intervention may be employed as a therapeutic strategy to prevent fuel substrate switching in the hypertrophied/failing heart [Friehs et al., 1999; Liao et al., 2002; Davila-Roman et al., 2002; Wallhaus et al., 2001; Stanley and Chandler, 2002]. However, this switch is not always observed. For example, Degens et al. (2006)

could not demonstrate the classic fuel substrate switch in a rat model of compensatory cardiac hypertrophy induced by aortic constriction for 4 weeks. They demonstrated modest elevation of glycolytic capacity associated with preserved fatty acid oxidative capacity [Degens et al., 2006].Thus the precise significance of this switch is not clear i.e. whether it is maladaptive or adaptive.

Although cardiac hypertrophy is associated with increased rates of glycolysis and glycolytic enzyme activities, glucose oxidation rates may be reduced and hence more lactate may accumulate [Bishop and Altschuld, 1970]. At the level of glucose uptake, cardiac hypertrophy and failure are associated with diminished expression of the insulin-dependent glucose transporter (GLUT4) and unchanged or increased expression of the fetal isoform (GLUT1) [Paternostro et al., 1995; Depre et al., 1998; Rosenblatt-Velin et al., 2001]. Razeghi et al. (2001a, 2002) showed that GLUT1 and GLUT4 mRNA are downregulated in the failing human heart. Sack et al. (1996) demonstrated downregulation of fatty acid oxidation in severe, end–stage heart failure. However, the decline in fatty acid oxidation and increased glucose oxidation that was observed during advanced end-stage failure was not observed during a period of moderately severe pacing induced heart failure [Recchia et al., 1998]. Increased GLUT1 expression may be an adaptive mechanism to sustain contractile function. For example, cardiac-specific overexpression of GLUT1 prevents the development of heart failure in a mouse model of LV pressure overload [Liao et al., 2002]. There has been conflicting views regarding alterations in glucose utilization in the human hypertrophied and/or failing heart. For instance, some studies reported diminished glucose utilization [Taylor et al., 2001], while others have demonstrated unchanged [Tadamura et al., 1998] or increased glucose utilization [Davila-Roman et al., 2002; Uehara et al., 1998]. Murray et al. (2006) demonstrated decreased GLUT4 levels, insulin resistance and decreased ischemic-stimulated glucose uptake in the failing rat heart.

End-stage pacing-induced heart failure in dogs exhibited repression of both the glucose and fatty acid metabolic enzymes [Lei et al., 2004]. This may represent a general "energy-deficient" state of the failing heart. In agreement, patients undergoing coronary artery bypass surgery exhibited increased UCP2/3 and decreased GLUT4 protein levels. If UCPs uncouple in the heart this would mean inefficient ATP production, while lower GLUT4 levels would translate into less myocardial glucose uptake [Murray et al., 2004]. A reduction in myocardial fatty acid oxidation is influenced by a reciprocal induction of transcription factors, including COUP-TF, and Sp1/ Sp3, repressing promoter activity of key fatty acid oxidation enzyme genes [Sack et al., 1997; Tian, 2003]. Expression of fatty acid oxidation genes and corresponding enzymatic activities are diminished in the failing rodent and human heart [Taegtmeyer and Overturf, 1988; Sack et al., 1996; Razeghi et al., 2001a]. Moreover, reduced fatty acid oxidation rates in rats subjected to coronary artery ligation are accompanied by lower expression of cardiac fatty acid transporters [Heather et al., 2006]. Conversely, other studies have reported an opposing metabolic profile, or no change in substrate utilization in humans and animal models of heart failure of mild-to-moderate severity [Paolisso et al., 1994; Wallhaus et al., 2001; Chandler et al., 2004]. These discrepancies suggest that the fuel substrate switch may be an end-stage phenomenon associated with cardiac decompensation and not moderate heart failure. Moreover, the switch may be a compensatory mechanism that occurs in end-stage heart failure in an attempt to rescue the myocardium only when the heart's function has become severely compromised [Chandler et al., 2004].

PPARα is a key regulator in the transcriptional control of myocardial fatty acid metabolism genes. Diminished fatty acid oxidation rates in the hypoxic, hypertrophied

and failing hearts are paralleled by reduced PPARα expression [Ngumbela et al., 2003; Razeghi et al., 2001a; Sharma et al., 2004] and activity [Izumo et al., 1988; Young et al., 2001a; Barger et al., 2000; Barger and Kelly, 2000]. Moreover, expression of PPARα is reduced in the hypertrophied and failing heart, and in hypertrophied cardiomyocytes [Barger et al., 2000; Lehman and Kelly, 2002; Barger and Kelly, 2000; Kanda et al., 2000; Garnier et al., 2003]. In agreement, expression of genes involved in fatty acid uptake / metabolism is diminished in experimental models of cardiac hypertrophy and failure [Remondino et al., 2000; Depre et al., 1998; Rosenblatt-Velin et al., 2001; Sack et al., 1997]. Expression of a coactivator of PPARα i.e. PGC-1α, is also altered in experimental models of cardiac hypertrophy and failure [Sano et al., 2004; Czubryt et al., 2003; Lehman and Kelly, 2002; Garnier et al., 2003; Feingold et al., 2004; Hsieh et al., 2005, 2006; Suliman et al., 2004; Finck et al., 2002].

Similar observations were made in left ventricular tissues of human cardiac transplant recipients [Razeghi et al., 2002] i.e. reduced PPARα mRNA expression. In addition, patients with compensated end-stage heart failure also displayed diminished PPARα protein expression in ventricular biopsies [Karbowska et al., 2003]. Therefore, PPARα/RXRα heterodimer activity and other nuclear receptors or transcription factors may contribute to the decreased expression of fatty acid oxidation enzymes in cardiac hypertrophy and failure, although RXRα expression has not been reported in heart failure patients [Stanley et al., 2005].

At the level of mitochondrial fatty acid uptake, Depre et al. (1998) reported reduced CPT-1β in human end-stage heart failure and in cardiac hypertrophy. However, CPT-1α expression was not altered. CPT-1α is less sensitive to malonyl-CoA inhibition compared to CPT-1β [McGarry and Brown, 1997; McGarry et al., 1983; Kerner et al.,

1994], which may explain increased CPT-1α levels and loss of malonyl-CoA sensitivity in the failing heart [Razeghi et al., 2002]. Expression of fatty acid oxidation enzyme-encoding genes i.e. MCAD and LCAD were reduced in explanted hearts from transplant recipients [Sack et al., 1996]. In agreement, MCAD protein levels were also downregulated. Similarly, MCAD and CPT-1 activity were decreased in the canine model of end-stage pacing-induced heart failure [Lei et al., 2004; Osorio et al., 2002], with no parallel reduction of PPARα [Osorio et al., 2002].

Several lines of evidence have implicated altered PGC-1α activity in the development of heart failure. For instance, in a chronic model of pressure-overload, PGC-1α levels are downregulated in parallel with PPARα-target genes regulating mitochondrial fatty acid oxidation [Lehman and Kelly, 2002a,b; Finck et al., 2002]. This may be an adaptive mechanism since cardiac-specific overexpression of PGC-1α leads to development of dilated cardiomyopathy and depressed contractile function in mice [Lehman et al., 2000].

Lastly, several lines of evidence have implicated altered PGC-1α activity to the development of pathophysiologic hypertrophy. For instance, in a chronic model of pressure-overload, PGC-1α levels are downregulated along with expression levels of PPARα-target genes involved in mitochondrial fatty acid oxidation [Lehman and Kelly, 2002a,b; Finck et al., 2002]. Furthermore, cardiac-specific overexpression of PGC-1α leads to development of dilated cardiomyopathy and depressed contractile function in mice [Lehman et al., 2000]. In an experimental model of heart failure, it has been demonstrated that the decrease in oxidative capacity and mitochondrial enzyme activities in both cardiac and skeletal muscles is paralleled by diminished

expression of nuclear- and mitochondrial-encoded subunits of complex IV of the respiratory chain in the absence of impaired mtDNA replication [Garnier et al., 2003].

1.7. Hypotheses

Having reviewed some of the key metabolic aspects related to the hypoxic and hypertrophied heart, I embarked on this study to investigate the following hypotheses:

1) With the onset of physiologic hypertrophy, there is adaptive remodeling that includes coordinate induction of mitochondrial respiratory genes that sustains mitochondrial energy production and cardiac contractile function.

2) Chronic PPARα activation and high fat availability will increase myocardial oxygen demand and uncouple mitochondrial oxidative phosphorylation, thereby resulting in impaired contractile function of the hypertrophied heart.

I investigated these hypotheses by employing a rat model of hypoxia-induced cardiac hypertrophy, previously demonstrated to be an adaptive phenotype.

1.8. REFERENCES

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Chapter 2

Identification of a novel cardioprotective program sustaining contractile function in the hypertrophied right ventricle in response to chronic hypobaric hypoxia

2.1. INTRODUCTION

It is well-described that chronic hypobaric hypoxia results in pulmonary vasoconstriction and an increase in pulmonary artery pressure, leading to the development of right ventricular (RV) hypertrophy. This trophic response is considered an adaptive mechanism to sustain RV cardiac output in response to increased load. For example, we recently reported a robust hypertrophic response in the right ventricles of rats exposed to chronic hypobaric hypoxia [Adrogue et al., 2004; Sharma et al., 2004]. Moreover, gene expression of atrial natriuretic factor (ANF), a marker of cardiac hypertrophy, was increased in parallel. In addition, we also found that increased RV weight was not associated with a greater degree of fibrosis after 2 weeks of hypobaric hypoxia, indicating a model of physiologic RV hypertrophy [Sharma et al., 2004].

Previous studies suggest that altered mitochondrial respiratory capacity may play a key role to sustain the contractile function of the hypertrophied heart. For example, in response to chronic hypobaric hypoxia maintained mitochondrial respiration was found in the hypertrophied right but not left ventricle (LV) [Rumsey et al., 1999]. Furthermore, Nishio et al. (1995) reported increased mitochondrial content in response to LV hypertrophy, while others found mitochondrial functional alterations in experimental models of volume overload- and pressure overload-induced cardiac hypertrophy [Janati-Idrissi et al., 1995]. Studies elucidating the RV mitochondrial respiration are scarce. However, Nouette-Gaulain et al. (2005) have shown that chronic high altitude hypoxia decreased ATP synthesis as a consequence of an alteration in mitochondrial respiratory chain complexes in both ventricles , albeit delay response in the RV. However, when the RV hypertrophy was fully developed, mitochondrial energy metabolism was decreased as in LV, suggesting specific

adaptive processes at the onset of right ventricular hypertrophy. An emerging paradigm therefore suggests that during the onset of moderate, physiologic cardiac hypertrophy, multiple adaptive pathways are triggered to sustain mitochondrial respiratory function thereby allowing the hypertrophied heart to meet higher energetic demands in response to increased load. However, the functional significance and molecular mechanisms underlying such changes are poorly understood. Studies elucidating the RV function of animals exposed to high altitude hypoxia and the comparison of the function of right and left heart are also still lacking. However, Kolar et al. (1993) demonstrated a higher resting RV systolic pressure in open chest rats exposed to high altitude compared to normoxic controls. Analysis of an isolated preparation of the RV working heart has shown that mechanical performance during the compensated phase of hypertrophy is increased in chronic high altitude hypoxic animals while the index of contractility remained unchanged, suggesting that the enhanced ventricular performance is merely the consequence of the increased muscle mass [Ostadal and Kolar, 2007]. La Padulla and Costa (2005) demonstrated increased contractility in LV isolated from papillary muscles in adult rats exposed to chronic high altitude hypoxia.

In the first part of the study, we hypothesized that exposure to chronic hypobaric hypoxia coordinately upregulates mitochondrial regulatory genes and mitochondrial content in the hypertrophied RV, as part of an adaptive response to sustain mitochondrial respiratory capacity and contractile function. To test our hypothesis, we exposed rats to 2 and 4 weeks of hypobaric hypoxia (11% O_2) and determined cardiac contractile and mitochondrial respiratory function for the right and left ventricles, respectively. Moreover, we performed real-time quantitative RT-PCR analysis to measure transcript levels of several mitochondrial regulators. Here, our data demonstrate the coordinate induction of several genes regulating mitochondrial respiratory function and increased mitochondrial DNA (mtDNA) content in the

hypertrophied RV after 2 weeks, linking the efficiency of mitochondrial oxidative phosphorylation and respiratory function to sustained RV contractile function in response to the increased load. Interestingly, these adaptations were not observed in the LV. Moreover, these changes were generally sustained in the RV after 4 weeks of hypoxic exposure. However, at this time-point the LV began to display diminished contractile and respiratory function.

For the second part of this study, I focused on further exploring the role of increased fatty acid utilization in the setting of right ventricular cardiac hypertrophy. As reviewed in Chapter 1 of this thesis, the hypertrophied and failing heart is associated with a pronounced degree of cardiac metabolic remodeling. These changes appear to manifest in a stage-dependent manner, with earlier stages of heart failure linked to increased fatty acid utilization [Chandler et al., 2004 and Stanley et al., 2005] while end-stage failure is associated with a switch away from fatty acid metabolism (fetal switch) [Sack et al., 1996]. It is also unclear whether these fuel substrate switches are part of an adaptive process or merely an epiphenomenon. In light of this, previous studies performed experiments to increase fatty acid utilization in the hypertrophied heart. However, conflicting data has thus far been generated. For example, Young et al. (2001) found that cardiac hypertrophy is associated with reduced peroxisome proliferator-activated receptor alpha (PPARα) associated with sustained cardiac contractile function, and that reactivation of PPARα was associated with impaired contractile function. These data therefore suggest that the fuel substrate switch away from fatty acid utilization may be an adaptive response. However, others have found that fenofibrate administration (a strategy to elevate fatty acid oxidation) to the failing heart resulted in no effect [Morgan et al., 2006] or a modest improvement in cardiac contractile function (Labinskyy et al., 2007). In light of the adaptive hypertrophic phenotype observed in our rat model at the 2 week time point, I devised a series of experiments to activate PPARα (using Wy-14, 643) in the hypertrophied RV and to

subsequently assess whether this intervention results in an adaptive or maladaptive phenotype. The major finding of the second part of this study is that in vivo Wy-14,643 administration elicited direct effects on mitochondrial respiratory function and cardiac contractility. The data show that Wy-14,643 treatment attenuated mitochondrial respiration in the RV and the LV while promoting uncoupling of mitochondrial oxidative phosphorylation in the LV.

2.2. MATERIALS AND METHODS

2.2.1. Animal studies

Six-week old male Wistar rats (weighing 190-230 g) were initially housed at room temperature on a 12-hour reverse light/dark cycle (lights off at 4 am zeitgeber time (ZT12); lights on at 4 pm (ZT0), with access to a conventional laboratory diet and water *ad libitum*. The hypoxic groups were housed inside a lexan chamber (45 kPa – 11% O_2) (SciTech, Cape Town, South Africa) for 2 or 4 weeks as described previously [Sharma et al., 2004], and compared to age-matched normoxic controls. In the hypoxic models, animals were immediately subjected to 11% O_2 and this is based on the previous protocol used in our laboratory [Essop et al., 2004, Sharma et al., 2004]. For PPARα activation studies, hypoxic and normoxic animals were treated either with Wy-14,643 (25 $mg.kg^{-1}day^{-1}$, Biomol, USA) or vehicle (1% Tween-80 and 0.5% hydroxypropylmethyl-cellulose) by oral gavage for 2 weeks starting on the first day of exposure to hypobaric hypoxia. During oral gavage the opening of the hypoxic chamber was limited to less than 5 minutes at a time to avoid excess exposure to normoxic air. Also we used the 2 week time point animals as the untreated normoxic and hypoxic groups, respectively, since these studies were performed concurrently. On the last day of the experiments, animals were anesthetized with sodium pentobarbital (100 $mg.kg^{-1}$ i.p.) whereupon the hearts were isolated and perfused for

functional assessment. For mitochondrial respiration, cardiac morphometrics and gene expression determination, the right ventricle (RV) was carefully separated from the left ventricle plus the inter-ventricular septum (LV+S). All rats were sacrificed at ZT16-ZT18 since they are most metabolically active during this period [Young, 2003]. The University of Cape Town's Animal Research Ethics Committee approved all animal experiments and the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2.2. Cardiac mitochondrial isolation and functional characterization

Mitochondria were isolated according to the method of Sordahl et al. [Sordahl et al., 1971] with modifications. Briefly, RV and LV tissue were homogenized separately in 10 ml ice-cold potassium-EDTA (KE) buffer (0.18 M KCl, 10 mM EDTA, [pH7.4]), whereafter the homogenate was centrifuged at 755 *x g* for 5 min. The supernatant was subsequently filtered through 41 µm nylon mesh (Spectrum, USA) and the filtrate centrifuged at 1,480 *x g* for 5 min. The mitochondrial pellet was resuspended in 50 µl KE buffer, and was subsequently used for mitochondrial respiration measurements. Mitochondrial outer membrane integrity of normoxic and hypoxic samples were validated using a spectrophotometric cytochrome c oxidase assay according to the manufacturer's instructions (Sigma, MO, USA).

Respiratory rates were polarographically measured using a Clark-type electrode (Hansatech Instruments, London, UK) at 25°C with constant stirring as previously described [Essop et al., 2004] with modifications. Our preliminary studies showed that conducting mitochondrial assays at temperatures above 25°C strongly inhibited mitochondrial respiration and we found 25°C to be the optimum temperature (data not shown). Furthermore, mitochondrial respiration studies have been conducted at

25°C in our laboratory [Essop et al., 2004, Zungu et al., 2006] as well as others [St-Pierre et al., 2000, Starnes et al., 2007, Fuller et al., 1985]. Briefly, isolated rat ventricular mitochondria (0.5 mg/mL) were added to the electrode chamber containing incubation medium (10 mM Tris-HCl, 0.25 M sucrose, 8.5 mM KH_2PO_4, [pH7.4]). We employed a mixture of 5 mM malate and 40 µM palmitoyl-L-carnitine as oxidative substrates. State 3 respiration was determined by measuring mitochondrial oxygen uptake after the addition of ADP to a final concentration of 350 µM. State 4 respiration was determined by measuring mitochondrial oxygen uptake upon complete phosphorylation of ADP to ATP. Basal proton leak in the isolated mitochondria was determined by measuring the rate of mitochondrial respiration after addition of 10 µg/ml oligomycin to state 4 mitochondria.

The ADP/O ratio, a measure of mitochondrial oxidative phosphorylation efficiency, was calculated as the ratio between the ADP added and oxygen consumed during ADP phosphorylation. The rate of ADP phosphorylation was calculated as nanomoles of ADP phosphorylated per minute during state 3 respiration as described before [Babsky et al., 2001]. Mitochondria were considered viable where the respiratory control index (RCI) (state 3/state 4) were ≥ 4. The RCI and the ADP/O ratios were calculated according to Estabrook [Estabrook, 1967] using 253 nmol O_2 /mL as the value for the solubility of oxygen at 25°C. All mitochondrial polarographic studies were normalized to total mitochondrial protein content, determined using the Lowry assay [Lowry et al., 1951].

2.2.3. Langendorff heart perfusions

Isolated hearts were perfused in the Langendorff mode with ice-cold Krebs-Henseleit buffer (11 mM Glucose, 118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 1.8 mM $CaCl_2.6H_2O$, pH 7.4). The aorta was located

and cannulated on the Langendorff perfusion rig, and a retrograde perfusion of the coronary arteries via the aorta was immediately initiated. The perfusion was performed with oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit buffer at a constant pressure (104 cm H_2O) and temperature (37°C). During perfusion, a latex balloon attached to a pressure transducer was first inserted into the left ventricular cavity for stabilization and inflated to produce a diastolic pressure of 4-12 mmHg, whereupon the balloon was then inserted into the right ventricle in order to determine the right ventricular developed pressure (RVDP) as described before [Thurich et al., 1999]. The RV and LV functional parameters were measured and included heart rate, systolic and diastolic pressure, developed pressure, coronary flow and rate pressure product (heart rate x developed pressure). The duration of the perfusion experiments was limited to 15 minutes for each ventricle.

2.2.4. RNA isolation and real-time quantitative RT-PCR analysis

RNA extraction and real-time quantitative RT-PCR of samples were performed in Dr. Martin Young's laboratory (Baylor College of Medicine, Texas) using previously described methods [Stavinoha et al., 2004]. Specific quantitative Taqman assays were designed from rat sequences available in GenBank. We determined transcript levels of: cytochrome c oxidase subunits II and IV (COXII and COXIV, respectively); nuclear respiratory factor 1 (NRF-1), a well-described transcriptional modulator regulating expression of several mitochondrial proteins; peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α), a transcriptional coactivator controlling cellular energy metabolic pathways [Wu et al., 1999]; and cardiac-enriched uncoupling proteins 2 and 3 (UCP2 and UCP3, respectively) [Teshima et al., 2003; Himms-Hagen et al., 2001]. Primer and probe sequences for PGC-1α, UCP2, and UCP3 have been published previously [Stavinoha et al., 2004; Lei et al., 2004], while

primer and probe sequences for COXII, COXIV, and NRF-1 are presented in Table 1. Standard RNA was made for all assays by the T7 polymerase method (Ambion, Austin, Texas), using total RNA isolated from rat hearts. The correlation between the C_t (the number of PCR cycles required for the fluorescent signal to reach a detection threshold) and the amount of standard was linear over at least a 5-log range of RNA for all assays (data not shown). Gene expression data are represented as mRNA molecules per ng total RNA.

Table 1: DNA primer sequences for cardiac metabolic genes

Gene	Primer/Probe	Sequence
COXII	Forward	5′-GCCCGAACCAAGCTACAGTC-3′
	Reverse	5′-AGCCGCAAATTTCAGAGCA-3′
	Probe	5′-FAM-CATCAAACCGACTAGGTCTATTCTATGGCCA-TAMRA-3′
COXIV	Forward	5′-TACTTCGGTGTCCTTCGG-3′
	Reverse	5′-CCTTCTGGCTGGCAGACAG-3′
	Probe	5′-FAM-CACATGGGAGTGTTGTGAAGAGTGAAGACTATG-TAMRA-3′
NRF-1	Forward	5′-TCTGCATCTCACCCTCCAAA-3′
	Reverse	5′-CTCCAGGTCTTCCAGGATCAT-3′
	Probe	5′-FAM-TACTTGCGCACCACATTCTCCAAAGG-TAMRA-3′

2.2.5. Citrate synthase activity

Citrate synthase activity was used as a marker for mitochondrial number and was determined according to an established spectrophotometric assay [Srere, 1969]. Isolated RV and LV mitochondria (100 µg) were incubated in 1 ml of a Tris-based buffer (100 mM Tris [pH 7.4], 10 mM acetyl-CoA, 1 mM DTNB [5, 5'–dithiobis-2-nitrobenzoic acid]). The assay is based on the reaction of citrate synthase with oxaloacetate and acetyl-CoA to produce CoASH (coenzyme A). DTNB reacts with sulfhydryls in CoASH producing a free thionitrobenzoate ion [Sheperd and Garland, 1969]. The absorbance of the suspension was measured at 412 nm (25°C). After a baseline setting, 100 µM oxaloacetate was added and measurements taken as before. The difference in absorbance before and after the addition of oxaloacetate was used as a measure of mitochondrial citrate synthase activity.

