The Energy Substrate Switch During Cardiac Development, with the Onset of Cardiac Hypertrophy and the Transition to Heart Failure. Delineation and Characterisation of Gene Regulatory Mechanisms

"Changing Concepts of Metabolic Regulation in the Heart"

University of Cape Town

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for the degree of Doctor of Philosophy (Medicine)

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February, 2000

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Acknowledgements

It is a pleasure to record my thanks to the many individuals who provided assistance during this research. Firstly, I express my gratitude to Daniel P. Kelly, MD, (Center for Cardiovascular Research, Washington University Medical School, St. Louis, MO, USA) my primary mentor and supervisor for the majority of this work. His excellent guidance and encouragement enabled me to begin the transition from clinician to that of physician-scientist. Moreover, the laboratory environment which he directed, was interactive, stimulating and challenging, a model I hope to replicate during my career.

To Lionel H. Opie, MBBCh, Ph.D. DSc (UCT - Cape Town) and Derek M. Yellon, Ph.D., D.Sc. (University College London Medical School, London, UK), the two mentors who not only promoted my scientific development and career, but encouraged me to undertake this Ph.D. I will be forever grateful, to the profound influence these two scientific leaders have had on me pursuing my career goals and objectives.

The focus of this dissertation, was on the study of the molecular regulation of genes encoding enzymes in the heart. These molecular biological studies, could not have be performed without the use of physiologic and pathophysiologic models of human disease. For this I am gratefully indebted to the physiologic studies performed and tissue

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obtained from Howard A. Rockman, MD (University of California, San Diego, CA, USA) and from Sylvia McCune, PhD (The Ohio State University, Columbus, OH, USA).

No researcher works in isolation, and to this end I would like to sincerely thank fellow researchers in the laboratories of Dan Kelly and Derek Yellon. Specifically I would like to acknowledge the support and teaching I received from Teresa Leone in Daniel Kelly's laboratory in the early part of these studies and to Louise Harrington in Derek Yellon's laboratory for her assistance.

I would like to express my gratitude to the Howard Hughes Medical Institute, for funding my laboratory experience at Washington University Medical School. The HHMI Physician Postdoctoral Research Fellowship afforded me the financial security to devote myself entirely to the pursuit of scientific discovery.

Finally, but not least of all, to the three generations of family, who provided me with encouragement and material support from start to finish, I record my gratitude to them. In partial, though inadequate, compensation of this debt, I dedicate this work to my parents, my wife - Karen, and our children - Danny and Josh.

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Abstract

Background. Substrate utilization for energy production is highly regulated during cardiac development, with the onset of cardiac hypertrophy and in the transition to heart failure. Glucose and lactate are the predominant fuel substrates utilised during cardiac development. Postnatally, a switch in energy substrate utilisation occurs, so that fatty acids become the chief energy substrate in the non-fed normal adult mammalian heart. However, with cardiac hypertrophy and failure, a transition reminiscent of foetal energy metabolism occurs with reduced fatty acid and increased glucose utilisation. Little is known, however, about the molecular regulatory mechanisms involved in this energy metabolic remodelling, nor whether this reversion towards foetal energy substrate utilisation contributes towards the development of cardiac hypertrophy and/or heart failure. **Objectives.** To begin to understand the role of this metabolic remodelling on cardiac development and disease we need to understand the regulatory mechanisms orchestrating these fluxes in substrate utilisation. Therefore, the focus of this dissertation addresses the study of molecular regulatory mechanisms directing these switches in energy substrate use, focussing on rate-controlling metabolic enzyme encoding genes during cardiac development and with the onset of cardiac hypertrophy and heart failure. The preparatory studies were on the gene

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expression patterns of numerous genes encoding for rate-controlling enzymes required for fatty acid and glucose catabolism in cardiac development and hypertrophy. The predominant focus is on the regulatory control of a nuclear-encoded mitochondrial β-oxidation enzyme gene responsible for medium-chain fatty acid catabolism in the heart during the development of cardiac hypertrophy and the transition to heart failure. **Results.** Coordinate regulation of genes encoding multiple metabolic enzymes required for fatty acid and glucose metabolism were demonstrated during both cardiac development and with the onset of cardiac hypertrophy. The fatty acid import and oxidation encoding enzyme gene expression patterns parallel the known utilization of fatty acids during cardiac development and with the onset of cardiac hypertrophy. In contrast, the adult-enriched glucose import and glycolytic enzyme encoding gene expression patterns are inversely regulated with respect to the known use of glucose with the development of cardiac hypertrophy. To further define these cardiac stage-specific gene expression patterns, we focused on the characterisation of the gene regulatory programme of medium chain acyl-CoA dehydrogenase, a representative mitochondrial fatty acid β-oxidation (FAO) enzyme gene. We initially assessed medium chain acyl-CoA dehydrogenase regulation at the level of transcription, via induction of right ventricular hypertrophy (RVH) in mice transgenic for the human medium chain acyl-CoA dehydrogenase promoter fused to a CAT

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reporter. Compared with sham controls, right ventricular medium chain acyl-CoA dehydrogenase and CAT mRNA levels were repressed by greater than 80% in the hypertrophied ventricles. Thus, the known reduction in FAO during the development of cardiac hypertrophy may be regulated in large part at the level of gene transcription. Using DNA:Protein interaction studies and gene promoter/reporter transfection studies, a number of putative nuclear regulatory proteins which orchestrate this regulation were identified. Interestingly, the repression of medium chain acyl-CoA dehydrogenase transcription with the development of pressure overload-induced cardiac hypertrophy appears to be via the reactivation of a foetal gene regulatory programme. The temporal pattern of this alteration in medium chain acyl-CoA dehydrogenase gene expression was then characterized in a rat model of progressive left ventricular hypertrophy (LVH) and heart failure (HF) [SHHF/Mcc-facp (SHHF) rat]. Medium chain acyl-CoA dehydrogenase enzyme mRNA level were downregulated (>70%) during both the LVH and HF stages in the SHHF rats compared with controls. In contrast, the activity and steady-state levels of the medium chain acyl-CoA dehydrogenase enzyme were not significantly reduced until the HF stage, indicating additional control at the translational or post-translational regulatory levels in the hypertrophied but non-failing ventricle. To evaluate whether medium chain acyl-CoA dehydrogenase gene expression could

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be modulated by attenuating the development of cardiac hypertrophy, we treated spontaneously hypertensive rats (SHR's) with the α- and β-adrenoreceptor antagonist, carvedilol. This therapy modestly attenuated the development of cardiac hypertrophy and significantly blunted the known downregulation of the metabolic gene expression pattern towards foetal levels in the treated SHR's compared to the placebo treated littermate controls. Finally, to determine whether this regulatory programme is applicable to human disease, medium chain acyl-CoA dehydrogenase protein and gene expression were delineated in the left ventricles of human cardiac transplant recipients. Medium chain acyl-CoA dehydrogenase enzyme and mRNA levels were coordinately downregulated (>40%) in failing human ventricles compared with controls. We have thus identified an energy metabolic gene regulatory programme that is regulated in part at the level of transcription during cardiac hypertrophy with additional translational or post-translational regulatory control. This translational/post-translational control is altered with the onset of heart failure and may contribute to the transition to this decompensated state. **Conclusions.** The medium chain acyl-CoA dehydrogenase gene regulatory control data elicited in this dissertation suggests a complex molecular regulatory programme directing metabolic remodelling during cardiac development, with the onset of cardiac hypertrophy, with the attenuation of hypertrophic growth in the heart and

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following the onset of heart failure. Further delineation and characterisation of these regulatory events are required to increase our understanding of the role of metabolic remodelling in cardiac development and disease. Finally, we propose that the data presented in this body of work advances the understanding of cardiac metabolism to include regulation at the genomic level.

