

**Regulation of Fatty Acid and Mitochondrial
Respiratory Chain Genes in a Hypobaric
Hypoxia-induced Right Ventricular
Hypertrophy Rat Model**

Kholiswa C. Ngumbela

**Submitted for the Degree of Doctor of
Philosophy in Medicine**

**Hatter Institute for Cardiology Research
Faculty of Health Sciences
University of Cape Town
2004**

Supervisors: Drs. M. Faadiel Essop & Michael N. Sack

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

Acknowledgements

This work was done in the Hatter Institute for Cardiology Research, located within the Department of Medicine at the University of Cape Town's Faculty of Health Sciences.

I would like to thank the following people and institutions for their help in making this thesis come into being:

- To Dr. M. Faadiel Essop, thank you for being the best supervisor (approachable, devoted, enthusiastic and challenging), for the scientific guidance, intellectual input, encouragement and support. Your help and dedication is highly appreciated.
- To Dr. Michael N. Sack (co-supervisor) - thanks for your supervision, scientific guidance and intellectual input.
- To Prof. Lionel Opie, thank you for your availability and patience each time I needed your intellectual input.
- To the late Dr. Cecile Muller, thanks for your much needed motherly advice and encouragement.
- To Dr. Bongani Mayosi and Dr. Nosisa Matsiliza - Dr. Mayosi thank you for introducing me to the Hatter Institute, and thanks to both of you for making sure that the Hatter Institute's doors stayed open until the completion of my study.

- A special thanks to Prof. Heinrich Taegtmeyer and Dr. Saumya Sharma (University of Texas Houston-Medical School, Houston, TX) for assisting by performing the quantitative RT-PCR work in their laboratory.
- The MCAD-CAT transgenic mice, MCAD and actin genes used in this study were received as a gift from Prof. Daniel P. Kelly (Washington University School of Medicine, St Louis, MO). I thank him for the wonderful gifts, which played an important part in the findings of the study.
- A special thanks to Dr. Michael Arand (University of Mainz, Germany) for the anti-PPAR used in this study.
- To Dr. Robert Smith, thank you for your help and technical assistance with the hypobaric hypoxic chamber used in this study.
- A special thanks to Joy McCarthy and Lydia Lacerda for their skillful technical assistance - your patience and availability was highly appreciated
- To Victor Claasen - thank you for always making me laugh during stressful times, always available to help and the skillful technical assistance especially with animals used in this study. I'll miss the fish braai's Claasen!
- To Elaine Richardson and Sylvia Dennis - assistance received from you ladies is highly appreciated.
- To Jeannie Walker, Pat van de Walt and Beauty Kom - your input didn't go unnoticed; thanks a lot!
- A special thanks to the "Tripartite" (Seeyah, Noush and Peter) and Tazz for making the lab all the more pleasant and their support in many different ways.

- I am grateful for the services rendered by UCT's Animal Unit, especially Noel Markgraaff and Jaap Visser for their technical assistance.
- To everyone else (for example Dr Nonkosi Ngumbela, Zola Makubalo) who assisted me all the way, I say without you I would not have made it.
- I would like to thank Aventis, the South African Medical Research Council and the University of Cape Town for financing this study.
- **To my parents - thank you for your financial support, encouragement, care, warmth and love. Without you I would not have made it.**
- A special thanks to my son, Uzile Uzzi Zingisa - for moral support and keeping me focused all these years.
- Lastly, to the Lord who gave me strength to carry on and make this thesis possible.

ABSTRACT

Regulation of Fatty Acid and Mitochondrial Respiratory Chain Genes in a Hypobaric Hypoxia-induced Right Ventricular Hypertrophy Rat Model: Kholiswa C. Ngumbela, February 2004

Cardiac metabolic remodeling is defined as a switch in cardiac substrate use in association with cardiogenesis and the development of pathophysiologic conditions. The question whether cardiac metabolic remodeling is beneficial, detrimental or an epiphenomenon has not been conclusively established. The aim of this study was to investigate this intriguing question and help unfold the mystery surrounding the contribution of metabolic remodeling to the development of cardiac disease. Here, we established and characterized a rat model of hypoxia-induced right ventricular cardiac hypertrophy. Wistar rats were exposed to hypobaric hypoxia (11% O₂) over a period of 24 weeks. We subsequently investigated, in a progressive manner, changes in the rat heart at the functional, and transcriptional levels. Our cardiac functional data demonstrate the development of compensated hypertrophy over the 24-week period, with no signs of cardiac dysfunction or heart failure. Moreover, we observed dynamic regulation of the expression of several fatty acid and glucose utilization enzyme-encoding genes. Mitochondrial respiration was maintained in the right ventricle despite reduced adenine nucleotide translocator 1 levels. Analysis of candidate redox-sensitive transcriptional regulators implicated Sp3 and PPAR α as important modulators of medium-chain acyl-CoA dehydrogenase (MCAD), a pivotal fatty acid utilization enzyme. Together these data highlight the complex interplay of multiple regulatory mechanisms that ensure adaptation of the rat heart in response to chronic hypobaric hypoxia. Our data therefore indicate that cardiac metabolic remodeling is a dynamic process in the hypertrophied right ventricle resulting in an adapted cardiac phenotype.

SUMMARY

The work reported in this thesis is divided into three sub-projects, namely (i) characterization of a hypobaric hypoxia-induced right ventricular hypertrophy rat model, (ii) investigation of adenine nucleotide translocator 1 (ANT1) and cardiac oxidative phosphorylation (oxygen consumption in isolated mitochondria) in response to chronic hypobaric hypoxia, and (iii) analysis of the effects of incremental hypoxia on the transcriptional regulation of a cardiac fatty acid oxidation enzyme-encoding gene.

(i) Characterization of hypobaric hypoxia-induced right ventricular hypertrophy rat model

During steady state conditions of oxygen demand hypoxia is caused by a reduction of the supply of oxygen to tissues, leading to an imbalance between oxygen supply and demand. Because of the high coronary arteriovenous difference, the myocardium is not able to bring about a substantial improvement in the oxygen supply by increased extraction of oxygen from the blood, and thus the only way of meeting the high demands is through an increased blood supply. Oxygen consumption depends on the heart rate, contractile performance and tension of the ventricular wall, as a result of the pressure volume relationship (Hisano et al., 1987). Theoretically, any of the known mechanisms leading to tissue hypoxia can be responsible for a reduced oxygen supply in the myocardium but the most common causes are undoubtedly:

- I. Ischemic hypoxia (often described as "cardiac ischaemia") induced by the reduction or interruption of the coronary blood flow,
- II. Systemic (hypoxia) hypoxia (cardiac hypoxia) characterised by a drop in PO_2 in the arterial blood but adequate perfusion,
- III. Anemic hypoxia in which the arterial PO_2 is normal but the oxygen transport capacity of the blood is increased.
- IV. Histotoxic hypoxia resulting from reduced intracellular utilization of oxygen in the presence of adequate saturation and adequate blood flow (i.e. inhibition of oxidative enzymes in cyanide poisoning).

[Ostadal et al., 1999]

In order to sustain metabolic processes, organisms have evolved adaptive regulatory mechanisms to cope with both acute and chronic exposure to hypoxia. Acute hypoxic exposure initiates hypoxia defenses via specific oxygen-sensing mechanisms. On the other hand, chronic exposure to hypobaric hypoxia causes pulmonary vasoconstriction and hypertension, right ventricular hypertrophy and right-sided heart failure. In this study, we investigated the progressive changes in the rat heart in response to chronic hypobaric hypoxia (11% O_2) with particular emphasis on the functional, metabolic and transcriptional levels. Here, we set up a robust working model of compensated right ventricular hypertrophy in response to chronic hypobaric hypoxia. We determined that metabolic gene regulation in the hypertrophied right ventricle is dynamic, fluctuating in response to the chronic hypoxic stimulus.

(II) Investigation of adenine nucleotide translocator 1 (ANT1) and cardiac oxidative phosphorylation in response to chronic hypobaric hypoxia

The cardiac-enriched adenine nucleotide translocator 1 (ANT1) is an integral inner mitochondrial membrane protein transporting ADP and ATP across the inner mitochondrial membrane. ANT is the only transport system currently known for the exchange of ADP and ATP, therefore regarded as a pivotal regulator of mitochondrial oxidative phosphorylation, linking ATP synthesis to cytoplasmic energy utilization. ANT1 deficiency has been proposed to be maladaptive, contributing to the pathogenesis of heart failure. To test this hypothesis, we evaluated the regulation of ANT1 in the right ventricle in response to 2 weeks chronic hypoxia. A robust hypertrophic response was induced as shown by significantly increased right ventricular weight and right ventricular developed pressure. Although oxygen consumption and proton leak were increased, the rate of ATP production and mitochondrial efficiency were maintained. ANT1 gene expression and ANT steady-state protein levels as assessed by Northern and Western blotting, respectively, were diminished while ANT functional content was increased. Furthermore, contractile function was maintained after 24 weeks exposure to hypobaric hypoxia despite decreased ANT levels. Our data suggest that reduction of ANT1 does not compromise energy production and cardiac function and we propose that augmented levels of alternate cardiac ANT isoforms may be part of this compensatory adaptive response.

(III) Analysis of the effects of incremental hypoxia on the transcriptional regulation of a cardiac fatty acid oxidation enzyme-encoding gene

The normal adult heart predominantly uses fatty acids to produce ATP. In contrast, the hypoxic heart switches energy substrate usage from fatty acids to glucose. To explore this metabolic switch, we evaluated the regulation of the medium-chain acyl-CoA dehydrogenase (MCAD), a pivotal fatty acid β -oxidation enzyme, in response to hypobaric hypoxia. We exposed transgenic mice, harboring 560-bp of the human MCAD gene promoter fused to the bacterial chloramphenicol acetyl transferase (CAT) gene, and wild type mice to either a moderate (14% O₂) or severe (8% O₂) hypobaric hypoxic stimulus vs. normoxic controls for a period of 48 hours and 1 week, respectively. We compared MCAD gene expression, MCAD-CAT activity and steady-state peptide levels of Sp3 and PPAR α . The latter are previously identified MCAD transcriptional modulators. Moderate hypoxia caused a downregulation of MCAD-CAT activity and MCAD gene expression. The steady-state levels of Sp3 and PPAR α were downregulated after 1 week of hypoxia. In contrast, severe hypoxia resulted in an increase in MCAD-CAT activity and MCAD gene expression at 48 hours, returning to baseline at 1 week. Sp3 steady-state levels were increased at 48 hours and returned to baseline at 1 week whilst PPAR α levels were increased at 48 hours and remained elevated at 1 week. We speculate that our data suggest that moderate hypoxia decreases Sp3 and PPAR α levels resulting in the downregulation of MCAD gene expression. On the other hand, severe hypoxia elevated Sp3 and PPAR α .

levels resulting in the upregulation of MCAD gene expression. These data suggest that redox-sensitive upstream factors are responsible for differences observed in the regulation of MCAD in response to moderate and severe hypoxic stimuli.

University of Cape Town

Table of Contents

INDEX	Page
Acknowledgments.....	ii
Abstract.....	v
Summary.....	vi
Chapter 1.....	1
1.1 Fatty Acid Metabolism.....	2
1.2 Glucose Metabolism.....	5
1.3 Oxidative Phosphorylation.....	7
1.4 Cardiac Metabolic Remodeling.....	11
1.4.1 Cardiac Metabolic Remodeling: Developmental.....	12
1.4.2 Cardiac Metabolic Remodeling: Cardiac Hypertrophy and Heart Failure.....	17
1.4.3 Is Metabolic Remodeling during Cardiac Hypertrophy and Heart Failure Beneficial or Detrimental.....	25
1.4.3.1 Cardiac Metabolic Remodeling: Evidence Favoring Detrimental Role.....	25
1.4.3.2 Cardiac Metabolic Remodeling: Evidence Favoring Beneficial Role.....	29
1.5 Aims of Study.....	32
1.6 References.....	33

Chapter 2.....	52
2.1 Introduction.....	53
2.2 Materials and Methods.....	58
2.2.1 Animals.....	58
2.2.2 Langendorff Perfusion.....	59
2.2.3 Determination of Metabolic Substrates and Hematocrit Levels.....	60
2.2.4 RNA Isolation and Northern Blot Analysis.....	61
2.2.5 Real-time Quantitative PCR.....	61
2.2.6 Statistical Analysis.....	62
2.3 Results.....	62
2.4 Discussion.....	74
2.5 Future Studies	81
2.6 References.....	83
 Chapter 3.....	 91
3.1 Introduction.....	92
3.2 Materials and Methods.....	95
3.2.1 Hypobaric Hypoxic Experiments	95
3.2.2 Determination of Right Ventricular Developed Pressure.....	95
3.2.3 Determination of Hematocrit.....	96
3.2.4 Mitochondrial Isolation and Functional Characterization.....	96
3.2.5 RNA Isolation and Northern Blot Analysis.....	98
3.2.6 Protein Immunoblotting Analysis.....	98
3.2.7 Statistical Analysis.....	99

3.3 Results.....	99
3.4 Discussion.....	109
3.5 References.....	114
 Chapter 4.....	 118
4.1 Introduction.....	119
4.2 Materials and Methods.....	121
4.2.1 Mice.....	121
4.2.2 Hypobaric Hypoxic Experiments.....	122
4.2.3 RNA Isolation and Northern Blot Analysis.....	122
4.2.4 Nuclear Protein Extraction.....	123
4.2.5 Protein Immunoblotting Analysis.....	124
4.2.6 CAT Activity Assay.....	125
4.2.7 Statistical Analysis.....	125
4.3 Results.....	125
4.4 Discussion.....	137
4.5 References.....	141
 Chapter 5.....	 146
5.1 Conclusion.....	146
5.2 References.....	150
5.3 Publications.....	150

Chapter 1

General Introduction

University of Cape Town

The normal heart has a high demand for energy that is predominantly supplied by ATP generated from the breakdown of fatty acids (FA), glucose and lactate [Bing et al., 1953]. Fatty acids are the major energy source for the normal, adult mammalian heart, providing approximately 60-80% of its energy requirements [Opie, 1968; Neely and Morgan, 1974; van Bilsen, 1998]. The rest is derived from glucose and lactate in equal proportions [van Bilsen, 1998]. This fuel supply is utilized by the heart to generate adequate ATP to ensure maintenance of essential cellular functions such as cardiac contraction, Ca^{2+} and $\text{Na}^{+}\text{-K}^{+}$ pump activity, and protein synthesis [Opie, 1998].

1.1 FATTY ACID METABOLISM

Fatty acids are stored as triacylglycerol reserves in adipose tissue and are transported to the myocardium in numerous ways i.e. (a) bound to albumin, (b) as triglycerides (bound to apolipoproteins in very low-density lipoproteins [VLDL].), or (c) contained in chylomicrons [Kantor et al., 2001]. VLDL and chylomicrons are hydrolyzed by lipoprotein lipase, releasing FAs for uptake by the heart [Kantor et al., 2001]. There are several FA transport proteins, including the CD36 homolog, fatty acid translocase (FAT), fatty acid transport proteins (FATPs) and plasma membrane fatty acid binding [Kantor et al., 2001]. The heart-type fatty acid binding protein (H-FABP) is then responsible for the transport of FAs to subcellular localizations such as the mitochondrion (Fig. 1) [Kantor et al., 2001].

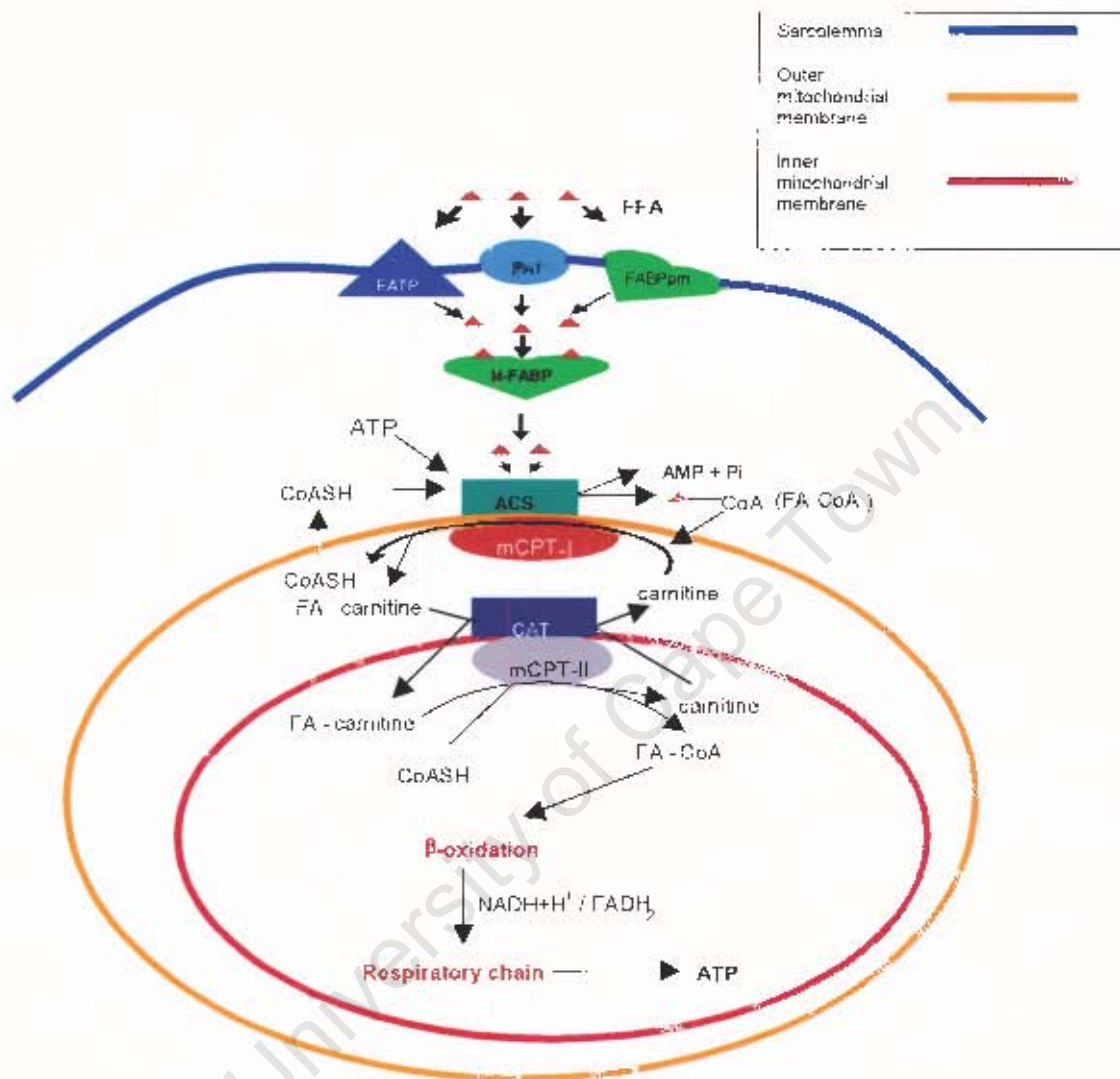


Figure 1: Simplified overview of fatty acid uptake and metabolism in the normal myocyte. Abbreviations: free fatty acids (FFA), fatty acid transport protein (FATP), fatty acid translocator (FAT), plasma membrane fatty acid binding protein (FABP_{pm}), heart-type fatty acid binding protein (H-FABP), muscle-type carnitine palmitoyl transferase I (mCPT-I), muscle-type carnitine palmitoyl transferase II (mCPT-II), carnitine acyl translocase (CAT), acyl-CoA synthetase (ACS), coenzyme A (CoASH).

Before entering the mitochondrion, FAs are activated by acyl-CoA synthetase (ACS), involving the substitution of a FA carboxylic acid terminus with a CoA thioester. Upon activation, fatty acyl-CoA's (FA-CoA) are ready to be transported into the mitochondrial matrix via the carnitine palmitoyl shuttle. The rate-limiting mitochondrial transfer enzyme, carnitine palmitoyltransferase-I (CPT-I) catalyzes the initial step in the import of FA-CoA into the mitochondrion by converting it to acyl carnitine. Two CPT-I isoforms have been described i.e. the liver-enriched, I-CPT-I, and the muscle-isoform, mCPT-I [Brown et al., 1995]. The latter isoform is predominantly expressed in tissues with high capacity for FA oxidation i.e. skeletal muscle, cardiac and brown adipose tissue. Acylcarnitines produced by this reaction are then transported across the inner mitochondrial membrane by carnitine acyl translocase (CAT). Inside the mitochondrial matrix, CPT-II converts acylcarnitine to FA-CoA, whilst the carnitine is shuttled back to the inter-mitochondrial membrane space by CAT (Fig. 1).

Inside the mitochondrial matrix, the imported FA-CoA undergoes β -oxidation via four enzymatic steps that make up the FA β -oxidation (FAO) spiral. Long-chain acyl-CoA dehydrogenase (LCAD) or medium-chain acyl-CoA dehydrogenase (MCAD) catalyzes the initial FAO step of long and medium chain FAs, respectively. The last three enzymatic steps are catalyzed by enoyl-CoA hydratase, L-3-hydroxyacyl CoA dehydrogenase and 3-oxoacyl-CoA thiolase, respectively. These reactions result in the sequential removal of acetyl-CoA from the FA-CoA. Furthermore, the MCAD, LCAD and L-3-hydroxyacyl CoA dehydrogenase steps all produce $\text{NADH} + \text{H}^+$ and FADH_2 .

reducing equivalents. After one cycle, the shortened acyl residue re-enters the FAO spiral and the cycle repeats until it is shortened to acetyl-CoA. Therefore, the final products of the β -oxidation spiral are acetyl-CoA and the reducing equivalents, $\text{NADH} + \text{H}^+$ and FADH_2 . Acetyl-CoA is further oxidized in the Krebs cycle yielding ATP and additional reducing equivalents, all which subsequently enter the mitochondrial respiratory chain for the production of ATP (Fig. 1).

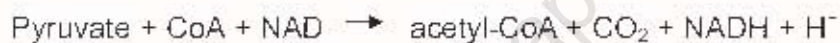
1.2 GLUCOSE METABOLISM

Glucose is the second major fuel substrate used by the heart and is transported across the sarcolemma by various glucose transporters (GLUTs) (Fig. 2) [Van Bilsen, 1998; Postic et al., 1994]. In the heart, the constitutively expressed (fetal) isoform, GLUT1, and the insulin-responsive transporter, GLUT4, are suggested to be the major glucose transporters [Postic et al., 1994].

Following uptake, glucose is catabolized to pyruvate in the cytosol. Glycolysis is initiated by the non-reversible reaction catalyzed by hexokinase (HK), phosphorylating glucose to glucose-6-phosphate which is later converted to fructose-6-phosphate by phosphofructokinase (PFK). This is the primary, irreversible step of glycolysis since PFK operates at the junction between glucose breakdown (glycolysis) and glucose storage (glycogenesis). Fructose biphosphate aldolase breaks down fructose-1, 6-bisphosphate (PFK product) into 2 x glyceraldehyde-3-phosphate, while glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate (Fig. 2).

Sequential reactions catalyzed by phosphoglycerate kinase, phosphoglyceromutase and enolase ultimately result in the production of 2 x phosphoenolpyruvate (PEP). Pyruvate kinase converts PEP to 2 x pyruvate that in turn is converted to acetyl-CoA by the rate-limiting pyruvate dehydrogenase (PDH) complex. PDH complex comprises 3 enzymes that mediate decarboxylation, acetylation and dehydrogenation of pyruvate in its passage into the mitochondrial matrix. It constitutes the rate limiting step for oxidation of glucose by the TCA cycle, catalyzing the reaction:



Acetyl-CoA subsequently enters the Krebs cycle resulting in the production of reducing equivalents, NADH + H⁺ and FADH₂. These in turn enter the respiratory chain complex leading to ATP production required to sustain normal cardiomyocyte function.

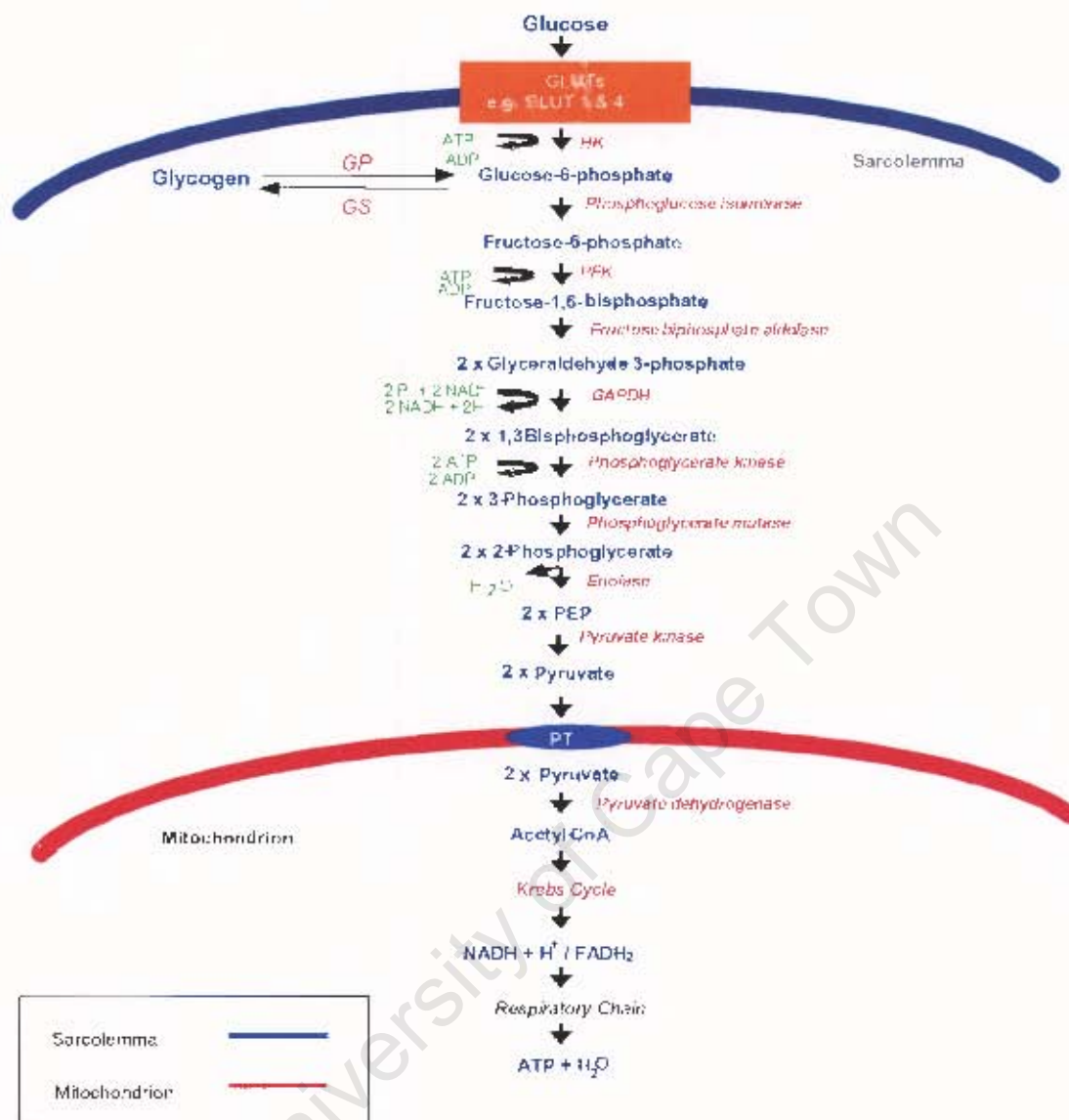


Figure 2: Simplified overview of glucose metabolism. Abbreviations: glucose transporter (GLUT), hexokinase (HK), phosphofructokinase (PFK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoenol pyruvate (PEP), pyruvate transporter (PT), glycogen synthase (GS), glycogen phosphorylase (GP).

1.3 OXIDATIVE PHOSPHORYLATION

Electron transport and oxidative phosphorylation are coupled reactions that result in the synthesis of ATP via the respiratory chain. As $\text{NADH} + \text{H}^+$ and FADH_2 produced by glucose and FA metabolic reactions are oxidized,

electrons pass through the respiratory chain complex until they are donated to oxygen, ultimately forming water (Fig. 3).

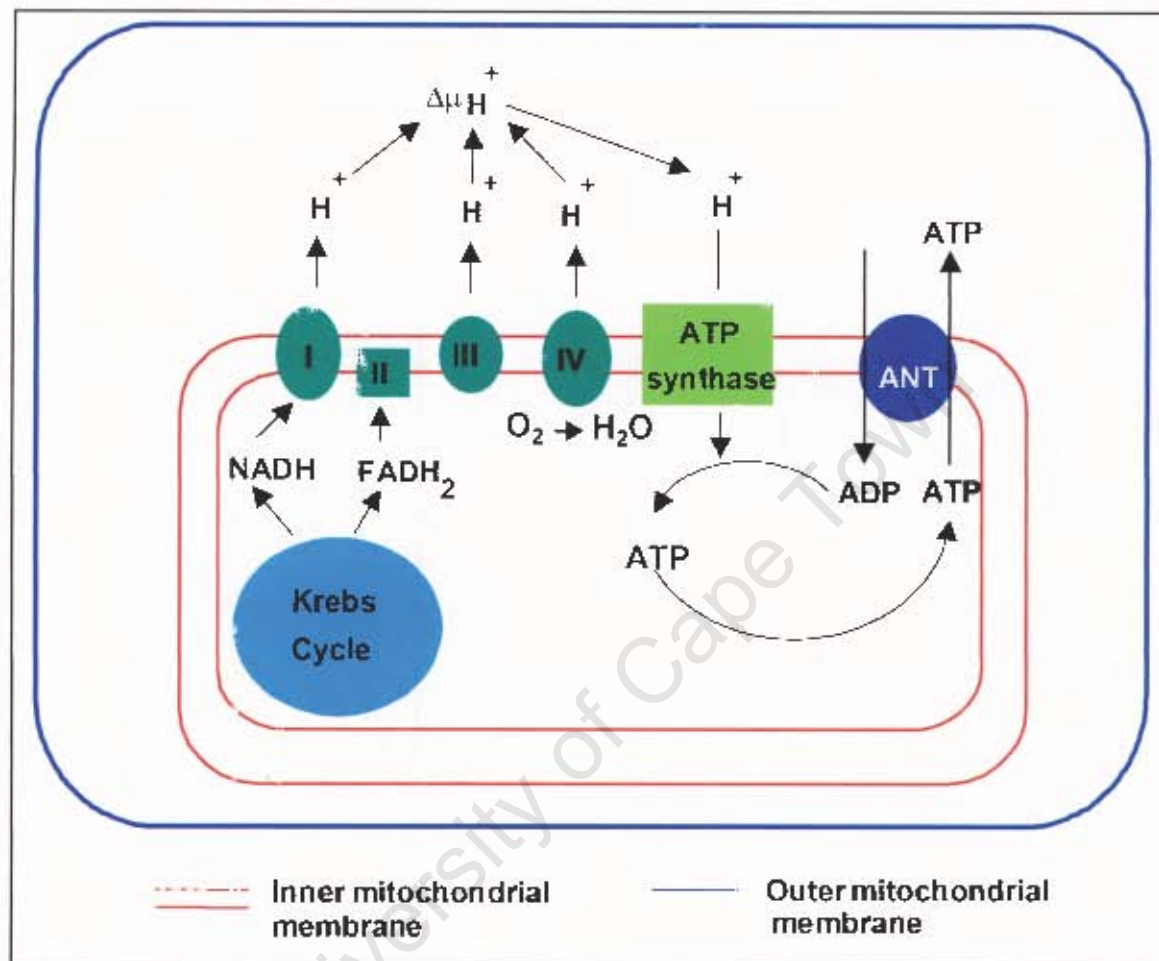


Figure 3: Simplified representation of oxidative phosphorylation. Abbreviations: electrochemical gradient across inner mitochondrial membrane ($\Delta\mu\text{H}^+$) adenine nucleotide translocase (ANT).

Briefly, the respiratory chain consists of four protein complexes (I, II, III, IV), and ATP synthase that span the inner-mitochondrial membrane. Electrons are carried from complex I (NADH-CoQ reductase) and complex II (succinate-coenzyme Q reductase) to complex III (CoQ-cytochrome c reductase) by coenzyme Q. Cytochrome c carries electrons from complex III to complex IV

(cytochrome c oxidase). Complex IV catalyzes the one-electron oxidation of four reduced cytochrome c molecules and concomitantly the four electron-reduction of one oxygen molecule, the final acceptor of electron transport.

This movement of electrons is linked to the extrusion of protons from the mitochondrial respiratory chain complexes into the inter-mitochondrial membrane space. This results in an electrochemical gradient forming across the inner membrane that is used to drive the phosphorylation of ADP to ATP, catalyzed by ATP synthase. In order for oxidative phosphorylation to occur, a continuous supply of ADP is essential. Since the inner mitochondrial membrane is impermeable to ATP and ADP, an exchange reaction is performed by an ATP-ADP translocase, ANT (adenine nucleotide translocase) that exports ATP to the cytosol while importing ADP into the mitochondrial matrix.

Sections 1.1-1.3 summarized the major fuel substrate pathways ultimately leading to the production of mitochondrial ATP in the heart. The present study focussed on the regulation of hypoxia in response to hypoxia. Transcription factors regulating cardiac fatty acid and glucose enzyme-encoding genes were investigated.

The cardiac fatty acid utilization pathways are regulated at multiple levels. For instance, it is well known that malonyl-CoA, the end-product of the first committed step in FA synthesis, inhibits CPT1 activity via an allosteric interaction [McGarry et al., 1989]. However the capacity for FA catabolism is

also exquisitely regulated at the level of gene transcription [Brown et al., 1995]. Moreover, acute or chronic changes in energy supply and demand, such as diabetes mellitus or starvation, also affect the expression of the key genes in fatty acid uptake and oxidation [Leone et al., 1999; Depre et al., 2000]. Furthermore the expression of genes encoding FA utilization enzymes is regulated, at least in part, at the level of transcription [Brown et al., 1995]. Interestingly, upstream DNA sequences termed peroxisome proliferator response elements (PPRE) are required for the transcriptional induction of these genes [Kelly et al., 1989]. PPREs are binding sites of peroxisome proliferator-activated receptors (PPARs), transcription factors that belong to the nuclear receptor superfamily for e.g PPAR α , β/δ and γ [Kelly et al., 1989]. After activation of PPARs by its ligands (i.e fibrates, thiazolidinediones, polyunsaturated FA), they heterodimerize with retinoid X receptors (RXR) and bind to PPREs, thereby exerting their effects on gene expression [Kliwer et al., 1992; Barger and Kelly, 2000]. Among PPAR α , β/δ and γ , the α receptor is abundantly expressed in the heart in rodents [Kliwer et al., 1994]. Barger and coworkers have revealed that the expression of most cardiac FA import and oxidation genes is regulated by the ligand-activated transcription factor, PPAR α [Barger and Kelly, 2000]. These findings suggest that PPAR α is a major regulator of myocardial fatty acid utilization pathways and that its reduced activity might be responsible for the downregulated expression of genes encoding FA utilization enzymes.

The transcription rates of glycolytic enzyme genes are co-ordinately induced in muscle cells subjected to hypoxia [Webster, 1987]. Studies have shown

that the hypoxia-inducible factor 1 (HIF-1) [Webster et al., 1988], originally identified as a factor in the regulation of erythropoietin [Bunn and Poyton, 1996], has been shown to play a role in the transcription of a number of genes by hypoxia [Firth et al., 1994; Semenza et al., 1994]. HIF-1 binds to an enhancer element containing the core sequence ACCTGC that is an obligatory and minimal component of HIF-1 response genes. The promoter elements of the Sp family (i.e Sp1, Sp3) is GC rich [Boulikas, 1994], making them part of hypoxia regulatory mechanism. Sp1 is a transcription activator whilst Sp3 can be either a repressor or activator depending on the promoter context as well as cellular background [Denning et al., 1996; Birnbaum et al., 1995]. Denning et al. (1996) have suggested that the repressor function of Sp3 is stronger there are multiple Sp1/Sp3 binding sites [Denning et al., 1996].

1.4 CARDIAC METABOLIC REMODELING

Fuel substrate utilization by the heart is dynamic and changes according to developmental status, substrate supply and pathophysiologic states [Reviewed, Sack and Kelly, 1998; Stanley and Chandler, 2002]. The term "metabolic remodeling" has been defined as a switch in cardiac substrate use in association with cardiogenesis and with the development of pathophysiologic conditions [van Bilsen et al., 1998; Stanley and Chandler, 2002]. Moreover, the metabolic remodeling concept may be extended to include dynamic switches in metabolic fuel utilization due to alterations in substrate availability during fed or fasted states. Examples of chronic cardiac metabolic remodeling include the diabetic and hypertrophied hearts that rely

on elevated FA and glucose utilization, respectively as primary energy sources [van Bilsen et al., 1998]. The functional role of metabolic remodeling in the development of cardiac disease is not clearly understood and is currently being investigated. I will now review two instances of metabolic remodeling i.e. (a) during development (fetal to adult) and (b) with cardiac hypertrophy and heart failure.

1.4.1 Cardiac Metabolic Remodeling: Developmental

The fetal myocardium functions in a relatively hypoxic environment with elevated lactate concentrations in the placenta, therefore relying mostly on glycolysis and lactate oxidation as sources for energy production [Lopaschuk et al., 1992; Makinde et al., 1998].

In the newborn heart, glycolysis and glucose oxidation remain the chief energy generating processes responsible for ATP production [Makinde et al., 1998]. In fact, both fetal and newborn rabbit hearts have a greater capacity to tolerate oxygen deprivation compared to the adult heart, which is suggested to be due to a greater anaerobic glycolytic capacity [Rolph and Jones, 1983; Lopaschuk et al., 1992]. Glucose utilization in this instance is suggested to be beneficial since it is a more oxygen-efficient fuel, producing 6-6.3 moles ATP per mole of oxygen oxidized compared to 5.6 moles ATP when FAs are utilized [Hochachka, 1985; Lundby and van Hall, 2002]. In agreement, studies on the high-altitude adult dwelling Quechuas and Sherpas have shown that glucose is a true metabolic defence adaptation in these chronic hypoxic conditions [Holden et al., 1995; Hochachka et al., 1999].