2.2.6. Mitochondrial DNA determination

Mitochondrial DNA content was measured in the laboratory of Dr. Francisco Garcia-Palmer (Instituto Salud Carlos III, Mallorca) by analyzing pre-treated homogenates using real-time PCR (primers sequences: 5'-TACACGATGAGGCAACCAAA-3'; 5'-GGTAGGGGGTGTGTTGTGAG-3') as described before [Colom et al., 2007]. Data are expressed as mtDNA per gram of heart tissue.

2.2.7. Statistical analysis

For the 2 and 4 week time points, data are presented as the mean ± standard error of mean (SEM). Statistically significant differences between normoxic and hypoxic

groups were calculated using Student t-test. For PPARα activation studies, statistically significant differences between interaction terms and main effects were calculated using the two-way analysis of variance (2-WAY ANOVA) using the SPSS version 15.0 (SPSS Inc., Chicago, Illinois, USA). Statistical significance was considered when $p<0.05$ for all studies.

2.3. RESULTS

A) 2 Week Time Point

2.3.1. Effects of hypoxia on morphometrics and cardiac contractile function

The body weight (BW) was lower in the hypoxic group but this difference did not reach statistical significance (Table 2). Heart weight (HW) was increased following exposure to hypobaric hypoxia (n=6, $p<0.01$ vs. normoxic control).

Table 2: Effects of hypobaric hypoxia on body and heart mass

	Normoxic	Hypoxic
BW (g)	313 ±13	281 ± 5.7
HW (mg)	850 ± 42.8	1,083 ± 30.7*
HW/BW (X1000)	2.71 ± 0.1	3.72 ± 0.1**
RV (mg)	266 ± 33.3	450 ± 34.2*
LV (mg)	583 ± 30.7	633 ± 33.3
RV/LV+S ratio	0.46 ± 0.1	0.78 ± 0.1*
LV+S/BW	1.86 ± 0.1	2.23 ± 0.1

Rats were exposed to hypobaric hypoxia (11% O_2) for 14 days and compared to control animals. BW: body weight, HW/BW: heart weight/body weight, RV/LV+S: right ventricle/ left ventricle + septum ratio, LV+S/BW: left ventricle plus septum/ body weight. Data are presented as mean ± SEM (standard error of the mean) for n=6. *$p<0.01$ and **$p<0.001$ vs. normoxic control.

The increase in RV weight (n=6, $p<0.01$ vs. normoxic control) reflects a robust hypertrophic response in the RV, as indicated by the higher RV/LV + S ratio (n=6, $p<0.01$ vs. normoxic control).

Exposure to chronic hypoxia did not significantly affect the heart rate (Table 3). However, coronary flow was increased in the RV in response to hypobaric hypoxia (n=6, $p<0.05$ vs. normoxic control).

Table 3: Effects of hypobaric hypoxia on cardiac contractile function

RV Cardiac Parameters	**Normoxic**	**Hypoxic**
Coronary Flow (ml/min)	7.22 ± 0.7	12 ± 0.7*
Heart Rate (beats.min^{-1})	250 ± 12.9	270 ± 13.4
Systolic Pressure (mmHg)	41 ± 3.1	59 ± 5.2
Developed Pressure (mmHg)	32 ± 3.6	50 ± 5.2**
Rate Pressure Product (mm Hg. beats.min^{-1})	7,870 ± 537	14,240 ± 1,756**
LV Cardiac Parameters	**Normoxic**	**Hypoxic**
Coronary Flow (ml/min)	10 ± 2.1	12 ± 0.6
Heart Rate (beats.min^{-1})	280 ± 14.1	288 ± 14.4
Systolic Pressure (mmHg)	91 ± 1.7	90 ± 2.7
Developed Pressure (mmHg)	82 ± 4.7	67 ± 6.1
Rate Pressure Product (mm Hg. beats.min^{-1})	19,020 ± 724	19,497 ± 2,215

Rats were exposed to hypobaric hypoxia (11% O_2) for 14 days and compared to control animals. Data are presented as mean ± SEM for n=6 per group. *$p<0.05$ and **$p<0.01$ vs. normoxic control.

Chronic hypoxia increased RV developed pressure (n=6, $p<0.01$ vs. normoxic control) (Table 3), accompanied by an 80 ± 9.9% increase in RV rate pressure product (RPP) (n=6, $p<0.01$ vs. normoxic control). LV functional parameters did not significantly change following exposure to hypobaric hypoxia.

2.3.2. Cardiac mitochondrial respiration

Following hypobaric hypoxia, RV mitochondrial state 3 respiration was increased by 32.9 ± 2.2% (n=6, $p<0.05$ vs. normoxic control) (Fig. 1A). State 3 mitochondrial respiration was not significantly increased in the LV in response to hypobaric hypoxia. The ADP/O ratio was not significantly different in the RV and LV after exposure to hypobaric hypoxia (Fig. 1B). There was no significant difference in the rate of mitochondrial ADP phosphorylation in the RV and LV (Fig. 1C). We next measured state 4 mitochondrial respiration as a marker for mitochondrial uncoupling. Here, we show that hypobaric hypoxia had no effect on state 4 respiration in the RV and LV (Fig. 1D). We further examined mitochondrial proton leak by adding oligomycin (inhibitor of mitochondrial electron transport chain complex V) to state 4 mitochondria. We found decreased proton leak in the RV in response to chronic hypoxia (n=6, $p<0.05$ vs. normoxic control) (Fig. 1E). In contrast, proton leak was not significantly altered in the LV following hypobaric hypoxia. Exposure to chronic hypoxia had no significant effect on the RCI (Fig. 1F).

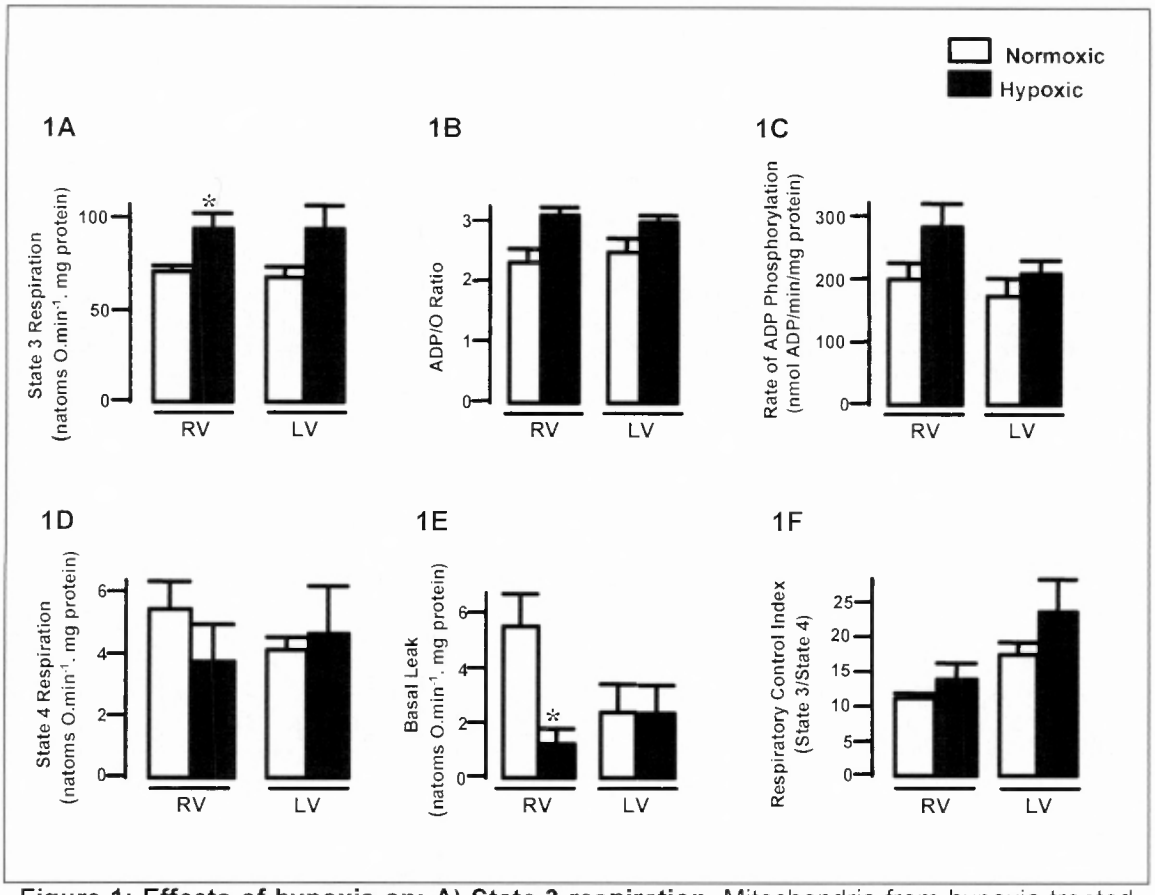

Figure 1: Effects of hypoxia on: A) State 3 respiration. Mitochondria from hypoxia-treated rats were isolated, supplied with 5 mM malate and 40 μM palmitoyl-L-carnitine and compared to age-matched controls. ADP (350 μM) was added to initiate state 3 respiration. **B) ADP/O ratio, C) Rate of ADP phosphorylation, D) State 4 mitochondrial respiration** was measured in the absence of ADP **E) Oligomycin-induced proton leak,** oligomycin (10 μg/ml) was added to state 4 respiring mitochondria to measure oligomycin-induced proton leak, and **F) respiratory control index (RCI).** Data are presented as mean ± standard error of the mean (SEM) for n=6 animals. *p<0.05 vs. normoxic control.

2.3.3. Effects of hypoxia on cardiac metabolic gene expression

Following exposure to chronic hypobaric hypoxia for 2 weeks, COXII transcript levels were increased by 72 ± 10.6% in the RV (n=6, $p<0.05$ vs. normoxic control) (Fig. 2A). RV COXIV transcript levels were not significantly increased while LV COXIV levels were elevated (n=6, $p<0.01$ vs. normoxic control) following exposure to hypobaric hypoxia (Fig. 2B). However, both PGC-1α and NRF-1 transcript levels were significantly increased in the hypertrophied RV (Figs. 2C and 2D). LV NRF-1 transcript levels were not altered for any of the experimental groups. UCP2 levels were significantly induced in the RV and LV following hypoxic exposure (Fig. 2E),

while UCP3 expression was reduced by 47 ± 8.4% in the hypertrophied RV (n=6, $p<0.05$) (Fig. 2F).

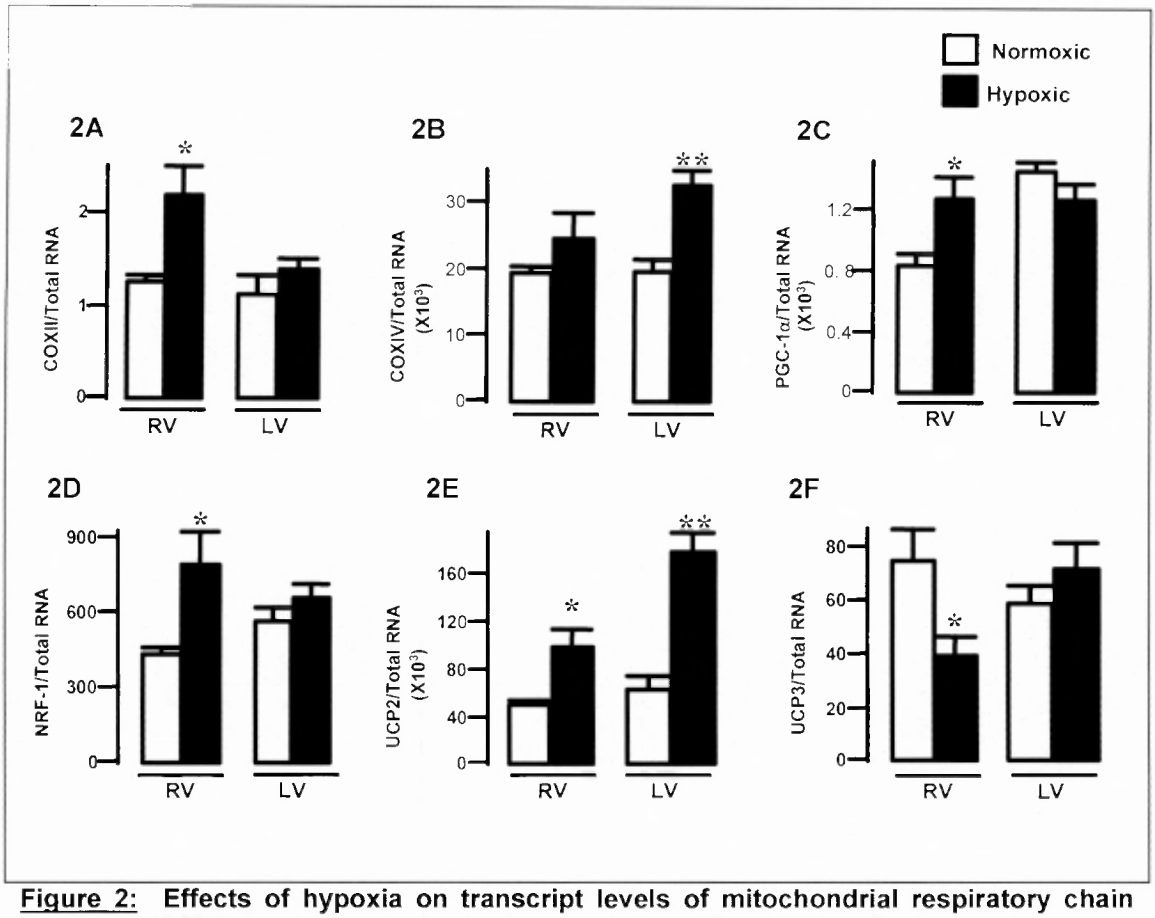

Figure 2: Effects of hypoxia on transcript levels of mitochondrial respiratory chain genes. A) COXII (cytochrome c oxidase subunit II), B) COXIV (cytochrome c oxidase subunit IV), C) PGC-1α (peroxisome proliferator-activated receptor gamma coactivator-1), D) NRF-1 (nuclear respiratory factor 1), E) UCP2 (uncoupling protein 2), and **F) UCP3 (uncoupling protein 3).** The data are presented as mean ± standard error of the mean (SEM) for n=6 animals. *p<0.05 and **p<0.001 vs. normoxic control.

2.3.4. Effects of hypoxia on citrate synthase activity and mtDNA content

We spectrophotometrically measured citrate synthase activity as a marker of mitochondrial content. In agreement with our transcript data, citrate synthase activity was markedly increased in the RV in response to chronic hypoxia (n=6, $p<0.01$ vs. normoxic control) (Fig. 3A). Although LV citrate synthase activity was higher after hypoxia this did not reach statistical significance. In agreement, mtDNA content was

higher in the hypertrophied RV versus control samples (n=4, p<0.05) (Fig. 3B). No significant changes were observed in the LV following the hypoxic exposure.

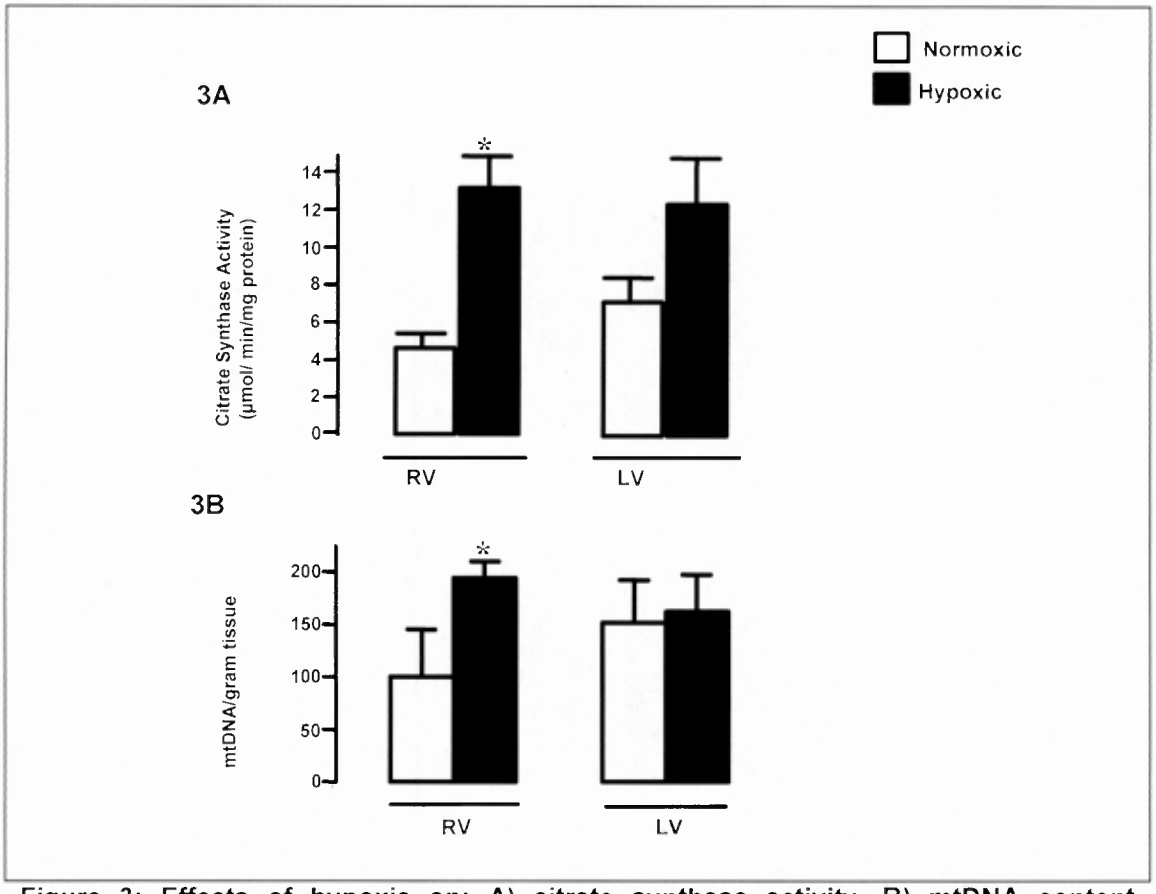

Figure 3: Effects of hypoxia on: A) citrate synthase activity, B) mtDNA content expressed per gram tissue. The data are presented as mean ± standard error of the mean (SEM) n=4 for mtDNA content and n=6 for citrate synthase content. *p<0.01 vs. normoxic control.

B) 4 Week Time Point

In rats exposed to hypobaric hypoxia for 4 weeks, the RV/LV+S ratio increased by 106 ± 17% (n=6, p<0.001 vs. normoxic control). The body weight was reduced by 21 ± 1.4% (n=6, p<0.001 vs. normoxic control). LV+S/BW was not significantly altered following exposure to hypobaric hypoxia for 4 weeks (data not shown).

Table 4: Effects of 4 weeks exposure to hypobaric hypoxia on cardiac contractile function

	Normoxic RV	Hypoxic RV
Coronary Flow (ml/min)	10 ±0.4	14 ±1.1**
Heart Rate (beats.min^{-1})	260±11.5	253±26
Systolic Pressure (mmHg)	55±2.1	66 ±5.1*
Developed Pressure (mmHg)	43±2.1	54 ±5.1*
Rate Pressure Product (beats.min^{-1}. mmHg)	11,257±842	13,993±2,214
	Normoxic LV	**Hypoxic LV**
Coronary Flow (ml/min)	12±0.97	17±1.5*
Heart Rate (beats.min^{-1})	266±12.9	306±21.7
Systolic Pressure (mmHg)	95±5.6	81±8.1*
Developed Pressure (mmHg)	83±5.6	69±8.1*
Rate Pressure Product (beats.min^{-1}. mmHg)	22,395±2,082	20,700±1,517

Rats were exposed to hypobaric hypoxia (11% O_2) for 4 weeks and compared to age-matched normoxic group. Isolated hearts were perfused in the Langendorff mode. Data are presented as mean ± SEM for n=6 animals. *p<0.05 vs. normoxic control; **p<0.01 vs. normoxic control.

Hypobaric hypoxia did not significantly affect the heart rate in any of the ventricles (Table 4). The HR values differ since the measurements were taken while the balloon was inserted in each ventricle separately under both normoxic and hypoxic conditions, respectively. However, coronary flow was significantly increased in the RV (n=6, p<0.01 vs. normoxic control) and LV (n=6, p<0.05 vs. normoxic control) in response to hypobaric hypoxia (Table 4). Chronic hypoxia also enhanced RV systolic

and developed pressures (n=6, $p<0.05$ vs. normoxic control). Conversely, LV systolic and developed pressures were reduced (n=6, $p<0.05$ vs. normoxic control). However, the RPP was not significantly changed in both ventricles following exposure to hypobaric hypoxia (Table 4).

Following 4 weeks hypobaric hypoxia, mitochondrial efficiency (as indicated by ADP/O) was increased by 36 ± 1.3% (n=6, $p<0.01$ vs. normoxic control) in the RV (Table 5). Also, the basal leak was reduced by 78 ± 18% (n=6, $p<0.01$ vs. normoxic control) in the RV. The increases in state 3 respiration, rate of ADP phosphorylation, state 4 respiration and RCI did not reach statistical significance in the RV following exposure to 4 weeks hypobaric hypoxia. In the LV, however, oxygen consumption was decreased by 36 ± 4.9% (n=6, $p<0.05$ vs. normoxic control) and RCI by 67 ± 14% (n=6, $p<0.05$ vs. normoxic control). Furthermore, this was associated with increased proton leak (n=6, $p<0.05$ vs. normoxic control) (Table 5).