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Glossary of Molecular Biologic Terminology:

Cis elements: DNA sequences within an individual gene that are responsible for directing the level of expression of the same gene.

Enhancer: Cis acting elements that can direct a significant increase in transcription independent of their respective position and orientation within a given gene; enhancers can play an important role in the regulation of tissue specific and inducible expression of individual gene.

Gene: The entire DNA sequence necessary for the synthesis of a functional protein (polypeptide) or RNA sequence.

Homology: DNA sequences appear partially identical when genes of different species are compared. It is estimated that the genes of mouse and man are 80% identical, which is considered high level of homology.

Isoforms: Proteins with a similar structure and function. They can be derived from a single gene or physically separate genes.

Knock-out: Gene targetting technique, to delete one allele of a gene through homologous recombination. The exact localization of this deletion is known. Lethality in homologous embryos indicates the essential function of the gene.

Messenger RNA (mRNA): The final RNA molecule formed through precursor RNA splicing and modification of the ends (polyA tail). This is

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the RNA molecule encompassing all coding sequences and capable of crossing the nuclear membrane to bring the message to the ribosomes for protein synthesis.

Poly A: The polymer of adenine nucleotides that are located at the end of mRNA.

Promoter: The region of a gene that binds RNA polymerase and initiates gene transcription; can include DNA elements that regulate various phases of development, target expression of a gene to selective tissues, and confer inducibility in response to defined stimuli.

Phenotype: Observable characteristics of an organism, resulting from the interaction of its genes and the environment in which development occurs.

Ribosomes: Consist of two unequal subunits bound together by magnesium ions. This cytosolic organelle translates mRNA into its appropriate protein.

Trans acting factor: The regulatory factors not located in the gene itself but direct and regulate the expression of a given gene (e.g., proteins that bind and regulate the promoter region of a gene).

Transcript: Product of transcription (RNA)

Transcription: The synthesis of RNA from a DNA template by the protein RNA polymerase.

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Transcription factor: Protein, that can modify transcription levels of a gene through direct binding of specific DNA sequences (cis-elements) of that gene.

Transfection: Introduction of foreign DNA into eukaryotic cells.

Transgenic: A technique used to transfer a gene into the germline of experimental animals that express the exogenous gene. The number of times and the localization of the transgene incorporation into the genome are unpredictable and random.

Translation: The synthesis of proteins from the mRNA template by the ribosomes.

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Abbreviations

DW	- Distilled Water
CAT	- Chloramphenicol acetyltransferase
CMP	- Cardiomyopathy
COUP-TF	- Chicken ovalbumin upstream promoter transcription factor
cTNI	- Cardiac troponin I
EMSA	- Electromobility shift assay
FAO	- Mitochondrial fatty acid β-oxidation
GAPDH	- Glyceraldehyde-3-phosphate dehydrogenase
GLUT4	- Insulin Sensitive Cardiac Enriched Glucose Transporter
HF	- Heart Failure
LCAD	- Long chain fatty acyl-CoA dehydrogenase
LCAS	- Long chain acyl-CoA synthetase
LVH	- Left Ventricular Hypertorphy
MCAD	- Medium chain acyl-CoA dehydrogenase
NRRE	- Nuclear Receptor Response Element
O_2	- Oxygen
PAB	- Pulmonary Artery Banding
PPARα	- Peroxisome Proliferator Activated Receptor (α isoform)
PFK-M	- Phosphofructokinase – Muscle enriched isoform.
RVH	- Right Ventricular Hypertrophy

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SHHF	-Spontaneously Hypertensive – Heart Failure Rat - SHHF/Mcc^{facp}
SHR	- Spontaneously Hypertensive Rat
Sp1	- Zinc finger nuclear regulatory protein acting as a transcriptional activator or repressor in a promoter specific context
Sp3	- Zinc finger nuclear regulatory factor which usually acts to repress transcriptional gene activity
WF	- Wistar Furth Rat

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Introduction

Cardiac Hypertrophy and Heart Failure

The development of ventricular hypertrophy constitutes one of the principal mechanisms by which the heart compensates for an increase in load.[1] One of the earliest cellular changes that occurs after a hypertrophic stimulus is the synthesis of mitochondria, presumably to provide the high-energy phosphates required to meet the increased energy demands of the hypertrophied cells.[2] The hypertrophied heart is usually considered to be a physiologically compensated state following an increased load on the heart. Compensated cardiac hypertrophy is associated with a proportional increase in the size of individual cardiac cells to maintain the systolic stress of the heart within normal limits.[3] The concept of the compensated heart may, however, be misleading as cardiac hypertrophy is an independent risk factor for sudden cardiac death.[4]

Heart failure is usually associated with progressive cardiac pump failure, with associated symptoms of dyspnoea and peripheral oedema. The pump failure may be from reduced contractility or an inability of the heart muscle to adequately relax between contractions. The underlying

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pathologies are multiple, and the end result is most often that of accelerated death.[5]

Hypothesis

A contributory role of altered energy production in the development of cardiac hypertrophy and the transition to heart failure has been an attractive, yet elusive hypothesis to prove. Evidence supporting this hypothesis includes the phenotypic presentation of cardiac hypertrophy and/or heart failure in individuals with inherited defects in metabolic genes;[6-9] and in some non-ischaemic diabetic patients (diabetic cardiomyopathy).[10] Moreover, the ablation of the genes encoding the adult cardiac enriched facilitative glucose transporter - (GLUT4),[11] and the gene encoding a putative intra-cytoplasmic heart specific fatty acid binding proten - (hFABP)[12] result in the development of cardiac hypertrophy in mice. In combination, these data implicate altered energy substrate utilization as a contributory factor in the pathogenesis of non-ichaemic cardiac hypertrophy and heart failure.

Substrate use for energy production is highly regulated during cardiac development and with the onset of cardiac hypertrophy and transition to heart failure.[13, 14] Glucose and lactate are the predominant

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fuel substrates utilized during cardiac development. Postnatally, a switch in energy substrate utilization occurs, so that fatty acids are the chief energy substrate in the non-fed normal adult mammalian heart. However, with cardiac hypertrophy and failure, a transition reminiscent of foetal energy metabolism occurs with reduced fatty acid and increased glucose utilization.[15, 16] Little is known, however, about the molecular regulatory mechanisms involved in this alteration in cardiac energy metabolism. Nor whether this reversion to the foetal energy substrate utilization contributes towards the development of cardiac hypertrophy and/or heart failure.