During hypoxia, mammalian cells enhance glycolytic capacity by increasing the expression of genes involved in glycolysis and glucose transport [Semenza et al., 1994; Ebert et al., 1995; Semenza et al., 1996; Semenza, 1998]. For example, hypoxia may increase glycolytic gene expression by stabilizing the redox-sensitive transcription factor, hypoxia-inducible factor-1 α (HIF-1 α) that is usually degraded during normoxic conditions. HIF-1 α in turn forms a heterodimer with HIF-1 β , resulting in its translocation to the nucleus where it binds to hypoxia-response promoter elements [Zhu and Bunn, 2001]. This may lead to the activation of several glucose transport and utilization genes for e.g. GLUT1 and phosphoglycerate kinase [Loike et al., 1992; Semenza et al., 1994]. Conversely, hypoxia diminishes expression of key FAO metabolic enzyme-encoding genes. For example, a recent *in vitro* study demonstrated that hypoxia prevented the known FA-induced induction of mCPT-I mRNA levels [Huss et al., 2001]. This reduction of mCPT-I should result in decreased FA uptake and FAO [Huss et al., 2001]. In agreement, an *in vivo* study on rats exposed to two different models of hypoxia (CoCl₂ and isovolemic hemodilution) demonstrated a reduction in the gene expression of several FA utilization enzyme-encoding genes [Razeghi et al., 2001a]. Here, rats received COCl₂ in their drinking H₂O [Badr et al., 1999]. Cobalt is a transition metal that replaces iron in heme protein. In contrast to iron, cobalt does not bind oxygen when incorporated into protoporphyrin [Goldberg et al., 1988]. When iron is replaced by cobalt, this oxygen sensor signals to the cell that there is a state of oxygen deficiency despite the normal oxygen levels. On the other hand, iso-volemic hemodilution was induced by bleeding rats,

followed by retransfusion of a plasma/saline solution. Anemia is characterized by a reduced oxygen binding capacity, resulting in lower tissue oxygenation. Elevated glycolytic capacity is also mediated by augmented activities of several glucose utilization proteins [Rolph and Jones, 1985; Lopaschuk et al., 1992]. For example, the activity of PFK, a key enzyme in the glycolytic pathway, is enhanced in the fetal heart and is less susceptible to inhibitory regulators such as citrate [Bristow et al., 1987; Ascuitto et al., 1989; Lopaschuk et al., 1992]. Moreover, hypoxia increases AMP/ATP and creatine/phosphocreatine (Cr/PCr) ratios, resulting in an increase in 5' adenosine monophosphate-activated protein kinase (AMPK) activity. AMPK activation in turn results in increased GLUT1 and 4 translocation to the cell membrane, leading to augmented glucose uptake during hypoxia [Coven et al., 2003]. Increased AMPK activity also leads to HK and PFK activation, thereby further increasing glycolytic capacity. In agreement with such increased glycolytic and reduced FAO capacity, several studies have reported lower mitochondrial content in fetal hearts [Hallman, 1971; Goodwin et al., 1976; Lopaschuk et al., 1992], and diminished activities of Krebs cycle and electron transport chain enzymes [Dallman and Schwartz, 1964; Glatz and Veerkamp, 1982; Werner et al., 1982; Lopaschuk et al., 1992]. Together, these findings underscore the fact that the relatively hypoxic fetal heart relies primarily on glucose utilization as a source of ATP production [Rolph and Jones, 1985; Lopaschuk et al., 1992].

The developmental shift from carbohydrates to FAs is caused by changes in circulating hormonal and energy substrate levels, expression of enzyme-

encoding genes and metabolic enzyme activities, collectively responsible for increased postnatal FA utilization and β -oxidation [Makinde et al., 1998]. During the first week of life, the newborn heart switches energy substrate preference towards FAO since oxygen levels are no longer a limiting factor [Lopaschuk et al., 1992; Makinde et al., 1998]. This fuel substrate switch allows mature isoforms of FAO enzyme-encoding genes to be expressed shortly after birth [Ascuitto et al., 1989; Lopaschuk et al., 1990; Lopaschuk et al., 1991; Lopaschuk et al., 1992]. Interestingly, levels of triiodothyronine (T3) increase significantly in the early newborn period. T3 has been shown to significantly increase basal metabolic rates, and induce the expression of some key enzymes involved in oxidative metabolism. The importance of increased T3 and its effect on energy substrate metabolism in the immediate newborn period remains to be determined [Makinde et al., 1998]. Furthermore, systemic insulin levels decrease postnatally and the constitutively active glucose transporter isoform, GLUT1, is replaced by the insulin-sensitive isoform GLUT4. This results in glucose transport becoming the rate-limiting step in myocardial glucose utilization [Santalucia et al., 1992; Montessuit et al., 2000]. Moreover, reduced postnatal circulating insulin levels result in the activation of AMPK [Makinde et al., 1998]. that in turn phosphorylates and inhibits its downstream target, acetyl-CoA carboxylase (ACC), the enzyme that catalyzes the production of malonyl-CoA, a potent inhibitor of CPT-I (see Fig. 4). Makinde et al. (1996) have shown that high levels of insulin inhibit AMPK activity, suggesting that a decrease in insulin levels *in vivo* has the potential to stimulate the observed AMPK activity [Makinde et al., 1997]. Moreover, AMPK activity can be stimulated

pharmacologically with 5-amino-4-imidazole carboxamide ribotide in 7 day old rabbit hearts [Makinde et al., 1996]. This results in a decrease in ACC activity and an increase in FAO rates. It therefore appears that AMPK activity is under hormonal control.

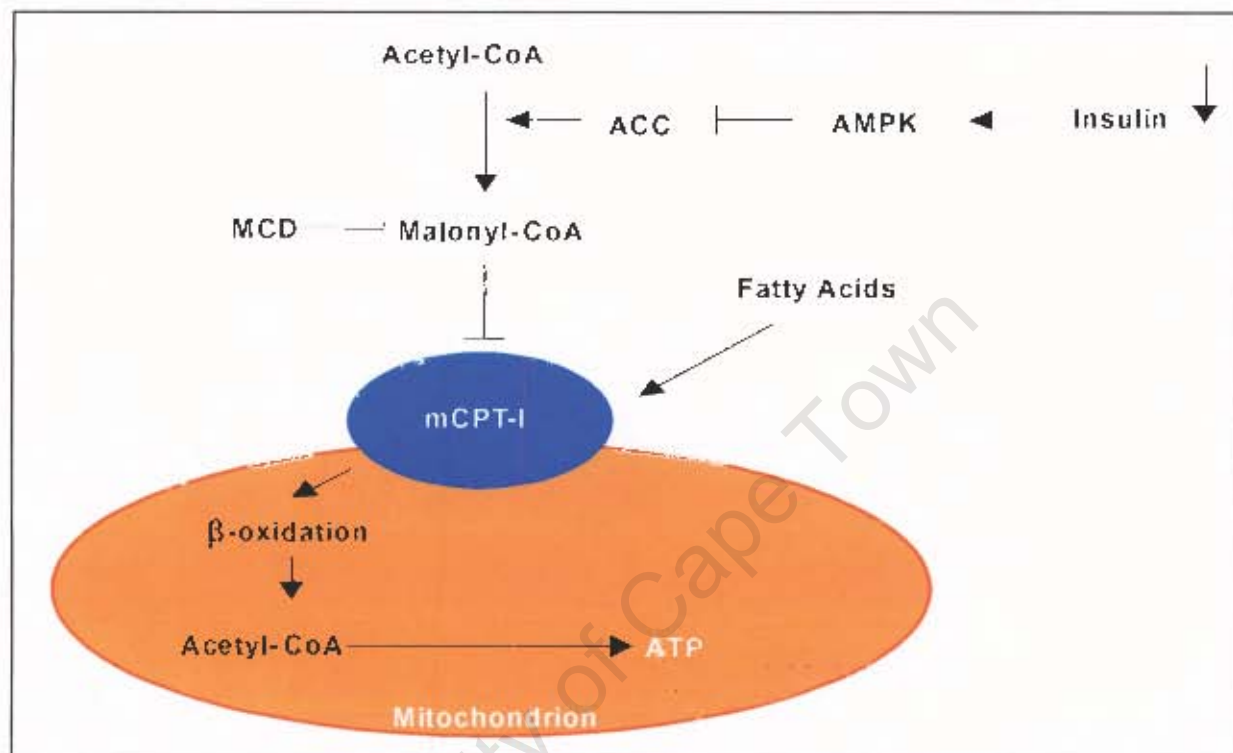


Figure 4: Upstream regulation of FA uptake via the mCPT-I shuttle in the mature heart. Abbreviations: acetyl-CoA carboxylase (ACC), muscle-type carnitine palmitoyl transferase-I (mCPT-I), 5' adenosine monophosphate-activated protein kinase (AMPK), malonyl-CoA decarboxylase (MCD).

Thus, the inhibition of ACC results in a decrease in malonyl-CoA levels thereby relieving the inhibitory effect on CPT-I [Makinde et al., 1998]. The release of CPT-I inhibition by malonyl-CoA leads to augmented transport of activated FAs into the mitochondrion, with a resultant increase in mitochondrial FAO [Makinde et al., 1998; Kantor et al., 1999].

In summary, the fuel substrate switches that occur during development (or physiologic metabolic remodeling) underscore (a) the omnivorous nature of the mammalian heart i.e. being able to select specific substrates at different stages of development, and also highlight (b) the complex, multiple regulatory steps required to rapidly and efficiently switch from one fuel substrate to another.

1.4.2 Cardiac Metabolic Remodeling: Cardiac Hypertrophy and Heart Failure

In an early attempt to establish the temporal onset of metabolic remodeling in heart disease, Taegtmeyer and Overton (1988) demonstrated that chronic mild hypertension in rabbits resulted in enhanced glycolytic enzyme activity under conditions that did not cause appreciable cardiac hypertrophy as measured by indexed heart weights [Taegtmeyer and Overton, 1988]. Although this is an insensitive measure of cardiac hypertrophy, these data suggested that cardiac metabolic remodeling may precede the development of hypertrophy and hence may be implicated in the development of this cardiac phenotype (cardiac growth). In a further study, Allard and coworkers (1994) evaluated functional substrate use by determining palmitate oxidation in isolated perfused hearts from sham operated and aortic-banded rats. Here, palmitate oxidation was almost halved in hypertrophic hearts [Allard et al., 1994]. These data were paralleled by *in vitro* studies demonstrating that palmitate oxidation was decreased in myocytes isolated from hypertrophic hearts compared to control hearts [Torielli et al., 1995].

In agreement, a number of studies employing animal models have shown that with severe hypertrophy, glycolysis and lactate (and sometimes glucose) oxidation increase with a concomitant decrease in FAO rates, representing a reversion to the fetal pattern of energy metabolism [Fisher, 1984; El Alaoui-Talibi et al., 1992; Allard et al., 1994; Christe and Rodgers, 1994; Kantor et al., 1999; Paternostro et al., 1999; Remondino et al., 2000].

Hypertrophy is usually characterized by an increase in energy demand (due to higher cardiac output required) and a decrease in energy supply (switch to fetal metabolic pattern), resulting in high AMP/ATP and decreased PCr/Cr ratios. These altered high-energy phosphate ratios may increase AMPK expression and enzyme activity in response to hypertrophy [Tian et al., 2001]. For example, Tian et al. (2001) demonstrated that high AMPK levels observed during left ventricular hypertrophy (LVH) are accompanied by elevated rates of glucose uptake and increased distribution of glucose transporters on the plasma membrane. These data suggest that increased AMPK activity in LVH may contribute to enhanced glucose uptake in these hearts [Tian et al., 2001]. In agreement, a similar phenomenon has been shown in various experimental models of cardiac hypertrophy i.e. increased myocardial glucose uptake is associated with elevated GLUT1 expression and a decrease / no change in GLUT4 levels [Weinberg et al., 1995; Paternostro et al., 1995; Abel et al., 1999; Allard et al., 2000; Sambandam et al., 2002].

Augmented glucose uptake in hypertrophied hearts is usually matched by elevated glycolytic rates [Allard et al., 1994; Schonekess et al., 1996; El

Alaoui-Talibi et al., 1997; Wambolt et al., 1999; Sambandam et al., 2002]. Such an increase in glycolytic rates may be due to the observed activation of several glycolytic enzymes in hearts exposed to pressure overload [Sambandam et al., 2002]. It has been suggested that augmented glycolysis in these instances may compensate for lower FAO in hypertrophied hearts [Allard et al., 1994; Al Alaoui-Talibi et al., 1997; Sambandam et al., 2002]. Increased heart work brought by hypertension results in an enzymatic shift favouring the oxidation of glucose over FA [Taegtmeyer and Overturn, 1988]. Increased heart work has been reported to increase intraventricular pressure development resulting in the increase in consumption of oxygen and glucose. Glucagon has been reported to be a significant source of glucose during low flow ischemia and possibly hypertrophy [Schonekess et al., 1997]. The mechanism of how the heart can have increase glycolytic rates and glycolytic enzymes is not clear.

An increase in ventricular pressure development accelerates the uptake and oxidation of glucose. More rapid rates of glucose transport and phosphorylation account for the faster glucose uptake. Transport of glucose and non-metabolised sugars is accelerated by higher ventricular pressure development, but measurable levels of intracellular free glucose do not accumulate indicating increased glycolytic flux. A 4-fold increase in glycolytic flux is accompanied by an increase in fructose-1-6-P but no glucose-6-phosphate, indicating that phosphofructokinase is activated. Since depletion of ATP and accumulation of ADP/ AMP occur in hypertrophied hearts, AMPK is the mechanism by which phosphofructokinase is increased during

hypertrophy [Morgan, 2003]. For example, AMPK is activated when oxygen supply is limited for e.g in hypoxia, ischemia and hypertrophy etc. In the heart, AMPK is involved in phosphorylating and activating 6-phosphofructokinase (PFK - 2), the enzyme responsible for the synthesis of fructose 2,6 bisphosphate, a potent stimulator of glycolysis. AMPK mediated PFK-2 activation is likely to be involved in the stimulation of glycolysis during ischemia and possibly hypertrophy. [Marsin et al, 2000].

However, glucose is catabolized by two tightly linked processes i.e. glycolysis and glucose oxidation (Fig.2) [Neely and Morgan, 1974; Stanley et al., 1997; Depre et al., 1999; Wambolt et al., 2000; Lydell et al., 2002]. Although glycolytic rates are increased in the hypertrophied heart, glucose oxidation is not always accordingly elevated, sometimes causing a dissociation between glycolysis and glucose oxidation [Allard et al., 1994; El Alaoui-Talibi et al., 1997; Wambolt et al., 2000; Lydell et al., 2002]. This dissociation has been proposed to result in diminished ATP production in the hypertrophied heart [Lydell et al., 2002]. Anaerobic glycolysis involves further commitment of pyruvate to lactate formation and the generation of ATP and NADH when oxygen is unavailable and mitochondrial uptake is forestalled [Kantor et al., 2001]. Randle and Morgan have established that the ensuing increase in glycolytic flux is governed by an acceleration of glucose transport into the cell and an increase in the conversion of fructose-6-phosphate to fructose 1,6-bisphosphate [Randle and Morgan, 1962, Kantor et al., 2001]. This is a reaction catalyzed by phosphofructo-1-kinase (PFK-1) [Dunaway et al., 1988]; Kantor et al., 2001]. PFK-1 is therefore a pivotal enzyme in the

hypertrophy [Morgan, 2003]. For example, AMPK is activated when oxygen supply is limited for e.g in hypoxia, ischemia and hypertrophy etc. In the heart, AMPK is involved in phosphorylating and activating 6-phosphofructokinase (PFK - 2), the enzyme responsible for the synthesis of fructose 2,6 bisphosphate, a potent stimulator of glycolysis. AMPK mediated PFK-2 activation is likely to be involved in the stimulation of glycolysis during ischemia and possibly hypertrophy. [Marsin et al, 2000].

However, glucose is catabolized by two tightly linked processes i.e. glycolysis and glucose oxidation (Fig.2) [Neely and Morgan, 1974; Stanley et al., 1997; Depre et al., 1999; Wambolt et al., 2000; Lydell et al., 2002]. Although glycolytic rates are increased in the hypertrophied heart, glucose oxidation is not always accordingly elevated, sometimes causing a dissociation between glycolysis and glucose oxidation [Allard et al., 1994; El Alaoui-Talibi et al., 1997; Wambolt et al., 2000; Lydell et al., 2002]. This dissociation has been proposed to result in diminished ATP production in the hypertrophied heart [Lydell et al., 2002]. Anaerobic glycolysis involves further commitment of pyruvate to lactate formation and the generation of ATP and NADH when oxygen is unavailable and mitochondrial uptake is forestalled [Kantor et al., 2001]. Randle and Morgan have established that the ensuing increase in glycolytic flux is governed by an acceleration of glucose transport into the cell and an increase in the conversion of fructose-6-phosphate to fructose 1,6-bisphosphate [Randle and Morgan, 1962, Kantor et al., 2001]. This is a reaction catalyzed by phosphofructo-1-kinase (PFK-1) [Dunaway et al., 1988]; Kantor et al., 2001]. PFK-1 is therefore a pivotal enzyme in the

glycolytic pathway [Kantor et al., 2001]. It operates at the junction of pathways of glucose degradation (glycolysis) or storage (glycogenesis). In addition, glucose-6-phosphate the reaction product, is in flux equilibrium to fructose 6-phosphate, which becomes the substrate for the "primary irreversible" reaction of the glycolytic pathway: the phosphorylation of fructose 6-phosphate by phosphofructokinase (PFK) [Kantor et al., 2001].

The importance of the AMPK mediated PFK-2 activation [Marsin et al, 2000] discussed above has been implicated in AMPK mutation studies. AMPK provides a central sensing mechanism that protects cells from exhaustion of ATP supplies. Blair et al. (2001) have mutations, one missense and one in-frame single codon insertion, occur in highly conserved regions of AMPK [Blair et al., 2001]. This group proposed that their AMPK mutation data substantiate energy compromise as a unifying pathogenic mechanism in all forms of hypertrophic cardiomyopathy (HCM) which is a widely studied as a genetic model of cardiac hypertrophy and sudden cardiac death [Blair et al., 2001]. Furthermore, during severe hypertrophy (preceding ventricular dilatation), glucose uptake has been shown to be significantly diminished in rabbit hearts [Friebs et al., 1999]. Since the hypertrophied heart appears to depend primarily on glucose for energy, glucose transport impairment may be a significant contributing factor towards the progression to heart failure in rabbits [Friebs et al., 1999]. In support, both GLUT1 and GLUT4 expression were shown to be downregulated in the failing human heart [Razeghi et al., 2002; Taegtmeyer, 2002]. Also, the relative contribution of glucose oxidation to energy production largely depends on PDH regulation. Interestingly, PDH

activity was found to be decreased with the development of compensated hypertrophy, suggesting that myocardial substrate delivery to the mitochondria may be impaired in hypertrophy, which may lead to the depleted energetic state observed with heart failure [Seymour and Chatham, 1997].

As previously discussed, the heart switches from FAs to glucose with the onset of cardiac hypertrophy and heart failure [Taegtmeyer and Overhof, 1988; Sack et al., 1996; Barger and Kelly, 1999]. However, this fuel substrate switch also includes decreased expression of mitochondrial FAO genes [de las Fuentes et al., 2003]. Tight coupling between cardiac work and substrate oxidation is a central feature of cardiac physiology. For example, the normal heart has a high demand for energy that is predominantly supplied by ATP generated from the breakdown of fatty acids, glucose and lactate [Bing et al., 1953]. Fatty acids are the major energy source for the normal, adult mammalian heart, providing approximately 60-80% of its energy requirements [Opie, 1968; Neely and Morgan, 1974; van Bilsen, 1998]. Therefore, the fuel substrate switch from fatty acids to glucose resulting in decreased expression of mitochondrial FAO genes may be the cause of the onset of cardiac hypertrophy and heart failure. Moreover, previous studies have shown that mRNA levels of rate-limiting FAO enzymes like mCPT-1 and MCAD were downregulated during left ventricular hypertrophy (LVH) and heart failure in spontaneous hypertensive and heart failure (SHHF) rats [Sack et al., 1996; Barger and Kelly, 1999; Remodino et al., 2000; Osorio et al., 2002]. These studies have also demonstrated, that in this instance, transcriptional repressors such as Sp1, Sp3 and chicken ovalbumin upstream promoter-

transcription factor (COUP-TF) are increased, resulting in the attenuated cardiac MCAD and CPT-1 expression [Sack et al., 1997; Barger and Kelly, 1999; Lehman and Kelly, 2002]. On the other hand, expression of the FA-induced transcriptional regulator PPAR α , a key activator of several FA utilization enzyme-encoding genes, is reduced during hypertrophy [Barger and Kelly, 2000; Barger et al., 2000; Kelly, 2002; Jamshidi et al., 2002; Finck and Kelly, 2002; Lehman and Kelly, 2002]. After activation of PPAR α by its ligands (i.e. fatty acids, Wy 14, 643), it dimerizes with retinoid X receptor (RXR) and binds to the peroxisome proliferator-activated receptor elements (PPREs), thereby exerting its effects on gene expression [Kliwer et al., 1992]. When fatty acids are available they act as ligands for PPAR α , the major regulator of myocardial fatty acid utilization pathways and that its reduced activity might be responsible for the downregulated expression of genes encoding fatty acid utilization enzymes. Moreover, a recent study has reported a decrease in RXR α , the obligatory heterodimeric partner of PPAR α , in the failing dog heart [Osorio et al., 2002]. Such downregulation of key FA utilization enzyme-encoding genes with hypertrophy and heart failure may explain why AMPK activation during hypertrophy is unable to increase FAO in this instance [Tian et al., 2001].

Razeghi and co-workers (2001b) investigated the hypothesis (based on several animal studies) that gene expression in the failing heart reverts to a fetal pattern in humans by characterizing the expression profile of several metabolic genes in heart failure patients [Razeghi et al., 2001b]. They showed that the fetal and failing human heart share similarities in their

expression profile, including atrial natriuretic factor (ANF), myosin heavy chain- α (MHC- α), MHC- β , and other regulators of energy metabolism. However, the human heart does not undergo a true switch to a fetal-like expression as observed in the rat heart. Here, metabolic genes are constitutively expressed or induced during the transition from the fetal to adult stage. Furthermore, they demonstrated that the failing human heart reverts to a fetal metabolic gene profile by downregulating adult gene transcripts rather than by the induction of fetal gene transcripts. These observations therefore challenge the prevailing idea that the hypertrophied and failing human heart reactivate the fetal gene program by upregulating fetal genes [Razeghi et al., 2001b].

In agreement with previously discussed gene studies, Sack et al. (1996) described the downregulation of key FAO enzymes in patients with severe heart failure undergoing transplantation [Sack et al., 1996]. It has been proposed that restricted FAO may lead to a lack of energy, ultimately compromising myocardial function [Lerch, 2001]. However, this hypothesis of diminished FAO rates with heart failure was challenged by data from a clinical study on patients with NYHA class II-III heart failure [Paolisso et al., 1994]. Here, heart failure patients displayed a 50% increase in lipid and 60% decrease in carbohydrate oxidation [Paolisso et al., 1994]. Paolisso et al. (1994) argued that deterioration of function in the failing heart may in fact be triggered by high rather than low FAO [Paolisso et al., 1994]. In agreement, some pharmacological studies have presented additional evidence that the inhibition of FAO in the failing heart may improve mechanical function and

prevent the progression to heart failure [D'hahan et al., 1997; Turcani and Rupp, 1997; Sabbah et al., 2000; Schmidt-Schweda and Holubarsch, 2000; Sabbah et al., 2001]. For example, Bristow et al. (2000) demonstrated improved cardiac function of failing human hearts when inhibiting FAO using the anti-anginal metabolic agent trimetazidine (TMZ) [Bristow, 2000].

Collectively, these studies suggest that metabolic remodeling may be important in the pathogenesis of cardiac hypertrophy and heart failure but have not yet clearly established its fundamental role in orchestrating the development of these cardiac phenotypes.

1.4.3 Is Metabolic Remodeling During Cardiac Hypertrophy and Heart Failure Beneficial or Detrimental?

1.4.3.1 Cardiac Metabolic Remodeling: Evidence Favoring Detrimental Role

Assessment of the functional consequences of metabolic remodeling may give us further insight into the role of this phenomenon in the development of cardiac disease. Allard et al. (1994), using an aorta-banded rat model of cardiac hypertrophy, have shown that reduced cardiac FAO was not fully compensated for by a corresponding increase in glucose oxidation [Allard et al., 1994]. The normal heart has a high demand for energy that is predominantly supplied by ATP generated from the breakdown of fatty acids, glucose and lactate [Bing et al., 1953]. Fatty acids are the major energy source for the normal adult mammalian heart, providing approximately 60-80

% of its energy requirements [Opie, 1968; Neely and Morgan, 1974; van Bilsen, 1998]. The rest is derived from glucose and lactate in equal proportions [van Bilsen, 1998]. This fuel supply is utilized by the heart to generate adequate ATP to ensure maintenance of essential cellular functions such as cardiac, Ca^{2+} , Na^{+} - K^{+} pump activity and protein synthesis [Opie, 1968]. The feature of cardiac physiology is that there is a coupling between cardiac work and substrate oxidation. Taken together, one could speculate that decreased ATP formation precedes heart failure. Interestingly, workload measurements from the Allard study (1994) suggested a decreased rate of ATP formation, implicating a role for diminished energy production in the transition from hypertrophy to heart failure. In this instance, metabolic remodeling may actually contribute to cardiac dysfunction.

At the mitochondrial level, interesting data has been generated for adenine nucleotide translocator (ANT), the mitochondrial ADP:ATP transporter suggesting that altered ATP production may contribute to cardiac pathology. Studies have been performed to investigate the role of ANT in heart failure for example two studies using the ANT1 knockout mouse have suggested that ANT protein deficiency may be maladaptive, playing a role in the pathogenesis of cardiac hypertrophy and heart failure [Graham et al., 1997; Ning et al., 2000]. For example, Graham et al. (1997) generated transgenic ANT1 knockout mice and demonstrated a severe defect in coupled respiration, mitochondrial myopathy and cardiomyopathy [Graham et al., 1997]. Here they suggested the lack of ANT1 (central role player in oxidative phosphorylation) leads to inhibition of respiration resulting

in decreased mitochondrial energy production, and the development of cardiac hypertrophy. In another study, Esposito et al. (1999) showed that ANT1 deficient mice produced high levels of reactive oxygen species (ROS), which may be detrimental to the heart [Esposito et al., 1999]. Together these studies suggest that lack of ANT1 result in oxidative phosphorylation inhibition, increased oxidative stress disruption of ATP production, eventually resulting in the development of cardiac hypertrophy and heart failure.

Genetic defects in metabolic enzymes also support the hypothesis that metabolic remodeling may contribute to cardiac hypertrophy and heart failure. Several studies have shown that chronic metabolic remodeling due to genetic defects in genes encoding enzymes or transport proteins involved in FAO are associated with the development of cardiomyopathies (dilated and hypertrophic) [reviewed by Kelly and Strauss, 1994]. For example, in subjects with genetic defects in very-long-chain acyl-CoA dehydrogenase (VLCAD), the enzyme that catalyzes the first step in β -oxidation of very long-chain FA metabolism, cardiomyopathy was the most common clinical phenotype detected [Mathur et al., 1999]. Interestingly, a study by Blair et al. (2001) described mutations within the AMPK locus in two families with severe hypertrophic cardiomyopathy (HCM) [Blair et al., 2001]. Furthermore, Gollob et al. (2001) evaluated two families with familial Wolff-Parkinson-White syndrome, a common cause of supraventricular tachycardia, and described a genetic defect within the AMPK locus that is believed to be responsible for the phenotype in these families [Gollob et al., 2001]. These studies support the argument that such genetic defects lead to an energy compromise that may

eventually result in cardiac hypertrophy and heart failure. However, the cardiac phenotype observed in humans harbouring genetic mutations in the gamma regulatory subunit (PRKAGZ) of AMPK is consistent with abnormal glycogen accumulation in the heart. In addition, mutations in gamma 2 and gamma 3 subunit isoforms lead to arrhythmias associated with abnormal glycogen storage in human heart. A glycogen binding domain (GBD) has now been identified in the B subunit. Co expression of truncated beta subunits lacking GBD with alpha and gamma subunits yielded complexes that were active and normally regulated. Hudson and co workers (2003) suggested that the GBD binds glycogen and can lead to abnormal glycogen-containing inclusions when the kinase is over expressed. These may be related to the abnormal glycogen storage bodies seen in heart disease patients with gamma 2 mutations.

These data still do not, however, unequivocally establish a causal role for metabolic remodeling in the development of cardiac hypertrophy and heart failure.

Evaluation of the temporal onset of metabolic remodeling in the context of cardiac muscle disease may give us insight into the role of these metabolic perturbations. In an attempt to define the temporal relationship between cardiac pathophysiology and metabolic remodeling, Sack and colleagues (1996) investigated the correlation between the expression pattern of genes encoding mitochondrial FAO enzymes, protein levels and enzyme activity in cardiac hypertrophy and heart failure in SHHF rats [Sack et al., 1996].

SHHF/Mcc - fa^{cp} (SHHF) is a well characterized rat model with temporal progression from the normal heart to compensated pressure overload - induced left ventricular hypertrophy (LVH) and ultimately to decompensated heart failure (HF). Interestingly, MCAD mRNA was downregulated during hypertrophy and heart failure but MCAD enzyme activity did not decrease significantly until heart failure was evident. These findings suggest that FAO enzyme expression is initially downregulated at the transcriptional level, but that the diminished enzyme activity manifested during heart failure may be an important mechanism by which energy substrate usage is detrimentally changed in the failing heart. These authors did not, however, precisely assess the transition time period, nor cardiac substrate utilization or levels of ATP production.

1.4.3.2 Cardiac Metabolic Remodeling: Evidence Favoring Beneficial Role

Pressure overload-induced hypertrophy has been previously reported to result in decreased PPAR α , mCPT-1 and MCAD expression, and a corresponding increase in glucose utilization [Sack et al., 1996; Barger and Kelly, 1999; Barger et al., 2000; Finck and Kelly, 2002; Jamshidi et al., 2002]. Young et al. (2001) tested whether PPAR α downregulation with hypertrophy is an adaptive or maladaptive event. Here, pressure-overload induced hypertrophied rats were administered WY-14, 643, a specific PPAR α ligand, resulting in PPAR α activation [Young et al., 2001]. As expected, PPAR α activation in hypertrophied rats resulted in increased expression of two PPAR α regulated genes i.e. MCAD and PDK4, resulting in elevated FAO while glucose

oxidation was decreased [Young et al., 2001]. The FA to glucose switch during hypertrophy was therefore prevented by the increase in PPAR α - regulated genes. Interestingly, prevention of this switch led to depressed cardiac power and efficiency of the hypertrophied heart. These data therefore suggest that FA utilization during hypertrophy is detrimental and that the switch to glucose utilization in response to hypertrophy is critical for the maintenance of contractile function in this instance [Young et al., 2001].

An increase in glucose uptake and glycolysis has been reported in both animal models and patients with cardiac hypertrophy and heart failure [Allard et al., 1994; Bishop and Altschuld, 1970; Kagaya et al., 1990; Nuutila et al., 1995; Taegtmeyer and Overturf, 1988; Zhang et al., 1995]. Liao et al. (2002) hypothesized that increased glucose utilization represents an adaptive response by hypertrophied hearts, improving cardiac function [Liao et al., 2002]. Recently, they tested this hypothesis by subjecting GLUT-1 overexpressing transgenic mice to ascending aortic constriction and showed that elevated glucose uptake protected against the progression to heart failure and increased the survival rate of mice subjected to chronic pressure overload [Liao et al., 2002]. Moreover, a recent study has suggested a novel mechanism whereby GLUT1 may protect against hypertrophy [Morissette et al., 2003]. Here, it was suggested that GLUT1 may also interact with intracellular signalling molecules that result in Akt phosphorylation and enhanced survival of cardiomyocytes [Morissette et al., 2003]. One cytoprotective pathway activated by insulin is via phosphatidylinositol - 3 - kinase (PI3 - kinase). PI3 - kinase appears to be part of a cascade that can

activate the serine/threonine kinase Akt/PKB (protein kinase B) and promote cell survival [Svendsen et al., 2001]. Once activated, Akt signals and activate p70s kinase (p70sk). P70s kinase phosphorylates BAD, inhibiting it. Dephosphorylation of BAD results in apoptosis. Therefore, Akt and /or p70sk mediate cytoprotection of the apoptotic regulator BAD [Jonassen et al., 2001].

Consistent with this beneficial outcome of metabolic remodeling, Hasenfuss and coworkers (2002) found that dose dependent (1-20 mmol/L) pyruvate administration in isolated myocardium from failing human hearts improved cardiac contractility [Hasenfuss et al., 2002]. It is thought that this effect is mediated by elevated sarcoplasmic reticulum- Ca^{2+} accumulation and release, resulting in increased Ca^{2+} transients and intracellular pH, leading to myofilament Ca^{2+} sensitivity [Hasenfuss et al., 2002]. Lastly, pharmacological reagents like TMZ and ranolazine (effective FAO inhibitors) have been used to inhibit FAO and stimulate glucose oxidation in the failing heart [McCormack et al., 1996; Kantor et al., 2000; Chandler et al., 2002]. For example, when TMZ was chronically administered to cardiomyopathic Syrian hamsters (a heart failure model with severely decreased PDH activity), there was a significant increase in survival time i.e. from 364 to 560 days [D'hahan et al., 1997; Stanley and Chandler, 2002]. Likewise, ranolazine treatment of dogs with microembolism-induced heart failure resulted in increased cardiac output, improved LV systolic function, external ventricular power and increased myocardial mechanical efficiency [Sabbah et al., 2000; Sabbah et al., 2001; Sabbah et al., 2002; Chandler et al., 2002]. In agreement, NYHA class II-III heart failure patients treated with etomoxir (a partial inhibitor of FAO acting on

CPT-I) showed an improvement in left ventricular function [Bristow et al., 2000]. Together these studies suggest that interventions that inhibit cardiac FAO and stimulate glucose oxidation may improve clinical outcome [Stanley and Chandler, 2002].

In conclusion, the question whether cardiac metabolic remodeling is beneficial, detrimental or an epiphenomenon has not been conclusively established. In light of this, additional studies need to be performed allowing energy substrate changes to be meticulously dissected out during the transition from the normal to the hypertrophic heart, and eventually to heart failure. This experimental approach may assist to clarify this intriguing question and help unfold the mystery surrounding the contribution of metabolic remodeling to the development of cardiac disease.

1.5 AIMS OF THIS STUDY

This thesis consists of several studies that investigate aspects of cardiac metabolic remodelling at a transcriptional level, with particular emphasis on FA metabolism in response to hypobaric hypoxic stress. In Chapter 2, a study is described that characterizes a hypoxia – induced rat model of right ventricular cardiac hypertrophy. In Chapter 3 we determined the regulation of mitochondrial respiration and adenine nucleotide translocator 1 (ANT1) expression in response to chronic hypobaric hypoxia. In Chapter 4, we delineated the molecular regulatory programs directing medium-chain acyl-CoA dehydrogenase (MCAD) gene expression in response to sub-acute and

chronic hypoxia. In Chapter 5, we conclude by summarizing the major findings of this study and their implications for cardiovascular research.

1.6 REFERENCES

1. Abel ED, Kaulbach HC, Tian R, Hopkins JCA, Duffy J, Doetschman T, Minnemann T, Boers ME, Hadro E, Oberste-Berghaus C, Quist W, Lowell BB, Ingwall JS, Kahn BB: Cardiac hypertrophy with preserved contractile function after selective deletion of GLUT-4 from the heart. *J Clin Invest* 104: 1703-1714, 1999.
2. Allard MF, Schonekess B, Henning SL, English D R, Lopaschuk G D: Contribution of oxidative metabolism and glycolysis to ATP production in hypertrophied hearts. *Am J Physiol* 267: H742-H750, 1994.
3. Allard MF, Wambolt RB, Longnus SL, Grist M, Lydell CP, Parsons HL, Rodrigues B, Hall JL, Stanely WC, Bondy GP: Hypertrophied rat hearts are less responsive to the metabolic and functional effects of insulin. *Am J Physiol* 279: E487-E493, 2000.
4. Ascuitto RJ, Ross-Ascuitto NT, Chen V, Downing SE: Ventricular function and fatty acid metabolism in neonatal piglet heart. *Am J Physiol* 256 (1PT2): H9-H15, 1989.
5. Badr GA, Zhang IZ, Tang I, Kern TS, Ismail-Beigi F: GLUT1 and GLUT 3 expression, but not capillary density is increased by cobalt chloride in rat cerebrum and retina. *Mol Brain Res* 64: 24-28, 1999 .
6. Barger PM, Brandt JM, Leone TC, Weinheimer CJ, Kelly DP: Deactivation of peroxisome proliferator-activated receptor -alpha

- receptor during cardiac hypertrophic growth. *J Clin Invest* 105 (12): 1723-1730, 2000.
7. Barger PM, Kelly DP: Fatty acid utilization in the hypertrophied and failing heart: molecular regulatory mechanisms. *Am J Med Sci* 318: 36-42, 1999.
 8. Barger PM, Kelly DP: PPAR signaling in the control of cardiac energy metabolism. *Trends Cardiovasc Med* 10: 238-245, 2000.
 9. Bing RJ, Siegel A, Vitale A, Balboni F, Sparks E, Taeschler M, Klapper M, Edwards S: Metabolic studies on the human heart in vivo. Studies on carbohydrate metabolism of the human heart. *Am J Med* 15: 284-96, 1953 .
 10. Birnbaum MJ, van Wijnen AJ, AJ, Odgren PR, Last TJ, Suske G, Stein GS, Stein JL: *Biochemistry* 34: 16503-16508, 1995.
 11. Bishop S, Altschuld R: Increased glycolytic metabolism in cardiac hypertrophy and congestive heart failure. *Am J Physiol* 218: 153-159, 1970.
 12. Blair E, Redwood C, Ashrafian H, Oliveira M, Broxholme J, Kerr B, Salmon A, Ostman-Smith I, Watkins H: Mutations in the gamma (2) subunit of AMP-activated protein kinase cause familial hypertrophic cardiomyopathy: evidence for the central role of energy compromise in disease pathogenesis. *Hum Mol Genet* 10 (11): 12-20, 2001.
 13. Boulikas T: A compilation and classification of DNA binding sites for protein transcription factors from vertebrates. *Crit Rev Eukaryotic Exp* 4: 117-321, 5519-5525, 1994.