Table 5: Effects of 4 weeks exposure to hypobaric hypoxia on mitochondrial respiration

	Normoxic RV	Hypoxic RV
State3/mg protein (nmol O_2/min/mg protein)	57±8.3	65±20.3
ADP/O Ratio	2.04±0.2	2.73±0. 1**
Rate of ADP Phosphorylation (nmol ADP/min/mg protein)	122±20.2	125±32.1
State 4 Respiration (nmol O_2/min/mg protein)	3.04±0.7	4.32±0.9
Basal Leak (nmol O_2/min/mg protein)	12±1.5	2.63±0.6**
RCI (State3/State4)	14±4.3	15±6.5
	Normoxic LV	**Hypoxic LV**
State3/mg protein (nmol O_2/min/mg protein)	118±11.6	75±10.5*
ADP/O Ratio	2.62±0.2	2.54±0.2
Rate of ADP Phosphorylation (nmol ADP/min/mg protein)	231±17.8	186±22.9
State 4 Respiration (nmol O_2/min/mg protein)	6.64±1.6	3.92±0.8
Basal Leak (nmol O_2/min/mg protein)	8.14±1.9	17±4.8*
RCI (State3/State4)	39 ±10	12 ±2.6*

Rats were exposed to hypobaric hypoxia (11% O_2) for 4 weeks and compared to age-matched normoxic groups. Mitochondria from normoxic and hypoxic rats were isolated, supplied with 5 mM malate and 40 µM palmitoyl-L-carnitine and compared to age matched normoxic controls. ADP (350 µM) was added to initiate state 3 respiration. Data are represented as mean ± SEM for n=6 animals. *$p<0.05$ vs. normoxic control; **$p<0.01$ vs. normoxic control.

At the gene level we found that NRF-1, COXII and UCP2 transcript levels were significantly increased in the hypertrophied RV, although PGC-1α was not different compared to controls (Fig. 4). For the LV, PGC-1α transcript levels were reduced by 38 ± 4% (n=6, p<0.001 vs. normoxic control) (Fig. 4B) while UCP2 expression was increased by 54 ± 3.2 % (n=6, p<0.01 vs. normoxic control) (Fig. 4D).

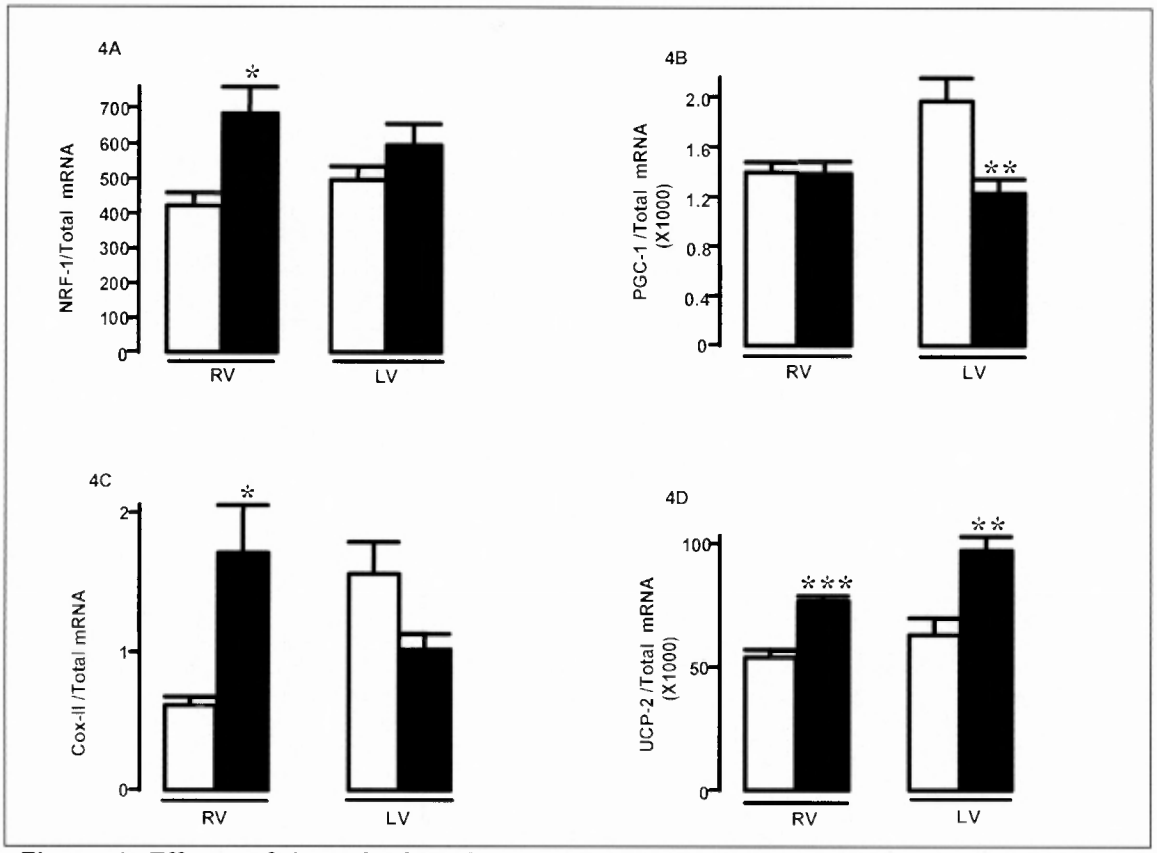

Figure 4: Effects of 4 weeks hypobaric hypoxia on transcript levels of mitochondrial respiratory chain genes. (A) NRF-1, (B) PGC-1α, (C) COXII and **(D) UCP2.** Bars represent mean ± standard error of the mean (SEM) (n=6). *p<0.05 vs. normoxic control **p<0.01 vs. normoxic control; ***p<0.001 vs. normoxic control.

C) PPARα Activation at the 2 Week Time Point

Exposure to chronic hypoxia alone did not significantly alter the heart rate (n=6, p=0.05) in the RV (Fig. 5A). However, heart rate was significantly decreased following hypoxia + Wy-14, 643 treatment (n=6, p<0.05). The coronary flow was increased in the RV in response to vehicle treatment under hypoxic conditions (n=6, p<0.01), remaining at relatively high levels following hypoxia + Wy-14,643 treatment

(n=6, p<0.01) (Fig. 5B). However, the LV coronary flow was increased in the vehicle + hypoxic group (n=6, p<0.05) (Fig. 5B).

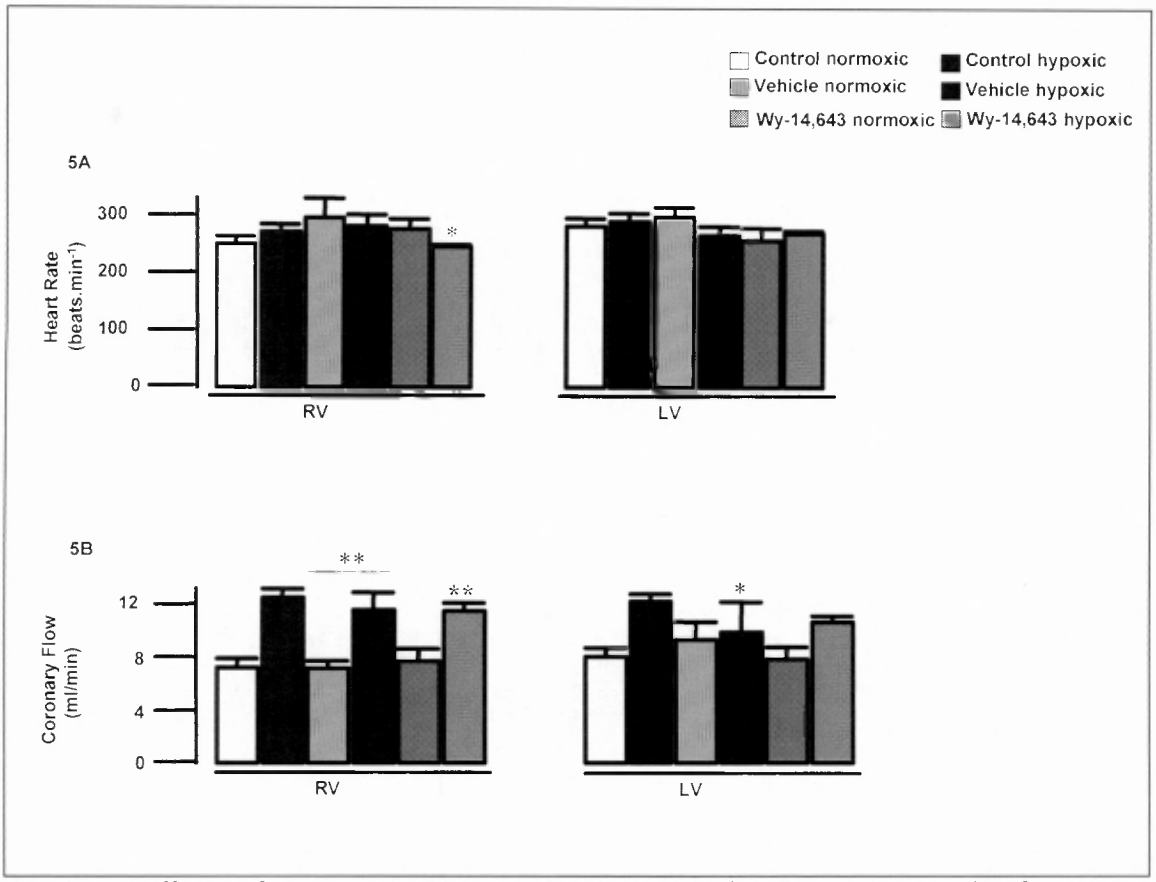

Figure 5: Effects of hypoxia ± Wy-14,643 treatment on: (A) Heart rate, and (B) Coronary flow. Bars represent mean ± standard error of the mean (SEM) for n=6 animals. *p<0.05; **p<0.01.

Vehicle treatment increased RV systolic pressure (n=6, p<0.01) (Fig. 6A), with no significant effect following Wy-14,643 treatment under hypoxic conditions. The developed pressure was not changed for all treatment groups in the RV (Fig. 6B). However, the RV rate pressure product (RPP) was increased following treatment with the vehicle (n=6, p<0.01) (Fig. 6C). Generally, Wy-14,643 treatment had no significant effects on cardiac functional parameters in the RV. LV heart rate was not significantly changed for all experimental groups (Fig. 6A). Interestingly, Wy-14,643 treatment significantly reduced the systolic pressure (n=6, p<0.01), developed pressure (n=6, p<0.01) and rate pressure product (n=6, p<0.05) in the LV (Figs. 6A - 6C).

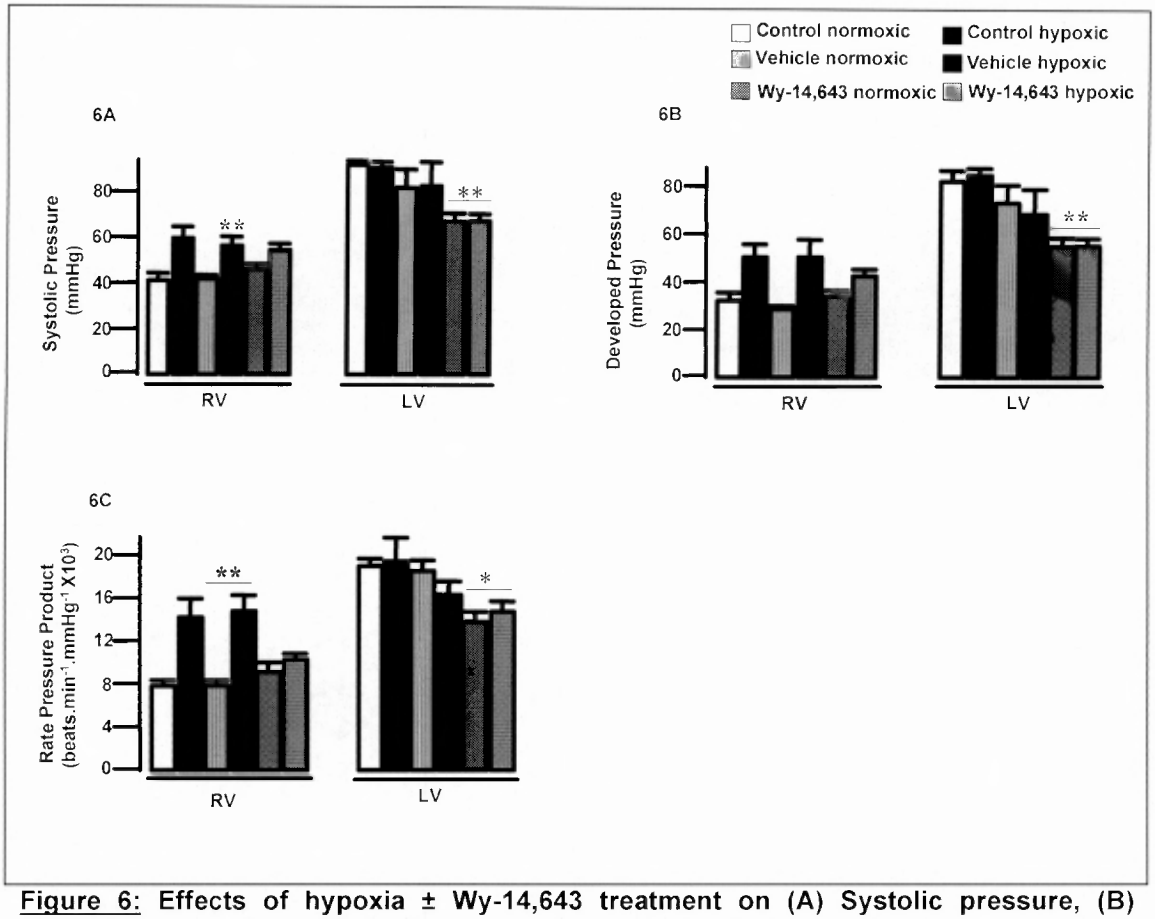

Figure 6: Effects of hypoxia ± Wy-14,643 treatment on (A) Systolic pressure, (B) Developed pressure, and C) Rate pressure product. Bars represent mean ± standard error of the mean (SEM) for n=6 animals. *p<0.05, **p<0.01.

Wy-14,643 treatment reduced RV state 3 respiration (n=6, p<0.01) (Fig. 7A) and rate of ADP phosphorylation (n=6, p<0.01) (Fig. 7C), but had no significant effect on ADP/O ratio (Fig. 7B).

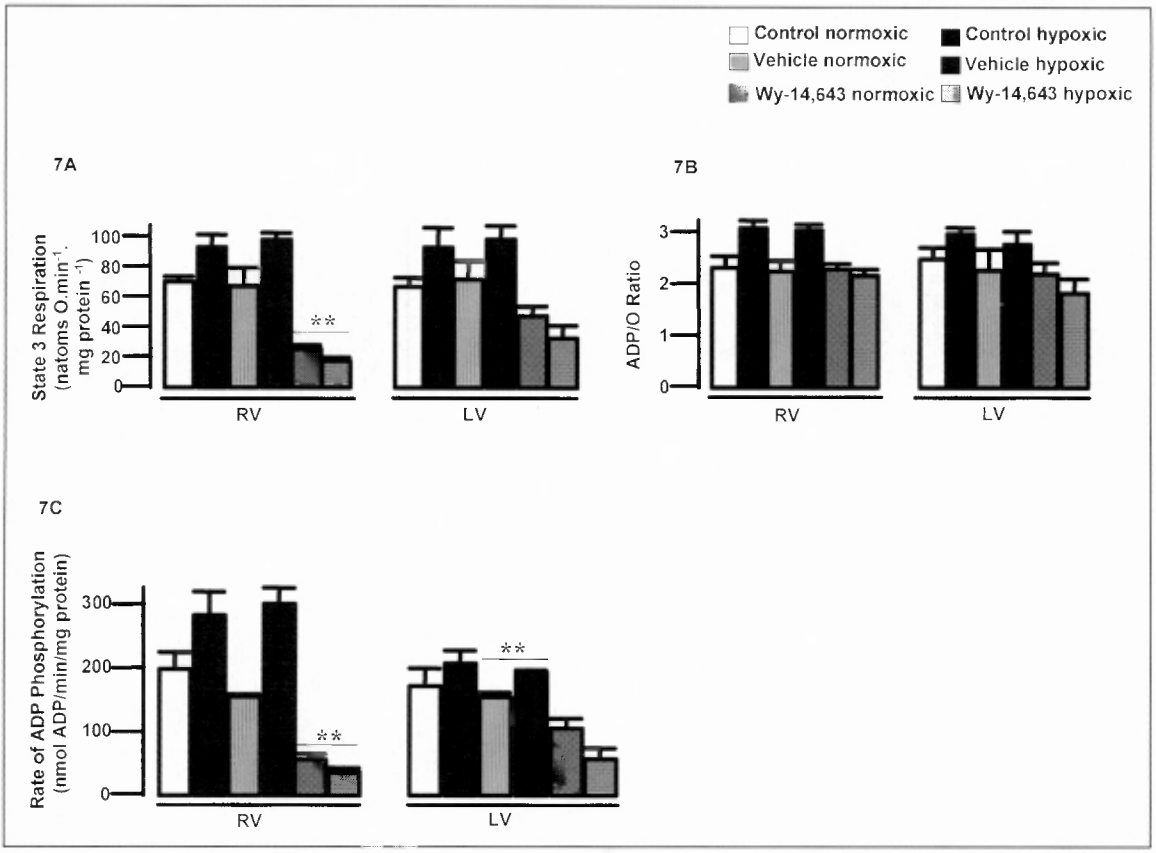

Figure 7: Effects of hypoxia ± Wy-14,643 treatment. Mitochondria from hypoxia ± Wy-14,643-treated rats were isolated, supplied with 5 mM malate and 40 μM palmitoyl-L-carnitine and compared to age matched controls. ADP (350 μM) was added to initiate state 3 respiration. **(A) State 3 mitochondrial respiration, (B) ADP/O ratio, and (C) Rate of ADP phosphorylation.** Bars represent as mean ± standard error of the mean (SEM) for n=6 animals **p<0.01.

State 3 mitochondrial respiration and ADP/O ratio were not significantly altered for all treatment groups in the LV (Figs. 7A, 7B). However, the vehicle treatment significantly increased the rate of ADP phosphorylation in the LV (n=6, $p<0.01$) (Fig. 7C).

We next measured state 4 mitochondrial respiration as a marker for mitochondrial uncoupling. Here, we show that the combination of hypoxia + Wy-14,643 significantly increased state 4 respiration in the RV (n=6, $p<0.01$), whilst in the LV increased state 4 respiration (n=6, $p<0.05$) was due to Wy-14,643 treatment alone (Fig. 8A). We further examined mitochondrial proton leak by adding oligomycin (inhibitor of mitochondrial electron transport chain complex V) to state 4 mitochondria. We found

increased proton leak in the RV (n=6, p<0.01) and LV (n=6, p<0.01) in response to Wy-14,643 treatment (Figs. 8A, 8B).

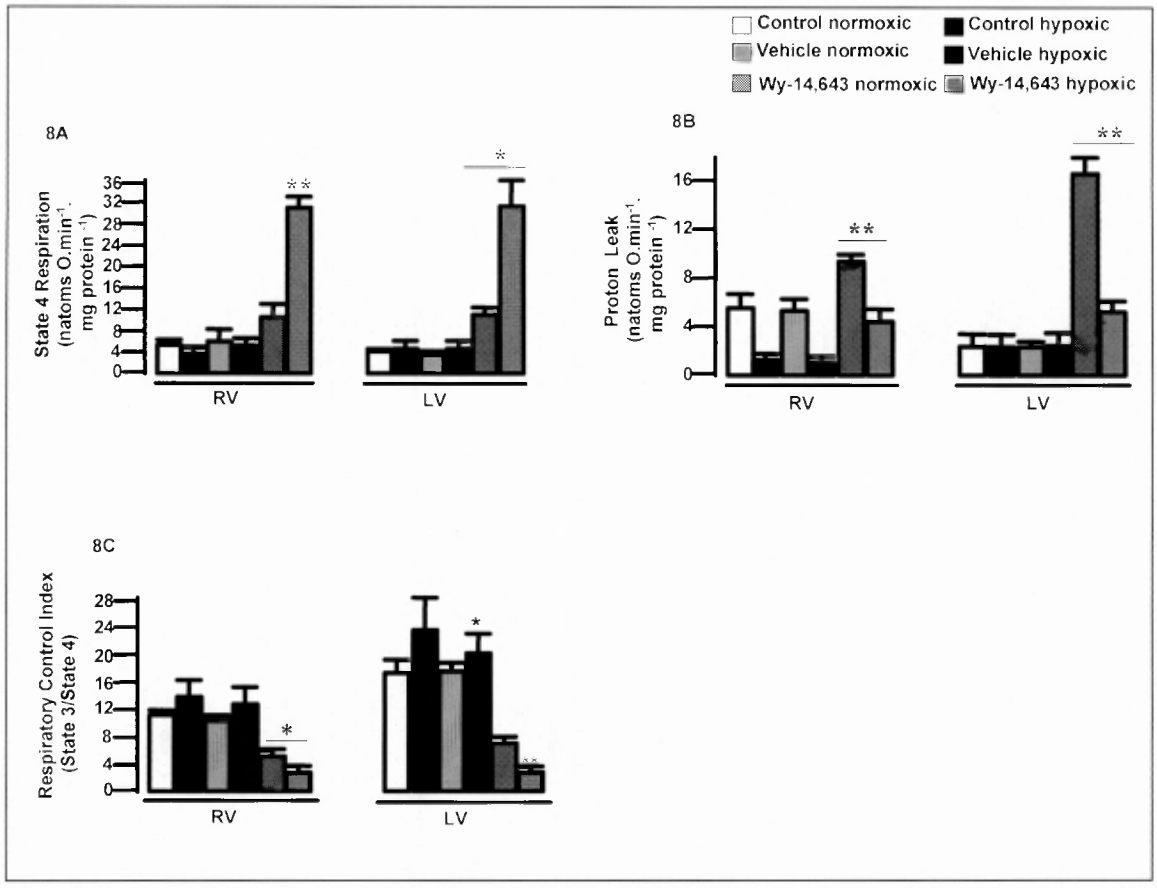

Figure 8: Effects of hypoxia ± Wy-14,643 treatment. Bars represent mean ± standard error of the mean (SEM) for n=6 animals **(A) State 4 mitochondrial respiration.** State 4 respiration was measured in the absence of ADP. Oligomycin (10 µg/ml) was added to state 4 respiring mitochondria. **B) oligomycin-induced proton leak,** and **C) respiratory control index (RCI).** *p<0.05; **p<0.01.

RCI was significantly reduced following treatment with Wy-14,643 in the RV (Fig. 8C). In the LV, vehicle treatment under hypoxia increased the RCI (n=6, p<0.05) but was reduced following Wy-14,643 treatment under hypoxic conditions (n=6, p<0.01) (Fig. 8C).