The hypothesis studied is that the regulation of genes encoding rate-controlling enzymes in fatty acid and glucose utilization pathways during the development of cardiac hypertrophy and heart failure are due to the reactivation of foetal gene regulatory programmes. Moreover, to begin to understand whether these gene regulatory programmes direct the known switch in energy substrate utilisation in cardiac hypertrophy and heart failure, these gene regulatory programmes need to be studied. Thus the further delineation and characterization of the regulatory programmes directing a gene encoding a metabolic enzyme during cardiac development and with the onset of cardiac hypertrophy/failure was studied at the molecular level.

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Energy Metabolism - A Perspective on the Focus of this Thesis

Prior to summarizing the known changes in energy metabolism in cardiac development, cardiac hypertrophy and heart failure, I will define the focus of this body of work in the context of the broad field of cardiac metabolism. A diverse array of studies of "cardiac metabolism" have been undertaken as energy metabolism is an integral component of cardiac function. The importance of metabolism in cardiac function is illustrated by the fact that the human heart accounts for only 0.5% of the total body weight, yet it claims 10% of the body's O_2 consumption.[14] Metabolic investigations in the heart include the study of: energy fuel substrate uptake and utilisation; metabolic pathway enzymatic activities; high-energy phosphate metabolism; cardiac oxygen consumption and the conversion of chemical energy to mechanical energy (cardiac efficiency). In this thesis the emphasis is on the utilization of fuel substrates by the heart for the production of chemical energy for the subsequent maintenance of cardiac contractile function. Many factors contribute to the selection of energy providing fuels for cardiac energy production. Krebs et al[17] classified these as: (i) the concentration of the direct fuel in the tissue, (ii) the presence in the tissue of the enzymes required for the degradation, and (iii) the kinetic properties of the key enzymes, especially those that initiate the release of energy. Each of these three main factors is, in turn,

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complex and depends on a variety of components. In this thesis, the focus is on the regulation of genes encoding representative energy metabolic enzymes in the context of pressure overload-induced cardiac hypertrophy and the subsequent transition to non-ischaemic cardiomyopathy. Thus, this body of work focuses on the gene regulatory programmes of category (ii), namely, directing the presence in the heart of the enzymes required for fuel substrate catabolism.

Fuel Substrate Utilization in Cardiac Development, Cardiac Hypertrophy and Heart Failure

During cardiogenesis the heart functions in a relatively hypoxic environment and glucose and lactate are the predominant fuel substrates.[18, 19] Postnatally, a switch in energy substrate utilisation occurs, so that fatty acids become the predominant fuel substrate utilised in the fasted state.[13] These switches in energy substrate utilisation for ATP production correspond to O_2 availability. When O_2 is rate-limiting, as in the foetal period, more ATP is generated from glucose and lactate per mole of O_2 consumed than from fatty acid oxidation.[14] Conversely, when there is an abundance of O_2, the energy yield from the oxidation of long-chain fatty acids is greater, on a mole-by-mole basis, than that obtained from glucose or lactate catabolism. Of note, this switch in energy substrate

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utilisation is paralleled by mitochondrial development, the main intracellular organelle for oxidative phosphorylation of ADP. Full mitochondrial maturation is evident in the late foetal to early postnatal period[20] and the postnatal heart is richly abundant in mitochondria, with the mitochondrial volume accounting for 25% of cardiocyte volume in humans to 38% in mice.[21]

The utilization of energy substrate and metabolic pathway enzymatic activities that have been observed in animals and humans demonstrate that glucose metabolism is increased with concurrent reduced fatty acid utilization in association with acquired cardiac hypertrophy and heart failure. These changes parallel the metabolism of the foetal heart and suggest a reversion to foetal metabolism in these pathologic states. However, the molecular regulation of cardiac energy-producing pathway enzymes during the development of cardiac hypertrophy and heart failure have not been previously studied. Moreover, characterisation of metabolic enzyme gene regulation during the development of compensated hypertrophy and in the decompensating failing heart may give us further insight into the contribution of energy metabolism in these disease processes.

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Genetic Defects in Energy Control and Cardiac Dysfunction

Identification and subsequent characterization of inherited defects in genes encoding fuel substrate utilization enzymes should give us greater insight into the functional role of altered fuel substrate utilization in the development of cardiac hypertrophy and non-ischaemic cardiomyopathy. Familial analyses have shown that cardiomyopathy may have a genetic or inherited basis in children and adults. For example, approximately 20 percent of patients with idiopathic dilated cardiomyopathy were found to have a first-degree relative with myocardial dysfunction.[22] Many descriptions of individual kindreds with cardiomyopathy of unknown cause transmitted as autosomal dominant, autosomal recessive, and X-linked traits have appeared.[23-25] Accordingly, a substantial proportion of idiopathic cardiomyopathies may have a genetic cause.

Molecular geneticists have concentrated primarily on identifying single-gene disorders resulting in human disease, including numerous cardiomyopathic syndromes. Recently, Kelly et al[7] reviewed the categories for which the genes causing cardiomyopathy have been identified (Table 1). Genetic defects have been predominantly identified in genes encoding cardiac contractile proteins. The characterization of the genetic defects resulting from the inherited defects in these contractile

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protein encoding genes have been extensively reviewed,[26, 27], and will not be discussed further.

However, a second subset of genetic disorders include defects in genes encoding enzymes required for mitochondrial oxidative phosphorylation and for mitochondrial fatty acid β-oxidation. The majority of cardiomyopathy due to defects in mitochondrial oxidative phosphorylation are concurrent with neuromuscular defects or mitochondrial myopathies (Table 1).

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Table 1

Contractile/Structural Protein Gene Defects	Disorders of Energy Metabolism	X-Linked Muscular Dystrophies
Defects have been identified in genes encoding myosin heavy and light chain isoforms, troponin I and C isoforms, myosin binding protein C, and in α tropomyosin The clinical features are variable, and include: asymetrical septal hyper-trophy, left ventricular hypertrophy, outflow tract obstruction. Sudden death is relatively common. Some subjects, however, are asymptomatic with or without ventricular hypertrophy	Defects have been identified in genes encoding enzymes required for mitochondrial fatty acid uptake, β-oxidation and mitochondrial oxidative phosphorylation The clinical features of the fatty acid oxidation enzyme defects are variable, and include: cardiomyopathy, fasting hypoglycemia, hepatic dysfunction and skeletal myopathies. The clinical features of disorders in mitochondrial oxidative phosphorylation include: neuromuscular manifestations and mitochondrial myopathies. Skeletal myopathy is more common than the cardiac variant	Duchenne's and Becker's are the most common inherited skeletal myopathies. Defects have been identified in the gene encoding dystrophin, a large membrane associated protein in both syndromes. The clinical features of both Duchenne's and Becker's muscular dystophy are predominantly skeletal myopathy. Most patients, however, have evidence of cardiomyopathy.

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Table 1. Classification of inherited defects presenting with cardiomyopathy as a clinical feature. This classification is based on a review by Kelly et al,[28] and summarizes the disorders. Disorders due to myocardial infiltration (e.g., glycogen storage diseases) are not reviewed.