14. Bristow J, Bie DM, Langer LG: Regulation of adult and fetal myocardial phosphofructokinase. *J Biol Chem* 262: 2172-2175, 1987.
15. Bristow M: Etomoxir: a new approach to treatment of chronic heart failure. *Lancet* 356: 1621-1622, 2000.
16. Brown N, Weis BC, Husti JE, Foster DW, McGarry JD: Mitochondrial carnitine palmitoyl transferase isoform switching in the developing rat heart. *J Biol Chem* 270: 8952-8957, 1995.
17. Bunn HF, Poyton RO: *Physiol Rev* 76: 839-885, 1996.
18. Chandler MP, Stanley WC, Morita H, Suzuki G, Roth BA, Blackburn B, Wolff A, Sabbah HN: Short-term treatment with ranolazine improves mechanical efficiency in dogs with chronic heart failure. *Circ Res*: 1-3, 2002.
19. Christie ME, Rogers RK: Altered glucose and fatty acid oxidation in hearts of the spontaneously hypertensive rat. *J MolCell Cardiol* 26: 1371-1375, 1994.
20. Coven DL, Hu X, Cong L, Bergeron R, Shulman GI, Hardie DG, Young LH: Physiological role of AMP-activated protein kinase in the heart: graded activation during exercise. *Am J Physiol Endocrinol Metab* 285 (3): E 629-36, 2003.
21. Dallman PR, Schwartz HC: Cytochrome c concentrations during rat and pig development. *Pediatrics* 33: 106-110, 1964.
22. de Las Fuentes L, Herrero P, Peterson LR, Kelly DP, Gropler RJ, Davila-Roman VG: Myocardial fatty acid metabolism: independent predictor of left ventricular mass in hypertensive heart disease. *Circulation* 41: 83-87, 2003.

23. Denning J, Beato M, Suske G: EMBO J 13: 3843-3851, 1994.
24. Depre C, Vanoverschelde JL, Taegtmeyer H: Glucose and the heart. *Circulation* 99:578-588, 1999.
25. Depre C, Young ME, Ying J, Ahuja HS, Han Q, Garza N, Davies PJA, Taegtmeyer H: Streptozotocin-induced changes in cardiac gene expression in the absence of severe contractile dysfunction. *J Mol Cell Cardiol* 32: 985-996, 2000.
26. D'hahan N, Taouil K, Dassouli A, Morel JE: Long-term therapy with trimetazidine in cardiomyopathic Syrian hamster *BIO* 14:6 : *Euro J Pharmacol* 328: 163-174, 1997.
27. Discher DJ, Bishopric NH, Wu X, Peterson CA: Hypoxia regulates β -Enolase and pyruvate kinase - M promoter by modulating Sp1/Sp3 binding to a conserved GC element. *J Biol Chem* 273 (40): 26087-26093, 1996.
28. Ebert BL, Firth JD, Ratcliffe PJ: Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter 1 via distinct cis-acting sequences. *J Biol Chem* 270 (49): 29083-29089, 1995.
29. El Alaoui-Talibi Z, Guendouz A, Moravec M, Moravec J: Control of oxidative metabolism in volume-overload rat hearts: effects of propionyl-L-carnitine. *Am J Physiol* 272: H1615-H1624, 1997.
30. El Alaoui-Talibi Z, Landormy S, loireau A, Moravec J: Fatty acid oxidation and mechanical performance of volume-overloaded rat hearts. *Am J Physiol* 262: H1068-H1074, 1992.

31. Esposito LA, Melov S, Panov A, Cottrell BA, Wallace DC. Mitochondrial disease in mouse results in increased oxidative stress. *Proc Natl Acad Sci USA* 96: 4820-4825, 1999.
32. Finck BN, Kelly DP: Peroxisome proliferator-activated receptor alpha signalling in the gene regulatory control of energy metabolism in the normal and diseased heart. *J Mol Cell Cardiol* 34 (10): 1249-1257, 2002.
33. Firth JB, Ebert BL, Pugh CW, Ratcliffe PJ: *Proc Natl Acad Sci USA* 91: 6496 - 6500, 1994.
34. Fisher DJ: Oxygenation and metabolism in the developing heart. *Semin Perinatol* 8: 217-225, 1984.
35. Friehs I, Moran AM, Stamm C, Colan SD, Takeuchi K, Cao-Danh H, Rader CM, McGowan FX, del Nido PJ: Impaired glucose transporter activity in pressure-overload hypertrophy is an early indicator of progression to failure. *Circulation* 100: 11187-11193, 1999.
36. Glatz JF, Veerkamp JH: Postnatal development of palmitate oxidation and mitochondrial enzyme activities in rat cardiac and skeletal muscle. *Biochim Biophys Acta* 711 (2): 327-335, 1982.
37. Goldberg MA, Donning SP, Bunn HF: Regulation of the erythropoietin gene: Evidence that the oxygen sensor is a heme protein. *Science* 242: 1412 - 1415, 1988.
38. Gollob MB: Glycogen storage disease as a unifying mechanism of disease in the PRKAG2 cardiac syndrome. *Biochem Soc Trans* 31 (pt 1), 228-31, 2003.

39. Gollob MH, Green MS, Tang AS, Gollo T, Karibe A, Ali Hassan AS, Ahmed F, Lozad R, Shan G, Fananapazir L, Bachinski LL, Roberts R, Hassan AS: Identification of a gene responsible for familial Wolff-Parkinson White syndrome. *N Engl J Med* 344 (24): 1823-31, 2001.
40. Goodwin C, Mela L, Deutsch C, Forster RE, Miller LD, Delivoria-Papadopoulos M: Development and adaptation of heart mitochondrial respiratory chain function in fetus and newborn. *Adv Exp Biol* 75: 713-719, 1976.
41. Hallman M: Changes in mitochondrial respiratory chain proteins during prenatal development. Evidence of the importance of environmental O₂ tension. *Biochim Biophys Acta* 253 (2): 360-372, 1971.
42. Hansford RG: Studies on inactivation of pyruvate dehydrogenase by palmitoylcarnitine oxidation in isolated rat heart metabolism. *J Biol Chem* 252: 1552-1560, 1972.
43. Hasenfuss G, Maier LS, Hermann HP, Luers C, Hunlich M, Zeitz O, Janssen PM, Pieske B: Influence of pyruvate on contractile performance and Ca²⁺ cycling in isolated failing human myocardium. *Circulation* 105: 194-199, 2002.
44. Hochachka PW, Clark CM, Matheson GO, Brown WD, Stone CK, Nickles RJ, Holden JE: Effects of regional brain metabolism of high altitude hypoxia: a study of six US marines. *Am J Physiol* 277 (1 Pt 2): R314-R319, 1999.
45. Hochachka PW: Fuels and pathways as designed systems for support of muscle work. *J Exp Biol* 115: 149-164, 1985.

46. Holden JE, Stone CK, Clark CM, Brown WD, Nickles RJ, Stanley C, Hochachka PW: Enhanced cardiac metabolism of plasma glucose in high-altitude natives: adaptation against chronic hypoxia. *J Appl Physiol* 79: 222-228, 1995.
47. Hudson ER, Pan DA, James J, Lucocq JM, Hawley SA, Green KA, Terashima T, Hardie DG: A novel domain in AMP-activated protein kinase causes glycogen storage bodies similar to those seen in hereditary cardiac arrhythmias. *Curr Biol* 13 (10): 861-6, 2003.
48. Huss JM, Levy FH, Kelly DP: Hypoxia inhibits the peroxisome proliferator-activated receptor α /retinoid X receptor gene regulatory pathway in cardiac myocytes: a mechanism for O₂ - dependent modulation of mitochondrial fatty acid oxidation. *J Biol Chem* 276 (29): 27605-27612, 2001 .
49. Itoi T, Lopaschuk GD: Ca²⁺ improves mechanical function and carbohydrate metabolism following ischaemia in isolated bi-ventricular working hearts from immature rabbits. *J Mol Cell Cardiol* 28: 1501-1514, 1996.
50. Jamshidi Y, Montgomery HE, Hense HW, Myerson SG, Torra IP, Staels B, World MJ, Doering A, Erdman J, Hengstenberg C, Humphries SE, Schunkert H, Flavel DM: Peroxisome proliferator-activated receptor alpha gene regulates left ventricular growth in response to exercise and hypertension. *Circulation* 105 (8): 950-955, 2002.
51. Kagaya Y, Kanno Y, Takeyama D, Ishide N, Maruyama Y, Takahashi T, Ido T, Takishima T: Effects of long-term pressure overload on

- regional myocardial glucose and free fatty acid uptake in rats: A quantitative autoradiographic study. *Circulation* 81: 1353-1361, 1990.
52. Kantor PF, Lopaschuk GD, Opie LH: Myocardial energy metabolism. In: *Heart Physiology and Pathophysiology*, 4th edition. Sperelakis N, Kurachi Y, Terzic A, Cohen MV. San Diego, Academic Press, p 543-569, 2001.
53. Kantor PF, Lucien A, Kozak R, Lopaschuk GD: The antianginal drug trimetazidine shifts cardiac energy metabolism from fatty acid oxidation to glucose oxidation by inhibiting mitochondrial long-chain 3-keto-acyl coenzyme A thiolase. *Circ Res* 86: 580-588, 2000.
54. Kantor PF, Robertson MA, Coe JY, Lopaschuk GD: Volume overload hypertrophy of the newborn heart slows the maturation of enzymes involved in the regulation of fatty acid metabolism. *J Am Coll Cardiol* 33: 1724-1734, 1999.
55. Kelly DP, Gordon JI, Alpers R, Strauss AW: The tissue-specific expression and development regulation of the two nuclear genes encoding rat mitochondrial proteins: medium-chain AcylCoA dehydrogenase and mitochondrial malate dehydrogenase. *J Biol Chem* 264: 18921-18925, 1989.
56. Kelly DP, Strauss AW: Inherited cardiomyopathies. *N Engl J Med* 330: 913-919, 1994.
57. Kelly DP: Peroxisome proliferator-activated receptor alpha as a genetic determinant of cardiac hypertrophic growth: culprit or innocent bystander? *Circulation* 105 (9): 1025-1027, 2002.

58. Kliewer SA, Forman BM, Blumberg B, Ong ES, Borgmeyer U, Mangelsdorf DJ, Umesono K, Evans RM: Differential expression and activation of a family of murine PPAR. *Proc Natl Acad Sci USA* 91: 7355-7359, 1994.
59. Kliewer SA, Umersono K, Noonan DJ, Heyman RA, Evans RM: Convergence of 9-cis-retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. *Nature* 358: 771-774, 1992.
60. Kobayashi K, Neely JR: Mechanisms of pyruvate dehydrogenase activation by increased heart work. *J Moll Cell Cardiol* 15: 369-382, 1983.
61. Lehman JJ, Kelly DP: Gene regulatory mechanisms governing energy metabolism during cardiac hypertrophic growth. *Heart Fail Rev* 7 (2): 175-185, 2002.
62. Leone TC, Weinheimer TC, Weinheimer CJ, Kelly. A critical role for the peroxisome proliferator - activated receptor alpha (PPAR α) in the cellular fasting in the cellular fasting response: The PPAR α -null mouse as a model of FAO disorders. *Prac Natl Acad Sci USA* 96: 7473-7478, 1999.
63. Lerch R: Is heart failure a metabolic disease? *Heart and Metabolism* 14: 2-3, 2001.
64. Liao R, Jain M, Cui L, D'Agostino BA, Aiello F, Luptak I, Ngoy S, Mortensen RM, Tian R: Cardiac-specific overexpression of GLUT-1 prevents the development of heart failure attributable to pressure overload in mice. *Circulation* 106: 2125-2131, 2002.

65. Loike JD, Cao L, Brett J, Ogawa S, Silverstein SC, Stern D: Hypoxia induces glucose transporter expression in endothelial cells. *Am J Physiol (Cell Physiol)* 263: C326-C333, 1992.
66. Lopaschuk GD, Collins-Nakai RL, Itoi T: Developmental changes in energy substrate use by the heart. *Cardiovasc Res* 26: 1172-1180, 1992.
67. Lopaschuk GD, Hamsen CA, Neely JR. Fatty acid metabolism in hearts containing elevated levels of CoA. *Am J Physiol* 250 (Heart Circ Physiol): H351-H359, 1986.
68. Lopaschuk GD, Spafford MA, Davies NJ, Walls SR: Glucose and palmitate oxidation in isolated working rat hearts reperfused after a period of transient global ischaemia. *Circ Res* 66: 546-553, 1990.
69. Lopaschuk GD, Spafford MA, Marsh DR: Glycolysis is predominant source of myocardial ATP production immediately after birth. *Am J Physiol* 261: H1698-H1705, 1991.
70. Lopaschuk GD, Witters LA, Itoi T, Barr A: Acetyl CoA carboxylase involvement in the rapid mutation of FAO in the newborn rabbit heart. *J Biol Chem* 269: 25871-25878, 1994.
71. Lundby C, van Hall G: Substrate utilization in sea level residents during exercise in acute hypoxia and after 4 weeks of acclimatization to 4100m. *Acta Physiol Scand* 176 (3): 195-201, 2002.
72. Lydell CP, Chan A, Wambolt RB, Sambandam N, Parsons H, Bondy GP, Rodrigues B, Popov KM, Harris RA, Brownsey RW, Allard MF: Pyruvate dehydrogenase and the regulation of glucose oxidation in hypertrophied rat hearts. *Cardiovasc Res* 53 (4): 841-851, 2002.

73. Makinde AO, Kantor PF, Lopaschuk GD: Maturation of fatty acid and carbohydrate metabolism in the newborn heart. *Mol Cell Biochem* 188: 49-56, 1998.
74. Makinde AO, Lopaschuk GD: Stimulating 5 AMP activated protein kinase increases fatty acid oxidation in newborn rabbit hearts. *J Mol Cell Cardiol* 28: A170, 1996.
75. Makinde AO, Gambia J, Lopaschuk GD: upregulation of 5 AMP activated protein kinase is responsible for the increase in myocardial fatty acid oxidation rates following the birth in the newborn rabbit. *Circ Res* 80: 482-489, 1997.
76. Marsin AS, Bertand L, Rider MH, Deprez J, Beauloye C, Vincent MF, van der Berghe S, Colling D, Hue L: Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. *Curr Biol* 10 (20): 1247-55, 2000.
77. Mathur A, Sims HF, Gopalakrishnan D, Gibson B, Rinaldo P, Vockley J, Hug G, Strauss AW: Molecular heterogeneity in very long chain acyl-CoA dehydrogenase deficiency causing pediatric cardiomyopathy and sudden death. *Circulation* 99: 1337-1343, 1999.
78. McCormack JG, Barr RL, Wolff AA, Lopaschuk GD: Ranolazine stimulates glucose oxidation in normoxic, ischemic and reperfused ischemic rat hearts. *Circulation* 93: 135-142, 1996.
79. McGarry JD, Woeltje KF, Kuwajima M, Foster DW: Regulation of ketogenesis and renaissance of carnitine palmitoyl. *Diabetes Metab Rev* 5: 271-284, 1989.

80. McGarry TD, Foster DW: Regulation of hepatic FAO and ketone body production. *Ann Rev Biochem* 49: 395-420, 1980.
81. Montessuit C, Papageorgiou I, Tardy-Cantalupi, Rosenblatt-Velin N, Lerch R: Postischemic recovery of heart metabolism and function: role of mitochondrial fatty acid transfer. *J Appl Physiol* 89 (1): 111-119, 2000.
82. Morissette MR, Howes AL, Zhang T, Brown JH: Upregulation of GLUT-1 expression is necessary for hypertrophy and survival of neonatal rat cardiomyocytes. *J Mol Cell Cardiol* 35: 1217-1227, 2003.
83. Neely JR, Morgan HE: Relationship between carbohydrate and lipid metabolism and the energy balance of heart muscle. *Annu Rev Physiol* 36: 413-459, 1974.
84. Nuutila P, Maki M, Laine H, Knuuti MJ, Ruotsalainen U, Luotolanti M, Haaparanta M, Solin O, Jula A, Koivisto VA, Voipio-Pulkki L-M, Yki-Järvinen H: Insulin action on heart and skeletal muscle glucose uptake in essential hypertension. *J Clin Invest* 96 (2): 1003-1009, 1995.
85. Opie LH, Owen P: Assessment of mitochondrial free NAD^+/NADH ratios and oxaloacetate concentrations during increased mechanical work in isolated perfused rat heart during production or uptake of ketone bodies. *Biochem J* 140: 403-415, 1975.
86. Opie LH: Fuels: Aerobic and anaerobic metabolism. In: *The Heart. Physiology, from cell to circulation*. 3rd edition. Philadelphia, Lippincott-Raven, p 295-340, 1998.
87. Opie LH: Metabolism of the heart in health and disease. *Am Heart J* 76: 685-698, 1968.

88. Osorio JC, Stanley WC, Linke A, Castellari M, Diep QN, Panchal AR, Hintze TH, Lopaschuk GD, Recchia FA: Impaired myocardial fatty acid oxidation and reduced protein expression of retinoid X receptor- α in pacing-induced heart failure. *Circulation* 106: 606-612, 2002.
89. Paolisso G, Gambarella A, Galzerano D, D'Amore A, Rubino P, Verza M, Teasuro P, Varricchio M, D'Onofrio F: Total body and myocardial substrate oxidation in congestive heart failure. *Metabolism* 43: 174-179, 1994.
90. Paternostro G, Clarke K, Heath J, Seymour AM, Radda GK: Decreased GLUT4 mRNA content and insulin-sensitive deoxyglucose uptake show insulin resistance in the hypertensive rat heart. *Cardiovasc Res* 30: 205-211, 1995.
91. Paternostro G, Pagano D, Gneccchi-Ruscione T, Bonser RS, Camici PG: Insulin resistance in patients with cardiac hypertrophy. *Cardiovasc Res* 42: 246-253, 1999.
92. Postic C, Leturque A, Printz RL, Maulard P, Loizeau M, Granner DK, Girard J: Development and regulation of glucose transporter and hexokinase expression in rat. *Am J Physiol* 266 (4 Pt 1), E548-E559, 1994.
93. Randle PJ, Morgan HE: Regulation of glucose uptake by muscle. *Vitam. Horm.* 20: 199-243, 1962.
94. Razeghi P, Young ME, Abbasi S, Taegtmeyer H: Hypoxia in vivo decreases peroxisome proliferator-activated receptor alpha regulated gene expression in rat heart. *Biochem Biophys Res Commun* 287: 5-10, 2001(a).

95. Razeghi P, Young ME, Alcorn JL, Moravec CS, Frazier OH, Taegtmeyer H: Metabolic gene expression in fetal and failing human heart. *Circulation* 104: 2923-2931, 2001(b).
96. Razeghi P, Young ME, Cockrill TC, Frazier OH, Taegtmeyer H: Downregulation of myocardial myocyte enhancer factor 2C and myocyte enhancer factor 2C-regulated gene expression in diabetic patients with nonischemic heart failure. *Circulation* 106:407-411, 2002.
97. Remondino A, Rosenblatt-Velin N, Montessuit C, Tardy I, Papageorgiou I, Dorsaz PA, Jorge-Costa M, Lerch R: Altered expression of proteins of metabolic regulation during remodeling of the left ventricle after myocardial infarction. *J Mol Cell Cardiol* 32: 2025-2034, 2000.
98. Rolph TP, Jones CT: Metabolism during fetal life: A functional assessment of metabolic development. *Physiol Rev* 65: 357-430, 1985.
99. Rolph TP, Jones CT: Regulation of glycolytic flux in the heart of the fetal guinea pig. *J Dev Physiol* 5: 31-49, 1983.
100. Sabbah HN, Hadda W, Mika Y, Nasso O, Avivi R, Sharov VG, Maltsev V, Felzen B, Undrovinas AI, Goldstein S, Darvish N, Ben-Haim SA: Cardiac contractility modulation with the impulse dynamic signal: studies in dogs with chronic heart failure. *Heart Fail Rev* 6 (1): 45-53, 2001.
101. Sabbah HN, Mishina T, Biesiadecki BJ, Suzuki G, Chaudhry P, Blackburn B, Huang HL, Stanley WC: Ranolazine improves left ventricular performance in dogs with chronic heart failure. *J Am Coll Cardiol* 35 (Suppl A): 218A, 2000.

102. Sabbah HN, Stanley WC: Partial fatty acid oxidation inhibitors: a potentially new class of drugs for heart failure. *Eur J Heart Fail* 4: 3-6, 2002.
103. Sack MN, Disch DL, Rockman HA, Kelly DP: A role for Sp and nuclear receptor transcription factors in a cardiac hypertrophic growth program. *Proc Natl Acad Sci USA* 94: 6438-6443, 1997.
104. Sack MN, Kelly DP: The energy substrate switch during development of heart failure: gene regulatory mechanisms. *Int J Mol Med* 1 (1): 17-24, 1998.
105. Sack MN, Rader TA, Park S, Bastin J, McCune SA, Kelly DP: Fatty acid oxidation enzyme gene expression is downregulated in the failing heart. *Circulation* 94: 2837-2842, 1996.
106. Sambandam N, Lopaschuk GD, Brownsey RW, Allard MF: Energy metabolism in the hypertrophied heart. *Heart Fail Rev* 7: 161-173, 2002.
107. Santalucia T, Camps M, Castello A, Munoz P, Nuel A, Testar X, Palacin M, Zarzani A: Developmental regulation of GLUT-1 (erythroid/HepG2) and GLUT-4 (muscle/fat) glucose transporter expression in rat heart, skeletal muscle and brown adipose tissue. *Endocrinology* 130 (2): 837-846, 1992.
108. Schmidt-Schweda S, Holubarsch C: First clinical trial with etomoxir in patients with chronic congestive heart failure. *Clin Sci (Lond)* 99 (1): 27-35, 2000 .

109. Schonekess BO, Allard MF, Lopaschuk GD: Recovery of glycolysis and oxidative metabolism during postischemic reperfusion of hypertrophied rat hearts. *Am J Physiol* 271: H798-H805, 1996.
110. Schonekess BO, Allona MF, Henning SL, Wambolt RB, Lopaschuk GD: Contribution of glycogen and exogenous glucose to glucose metabolism during ischaemia in the hypertrophied heart. *Circ Res* 81 (4): 540-9, 1997.
111. Semenza GL, Agani F, Iyer N, Jiang BH, Leung S, Wiener C, Yu A: Hypoxia-inducible factor 1: from molecular biology to cardiopulmonary physiology. *Chest* 114 (1 Suppl) : 405-455, 1998.
112. Semenza GL, Jiang BH, Leung SW, Passantino R, Concordet JP, Maire P, Giallongo A: Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *J Biol Chem* 271: 32529-32537, 1996.
113. Semenza GL, Roth PH, Fang HM, Wang GL: Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J Biol Chem* 269: 23757-23763, 1994.
114. Seymour AM, Chatham JC: The effects of hypertrophy and diabetes on cardiac pyruvate dehydrogenase activity. *J Mol Cell Cardiol* 29 (10): 2771-2778, 1997.
115. Stanley WC and Chandler MP: Energy metabolism in the normal and failing heart: potential for therapeutic interventions. *Heart Fail Rev* 7: 115-130, 2002.

116. Stanley WC, Lopaschuk GD, Hall JL, McCormack JG: Regulation of myocardial carbohydrate metabolism under normal and ischaemic conditions. Potential for pharmacological interventions. *Cardiovasc Res* 33: 243-357, 1997.
117. Taegtmeyer H, Overton ML: Effects of moderate hypertension on cardiac function and metabolism in the rabbit. *Hypertension* 11: 416-426, 1988.
118. Taegtmeyer H: Switching metabolic genes to build a better heart. *Circulation* 106: 2043-2045, 2002.
119. Tian R, Musi N, D'Agostino J, Hirshman MF, Goodyear LJ: Increased adenosine monophosphate-activated protein kinase activity in rat hearts with pressure-overload hypertrophy. *Circulation* 104: 1664-1669, 2001.
120. Torielli L, Conti F, Cinato E, Ceppi E, Anversa P, Bianchi G, Ferrari P: Alterations in energy metabolism of hypertrophied rat cardiomyocytes: influence of propionyl-L-carnitine. *J Cardiovasc Pharmacol* 26 (3): 372-380, 1995.
121. Turciani M, Rupp H: Etomoxir improves left ventricular performance of pressure-overloaded rat heart. *Circulation* 96 (10): 3681-3686, 1997.
122. van Bilsen M, van der Vusse GJ, Reneman RS: Transcriptional regulation of metabolic processes: implications for cardiac metabolism. *Pflugers Arch* 437 (1): 2-14, 1998.
123. Wambolt RB, English DR, Lopaschuk GD, Brownsey RW, Allard MF: Dichloroacetate improves post-ischemic function of hypertrophied rat hearts. *J Am Coll Cardiol* 36: 1378-1385, 2000.

124. Wambolt RB, Henning SL, English DR, Dyachkova Y, Lopaschuk GD, Allard MF: Glucose utilization and glycogen turnover are accelerated in hypertrophied rat hearts during severe low-flow ischemia. *J Mol Cell Cardiol* 31: 493-502, 1999.
125. Webster KA, Murphy BJ: *Can J* 2001 66: 1046-1058, 1988.
126. Webster KA: Regulation of glycolytic enzyme RNA transcriptional rates by oxygen availability in skeletal muscles. *Mol Cell Biochem* 77: 19-28, 1987.
127. Weinberg RS, Acosta R, Knobloch ME, Garber M, Alter BP: Low O₂ enhances sickle and normal erythropoiesis and fetal hemoglobin synthesis *in vitro*. *Hemoglobin* 19 (5): 263-275, 1995.
128. Werner JC, Sicard RE, Schuler HG: Palmitate by isolated working fetal newborn pig hearts. *Am J Physiol* 256 (Endocrine Metab 19): E315-E21, 1989.
129. Werner JC, Whitman V, Musselman J, Schuller HG: Perinatal changes in mitochondrial respiration of the rabbit heart. *Biol Neonate* 42: 208-216, 1982.
130. Young ME, Laws FA, Goodwin GW, Taegtmeyer H: Reactivation of peroxisome proliferator-activated receptor alpha is associated with contractile dysfunction in hypertrophied rat heart. *J Biol Chem* 276: 44390-44395, 2001.
131. Zhang J, Duncker DJ, Ya X, Zhang Y, Pavek T, Wei H, Merkle H, Ugurbil K, From AH, Bache RJ: Effect of left ventricular hypertrophy secondary to chronic pressure overload on transmural myocardial 2-

deoxy-glucose uptake. A ^{31}P NMR spectroscopic study. *Circulation* 92: 1274-1283, 1995.

132. Zhu H and Bunn HF: How do cells sense oxygen? *Science* 292: 449-451, 2001.

University of Cape Town

Chapter 2

Characterization of Hypobaric Hypoxia-Induced Right Ventricular Hypertrophy Rat Model

University of Cape Town

2.1 INTRODUCTION

Hypoxia is caused by a reduction in the supply of arterial oxygen to tissues, leading to an imbalance between oxygen supply and demand [Abel-aleem et al., 1999]. Some of the most common causes of tissue hypoxia are the following: (i) ischemic hypoxia - due to reduced coronary blood flow, (ii) systemic hypoxia - induced by a fall in arterial blood PO_2 or (iii) a combination of either (i) or (ii) and increased oxygen demand [Ostadal et al., 1999].

In order to sustain metabolic processes, organisms have evolved adaptive regulatory mechanisms to cope with both acute and chronic exposure to hypoxia. In mammals the most common cause of hypoxia is residence at high altitude. Acute high altitude exposure initiates hypoxia defences via specific oxygen-sensing mechanisms [Hochachka, 1998]. Five hypoxia response systems have been identified in this regard. Firstly, the carotid body oxygen sensor increases the hypoxic ventilatory response (HVR), elevating oxygen uptake by the lungs to compensate for the oxygen shortage [Acker and Xue, 1995]. In the second instance, the pulmonary vasculature sensors activate the hypoxic pulmonary vasoconstrictor response (HPVR) and increase efficiency of gas exchange [Youngson et al., 1993; Weir and Archer, 1995; Heath and William, 1991]. Thirdly, general vasculature oxygen sensors i.e., hypoxia-inducible factor 1 (HIF-1) activate expression of vascular endothelial growth factor 1 (VEGF 1) together with its receptor, promoting angiogenesis and improving oxygen carrying capacity in the heart [Forsythe et al., 1996; Detmar et al., 1996; Ogita et al., 1995; Ladoux and Felin, 1993]. Fourthly,

oxygen sensors like HIF-1 activate the expression of erythropoietin (EPO) in the kidney and liver increasing red blood cell mass and thereby improving oxygen carrying capacity [Goldberg et al., 1988; Maxwell et al., 1993; Wang et al., 1995; Wenger and Gassman, 1997]. Lastly, oxygen-sensing mechanisms may lead to metabolic reorganization by changing the rate of expression of hypoxia-sensitive genes (for e.g. glycolytic enzymes) resulting in more ATP being produced per oxygen molecule compared to FAs [Hochachka et al., 1991, 1995, 1996 a, b, c; Bunn and Poyton, 1996; Wenger and Grassman, 1997].

Continued exposure of lowlanders to hypoxia causes an acclimation process to occur. Acclimation maintains angiogenesis [Heath and Williams, 1991; Harik et al., 1996]. and erythropoiesis, further increasing the red blood cell mass [Winslow and Monge, 1987]. Moreover, it sustains metabolic reorganization, increasing carbohydrate preference [Hochachka et al., 1996 (b)]. In addition, an increase in hypoxic ventilatory response (HVR) resulting in an increased ventilatory response occur as a consequence of acclimation [Bunn and Poyton, 1996]. This process extends the HPVR, sometimes leading to hypertension [Heath and Williams, 1991]. Pulmonary hypertension in turn may create a high afterload against which the right ventricle must work, and if sustained, may eventually lead to the development of right ventricular hypertrophy. This is thought to be an adaptive mechanism allowing normal cardiac output despite the high afterload. However, right-sided heart failure may eventually occur when the hypertrophied heart is no longer able to compensate for the increased afterload.

The Himalayan Sherpas are generally considered to be the best adapted to living at high altitude. However, most experimental data has been collected on the high-dwelling Andean natives i.e. the Quechuas [Monge et al., 1990]. Highlanders have been adapted for many generations to live at high altitudes and have successfully evolved adaptive mechanisms to sustain normal functions [Monge et al., 1990; Hochachka, 1992 (a, b)]. For example, Holden et al. (1995) have shown that high altitude natives utilize glucose as their preferred fuel and that this is a true biochemical adaptation in humans exposed to chronic hypoxia over generations [Holden et al., 1995]. Furthermore, Hochachka et al. (1996 b) supported this notion by showing that elevated carbohydrate supply to the heart's energy requirements is advantageous under hypobaric hypoxic conditions since the ATP yield per oxygen molecule is 25-60% higher compared to free fatty acids [Hochachka et al., 1996 b]. The *in vivo* biochemical properties of skeletal muscle in Quechuas are predominantly those of slow twitch fibres [Rosser and Hochachka, 1994], consistent with the adjustment of glycolytic vs. oxidative contributions to energy supply, thus improving the yield of ATP per mole of carbon fuel utilized [Hochachka, 1996c]. Slow twitch muscle fibres have more extensive blood vessels system and capillaries to supply extra amount of oxygen in a hypoxic environment. In addition slow twitch muscle fibres contain large amounts of myoglobin, an iron-containing protein similar to hemoglobin in red blood cells. Myoglobin combines with oxygen and stores it until needed, and it greatly speeds oxygen transport to the mitochondria during hypoxia. The combination of extensive blood vessels and myoglobin in

slow twitch muscle fibres increase their oxygen levels. Furthermore, these muscle fibres are rich in mitochondria, which supports high levels of oxidative metabolism. This is an adaptation to hypoxia because slow twitch muscle fibres increase oxygen levels and increase ATP production with increased mitochondrial numbers.

Previous hypobaric hypoxic studies using animal models have demonstrated that Wistar rats developed pulmonary hypertension in response to $\approx 11\%$ oxygen (46 kPa) after 4 weeks [Thurich et al., 1999]. Moreover, a robust right ventricular hypertrophic response was evident during the exposure to chronic hypobaric hypoxia [Thurich et al., 1999]. After 4 weeks exposure (11%, 46 kPa) these hearts began to reach a state of decompensation [Thurich et al., 1999]. The predominant cardiac pathologies are myocardial hypertrophy for which there is no external cause such as hypertension or aortic valve stenosis [Roberts et al., 1982]. Myocardial hypertrophy involves predominantly the interventricular septum, but may also involve the left ventricle symmetrically, or apical portion of the left ventricle, or is located in the right ventricle [Seidman et al., 1995]. Therefore, when pathology exceeds these limits, the heart reaches a state of decompensation.

In another study, Sprague-Dawley rats were exposed to 10 % oxygen (50 kPa) for 1, 2, 3 and more than 6 weeks, and metabolic changes in the heart associated with long term exposure to hypoxia were characterized [Rumsey et al., 1999]. Here, they found that fuel substrate oxidation in the right ventricle (RV) was not altered by hypoxia, the only exception being a reduction in fatty

acid oxidation after 2 weeks [Rumsey et al., 1999]. In contrast, oxidation of pyruvate, palmitoylcarnitine and glutamate was compromised in the LV within 24 hours of hypoxia and remained reduced in response to the hypoxic stimulus [Rumsey et al., 1999]. Chronic exposure to hypoxia results in RV hypertrophy. The RV develops higher pressure under hypoxic conditions. A response to this chronic load is a compensatory increase in right ventricular mass. Therefore, the RV is affected by hypoxia and hypertrophy. On the hand, the LV does not develop hypertrophy in response to chronic hypoxia and is likely to be only affected by decreased oxygen levels.

Previous studies have characterized limited biochemical, morphological and metabolic changes in response to hypobaric hypoxia and included only a few experimental time points. In this study, we set out to investigate the progressive changes in the rat heart in response to long-term hypobaric hypoxia and with particular emphasis on regulation at the functional, metabolic and transcriptional levels. Here, we aimed to set up a working model to (i) investigate progressive changes in the heart during the development of right ventricular hypertrophy and (ii) dissect molecular regulatory changes in the transition from compensated hypertrophy to heart failure.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Six-week-old male Wistar rats (200-250 g) were housed at room temperature in a 12-hour light/12-hour dark cycle with access to food and water *ad libitum*. For the hypoxic experiments, rats were placed inside a lexan chamber (650 mm x 1000 mm x 1700 mm) at a pO_2 of 11% (45 kPa) for 48 hours, 1, 2, 4, 6, 8, 10, 12, 16 and 24 weeks, respectively. The hypobaric hypoxic chamber simulates high altitude, with lower pressures resulting in decreased oxygen levels. The pO_2 was calculated using the formula: $pO_2 \text{ (mmHg)} = 0.2093 \times [\text{pressure within the chamber (mmHg)} - \text{the vapour pressure at room temperature (mmHg)}]$. Appropriate age and sex matched normoxic controls were used for all experiments and were housed in same location as the hypoxic animals. The chamber was opened every 48 hours for no more than 20 minutes to replace bedding, clean cages and provide fresh food and water. After each experimental time point, rats were weighed and then sacrificed using carbon dioxide. Hypoxic and control rats were sacrificed by inhaling CO_2 and these tissues were used to measure gene expression. The effects of the CO_2 method were experienced by the hypoxic and control rats. In addition, the mRNA levels are reported in arbitrary units (AU) determined by densitometry normalized to normoxic controls (mean control value set at 100). Therefore, this might correct effects of CO_2 on humoral and gene expression measurements. (Pentobarbital was used in all perfusion studies to assess *ex vivo* contractile function – refer below). Right and left ventricles were carefully dissected out and weighed, snap frozen in liquid nitrogen and stored at –

80°C for subsequent analyses. The University of Cape Town's Animal Research Ethics Committee approved all animal experiments.

2.2.2 Langendorff Perfusion

In a separate set of experiments, normoxic and hypoxic Wistar rats were anesthetized by pentobarbital sodium (100mg/kg^{-1} i.p), their hearts removed and placed in ice-cold Krebs-Henseleit buffer (11 mM Glucose, 118 mM NaCl, 25 mM NaHCO_3 , 4.7 mM KCl, 1.21 mM KH_2PO_4 , 1.22 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.84 mM $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$). The aorta was located and cannulated on the Langendorff perfusion rig, and the heart suspended from this cannula. After cannulation, a retrograde (Langendorff) perfusion of the coronary arteries via the aorta was immediately initiated. The perfusion was performed at a constant pressure (104 cm H_2O) and temperature (37°C) [Langendorff O, 1895].

Hearts were perfused for 30 minutes with oxygenated (95% O_2 , 5% CO_2) Krebs-Henseleit buffer (11 mM Glucose, 118 mM NaCl, 25 mM NaHCO_3 , 4.7 mM KCl, 1.21 mM KH_2PO_4 , 1.22 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.84 mM $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$). During perfusion, a latex balloon was inserted through the right atrium into the right ventricle in order to determine right ventricular developed pressure (RVDP) [Thurich et al., 1999]. Heart functional parameters were measured using a chart recorder and included heart rate, systolic and diastolic pressure, RVDP and coronary flow. The exclusion criteria used in this study were as follows: heart rate (140–400 beats/min), coronary flow (8–18 ml), and RVDP (50–120 mmHg using a right ventricular end diastolic pressure of 2–12 mmHg). These criteria were used to exclude values not obtained within these ranges.