Following exposure to chronic hypobaric hypoxia for 2 weeks, COXII transcript levels were increased ($n=6$, $p<0.05$) in the RV (Fig. 9A). Likewise, the vehicle treatment also increased COXII levels in the RV ($n=6$, $p<0.05$) (Fig. 9A). COXII transcript levels were not changed following Wy-14,643-treatment, i.e. either alone or in the presence of hypobaric hypoxia in the RV. For the LV, COXII transcript levels were increased in the vehicle + hypoxic group ($n=6$, $p<0.05$). However, this effect was abolished following Wy-14,643 treatment in the presence of hypoxia ($n=6$, $p<0.01$) (Fig. 9A). COXIV levels were not significantly changed in all treatment groups in the RV (Fig. 9B). However, COXIV levels were increased following exposure to hypobaric hypoxia ($n=6$, $p<0.01$) and vehicle treatment ($n=6$, $p<0.05$) in the LV (Fig. 9B). These effects were abolished following Wy-14,643 treatment ($n=6$, $p<0.01$). PGC-1α transcript levels were increased following exposure to hypobaric hypoxia and vehicle treatment ($n=6$, $p<0.05$), respectively, in the RV (Fig. 9C). PGC-1α transcript levels were further increased following Wy-14,643 treatment ($n=6$, $p<0.01$) in the RV (Fig. 9C). However, PGC-1α levels were reduced in the LV following exposure to hypobaric hypoxia ($n=6$, $p<0.01$) and Wy-14,643 treatment ($n=6$, $p<0.01$), respectively.

NRF-1 transcript levels were significantly increased in response to exposure to hypobaric hypoxia in the RV ($n=6$, $p<0.01$) (Fig. 9D). However, this effect was significantly reduced following treatment with Wy-14,643 ($n=6$, $p<0.05$). LV NRF-1 transcript levels were not altered for any of the experimental groups (Fig. 9D).

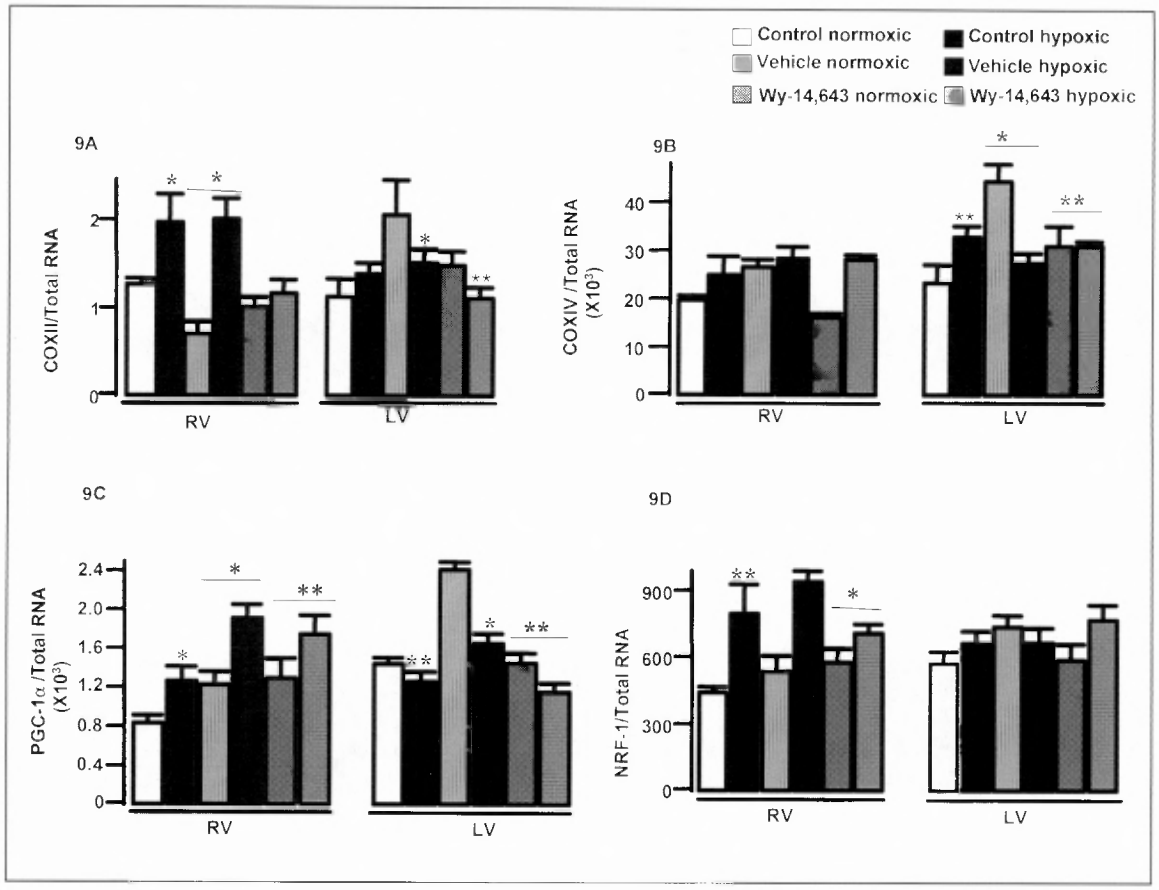

Figure 9: Effects of hypoxia ± Wy-14,643 treatment on transcript levels of mitochondrial respiratory chain genes. Bars represent mean ± standard error of the mean (SEM) for n=6 animals. **A) COXII (cytochrome c oxidase subunit II), B) COXIV (cytochrome c oxidase subunit IV), C) PGC-1α (peroxisome proliferator-activated receptor gamma coactivator-1 α),** and **D) NRF-1 (nuclear respiratory factor 1)**.*p<0.05; **p<0.01

PDK4 transcript levels were significantly increased following a combination of Wy-14,643 treatment and hypobaric hypoxic exposure (n=6, p<0.01) in both ventricles (Fig. 10A). MCAD levels were not significantly altered in the RV in all experimental groups (Fig. 10B). However, in the LV the combination of vehicle treatment and hypobaric hypoxic exposure increased MCAD levels (n=6, p<0.01) (Fig. 10B). Wy-14,643 treatment in combination with hypobaric hypoxic exposure markedly induced expression UCP3 levels in the RV (n=6, p<0.05) (Fig. 10C). However, in the LV exposure to hypobaric hypoxia increased UCP3 levels (n=6, p<0.05), an affect which was abolished following vehicle treatment under hypoxic conditions (n=6, p<0.05) (Fig. 10C). PPARα transcript levels were not significantly altered in all experimental groups in the RV (Fig. 10D). However, LV PPARα transcript levels were reduced

following exposure to hypobaric hypoxia (n=6, p<0.05) (Fig. 10D). Wy-14,643 treatment had no significant effect on PPARα transcript levels in both ventricles (Fig. 10D).

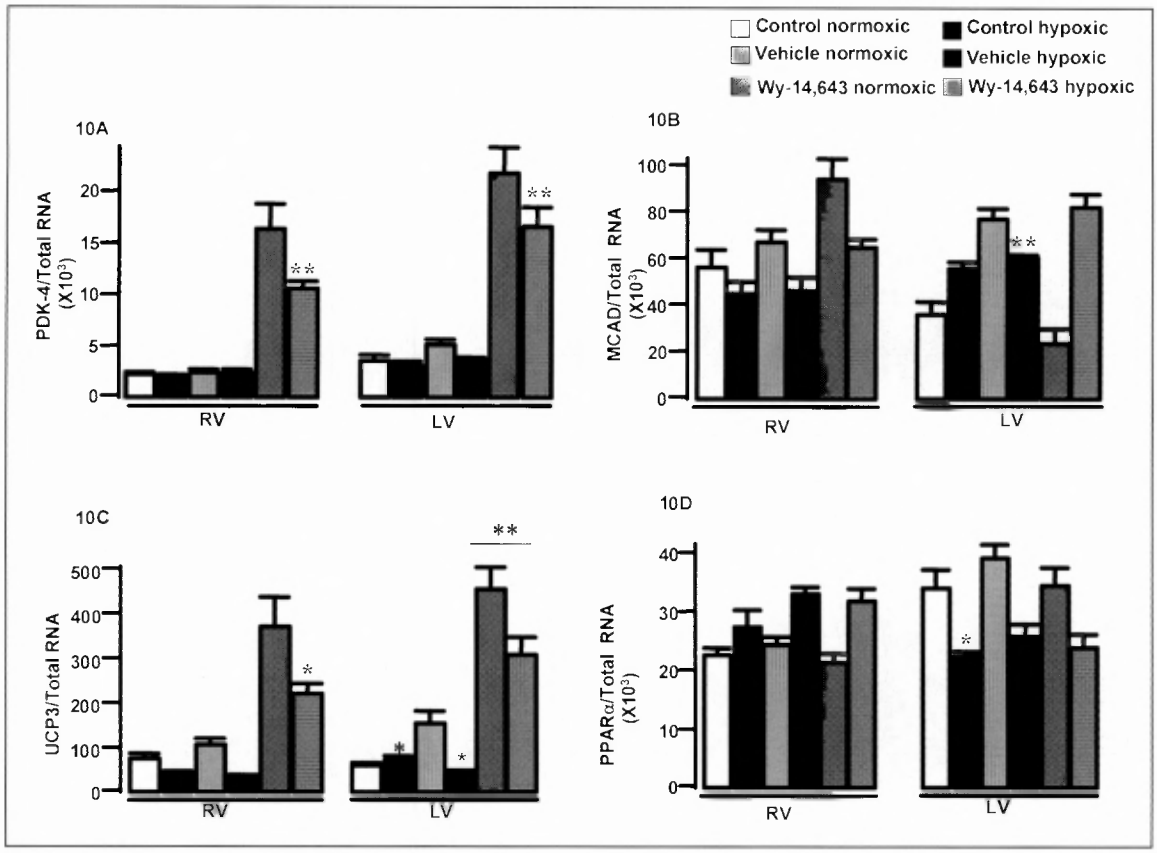

Figure 10: Effects of hypoxia ± Wy-14,643 treatment on transcript levels of PPARα-regulated genes. Bars represent mean ± standard error of the mean (SEM) for n=6 animals **A) PDK-4 (pyruvate dehydrogenase kinase-4)**, **B) MCAD (medium-chain acyl-CoA dehydrogenase)**, **C) UCP3 (uncoupling protein 3)**, and **D) PPARα (peroxisome proliferator activator receptor α)** *p<0.05; **p<0.01

UCP2 levels were upregulated in the RV following hypobaric hypoxic exposure (n=6, p<0.01) (Fig. 11A). For the LV, UCP2 levels were increased following exposure to hypobaric hypoxia (n=6, p<0.01) and vehicle treatment (n=6, p<0.01) (Fig. 11B). Wy-14,643 treatment had no significant effect on UCP2 transcript levels in both ventricles (Fig. 11A).

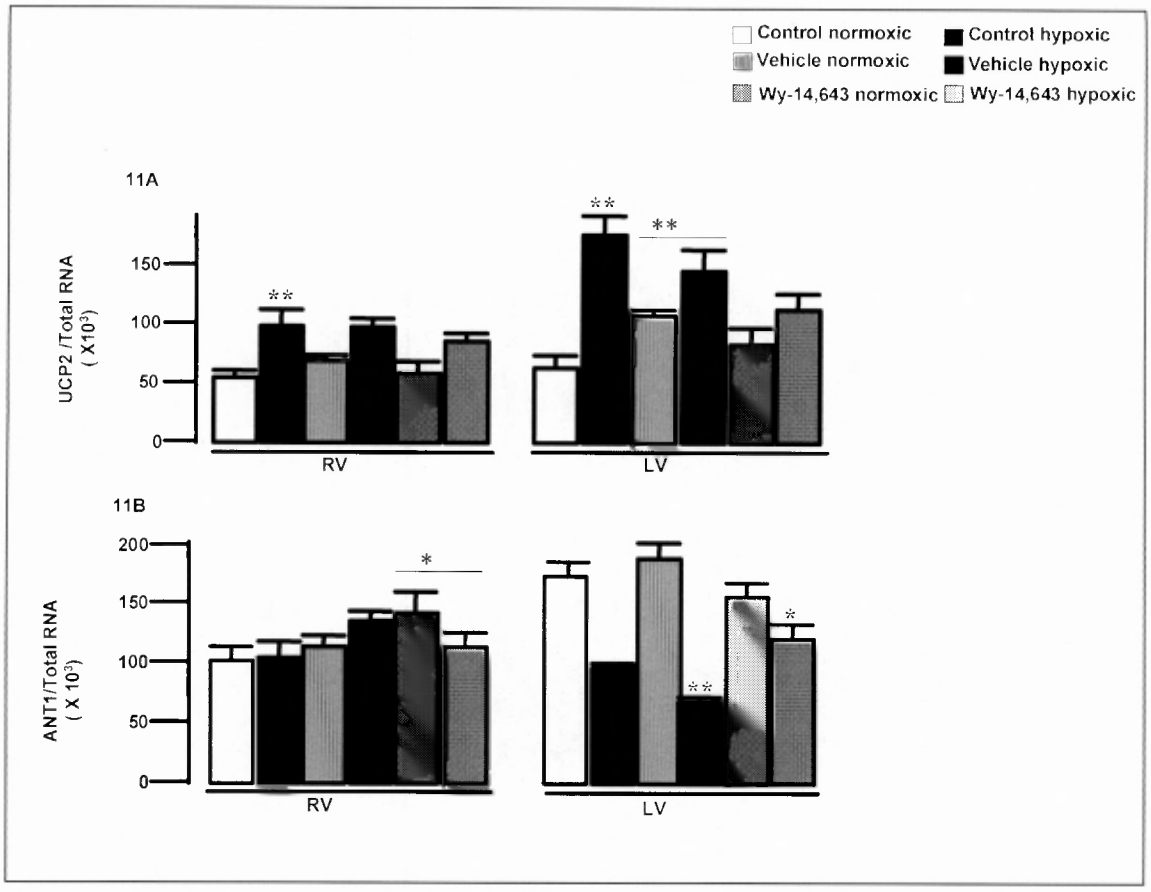

Figure 11: Effects of hypoxia ± Wy-14,643 treatment on transcript levels of mitochondrial membrane carrier proteins. Bars represent mean ± standard error of the mean (SEM) for n=6 animals. **A) UCP2 (uncoupling protein 2), B) ANT1 (adenine nucleotide translocator).** *p<0.05; **p<0.01

ANT1 transcript levels remained unchanged in the RV following exposure to hypobaric hypoxia alone (Fig. 11B). However, Wy-14,643 treatment significantly increased ANT1 levels in the RV (n=6, $p<0.05$). In the LV ANT1 transcript levels were reduced following combination of hypobaric hypoxia and vehicle treatment (n=6, $p<0.01$), and was increased following treatment with Wy-14,643 under hypoxic conditions (n=6, $p<0.05$) (Fig. 11B).

DISCUSSION

We hypothesized that mitochondrial regulatory genes are induced in response to hypoxia-mediated RV hypertrophy as part of an adaptive response to sustain mitochondrial respiratory capacity and contractile function in response to increased load. To test our hypothesis, we exposed rats to 2 weeks of hypobaric hypoxia and determined cardiac contractile and mitochondrial respiratory function for the right and left ventricles, respectively. The main finding of this study is the coordinate induction of several genes regulating mitochondrial function and increased mtDNA content in the hypertrophied RV, linking the efficiency of mitochondrial oxidative phosphorylation and enhanced respiratory function to increased RV contractile function. Coordinate induction of genes regulating mitochondrial function and increased mtDNA content in hypertrophied right ventricle is associated with enhanced respiratory and contractile function.

Identification of a novel gene program in the hypertrophied right ventricle linking enhanced mitochondrial respiratory function to increased cardiac contractile function.

Exposure to chronic hypobaric hypoxia results in pulmonary hypertension and increased RV load. Our data demonstrate a robust hypertrophic response in the RV in response to chronic hypobaric hypoxia. These data are in agreement with our earlier findings showing increased myocyte diameter size and ANF expression after 2 weeks of hypobaric hypoxia [Sharma et al., 2004]. Moreover, we also reported a lack of fibrosis, suggesting adaptive physiologic remodeling in the RV in response to pulmonary vasoconstriction. Since the LV is not challenged by increased load in this

instance, higher hematocrit levels and/or neuroendocrine regulation may be key factors influencing adaptation.

Our respiration and gene expression studies show distinct remodeling in the right and left ventricles in response to the hypoxic stimulus. For example, at the 2-week time point we found that RV mitochondrial oxygen consumption was increased with chronic hypoxia. However, this returned to normoxic levels at the 4-week time point. These data are in agreement with previous studies reporting sustained respiratory capacity in the RV in response to chronic hypobaric hypoxia [Rumsey et al., 1999; Nouette-Gaulain et al., 2005]. Moreover, others have shown increased and/or sustained rates of ATP synthesis in response to chronic hypoxia [Reynafarje and Marticorena, 2005; Costa et al., 1997; Costa et al., 1988]. We also found that exposure to hypobaric hypoxia improved the efficiency of mitochondrial respiration (↓ proton leak) in the hypertrophied RV at both 2- and 4- week time points. In parallel, transcript levels of NRF-1, PGC-1α, COX II and UCP2 were coordinately upregulated while UCP3 was downregulated in the RV. Likewise, expression of these genes remained elevated in the RV (except PGC-1α) after 4 weeks of hypoxic exposure.

COX is the terminal enzyme of the mitochondrial electron transport chain catalyzing the transfer of electrons from cytochrome c to molecular oxygen. Its subunits are encoded by both nuclear (e.g. COXIV) and mitochondrial genomes (e.g. COXII) [Grossman et al., 1998]. NRF-1 and PGC-1α are central transcriptional regulators of mitochondrial biogenesis and are good candidate factors for further investigation [Vega et al., 2000]. Interestingly, a recent study suggested that cyclic-nucleotide regulatory element binding protein (CREB) may be a pivotal transcriptional modulator that regulates physiological hypertrophy by enhancing expression of genes important for efficient oxidative capacity and resistance to apoptosis [Watson et al., 2007].

Moreover, CREB is proposed to mediate such effects via control of PGC-1 expression and subsequent mitochondrial biogenesis [Wu et al., 2006]. In addition, other transcriptional modulators such as PGC-1β, NRF-2, Sp1, AP1, YY1 and TFAM are also likely candidates that may play a role in the induction of this gene program [reviewed in Scarpulla, 2006; Goffart and Wiesner, 2003].

Exposure to 2 weeks of chronic hypobaric hypoxia had no significant effects on LV mitochondrial respiration or contractile function. However, mitochondrial oxygen consumption, RCI and basal leak were all reduced in the LV after 4 weeks exposure. This was associated with lowered systolic and developed pressures. A possible reason for the modest blunting of left ventricular mitochondrial function at the 4 week time point may purely be in response to diminished energy demand. It is possible that the modest attenuated function at 4 weeks may be in response to incomplete adaptation to the increased pulmonary pressure with reduced left sided contractile demand and therefore diminished supply. Nouette-Gaulain et al. (2005) demonstrated reduced mitochondrial electron transport chain enzyme activities in the LV following 14 days of chronic hypoxia. Furthermore, Rumsey et al. (1999) have shown that oxidation of pyruvate and glutamate was diminished in LV as early as 7 days of hypoxic exposure in a 10% O_2 atmosphere. Adaptation of enzymes involved in cardiac energetics, total creatine kinase activity and mitochondrial creatine kinase were also reduced in LV of rats exposed for 4 weeks to a simulated altitude of 5500m [Pissarek et al., 1997]. These data suggest that decrease in oxidative capacity is an early adaptation mechanism to chronic hypoxia, at least in the LV. Oxidative capacity of both ventricles was decreased following 21 days of chronic hypoxia. At the gene level, LV COXIV, MCAD and UCP2 expression were increased while PGC-1α levels were unaltered after 2 weeks. A similar gene profile was observed at the 4-week time point, except that LV PGC-1 α transcript levels were significantly reduced. Since

the LV is not exposed to increased load in our experimental model, we propose that these genes are regulated by hypoxia-mediated transcriptional mechanisms that may result in a lesser degree of mitochondrial biogenesis

Focusing on the hypertrophied RV, our data suggest that the adaptive mitochondrial phenotype observed is largely mediated via load-dependent mechanisms. In support, we found significantly increased citrate synthase activity and mtDNA content only in the hypertrophied RV. As the trophic response results in a larger cell volume, mitochondria must proliferate to ensure an adequate supply of mitochondrial energy required for cell maintenance and contractile purposes. In agreement, increased mitochondrial numbers have been found in hypertrophic hearts [reviewed in Goffart et al., 2004]. However, it has been proposed that an imbalance between inadequate mitochondrial proliferation and increased energy demands (due to high workloads) may contribute to the onset of pathologic hypertrophy. Here, we suggest that the hypertrophied RV adapts by increasing the expression of mitochondrial proteins leading to enhanced respiratory capacity and more efficient mitochondrial energy production.

What are the mechanisms responsible for increased efficiency of mitochondrial ATP production? We found reduced UCP3 expression in the hypertrophied RV thus providing a potential mechanism whereby proton leak may be reduced. However, further studies are required to confirm this observation since it has also been proposed that UCP3 does not necessarily act as a classic uncoupler of oxidative phosphorylation in the heart [Himms-Hagen J and Harper, 2001]. Recently, it was reported that exposure to hypobaric hypoxia resulted in reduced opening of the mitochondrial permeability transition pore (MPTP) [Zhu et al., 2006]. Since opening of the MPTP will lead to the dissipation of the electrochemical proton gradient across the inner mitochondrial membrane, we propose that increased MPTP closure could

also potentially contribute to improved efficiency of mitochondrial ATP production in the hypertrophied RV. In agreement with such an adaptive mitochondrial phenotype, we found a hypoxia-mediated induction of UCP2 transcript levels in the RV. Previous studies suggest that UCP2 is more likely to be part of a defense mechanism against damaging ROS instead of a true uncoupler of mitochondrial oxidative phosphorylation in the heart [Teshima et al., 2003; Horimoto et al., 2004].

What are the broader implications of the findings of these findings? Since most changes were observed only in the hypertrophied RV (and not the LV) we believe our data may be extended to physiologic hypertrophied hearts in general. Our data are in agreement with views expressed in a recent review article (Finck and Kelly, 2007] where it is proposed that physiologic hypertrophy is coupled with increased PGC-1α expression and greater mitochondrial oxidative capacity. Furthermore, the authors suggested that pathologic hypertrophy is associated with reduced PGC-1α and dysfunctional mitochondria. These interesting possibilities require further investigation.

In summary, the first part of this chapter demonstrates that the hypertrophied RV induces expression of several mitochondrial regulatory genes that are associated with increased respiratory function and contractility. On the other hand, the LV displayed a progressive decline in respiratory function and cardiac contractility. In addition, LV PGC-1α transcript levels were reduced at the 4-week point supporting the argument that enhanced mitochondrial respiratory function better sustains cardiac output in the hypertrophied RV compared to the hypoxic LV. In light of this, I propose that increased mitochondrial biogenesis and respiratory function plays an important role to sustain contractile function of the hypertrophied heart.

Chronic Wy-14,643 administration uncouples cardiac mitochondrial respiration.