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Autosomal recessive defects in genes encoding multiple fatty acid β-oxidation enzymes cause cardiac hypertrophy, heart failure, and sudden death in human subjects.[7] The most commonly inherited gene defect of the mitochondrial fatty acid β-oxidation (FAO) enzyme encoding genes is that of medium chain acyl-CoA dehydrogenase (MCAD). The MCAD gene defect is an autosomal recessive inherited disorder, and cardiac phenotypic manifestations include cardiac hypertrophy and dilated cardiomyopathy.[29] The inherited FAO enzyme defects which most commonly manifest with cardiomyopathy are defects in the genes encoding the enzymes required for long and very long chain fatty acid metabolism. Moreover, in a recent study screening subjects with hereditary hypertrophic cardiomyopathy, a high incidence of a deficiency of a putative long chain fatty acid transporter (CD36) was noted in the subset of subjects with asymmetrical hypertrophy of the cardiac septum. Furthermore, those individuals with deficiency in CD36 and asymmetrical septal hypertrophy also had decreased long chain fatty acid uptake into the hypertrophied myocardium despite normal myocardial perfusion.[9] These data suggest that in a subset of subjects with genetic defects disrupting fatty acid metabolic genes, the reduced capacity to utilize fatty acids for energy production, can directly result in cardiac contractile

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dysfunction. However, whether a reduction in fatty acid utilization capacity, contributes to cardiac decompensation in acquired forms of heart disease is unknown.

Acquired / Induced Defects in Fuel Substrate Utilisation and Myocardial Dysfunction

Diabetes mellitus is the most common metabolic disease in which cardiac dysfunction may present as a distinct manifestation independent of coronary artery disease. Primarily, cardiac contractile dysfunction presents in diabetic patients in association with myocardial ischaemia. This combination of diabetes (hyperglycemia, elevated free fatty acids and ketone bodies) and myocardial ischaemia (hypoxia, lactic acidosis, fuel substrate limitations) has, not surprisingly, been recognized as resulting in a higher incidence of, and more pronounced myocardial cell death, contractile dysfunction and a worse prognosis compared to non-diabetics. To dissect out the functional significance of the metabolic pertubations in these circumstances, is very complex and not discussed further in this monograph. However, it has been determined that ventricular performance can be impaired (diabetic cardiomyopathy) in the presence of minimal, or in the absence of ischaemic heart disease in subjects with diabetes mellitus.[10] Whether the reduced capacity to utilise glucose, the

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elevated levels of circulating free fatty acids or the high myocardial triacylglycerol content in diabetes contribute to contractile dysfunction in these subjects is unknown. Molecular approaches to address these question are being pursued and are addressed briefly in the introductory section on the manipulation of metabolic enzyme encoding genes.

Finally, the impairment of cardiac fatty acid utilization by pharmacologic inhibition of mitochondrial fatty acid importation in animal studies has been shown to result in the development of cardiac hypertrophy and contractile dysfunction.[30, 31]

Thus, cardiac dysfunction can be a manifestation of defective substrate utilization in humans (diabetes mellitus) and in animals subjected to pharmacologic inhibition of mitochondrial fatty acid import. Taken together these data suggest a fundamental role of substrate utilization in the induction of myocyte growth and dysfunction.

Candidate Enzyme Encoding Genes Studied - An Overview of Metabolic Pathways

The focus of this thesis is on the gene regulatory programmes directing the presence of cardiac rate-controlling enzymes required for major fuel substrate catabolism during cardiac development and with the onset of

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cardiac hypertrophy. I will therefore, briefly review these energy metabolic pathways, concentrating on these key enzymes.

During cardiogenesis the major fuel substrates are glucose (exogenous and intracellular glycogen) and lactate. Perinatally, in parallel with mitochondrial genesis the contribution of fatty acid oxidation is enhanced with reduced glucose and lactate utilisation. In the adult it has been well characterised that fatty acid oxidation accounts for between 60 and 100% of oxygen consumption with a lesser contribution from lactate and glucose (0-20% of each).[32] The switches and variability in substrate use are dependent in part on the nutritional status with increased glucose use in the acute post food-consumption period and predominantly fatty acid use during the greater part of the day. Moreover, switches in fuel substrate are also precipitated by acute increase in work load, when glycogen and glucose become preferentially oxidized in the heart.[33]

Regulation of Glycolysis and Glycogen Metabolism

The uptake of extracellular glucose is regulated by the transmembrane gradient and the concentration and activity of glucose transporters (See Figure 1) in the plasma membrane. Two isoforms from the glucose transporter family have been identified in the myocardium, GLUT1 and GLUT4.[34, 35] Both transporters are located in the sarcolemmal

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membrane and in intracellular microsomal vesicles[36] and are regulated at the levels of gene transcription through post-translational control [37, 38]. Upon entering the cell, free glucose is rapidly phosphorylated by hexokinase to form glucose-6-phosphate (G-6-P), which is impermeable to the cell membrane (Figure 1). G-6-P can be used for either glycogen synthesis or it can proceed down the glycolytic pathway to form pyruvate (Figure 1).

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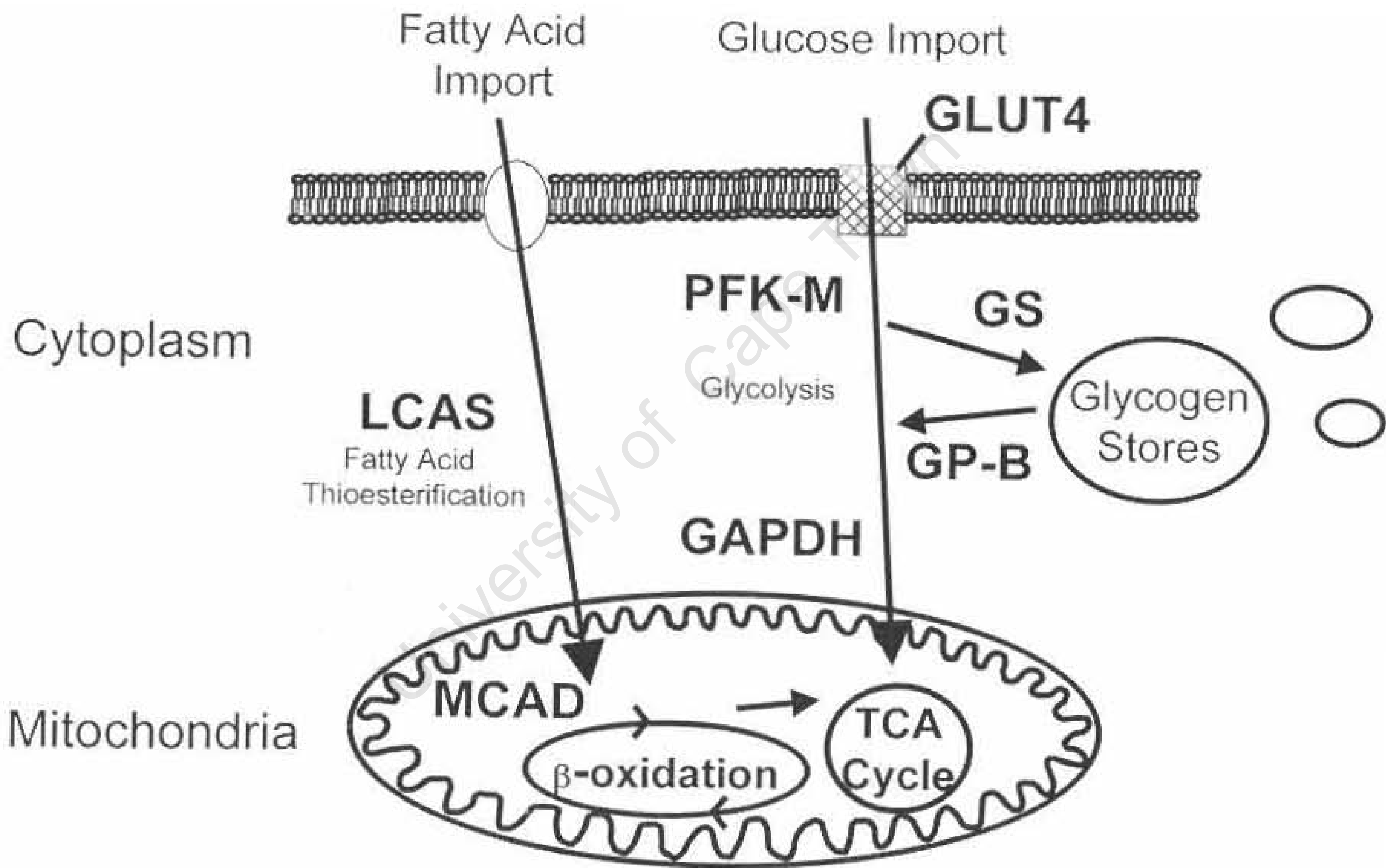