2.2.3 Determination of Metabolic Substrates and Hematocrit Levels

At the end of each experimental time point, blood was collected from the normoxic and hypoxic rats by cardiac puncture. In order to prevent any bias due to diurnal variations, blood collection was routinely performed between 14h00 and 16h00. The metabolic flux and the expression of metabolic genes (carbohydrate utilization, FAO and mitochondrial functions) show diurnal variation with a general peak at night. Over the course of the day, the normal heart anticipates, responds and adapts to physiological alterations within its environment [Young et al., 2001]. Taking the above into consideration, collection of samples at only a single time point may be regarded a limitation of the present study. However, the 14h00 - 16h00 time point was used for consistency in our results and try to prevent any additional bias in our experimental protocol. Blood samples were centrifuged in a microfuge at 3,500 rpm for 10 minutes, and the supernatant used to determine systemic glucose and non-esterified fatty acid (NEFA) levels. Plasma (50 μ l) glucose levels were measured using a spectrophotometric glucose oxidase method described previously [Trinder, 1969]. A commercially available kit (Roche Mannheim) was used to spectrophotometrically determine plasma NEFA levels [Harris, 1974]. Briefly, plasma (10 μ l) is reacted in three steps, incubated at 24°C at each step and NEFA levels are then spectrophotometrically determined as previously described by Harris et al. (1974). In order to determine hematocrit levels, collected blood was put into heparinized capillary tubes and subsequently spun in a Hereaus hemofuge

(Hanau, Germany) at 12,000 rpm for 3 minutes, allowing separation of red blood cells and serum.

2.2.4 RNA Isolation and Northern Blot Analysis

Total RNA was extracted from hypoxic and normoxic RV tissue (≈ 100 mg) as previously described [Disch et al., 1996]. Northern blot analysis was performed by loading 20 μ g of total RNA per lane, and blots probed using radiolabeled cDNA fragments encoding MCAD, PPAR α , GLUT1, GLUT4 and PDK4. 28S rRNA transfer to nitrocellulose was used to assess RNA integrity and to control for variance in gel loading. The relative abundance of mRNA was quantified by laser densitometric analysis using the UVLTec autoradiography analyzer with uviband software v. 97 (Cambridge, UK). The composite data is represented as mean $\pm$ standard error of the mean. The mRNA levels are reported in arbitrary units (AU) determined by densitometry normalized to normoxic controls (mean control value set at 100).

2.2.5 Real-time Quantitative PCR

The methods for RNA extraction and real-time quantitative PCR have been described previously [Depre et al., 1998]. The nucleotide sequences for primers and probes have been previously published [Depre et al., 1998; Young et al., 2001]. The transcript for the constitutive gene 18S was used as housekeeping gene for data normalization. Internal standards were prepared using T7 RNA polymerase method (Ambion, Austin). The real-time quantitative PCR analyses were performed in collaboration with Prof. H. Taegtmeyer and Dr. S. Sharma (Houston, TX, USA).

2.2.6 Statistical Analysis

The data is presented as mean $\pm$ standard error of the mean (SEM). Statistical significance between the controls and experimental groups was performed using Student's t-test and Student-Newman-Keuls Multiple comparison test. Values were considered significant when $p < 0.05$.

2.3 RESULTS

In order to characterize the progressive development of right ventricular hypertrophy in response to chronic hypoxia, we exposed Wistar rats to hypobaric hypoxia (11% O₂) for a period ranging from 48 hours to 24 weeks. Age and sex-matched controls were included for each time point.

Evidence for a robust hypertrophic response was observed in the rats exposed to chronic hypobaric hypoxia. The right ventricular response to hypobaric hypoxia was measured by comparing right ventricle (RV) to left ventricle plus septum (LV + S) ratio weights in normoxic controls and hypoxic rats over the course of the 24 week study (Table 1). A significant increase in RV/LV+S ratio was observed after 1 week, further increasing after 2 weeks and thereafter maintained until 24 weeks compared to normoxic controls ($p < 0.001$) (Fig. 1A). The left ventricular response to hypobaric hypoxia was measured by comparing LV + S to heart mass (HM) ratio weights in normoxic controls and hypoxic rats over the course of the 24 weeks study. Here, an inverse pattern vs. the RV was observed i.e. the LV+S/HM ratio initially

decreased until 2 weeks, thereafter being more or less maintained over the 24- week period ($p < 0.001$) (Fig. 1B). Moreover, LV+S/body weight ratios remained constant for the duration of the experiment.

University of Cape Town

	control	48 h	control	1 wk	control	2 wk	control	4 wk	control	6 wk	control	8 wk	control	10 wk	control	12 wk	control	16 wk	control	24 wk
BM (g)	200	186 ± 1.4	244 ± 2.5	229 ± 2.2	256 ± 3.2	238 ± 4.3	298 ± 0.9	268 ± 4.3	399 ± 2.9	362 ± 8.4	423 ± 1.3	375 ± 4.9	432 ± 15.6	394 ± 7.9	483 ± 3.2	440 ± 10.7	536 ± 6.3	465 ± 10	667 ± 10.5	501 ± 6.6
RV (g)	0.2	0.2 ± 0.001	0.2	0.33 ± 0.01	0.2	0.38 ± 0.01	0.25 ± 0.01	0.45 ± 0.1	0.3 ± 0.01	0.6	0.3 ± 0.01	0.6	0.3	0.6	0.3	0.6 ± 0.1	0.4 ± 0.01	0.6 ± 0.1	0.3	0.6
LV+S	0.5	0.5	0.5	0.6	0.6	0.65 ± 0.03	0.9 ± 0.06	0.78 ± 0.05	1.05 ± 0.03	0.85 ± 0.03	1.03 ± 0.03	0.98 ± 0.03	0.95 ± 0.03	0.9 ± 0.04	1.15 ± 0.03	0.98 ± 0.05	1.3 ± 0.06	0.98 ± 0.03	1.13 ± 0.03	1.05 ± 0.05
RV / LV+S	0.4	0.45 ± 0.03	0.4	0.54 ± 0.02	0.33	0.58 ± 0.03	0.28 ± 0.04	0.58 ± 0.02	0.27 ± 0.02	0.71 ± 0.02	0.27 ± 0.02	0.62 ± 0.02	0.32 ± 0.01	0.67 ± 0.03	0.26 ± 0.01	0.65 ± 0.05	0.27 ± 0.03	0.65 ± 0.05	0.27 ± 0.01	0.58 ± 0.03
HM (g)	0.7	0.73 ± 0.002	0.7	0.93 ± 0.03	0.8	1.03 ± 0.05	1.15 ± 0.06	1.23 ± 0.07	1.33 ± 0.03	1.45 ± 0.03	1.3 ± 0.04	1.58 ± 0.02	1.25 ± 0.03	1.5 ± 0.04	1.45 ± 0.03	1.6 ± 0.04	1.65 ± 0.03	1.6	1.45 ± 0.03	1.65 ± 0.05
HM / BM	0.0035	0.0039 ± 0.0002	0.0039 ± 0.0002	0.0041 ± 0.0001	0.0032	0.0044 ± 0.0002	0.0039 ± 0.0003	0.0045 ± 0.0003	0.0033 ± 0.0001	0.004 ± 0.0001	0.0031 ± 0.0001	0.0042 ± 0.0001	0.0029 ± 0.0001	0.0039 ± 0.0001	0.003	0.0036	0.0031 ± 0.0001	0.0034 ± 0.0001	0.0022 ± 0.0001	0.0033 ± 0.0001

Table 1: Heart weights measurements in response to hypobaric hypoxia. Abbreviations: body mass (BM), right ventricle (RV), left ventricle plus septum (LV+S), heart mass (HM) - (n = 4, * p<0.05 vs normoxic control).

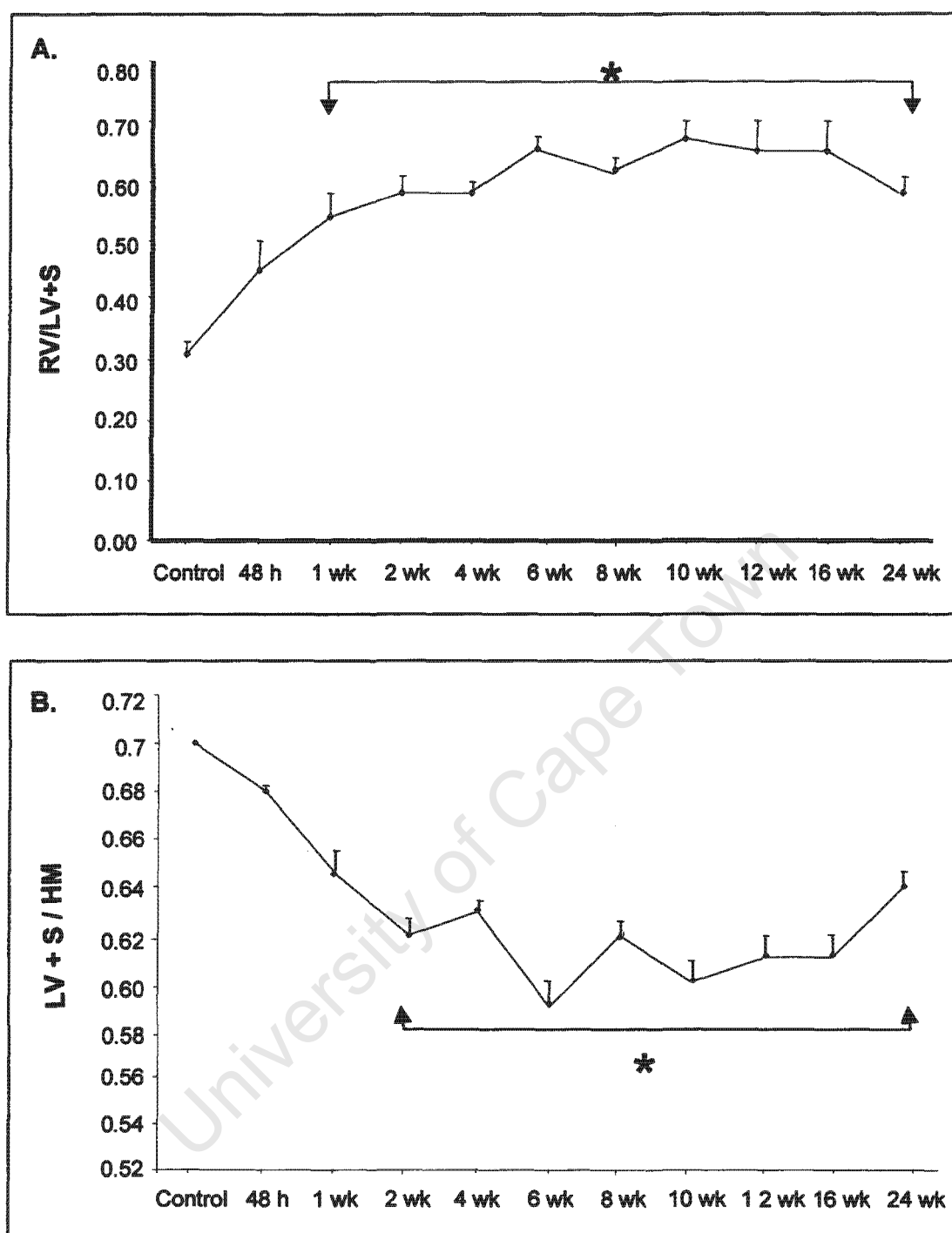


Figure 1: (A) Right ventricle (RV) to left ventricle plus septum (LV + S) weight ratios and (B) LV+S/heart mass (HM) ratios in rats exposed to hypobaric hypoxia over a period of 24 weeks. (n = 4; * p < 0.001 vs. normoxic animals).

The classic adaptive response to hypoxia is an increase in the number of red blood cells. In our model, a significant rise in hematocrit was observed after 1 week's exposure to hypobaric hypoxia and thereafter maintained up to the 24 week point compared to matching controls ($p < 0.001$) (Fig. 2).

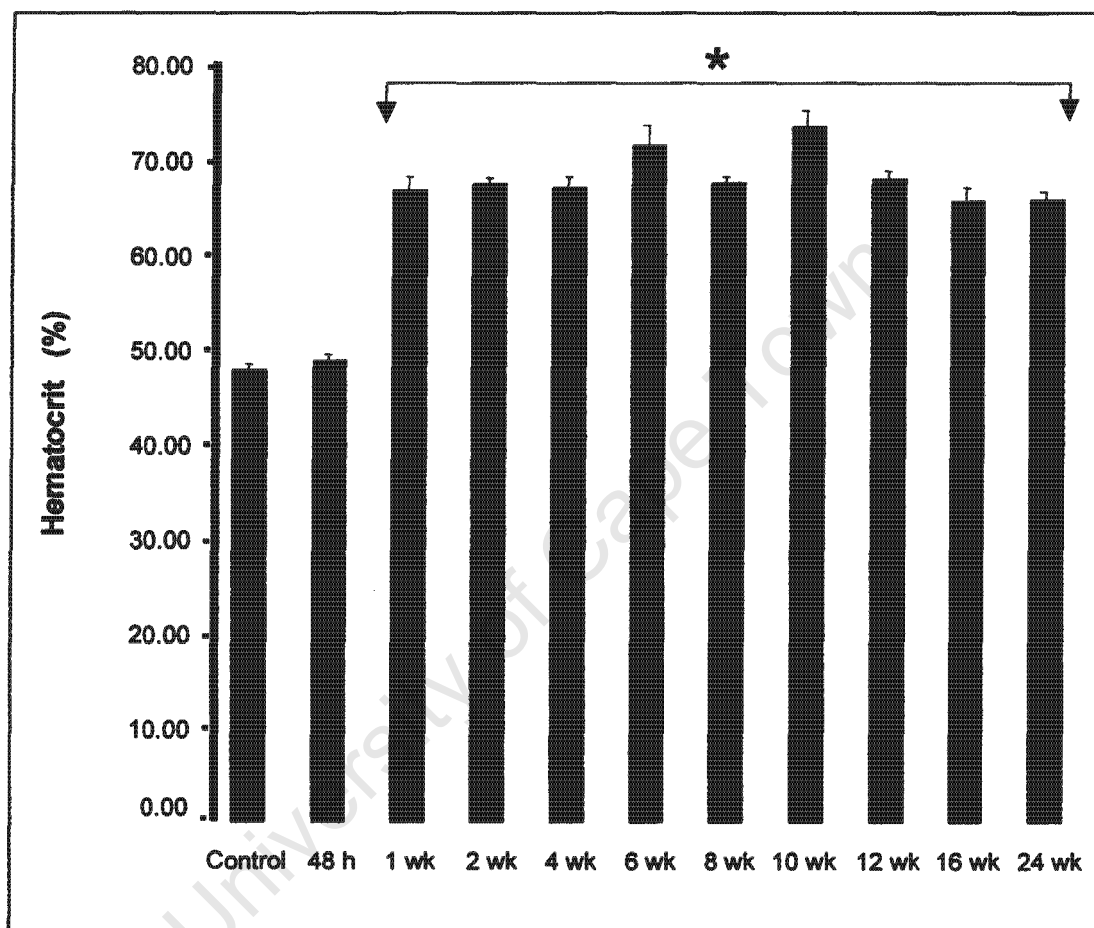


Figure 2: Hematocrit levels (%) in response to 24 weeks chronic hypobaric hypoxia. The asterisk denotes a significant difference vs. 48 hr hypoxia and normoxic controls ($n = 4$; $*p < 0.001$).

The heart rate was significantly increased after 48 hours hypoxia, subsequently decreasing by $32 \pm 4.1\%$ after 2 weeks ($p < 0.01$). Thereafter it

returned to control levels (4 weeks) until the 10 week point, then moderately declining towards the 24-week point (Fig. 3).

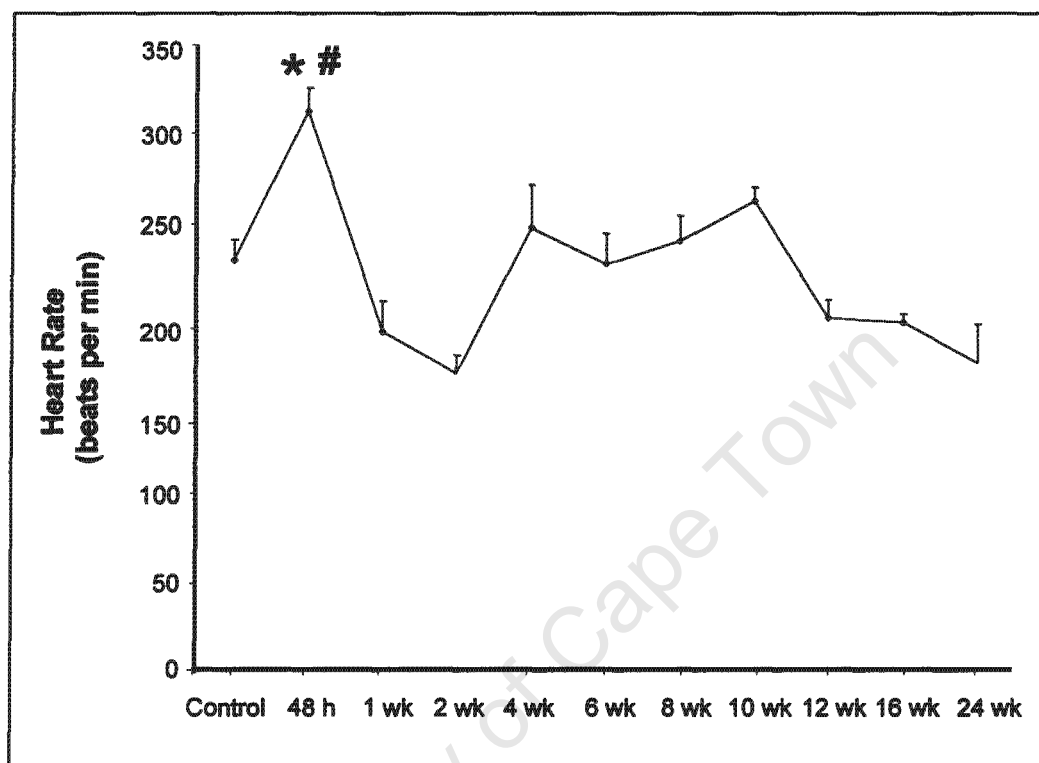


Figure 3: Heart rate in response to 24 weeks hypoxia. The asterisk denotes a significant difference between control vs. 48 hours and # denotes a significant difference between 48 hours vs. 2, 4, 10 and 24 weeks. (n = 4; *p < 0.01; #p < 0.01).

To investigate the effects of chronic hypoxia on heart function, we measured the right ventricular developed pressure (RVDP) over the course of 24 weeks exposure to hypoxia. Our data showed an increase in RVDP in the hypoxic rats compared to matching normoxic controls, steadily increasing and peaking at 6 weeks, followed by a decline (8 weeks - 12 weeks) and finally a secondary increase at 16 and 24 weeks (p < 0.01) (Fig. 4).

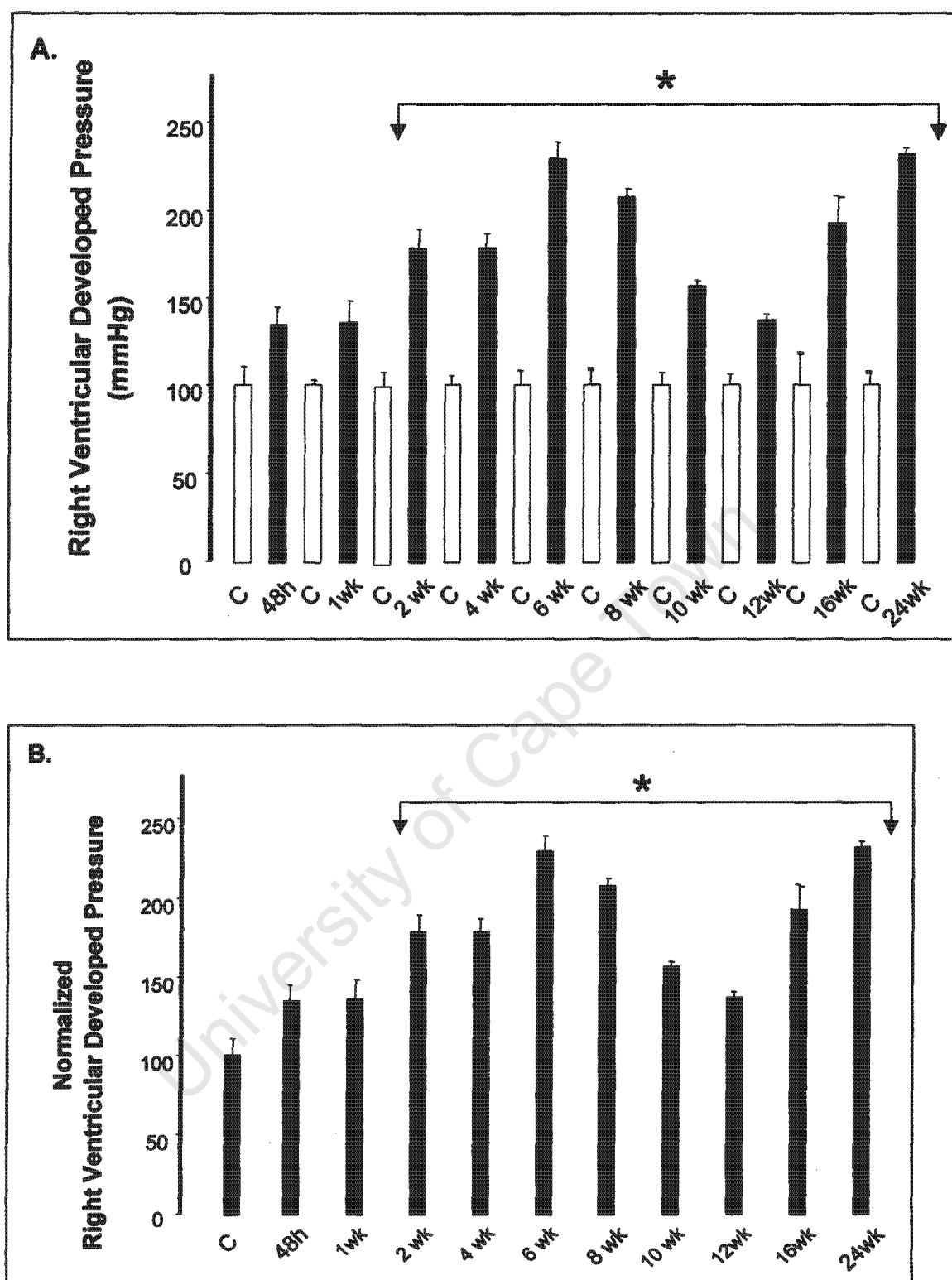
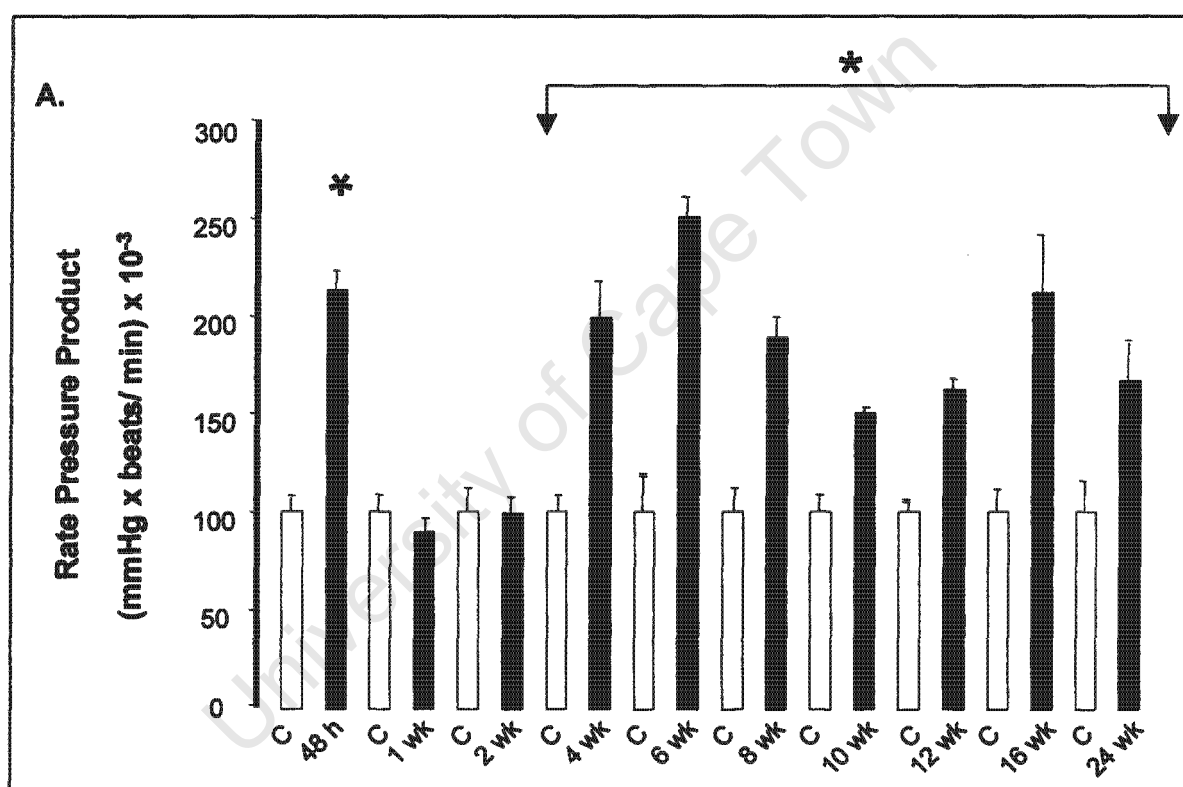


Figure 4: Right ventricular developed pressure (RVDP) in rats exposed to hypobaric hypoxia. (A) RVDP for each time point with matching control. (B) RVDP normalized to control values. (n = 4; *p < 0.01 vs. control).

To obtain another parameter of cardiac function, we calculated the rate pressure product (RPP) i.e. HR x RVDP (Fig. 5). The RPP was markedly elevated after 48 hours ($p < 0.01$), normalizing by 1 week. Thereafter it increased after 4 weeks being maintained until 10 weeks. A modest decline in RPP was observed between 10 and 24 weeks, although still significantly above control levels.



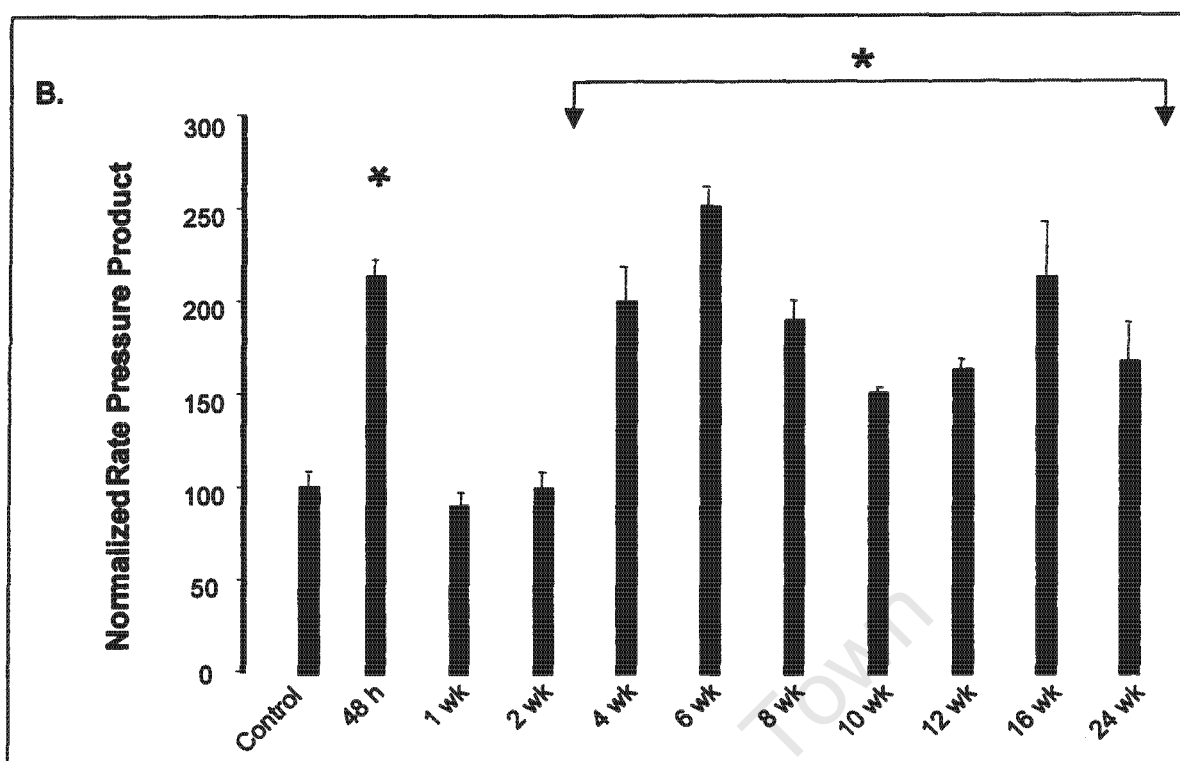


Figure 5: Rate pressure product (RPP) in response to 24 weeks hypobaric hypoxia. (A) RPP for each time point with matching control. (B) RPP normalized to control values. (n = 4; *p < 0.05 vs. control)

We next determined the effects of chronic hypobaric hypoxia on plasma glucose and NEFA levels. Plasma glucose levels were unchanged up to 4 weeks hypoxia (Fig. 6A). This was followed by a decrease at 6 weeks ($p < 0.05$), and a return to control levels at 8 and 10 weeks. Lastly, glucose levels decreased at 12 and 16 weeks and increased again at 24 weeks ($p < 0.05$). On the other hand, systemic NEFA levels were maintained up to 2 weeks. A decline in NEFA at 4 weeks was observed ($p < 0.05$), returning to baseline at 6 and 8 weeks. This was followed by a decrease at 10 /12 weeks and an increase at 16 / 24 weeks ($p < 0.05$).

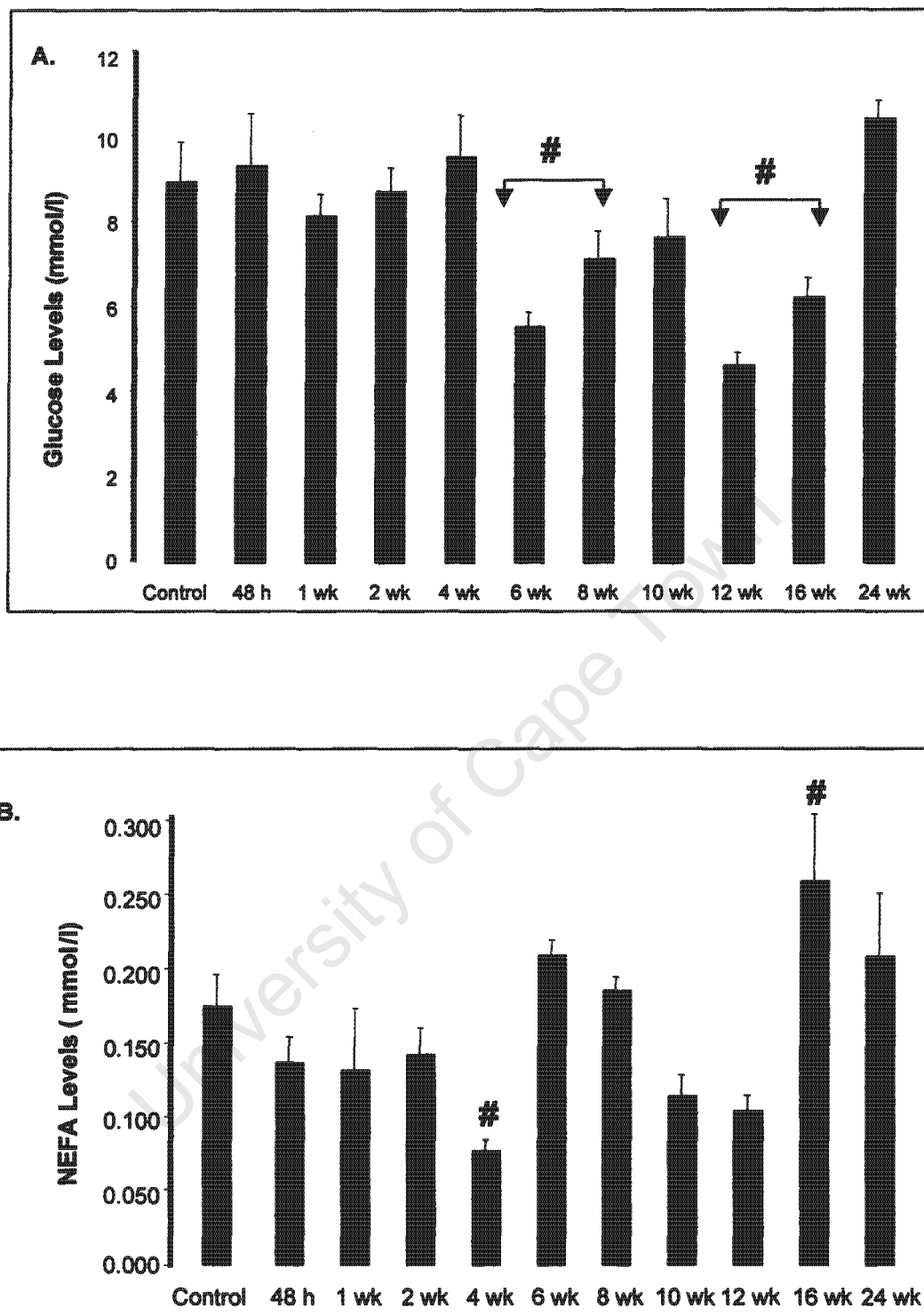


Figure 6: Plasma glucose and NEFA levels in response to 24 weeks hypobaric hypoxia. (A) Glucose levels and (n = 4; #p < 0.05 vs. control to 4 wk and 24 wk point) (B) NEFA levels, (n = 4; #p < 0.05 vs. control),

In light of these interesting functional and humoral changes, we next determined the effects of hypobaric hypoxia on the expression (by real-time quantitative RT-PCR) of key fatty acid and glucose utilization metabolic enzyme-encoding genes i.e. MCAD, GLUT1 and 4, PDK4 and PPAR α in the RV at the 48 hour, 1 week, 2, 4 and 12 week time points. Here, MCAD and PPAR α expression were downregulated after 1 week hypoxia, upregulated at 2 weeks thereafter returning to baseline (4 and 12 weeks) (Fig. 7 A - 7D).

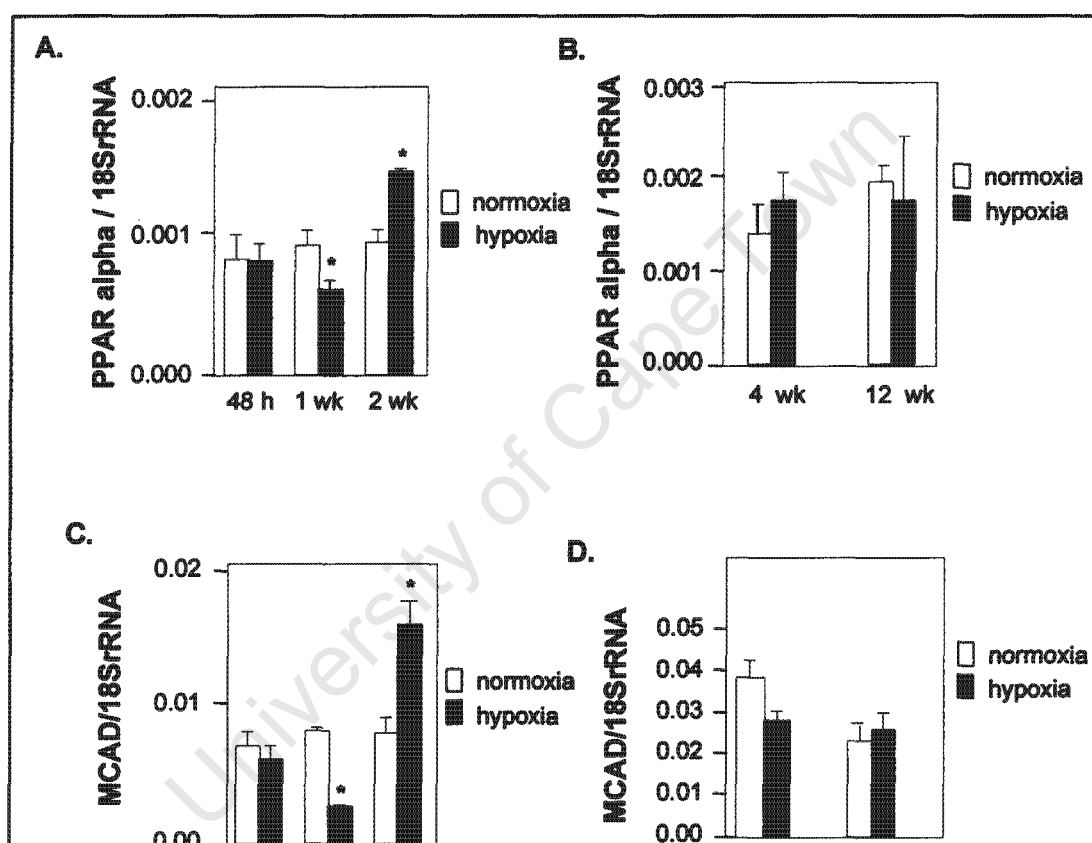


Figure 7: MCAD and PPAR α gene expression (by real-time quantitative PCR) in response to acute and chronic hypobaric hypoxia. (A) PPAR α gene expression at 48 h, 1 and 2 weeks hypoxic exposure. (B) PPAR α gene expression in response to 4 and 12 weeks exposure to hypoxia. (C) MCAD gene expression in response to 48 h, 1 and 2 weeks hypoxic exposure. (D) MCAD gene expression in response to 4 and 12 weeks exposure to hypoxia. (n = 5; * p < 0.05 vs. normoxic controls).

The gene expression patterns of the glucose utilization enzyme-encoding genes showed that RV GLUT 1 was significantly decreased at 2 weeks,

thereafter returning to baseline levels (12 weeks) (Fig. 8 A and B). On the contrary, GLUT 4 expression was elevated after 2 weeks and subsequently markedly downregulated at 12 weeks (Fig. 8 C and D). Interestingly, PDK4 was significantly reduced after 48 hours, increased at 2 weeks, thereafter downregulated after 4 and 12 weeks (Fig. 8 E and F).