Since we proposed that increased respiratory function is an adaptive response to sustain cardiac contractile function in the hypertrophied RV, we next tested whether PPARα activation (via Wy-14,643 administration) would result in a maladaptive phenotype. Here we proposed that PPARα activation (and subsequent increased fatty acid utilization) in the hypertrophic context would result in decreased efficiency of mitochondrial respiration, thereby leading to impaired contractile function.

The major finding of the second part of this study is that in vivo Wy-14,643 administration elicited direct effects on mitochondrial respiratory function and cardiac contractility. The data show that Wy-14,643 treatment attenuated mitochondrial respiration in the RV and the LV while promoting uncoupling of mitochondrial oxidative phosphorylation in the LV. Previously, it was reported that Wy-14,643 uncouples mitochondrial respiration in isolated hepatic mitochondria [Keller et al., 1992]. Our laboratory has shown that acute and direct administration of Wy-14,643 impairs mitochondrial respiration and promote uncoupling of oxidative phosphorylation [Zungu et al., 2006]. It has been reported that mitochondrial proton leak may occur through a number of mechanisms: (i) via uncoupling proteins (UCPs) [Brand et al., 1994; Stuart et al., 2001], (ii) inhibiting adenine nucleotide translocator via allosteric stimulation by adenine monophosphate [Cadenas et al., 2000], (iii) trans-membrane cycling of protonated/unprotonated free fatty acids [Garlid et al., 2000; Jezek et al., 2004], and (iv) inducing permeability transition pore opening [Crompton, 1999; Broekemeier et al., 1998; Huser and Blatter, 1999]. For example, a previous study on isolated rat liver mitochondria reported that Wy-14,643 induces permeability transition while fenofibrate directly inhibits mitochondrial respiration [Zhou and Wallace, 1999]. For the first time as far as I am aware, we have shown

that chronic in vivo Wy-14,643 administration uncouples and impairs respiration in the rodent heart.

Wy-14,643 elicited direct effects on LV respiration. Since the LV does not undergo an adaptive hypertrophic response during exposure to chronic hypoxia, one can speculate that it may be more susceptible to the direct effects of Wy-14,643. Our findings therefore show that care must be taken when evaluating cardiac functional data generated by in vivo Wy-14,643 administration since these effects may not occur as a result of higher FAO but instead due to uncoupling of mitochondrial oxidative phosphorylation.

Because of direct effects of vehicle on mitochondrial respiratory function it is difficult to analyze the gene data and to make meaningful conclusions. The combination of Wy-14,643 and hypoxia resulted in the coordinate induction of PPARα-target genes (PDK-4 and UCP3) in the RV. Moreover, Wy-14,643 treatment increased PGC-1α, NRF-1, and ANT1 levels in the right ventricle. However, this was associated with impaired respiratory capacity. The Wy-14,643-hypoxia combination increased transcript levels of PDK-4, ANT1 and decreased COXII in the LV. Also, LV, COXIV and UCP3 levels were increased, whereas PGC-1α levels were decreased in response to Wy-14,643 treatment. Reduced PGC-1α was associated with uncoupling and impaired LV function. However, as mentioned before it is difficult to make any reasonable deductions from these findings.

Limitations: There are few limitations in this study that need to be addressed. Firstly, we did not measure citrate synthase and mtDNA content at the 4 week time point or in the vehicle/ Wy-14,643 treatment groups due to limited number of animals and therefore tissue availability. Secondly, the gene data was not accompanied by protein

analysis. This would have elucidated the mechanism of mitochondrial regulatory gene expression in response to chronic hypobaric hypoxia. Lastly, in the basal studies i.e. normoxia versus hypoxia, we did not employ pyruvate and or glutamate and malate as substrates to feed the mitochondrial electron transport chain. This would have demonstrated whether glucose oxidation or composite electron transport chain flux contributed to the energy supply supporting improved contractile function. We are currently in the process of planning these experiments to further help our understanding of these findings.

In summary, in the first part of this study we found coordinate induction of several genes regulating mitochondrial function and mitochondrial content in a model of physiologic RV hypertrophy, linking the efficiency of mitochondrial oxidative phosphorylation and respiratory function to sustained RV contractile function in response to increased load. For the second part of this study we increased PPARα activation by administering Wy-14,643 in our model of hypoxia-induced right ventricular hypertrophy. Here, we found that chronic in vivo Wy-14,643 treatment elicited direct effects on mitochondrial function (in the LV and RV) and cardiac contractility (in the LV). These data suggest that Wy-14,643 (at the doses employed) may not be the ideal experimental agent to use for in vivo studies aiming to increase myocardial fatty acid oxidation rates.

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Chapter 3

Chronic PPARα Activation Impairs Contractile Function of the Hypertrophied Heart by Increased Uncoupling of Mitochondrial Oxidative Phosphorylation

3.1. INTRODUCTION

Chronic exposure to hypoxia is characterized by selective pressure overload on the right ventricle (RV) and subsequent development of RV hypertrophy due to pulmonary hypertension. However, unlike the RV, the left ventricle (LV) does not hypertrophy since it is not exposed to increased load during hypoxia. Previous studies have reported altered cardiac energy metabolism in response to cardiac hypertrophy. Here, the hypertrophied heart is characterized by a switch away from fatty acids to increased glucose utilization, thus a resemblance of fetal fuel substrate utilization [Taegtmeyer et al., 1998; Sambandam et al., 2002; Barger and Kelly, 1999; Razeghi et al., 2001]. For example, studies have shown that fatty acid oxidation rates may be ~30-40% lower in hypertrophied than in normal hearts [El Alaoui-Talibi et al., 1992, 1997; Allard et al., 1994; Schonekess et al.,1995]. Since myocardial capillaries of the hypertrophied heart are exposed to some degree of hypoxia, it is proposed that such a switch forms part of an adaptive remodeling process whereby higher myocardial glucose utilization decreases oxygen consumption per mole of ATP generated.

At the transcriptional level, previous studies have demonstrated that expression of peroxisome proliferator-activated receptor alpha (PPARα), a pivotal transcriptional modulator of numerous cardiac fatty acid metabolism genes, is downregulated during pressure overload-induced cardiac hypertrophy [Sack et al., 1997; Barger et al., 2000]. Furthermore, this was associated with the coordinate reduction of fatty acid metabolic gene expression, including muscle-type carnitine palmitoyl transferase-1 (mCPT-1), medium-chain acyl-CoA dehydrogenase (MCAD), and acyl-CoA oxidase (ACO) [Barger et al., 2000]. These data therefore suggest an essential role for PPARα downregulation in lowering fatty acid utilization during cardiac hypertrophy.

Moreover, reduced PPARα activity and fatty acid metabolism are proposed to be an adaptive response to prevent the progression to pathological hypertrophy and heart failure [Huss and Kelly, 2004]. In agreement, Young et al. (2001) found that PPARα activation in the hypertrophied heart resulted in impaired contractile function.

Fatty acids [Huss and Kelly, 2004; Stanley et al., 2005] and fenofibrates [Devchand et al., 1996; Forman et al., 1996; Kliewer et al., 1997] are well-known activators of PPARα. Moreover, fatty acids are known uncouplers of mitochondrial oxidative phosphorylation, resulting in diminished bioenergetic capacity [Essop and Opie, 2004]. In light of this, we investigated our hypothesis i.e. that elevated myocardial fatty acid utilization by the hypertrophied heart uncouples mitochondrial respiration, thereby resulting in reduced contractile function. We investigated our hypothesis in a rat model of hypoxia-induced RV hypertrophy. Rats were exposed rats to 12 weeks of hypobaric hypoxia (11% O_2) with two major aims: 1) To investigate whether adaptive cardiac changes we earlier observed (i.e. after 2 and 4 weeks) in the hypertrophied RV are sustained at a later time point (12 weeks), and 2) To increase cardiac fatty acid utilization in the hypertrophied heart using two approaches i) enhancing fatty acid delivery by chronic supply of a high fat diet (cocoa butter), and ii) activating PPARα using fenofibrate as a ligand.

3.2. MATERIALS AND METHODS

3.2.1. Animals

Six-week old male Wistar rats (weighing 190-230 g) were initially housed at room temperature on a 12-hour reverse light/dark cycle (lights off at 4 am, zeitgeber time (ZT12); lights on at 4 pm (ZT0), with access to a conventional laboratory diet and

water *ad libitum*. After one week of acclimatization, rats were initially exposed to hypobaric hypoxia (45 kPa – 11% O_2) for 10 weeks and compared with age-matched normoxic groups. In the hypoxic models, animals were immediately subjected to 11% O_2 and this is based on the previous studies used in our laboratory [Essop et al., 2004, Sharma et al., 2004]. At the 10 week time point, rats were divided into 4 experimental treatment groups and exposed to a further 2 weeks of hypobaric hypoxia: 1) vehicle (1% Tween-80 and 0.5% hydroxypropylmethyl-cellulose); 2) cocoa butter; 3) fenofibrate; and 4) fenofibrate ± cocoa butter. The predominant fatty acids in cocoa butter are saturated, namely, stearic (18:0, 35%), palmitic (16:0, 25%), and monounsaturated oleic (18:1, 35%), with the remaining fat being primarily polyunsaturated linoleic (3%) [Bracco,1994]. All agents were administered by oral gavage. Fenofibrate (Sigma, St. Loius, MO) was administered at a dose of 150 $mg.kg^{-1}.day^{-1}$ as previously described [Morgan et al., 2005]. During oral gavage the opning of the chamber was minimized to less than 5 minutes at a time. The volume for vehicle and cocoa butter was 250 µl, respectively. All rats were sacrificed at ZT16-ZT18 since they are most metabolically active during this period [Young, 2003]. The hypoxic chamber was opened for not more than 10 minutes three times weekly for animal feeding and cleaning of the cages. At the end of the treatment, animals were anesthetized with sodium pentobarbital (100 $mg.kg^{-1}$ i.p.) whereupon hearts were isolated and perfused for functional assessment. For mitochondrial respiratory functional studies, the RV was carefully separated from the left ventricle plus the inter-ventricular septum (LV+S). The University of Cape Town's Animal Research Ethics Committee approved all animal experiments and the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

3.2.2. Cardiac Mitochondrial Isolation and Functional Characterization

Mitochondria were isolated according to the method of Sordahl et al. (1971) with modifications. Briefly, RV and LV tissue were separately homogenized in 10 ml ice-cold potassium-EDTA (KE) buffer (0.18 M KCl, 10 mM EDTA, [pH7.4]), whereafter the homogenate was centrifuged at 755 *x g* for 5 min. The supernatant was subsequently filtered through 41 µm nylon mesh (Spectrum, USA) and the filtrate centrifuged at 1,480 *x g* for 5 min. The mitochondrial pellet was resuspended in 50 µl KE buffer, and was subsequently used for mitochondrial respiration measurements.

Respiratory rates were polarographically measured using a Clark-type electrode (Hansatech Instruments, London, UK) at 25°C with constant stirring as previously described [Essop et al., 2004] with modifications. Our preliminary studies showed that conducting mitochondrial assays at temperatures above 25°C strongly inhibited mitochondrial respiration and we found 25°C to be the optimum temperature (data not shown). Furthermore, mitochondrial respiration studies have been conducted at 25°C in our laboratory [Essop et al., 2004, Zungu et al., 2006] as well as others [St-Pierre et al., 2000, Starnes et al., 2007, Fuller et al., 1985]. Briefly, isolated rat ventricular mitochondria (0.5 mg/mL) were added to the electrode chamber containing incubation medium (10 mM Tris-HCl, 0.25 M sucrose, 8.5 mM KH_2PO_4, [pH7.4]). We employed a mixture of 5 mM malate and 40 µM palmitoyl-L-carnitine as oxidative substrates. State 3 respiration was determined by measuring mitochondrial oxygen uptake after the addition of ADP to a final concentration of 350 µM. State 4 respiration was determined by measuring mitochondrial oxygen uptake upon complete phosphorylation of ADP to ATP. Basal proton leak in the isolated mitochondria was determined by measuring the rate of mitochondrial respiration after addition of 10 µg/ml oligomycin to state 4 mitochondria.

The rate of ADP phosphorylation was calculated as nanomoles of ADP phosphorylated per minute during state 3 respiration as described before [Babsky et al., 2001]. All mitochondrial polarographic studies were normalized to total mitochondrial protein content, determined using the Lowry assay [Lowry, 1951].

3.2.3. Determination of Right and Left Ventricular Developed Pressures

Isolated hearts were perfused in the Langendorff mode with ice-cold Krebs-Henseleit buffer (11 mM Glucose, 118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 1.8 mM $CaCl_2.6H_2O$, pH 7.4). The aorta was located and cannulated on the Langendorff perfusion rig, and a retrograde perfusion of the coronary arteries via the aorta was immediately initiated. The perfusion was performed with oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit buffer at a constant pressure (104 cm H_2O) and temperature (37°C). During perfusion, a latex balloon attached to a pressure transducer was first inserted into the left ventricular cavity for stabilization and inflated to produce a diastolic pressure of 4-12 mmHg, whereupon the balloon was then inserted into the right ventricle in order to determine the right ventricular developed pressure (RVDP) as described before [Thurich, 1999]. The RV and LV functional parameters were measured and included heart rate, systolic and diastolic pressure, developed pressure, total coronary flow and rate pressure product (heart rate x developed pressure). The duration of perfusion protocol was 15 minutes fro each ventricle.

3.2.4. Statistical Analysis

Data are presented as the mean ± standard error of mean (SEM). Statistically significant differences between interaction terms and the main effects were calculated using the two-way analysis of variance (2-WAY ANOVA) using the SPSS version 15.09 SPSS Inc., Chicago, Illinois, USA). Statistical significance was considered when $p<0.05$.

3.3. RESULTS

Exposure to chronic hypoxia reduced body weight (BW) gain (n=6; $p<0.01$), which remained unaltered in the treated groups (data not shown). We initially measured cardiac contractile function in our model. Coronary flow was not significantly altered in the RV in response to hypobaric hypoxia (Fig. 1A). However, it was increased in the LV following exposure to hypobaric hypoxia and cocoa butter-fenofibrate treatment (n=6, $p<0.05$). The heart rate was not significantly altered for any of the experimental groups (Fig. 1B).

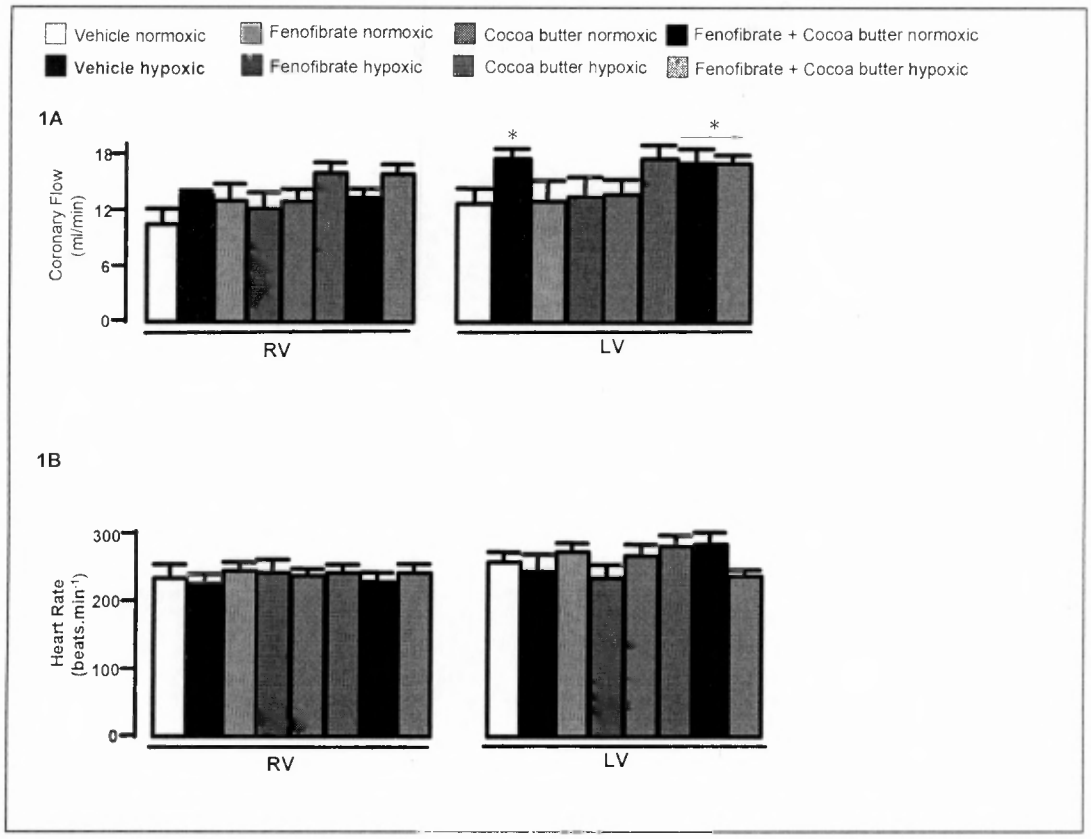

Figure 1: Effects of hypoxia, PPARα activation and high fat diet on: A) coronary flow, and B) heart rate. Isolated hearts were perfused in the Langendorff mode. Bars represent mean ± standard error of the mean (SEM) for n=6 animals.*p<0.05.

Right ventricular systolic pressure, developed pressure, and rate pressure product were all increased in response to 12 weeks of hypobaric hypoxia before the treatments (n=6, p<0.01) (Figs. 2A-2C). Although RV contractile parameters remained increased (n=6, p<0.01) following administration of fenofibrate under hypoxic conditions, this occurred to a much lesser extent compared to the hypoxic control. Coco-butter administration under hypoxic conditions also increased RV contractile functional parameters, similar to hypoxic controls (Figs. 2A-2C). However, when fenofibrate was jointly administered with coco-butter under these conditions, this lead to a reduction in RV contractile function (n=6, p<0.01).

As for the RV, the LV displayed enhanced systolic pressure, diastolic pressure (n=6, p<0.01) and rate pressure product (n=6, p<0.05) after 12 weeks of hypobaric hypoxia

before the treatments (Figs. 2A-2C). However, both fenofibrate and coco-butter administration resulted in direct effects on LV function i.e. under normoxic conditions fenobitrate and cocoa butter individual treatments reduced LV function (n=6, $p<0.01$), whereas the increase under hypoxic conditions did not reach vehicle hypoxic levels. Also, the hypoxia-mediated increase in systolic and developed pressures was attenuated following treatment with the coco butter-fenofibrate regimen under hypoxic conditions (n=6, $p<0.01$) (Figs. 2A, 2B).

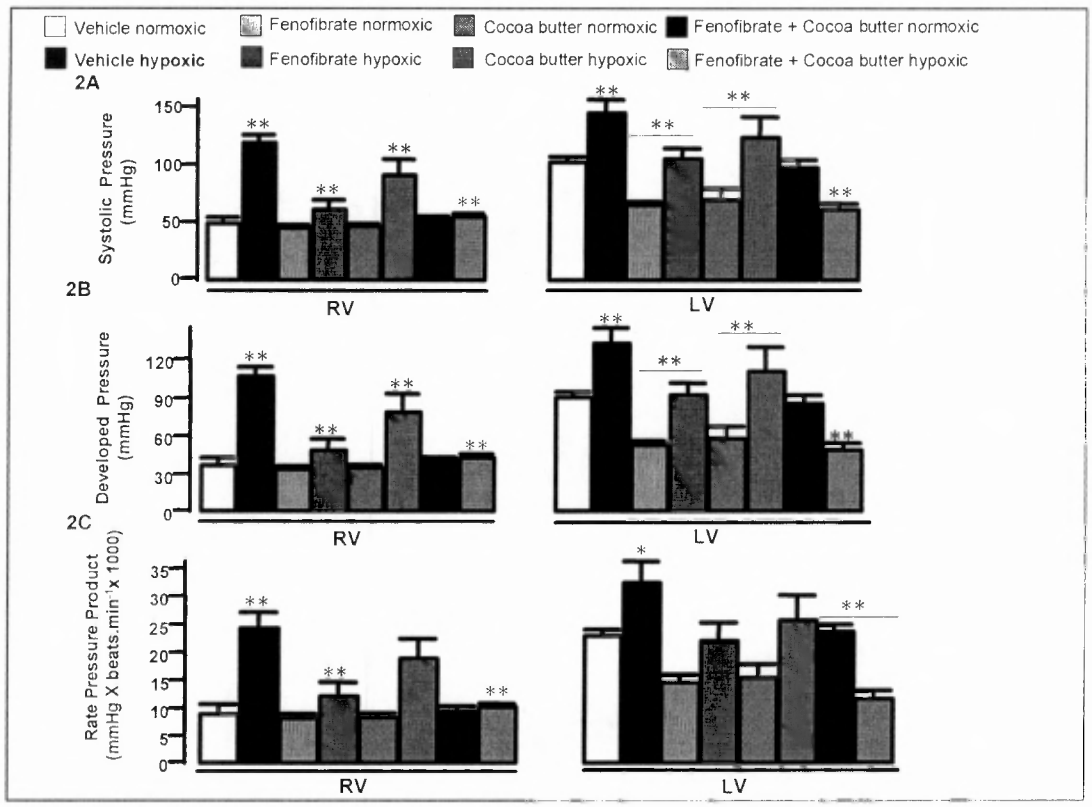

Figure 2: Effects of hypoxia, PPARα activation and high fat diet on: A) Systolic pressure. Isolated hearts were perfused in the Langendorff mode **B) Developed pressure,** and **C) Rate pressure product.** Bars represent mean ± standard error of the mean (SEM) for n=6 animals. $^{*}p<0.05$; $^{**}p<0.01$.

We next determined state 3 respiration as a measure of mitochondrial oxygen consumption. Here, 12 weeks exposure to hypobaric hypoxia reduced both RV (n=6, $p< 0.05$) and LV (n=6, $p<0.01$) state 3 mitochondrial respiration (Fig. 3A). The ADP/O

ratios did not significantly change following hypoxia. However, the ADP phosphorylation rate was reduced in both ventricles (n=6, p<0.01) (Figs. 3B, 3C).

Fenofibrate administration resulted in direct effects on state 3 respiration, i.e. it increased state 3 respiration under hypoxic conditions compared to vehicle hypoxic value (n=6,p<0.05) and reduced state 3 respiration under normoxic conditions compared to vehicle normoxic value (n=6,p<0.05) (Fig. 3A). Moreover, addition of coco-butter to fenofibrate under these circumstances elicited similar effects. Unfortunately, these direct effects make it very difficult to analyze LV respiration data generated in this study. On the other hand, fenofibrate administration did not result in direct effects on RV respiratory function (Figs. 3A-3C).