Figure 1

Figure 1. Schematic Representation of the Major Cardiac Metabolic Pathways.

The expression patterns of genes encoding the following substrate uptake and catabolism enzymes were assessed during cardiac development and with the onset of hypoxia induced RVH: (i) Long-chain acyl CoA synthetase (LCAS), a thioesterification enzyme that facilitates the transfer of long chain fatty acids across the cytosolic compartment; (ii) medium chain acyl-CoA dehydrogenase (MCAD), a representative enzyme in the mitochondrial fatty acid β-oxidation cycle; (iii) GLUT4, an adult cardiac enriched facilitative glucose transporter; (iv) the adult cardiac enriched isoform of phosphofructokinase (PFK-M); and the ubiquitous glycolytic enzyme - glyceraldehyde 3-P dehydrogenase (GAPDH). Additional abbreviations on figure: TCA - tricarboxylic acid, GS - glycogen synthase, GP-B - glycogen phosphorylase - brain isoform.

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Understanding the regulation of cardiac glycogen metabolism is important as glycogen has been shown to account for up to 41% of glucose metabolism and is preferentially oxidized compared with exogenous glucose in the adult heart under aerobic conditions.[39, 40] Moreover, the role of glycogen during cardiogenesis is proposed to extend beyond metabolic requirements, and is potentially thought to play a role in mechanical cardiocyte stability.[20, 41] The rate of glycogen synthesis is regulated by the concentration of G-6-P and the activity of glycogen synthase (GS). GS activity and glycogen storage are increased by insulin.[42] Glycogenolysis results in the formation of G-6-P, and is regulated by the activity of glycogen phosphorylase (GP). GP activity is controlled by both hormonal stimulation (e.g. β-adrenergic receptor stimulation) and via energy demand (contractile function).[43] Animal and human studies indicate that there is simultaneous synthetase and phophorylase activities in-vivo.[39, 44] In addition, the cardiac glycogen content is highly dependent on substrate availability, energy demand and on the species studied (Reviewed [43]).

The overall rates of glucose uptake, glycogen synthesis and breakdown, and the rate of glycolysis are controlled by multiple steps distributed along these pathways, and not subject to control at a discrete point.[45] Given a constant supply of G-6-P, the primary regulators of

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glycolytic rate are the activity of phosphofructokinase (PFK)(EC 2.7.1.11), and the ability to form reduced NADH.[46] NAD^+ is reduced to NADH by the conversion of glyceraldehyde 3- phosphate to 3-phosphoglycerol by the enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH, was thought to be a constitutively expressed, but now appears to be regulated by contractile work,[47] during myocardial ischaemia[48] and with the circadian rhythm.[49] The final fuel product of the glycolytic pathway is the formation of pyruvate. Of note, lactate is an additional major in-vivo source of pyruvate formation in the heart.[50]

Pyruvate decarboxylation is the key irreversible step in carbohydrate oxidation and is catalysed by pyruvate dehydrogenase (PDH). Pyruvate oxidation and the activity of PDH in the heart are decreased by elevated rates of fatty acid oxidation and enhanced by the suppression of fatty acid oxidation.

Glucose Utilisation Enzyme Isoforms

Multiple isoforms of genes encoding rate-controlling glucose utilization enzymes have been described. The expression patterns of the respective isoforms are relatively tissue specific, thereby, directing the presence of these enzymes in an appropriate developmental and tissue specific

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manner. The developmental expression patterns of gene isoforms encoding the representative rate-controlling glucose utilization enzymes used in this study are described in the results section of this dissertation.

Two of the facilitative glucose transporters are known to be enriched in the heart, these include GLUT1 and GLUT4. GLUT1, the more ubiquitous isoform, is thought to be the major determinant of basal glucose transport in cardiac muscle.[51] GLUT1 is developmentally regulated in the heart with high mRNA expression in the foetus and reduced expression in the adult myocardium.[52] GLUT4 is the predominant cardiac adult isoform and is highly regulated during acute cardiac stress.[53] GLUT4 is in addition, insulin-responsive[34] and highly regulated at the pre- and post-translational levels.[35, 36, 54] The cardiac developmental mRNA expression pattern of GLUT4 is inverse to that of GLUT1, with low expression in the foetal heart and high expression in the normal adult heart.[52]

Four hexokinase isoforms have been characterized, and, like the glucose transporters, they show differences in tissue distribution. HK I is thought to be ubiquitous, but in a similar pattern to GLUT1, is more highly expressed in the foetal heart and downregulated at the level of gene expression in the adult heart.[52] HK II, is seemingly coordinately regulated

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with GLUT4 and is higher in the adult than foetal heart and is expressed in insulin-sensitive tissue.[52]

PFK contributes to the regulation of intermediary steps of glycolysis and three isoenzymes of PFK-1 have been described in rodents.[55] The muscle isoform (PFK-M) is enriched in cardiac and skeletal muscle and is known to be markedly regulated during skeletal muscle differentation, with a 5-7 fold induction during myoblast to myotube differentiation of C2 cells.[56] The cardiac developmental expression patterns of the PFK encoding gene isoforms have not yet been described. A second phosphofructokinase, PFK-2 is also an important enzyme in glycolytic flux. The activity of this enzyme has been shown to increase when glycolytic flow is increased in response to insulin or increased heart work.[57]

To advance the understanding of the molecular regulation of the genes encoding the adult-enriched glucose transporter (GLUT4) and of PFK-M during cardiac development, with the onset of cardiac hypertrophy, and following antihypertensive treatment in hypertensive rats the mRNA expression patterns of these genes were evaluated in our studies. Although these data are a minor aspect of the dissertation, they do contribute to the broader concept of the role of gene regulation in cardiac metabolism. The new paradigm that genomic regulation plays a role in

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directing cardiac metabolism is considered further in the discussion section of this dissertation.