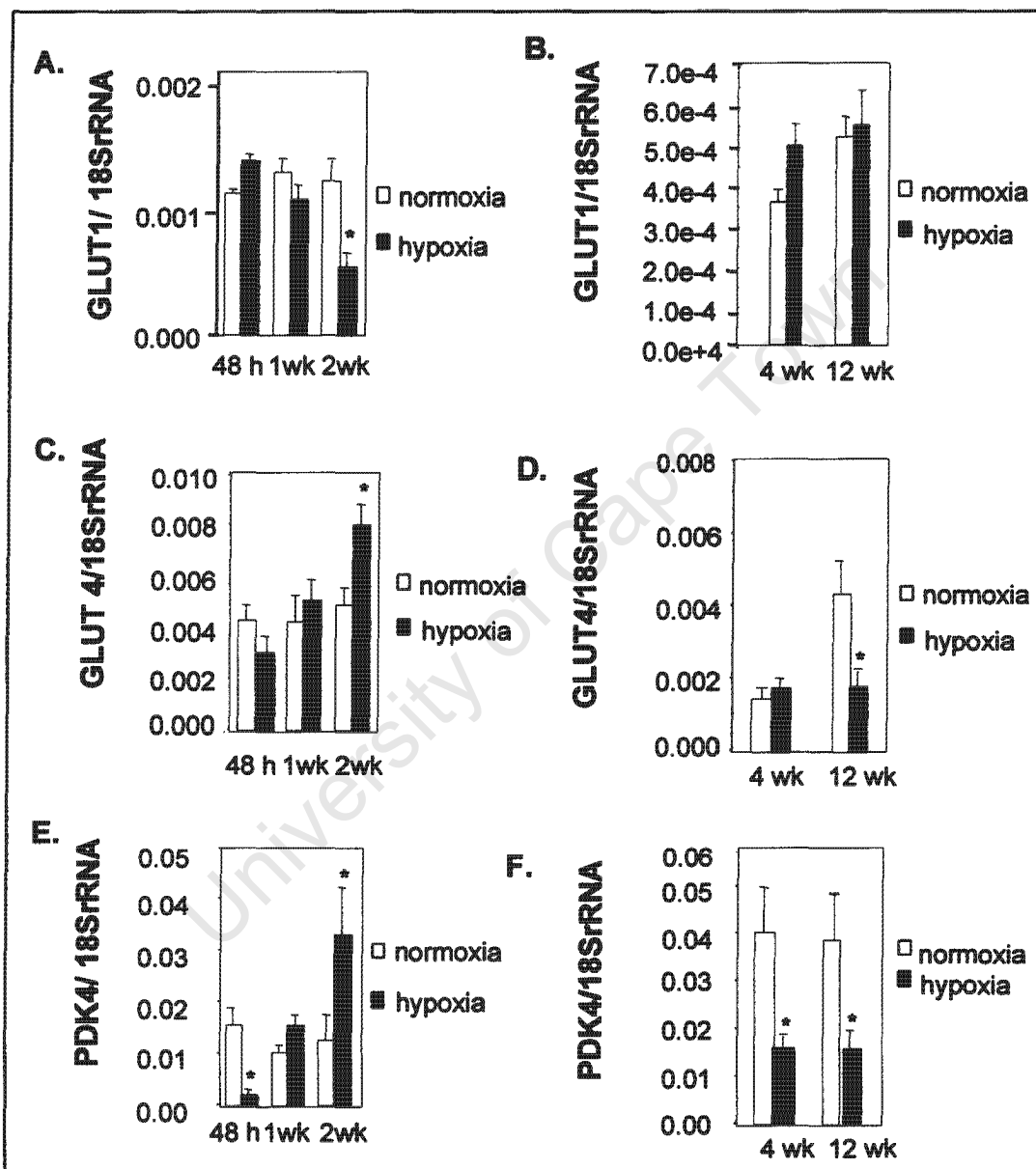


Figure 8: Glucose utilization gene expression (by real-time RT-PCR) in response to hypobaric hypoxia. (A, B) GLUT1 gene expression in response to 48 h to 12 weeks hypoxic exposure. (C, D) GLUT4 gene expression in response to 48 h to 12 weeks hypoxic exposure. (E, F) PDK4 gene expression in response to 48 h to 12 weeks hypoxic exposure. (n = 5; *p < 0.05 vs. normoxic controls)

2.4 DISCUSSION

Chronic exposure to hypobaric hypoxia is well reported to cause pulmonary hypertension, which may eventually lead to the onset of right ventricular hypertrophy [Thurich et al., 1999]. This represents an initial, adaptive process that allows increased contractility in response to the higher afterload. However, this compensatory effect may be lost in response to sustained hypertension, eventually leading to the onset of right-sided heart failure [Thurich et al., 1999; Hida et al., 2002; Brusselmans et al., 2003]. Although previous studies have investigated contractile function and some biochemical parameters in response to chronic hypobaric hypoxia, analyses of functional and molecular changes have not been well studied. In light of this, we investigated, in a progressive manner, the functional and molecular changes in rats exposed to 24 weeks chronic hypobaric hypoxia in order to (i) establish a well-characterized model of right ventricular hypertrophy and (ii) to dissect changes in the transition from right ventricular hypertrophy to heart failure.

We initially determined that the heart rate was markedly increased after 48 hours exposure to hypoxia, thereafter remaining more or less stable until the 24 week time point. This rapid increase was paralleled by a peak observed in the rate pressure product after 48 hours. Metabolically active tissues such as the heart are responsive to acute episodes of hypoxia [Rumsey et al., 1999]. For example, previous studies have shown that acute hypoxia elevates adrenergic stimulation resulting in an increased heart rate [Cohn et al., 1982;

Bryan, 1990]. The observed increase in contractility after 48 hours probably reflects the initial stress of the rats when exposed to diminished oxygen supply. Interestingly, PDK4 an indirect inhibitor of pyruvate dehydrogenase, is downregulated at this point. Our data is in agreement with Razeghi et al. (2001) who demonstrated that hypoxia significantly decreases PDK4 expression [Razeghi et al., 2001]. We suggest that this attempt to increase glucose oxidation may be part of an adaptive response to the initial, rapid reduction in oxygen supply. Glucose utilization in this instance is suggested to be beneficial since it is a more oxygen-efficient fuel, producing approximately 6-6.3 moles of ATP per mole of oxygen oxidized compared to 5.6 moles of ATP when fatty acids are utilized [Hochachka, 1985; Lundy and van Hall, 2002].

After 1 week contractile function was restored and also maintained over the course of the 24-week period. Moreover, the rats exposed to chronic hypobaric hypoxia showed evidence for the development of right ventricular hypertrophy after 1 week. Here, the right ventricular mass doubled versus normoxic controls, representing a compensatory response to the chronic elevated afterload [Rumsey et al., 1999]. Increased right ventricular mass was matched by elevated cardiac output i.e. elevated RVDP. This reflects increased thickening of the right ventricle required to maintain similar cardiac output compared to normoxic controls. These data are in agreement with previous studies. For example, Thurich et al. (1999) showed an increase in RVDP in rats exposed to 11% oxygen (46 kPa) for 4 weeks [Thurich et al., 1999]. Furthermore, Rumsey et al. (1999) also demonstrated an increase in

right ventricular systolic pressure in rats exposed to 6 weeks normobaric hypoxia (10% O₂, 50 kPa) [Rumsey et al., 1999].

As part of this adapted state, hematocrit levels were significantly increased in an attempt to restore oxygen delivery to the heart. Interestingly, by 1 week some metabolic remodeling seems to have occurred with the heart reducing reliance on fatty acids and hence increasing glucose utilization. However, it should be borne in mind that these conclusions are based only on gene expression data and may not necessarily apply at the protein/enzyme level. GLUT1 and GLUT4 mRNA levels remained unaltered compared to matching controls suggesting that glucose transport into the cardiomyocyte is maintained despite reduced oxygen supply. Although these data are not in complete agreement with Sivitz et al. (1992) who showed increased expression of GLUT1 in the hypoxic rat heart (higher in the right ventricle than left ventricle), we propose that in both instances glucose utilization is at least maintained. Differences in expression data may be due to different degrees of stress stimuli and/or variations in animal models employed.

The switch to increased glucose utilization appears to be driven by diminished expression of fatty acid enzyme-encoding genes. Here, expression of MCAD, a representative fatty acid oxidation enzyme, was downregulated. In support, transcript levels of PPAR α , a pivotal *trans*-activator of several cardiac fatty acid utilization genes, were downregulated. These data suggest diminished expression of several PPAR α -target FA-utilization genes by 1 week's exposure to hypoxia. In agreement, Razeghi et al. (2001) have demonstrated

that PPAR α and PPAR α -regulated genes (MCD, mCPT-I) were significantly decreased by *in vivo* hypoxia [Razeghi et al., 2001]. They suggested that this represents a transcriptional mechanism for reducing cardiac fatty acid oxidation and increasing glucose utilization during hypoxia [Razeghi et al., 2001]. On the other hand, Huss et al. (2001) reported that *in vitro* hypoxia deactivated PPAR α not by reducing its levels but by diminishing the availability of its obligate partner, RXR α , resulting in the downregulation of mCPT-I [Huss et al., 2001].

After 2 weeks hypobaric hypoxia the adaptive process appears to have been completed and oxygen delivery, via increased hematocrit, restored to a reasonable degree. In support, MCAD and PPAR α expression levels were significantly upregulated suggesting a reversion to more adult-like metabolism i.e. fatty acids being the primary fuel substrate of the heart. In agreement, GLUT1 was downregulated and expression of the insulin-responsive GLUT4 elevated, consistent with a postnatal gene expression profile. Moreover, PDK4 transcript levels were increased, which in turn should lead to the inhibition of pyruvate dehydrogenase and glucose oxidation. Together these data support a switch to increased fatty acid oxidation at the two-week point. However, it should be borne in mind that we did not perform substrate utilization studies to confirm our gene studies.

Interestingly, our data also show a dissociation between cardiac hypertrophy and the fetal gene expression profile. Previous studies have highlighted the gene expression switch from fatty acid to glucose utilization with the onset of

hypertrophy. For example, a number of studies employing animal models have shown that with hypertrophy, glycolysis and glucose/lactate oxidation increase together with a concomitant decrease in fatty acid oxidation rates, representing a reversion to the fetal pattern of energy metabolism [Fisher, 1984; El-Alaoui et al., 1992; Allard et al., 1994; Christe and Rogers, 1994; Kantor et al., 1999; Paternostro et al., 1999; Remodino et al., 2000]. However, our data show that mere gene profiling may not necessarily be a true indicator for the onset of cardiac hypertrophy. Moreover, our data questions the role of metabolic gene remodeling as a causative factor in the development of cardiac hypertrophy.

After 4-12 weeks, cardiac contractile function was maintained together with a degree of normalization to baseline of MCAD expression and its transcriptional regulator, PPAR α . After 12 weeks GLUT1 expression levels are maintained while GLUT4's expression is diminished. These data suggest a secondary reversion to the fetal gene expression pattern, but in this instance not by the downregulation of fatty acid enzyme-encoding genes. Rather, glucose utilization genes revert to a fetal expression i.e. reduced GLUT4 levels. This switch is unlike previous studies that have shown downregulation of mRNA levels of rate-limiting fatty acid oxidative enzymes like mCPT-I and MCAD during the onset of left ventricular hypertrophy and heart failure [Sack et al., 1996; Barger and Kelly, 1999; Remodino et al., 2000; Osorio et al., 2002]. However, dynamic changes in the gene expression profile as shown here may not have been detected in these studies since expression levels were not progressively monitored.

Our study underscores the basic premise that the heart is able to switch substrate utilization in response to multiple stimuli, for e.g. workload, substrate availability, hypoxia etc [Taegtmeyer, 2002]. Here, this 'metabolic flexibility' is emphasized by dynamic changes of both systemic fuel substrate supply and metabolic gene regulation. We believe that this transcriptional flexibility of the right ventricle in response to hypoxia represents another facet of the overall adaptive response. It is possible that longer duration of hypoxia may eventually result in the loss/impairment of such transcriptional flexibility and that this may be an important contributing factor to the development of decompensated hypertrophy and heart failure. However, to prove this hypothesis would require further investigation. Moreover, it should be kept in mind that additional humoral factors (not measured in the current study) for e.g. thyroid hormone levels may contribute to the complex temporal changes observed. For example, Zayour and co-workers (2003) have investigated endocrine changes in a rat model of chronic hypoxia mimicking cyanotic heart disease. They found that hypoxia resulted in thyroid hormone axis suppression [Zayour et al., 2003]. Also, additional mediators of the effects of hypoxia on cardiac gene expression may play a role. For example, the NAD^+/NADH ratio influences timing of transcriptional mechanisms. We expect hypoxia to increase the NAD^+/NADH ratio and hence this parameter may affect transcription of several cardiac metabolic enzyme-encoding genes, particularly those sensitive to diurnal variations for e.g. PDK4.

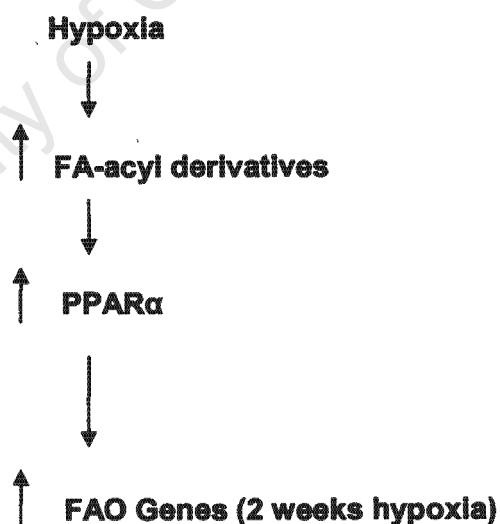
Despite these unanswered questions, our data suggest that we have successfully established a robust model of right ventricular hypertrophy in rats. We speculate that the adapted state of the heart is maintained from 12 weeks onwards (up to 24 weeks) since cardiac contractile output was not significantly altered. We did not, however, succeed in generating a model of right ventricular heart failure and suggest that this may be achieved by either increasing the severity of the oxidant stress and/or the length of time animals spend in the hypobaric hypoxic chamber. A similar study was performed by Thurich et al. (1999) who exposed Wistar rats to hypobaric hypoxia (11% O₂) for 4 weeks. Here, rats initially developed right ventricular hypertrophy and after 4 weeks approached a state of decompensation [Thurich et al., 1999]. The reason for this discrepancy compared to our model is not apparent. However, several factors for e.g. chamber dimensions, animal housing, variation in animal breeding etc. may all have contributed to differences between these studies. For example, Pissarek et al. (1997) employed a similar methodology to our study and others [Thurich et al., 1999]. but animals did not develop significant hypertrophy after 4 weeks of hypobaric hypoxia [Pissarek et al., 1997]. Moreover, Rumsey et al. (1999) exposed Sprague-Dawley rats to normobaric hypoxia (10% O₂) for 6 weeks with rats showing adaptive right ventricular hypertrophy [Rumsey et al., 1999]. These data therefore suggest that each animal model requires exact optimization in the host laboratory to ensure that the aims of projects are successfully achieved.

In conclusion, in this study we have succeeded in establishing a robust rat model for compensated right ventricular hypertrophy. This was matched by sustained cardiac output and a dynamic regulation of both metabolic fuel substrate supply and fatty acid and glucose utilization enzyme-encoding genes. Moreover, our data highlights the importance of doing progressive analyses in this regard i.e. carefully tracking changes in contractile function and gene expression during the onset of right ventricular hypertrophy.

2.5 FUTURE STUDIES

Future studies could investigate 3 interesting areas:

1. In our study we were not successful in establishing the transition from decompensated hypertrophy to heart failure.



However, the 2 week hypoxic data may provide clues how to accelerate the transition to heart failure. The increased expression of FA utilization genes

could be an attempt by the heart to normalise FAO. This would mediate maladaptation (i.e. contractile dysfunction). Therefore, future studies aimed at reaching RV heart failure in response to chronic hypoxia could use FAs or other PPAR α ligands to increase FAO in the hypoxic environment. Wistar rats should be exposed to chronic hypoxia and specific PPAR α activators (i.e. FAs, Wy 14,643, etc.) or high fat diet given instead of normal chow used in the present study. The idea is that high fat diet will activate PPAR α , increasing FAO in an hypoxic environment. Increased FAO in this instance could mediate maladaptation and accelerate right-sided heart failure.

2. Thyroid function involves TSH, T3 and T4. Makinde et al. (1998) have reported that triiodothyronine (T3) levels were increased significantly in the early newborn period. T3 has also been shown to significantly increase basal metabolic rates and induced the expression of some key enzymes involved in oxidative metabolism [Makinde et al., 1998]. Hypoxia-inducible factor 1 (HIF-1) expression is increased during hypoxia and its involved in transcriptional activation of a number genes by hypoxia [Semenza et al., 1994]. Future studies could determine the effect of chronic hypoxia, right ventricular hypertrophy on HIF - 1 and thyroid hormone.

3. Future studies could also investigate the transcription factors regulating oxidative phosphorylation in response to chronic hypobaric hypobaric hypoxia.

2.6 REFERENCES

1. Abdel-aleem S, St Louis JD, Hughes GC, Lowe JE: Metabolic changes in the normal and hypoxic neonatal myocardium. *Annals NY Acad Sciences* 874: 254-261, 1999.
2. Acker H, Xue D: Mechanism of O₂ sensing in the carotid body in comparison with other O₂ sensing cells. *News Physiol Sci* 10: 211-216, 1995.
3. Allard MF, Schonekesss B, Henning SL, English DR, Lopaschuk GD: Contribution of oxidative metabolism and glycolysis to ATP production in hypertrophied hearts. *Am J Physiol* 267: H742-H750, 1994
4. Barger PM, Kelly DP: Fatty acid utilization in the hypertrophied and failing heart: molecular regulatory mechanisms. *Am J Med Sci* 318: 36-42, 1999.
5. Brusselmans K, Compernelle V, Tjwa M, Wiesener MS, Maxwell PH, Collen D, Carmeliet P: Heterozygous deficiency of hypoxia-inducible factor -2 α protects mice against pulmonary hypertension and right ventricular dysfunction during prolonged hypoxia. *J Clin Invest* 111: 1519-1527, 2003.
6. Bryan RM Jr: Cerebral blood flow and energy metabolism during stress. *Am J Physiol* 259 (2 Pt 2): H269-H280, 1990.
7. Bunn HF, Pyton RO: O₂ sensing and molecular adaptation to hypoxia. *Physiol Rev* 76: 839-885, 1996.

8. Christe ME, Rogers RK: Altered glucose and fatty acid oxidation in hearts of the spontaneously hypertensive rat. *J Mol Cell Cardiol* 26: 1371-1375, 1994.
9. Cohn HE, Piasecki GJ, Jackson BT: The effects of beta-adrenergic stimulation on fetal cardiovascular function during hypoxemia. *Am J Obstet Gynecol* 144 (7): 810-816, 1982.
10. Depre C, Shipley GL, Chen W, Han Q, Doenst T, Moore ML, Stepkowski S, Davies PJA, Taegtmeyer H: Unloaded heart *in vivo* replicates fetal gene expression of cardiac hypertrophy. *Nature Medicine* 4: 1269-1275, 1998.
11. Detmar M, Brown LF, Berse B, Jackman RW, Elicker BM, Dvorak HF, Claffey KP: Hypoxia regulates the expression of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) and its receptor in human skin. *J Invest Dermatol* 108: 263-268, 1996.
12. Disch DL, Rader TA, Cresci S, Leone TC, Barger PM, Vega R, Wood PA, Kelly DP: Transcriptional control of a nuclear gene encoding a mitochondrial fatty acid oxidation enzyme in transgenic mice: role for nuclear receptors in cardiac and brown adipose tissue expression. *Mol Cell Biol* 16: 4043-4051, 1996.
13. El Alaoui-Talibi Z, Landormy S, Ivoireau A, Moravec J: Fatty acid oxidation and mechanical performance of volume-overloaded rat hearts. *Am J Physiol* 262: H1068 - H1074, 1992.
14. Fisher DJ: Oxygenation and metabolism in the developing heart. *Semin Perinatol* 8: 217-225, 1984.

15. Forsythe JS, Hang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL: Activation of vascular endothelial growth factor gene transcription by hypoxia inducible factor-1. *Mol Cell Biol* 16: 4604-4613, 1996.
16. Goldberg MA, Dunning SP, Bunn HF: Regulation of the erythropoietin gene: evidence that the O₂ sensor is a heme protein. *Science* 242: 1412-1415, 1988.
17. Harik N, Harik SI, Kuo NT, Sakai K, Przybylski RJ, Lamanna JC: Time course and reversibility of the hypoxia induced alterations in cerebral vascularity and cerebral capillary glucose transporter density. *Brain Res* 737: 335-338, 1996.
18. Harris RJ: Plasma nonesterified fatty acid and blood glucose levels in healthy and hypoxemic newborn infants. *J Pediatr* 84 (4): 578-84, 1974.
19. Heath D, William DR: Man at high altitude. London: Churchill Livingstone. p 3-23, 1991.
20. Hida W, Tun Y, Kikuchi Y, Okabe S, Shirato K: Pulmonary hypertension in patients with chronic obstructive pulmonary disease: recent advances in pathophysiology and management. *Respirology* 7: 3-13, 2002.
21. Hochachka PW, Buck LT, Doll C, Land SL: Unifying theory of hypoxia tolerance: Molecular/metabolic defence and rescue mechanisms for surviving O₂ lack. *Proc Natl Acad Sci USA* 93: 9493-9498, 1996(a).
22. Hochachka PW, Clark CM, Brown WD, Stanley C, Stone CK, Nickles RJ, Zhu G, Allen PS, Holden JE: The brain at high altitude:

- hypometabolism as a defence against chronic hypoxia? *J Cerebral Blood Flow Metab* 14: 671-679, 1995.
23. Hochachka PW, Clark CM, Holden JE, Stanley C, Ugurbil K, Menon RS: ^{31}P magnetic resonance spectroscopy of the Sherpa heart: A PCr/ATP signature of metabolic defence against hypobaric hypoxia. *Proc Natl Acad Sci USA* 93: 1215-1220, 1996(b).
 24. Hochachka PW, Clark CM, Monge C, Stanley C, Brown WD, Stone CK, Nickles RJ, Holden JE: Sherpa brain glucose metabolism and defence adaptations against chronic hypoxia. *J Appl Physiol* 81: 1355-1361, 1996(c).
 25. Hochachka PW, Stanley C, Matheson GO, McKenzie DC, Allen PS, Parkhouse WS: Metabolic and work efficiencies during exercise in Andean natives. *J Appl Physiol* 70: 1720-1729, 1991 .
 26. Hochachka PW, Stanley C, McKenzie DC, Villena A, Monge C: Enzyme mechanisms for pyruvate-to-lactate flux attenuation: A study Sherpas, Quechuas and humming birds. *Int J Sports Med* 13: S119-S123, 1992 (b) .
 27. Hochachka PW: Fuels and pathways as designed systems for support of muscle work. *J Exp Biol* 115: 149-164, 1985.
 28. Hochachka PW: Mechanism and evolution of hypoxia-tolerance in humans. *J Exp Biol* 201: 1243-1254, 1998.
 29. Hochachka PW: Muscle enzymatic composition and metabolic regulation in high altitude adapted natives. *Int J Sports Med* 13: S89-S91, 1992(a).

30. Holden JE, Stone CK, Clark CM, Brown WD, Nickles RJ, Stanley C, Hochachka PW: Enhanced cardiac metabolism of plasma glucose in high altitude natives: adaptation against chronic hypoxia. *J Appl Physiol* 79: 222-228, 1995.
31. Huss JM, Levy FH, Kelly DP: Hypoxia inhibits the peroxisome proliferator-activated receptor α /retinoid X receptor gene regulatory pathway in cardiac myocytes: a mechanism for O₂-dependent modulation of mitochondrial fatty acid oxidation. *J Biol Chem* 276 (29): 27605-27612, 2001.
32. Kantor PF, Robertson MA, Coe JY, Lopaschuk GD: Volume overloaded hypertrophy of the newborn heart slows the maturation of enzymes involved in the regulation of fatty acid metabolism. *J Am Coll Cardiol* 33: 1724-1734, 1999.
33. Ladoux A, Felic C: Hypoxia is a strong inducer of vascular endothelial growth factor mRNA expression in the heart. *Biochem Biophys Res Commun* 195: 1005-1010, 1993.
34. Langendorff O: Untersuchungen am überlebenden Säugethierherzen. *Arch Ges Physiol Mensch Tiere* 61: 291-332, 1895.
35. Lundby C, van Hall G: Substrate utilization in sea level residents during exercise in acute hypoxia and after 4 weeks of acclimation to 4100 m. *Acta Physiol Scand* 176 (3): 195-201, 2002.
36. Maxwell PH, Pugh CW, Ratcliffe PJ: Inducible operation of the erythropoietin 3' enhancer in multiple cell lines: evidence for widespread O₂ sensing mechanism. *Proc Natl Acad Sci USA* 90: 2423-2427, 1993.

37. Monge C, Bonavia D, Leon-Verlade F, Arregui A: The adaptations. In: Hypoxia: eds. Sutton JR, Coates G, Remmers JE. Toronto: Decker. p 53-58, 1990.
38. Ogita HE, Nakaoka T, Matsuoka R, Takao A, Kira Y: Rapid induction of vascular endothelial growth factor expression by transient ischaemia in rat heart. *Am J Physiol* 267: H1948-H1954, 1995.
39. Osorio JC, Stanley WC, Linke A, Castellari M, Diep QN, Panchal AR, Hintze TH, Lopaschuk GD, Recchia FA: Impaired myocardial fatty acid oxidation and reduced protein expression of retinoid X receptor- α in pacing-induced heart failure. *Circulation* 106: 606-612, 2002.
40. Ostadal B, Ostadalova I, Dhalla NS: Development of cardiac sensitivity to O₂ deficiency: comparative and ontogenetic aspects. *Physiol Rev* 79: 635-659, 1999.
41. Paternostro G, Pagano D, Gneccchi-Ruscione T, Bonser RS, Camici PG: Insulin resistance in patients with cardiac hypertrophy. *Cardiovasc Res* 42: 246-253, 1999.
42. Pissarek M, Bigard X, Mateo P, Guezennec CJ, Hoerter JA: Adaptation of cardiac myosin and creatine kinase to chronic hypoxia: role of anorexia and hypertension. *Am J Physiol* 272 (4 Pt 2) : H1690-H1695, 1997.
43. Razeghi P, Young ME, Abbasi S, Taegtmeyer H: Hypoxia *in vivo* decreases peroxisome proliferator-activated receptor α -regulated gene expression in rat heart. *Biochem Biophys Res Commun* 287: 5-10, 2001 .

44. Remondino A, Rosenblatt-Velin N, Montessuit C, Tardy I, Papageorgiou I, Dorsaz PA, Jorge-Costa M, Lerch R: Altered expression of proteins of metabolic regulation during remodeling of the left ventricle after myocardial infarction. *J Moll Cell Cardiol* 32: 2025-2034, 2000.
45. Roberts et al., 1982 – MSc thesis.
46. Rosser BWC, Hochachka PW: Metabolic capacity of muscle fibers from high-altitude natives. *Eur J Appl Physiol* 67: 513-517, 1994.
47. Rumsey ML, Abbott B, Bertelsen D, Mallamaci M, Hagan K, Nelson D, Erecinska M: Adaptation to hypoxia alters energy metabolism in rat heart. *Am J Physiol* 276: H71-H80, 1999 .
48. Sack MN, Rader TA, Park S, Bastin J, McCune SA, Kelly DP: Fatty acid oxidation enzyme gene expression is downregulated in the failing heart. *Circulation* 94: 2837-2842, 1996.
49. Seidman et al., 1995 – MSc thesis.
50. Sivitz WI, Lund DD, Yorek B, Grover-McKay M, Schmid PG: Pretranslational regulation of two cardiac glucose transporters in rats exposed to hypobaric hypoxia. *Am J Physiol* 263 (Endocrinol Metab): E562-E569, 1992.
51. Taegtmeyer H: Switching metabolic genes to build a better heart. *Circulation* 106: 2043-2045, 2002.
52. Thurich T, Bereiter-Hahn J, Schneider M, Zimmer G: Hemodynamics and mitochondrial energy metabolism in right heart hypertrophy after acute hypoxic stress. *Drugs Res* 49 (1): 212-220, 1999.

53. Trinder P: Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *Ann Clin Biochem* 6: 24-27, 1969.
54. Wang GL, Jian BH, Rue EA, Semenza GL: Hypoxia inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular oxygen tension. *Proc Natl Acad Sci USA* 92: 5510-5514, 1995.
55. Weir EK, Archer SL: The mechanism of acute hypoxic pulmonary vasoconstriction: a tale of two channels. *Faseb J* 9: 183-189, 1995.
56. Wenger RH, Gassman M: Oxygen (s) and the hypoxia inducible factor 1. *J Biol Chem* 378: 609-616, 1997.
57. Winslow RM, Monge C: Hypoxia, polycythemia and chronic mountain sickness. Baltimore MD: John Hopkins University Press. p255, 1987.
58. Young ME, Razeghi P, Cedars AM, Guthrie PH, Taegtmeyer H: Intrinsic diurnal variations in cardiac metabolism and contractile function. *Circ Res* 89: 1199-1208, 2001.
59. Youngson C, Nurse C, Yeger H, Cutz E: O₂ sensing in airway chemoreceptors. *Nature* 365: 153-155, 1993.

Chapter 3

Maintenance of Cardiac Oxidative Phosphorylation despite Chronic Reduction in Adenine Nucleotide Translocator 1 Levels

3.1 INTRODUCTION

Mitochondria are regarded as the cell's powerhouse, continually generating ATP. The abundant and continuous supply of mitochondrial ATP is utilized by the mammalian heart to sustain its contractility and function [Taegtmeyer, 1994]. Oxidative phosphorylation is an important component of mitochondrial fuel metabolism. It is linked to the electron transport chain, ultimately resulting in the phosphorylation of ADP to ATP. The inner mitochondrial membrane is impermeable to both ADP and ATP. A nucleotide carrier, the adenine nucleotide translocator (ANT) transports ADP and ATP across the inner mitochondrial membrane [Klingenberg and Nelson, 1994]. Here, cytosolic ADP is transported into the matrix while mitochondrial ATP is exported to the cytosol providing intracellular energy [Brown and Wallace, 1994]. ANT exchanges mitochondrial ATP for ADP in a 1:1 ratio [Dörner et al., 1997]. Furthermore, ANT regulation is coupled to the mitochondrial ATP synthase that catalyzes phosphorylation of ADP to ATP in conjunction with proton flux from the inter-mitochondrial membrane space into the mitochondrial matrix [Jacobus et al., 1982; Heineman and Balaban, 1990]. Oxidative phosphorylation is an important component of mitochondrial fuel metabolism. It is linked to the electron transport chain, ultimately resulting in the phosphorylation of ADP and ATP. For oxidative phosphorylation to occur, a constant supply of ATP/ADP is required. ANT transports ADP and ATP across the inner mitochondrial membrane [Klingenberg and Nelson, 1994]. ANT exchanges mitochondrial ATP for ADP in a 1:1 ratio [Dörner et al., 1997]. ANT regulation is therefore linked or 'coupled' to the mitochondrial ATP synthase that catalyses phosphorylation of ADP to ATP in conjunction with

proton flux from the inter-mitochondrial membrane space into the mitochondrial matrix [Jacobus et al., 1982; Heineman and Balaban, 1990]. ANT is an important transport system for ATP/ADP, therefore regarded as a pivotal regulator of mitochondrial respiration linking mitochondrial ATP synthesis to cytoplasmic energy utilization [Dörner et al., 1997]. ANT is an important transport system for these nucleotides, therefore regarded as a pivotal regulator of mitochondrial respiration linking mitochondrial ATP synthesis to cytoplasmic energy utilization [Dörner et al., 1997].

ANT is a nuclear-encoded gene and consists of two identical 30 kDa subunits to form a homodimer. ANT is located within the inner mitochondrial membrane [Klingenberg, 1992], and exists in multiple isoforms in a number of species [Graham et al., 1997]. In humans, there are three ANT isoforms, designated ANT1, ANT2 and ANT3, which are expressed in a tissue-specific pattern [Cozen et al., 1989; Ku et al., 1989]. ANT1 is highly expressed in the heart and skeletal muscle and its expression is postnatally induced [Li et al., 1989; Stepien et al., 1992; Portman et al., 1997]. On the other hand, ANT2 is weakly expressed in most tissues. ANT2 is abundant in the fetal heart and its expression declines after birth. However, increased levels of ANT2 have been observed in cells with a high glycolytic rate for e.g. C2C12 myoblasts, leading Stepien et al. (1992) to propose that it may mediate ATP/ADP transport during glycolytic conditions (for e.g. hypoxia) [Stepien et al., 1992]. ANT3 is expressed ubiquitously and has been proposed to form ANT1/ANT3 heterodimers [Stepien et al., 1992].

Recent studies using the ANT1 knockout mouse have suggested that ANT protein deficiency may be maladaptive, playing a role in the pathogenesis of cardiac hypertrophy and heart failure [Graham et al., 1997; Ning et al., 2000]. For example, Graham et al. (1997) generated transgenic ANT1 knockout mice and demonstrated that they developed a severe defect in coupled respiration, mitochondrial myopathy and cardiomyopathy [Graham et al., 1997]. Here they suggested that lack of ANT1 (central role player in oxidative phosphorylation) leads to inhibition of respiration resulting in decreased mitochondrial energy production, and the development of cardiac hypertrophy. In a further study, Esposito et al. (1999) showed that ANT1 deficient mice produced high levels of reactive oxygen species (ROS), which may be detrimental to the heart [Esposito et al., 1999]. Together these data suggest that lack of ANT1 results in oxidative phosphorylation inhibition, increased oxidative stress and disruption of ATP production, eventually resulting in the development of cardiac hypertrophy and heart failure.

To further delineate the role of ANT1 in the heart, we investigated whether ANT1 is regulated in parallel with the development of acquired forms of heart disease in a more physiologically relevant model than complete knockout.

3.2 MATERIALS AND METHODS

3.2.1 Hypobaric Hypoxic Experiments

For the hypoxic experiments, six-week old male Wistar rats were housed inside a lexan chamber (SciTech, Cape Town, South Africa) as described previously [Smith et al., 2001]. Rats were exposed to a hypobaric hypoxic stimulus (45 kPa – 11% O₂) for a period of 2 weeks whereafter animals were sacrificed using CO₂. Intact hearts were removed and the right ventricular (RV) tissues were carefully dissected out, weighed and stored at – 80°C for subsequent molecular analyses. Appropriate age and sex matched normoxic controls were used for all experiments. The chamber was opened every 48 hours for no more than 20 minutes to replace bedding, clean cages and provide fresh food and water. The University of Cape Town's Animal Research Ethics Committee approved all animal experiments and the investigation conforms with 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 Determination of Right Ventricular Developed Pressure

Normoxic and hypoxic rats were anesthetized by pentobarbital sodium (100 mgkg⁻¹; ip), whereafter their hearts were removed and placed in ice-cold Krebs-Henseleit buffer (11 mM Glucose, 118 mM NaCl, 25 mM NaHCO₃, 4.7 mM KCl, 1.21 mM KH₂PO₄, 1.22 mM MgSO₄·7H₂O, 1.84 mM CaCl₂·6H₂O). The aorta was located and cannulated on the Langendorff perfusion rig, and a retrograde perfusion of the coronary arteries via the aorta was initiated

immediately [Langendorff, 1895]. The perfusion was performed for 30 minutes with oxygenated (95% O₂, 5% CO₂) Krebs-Henseleit buffer at a constant pressure (104 cm H₂O) and temperature (37°C). During perfusion, a latex balloon was inserted into the right ventricle in order to determine the right ventricular developed pressure (RVDP) as described before. Heart functional parameters were measured using a chart recorder and included heart rate, systolic and diastolic pressure, RVDP and coronary flow. The exclusion criteria used in this study were as follows: heart rate (140–400 beats/min), coronary flow (8–18 ml), and RVDP (50–120 mmHg using a right ventricular end diastolic pressure of 2–12 mm Hg). These criteria were used to exclude values not obtained within these ranges since readings taken at greatly different diastolic pressures will give faulty results.

3.2.3 Determination of Hematocrit Levels

In order to determine hematocrit levels, collected blood was put into heparinized capillary tubes and subsequently spun in a Heraeus hemofuge (Hanau, Germany) at 12,000 rpm for 3 minutes, allowing separation of red blood cells and serum.

3.2.4 Mitochondrial Isolation and Functional Characterization

Mitochondria were isolated by homogenizing RV tissue (using hand-held 5 ml glass homogenizer) in 10 ml ice-cold potassium-EDTA (KE) buffer (0.18 M KCl, 10 mM EDTA, [pH7.4].), whereafter the homogenate was centrifuged (Sigma M2K, Sigma, MO) at 755 x g for 5 minutes at 4°C. The supernatant was subsequently filtered through 41 µm nylon mesh (Spectrum, USA) and

the filtrate centrifuged at 18,800 x g for 5 minutes at 4°C. The pellet (containing isolated mitochondria) was resuspended in 200-500 µl of KE buffer, and was subsequently used for mitochondrial respiration measurements and protein immunoblotting analyses.

Respiratory rates were polarographically measured using a Clark-type electrode (Hansatech Instruments, London, UK) at 25°C with constant stirring. State 3 respiration was estimated by measuring mitochondrial oxygen uptake after the addition of 10 mM ADP to the electrode chamber containing 650 µl of incubation medium (10 mM Tris-HCl, 0.25 M sucrose, 8.5 mM K₂HPO₄, 5 mM glutamate, [pH7.4].), and glutamate to a final concentration of 15 mM (substrate for complex I), and 35 µl of isolated mitochondria.

Proton leak in the isolated mitochondria was determined by measuring state 3 respiration in the presence of 1 mM oligomycin and 50 nM ADP. Oligomycin inhibits ATP synthase and allows determination of proton flux into the matrix other than through ATP synthase. Initially, state 3 respiration was measured in the absence of oligomycin and at the end of state 4, 1 mM oligomycin and 50 nM ADP were added and respiration determined again.

To measure the active ANT protein content, we titrated state 3 mitochondrial respiration with atractyloside (Atr), a specific and irreversible inhibitor of ANT. Isolated mitochondria were incubated in the presence of Atr (10 pmol/mg protein/min) until state 3 respiration was completely inhibited. The amount of Atr required to completely inhibit state 3 respiration was used as a measure of

functional ANT content. All mitochondrial polarographic studies were normalized to total mitochondrial protein content (using the Lowry assay) to determine proton leak.