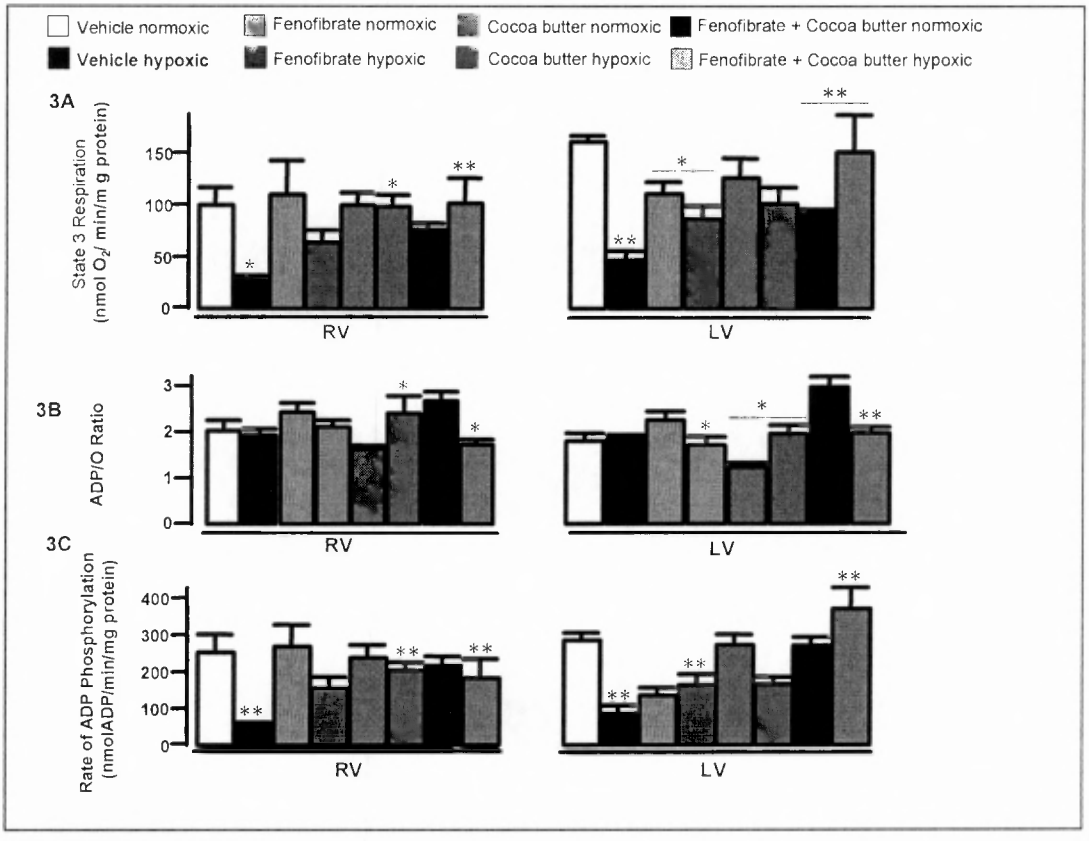

Figure 3: Effects of hypoxia, high fat diet and PPARα activation on: A) State 3 respiration: Mitochondria from hypoxic group ± treatment regiments rats were isolated, supplied with 5 mM malate and 40 μM palmitoyl-L-carnitine and compared to age-matched controls. ADP (350 μM) was added to initiate state 3 respiration **B) ADP/O ratio, and C) Rate of ADP phosphorylation.** Bars represent mean ± standard error of mean (SEM) for n=6 animals. *p<0.05; **p<0.01.

Administration of coco-butter under hypoxic conditions reversed the hypoxia-mediated attenuation of state 3 respiration in the RV ($n=6$, $p<0.05$). A similar result was obtained after cocoa butter-fenofibrate-hypoxia treatment ($n=6$, $p<0.01$). Cocoa butter administration increased the ADP/O ratio in the RV under hypoxic conditions ($n=6$, $p<0.05$). However, this effect was abolished for the cocoa butter-fenofibrate treatment group under hypoxic conditions ($n=6$, $p<0.05$) (Fig. 3B). The rate of ADP phosphorylation largely followed this pattern. Here, the hypoxia-mediated decline in rate of ADP phosphorylation was reversed following treatment with cocoa butter ($n=6$, $p<0.01$) and cocoa butter-fenofibrate treatment under similar conditions ($n=6$, $p<0.01$) (Fig. 3C). Coco-butter treatment under normoxic and hypoxic conditions had direct effects on LV ADP/O ratio ($n=6$, $p<0.05$) (Fig. 3B). In the LV, the hypoxia-mediated decline in rate of ADP phosphorylation was reversed following treatment with fenofibrate under similar conditions ($n=6$, $p<0.01$) and cocoa butter-fenofibrate-hypoxia treatment ($n=6$, $p<0.01$) (Fig. 3C).

We also assessed state 4 respiration and oligomycin-insensitive proton leak (referred to as basal leak) as indicators of uncoupling of mitochondrial oxidative phosphorylation. Neither state 4 respiration nor basal leak were significantly altered in any of the ventricles in response to hypobaric hypoxia (Figs. 4A, 4B). However, state 4 respiration was increased in the LV under normoxic and hypoxic conditions following fenofibrate treatment ($n=6$, $p<0.01$) (Fig. 4A). In agreement, fenofibrate increased mitochondrial basal leak under the same conditions ($n=6$; $p<0.05$) (Fig. 4B). Interestingly, coco-butter administration under hypoxic conditions reduced state 4 respiration in the LV ($n=6$, $p<0.01$).

For the RV, fenofibrate did not significantly alter state 4 respiration under normoxic or hypoxic conditions. However, it did increase basal proton leak under these

conditions (n=6, p<0.05). RV state 4 respiration and proton leak were decreased in the cocoa butter-hypoxic group (n=6, p<0.01). It was not, however, significantly altered in other experimental groups in the RV (Fig. 4A).

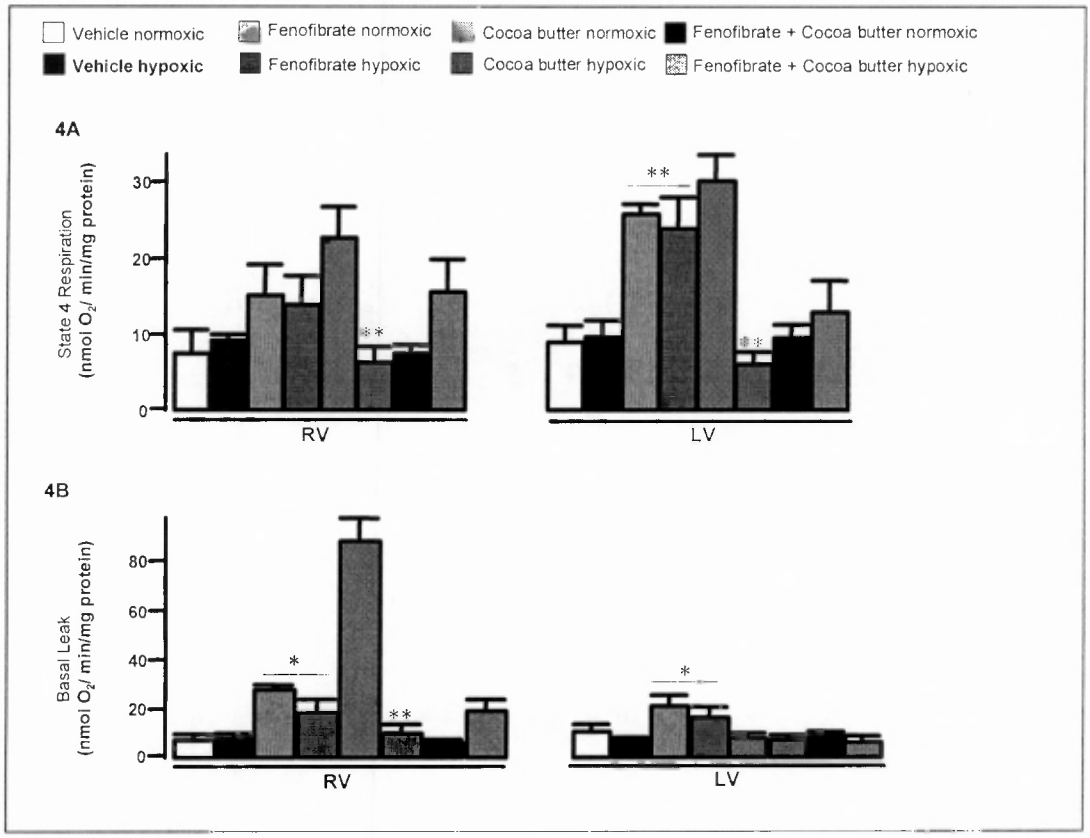

Figure 4: Effects of hypoxia ± treatment regiments on: A) State 4 mitochondrial respiration. State 4 mitochondrial respiration was measured in the absence of ADP, and **B) Oligomycin-induced proton leak.** Oligomycin (10 µg/ml) was added to state 4 respiring mitochondria. Bars represent mean ± standard error of the mean (SEM) for n=6 animals. *p<0.05; **p<0.01.

3.4. DISCUSSION

The major findings of this study are: *1) Increased RV and LV contractile function in response to 12 weeks of hypobaric hypoxia, associated with lowered mitochondrial respiratory functional capacity, and 2) Fenofibrate administration results in more pronounced effects on contractile and respiratory function than cocoa butter treatment*

1) Sustained RV and LV contractile function in response to 12 weeks of hypobaric hypoxia, associated with lowered mitochondrial respiratory functional capacity

Relatively few studies [Rumsey et al., 1999; Nouette-Gaulain et al., 2002, 2005; Py et al., 2005] have examined cardiac function and metabolism in response to chronic hypobaric hypoxia [La Padula and Costa; 2005]. Exposure to chronic hypobaric hypoxia is characterized by a selective pressure overload on the RV and the subsequent development of a robust hypertrophic response due to pulmonary hypertension. Conversely, the LV is not exposed to pressure overload and does not hypertrophy. In this thesis, I found increased RV and LV systolic and developed pressure in response to 2 and 4 weeks of hypobaric hypoxia (Chapter 2). The current data therefore demonstrate that cardiac contractile function is increased (in both RV and LV) at later time points, i.e. 12 weeks. Furthermore, we found that LV contractile function is now increased versus the earlier time points (2/4 weeks) (Chapter 2). We are not precisely clear regarding the underlying reasons for the latter increase, but propose that this may be due to increased volume overload i.e. higher hematocrit levels previously reported [Adrogue et al., 2005]. Our data, especially for the RV, are in agreement with longer term studies. For example, we (Chapter 2) and also Thurich et al. (1999) reported increased RVDP in rats exposed to 11 % oxygen

for a 4 week period, while Rumsey et al. (1999) demonstrated increased RVSP in rats exposed to 6 weeks normobaric hypoxia (10% O_2, 50 kPa). Together, these data show that both RV and LV contractile function is enhanced in response to moderate chronic hypobaric hypoxia. Since different stimuli i.e. hypertrophy and hypoxia *per se* are influencing the RV and LV, respectively, it is likely that regulatory mechanisms mediating RV and LV contractile function are of a distinct nature. These possibilities are the subject of ongoing research work in our laboratory.

Exposure to 12 weeks hypobaric hypoxia reduced body weight gain. It has been demonstrated that exposure to high altitude is associated with the decrease in food intake [Barrie and Harris, 1976; Singh and Selvamurthy, 1993; Schwartz et al., 1998]. To gain further insight into regulatory mechanisms underlying these observations, we also measured mitochondrial respiratory function. Unlike the 2- and 4-week time points (Chapter 2), we found that exposure to 12 weeks of hypobaric hypoxia resulted in diminished palmitoyl-L-carnitine oxidation and ADP phosphorylation rates (both ventricles). These data highlight the temporal regulation of mitochondrial respiration in response to chronic hypobaric hypoxia. For example, we found sustained respiratory function and increased mitochondrial efficiency in the RV at the 2-week point (Chapter 2). Also, at the earlier time points we found that the LV was characterized by reduced oxygen consumption and increased basal leak. Thus, it appears as if, initially, the hypertrophied RV better adapts (at the mitochondrial level) to the chronic stimulus compared to the LV. However, with increased hypoxic exposure both ventricles display similar respiratory capacity. Likewise, others found fluctuations in mitochondrial respiratory function in response to hypobaric hypoxia. For example, Nouette-Gaulain et al. (2002) reported reduced rates of ATP synthesis and oxygen consumption in the LV, with no significant changes in RV in response to 2 weeks exposure of hypobaric hypoxia. Furthermore, they later reported lower mitochondrial rates of ATP synthesis and oxygen consumption in both ventricles

following 2 weeks of chronic hypoxia, returning to normoxic values after 3 weeks [Nouette-Gaulain et al., 2005].

What is the significance of this decline in mitochondrial respiratory function? I am not completely clear but am of the opinion that two possibilities require further investigation. In the first instance, this may be an adaptive response to slow down respiratory function, i.e. as a defensive measure. For example, Casey et al. (2002) showed that cardiomyocytes exposed to moderate hypoxia markedly reduced rates of mitochondrial oxygen consumption and energy production. Moreover, they reported reduced rates of mitochondrial energy production. The authors proposed this to be an adaptive measure to conserve intracellular energy and thus prolonging the cell's survival.

Alternatively, this reduction may be a prelude to detrimental effects that will only later manifest itself at the heart's functional level. Additional longer-term studies are required to investigate this possibility. Another contributory factor to attenuated mitochondrial respiration with chronic hypoxia may be a reduction in mitochondrial mass. Indeed, studies have shown that exposure of rats to hypoxia for 21 days [Nouette-Gaulain et al., 2005], 9 -11 months [Costa et al., 1988], and to normobaric hypoxia [Cervos-Navarro et al., 1999] resulted in reduced mitochondrial mass in the heart. Furthermore, decreased mitochondrial mass was observed in fetal sheep hearts exposed to hypobaric hypoxia for 100 days [Lewis et al., 1999]. However, we found an induction of mitochondrial biogenesis genes at the 2-week time point (Chapter 2). Differences in published results may depend on the severity and duration of the hypoxic stimulus. It is therefore possible that the mitochondrial biogenesis gene program we observed earlier, especially (in the RV), may be attenuated at the 12-week point. Unfortunately, we could not investigate this possibility due to limited tissue availability.

2) Fenofibrate administration results in more pronounced effects on contractile and respiratory function than cocoa butter treatment

Cardiac hypertrophy or hypoxia is associated with downregulation of PPARα expression, thought to be an adaptive process [Barger et al., 2000; Razeghi et al., 2001] by which the heart can switch from fatty acid to glucose utilization. Downregulation of PPARα may be required to maintain contractile function of the hypertrophied heart. For example, activation of PPARα in the setting of pressure-overload induced by aortic banding [Young et al., 2001], and during an ischemic insult [Dewald et al., 2005] resulted in cardiac contractile dysfunction. Furthermore, mice with cardiac-specific overexpression of PPARα exhibit signs of diabetic cardiomyopathy, including ventricular hypertrophy and dysfunction [Finck et al., 2002]. The fuel substrate switch away from fatty acids is proposed to be beneficial for the heart by decreasing cardiac oxygen consumption [Barger and Kelly, 2000].

Previous work in our model showed that GLUT4 expression is downregulated, whereas GLUT1 expression is maintained following exposure to 12 weeks of hypobaric hypoxia [Adrogue et al., 2005]. Thus, these data reflect a reversal to a fetal gene expression profile. Of note, no alterations were observed in fatty acid enzyme-encoding gene expression [Adrogue et al., 2005]. It is therefore possible that increased glucose utilization may be the reason for the reduced oxidation of palmitoyl-L-carnitine in RV/ LV mitochondria we observe at the 12-week point (discussed earlier). In light of this, we here employed fenofibrate and coco-butter as agents to increase myocardial fatty acid utilization in the hypertrophied heart, and subsequently assessed its effects on RV and LV contractile and respiratory function.

In this study, we found that sole treatments with fenofibrate and cocoa butter elicited more direct effects on the LV compared to the RV. In the RV, the hypoxia-mediated increase in systolic pressure, developed pressure and RPP was reduced following fenofibrate administration under hypoxic conditions. In contrast, RV systolic and developed pressures remained relatively high following cocoa butter treatment. The cocoa butter-fenofibrate combination under hypoxic conditions largely reduced cardiac contractile function in both ventricles.

Increased reliance on fatty acid oxidation for ATP production pushes up mitochondrial oxygen consumption due to high mitochondrial oxidative flux compared to glycolysis and glucose oxidation. In agreement, we found higher mitochondrial oxygen consumption following administration of coco-butter and cocoa butter-fenofibrate treatment in the hypertrophied RV, thus reversing the hypoxia-mediated reduction at 12 weeks. Likewise, Rennison et al. (2007), showed increased mitochondrial state 3 respiration which was associated with greater activities of complexes II and IV of the electron transport chain in failing rat hearts fed a high-saturated fat diet. Coco-butter treatment under hypoxic conditions also increased ADP/O ratio and the rate of ADP phosphorylation in the RV. For the LV, however, fenofibrate and cocoa butter treatments resulted in direct effects on oxygen consumption under normoxic and hypoxic conditions. Again, this makes interpretation of LV respiration data very difficult. However, fenofibrate-coco-butter administration under hypoxic conditions reduced both systolic and developed pressure in the LV. Together these data show that coco-butter, unlike fenofibrate administration, elicited a lesser reduction in contractile function in the hypertrophied RV, suggesting some degree of adaptive remodeling.

Fatty acids and PPARα agonists are able to uncouple mitochondrial oxidative phosphorylation [Keller et al., 1992, 1993; Essop and Opie, 2004]. We found that

fenofibrate increased uncoupling (basal leak) in both ventricles, albeit to a lesser extent in the RV. This is in agreement with our previous work showing that treatment with another PPARα agonist, Wy-14,643, in the setting of hypobaric hypoxia increased uncoupling in the LV (Chapter 2). However, the Wy-14, 643 direct effects were more severe (Chapter 2) than fenofibrate effects shown here. Our laboratory and others have demonstrated that fibrates can exert direct effects on cardiac mitochondrial respiration independent of PPARα action [Zungu et al., 2006; Keller et al., 1992, 1993; Zhou and Wallace, 1999]. Why the differences between Wy-14, 643 and fenofibrate effects in our studies? We propose that since Wy-14,643 was administered from the onset of the 2-week hypobaric hypoxic exposure period this may have resulted in the more severe and direct effects earlier discussed (refer Chapter 2). However, for the 12-week study, we first exposed rats to low oxygen levels for 10 weeks before administering the PPARα agonist for an additional 2 weeks.

Our data therefore show distinct effects of coco-butter and fenofibrate on cardiac and respiratory function in the RV and LV following hypoxic exposure. How does one explain these differences? I propose several reasons. It is likely that fenofibrate may have more direct uncoupling effects (i.e. not necessarily via increased fatty acid oxidation) in the LV since it is the major ventricle of the heart and therefore more exposed to circulating fenofibrate molecules than the RV. Secondly, cocoa butter is rich in saturated fatty acids [Bracco, 1994], and current evidence suggest that saturated and monounsaturated fatty acids have a lower affinity for PPARα compared to long-chain polyunsaturated fatty acids and eicosanoids [Forman et al., 1996]. Therefore, one may expect that coco-butter administration may result in less activation of fatty acid oxidation compared to fenofibrate. This may be beneficial since greater fatty acid oxidation under hypoxic conditions is less energetically

favorable versus glucose utilization. Also, lower fatty acid oxidation under these conditions may mean less reactive oxygen species production and attenuated opening of the mitochondrial permeability transition pore (MPTP). Previous work has shown that the MPTP is found in a more closed configuration in response to hypobaric hypoxia [Zhu et al., 2006], therefore meaning that there would be lowered proton leak and apoptosis. One could therefore speculate that high fat supply may limit MPTP opening to a greater extent when compared to fenofibrate, particularly in the hypertrophied RV. This interesting question requires further investigation.

Our data are in agreement with recent studies employing high fat diets in the setting of cardiac hypertrophy. For example, Okere et al. (2005) showed that administration of a high saturated fat diet in a model of hypertension-induced cardiomyopathy reduced LV hypertrophy, improved contractile function, and prevented LV dilation despite elevated plasma FFAs and myocardial triglycerides. Moreover, they also reported that feeding Wistar rats with high-fat diets for 8 weeks [Okere et al., 2007], or for more than 2 months [Okere et al., 2006a] did not adversely affect the cardiac function. Likewise, a high-fat diet in Dahl salt-sensitive rats attenuated cardiac hypertrophy, LV chamber remodeling, systolic dysfunction and induction of molecular markers of cardiac hypertrophy and dysfunction [Okere et al., 2006b]. Together these data suggest that high fat diets may trigger signaling pathways that may be crucial for prevention of LV remodeling and heart failure in response to pressure overload. However, we cannot exclude the possibility that the administered dose of cocoa butter was not enough to elicit a response in cardiac function and respiration. This question is being further investigated.

In contrast, it has recently been shown that prolonged administration of a PPARα agonist (fenofibrate) induced the fatty acid metabolic pathway, causing left ventricular hypertrophy without affecting cardiac contractile dysfunction [Morgan et al., 2006].

This study also demonstrated that high fat feeding increased cardiac triglyceride stores without exacerbating LV dysfunction or remodeling. Furthermore, they reported that fenofibrate, and not the high fat diet, induced upregulation of fatty acid metabolic pathways and transcription of PPARα–regulated genes. In addition, Labinskyy et al. (2007) showed that fenofibrate prevented a myocardial substrate switch and caused a modest improvement in cardiac function during the progression of heart failure but had had no effect on the onset of decompensation in the canine model of pacing-induced heart failure. Thus, chronic PPARα activation may not necessarily impair contractile function of the hypertrophied heart and may depend on particular experimental models employed and the duration/dose of the agonist administered. However, we found that fenofibrate increased uncoupling of mitochondrial respiration in the hypertrophied RV and hypoxic LV after 12 weeks of hypoxia. These studies therefore show the difficulty in trying to unravel whether the fetal switch in the hypertrophied heart is an adaptive or maladaptive phenomenon. Differences between studies may include variations in experimental protocols, animal models of hypertrophy/failure, composition of diets, and mode/duration of PPARα agonist administration.

Limitations: There are several limitations of the present study that need to be addressed. Firstly, we did not measure *ex vivo* myocardial fatty acid uptake /oxidation directly. Secondly, we did not determine the expression of a panel of mitochondrial regulatory genes as before (refer Chapter 2). Thirdly, we did not perform any histological analyses for e.g. electron microscopy to assess the arrangement and number of mitochondria in response to treatment regimens. These studies were not performed due to tissue limitations and the limited availability of laboratory rats. In the basal studies i.e. normoxia versus hypoxia, we did not employ pyruvate and or glutamate and malate as substrates to feed the mitochondrial

electron transport chain. This would have demonstrated whether glucose oxidation or composite electron transport chain flux contributed to the energy supply supporting improved contractile function. Lastly, we did not perform a dose-response curve to determine the dose of cocoa-butter that would elicit a maximal response on cardiac function and mitochondrial respiration. We are currently in the process of planning these experiments to further help our understanding of these findings.