Regulation of Fatty Acid Metabolism

Lipids are a major source of energy in differentiated cardiac muscle and are used in preference to glucose in aerobic conditions. However, compared to glucose metabolism, the control of lipid metabolism in muscle is less well understood. The heart does contain a certain amount of stored triacylglycerol, however, the activity of triacylglycerol lipase, which is required to mobilize these stores are low. In the rat heart, for example, the activity of triacylglycerol lipase is less than one percent of the activity of phosphofructokinase.[58] However, this endogenous triglyceride pool may account for up to 50% of the myocardial energy requirement in the absence of exogenous fatty acids.[59] This would be a very rare event and accordingly the main source of lipids for oxidation in the heart is derived from the circulation. These are carried in the circulation as either free fatty acids bound to albumin, as triacylglycerol or in lipoprotein particles.

A major factor in the control of lipid oxidation appears to be substrate supply. This concept was initially introduced by Randle and colleagues in 1963 and was described as the 'glucose fatty-acid cycle'.[60] In these experiements, Randle demonstrated that the metabolic

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relationship between glucose and fatty acids were both reciprocal and partially dependent (reviewed[61]). An additional convincing mechanism, which has been suggested, is at the stage of acetyl CoA. It seems highly unlikely, however, that regulation of the supply of circulating lipid and by feedback inhibition by the end-product on an enzyme distal in the β-oxidation pathway are sufficient control points for exquisite regulation of this pivotal cardiac fuel substrate. Data implicates additional regulatory control at the level of fatty acid import into the mitochondria via carnitine palmitoyaltransferase enzymes.[62-64] The regulation of carnitine palmitoyltransferase is exquisite, and is dependent on numerous enzymes, kinases and substrates (described in detail in the referenced manuscripts[65-68]). Other mechanisms to coordinate the rate of fatty acid oxidation with the energy demand of the contracting myocyte are expected to be operational. However, if there are other sites of regulation they have yet to be well defined and characterized. The fatty acid β-oxidation cycle is illustrated in Figure 2.

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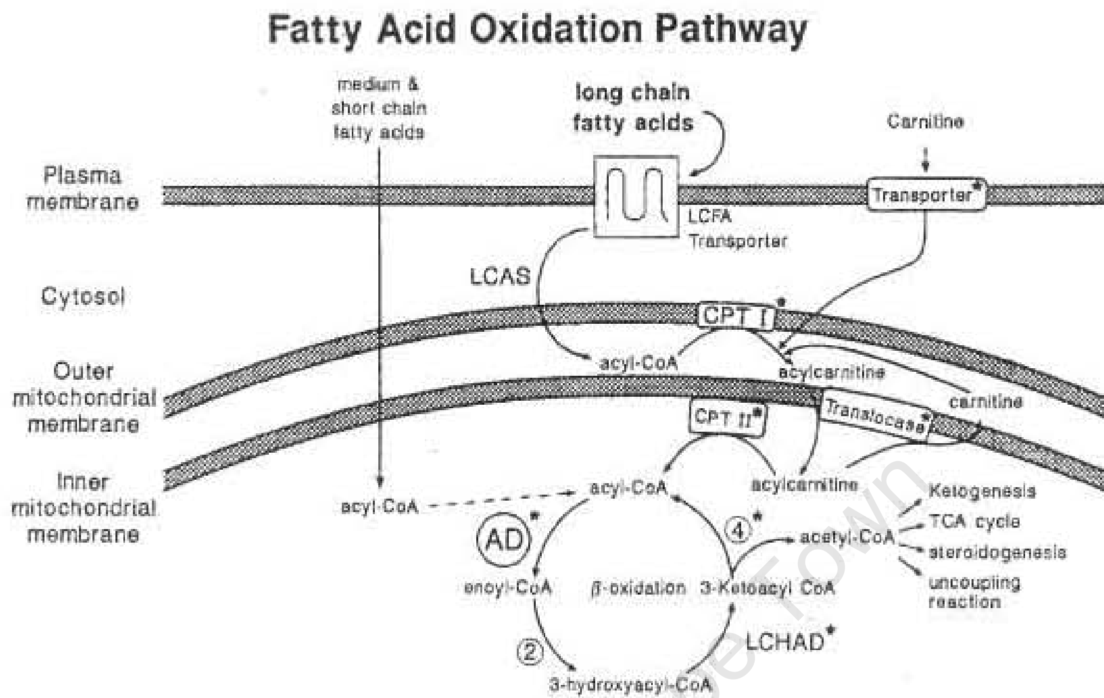

Figure 2. The β-oxidation pathway of fatty acids and specific defects causing cardiomyopathy.

The pathways of fatty acid oxidation and cellular carnitine metabolism are shown. Defects known to cause cardiomyopathy (indicated by the asterisks) include abnormalities in carnitine and acylcarnitine transport, carnitine palmitoyltransferase I and II (CPT I and II) deficiency, and carnitine-acylcarnitine translocase (translocase) deficiency - and errors in steps 1 and 3 of mitochondrial β-oxidation, long-chain and medium-chain acyl coenzyme A dehydrogenase (AD) deficiencies and long-chain 3-hydroxyacyl-coenzyme A dehydrogenase (LCHAD) deficiency, respectively. LCFA, long -chain fatty acids; LCAS, long-chain acyl-CoA synthetase.

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The Foetal Gene Programme and Cardiac Dysfunction

Numerous investigators have demonstrated, while studying the molecular regulatory events associated with cardiac hypertrophy, that there is a re-expression of the foetally enriched isoforms of numerous genes encoding cardiac contractile and ion regulatory proteins.[69-74] The current paradigm, therefore implies that the activation of the "foetal gene" programmes induce adaptive structural and metabolic responses of the overloaded ventricle to maximize chemomechanical energy conversion efficiency, and thereby decrease oxygen consumption in the hypertrophied heart. This reversion to the expression of foetal isoforms is paralleled at the metabolic level in the switch in energy substrate utilisation in the heart during the development of cardiac hypertrophy and failure. In this body of work we have begun to explore and characterise the regulation of the genes encoding metabolic enzymes required to orchestrate the use of specific fuel substrate during cardiac development and with the onset of cardiac hypertrophy and the transition to heart failure.

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The Gene Regulatory Programme

The study of inherited disorders resulting in cardiomyopathy discussed above has given us insight into multiple potential mechanisms in the development of cardiac contractile dysfunction. To further enhance the understanding of the development of cardiac hypertrophy and the transition to heart failure, an approach would be to delineate and characterize the gene regulatory programmes activated during the development of these pathologies. Using this strategy, one can focus on cardiac gene/s thought to be regulated by relevant pathologic cardiac stimuli such as pressure overload. Subsequently, the components of the pathway upstream of the candidate gene of interest can be characterized in "reverse" from the gene's promoter (on/off switch), to the promoter binding transcription factors (gene regulatory proteins), to intracellular signalers and finally to the membrane receptor and their ligand/s (Figure 3).