3.2.5 RNA Isolation and Northern Blot Analysis

Total RNA was extracted from hypoxic and normoxic RV tissue (≈ 100 mg) as previously described [Sack et al., 1996]. Northern blot analysis was performed by loading 20 μ g of total RNA per lane, whereafter blots were probed using a radiolabeled cDNA fragment encoding for ANT1. A cDNA fragment encoding the atrial natriuretic factor (ANF) gene was included as an indicator of cardiac hypertrophy. Also, a cDNA fragment encoding the β -actin gene (a gift from Dr. Dan Kelly, Washington University School of Medicine, St. Louis, MO) was used to demonstrate unchanged mRNA expression levels in response to hypobaric hypoxia. 28S RNA transfer to nitrocellulose membrane was used to assess RNA integrity and to control for variance in gel loading. The relative abundance of mRNA was quantified by laser densitometric analysis using the UVLTec autoradiography analyzer with uviband software v.97 (Cambridge, U.K.). The composite data is represented as mean $\pm$ standard error of the mean. The mRNA levels are reported in arbitrary units (AU) determined by densitometry normalized to normoxic controls (mean control value set at 100).

3.2.6 Protein Immunoblotting Analysis

Protein immunoblot analysis was done as described previously using mitochondrial extracts prepared from normoxic and hypoxic RV heart tissues

(≈ 100 mg) [Sack et al., 1996]. Briefly, $100\ \mu\text{g}$ of mitochondrial protein extracts were loaded per lane, resolved by SDS-polyacrylamide gel electrophoresis (10%), and probed using an anti-ANT antibody (Santa Cruz Biotechnology Inc.). Equal protein loading was confirmed using Ponceau Red staining as described [Jonassen et al., 2001]. The relative abundance of peptides was determined using a chemiluminescence detection system (Amersham Pharmacia Biotechnology) and quantified by laser densitometric analysis using the UVLTec autoradiography analyzer with uviband software v.97 (Cambridge, U.K.). The composite data is represented as mean $\pm$ standard error of the mean. The peptide levels are reported in arbitrary units (AU) determined by densitometry normalized to normoxic controls (mean control value set at 100).

3.2.7 Statistical Analysis

The data is presented as mean $\pm$ standard error of the mean (S.E.M.). Statistical significance between the controls and experimental groups was assessed using Student's *t*-test. Values were considered significant when $p < 0.05$.

3.3 RESULTS

To determine the effects of chronic hypoxia on ANT1 expression, we exposed Wistar rats to hypobaric hypoxia (11% O_2) for a period of 2 weeks. We selected the 2 week experimental point since here we observed a peak in the hypertrophic response, thereafter maintained (refer Chapter 2). Appropriate

age and sex matched controls were included. In response to the chronic hypobaric hypoxic stimulus, hematocrit levels increased by $36.5 \pm 0.4\%$ ($p < 0.001$) (Fig. 1).

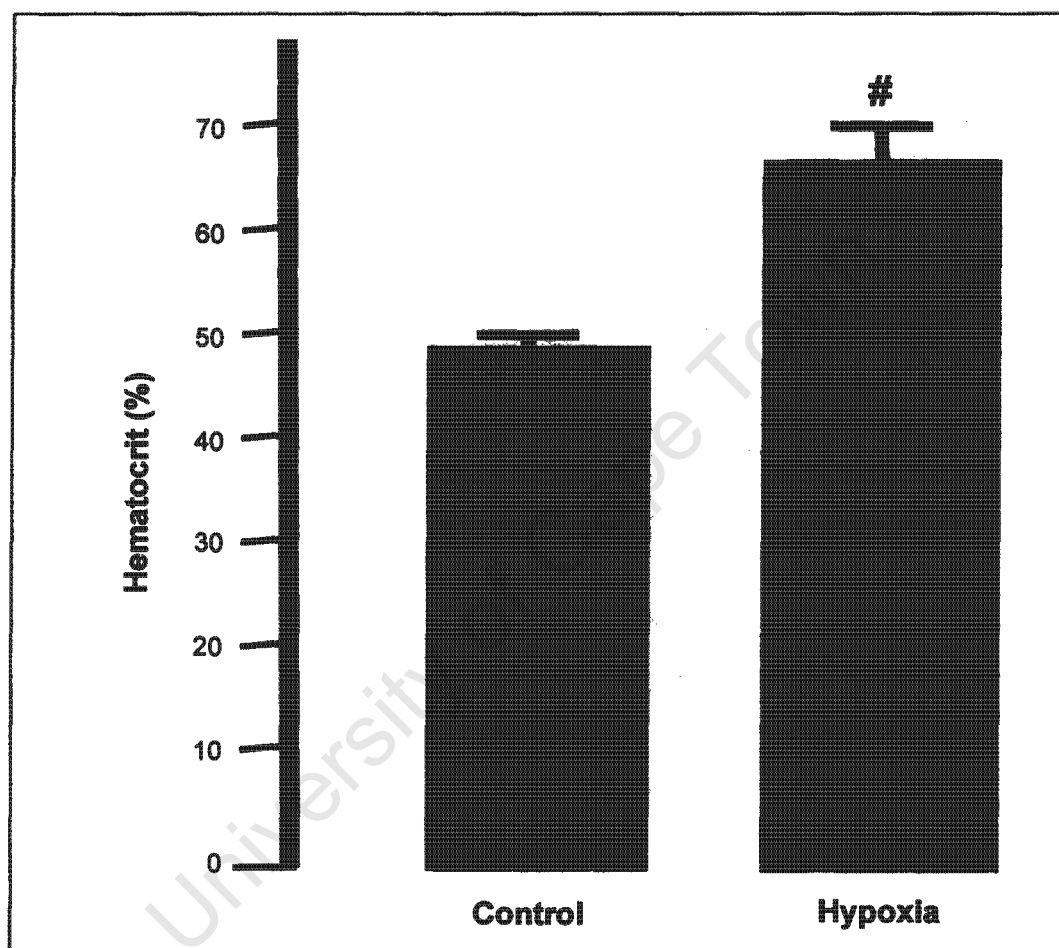
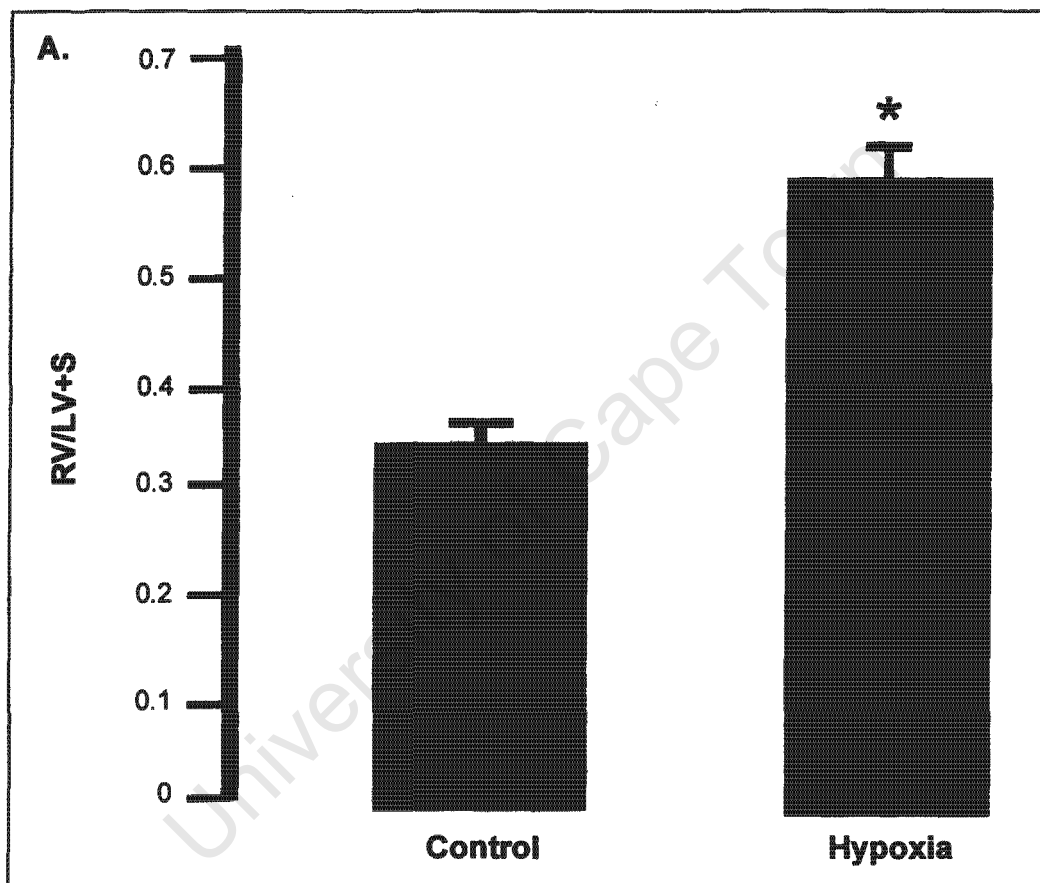


Figure 1: Hematocrit levels in response to 2 weeks hypobaric hypoxia. Values are means $\pm$ S.E.M. for 12 animals. #Significant difference between normoxic controls and 2 weeks hypoxia ($p < 0.001$).

A robust hypertrophic response was observed in the right ventricle after 2 weeks exposure to hypoxia. Supporting evidence for RV hypertrophy

include:(i) RV to left ventricle (LV) plus septum weight ratio was increased by $75.8 \pm 3.9\%$ ($p < 0.05$) (Fig. 2A), (ii) the right ventricular developed pressure (RVDP) was elevated by $78.3 \pm 4.9\%$ ($p < 0.05$) (Fig. 2B) and (iii) atrial natriuretic factor (ANF) gene expression, a gene marker for cardiac hypertrophy, was significantly increased by 6-fold ($p < 0.05$) (Fig. 2C).



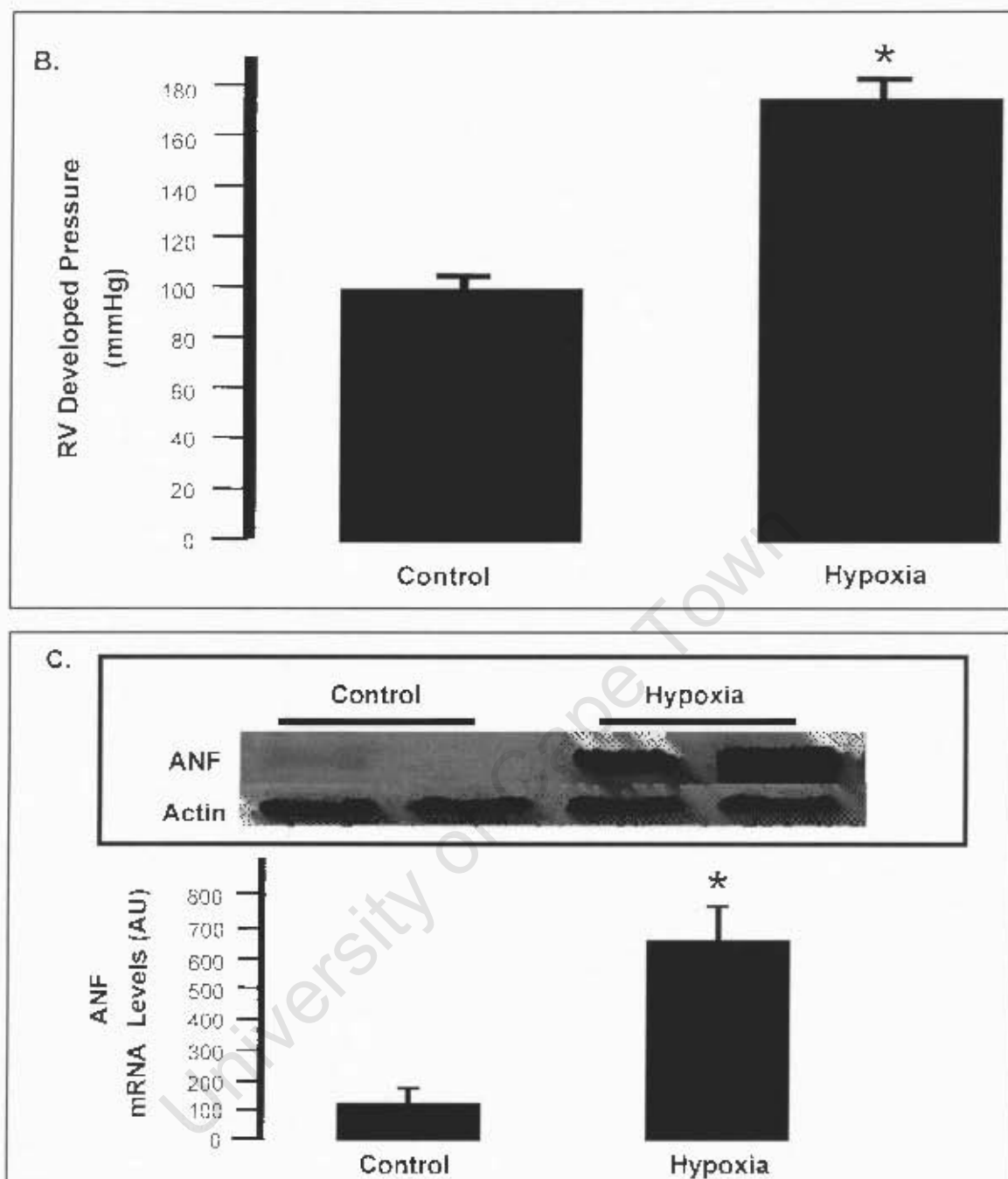
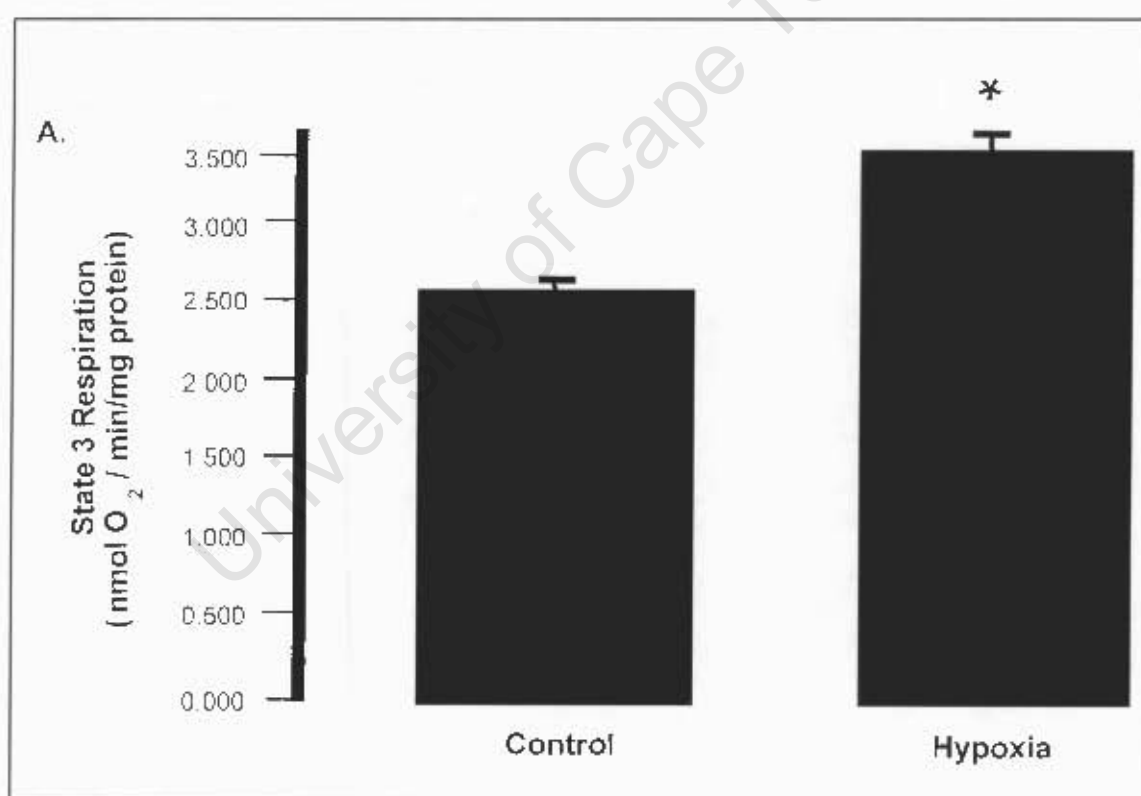
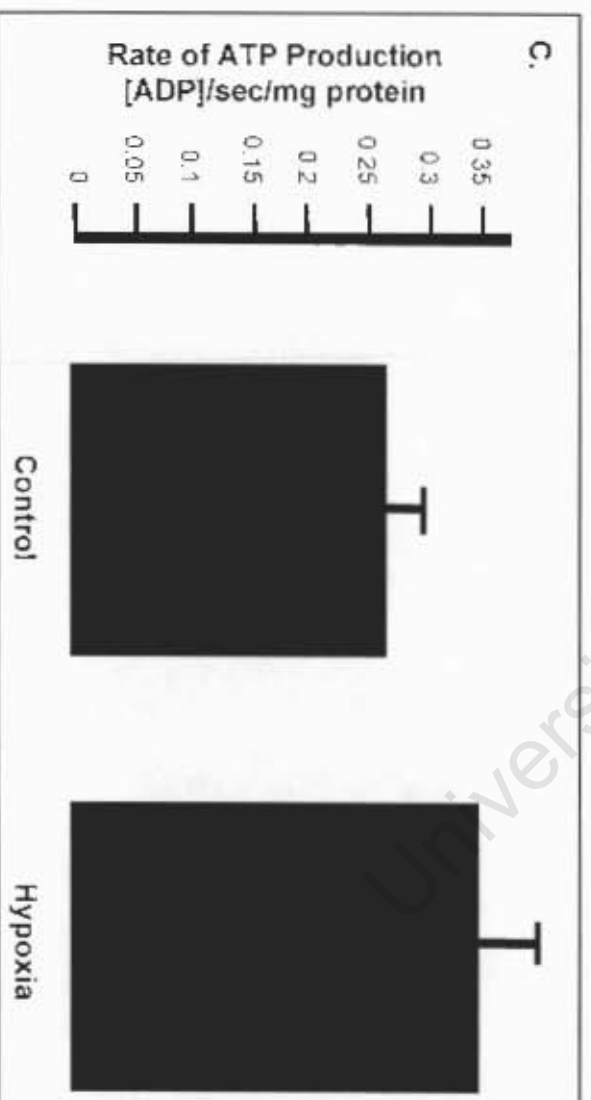
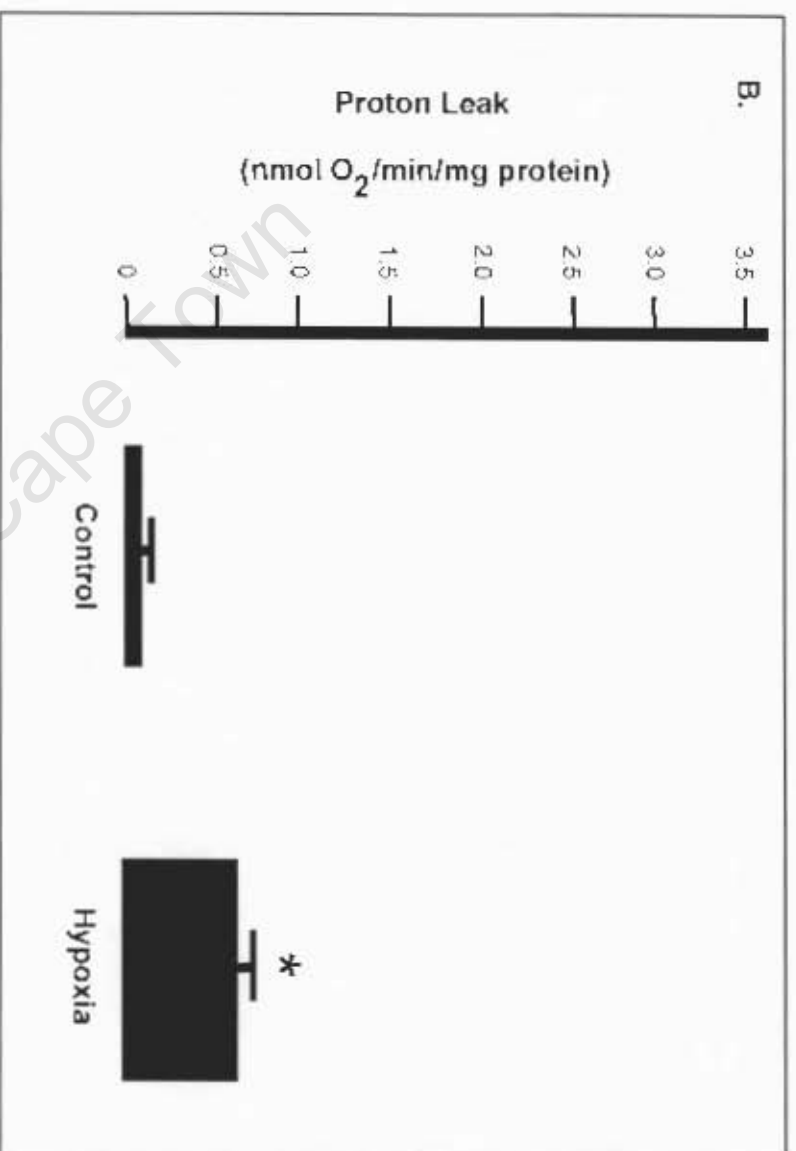


Figure 2: Physiologic and molecular evidence of right ventricular cardiac hypertrophy in response to 2 weeks hypobaric hypoxia (11%O₂). (A) Right ventricle (RV) to left ventricle plus septum (LV+S) ratio in normoxic controls and rats exposed to 2 weeks hypobaric hypoxia. Bars represent mean ($\pm$ S.E.M.) weight ratio for 4 animals. *Significant difference between 2 weeks hypoxia and normoxic controls ($p < 0.05$). (B) Right ventricular developed pressure (RVDP) measurements. The bars represent mean $\pm$ S.E.M. (mean control at 100) for 4 animals. *Significant difference between 2 weeks hypoxia vs. normoxic controls ($p < 0.05$). (C) ANF gene expression. Inset – a representative Northern blot is shown for ANF. Actin is included to show unchanged mRNA expression. Densitometric analysis of Northern blots was performed and data normalized to the 28S RNA. The bars represent mean $\pm$ S.E.M. (mean control value set at 100) ($n=6$). *Significant difference between 2 weeks hypoxia vs. normoxic controls ($p < 0.05$).

To assess the energetic status of the heart in response to hypobaric hypoxia, mitochondria were isolated from RV tissue and state 3 respiration (an experimental measure of oxygen consumption) determined. Baseline oxygen consumption was increased by ($41.7 \pm 6.7\%$) in isolated mitochondria exposed to 2 weeks hypobaric hypoxia versus normoxic controls (Fig. 3A). Mitochondrial proton leak, determined by measuring state 3 respiration in the presence of oligomycin, was elevated after 2 weeks hypobaric hypoxia (Fig. 3B). The rate of ATP production and mitochondrial efficiency was not significantly altered (Fig. 3C and Fig. 3D).





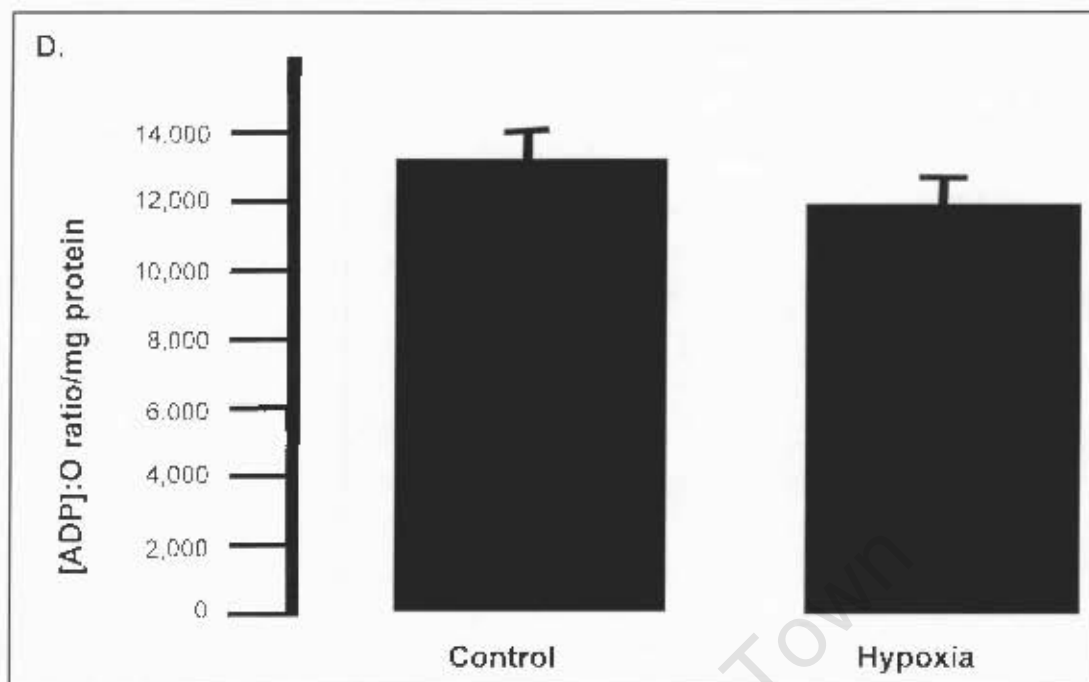
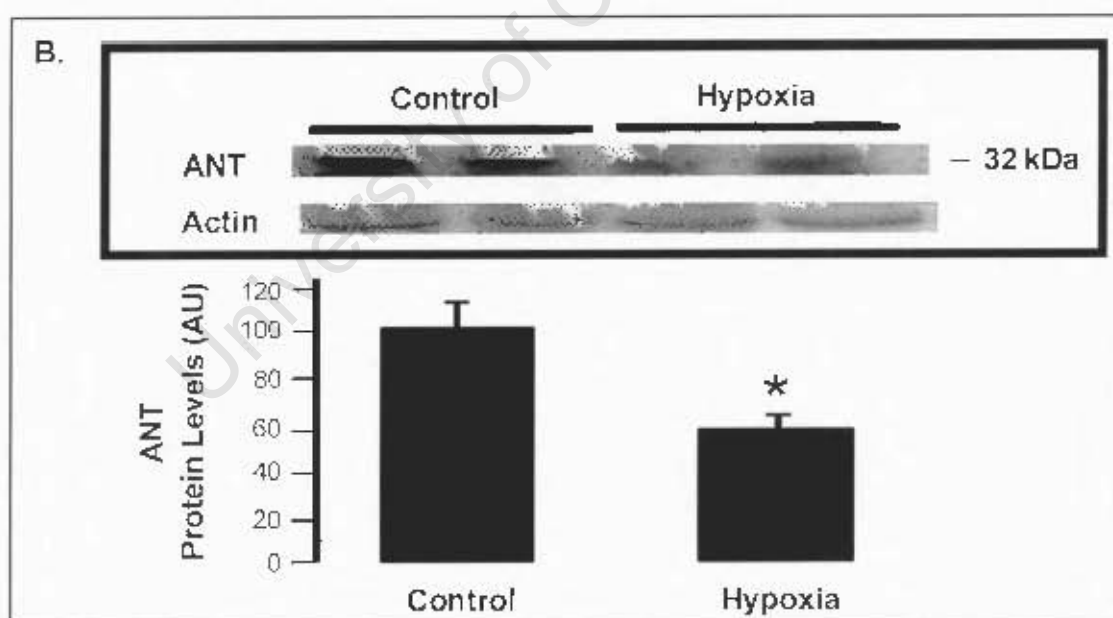
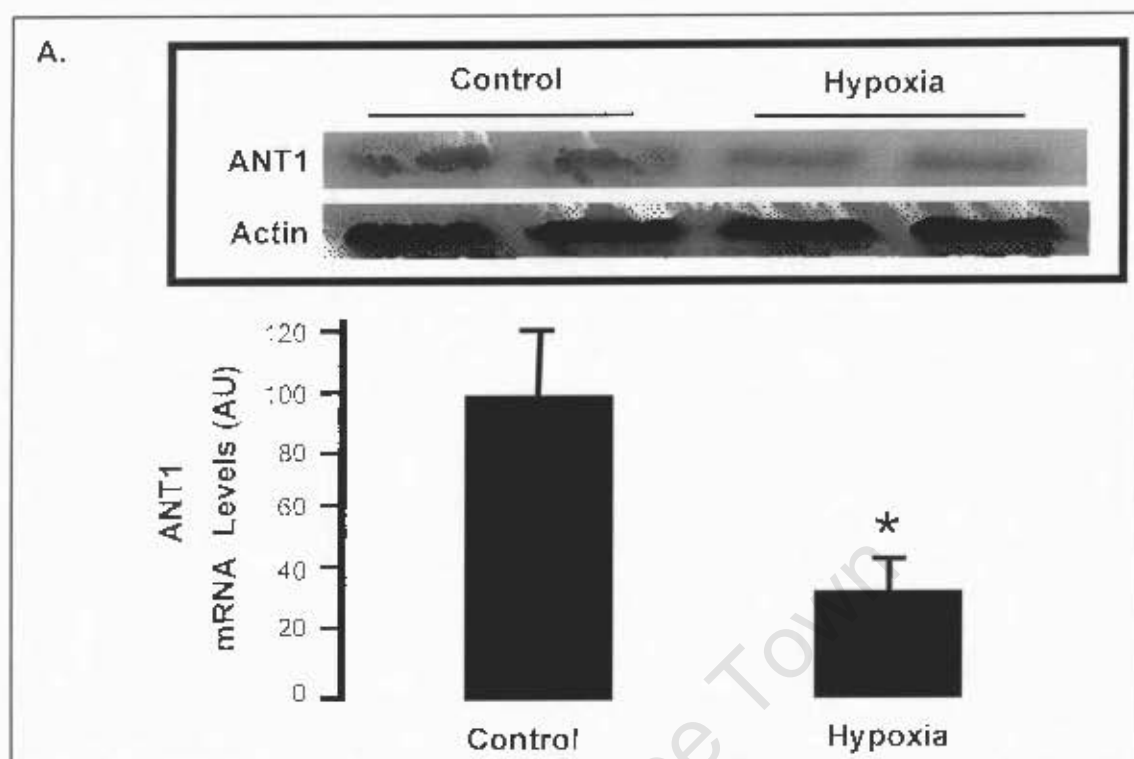


Figure 3: Right ventricular mitochondrial respiration in response to 2 weeks hypobaric hypoxia (11% O₂). (A) **Baseline oxygen consumption.** The bars represent mean (± S.E.M.) state 3 respiration (nmol O₂/min/mg protein) (n= 12). *Significant difference between 2 weeks hypoxia vs. normoxic controls (p < 0.05). (B) **Determination of mitochondrial proton leak.** Bars represent mean ± (S.E.M.) ratio of state 3 respiration in the presence of 1 mM oligomycin/ uninhibited state 3 respiration (n=7). *Significant difference between normoxic controls and 2 weeks hypoxia (p < 0.05). (C) **Rate of ATP production.** The bars represent mean (+ S.E.M) rate of ATP production ([ADP]/sec/mg protein) (n=12). (D) **[ADP]:O ratio.** The bars represent mean (± S.E.M.) [ADP] :O ratio ([ADP]:O₂/mg protein) (n=12).

The regulation of ANT1 in response to chronic hypobaric hypoxia was determined by measuring RV ANT gene expression and protein levels. ANT1 gene expression was decreased by 66.4 ± 25% (p < 0.05) (Fig. 4A). In parallel, ANT mitochondrial protein levels were reduced by 39.1 ± 9.8 % (p < 0.05) (Fig. 4B). Unexpectedly, ANT functional content was upregulated since more atractyloside was required to inhibit ANT function in mitochondria isolated from the hypoxic RV (Fig. 4C).



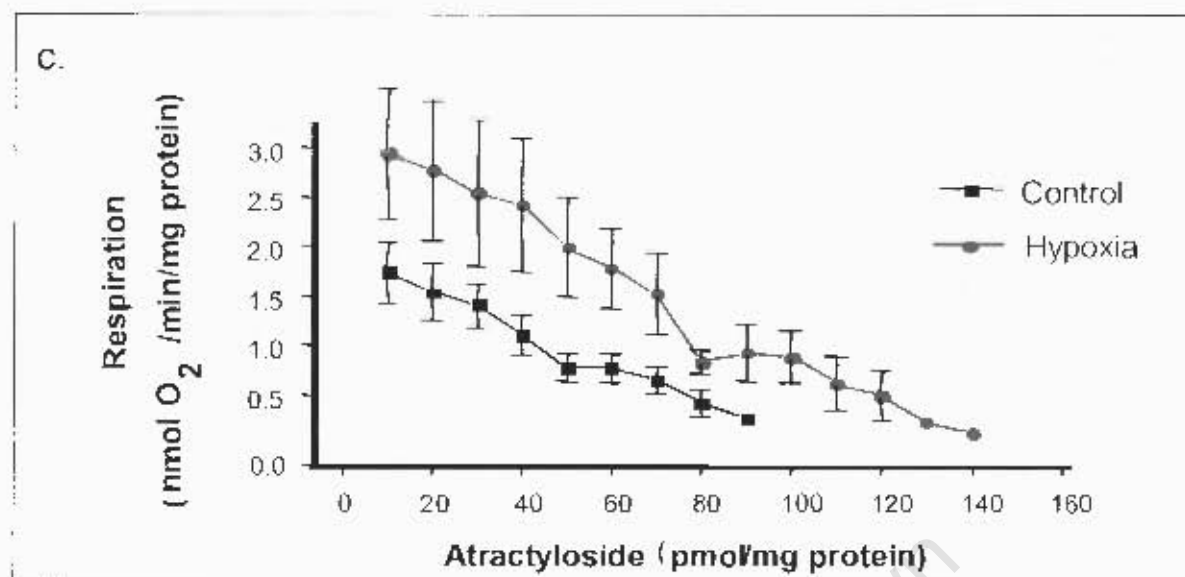


Figure 4: Regulation of ANT gene expression, protein steady-state levels and functional ANT content in response to 2 weeks hypobaric hypoxia (11% O₂). (A) **ANT1 gene expression.** Inset-a representative Northern blot is shown for ANT1, the cardiac-enriched isoform. Actin is included to show unchanged mRNA expression. Densitometric analysis of Northern blot and data normalized to the 28S RNA. The bars represent mean $\pm$ S.E.M. (mean control value set at 100 (n=6)). * Significant difference between 2 weeks hypoxia vs. normoxic controls ($p < 0.05$). (B) **Mean steady-state ANT peptide levels.** ANT peptide levels (in arbitrary units) normalized to Ponceau-stained protein bands (mean control set at 100) (n=12). * Significant difference between 2 weeks hypoxia vs. normoxic controls ($p < 0.05$). Inset – a representative Western blot for ANT. Actin is included to show unchanged steady-state levels. (C) **Determination of functional ANT content.** The graph depicts uninhibited state 3 mitochondrial respiration (nmol O₂/min/mg protein) as a function of the percentage of ANT inhibited by atractyloside (n=9). *Significant difference between normoxic controls and 2 weeks hypoxia ($p < 0.05$).

In order to determine the long-term effects of reduced ANT1 levels in the RV we exposed Wistar rats to 24 weeks hypobaric hypoxia and determined RVDP and ANT steady-state peptide levels. RVDP was maintained after 24 weeks hypobaric hypoxia i.e. similar to 2 week experimental point (Fig. 5A). ANT steady-state protein levels were markedly downregulated after 24 weeks hypobaric hypoxia ($65 \pm 5.3 \%$; $p < 0.001$) (Fig. 5B).

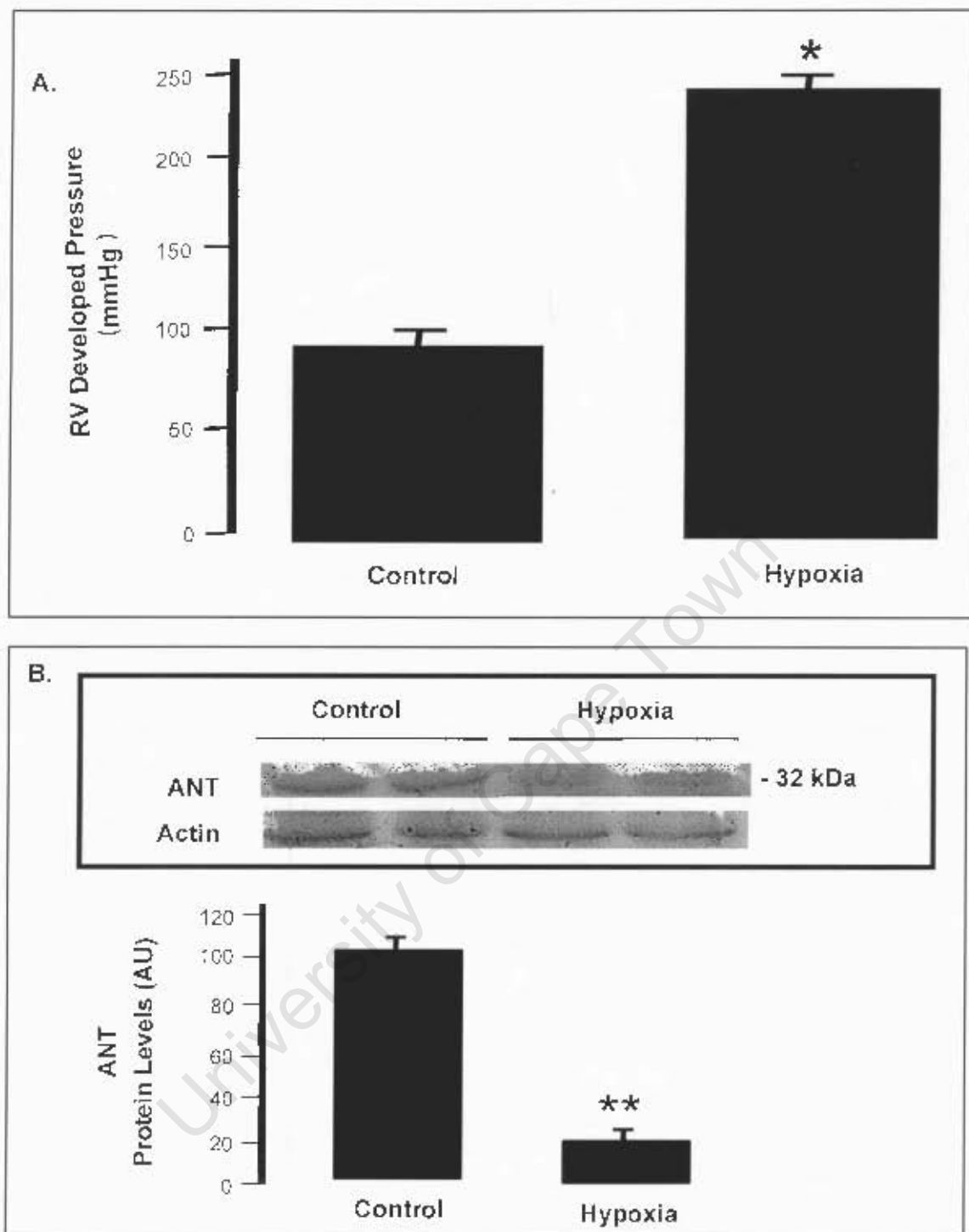


Figure 5: Effects of chronic hypobaric hypoxia on cardiac output and ANT protein steady-state levels in response to 24 weeks hypobaric hypoxia (11 % O₂). **(A)** Right ventricular developed pressure (RVDP) measurements. The bars represent mean $\pm$ S.E.M. (mean control at 100) for 4 animals. **Significant difference between 24 weeks hypoxia vs. normoxic controls ($p < 0.001$). **(B)** Mean steady-state ANT peptide levels. ANT peptide levels (in arbitrary units) normalized to Ponceau-stained protein bands (mean control set at 100) ($n=12$). ** Significant difference between 24 weeks hypoxia vs. normoxic controls ($p < 0.001$). Inset – a representative Western blot for ANT. Actin is included to show unchanged steady-state levels.