In summary, we report that contractile function was increased in both ventricles in response to 12 weeks of hypobaric hypoxia. Furthermore, we found that mitochondrial respiratory function and bioenergetic capacity were reduced in the hypertrophied RV and hypoxic LV. I propose that this may be part of an adaptive response to conserve energy for contractile purposes. For the second part of the study, we demonstrated that fenofibrate treatment under hypoxia resulted in more severe effects on contractile function, particularly in the LV. Furthermore, we found that adaptive mitochondrial pathways and function of the hypertrophied RV triggered in response to hypobaric hypoxia were not blunted in the presence of cocoa butter. These data therefore indicate that chronic hypobaric hypoxia triggers adaptive pathways that may rescue organisms from the potentially damaging effects of higher fatty acid supply (e.g. cocoa butter treatment). Lastly, our study extends previous work by providing a potential mechanism whereby PPARα activation, and by implication increased fatty acid oxidation, may impair contractile function of the hypertrophied heart i.e. by uncoupling mitochondrial oxidative phosphorylation. Furthermore, our data support the concept that the fuel substrate switch away from fatty acids in the hypertrophied heart may be an adaptive mechanism. The precise mechanisms underlying these processes are currently being investigated in our laboratory.

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Chapter 4

Wy-14,643 and Fenofibrate Inhibit Mitochondrial Respiration in Isolated Rat Cardiac Mitochondria

4. 1. INTRODUCTION

Having shown direct effects of chronic administrations of Wy-14, 643 (Chapter 2) as well as fenofibrate (Chapter 3) on mitochondrial uncoupling, we next decided to further investigate this phenomenon by employing isolated rat heart mitochondria.

Peroxisome proliferators are a structurally diverse group of compounds that cause an increase in the size and number of peroxisomes [Reddy et al., 1980]. Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear hormone receptor superfamily of ligand-activated transcription factors [Lee and Lee, 2001]. PPARs are activated both by natural ligands for e.g. fatty acids (FA) and eicosanoids, and by pharmacological agonists such as fenofibrate, clofibrate, and Wy-14,643 [Kersten et al., 2000]. Upon activation by its ligands, PPARs heterodimerize with retinoid X receptors (RXRs) and thereafter bind peroxisome proliferator response elements in the promoter regions of target genes [Latruffe et al., 2001]. Three major PPAR isoforms, with distinct tissue expression patterns, have thus far been identified namely PPARα, PPARδ, and PPARγ [Issemann and Green, 1990; Dreyer et al., 1992]. PPARα is abundantly expressed in tissues with high rates of FA β-oxidation (FAO), including liver, brown adipose tissue, heart, skeletal muscle and kidneys [Issemann and Green, 1990; Schoonjans et al., 1995; Braissant et al., 1996]. In contrast, PPARγ is predominantly enriched in adipose tissue while PPARδ is ubiquitously expressed.

Numerous studies have demonstrated that PPARα plays a major role in cardiac energy metabolism through regulation of target genes involved in intracellular and mitochondrial

FA uptake and FAO [Barger and Kelly, 2000]. However, in addition to its effects on transcriptional regulation and as hypolipidemic and hypotriglyceridemic drugs [Vamecq et al., 1999; Kersten et al., 2000], several in vitro studies have shown that peroxisome proliferators also have direct actions on mitochondrial respiratory function [Keller et al., 1992; Brunmair et al., 2004; Scatena et al., 2004]. For example, studies performed on the human HL-60 cell line demonstrated that fibrates perturb mitochondrial respiration by inhibiting NADH-cytochrome c reductase activity [Scatena et al., 2004]. Likewise, others have reported that fenofibrate and clofibrate inhibit respiratory complex I of the mitochondrial respiratory chain in isolated homogenates of rat skeletal muscle [Brunmair et al., 2004]. Furthermore, it has been reported that the selective PPARα ligand, Wy-14,643, uncouples mitochondrial oxidative phosphorylation in isolated hepatic mitochondria [Keller et al., 1992]. Consistent with this notion, we found that Wy-14,643 administration in vivo resulted in direct effects on mitochondrial respiration (Chapter 2). To our knowledge, the acute effects of peroxisome proliferators on mitochondrial respiration in the heart have not been studied. In light of this, we therefore investigated the direct effects of two selective PPARα ligands, fenofibrate and Wy-14,643, on mitochondrial respiratory function using isolated rat cardiac mitochondria. We selected these particular agents i.e. Wy-14,643 and fenofibrate because it has been previously suggested that different peroxisome proliferators exhibit distinct mechanisms of mitochondrial inhibition [Zhou and Wallace, 1999]. Therefore, this study should provide additional insight whether this is indeed the case for cardiac mitochondrial respiration.

4.2. Materials and Methods

4.2.1. Animal studies

Seven-week-old male Wistar rats (weighing 200-230 g) were housed for 7 days at room temperature on a 12-hour reverse light/dark cycle (lights off at 4 am, zeitgeber time (ZT12); lights on at 4 pm (ZT0), with access to a conventional laboratory diet and water *ad libitum*. Rats were anesthetized using sodium pentobarbital (100 $mg.kg^{-1}$, ip), whereupon the left ventricles (LV) were dissected out and used for mitochondrial functional analyses. The reverse cycle allowed us to easily sacrifice rats in the middle of their night phase during our normal laboratory hours. This approach was followed because rats are most metabolically active during the night period. The University of Cape Town's Animal Research Ethics Committee approved all animal experiments and the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

4.2.2. Mitochondrial isolation and functional characterization

Mitochondria were isolated as described before, with modifications [Sordahl et al., 1971]. Briefly, LV tissue was homogenized in 10 ml ice-cold potassium-EDTA (KE) buffer (0.18 M KCl, 10 mM EDTA, [pH7.4]), whereafter the homogenate was centrifuged at 755 *x g* for 5 min. The supernatant was subsequently filtered through 41 µm nylon mesh (Spectrum, USA) and the filtrate centrifuged at 1,480 *x g* for 5 min. The mitochondrial

pellet was resuspended in 400 µl KE buffer, and was subsequently used for mitochondrial respiration measurements.

Respiratory rates were polarographically measured using a Clark-type electrode (Hansatech Instruments, London, UK) at 25°C with constant stirring as previously described [Essop et al., 2004] with modifications. Our preliminary studies showed that conducting mitochondrial assays at temperatures above 25°C strongly inhibited mitochondrial respiration and we found 25°C to be the optimum temperature (data not shown). Furthermore, mitochondrial respiration studies have been conducted at 25°C in our laboratory [Essop et al., 2004, Zungu et al., 2006] as well as others [St-Pierre et al., 2000, Starnes et al., 2007, Fuller et al., 1985]. Briefly, isolated rat ventricular mitochondria (0.5 mg/mL) were added to the electrode chamber containing incubation medium (10 mM Tris-HCl, 0.25 M sucrose, 8.5 mM KH_2PO_4, [pH7.4]), and thereafter aliquots of isolated mitochondrial suspension (0.5 mg/mL) were incubated for 3 min each with separate concentrations of fenofibrate (Sigma, USA) or Wy-14, 643 (Biomol, USA) i.e. 10 µM, 100 µM and 500 µM. Dose-response experiments were performed to establish a dose that resulted in acute and moderate inhibition of mitochondrial respiratory capacity within 3 minutes. Stock solutions of Wy-14,643 and fenofibrate were dissolved in DMSO. Mitochondrial respiration was induced by the addition of: a) 5 mM malate + 5 mM glutamate (substrates for complex I), b) 10 mM succinate (complex II-dependent substrate) in the presence of 4.5 µM rotenone (complex I inhibitor) and c) 5 mM malate + 40 µM palmitoyl-L-carnitine (mitochondrial FAO substrate). State 3 respiration was determined by measuring mitochondrial oxygen uptake after the addition of ADP to a final concentration of 350 µM. State 4 respiration was determined by measuring mitochondrial oxygen uptake upon complete ADP phosphorylation to ATP. The ADP/O ratio, a measure of mitochondrial oxidative phosphorylation efficiency, was

calculated as the ratio between the ADP added and oxygen consumed during ADP phosphorylation. The rate of oxidative phosphorylation was calculated as nanomoles of ADP phosphorylated per minute during state 3 respiration as described before [Babsky et al., 2001]. For respiration studies, only mitochondria with a respiratory control index (RCI) (state 3/state 4) value ≥ 4 were considered viable enough to be used under control conditions. The RCI and the ADP/O ratios were calculated according to Estabrook [Estabrook, 1967] using 253 nmol O_2 /mL as the value for the solubility of oxygen at 25°C. All mitochondrial polarographic studies were normalized to total protein content, determined using the Lowry assay [Lowry et al., 1951].

4.2.3. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). The statistical significance between groups was determined using one-way analysis of variance (1-WAY ANOVA) followed by the Bonferroni post hoc test. Statistical significance was set at $p<0.05$.

4.3. RESULTS

Wy-14,643 inhibited state 3 mitochondrial respiration in a dose-dependent manner in the presence of complex I (malate and glutamate) and complex II (succinate, in the presence of rotenone) oxidative substrates (Fig. 1A). The 100 μM and 500 μM doses of Wy-14,643 inhibited state 3 mitochondrial respiration by 58.3 ± 12.5% and 80 ± 10.2%, respectively in the presence of malate and glutamate ($n=5$; $p<0.001$ vs. controls) (Fig. 1A). However, much higher doses of Wy-14,643 were required to inhibit state 3

respiration when a complex II substrate (succinate) was employed i.e. the 500 µM dose reduced state 3 respiration by 45.5 ± 6.1% (n=5; $p<0.05$ vs. controls) (Fig. 1B). When palmitoyl-L-carnitine was employed as oxidative substrate, the 100 µM and 500 µM doses of Wy-14,643 inhibited state 3 mitochondrial respiration by 65 ± 12% (n=5; $p<0.01$ vs. controls) and 72 ± 19% (n=5; $p<0.001$ vs. controls), respectively (Fig. 1C).

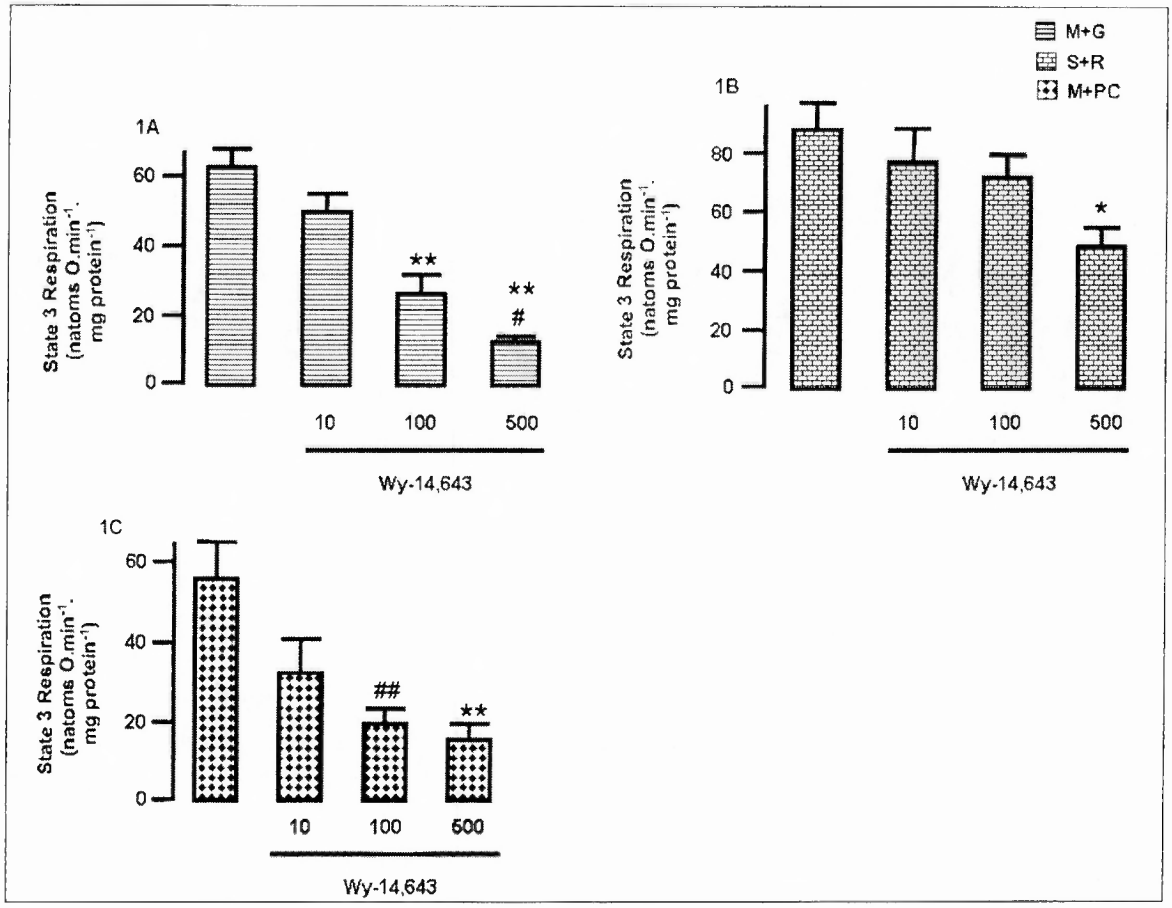

Figure 1: Functional assessment of isolated LV mitochondria, measuring state 3 mitochondrial respiration in response to 10, 100 and 500 µM Wy-14, 643. Bars represent mean ± standard error of the mean (SEM) for n=5 animals. **(A) 5 mM malate and 5 mM glutamate (M+G)** $^{**}p<0.001$ vs. control; $^{\#}p<0.01$ vs. 10 µM dose, **(B) 10 mM succinate and 4.5 µM rotenone (inhibitor of complex I) (S+R)** $^{*}p<0.05$ vs. control, and **(C) 5 mM malate and 40 µM palmitoyl-L-carnitine (M+PC) as oxidative substrates** $^{\#\#}p<0.01$ vs. control; $^{**}p<0.001$ vs. control.

On the other hand, fenofibrate markedly inhibited cardiac state 3 mitochondrial respiration (Figs. 2A-2C). However, as with Wy-14,643, higher fenofibrate doses were required to inhibit complex II-dependent mitochondrial respiration. These findings indicate that both Wy-14,643 and fenofibrate attenuate cardiac mitochondrial respiration, especially at complex I. To determine the acute effects of Wy-14,643 and fenofibrate on mitochondrial bioenergetic capacity, the rate of mitochondrial oxidative phosphorylation was determined.

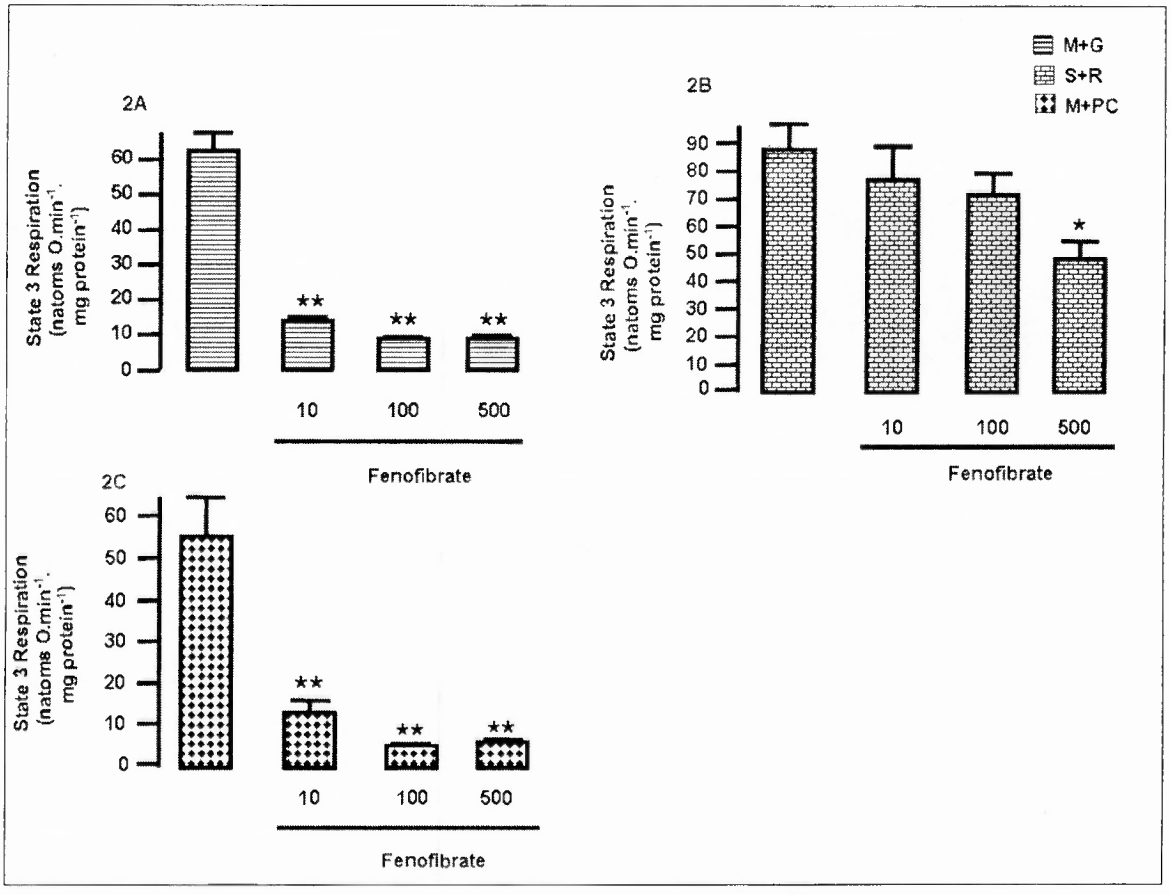

Figure 2: Functional assessment of isolated LV mitochondria, measuring state 3 respiration in response to 10, 100 and 500 µM additions of fenofibrate. Bars represent mean ± standard error of the mean (SEM) for n=5 animals **(A) 5 mM malate and 5 mM glutamate (M+G)** $^{**}p<0.001$ vs. control **(B) 10 mM succinate and 4.5 µM rotenone (inhibitor of complex I) (S+R)** $^{*}p<0.05$ vs. control, and **(C) 5 mM malate and 40 µM palmitoyl-L-carnitine (M+PC) as oxidative substrates** $^{**}p<0.001$ vs. control.

Consistent with our state 3 respiration data, Wy-14,643 dose-dependently reduced the rate of ADP phosphorylation in the presence of malate and glutamate at the 100 μM and 500 μM doses (Fig. 3A).Here, the rate of oxidative phosphorylation was reduced by 71.9 ± 23.1% in response to the 100 μM dose (n=5; $p<0.001$ vs. control), and further declined to 80 ± 10.4% in response to the 500 μM dose (n=5; $p<0.001$ vs. control) (Fig. 3A). Reduced rates of oxidative phosphorylation at high doses of Wy-14,643 were not statistically significant when succinate was employed as an oxidative substrate (Fig. 3B). However, the 100 μM and 500 μM doses of Wy-14,643 also inhibited the rate of ADP phosphorylation by 74.9 ± 18.8% and 76.8 ± 25.6% (n=5, $p<0.01$ vs. controls), respectively, when palmitoyl-L-carnitine was employed as oxidative substrate (Fig. 3C).

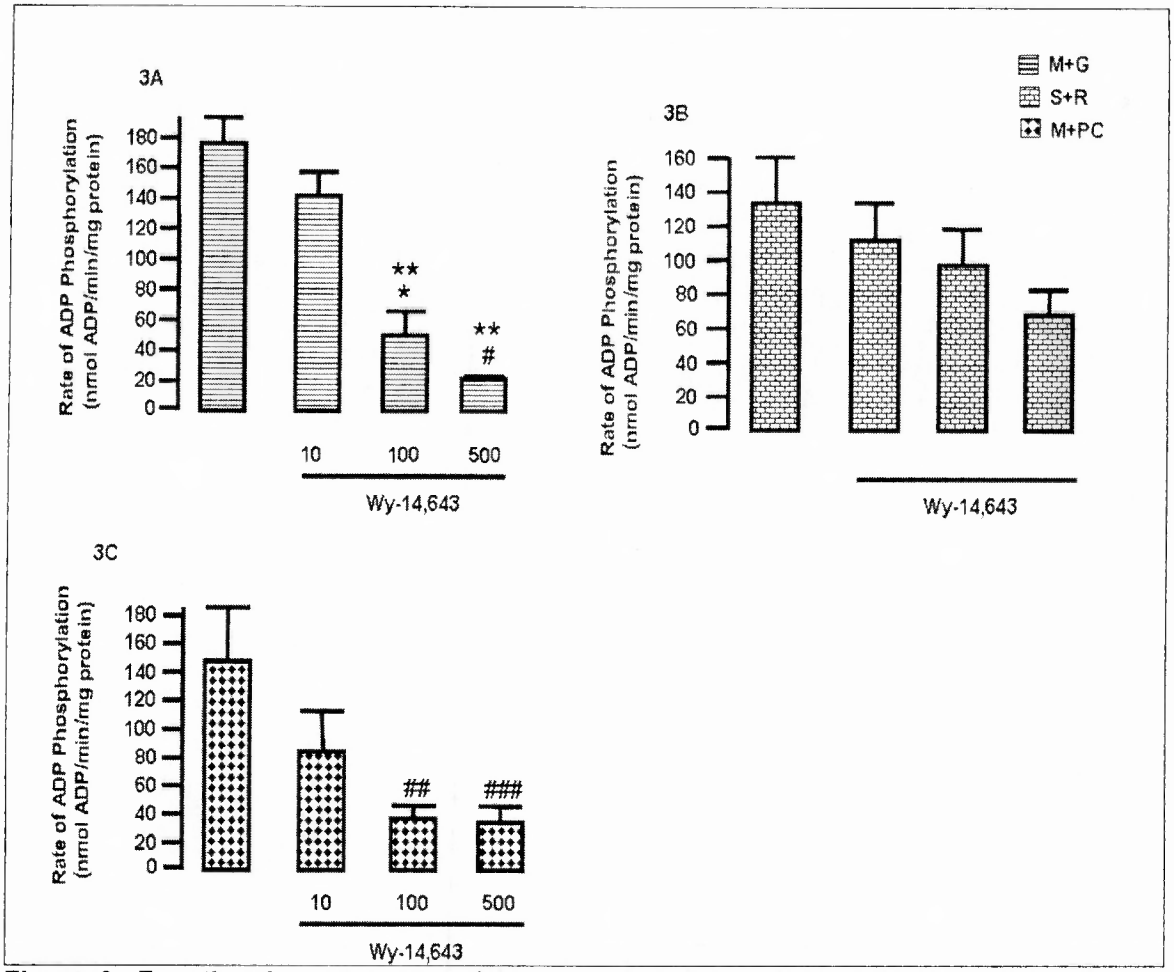

Figure 3: Functional assessment of isolated LV mitochondria, measuring rate of ADP phosphorylation in response to 10, 100 and 500 µM additions of Wy-14,643 Bars represent mean ± standard error of the mean (SEM) for n=5 animals. **(A) 5 mM malate and 5 mM glutamate (M+G)** $^{*}p<0.05$ vs. control; $^{**}p<0.001$ vs. control, $^{\#}p<0.05$ vs. 10µM dose, **(B) 10 mM succinate and 4.5 µM rotenone (inhibitor of complex I) (S+R),** and **(C) 5 mM malate and 40 µM palmitoyl-L-carnitine (M+PC) as oxidative substrates** $^{\#\#}p<0.01$ vs. control, $^{\#\#\#}p<0.05$ vs. 10µM dose.