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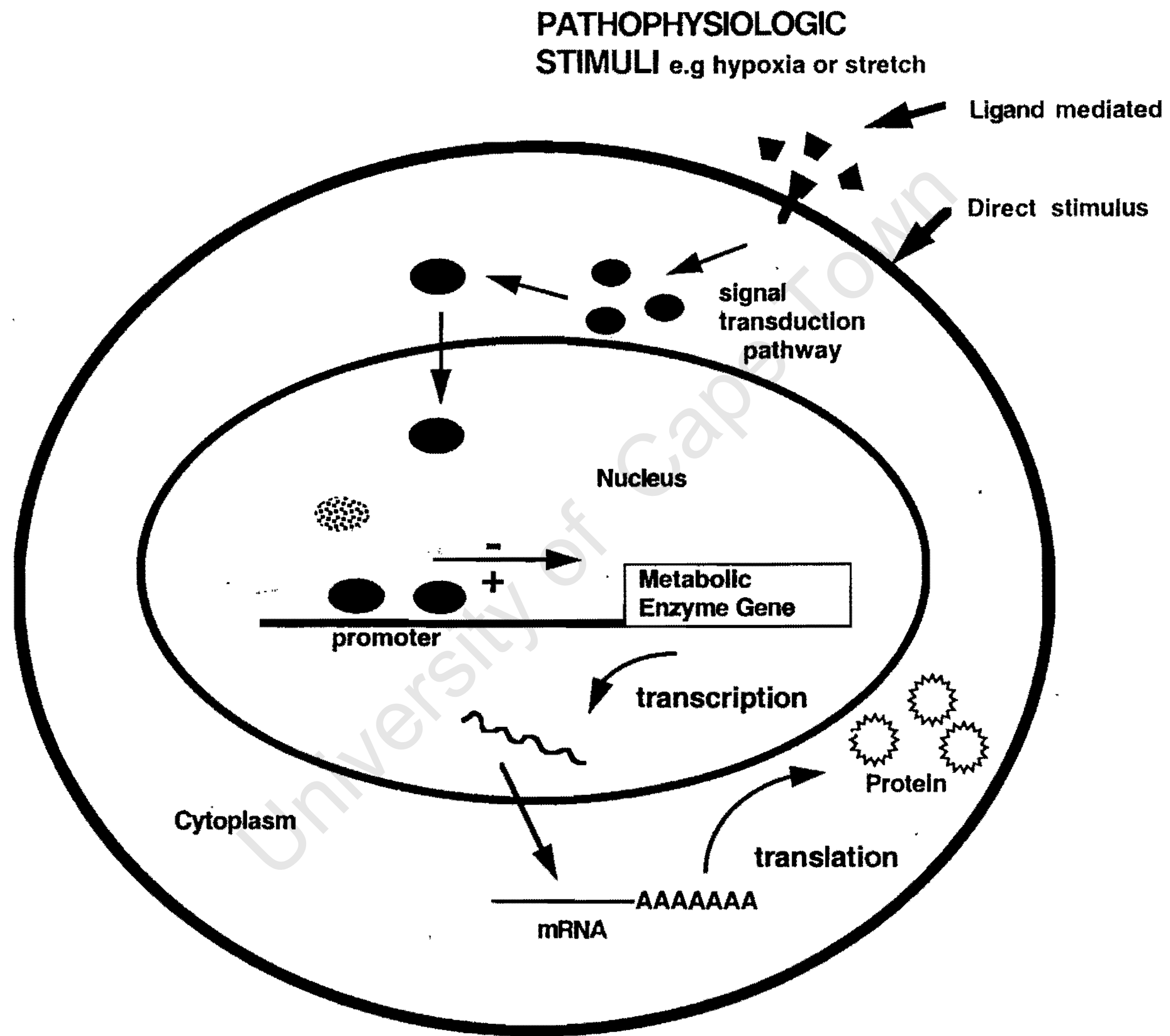

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Figure 3

Figure 3. Schematic representation of a gene regulatory programme in response to a pathophysiologic stimulus.

The extracellar stimulus elicits a direct or a ligand/receptor-mediated response which couples to appropriate signal-transduction pathways. The intracellular signal transduction pathways activate specific transcription factors (regulatory proteins) to increase or decrease the transcription of target genes. These transcription factors bind to defined regions of the candidate gene promoter to modulate gene transcription. Translation of these genes result in the production of proteins in response to the initial extracellular stimulus.

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This reverse approach has been used recently to elucidate adaptive and maladaptive responses of the cell during hypertrophy and in the failing heart (Reviewed [28, 74]). We have used this reverse approach strategy in this body of work by focussing on a single rate-controlling energy substrate pathway enzyme encoding gene (medium chain acyl-CoA dehydrogenase [see figure 2]).

However, we firstly evaluated the expression patterns of numerous genes encoding multiple fatty acid and glucose metabolic enzymes during cardiac development and with the onset of cardiac hypertrophy (Illustrated in figures 1 and 2). As will be described, we demonstrated a coordinate expression pattern of genes encoding multiple metabolic enzymes during cardiac development and with the onset of cardiac hypertrophy. Thereafter using this 'reverse' strategy to understand gene regulation we focussed on evaluating the gene regulatory programme directing medium chain acyl-CoA dehydrogenase in cardiac development, with the onset of cardiac hypertrophy and during established heart failure.

In the preliminary studies we established that medium chain acyl-CoA dehydrogenase was highly regulated at the gene expression level during cardiac development and with the onset of cardiac hypertrophy. Further characterization of medium chain acyl-CoA dehydrogenase was therefore directed at evaluating the regulation of this gene at the

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transcriptional level. Transcriptional regulation is, in part, dependent on the regulatory elements contained within the gene's promoter region. This structural region (promoter) directs the expression of a gene in response to physiologic or pathologic signals (see Figure 3). Thus we can construct modified genes which include the candidate gene's promoter region fused to a reporter gene (see schematic diagram in Figure 4). By measuring the activity of this reporter gene in-vitro we can begin to understand which transcriptional factors modulate the regulation of this gene in response to different stimuli. Moreover, this transgenic construct, can be randomly inserted into the mouse genome. Subsequent measurement of the activity of the reporter gene allows us to determine the ability of the incorporated promoter region to turn on (upregulate) or off (downregulate) the reporter in response to physiologic and pathologic stimuli in-vivo. Subsequent promoter region DNA:protein binding studies may identify the transcription factors that bind to the promoter and direct the appropriate regulation of the candidate gene. All of these types of studies were performed by myself to evaluate and characterize the regulation of medium chain acyl-COA dehydrogenase during cardiac development and with the development of cardiac hypertrophy and are described in detail during the remainder of the text. Of note multiple studies to characterize the regulation of medium chain acyl-CoA both in-vitro and in-vivo have previously been performed in Dr. Kelly's

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laboratory[75-80] prior to my work and these studies were the essential foundation to enable me to advance this area of study.