3.4 DISCUSSION

In this study we further investigated the *in vivo* role of ANT1 in the heart by determining its regulation in rats exposed to 2 weeks hypobaric hypoxia (11% O_2). Our major findings are cardiac contractile function and right ventricular cardiac hypertrophy was maintained over a period of 24 weeks despite a significant decrease in ANT1 level. Moreover, after 2 weeks of hypobaric hypoxia, mitochondrial energetic status was sustained despite a significant reduction in ANT1 levels. Taken together, these data suggest that modest ANT1 depletion does not compromise mitochondrial respiratory capacity or cardiac function.

In order to maintain the energetic homeostasis of the cardiac myocyte, a balance between intracellular ATP supply and demand is important [Klingenberg, 1992]. Previous studies have demonstrated that ANT1 deficiency results in a defect in coupled respiration that inhibits oxidative phosphorylation, impairing ATP production and exhibiting progressive cardiac hypertrophy [Graham et al., 1997]. In contrast, our data demonstrate that the reduction in ANT1 expression in response to hypoxia-induced cardiac hypertrophy is associated with an increase in state 3 respiration. We propose that this response is mediated by a compensatory upregulation of ANT2 and/or ANT3 levels. In support, ANT2 has been implicated as an important regulator during high glycolytic conditions i.e., fetal state and during hypoxia [Stepien et al., 1992].

We found that ANT1 gene expression was significantly reduced in response to chronic hypobaric hypoxia. In parallel ANT protein expression was also downregulated. Unfortunately the antibody used in this study could not discriminate between the 3 ANT isoforms expressed in the rat heart. We could not therefore establish whether ANT2 or ANT3 were induced to compensate for ANT1 depletion. The latter is supported by the fact that total ANT protein levels were reduced to a lesser extent (~39%) compared to ANT1 gene expression.

Despite decreased ANT1 gene expression and ANT protein levels, we observed increased ANT functional content accompanied by maintained mitochondrial efficiency and ATP production. These data were surprising as Graham et al. (1997) have shown that ANT1 knockout mice have mitochondrial oxidative phosphorylation inhibition, impaired ATP synthesis and pathologic hypertrophy [Graham et al., 1997]. In order to determine the long-term effects of reduced ANT1 expression on cardiac output, we exposed rats to 24 weeks of chronic hypobaric hypoxia (11% O₂). In our study, cardiac output was maintained despite the chronic reduction in ANT protein levels. The differences between our long-term study and the ANT1 knockout mouse may be due to the degree of ANT1 deficiency i.e. approximately a 50-60% reduction in our model compared to the total ablation with the ANT1 knockout mouse. The remainder of ANT1, augmented ANT2 and/or ANT3 protein levels may be part of a compensatory adaptive response in our study. Rosser and Hochachka (1994) have found that in vivo biochemical properties of

skeletal muscle in Quechuas (high-dwelling Andean native) are predominantly those of slow twitch fibres [Rosser and Hochachka, 1994]. consistent with the adjustments of glycolytic versus oxidative contributions to energy supply, thus improving the yield of ATP per mole of carbon fuel utilized [Hochachka, 1996c]. Slow twitch fibres are rich in mitochondria which supports high levels of oxidative metabolism. This is an adaptation to hypoxia because slow twitch muscle fibers increase O_2 levels and ATP production with increased mitochondria numbers. During chronic hypoxia, increased mitochondrial numbers could maintain mitochondrial oxidative phosphorylation (ATP production) despite reduced oxygen availability.

ANT 2 is weakly expressed in most tissues. It is abundant in the fetal (hypoxic environment) heart and its expression declines after birth. Increased levels of ANT2 have been observed in cells with a high glycolytic rate leading [Stepien et al., 1992]. to propose that it may mediate ATP/ADP transport during glycolytic conditions (for e.g hypoxia) [Stepien et al., 1992]. Therefore, it is possible that right hypertrophy due to chronic hypoxia results in ANT isoform alteration for e. g from ANT1 to ANT2.

Previous studies have shown that chronic hypobaric hypoxic exposure results in various adaptations i.e. an increase in mitochondrial number (even though size is small), erythropoiesis and maintained oxygen consumption [Costa et al., 1988]. Increased erythropoiesis improves oxygen transport whereas an increase in the number of mitochondria would be a specific adaptation to chronic hypoxia [Lund and Tomanek, 1980]. During chronic hypoxia,

increased mitochondrial numbers could maintain mitochondrial oxidative phosphorylation (ATP production) despite reduced oxygen availability. In this study, we show elevated mitochondrial oxygen consumption and proton leak in the right ventricle in response to 2 weeks hypobaric hypoxia. Previous studies have suggested that ANT may act as an uncoupler of mitochondrial respiration in the presence of low intracellular free fatty acid (FFA) concentrations. In agreement, ANT shares several similarities with UCPI for example similar sequence and domain composition and molecular mass. Simonyan and Skulachev (1998) demonstrated that mild uncoupling inhibited ROS formation in heart mitochondria and that this cardioprotective effect was primarily mediated by ANT. They therefore hypothesised that mild uncoupling (for e.g. by FFAs) may be advantageous by reducing mitochondrial membrane potential and thereby preventing damaging ROS production. Increasingly, we found that plasma FFA levels were reduced after 2 weeks of hypobaric hypoxia, suggesting increased cellular FFA uptake. In light of this, we propose that in response to hypobaric hypoxia, elevated intracellular FFAs levels drive the increased proton leak in the RV via ANT and/or alternatively by one of the UCPs expressed in the heart resulting in a reduction of membrane potential and limiting damaging mitochondrial ROS production. However, mitochondrial oxidative phosphorylation (ADP/O ratio) and the rate of ATP synthesis were maintained in the hypertrophied RV in response to chronic hypoxia. We speculate that a switch-back to fatty acids as energy source after 2 weeks exposure to hypoxia is responsible for the observed elevation of oxygen consumption and proton leak (as discussed in the Discussion of Chapter 2). This switch-back to fatty acid utilization may be a

less efficient energy source compared to glucose utilization but we propose that it may form part of a compensatory response ensuring maintenance of mitochondrial ATP generation and cardiac function. However, a limitation of this study is that we did not perform mitochondrial respiration studies with a fatty acid substrate for e.g. fatty acyl-CoA or fatty acylcarnitine. These will need to be performed to confirm our hypothesis in this regard. A further limitation is that we did not measure ANT2 and ANT3 expression levels. We have attempted to perform Northern blotting analyses employing cDNAs for ANT2 and ANT3, but were not successful in optimizing experimental conditions.

In conclusion, we report the development of right ventricular cardiac hypertrophy in rats exposed to 2 weeks hypobaric hypoxia vs. normoxic controls. Mitochondrial ATP production and cardiac output was sustained despite a decrease in ANT gene and protein expression. However, functional ANT content was elevated. Moreover, we found that in response to 24 weeks hypobaric hypoxia, cardiac output was maintained despite the long-term reduction of ANT protein levels. Our data therefore suggest an adaptive response to hypobaric hypoxia that may be mediated by altered ANT isoform expression, ensuring sustained mitochondrial ATP production in response to reduced oxygen availability.

Postscript: At the time of submission of this thesis, Wallace's group genetically inactivated two isoforms of ANT i.e. ANT1 and ANT2 in the mouse liver (mice only have ANT1/2 isoforms) [Kokoszka et al., 2004]. Interestingly, mice

survived suggesting that these proteins may not be solely responsible for exchange of ADP / ATP across the mitochondrial membrane [Kokoszka et al., 2004].

3.5 REFERENCES

1. Brown MD and Wallace DC: Molecular basis of mitochondrial DNA disease. *J Bioenerg Biomembr* 26: 273-289, 1994.
2. Costa LE, Boveris A, Koch OR, Taquini AC: Liver and heart mitochondria in rats submitted to chronic hypobaric hypoxia. *Am J Physiol (Cell Physiol)* 255: C123-C129, 1988.
3. Cozen AL, Runswick MJ, Walker JE: DNA sequences of two expressed nuclear genes for human mitochondrial ADP/ATP translocase. *J Mol Biol* 206: 261-280, 1989.
4. Dörner A, Schulze K, Rauch U, Schulthesiss HP: Adenine nucleotide translocator in dilated cardiomyopathy: pathophysiological alterations in expression and function. *Mol Cell Biochem* 174: 261-269, 1997.
5. Esposito LA, Melov S, Panov A, Cottrell BA, Wallace DC: Mitochondrial disease in mouse results in increased oxidative stress. *Proc Natl Acad Sci USA* 96 (9): 4820-4825, 1999.
6. Graham BH, Waymire KG, Barbara C, Trounce TA, MacGregor GR, Wallace DC: A mouse model for mitochondrial myopathy and cardiomyopathy resulting from a deficiency in the heart/muscle isoform of the adenine nucleotide translocator. *Nat Genet* 16 (3): 226-234, 1997.

7. Heineman FW, Balaban RS: Control of mitochondrial respiration in the heart in vivo. *Annu Rev Physiol* 52: 523-542, 1990.
8. Jacobus WE, Moreadith RW, Vandegaer KM: Mitochondrial respiratory control. Evidence against the regulation of respiration by extramitochondrial phosphorylation potential or by [ATP]/[ADP] ratios. *J Biol Chem* 257 (5):2397-402, 1982.
9. Jonassen AK, Sack MN, Mjos OD, Yellon DM: Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. *Circ Res* 89 (12): 1191-1198, 2001.
10. Klingenberg M, Nelson DR: Structure-function relationships of the ADP/ATP carrier. *Biochim Biophys Acta* 1187 (2): 241-244, 1994.
11. Klingenberg M: Structure-function of the ADP/ATP carrier. *Biochem Soc Trans* 20 (3):547-550, 1992.
12. Kokoszka JE, Waymire KG, Levy SE, Sligh JE, Cai J, Jones DP, MacGregor GR, Wallace DC: The ADP /ATP translocator is not essential for the mitochondrial permeability transition pore. *Nature* 427: 461-465, 2004.
13. Ku DF, Kagan J, Chen ST, Chang CD, Basergo R, Wurzel J: The human fibroblast adenine nucleotide translocator gene. *J Biol Chem* 264:13998-14004, 1989.
14. Langednorff O: Untersuchungen am uberlebenden Saugethierherzen. *Arch Ges Menschen Tiere* 61: 291-332, 1895.
15. Li K, Warner CK, Hodge JA, Minoshima S, Kudob J, Fukuyama R, Maekawa M, Shimizu Y, Shimizu N, Wallace DC: A human muscle

- adenine nucleotide translocator gene has four exons, is located to chromosome 4, and is differentially expressed. *J Biol Chem* 264 (24): 13996-4004, 1989.
16. Lund DD, Tomanek RJ: The effects of chronic hypoxia on the myocardial cells of normotensive and hypertensive rats. *Anat Rec* 196:421-430, 1980.
 17. Ning X H, Zhang J, Liu J, Ye Y, Chen SH, From AH, Bache RJ, Portman MA: Signaling and expression for mitochondrial membrane proteins during left ventricular remodeling and contractile failure after myocardial infarction. *J Am Coll Cardiol* 36 (1): 282-287, 2000.
 18. Portman MA, Xiao Y, Song Y, Ning XH: Expression of adenine nucleotide translocator parallels maturation of respiratory control in heart in vivo. *Am J Physiol* 273 (4 Pt 2): H1977-H1983, 1997.
 19. Sack MN, Rader TA, Park S, Bastin J, McCune SA, Kelly DP: Fatty acid enzyme encoding gene expression is downregulated in the failing heart. *Circulation* 94: 2837-2842, 1996.
 20. Simonyan RA, Skulachev VP: Thermoregulatory uncoupling in heart muscle mitochondria: involvement of the ATP/ADP antiporter and uncoupling protein. *FEBS Lett* 436: 81-84, 1998.
 21. Smith RM, McCarthy J, Sack MN: TNF alpha is required for hypoxia-mediated right ventricular hypertrophy. *Mol Cell Biochem* 219: 139-143, 2001.
 22. Stepien G, Torroni A, Chung AB, Hodge JA, Wallace DC: Differential expression of adenine nucleotide translocator isoforms in mammalian

tissues and during muscle cell differentiation. *J Biol Chem* 267 (21): 14592-4597, 1992.

23. Taegtmeyer H: Energy metabolism of the heart: from basic concepts to clinical applications. *Curr Probl Cardiol* 19 (2): 59-113, 1994.

University of Cape Town

Chapter 4

Counter-regulatory Effects of Incremental Hypoxia on the Transcription of a Cardiac Fatty Acid Oxidation Enzyme-Encoding Gene

4.1 INTRODUCTION

The normal adult heart predominantly uses fatty acids (FAs) to produce ATP [Taegtmeyer, 1980]. In contrast, the hypoxic heart switches energy substrate usage from FAs to glucose [Holden et al., 1995]. In this instance, increased glucose utilization is considered an adaptive response since it ensures more efficient production of ATP per molecule oxygen compared to fatty acid oxidation (FAO) [Holden et al., 1995]. Hypoxia results in enhanced glycolytic capacity by increasing expression of genes involved in glycolysis and glucose transport [Semenza et al., 1994; Semenza et al., 1996; Semenza et al., 1998]. For example, hypoxia increases glycolytic gene expression by stabilizing the redox-sensitive transcription factor, hypoxia-inducible factor-1 α (HIF-1 α) that otherwise is degraded during normoxic conditions [Zhu et al., 2001]. HIF-1 α forms a heterodimer with HIF-1 β , subsequently translocating to the nucleus where it binds to hypoxia-response elements (HRE), resulting in elevated transcription of glycolytic genes for e.g. GLUT-1 and phosphoglycerate kinase [Loike et al., 1992; Semenza et al., 1994]. On the other hand, depletion of transcriptional repressors by hypoxia for e.g. Sp3 results in an induction of glycolytic genes lacking HREs (β -enolase and pyruvate kinase-M) [Discher et al., 1998].

The pivotal FAO mitochondrial enzyme, medium-chain acyl-CoA dehydrogenase (MCAD), catalyzes a pivotal step in the mitochondrial FAO pathway. It has been previously demonstrated that MCAD gene expression is

downregulated with cardiac hypertrophy and heart failure [Sack et al., 1996; Sack et al., 1997]. While it has been shown that FAO decreases during hypoxia, the regulatory mechanisms responsible for such a downregulation have not been clearly defined [Abel-aleem et al., 1998; Rumsey et al., 1999]. Previous studies have reported that the PPAR α /retinoid X receptor (RXR α) heterodimer, a key modulator of several FA target genes, is reduced in response to *in vitro* hypoxia [Huss et al., 2001; Razeghi et al., 2001]. For example, Huss et al. (2001) reported a decrease in steady-state nuclear levels of RXR α in response to hypoxia, resulting in reduced mCPT-I gene expression in cardiomyocytes [Huss et al., 2001]. Here, PPAR α steady-state nuclear levels remained unaltered. In contrast, Razeghi et al. (2001) showed a reduction of *in vivo* PPAR α and several target FA utilization genes for e.g. mCPT-I and MCD in response to hypoxia - 10 days CoCl₂ and 5 days isovolemic hemodilution [Razeghi et al., 2001].

In light of these conflicting PPAR α data, we evaluated the regulation of the MCAD gene promoter (a PPAR α target gene) in an *in vivo* hypobaric hypoxic system. We exposed transgenic and wild type mice to either a moderate (14% O₂) or severe (8% O₂) hypobaric hypoxic stimulus vs. normoxia for a period of 48 hours and 1 week, respectively. We compared gene expression, MCAD promoter activity and steady-state levels of the regulatory peptides PPAR α and Sp3.

4.2 MATERIALS AND METHODS

4.2.1 Mice

We used the B6SJLF1/J mouse strain in this study. The transgenic and the wild type mice were a gift from Dr. Dan Kelly (Washington University School of Medicine, St. Louis, MO). To assess MCAD promoter activity, we employed the MCADCAT.371 (line 10-4) and MCADCAT.371 (line 10-1) transgenic mouse strains containing 14 and 55 transgene copies, respectively [Disch et al., 1996]. The MCADCAT.371 transgenic mouse has been previously described and characterized in response to a hypertrophic stimulus, and harbors a functional human MCAD gene promoter (560-bp) fused to the bacterial chloramphenicol acetyl transferase (CAT) reporter gene [Sack et al., 1997]. In order to establish a sustained breeding colony in our animal laboratory, MCADCAT mice were bred by mating a male transgene (TG) with a female wild type (WT) mouse. At age three weeks, mice ears were clipped for identification purposes, and tails cut (1 cm) and stored at -80°C . DNA was extracted from mice tails as previously described [Disch et al., 1996], and mice genotyped using the polymerase chain reaction (PCR). Previously described CAT and actin primers were utilized in order to distinguish between WT and TG mice [Disch et al., 1996].

4.2.2 Hypobaric Hypoxic Experiments

For the hypoxic experiments, 7-week old male and female transgenic and wild type mice were housed in a lexan hypobaric chamber as described previously [Smith et al., 2001]. We performed two sets of experiments by exposing animals to either a moderate (30 kPa - 14% O₂) or severe (60 kPa - 8% O₂) hypobaric hypoxic stimulus for a period of 48 hours and 1 week, respectively. Appropriate age and sex matched normoxic controls were used for all experiments. The chamber was opened every 48 hours for no more than 10 minutes to replace bedding and clean cages. Mice were sacrificed after each experimental time point using carbon dioxide, and intact hearts dissected out and stored at -80°C. RNA and peptide analyses were performed on the collected biventricular tissue. In order to avoid any bias due to diurnal variations, preparations such as blood and tissue collection were routinely performed between 14h00 and 16h00. The University of Cape Town's Animal Research Ethics Committee approved all animal experiments.

4.2.3 RNA Isolation and Northern Blot Analysis

Northern blotting analysis was performed using total RNA isolated from normoxic, control hearts and from mice exposed to 48 hours and 1 week of hypobaric hypoxia. RNA extraction was performed by homogenizing mouse hearts (100 mg) in 2 ml Total RNA Isolation Reagent (Abgene House, UK). The homogenate was centrifuged at 10,500 rpm for 15 minutes and the supernatant collected. Twenty µg of total RNA was loaded per lane and the gel (1.2%) run at 80 V for 4 hours. RNA in the gel was transferred onto a nylon membrane (Amersham Pharmacia Biotech, UK), whereafter it was

probed for 1 hour with radiolabeled cDNA fragments encoding the MCAD gene (a gift from Dr. Dan Kelly, Washington University School of Medicine, St. Louis, MO) [Sack et al., 1996]. A cDNA fragment encoding the β -actin gene (a gift from Dr. Dan Kelly, Washington University School of Medicine, St. Louis, MO) was used to demonstrate unchanged mRNA expression levels in response to hypobaric hypoxia [Sack et al., 1996]. 28S RNA transfer to nitrocellulose membrane was used to assess RNA integrity and to control for variance in gel loading.

The relative abundance of mRNA was quantified by laser densitometric analysis using the UVLTec autoradiography analyzer with uviband software v.97 (Cambridge, U.K.). The composite data is represented as mean $\pm$ standard error of the mean. The mRNA levels are reported in arbitrary units (AU) determined by densitometry normalized to normoxic controls (mean control value set at 100).

4.2.4 Nuclear Protein Extraction

Crude nuclear proteins were prepared as previously described [Deryckere F. and Gannon F, 1994]. Here, heart tissue (50-100 mg) was homogenized in 3 ml of solution A (0.6 % Nonidet P-40, 50 mM NaCl, 10 mM Hepes [pH 7.9], 1 M EDTA, 0.5 mM phenylmethylsulfonyl fluoride [PMSF].) using a glass hand-held tissue homogenizer. The homogenate was centrifuged and the pelleted nuclei was resuspended in 100-500 μ l of solution B (25 % glycerol, 20 mM Hepes [pH 7.9], 420 mM NaCl, 1.2 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 2 mM benzamide and 5 μ g/ml of each pepstatin,

leupeptin and aprotinin). Nuclei were incubated on ice for 20 minutes for high salt extraction, and then centrifuged at 5,000 rpm for 5 minutes. The supernatant containing the nuclear protein fraction was aliquoted (50 μ l), snap-frozen in liquid nitrogen and stored at -80°C. All other procedures were carried out on ice and working in a cold room (4°C). Protein quantitation of nuclear protein extracts was performed using the Lowry assay [Lowry et al., 1951].

4.2.5 Protein Immunoblotting Analysis

Protein immunoblot analysis was done as described previously [Sack et al., 1996], using crude nuclear protein extracts prepared from normoxic and hypoxic mouse hearts. Briefly, forty μ g of protein extracts were loaded per lane, and resolved by SDS-polyacrylamide gel electrophoresis (10%). Blots were probed using anti-PPAR α (a gift from Dr. Michael Arand, University of Mainz, Germany) and anti-Sp3 antibodies (Santa Cruz Biotechnology Inc.). Equal protein loading was confirmed using Ponceau Red staining as described [Jonassen et al., 2001].

The relative abundance of peptides was determined using a chemiluminescence detection system (Amersham Pharmacia Biotechnology) and quantified by laser densitometric analysis using the UVLTec autoradiography analyzer with uviband software v.97 (Cambridge, U.K.). The composite data is represented as mean $\pm$ standard error of the mean. The peptide levels are reported in arbitrary units (AU) determined by densitometry normalized to normoxic controls (mean control value set at 100).

4.2.6 CAT Activity Assay

CAT enzyme activities were determined with protein extracts prepared from normoxic and hypoxic animals. Here, protein extracts for the CAT activity assay were prepared by sonicating frozen heart tissue (50-100 mg) in 0.25 M Tris buffer [pH 8]. The homogenate was centrifuged at 13,000 rpm for 10 minutes and protein quantitation of the supernatant performed using the Lowry assay [Lowry et al., 1951]. The CAT activity assay was performed on duplicate samples by mixing 100 µg/µl of total protein with n-butyl coenzyme A and ¹⁴C-chloramphenicol substrates as described by Raisher et al. (1992) [Raisher et al., 1992]. The CAT activity for each sample was determined using the standard scintillation counting procedures (United Technologies Packard, UK).

4.2.7 Statistical Analysis

The data is presented as mean – standard error of the mean (S.E.M.). Statistical significance between the controls and experimental groups was analyzed by a one-factor analysis of variance (ANOVA) followed by pair-wise comparison using the Student-Newman-Keuls test. Values were considered significant when $p < 0.05$.

4.3 RESULTS

In order to determine the effect of hypoxia on MCAD gene expression and MCAD-CAT activity, we exposed transgenic mice to a moderate hypobaric

hypoxic stimulus (14% O₂). A modest increase ($4.4 \pm 0.7\%$, $p = \text{NS}$) in hematocrit levels was observed in animals exposed to 1 week of hypobaric hypoxia. (Fig. 1)

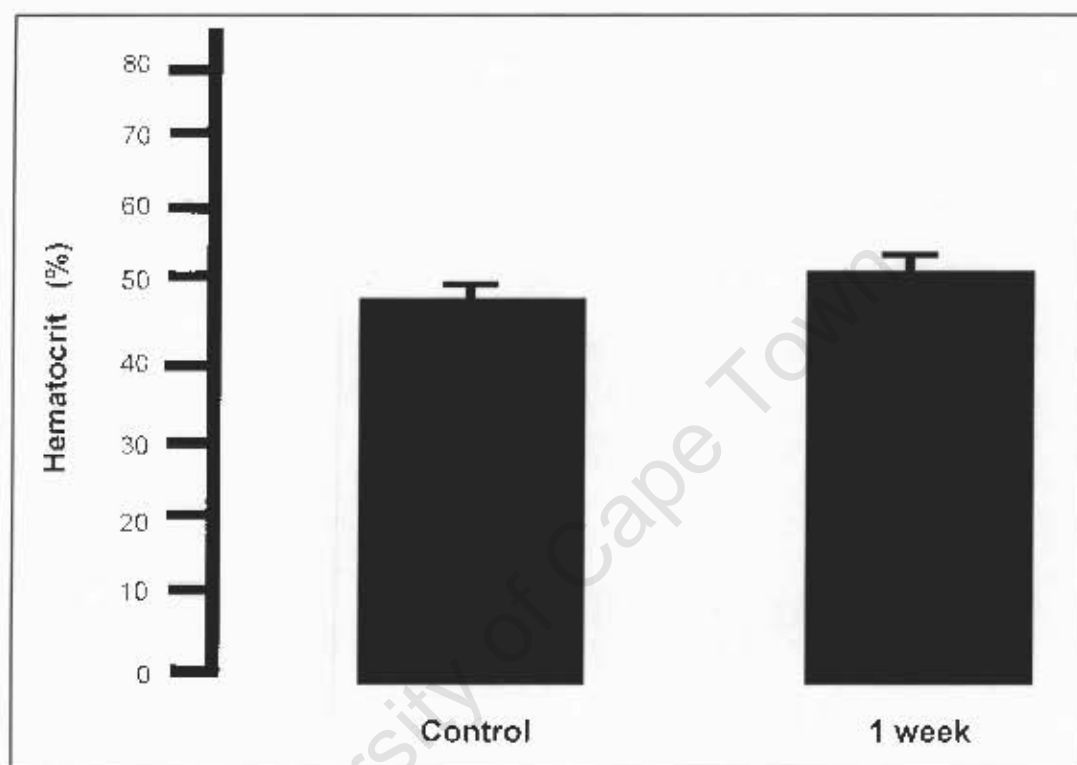


Figure 1: Hematocrit levels in response to moderate hypobaric hypoxia. Values are means $\pm$ S.E.M for 10 animals.

The effect of moderate and severe hypoxia on hematocrit, glucose and fatty acid levels are summarized in Tables 1 – 5.

Table 1: The effects of moderate hypoxia (30 kPa) on hematocrit, glucose and fatty acid levels.

	Haematocrit (%)			Glucose (mmol/L)			Free Fatty Acids (mmol/L)		
	Control	48 hours	1 week	Control	48 hours	1 week	Control	48 hours	1 week
	49		53	8.6	6.4	7.4	1.62	2.45	2.9
	46		52	8.5	6.9	11	3.13	1.89	1.5
	52		56	8.6	7	7.9	2.54	2.47	1.11
	49		49	12.5	6.2	9.1	1.64	2.6	1.87
	49.6		56	11.5	7.7	8.3	2.76	2.52	2.78
	49.2		54	10.5	8.2	7.7	2.88	1.92	2.84
	48		57	8.3	8.1	9.3	1.74	0.89	2.38
	51.4		52	7.3	6.1	9	2.42	0.65	2.2
	51.7		54	11	7	8	2.34	2.3	1.99
	54		58	12	6.5	7.1	2.2	1.95	1.85
	54		56	7.8	6.2	8.9	1.63	2.45	2.65
	52		52	10	7.2	8.8	98.25	1.8	2.5
Average	50.5		54.3	9.7	7.0	8.5	10.3	2.0	2.2
SD	2.4		2.8	1.8	0.7	1.1	27.7	0.6	0.6
SEM	0.7		0.8	0.5	0.2	0.3	8.0	0.2	0.2

NB: Heart weight = 0.1 g in all animals

Table 2: The effects of severe hypoxia (60 kPa) on hematocrit, glucose and fatty acid levels.

	Haematocrit (%)			Glucose (mmol/L)			Free Fatty Acids (mmol/L)		
	Control	48 hours	1 week	Control	48 hours	1 week	Control	48 hours	1 week
	55		72	8.2	6.8	2.8	1.16	0.39	1.06
	51.8		73.7	9.2	7.2	2.2	1.35	0.46	0.7
	55		74	10.8	14.6	2.5	1.1	0.63	0.93
	54		73	8.5	6.4	3.3	0.73	1.06	1.52
	56.5		77	11.7	7.8	0.3	1.14	0.89	1.7
	50		70.6	9.1	8.1	6.6	1.25	1.05	2.11
	50		78	7.3	8.6	5.8	1.12	0.98	2.14
	54		73.7	8.3	7.9	4	0.98	0.61	1.45
	53.6		67.5	8.8	7.5	3	0.97	1.02	1.98
	51.1		74.6	9.5	7.7	2.1	0.99	0.78	1.58
Average	53.1		73.4	9.1	8.3	3.3	1.1	0.8	1.5
Std Dev	2.1		2.8	1.2	2.2	1.7	0.2	0.2	0.5
SEM	0.7		0.9	0.4	0.7	0.5	0.1	0.1	0.1

NB: Heart weight = 0.1 g in all animals

Table 3: The effects of moderate hypoxia (30 kPa) on body measurements.

	Body weight (before) gr			Body weight (after) gr		
	Control	48 hours	1 week	Control	48 hours	1 week
	23.2	24.9	17.7	22.3	25.1	17.2
	19.3	26.3	18	20.2	26.5	17.7
	18.3	27.6	19.1	18.4	27.5	16.7
	19.9	22.2	19.2	20.2	22	19.4
	20.3	24.2	19	20	23.5	19.7
	20	30.6	17.8	19.9	29.7	16.5
	21.3	27.8	18	20.5	27.1	16.8
	19.6	24.4	19.4	19.2	23.6	18.8
	21.2	26.2	20.5	19.6	26.2	20.3
	19.8	28.6	19.7	19	26.7	19.6
	19.5	28	20.1	18.6	26.2	19.9
	18.9	25	18.8	18.1	24.5	17.6
	19.9	24.1	18.1	19.3	24.1	17.2
	19.2	26.6	19.6	18.9	25.8	18.1
Average	20.0	26.2	18.9	19.6	25.6	18.3
Std Dev	1.2	2.2	0.9	1.1	2.0	1.3
SEM	14.0					

NB: Heart weight = 0.1 g in all animals

Table 4: The effects of severe hypoxia (60 kPa) on body measurements.

	Body weight (before) gr			Body weight (after) gr		
	Control	48 hours	1 week	Control	48 hours	1 week
	18	26	22.5	17.1	22.3	14.3
	20.7	25	21.2	20.4	20.2	13
	19.2	30.46	19.3	18.8	25.7	12.5
	18.2	25.4	19.65	17.9	20.8	12.8
	18.1	23.66	17.97	17.9	18.1	12.1
	16.35	24.9	20.9	16.2	19	14.1
	17.05	26.45	18.7	15.4	21.2	12.7
	18.36	27.76	16.8	16.7	22.2	10.8
	17.87	28.24	17.4	16.5	22.4	12.1
	18.9	26.03	17.4	18.1	23	12.1
	19.97	25.98	18.36	19	22	13.2
	18.3	24.86	17.18	16.6	21.3	12.4
	20.1	26.66	19.3	19.3	20.9	13.3
	17.1	27.4	18	16.3	23.6	13.3
Average	18.4	26.3	18.9	17.6	21.6	12.8
Std Dev	1.2	1.7	1.7	1.4	1.9	0.9
SEM						

NB: Heart weight for all animals = 0.1 g

Table 5: The effects of severe hypoxia (60 kPa) on body measurements.

	Body weight (before) gr			Body weight (after) gr		
	Control	48 hours	1 week	Control	48 hours	1 week
	23.6	17.75	17.8	21.9	14.1	21.9
	17.25	16.1	18.38	17.25	12.9	17.3
	15.22	19.06	14.6	15.22	15.8	16.7
	18.7	22.14	17	20.8	17.3	20.8
	17.8	18.8	18.1	16.9	15	16.9
	22.5	17.5	15.2	15.5	16.5	20.2
Average	19.2	18.6	16.8	17.9	15.3	19.0
Std Dev	3.2	2.0	1.6	2.8	1.6	2.3
SEM						

Heart weight for all animals = 0.1 g

Mouse MCAD mRNA expression showed no change at 48 hours but a significant reduction of $29.1 \pm 5.4\%$ after 1 week of hypoxia ($p < 0.05$) (Fig. 2A). We used both MCADCAT.371 transgenic mouse strains (lines 10-1 and 10-4) [Disch et al., 1996]. As the regulation was the same in both lines, we only report the data from the 10-4 mouse line. The CAT activity of the MCADCAT.371 transgene paralleled the mRNA regulation with no change at day 2 and reduced by $19.4 \pm 0.6\%$ in transgenic mice after 1 week of moderate hypoxia ($45,553 \pm 1,376$ cpm) compared to the normoxic controls ($56,526 \pm 3,219$ cpm; $p < 0.05$) (Fig. 2B).

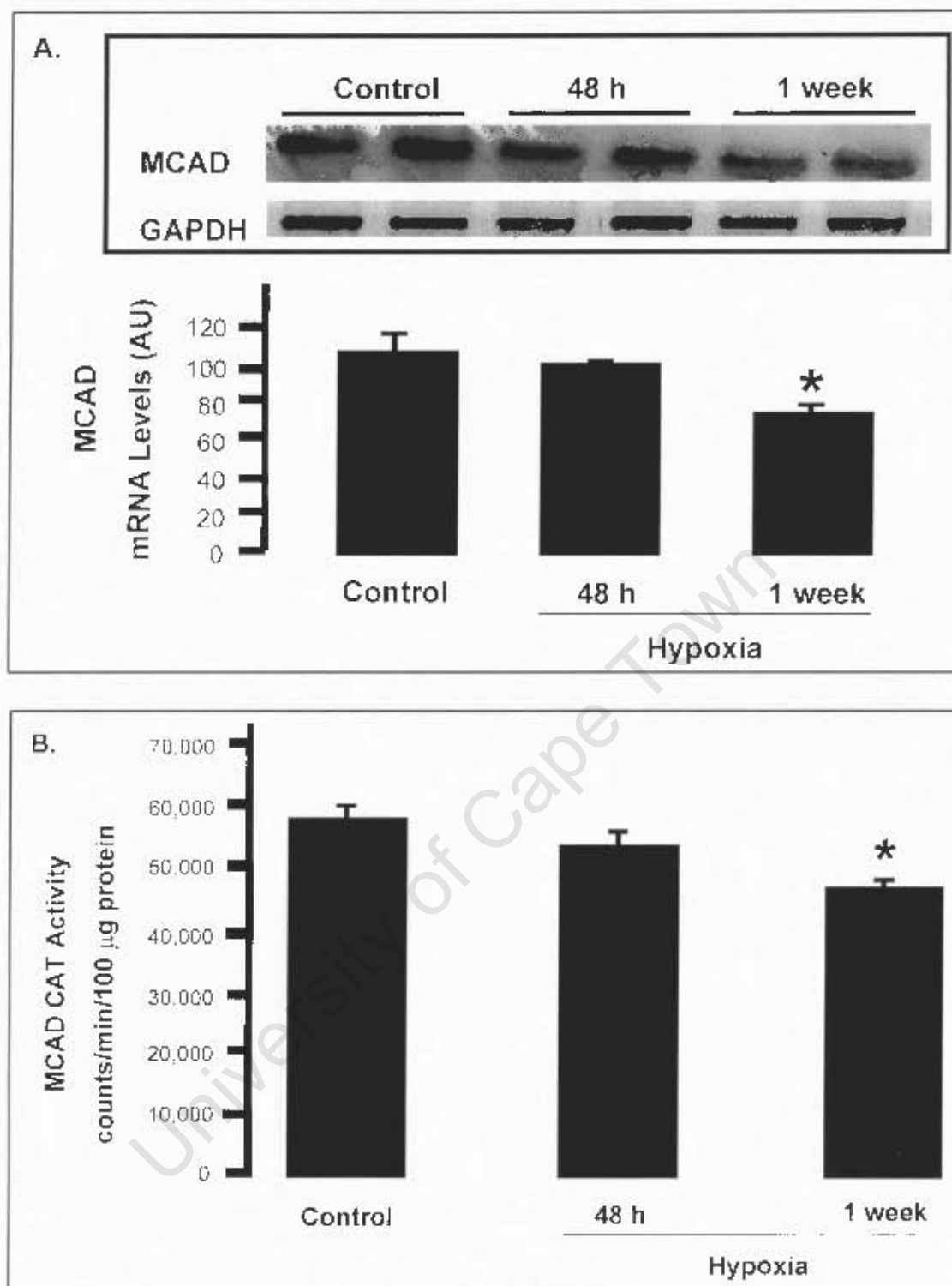


Figure 2: Regulation of MCAD promoter activity and gene expression in response to moderate hypobaric hypoxia (14% O₂). (A) **MCAD gene expression.** Inset - representative Northern blot is shown for MCAD. GAPDH is included to show unchanged mRNA expression. Densitometric analysis of Northern blots was performed and data normalized to the 28S RNA. The bars represent mean $\pm$ S.E.M. ($n = 5$). The asterisk denotes a significant difference between 1 week hypoxia with normoxic controls ($p < 0.05$). AU - arbitrary units. (B) **MCAD-CAT promoter activity.** Bars represent mean ($\pm$ S.E.M.) counts per minute / 100 µg of total protein ($n = 8$). The asterisk denotes a significant difference between 1 week of hypoxia vs. normoxic controls ($p < 0.05$).