As before, acute exposure of cardiac mitochondria to fenofibrate elicited a marked response i.e. it significantly attenuated the rates of ADP phosphorylation in the presence of all three oxidative substrates employed (Figs. 4A-4C). The rate of ADP phosphorylation in the presence of malate and glutamate was reduced by 82.1 ± 14.5% with a low fenofibrate dose (10 µM), remaining at similar levels at higher fenofibrate doses (Fig. 4A).

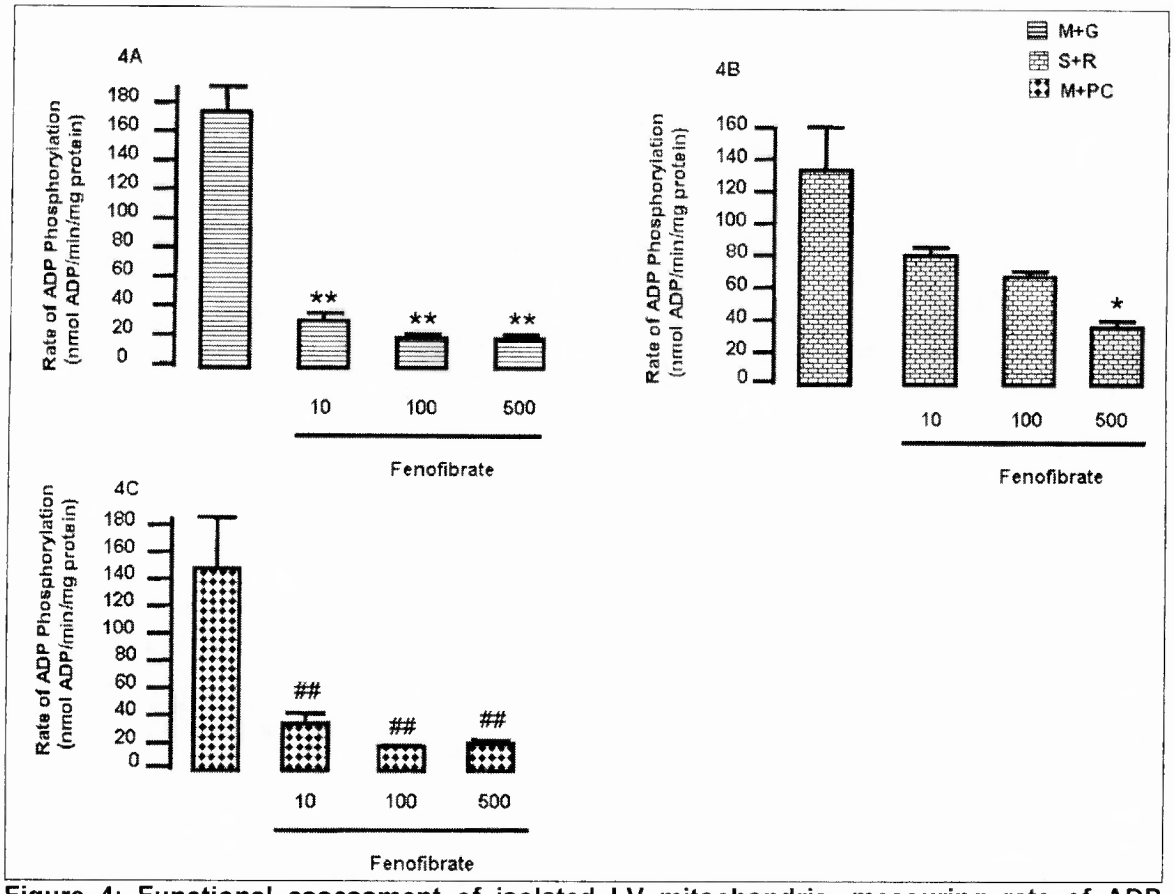

Figure 4: Functional assessment of isolated LV mitochondria, measuring rate of ADP phosphorylation in response to 10, 100 and 500 µM additions of fenofibrate. Bars represent mean ± standard error of the mean (SEM) for n=5 animals **(A) 5 mM malate and 5 mM glutamate (M+G)** $^{**}p<0.001$ vs. control, **(B) 10 mM succinate and 4.5 µM rotenone (inhibitor of complex I) (S+R)** $^{*}p<0.05$ vs. control, and **(C) 5 mM malate and 40 µM palmitoyl-L-carnitine (M+PC) as oxidative substrates** $^{\#\#}p<0.01$ vs. control.

However, higher fenofibrate doses were required to inhibit the rate of phosphorylation at complex II, i.e. the 500 µM dose attenuated it by 73.3 ± 8% (n=5; $p<0.05$ vs. control) (Fig. 4B). When palmitoyl-L-carnitine was used as oxidizing substrate, the rate of ADP phosphorylation was markedly reduced by 77.3 ± 18.5% with a low fenofibrate dose (10 µM), remaining at similar levels with higher doses (Fig. 4C). Together these data show that Wy-14,643 and fenofibrate acutely inhibit mitochondrial oxidative phosphorylation, particularly at complex I.

We next determined state 4 respiration as marker of proton leak. With malate and glutamate, we found that state 4 mitochondrial respiration was significantly increased for 10 µM Wy-14,643, thereafter remaining at similar levels for higher Wy-14,643 doses (Table 1). A similar pattern was observed when succinate was used as oxidative substrate (Table 1). Of note, malate and palmitoyl-L-carnitine significantly increased state 4 respiration rate only at the 500 µM Wy-14,643 dose (Table 1).

Table 1: Acute effects of Wy-14,643 on state 4 mitochondrial respiration, ADP/O and RCI

State 4 Respiration (natoms O.ml^{-1}.min^{1}.mg protein $^{-1}$)	**Untreated Control**	**10 µM Wy-14,643**	**100 µM Wy-14,643**	**500 µM Wy-14,643**
Malate+glutamate	0.91 ± 0.3	8.38 ± 2.2*	13±1.9**	13± 0.9**
Malate+ palmitoyl-L-carnitine	2.40 ± 0.6	6.93 ± 1.2	11 ±1.9	16 ± 5.4*
Succinate+rotenone	4.82 ±1.6	34± 2.8*	36±2.4**	36 ± 4.3**
ADP/O ratio				
Malate+glutamate	2.92 ± 0.3	2.58 ± 0.3	1.69 ± 0.3	1.53 ± 0.3
Malate+ palmitoyl-L-carnitine	3.05 ± 0.2	2.37 ± 0.3	1.91 ± 0.3*	1.59 ± 0.1***
Succinate+rotenone	1.58 ± 0.1	1.36 ± 0.3	1.23 ± 0.2	1.11 ±0.2
Respiratory Control Index (State 3/State 4)				
Malate+glutamate	34 ± 8.6	6.01 ± 1.4**	1.85 ± 0.5**	0.96 ± 0.1**
Malate+ palmitoyl-L-carnitine	20 ± 6.1	2.45 ± 0.5**	1.88 ±0.3**	1.23 ±0.1**
Succinate+rotenone	16 ± 2.1	3.44 ±1.2**	1.86 ±0.2**	1.63 ± 0.1**

Data are presented as mean ± standard error of the mean (SEM). *p<0.05 vs. control, **p<0.001 vs. control, ***p<0.0.01 vs. control.

When fenofibrate was administered, significant increases were observed at the 100 µM and 500 µM doses (Table 2). We also found that RCI was dose-dependently reduced for both Wy-14,643 (Table 1) and fenofibrate groups (Table 2) when complex I and complex

II-dependent substrates were used. In agreement, the ADP/O ratio progressively declined in the presence of Wy-14,643 and fenofibrate (Tables 1 and 2), reaching significance for 100 and 500 μM doses of Wy-14,643 when palmitoyl-L-carnitine was employed as oxidative substrate.

Table 2: Acute effect of fenofibrate on state 4 mitochondrial respiration, ADP/O and RCI

State 4 Respiration (natomsO.ml^{-1}.min^{1}.mg protein $^{-1}$)	Untreated Control	10 μM Fenofibrate	100 μM Fenofibrate	500 μM Fenofibrate
Malate+glutamate	0.96 ± 0.3	1.76 ± 0.9	5.13 ± 1.2	5.87± 0.9**, #
Malate+ palmitoyl-L-carnitine	2.42 ± 0.6	2.98 ± 0.3	3.74 ± 0.6	6.06 ± 0.3#,###
Succinate+rotenone	4.81 ± 1.6	14 ±0.6	29 ±2.6	30 ± 5.6***
ADP/O				
Malate+glutamate	2.91 ± 0.3	2.32 ± 0.4	1.83 ± 0.4	1.66 ± 0.4
Malate+ palmitoyl-L-carnitine	3.05 ± 0.2	2.51 ± 0.4	2.47 ± 0.3	1.84 ± 0.4
Succinate+rotenone	1.58 ± 0.1	1.53 ± 0.1	1.16 ± 0.1	1.12 ± 0.1
Respiratory Control Index (State 3/State 4)				
Malate+Glutamate	34 ± 8.5	4.33 ± 1.7**	2.16 ±0.5**	1.78±0.6**
Malate+ palmitoyl-L-carnitine	20 ± 6.1	4.54 ± 2.4*	1.55 ±0.2**	1.05±0.1*
Succinate+Rotenone	16 ± 2.1	1.26 ±0.12***	0.55±0.2***	0.55±0.1**

Data are presented as mean ± standard error of the mean (SEM). *p<0.05 vs. control, **p<0.01 vs. control, ***p<0.001 vs. control, #p<0.05 vs. 10 μM dose, ##p<0.05 vs. 100 μM dose .

Because the dose-response curves were performed using identical mitochondrial preparations, we analyzed these differences in a paired manner and found that the 500 μM fenofibrate dose in the presence of palmitoyl-L-carnitine caused a statistically significant decrease. Together these data suggest that acute exposure to both Wy-14,643 and fenofibrate induces uncoupling of mitochondrial oxidative phosphorylation.

4.4. DISCUSSION

Wy-14,643 and fenofibrate are synthetic ligands for the transcriptional regulator PPARα, inducing the expression of a number of cardiac FA utilization genes (Barger and Kelly, 2000). However, previous studies have shown that these agents also exert direct effects at the mitochondrial level, including inhibition of the mitochondrial respiratory chain complexes and state 3 respiration, and the uncoupling of mitochondrial oxidative phosphorylation [Keller et al., 1992; Keller et al., 1993; Zhou and Wallace, 1999]. To assess whether peroxisome proliferators exert direct effects on cardiac mitochondrial function, we acutely exposed isolated rat LV mitochondria to increasing concentrations of Wy-14,643 and fenofibrate and subsequently evaluated mitochondrial respiratory function.

Our data show that Wy-14,643 and fenofibrate differentially inhibit mitochondrial respiratory function in isolated cardiac mitochondria, with fenofibrate effects occurring at lower concentrations. Of note, in our study we report lower state 3 mitochondrial respiration values since we used isolated mitochondria from LV tissue instead of the whole heart. We found that acute exposure to fenofibrate markedly inhibited state 3 respiration and the rate of ADP phosphorylation, particularly at complex I. On the other hand, Wy-14,643 elicited dose-dependent effects on respiratory capacity and phosphorylation rate. It appears as if particularly complex I was more severely affected since we only observed inhibition of complex II-dependent respiration activity at higher doses of both agents. We therefore speculate that fenofibrate has a higher affinity for complex I compared to Wy-14,643. Our data raise the possibility these agents have

direct effects on mitochondrial respiration, resulting in impaired bioenergetic capacity. However, further studies are needed to confirm this.

Our findings are in agreement with previous reports demonstrating the inhibition of respiration by fenofibrate in muscle and liver mitochondria [Keller et al., 1992; Brunmair et al., 2004]. For example, Brunmair et al., showed that the catalytic activity of complex I was inhibited by 100 μM fenofibrate in homogenates of skeletal muscle and liver [Brunmair et al., 2004]. Furthermore, they found that 100 μM fenofibrate in isolated rat hepatocyte mitochondria inhibited state 3 respiration. Also, they found that complex II-dependent state 3 respiration was not affected [Brunmair et al., 2004], while we observed that complex II was only inhibited at higher doses of fenofibrate and Wy-14,643. Reduced state 3 mitochondrial respiration is thought to be due to respiratory chain dysfunction through intrinsic damage of its oxido-reductases and/or a limitation of reducing equivalents [Willet et al., 2000 a, b]. This is proposed to result in reduced supply of electrons to the mitochondrial electron transport chain. Alternatively, it could result in damage to the mitochondrial phosphorylation system i.e. ATP synthase, phosphate transporters or adenine nucleotide translocators [Ascensao et al., 2006].

Previously, it was reported that Wy-14,643 uncouples mitochondrial respiration in isolated hepatic mitochondria [Keller et al., 1992]. Since we observed that state 3 respiration and oxidative phosphorylation were impaired, we reasoned that these agents may result in uncoupling of mitochondrial oxidative phosphorylation. We found that both Wy-14,643 and fenofibrate increased state 4 mitochondrial respiration for all substrates utilized. Furthermore, ADP/O was reduced at higher concentrations of Wy-14,643 and fenofibrate. Because state 4 respiration represents mitochondrial oxygen consumption that is not coupled to ATP synthesis and both agents resulted in reduced RCI (↓state 3/

↑state 4) and ADP/O, these data suggest mitochondrial uncoupling. It has been reported that mitochondrial proton leak may occur through a number of mechanisms: (i) via uncoupling proteins (UCPs) [Brand et al., 1994; Stuart et al., 2001], (ii) inhibiting adenine nucleotide translocator via allosteric stimulation by adenine monophosphate [Cadenas et al., 2000], (iii) trans-membrane cycling of protonated/unprotonated free fatty acids [Garlid et al., 2000; Jezek et al., 2004], and (iv) inducing permeability transition pore opening [Crompton, 1999; Broekemeier et al., 1998; Huser and Blatter, 1999]. For example, a previous study on isolated rat liver mitochondria reported that Wy-14,643 induces permeability transition while fenofibrate directly inhibits mitochondrial respiration [Zhou and Wallace, 1999]. However, additional studies are required to determine the mechanisms whereby Wy-14,643 and fenofibrate uncouple oxidative phosphorylation in heart mitochondria.

What are the reasons for the different responses elicited by fenofibrate and Wy-14,643? It is not immediately clear from our study. However, it has been shown that Wy-14,643 impairs mitochondrial function by inducing mitochondrial permeability transition, whereas fenofibrate is thought to exert a more direct effect i.e. via inhibition of the mitochondrial respiratory chain complexes [Scatena et al., 2003]. This may be the case in our model since we found that fenofibrate exhibited a stronger inhibitory effect on mitochondrial respiration compared to Wy-14,643. However, additional studies are required to confirm these suggestions.

In conclusion, our data show that acute exposure to Wy-14,643 and fenofibrate differentially perturb cardiac mitochondrial respiration i.e. fenofibrate more potently inhibited mitochondrial respiratory function compared to Wy-14,643. These findings may have implications for researchers and in clinical settings since Wy-14,643 and

fenofibrate are often used to activate PPARα and its target genes *in vivo* and in cell culture studies [Hamano et al., 2001; Toyama et al., 2004]. Moreover, fenofibrate is also used as therapeutic agent against hypertriglyceridemia and dyslipidemia [Vamecq and Latruffe, 1999; Kersten at al., 2000]. Of note, the effects of Wy-14, 643 and fenofibrate in this study were of a direct and acute nature on isolated mitochondria from the left ventricle only and therefore may not necessarily reflect changes resulting from chronic in vivo administration. Further studies are required to determine the effects of chronic administration of Wy-14,643 and fenofibrate on cardiac mitochondrial respiration.

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Chapter 5

Concluding Remarks

This thesis highlights several interesting concepts and also opens up new frontiers to be further investigated. In the first instance, it demonstrates ***distinct responses of the right and left ventricles to chronic hypobaric hypoxia.*** Although previous studies have documented this before, for e.g. pulmonary hypertension leading to right ventricular hypertrophy, this concept is further extended by the current work. Here, I report the coordinate induction of several mitochondrial respiratory genes in response to hypoxia-mediated physiologic right ventricular hypertrophy. Moreover, this gene program was associated with sustained mitochondrial respiratory and right ventricular contractile function after 2 and 4 weeks of hypoxic exposure. Furthermore, increased citrate synthase content (a measure of mitochondrial number) in the RV was paralleled by increased mtDNA content in response to 2 weeks hypobaric hypoxia. These changes were not observed in the LV.

Conversely, the LV (not exposed to increased load) initially coped well with the chronic hypoxic stimulus (after 2 weeks), but displayed reduced respiratory and contractile function later-on (4 weeks). A possible reason for the modest blunting of left ventricular mitochondrial function at the 4 week time point may be in response to diminished energy demand. It is possible that the modest attenuated function at 4 weeks may be in response to incomplete adaptation to the increased pulmonary pressure with reduced left sided contractile demand and therefore diminished supply.

Recent studies have implicated the PI3-kinase signaling pathway in the development of physiologic hypertrophy and this may be an ideal candidate to begin to investigate in this experimental model of right ventricular hypertrophy. Another intriguing question that arises is whether activation of classic pathophysiologic hypertrophic signaling pathways in our model would result in loss of the physiologic phenotype observed in the hypertrophied right ventricle.

The study also highlights the temporal nature of metabolic changes in this experimental model. Thus, I believe a general principle is established i.e. it is important to study the progression of changes in an experimental model at several time points in response to chronic stimuli. In this manner, greater insight into the progression of disease, or the monitoring of adaptive changes would be achieved. For example, at the later time point (12 weeks) mitochondrial respiration is impaired, in contrast to the earlier experimental time points, although cardiac contractile function is still maintained. Such metabolic flexibility, in my opinion, allows the organism to continuously adjust and fine tune its metabolic machinery to better cope with an acute or chronic stimulus. In this instance I propose that the general impairment of mitochondrial respiration in both ventricles may be an attempt to preserve ATP, thereby sustaining contractile function. However, this newly-spawned hypothesis requires further investigation. Interestingly, the right hypertrophied ventricle generally performed better than the left ventricle in response to the longer-term time points (4 and 12 weeks). I believe that mitochondrial changes in the hypertrophied heart may be responsible for these differences. Future studies should investigate whether specific signaling pathways are activated in the hypertrophied heart that limits the opening of the mitochondrial permeability transition pore, further eliciting an adaptive response.

For the second part of this study, I focused on further exploring the role of increased fatty acid utilization in the setting of right ventricular cardiac hypertrophy. I devised a series of experiments to activate PPARα (using Wy-14, 643) in the hypertrophied RV (2 weeks of hypobaric hypoxia) and to subsequently assess whether this intervention results in an adaptive or maladaptive phenotype. ***The major finding of the second part of this study is that in vivo Wy-14,643 administration elicited direct effects on mitochondrial respiratory function and cardiac contractility.*** The data show

that Wy-14,643 treatment attenuated mitochondrial respiration in the RV and the LV while promoting uncoupling of mitochondrial oxidative phosphorylation in the LV.

We next investigated whether adaptive cardiac changes we earlier observed (i.e. after 2 and 4 weeks) in the hypertrophied RV are sustained at a later time point (12 weeks). We also further investigated our hypothesis i.e. that elevated myocardial fatty acid utilization by the hypertrophied heart uncouples mitochondrial respiration, thereby resulting in reduced contractile function. We pursued our hypothesis in a rat model of hypoxia-induced RV hypertrophy by exposing rats to 12 weeks of hypobaric hypoxia (11% O_2), and increased cardiac fatty acid utilization in the hypertrophied heart by enhancing fatty acid delivery by chronic supply of a high fat diet (cocoa butter), and activating PPARα using fenofibrate as a ligand. The major findings of this part of my thesis are: ***1) Increased RV and LV contractile function in response to 12 weeks of hypobaric hypoxia, associated with lowered mitochondrial respiratory functional capacity, and 2) Fenofibrate administration results in more pronounced effects on contractile and respiratory function than cocoa butter treatment***

I propose that the reduction of mitochondrial respiratory function at the 12-week point may either be part of an adaptive response to conserve energy for contractile purposes, or the beginning of a maladaptive phenotype. These possibilities require further investigation. For the fenofibrate treatment studies, fenofibrate administration under hypoxia resulted in more severe effects on contractile function, particularly in the LV. Moreover, we found that adaptive mitochondrial pathways and function of the hypertrophied RV triggered in response to hypobaric hypoxia were not blunted in the presence of cocoa butter. These data therefore indicate that chronic hypobaric hypoxia triggers adaptive pathways that may rescue organisms from the potentially damaging effects of higher fatty acid supply (e.g. cocoa butter treatment). Lastly,

our study extends previous work by providing a potential mechanism whereby PPARα activation, and by implication increased fatty acid oxidation, may impair contractile function of the hypertrophied heart i.e. by uncoupling mitochondrial oxidative phosphorylation. Thus our data support the concept that the fuel substrate switch away from fatty acids in the hypertrophied heart may be an adaptive mechanism.

Lastly, this thesis ***highlights the inhibitory effects of PPARα ligands on mitochondrial respiration.*** For example, both fenofibrate and Wy-14,643 inhibited cardiac mitochondrial respiration, albeit to different degrees. Since these agents are often used by researchers for *in vivo* studies, these data indicate that greater attention should be given to the dosage given to laboratory animals and also to the duration of the compound's administration.

5.2. PUBLICATIONS

1. **Zungu M**, Felix R, Essop MF: Wy-14,643 and fenofibrate inhibit mitochondrial respiration in isolated rat cardiac mitochondria. Mitochondrion 6:315-322, 2006.
2. **Zungu M**, Alcolea MP, Garcia-Palmer FJ, Young ME, Essop MF: Genomic modulation of mitochondrial respiratory genes in the hypertrophied heart reflects adaptive changes in mitochondrial and contractile function. Am J Physiol Heart Circ Physiol 293(5):H2819-H2825, 2007.