To investigate transcriptional regulatory mechanisms responsible for MCAD regulation, we determined the steady-state peptide levels of PPAR α and Sp3. PPAR α levels were moderately decreased after 48 hours ($22 \pm 6.5\%$; $p < 0.05$) and remained downregulated after 1 week (Fig. 3A). Sp3 peptide levels were unchanged after 48 hours but significantly reduced ($65 \pm 9.7\%$) following 1 week of hypoxia ($p < 0.01$) (Fig. 3B).

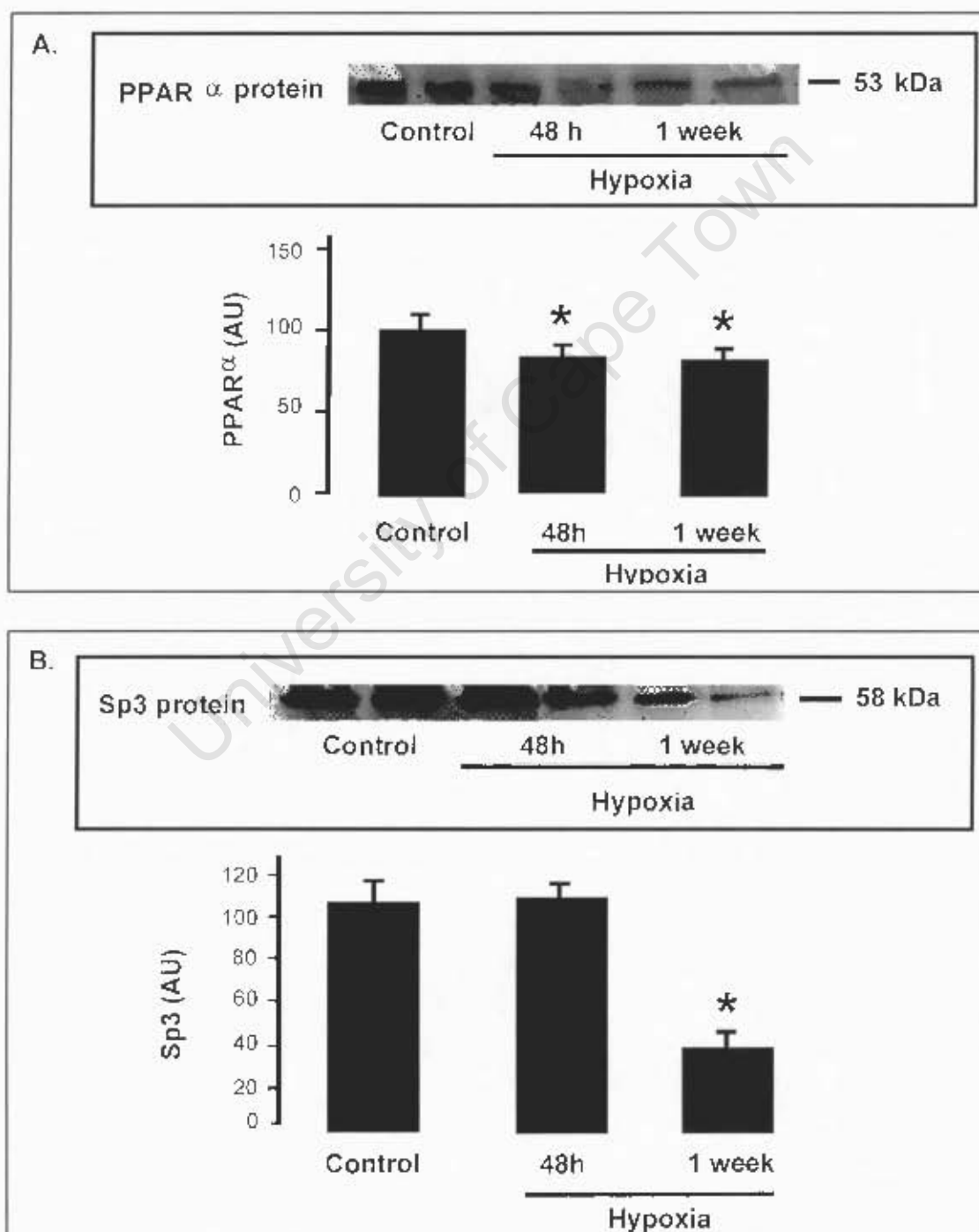


Figure 3: Regulation of PPAR α and Sp3 steady-state protein levels with moderate hypobaric hypoxia (14% O $_2$). (A) **Mean steady-state PPAR α peptide levels following moderate hypobaric hypoxia.** PPAR α peptide levels (in arbitrary units) normalized to Ponceau-stained protein bands (mean control value set at 100) ($n \geq 5$). The asterisk denotes a significant difference between 48 h and 1 week of hypoxia vs. normoxic controls ($p < 0.05$). Inset - A representative Western blot for PPAR α . (B) **Sp3 steady-state peptide levels.** A representative Western blot for Sp3 is shown and composite data of $n = 6$ per group are represented on the bar graph. The asterisk denotes a significant difference between 1 week of hypoxia vs. normoxic controls ($p < 0.01$).

In light of the modest downregulation of MCAD gene expression, we hypothesized that the downward MCAD trend could be enhanced by a more severe hypoxic stimulus. In light of this, transgenic mice were exposed to a severe hypobaric hypoxic stimulus (8% O $_2$). Here, hematocrit levels were increased by $37.9 \pm 1.7\%$ after 1 week compared to normoxic controls ($72.9 \pm 0.9\%$ vs. $52.9 \pm 0.7\%$; $p < 0.0001$) (Fig. 4).

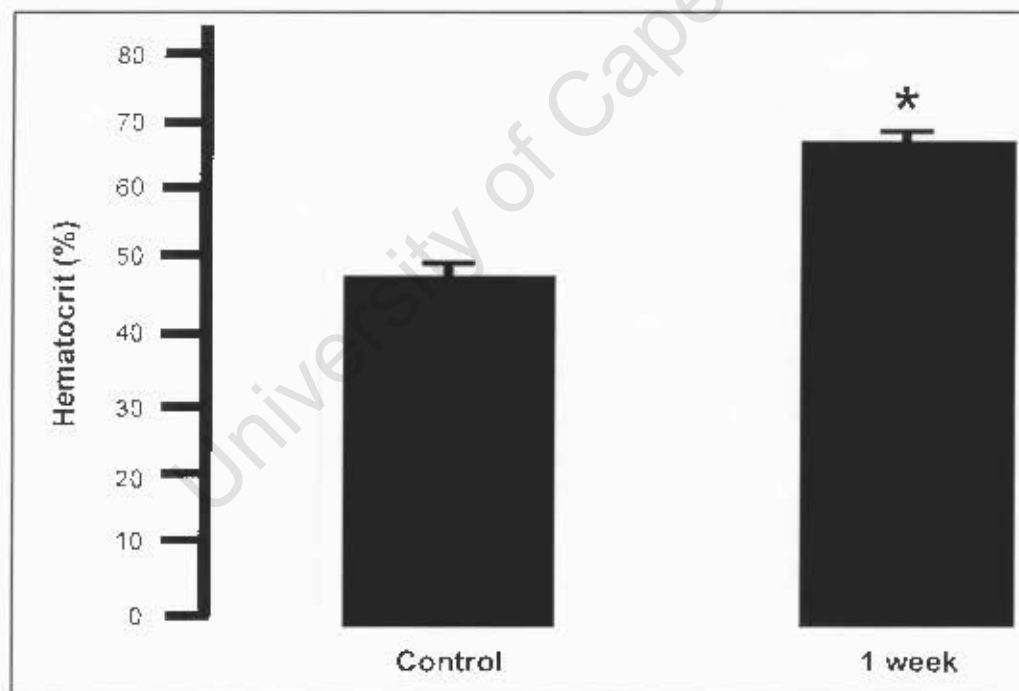
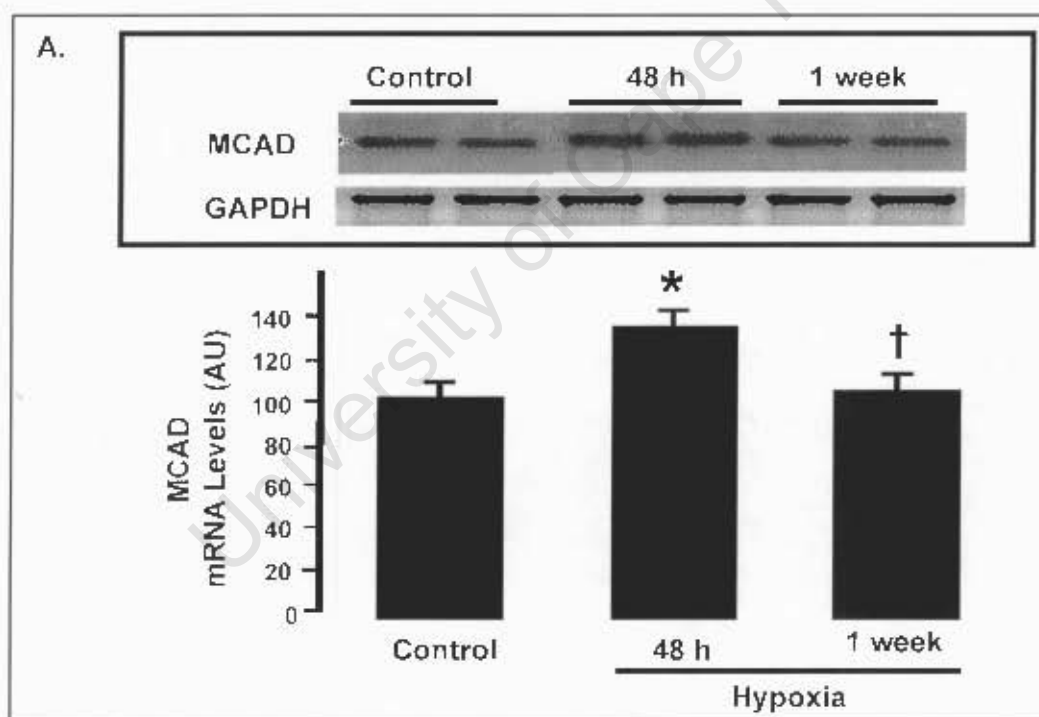


Figure 4: Hematocrit levels in response to severe hypobaric hypoxia. Values are means $\pm$ S.E.M. for 12 animals. * Significant difference between normoxic controls and 1 week hypoxia. ($p < 0.0001$).

Surprisingly, the MCAD downregulation previously observed in response to moderate hypoxia was attenuated, with an increase in MCAD gene expression after 48 hours of severe hypoxic exposure ($33.9 \pm 9.1\%$) compared to normoxic controls ($p < 0.05$) with a return to control levels at 1 week (Fig. 5A). CAT activity matched the regulation of MCAD gene expression by increasing after 48 hours ($31 \pm 5.8\%$) exposure to severe hypoxia compared to normoxic controls ($65,281 \pm 2,898$ cpm vs. $52,819 \pm 1,720$ cpm; $p < 0.001$) (Fig. 5B). This activity returned to baseline levels after 1 week exposure.



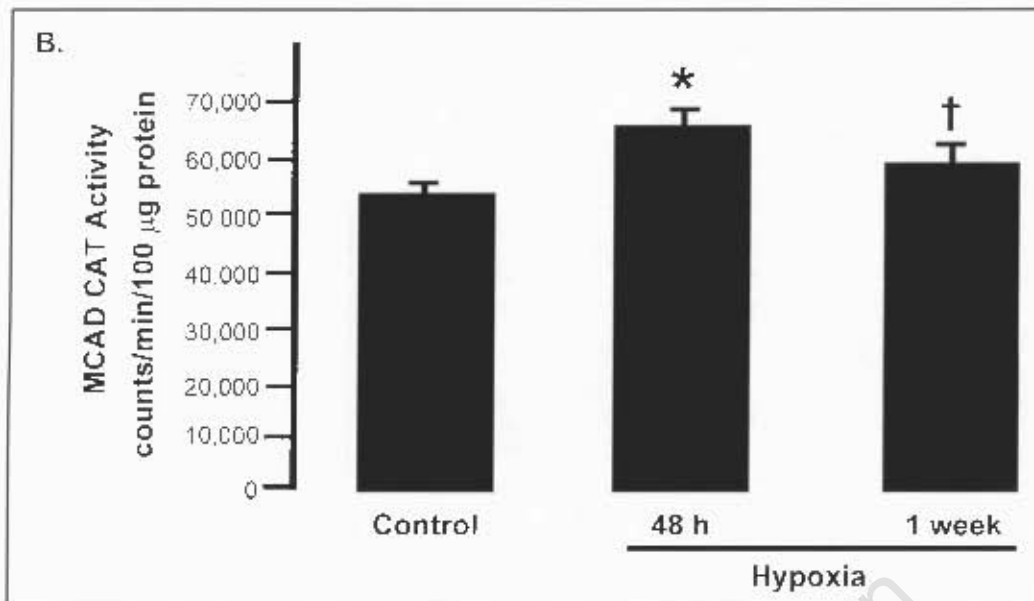


Figure 5: Regulation of MCAD promoter activity and gene expression in response to severe hypobaric hypoxia (8% O₂). (A) **MCAD gene expression.** GAPDH is included to show unchanged mRNA expression. The bars represent mean $\pm$ S.E.M. (mean control value set at 100) ($n = 5$). The asterisk indicates a significant difference between 48 hrs hypoxia vs. normoxic controls ($p < 0.05$). The † indicates a significant difference between 48 hrs and 1 week hypoxia ($p < 0.05$). Inset - representative Northern blot is shown for MCAD. (B) **MCAD-CAT promoter activity.** Bars represent mean ($\pm$ S.E.M.) counts per minute / 100 µg of total protein ($n = 7$). The asterisk indicates a significant difference between 48 hrs hypoxia vs. normoxic controls ($p < 0.001$). The † indicates a significant difference between 48 hrs and 1 week hypoxia ($p < 0.05$).

We also measured the steady-state peptide levels of PPAR α and Sp3, two candidate transcription factors regulating MCAD transcription. PPAR α steady-state protein levels were significantly increased after 48 hours exposure to the severe hypoxic stimulus compared to controls ($92 \pm 20.2\%$; $p < 0.01$) and remained elevated after 1 week (Fig. 6A). Sp3 levels were increased after 48 hours ($33.4 \pm 4\%$; $p < 0.001$) and returned to control levels after 1 week hypoxia (Fig. 6B).

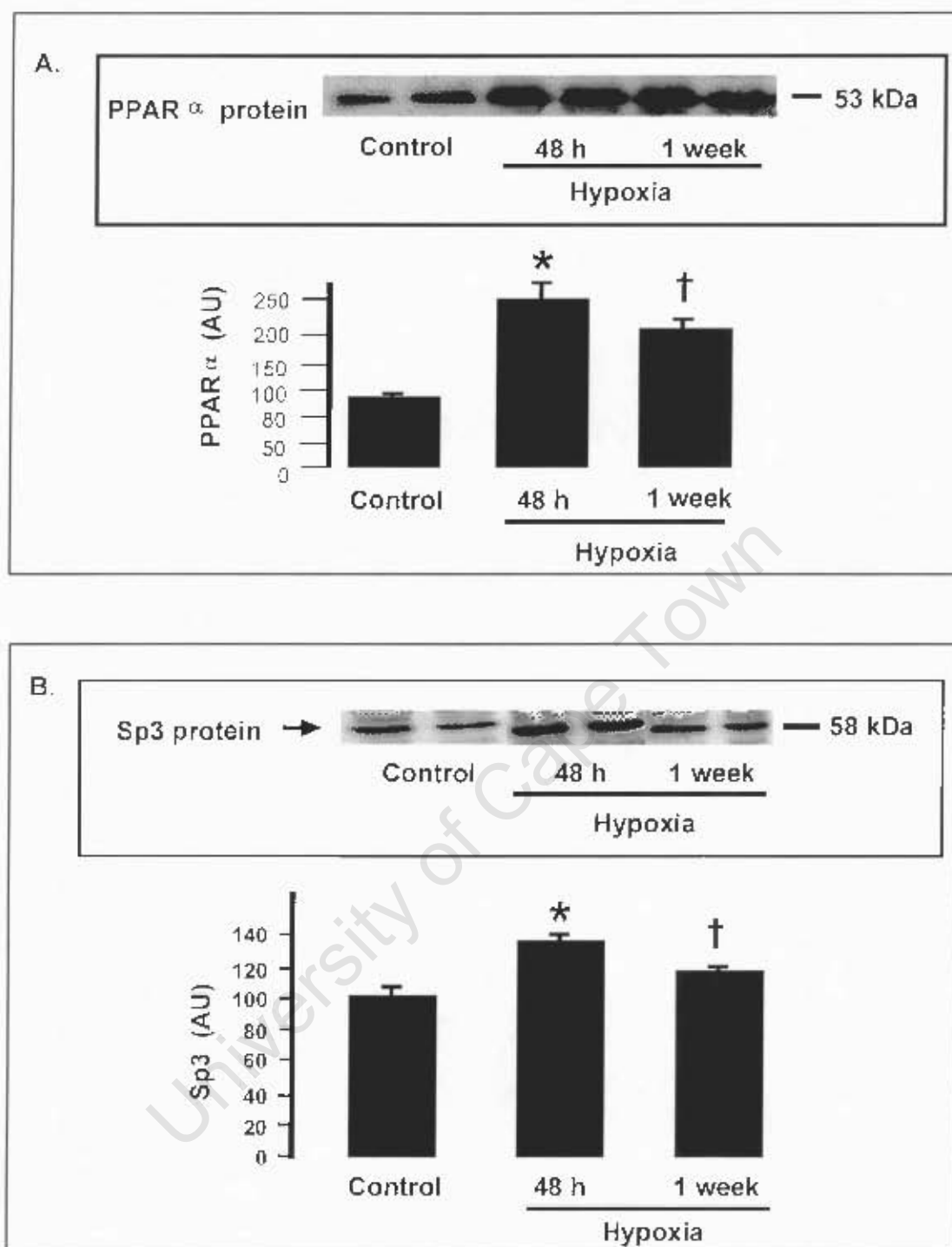


Figure 6: Expression of transcription factors following severe hypobaric hypoxia (8% O_2). **(A) PPAR α peptide levels.** The asterisk indicates a significant difference between 48 h hypoxia vs. normoxic controls ($p < 0.01$). The † indicates a significant difference between normoxic controls and 1 week of hypoxia ($p < 0.05$). Inset - A representative Western blot for PPAR α is shown ($n = 9$). **(B) Sp3 peptide levels.** The asterisk indicates a significant difference ($p < 0.001$) between 48 h hypoxia and normoxic controls. The † indicates a significant difference between 48 h and 1 week hypoxia ($p < 0.05$). Inset - A representative Western blot for Sp3 is shown ($n > 6$).

Since fuel substrate levels, especially FAs, are known to regulate MCAD gene expression [Gulick et al., 1994], we next determined the difference between caloric intake and substrate levels in the moderate and severe hypoxic animals compared to normoxic controls. The body weights and dietary intake of animals exposed to moderate hypoxia were similar to normoxic control animals as measured by residual (desiccated) food.

In contrast, animals exposed to 48 hours and 1 week severe hypoxia consumed less chow (16 and 60%, respectively), compared to what is usually consumed by normoxic control animals. The reduced dietary intake resulted in a decrease in body weight of mice after 48 hours of exposure to severe hypobaric hypoxia vs. normoxic controls (19.36 ± 0.14 vs. 14.72 ± 0.18 g; $p < 0.001$). The body weight was maintained after 1 week vs. normoxic control mice (19.6 ± 0.43 vs. 15.6 ± 0.48 g; $p < 0.001$). To further explain the reduced dietary intake and body weight, we determined plasma glucose and NEFA levels in these mice. After exposure to severe hypoxia, glucose levels were similar to control levels after 48 hours, but were significantly reduced compared to controls after 1 week of severe hypoxia (9.1 ± 0.5 vs. 3.5 ± 0.1 mmol/L; $p < 0.001$). Surprisingly, no significant increase in plasma NEFA levels was observed after 48 hours and 1 week of severe hypoxia as would be expected in response to the reduced caloric intake.

4.4 DISCUSSION

The hypoxia stimulated induction of the glycolytic enzyme-encoding genes has been well characterized [Semenza et al., 1994; Semenza et al., 1996; Discher et al., 1998]. However, the molecular regulation of cardiac FA utilization enzyme-encoding genes still needs additional clarification. To further elucidate the transcriptional regulating mechanisms controlling FA utilization enzyme-encoding genes, we investigated the regulation of MCAD, a representative enzyme of FAO, in response to progressive hypobaric hypoxia. In this study we demonstrate decreased MCAD gene expression and promoter activity in response to moderate hypoxia (14% O₂). Unexpectedly, severe hypoxia (8% O₂) caused increased MCAD gene expression and promoter activity. The two known MCAD transcriptional regulators, PPAR α and Sp3, are both decreased in response to moderate hypoxia and increased in response to severe hypoxia.

Previous studies have shown downregulation of cardiac mRNA levels of the rate-limiting mitochondrial FA transport carrier, mCPT-I, in response to hypoxia [Huss et al., 2001; Razeghi et al., 2001]. In this study we demonstrate the downregulation of cardiac MCAD gene expression and MCAD-CAT promoter activity following 1 week of moderate hypobaric hypoxia. We reasoned that this modest MCAD downregulation could be further enhanced in response to a severe hypobaric hypoxic stress. The severe hypoxic experiments showed a classic adaptive response i.e. erythrocytosis as evident by the significant increase in hematocrit. Surprisingly, MCAD gene expression and MCAD-CAT promoter activity were

significantly elevated after 48 hours of severe hypoxia, returning to baseline after 1 week's exposure. Our data suggest divergent regulation of MCAD gene expression in response to different hypoxic stimuli.

To begin to investigate the molecular regulatory mechanisms responsible for this divergent regulation, we determined steady-state nuclear levels of factors known to regulate MCAD gene expression i.e. PPAR α (a known MCAD trans-activator) and Sp3 [Gulick et al., 1994]. In response to moderate hypoxia, PPAR α steady-state peptide levels were decreased after 48 hours and 1 week of moderate hypoxia. In contrast, severe hypoxia caused an increase of PPAR α steady-state peptide levels after 48 hours and levels remained elevated after 1 week. These findings suggest that PPAR α levels correlate with, and hence putatively play a regulatory role, in MCAD gene expression in response to different levels of hypoxia. Our moderate hypoxic data are in agreement with Razeghi et al. (2001) who demonstrated a downregulation of cardiac PPAR α mRNA levels using two different models of *in vivo* hypoxia [Razeghi et al., 2001]. Collectively the results support the role of PPAR α as a *trans*-activator of MCAD gene expression in the rat heart.

The severe hypoxic data suggest that increased PPAR α levels are responsible for activating MCAD gene expression and MCAD-CAT activity after 48 hours. As FAs are suggested to be endogenous ligands for PPAR α [Gulick et al., 1994], we measured substrate levels and caloric intake for hypoxic and normoxic mice. Severe hypoxic exposure (48 hours and 1 week) resulted in the reduced caloric intake, leading to a significant decline in body

weight. These data suggest a fasted state where one would expect elevated systemic NEFA levels. However, severe hypoxia did not result in increased NEFA levels after 48 hours and 1 week. Although these results are not directly enlightening, they illustrate the complexity of studying gene regulation in the *in vivo* setting and cannot rule out a putative role of substrate metabolism in these gene regulatory programs. Alternatively, other hypoxia-induced effects for e.g. neuro-hormonal or mechanical strain on the right ventricle may be implicated in these responses.

Sack et al. (1997) identified Sp3 as a putative repressor of MCAD in response to pressure-overload induced ventricular hypertrophy [Sack et al., 1997]. In light of this, we would have expected Sp3 to be upregulated with moderate hypoxia and downregulated in response to severe hypoxia. However, we found that Sp3 levels were significantly reduced after 1 week of moderate hypoxia in association with diminished MCAD gene expression. Also, Sp3 levels were significantly augmented after 48 hours of severe hypoxia in association with increased MCAD gene expression. These data suggest that in this instance Sp3 acts like a transcriptional activator of MCAD gene expression in the rat heart. Previous studies have shown that Sp3 can function either as a repressor or activator, depending on the promoter content [Majello et al., 1997; Kennett et al., 2002]. Our study suggests that Sp3 may not function as a transcriptional repressor in response to hypobaric hypoxia. Alternatively, the PPAR α nuclear hormone-signaling pathway may override Sp3 functional control of MCAD gene expression in this context. We cannot rule out other *trans*-activating redox-sensitive factors such as COUP-TF, HNF-

4, and Sp1 in regulating MCAD gene expression in response to hypoxia [Carter et al., 1993; Carter et al., 1994; Leone et al., 1995; Disch et al., 1996; Sack et al., 1996]. The moderate hypoxic data correlate with that of Discher et al. (1998) who demonstrated Sp3 depletion with hypoxia in muscle cells [Discher et al., 1998].

Lastly, it should be borne in mind that multiple intra- and extracardiac mediators may be involved in the effects of moderate and severe hypoxia on cardiac transcriptional regulation. For example, it has been demonstrated in chronic experiments on animals (rabbits, rats) that in the presence of a predominance of sympathetic extracardiac influences on the heart during emotional stress (hypoxia, ischemia), the disturbances that appear in metabolism and myocardial ultrastructure promote a decrease in the electrical stability of the heart, the development of disturbances in cardiac rhythm and arterial hypertension. A predominance of parasympathetic (intracardiac) influences on the heart during stress, to the contrary, leads to an increase in the content of catecholamines and the development of structural injuries in the myocardium. At the same time, arterial hypertension is absent. An inference is drawn regarding the adaptive - trophic, protective action of the vagus nerves on the heart during emotional stress. [Ul'yaniskii, 1995]

In summary, we speculate that our data suggest that with moderate hypoxia, reduced PPAR α levels result in decreased MCAD gene expression in the mouse heart. However, with severe hypoxia elevated PPAR α levels result in

increased MCAD gene expression. Whether Sp3 acts as a repressor or an activator in this instance requires further investigation.

4.5 REFERENCES

1. Abdel-aleem S, St Louis J, Hendrickson SC, El-Shewy HM, El-Dawy K, Taylor DA, Lowe JE: Regulation of carbohydrate and fatty acid utilization by L-carnitine during cardiac development and hypoxia. *Mol Cell Biochem* 180: 95-103, 1998.
2. Carter ME, Gulick T, Raisher BD, Caira T, Ladias JAA, Moore DD, Kelly DP: Hepatocyte nuclear factor-4 activates medium-chain acyl-CoA dehydrogenase gene transcription by interacting with a complex regulatory element. *J Biol Chem* 268: 13805-13810, 1993.
3. Carter ME, Gulick TG, Moore DD, Kelly DP: A pleiotropic element in the medium-chain acyl coenzyme A dehydrogenase gene promoter mediates transcriptional regulation by multiple nuclear receptor transcription factors and defines novel receptor-DNA binding motifs. *Mol Cell Biol* 14: 4360-4372, 1994.
4. Deryckere F, Gannon F: A one hour miniprep technique for extraction of DNA-binding proteins from animal tissues. *Biotechniques* 16 (3): 405, 1994.
5. Disch DL, Rader TA, Cresci S, Leone TC, Barger PM, Vega R, Wood PA, Kelly DP: Transcriptional control of a nuclear gene encoding a mitochondrial fatty acid oxidation enzyme in transgenic mice: role for

- nuclear receptors in cardiac and brown adipose tissue expression. *Mol Cell Biol* 16: 4043-4051, 1996.
6. Discher DJ, Bishopric NH, Wu X, Peterson CA, Webster KA: Hypoxia regulates β -enolase and pyruvate kinase-M promoters by modulating Sp1/Sp3 binding to a conserved GC element. *J Biol Chem* 273: 26078-26093, 1998.
 7. Gulick T, Cresci S, Caira T, Moore DD, Kelly DP: The peroxisome proliferator-activated receptor regulates mitochondrial fatty acid oxidative enzyme gene expression. *Proc Natl Acad Sci USA* 91: 11012-11016, 1994.
 8. Holden JE, Stone CK, Clark CM, Brown WD, Nickles RJ, Stanley C, Hochachka PW: Enhanced cardiac metabolism of plasma glucose in high-altitude natives: adaptation against chronic hypoxia. *J Appl Physiol* 79: 222-228, 1995.
 9. Huss JM, Levy FH, Kelly DP: Hypoxia inhibits the peroxisome proliferator-activated receptor α /retinoid X receptor gene regulatory pathway in cardiac myocytes: a mechanism for O₂-dependent modulation of mitochondrial fatty acid oxidation. *J Biol Chem* 276: 27605-27612, 2001.
 10. Jonassen AK, Sack MN, Mjos OD, Yellon DM: Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. *Circ Res* 89: 1191-1198, 2001.

11. Kennett SB, Moorefield KS, Horowitz, JM: Sp3 represses gene expression via the titration of promoter-specific transcription factors. *J Biol Chem* 277: 9780-9789, 2002.
12. Leone TC, Cresci S, Carter ME, Zhang Z, Lala DS, Strauss AW, Kelly DP: The human medium chain acyl-CoA dehydrogenase gene promoter consists of a complex arrangement of nuclear receptor elements and Sp1 binding sites. *J Biol Chem* 270: 16309-16314, 1995.
13. Loike JD, Cao L, Brett J, Ogawa S, Silverstein SC, Sten D: Hypoxia induces glucose transporter expression in endothelial cells. *Am J Physiol (Cell Physiol)* 263: C326-C333, 1992.
14. Lowry R, Rosenbrough N, Farr A, Randall R: Protein measurement with the folin phenol reagent. *J Biol Chem* 193: 265-275, 1951.
15. Majello B, De Luca P, Lania L: Sp3 is a bifunctional transcription regulator with modular independent activation and repression domains. *J Biol Chem* 272: 4021-4026, 1997.
16. Raisher BD, Gulick T, Zhang Z, Stauss AW, Moore DD, Kelly DP: Identification of a novel retinoid-response element in the promoter region of the medium chain acyl-Coenzyme A dehydrogenase gene. *J Biol Chem* 267 (28): 20264-20269, 1992 .
17. Razeghi P, Young ME, Abbasi S, Taegtmeyer H: Hypoxia *in vivo* decreases peroxisome proliferator-activated receptor alpha-regulated gene expression in rat heart. *Biochem Biophys Res Commun* 287: 5-10, 2001.

18. Rumsey WL, Abbott B, Bertelsen D, Mallamaci M, Hagan K, Nelson D, Erecinska M: Adaptation to hypoxia alters energy metabolism in rat heart. *Am J Physiol* 276: H71-H80, 1999.
19. Sack MN, Disch DL, Rockman HA, Kelly DP: A role for Sp and nuclear receptor transcription factors in a cardiac hypertrophic growth program. *Proc Natl Acad Sci USA* 94: 6438-6443, 1997.
20. Sack MN, Rader TA, Park S, Bastin J, McCune SA, Kelly DP: Fatty acid enzyme encoding gene expression is downregulated in the failing heart. *Circulation* 94: 2837-2842, 1996.
21. Semenza GL, Agani F, Iyer N, Jiang BH, Leung S, Wiener C, Yu A: Hypoxia-inducible factor 1: from molecular biology to cardiopulmonary physiology. *Chest* 114 (1 Suppl): 405-455, 1998.
22. Semenza GL, Jiang BH, Leung SW, Passantino R, Concordet JP, Maire P, Giallongo A: Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *J Biol Chem* 271: 32529-32537, 1996.
23. Semenza GL, Roth PH, Fang HM, Wang GL: Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J Biol Chem* 269: 23757-23763, 1994.
24. Smith RM, McCarthy J, Sack MN: TNF alpha is required for hypoxia-mediated right ventricular hypertrophy. *Mol Cell Biochem* 219: 139-143, 2001.

25. Taegtmeyer H, Hems R, Krebs HA: Utilization of energy-providing substrates in the isolated working rat heart. *Biochem J* 186: 701-711, 1980.
26. Ul'yaniskii LS: Emotional stress and extracardial regulation. *Neurosc Behav Physiol* 25 (3): 257-65, 1995.
27. Zhu H and Bunn H. F: How do cells sense oxygen? *Science* 292: 449-451, 2001.

University of Cape Town

Chapter 5

Conclusion

University of Cape Town

Substrate utilization by the heart is dynamic and changes according to developmental status, substrate supply and pathophysiologic states [Sack and Kelly, 1998; Stanley and Chandler, 2002]. These fuel substrate switches underscore the omnivoric nature of the mammalian heart i.e. being able to select specific substrates at different stages of development and also highlights the complex, multiple regulatory steps required to rapidly and efficiently switch from one fuel substrate to another. The term metabolic remodeling has been defined as a switch in cardiac substrate use in association with cardiogenesis and with the development of pathophysiologic conditions [van Bilsen et al., 1998]. The functional role of metabolic remodeling in the development of cardiac disease is not clearly understood. Moreover, the question whether cardiac metabolic remodeling is beneficial, detrimental or an epiphenomenon has not been conclusively established. The aim of this study was to further investigate this intriguing question and help unfold the mystery surrounding the contribution of metabolic remodeling to the development of disease.

We initially set out to investigate progressive changes in the rat heart in response to chronic hypobaric hypoxia with emphasis on regulation at functional, metabolic and transcriptional levels. In particular, we aimed to set up (i) a robust model of RV hypertrophy and (ii) a working model to dissect molecular regulatory changes during the transition from compensated hypertrophy to heart failure. Here, we have succeeded in establishing and characterizing a robust rat model of compensated right ventricular hypertrophy. However, we were not successful in establishing the transition

from decompensated hypertrophy to heart failure. We suggest that this important question be further investigated by employing amended research protocols. In our model, right ventricular hypertrophy developed by 1 week and was maintained for the 24-week period. This was matched by a dynamic regulation of fatty acid and glucose utilization enzyme-encoding genes. Initially, gene regulation switched from fatty acids to glucose, followed by an upregulation of fatty acid enzyme-encoding genes. This in turn was followed by normalization to baseline of the fatty acid genes and glucose utilization genes reverting to a fetal pattern. A switch from fatty acids to glucose utilization is often associated with the development of cardiac hypertrophy [Sambandam et al., 2002]. This study has demonstrated that the fatty acid to glucose switch at the gene expression level does not necessarily parallel the onset of cardiac hypertrophy. Rather, we demonstrate that metabolic gene expression is a dynamic process fluctuating independently of the trophic response. The complex interaction between compensatory mechanisms for e.g. elevated hematocrit levels that attempt to restore normoxia and the trophic adaptation of the right ventricle in response to increased load may contribute to the observed fluctuations in gene expression. Therefore, our data suggest that metabolic gene expression in the hypertrophied heart is a complex process that can rapidly alternate between fetal and adult gene expression patterns.

We also found that cardiac contractile function was sustained over the 24-week period. We believe that this represents a compensatory hypertrophic response. Interestingly, after 2 weeks of hypobaric hypoxia, mitochondrial

energetic status was sustained despite a significant reduction in ANT1 levels. During chronic hypoxia, increased mitochondrial numbers and erythropoiesis could maintain mitochondrial oxidative phosphorylation despite reduced oxygen availability. We speculate that compensatory upregulation of ANT 2/3 may be responsible for sustained mitochondrial energetic status. Moreover, cardiac output was maintained after 24 weeks despite a significant decrease in ANT1 levels. These data suggest sustained mitochondrial respiratory capacity over this period.

We have also identified key transcription factors that may regulate the gene expression of several metabolic enzyme-encoding in response to right ventricular hypertrophy. In particular, we found that Sp3 and PPAR α levels were altered in response to hypoxia, implicated in the regulation of MCAD gene expression in this instance. Our data suggest that redox-sensitive transcriptional factors are responsible for differences in the regulation of MCAD in response to different hypoxic stimuli. Moreover, both these transcriptional modulators may also play a regulatory role in determining expression of some of the other metabolic genes investigated in this study.

In conclusion, advances in the understanding of the progression of right ventricular hypertrophy should eventually allow for the design of novel metabolic therapeutic interventions, leading to improved patient care. Moreover, elucidation of such mechanisms may have broader implications regarding our general understanding of heart disease and may open up several interesting and intriguing research avenues.

5.2 REFERENCES

1. Sack MN, Kelly DP: The energy substrate switch during development of heart failure: gene regulatory mechanisms. *Int J Mol Med* 1 (1): 17-24, 1998.
2. Sambandam N, Lopaschk GD, Brownsey RW, Allard MF: Energy metabolism in the hypertrophied heart. *Heart Fail Rev* 7: 161-173, 2002.
3. Stanley WC, Chandler MP: Energy metabolism in the normal and failing heart: potential for therapeutic interventions. *Heart Fail Rev* 7: 115-130, 2002.
4. Van Bilsen M, van der Vusse GT, Reneman RS: Transcriptional regulation of metabolic processes: implication for cardiac metabolism. *Pflugers Arch* 437 (1): 2-14, 1998.

5.3 PUBLICATIONS

1. Ngumbela KC, Sack MN, Essop MF: Counter-regulatory effects of incremental hypoxia on the transcription of a cardiac fatty acid oxidation-encoding gene. *Mol Cell Biochem*, 250: 151-158, 2003.
2. Sharma S, Taegtmeyer H, Adroque J, Razeghi P, Sen S, Ngumbela K, Essop MF: Dynamic changes of gene expression in hypoxia-induced right ventricular hypertrophy. *Am J Physiol*, 286: H1185-H1192, 2004.
3. Adroque J, Sharma S, Ngumbela K, Essop MF, Taegtmeyer H: Chronic hypobaric hypoxia induces differential transcriptional response

in the right and left ventricle of rat heart. Submitted to J Appl Physiol, 2004.

University of Cape Town