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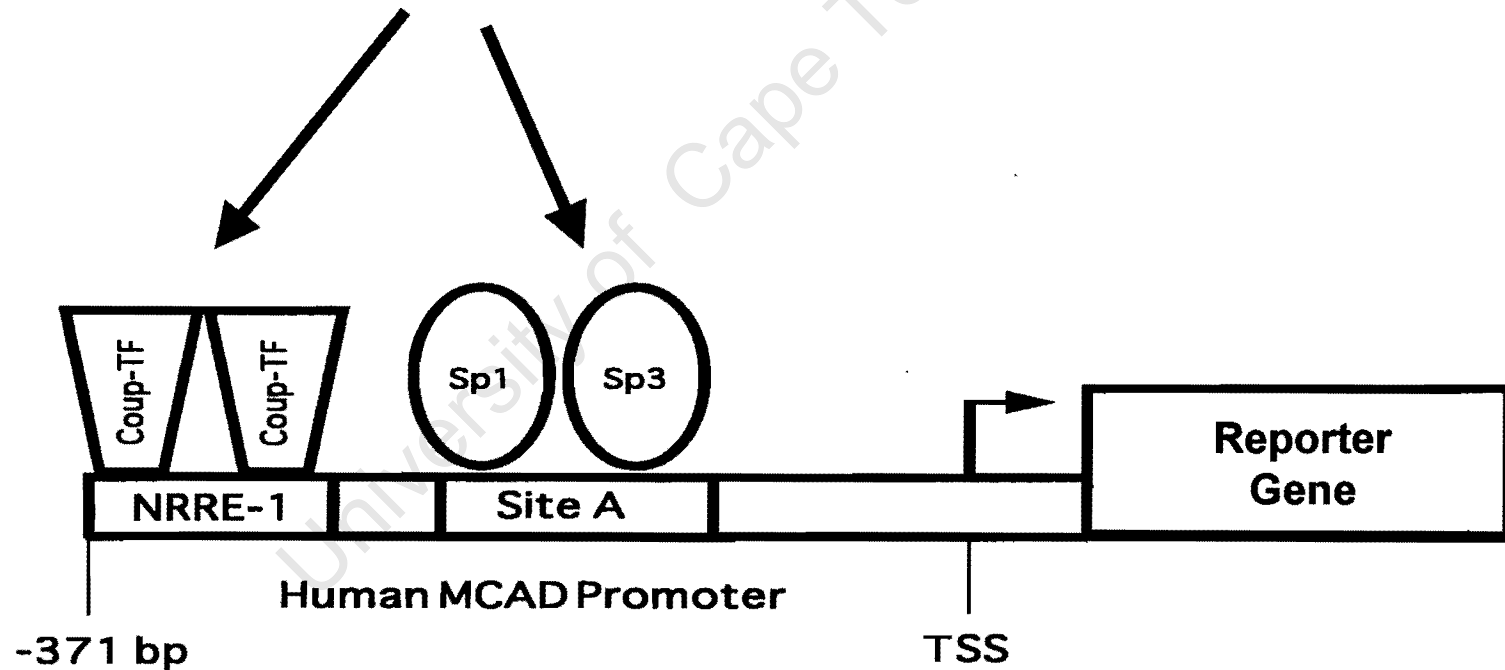

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Figure 4

Figure 4. Schematic representation of a transgene generated which incorporates the human medium chain acyl-CoA dehydrogenase promoter and a reporter gene.

The human MCAD promoter region from - 371 base pairs (bp) upstream of the transcription start site (TSS) to + 189 bp downstream of the TSS is shown fused to an exogenous reporter gene. The reporter gene activity is a measure of the activity of the human MCAD promoter in-vivo. The nuclear receptor response element 1 (NRRE-1) and the GC nucleotide rich sequence (Site A) are two distinct regulatory regions of the promoter. The functional significance of these regulatory elements are discussed in the text. The nuclear regulatory transcription factors, Coup-TF, Sp1 and Sp3 are described in greater detail in the results section of the text.

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Manipulation of Candidate Genes to Evaluate Their Functional Role in the Development of Cardiac Hypertrophy and Heart Failure

An alternate molecular approach to evaluate the functional role of metabolic genes in the development of cardiac hypertrophy and heart failure is the use of genetic manipulation of candidate genes in mice with a careful evaluation of the cardiac phenotype. Of note, we did not undertake any genetic ablation or gene overexpression studies for this body of work. However, to illustrate how this approach can also be used to evaluate the role of metabolic genes in the development of cardiac hypertrophy I have briefly described the studies of the genetic ablation of the cardiac enriched glucose transporter encoding gene - GLUT4 in the following section.

Genetic Manipulation of GLUT4 Transporter Encoding Gene

With the development of cardiac hypertrophy, aerobic glycolysis from exogenous glucose is accelerated.[81, 82] The uptake of exogenous glucose is regulated by the transmembrane gradient and by the concentration and activity of the facilitative glucose transporters. The GLUT4 isoform of the glucose transporter family, is insulin-sensitive and highly expressed in the adult heart, skeletal muscle and adipose tissue.[83]

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The process of insulin-stimulated glucose uptake is defective in the muscle of patients with type II diabetes[84]. Thus there is considerable interest in the mechanism of GLUT4 activation and the establishment of methods by which this 'insulin-resistance' can be overcome. The ablation of the GLUT4 gene was undertaken by homologous recombination [(GLUT4-/-)[11]]. Using these mice investigators were able to determine the developmental and metabolic consequences of abolishing the most abundant glucose transporter in insulin target tissues including the heart.

The mice deficient in GLUT4 are growth-retarded, experience decreased longevity, develop cardiac hypertrophy and exhibit severely reduced adipose tissue deposits. In addition, these mice exhibit postprandial hyperinsulinaemia, indicating possible insulin resistance and increased expression of cardiac GLUT1. The authors conclude that functional GLUT4 protein is essential for sustained growth, normal cellular glucose and fat metabolism, and expected longevity.[11] The significant cardiac hypertrophy in these GLUT4 null mice is thought to be due to the hyperinsulinaemia (functioning as a growth factor) in these animals, and/or the due to the decreased supply of free fatty acids to the heart. Importantly, insulin-resistance has been described in hypertensive and failing heart conditions.[85, 86] Likewise, reduced free fatty acid utilization by the heart as exhibited via pharmacologic inhibition of fatty acid β-

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oxidation[31, 87, 88] and in the genetic defects in fatty acid utilization[29] is known to induce cardiac hypertrophy. The mechanisms of these phenomena are, however, unknown. Further characterization of the GLUT4 deficient mice has suggested that another probable hypertrophic inducing factor is the development of hypertension as shown in the heterozygous knockout GLUT4 mice (GLUT4+/-).[89]

Interestingly, as with the fatty acid utilizing enzyme genes, GLUT4 is highly regulated during development, with low expression in the foetal and neonatal stages, and high expression in the adult myocardium.[52] We and others have demonstrated that at the level of gene expression and whole cell protein levels, GLUT4 reverts to foetal levels with the development of pressure overload-induced cardiac hypertrophy.[90] This seems counterintuitive, as GLUT4 is the predominant cardiac glucose transporter, and glucose utilisation is known to be increased in cardiac hypertrophy. The metabolic substrate utilization studies in the GLUT4 ablation studies described above, however, demonstrate that despite a complete absence of GLUT4, glucose uptake and utilization are increased in the hypertrophic GLUT4 deficient mice compared to normal controls.[91] In these studies, the level of GLUT1 is augmented and probably overcompensates for the loss of GLUT4.[11]

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Thus, although not conclusive these studies into the GLUT4 knockout mice do illustrate an alternative and complementary approach to delineate the genomic role of energy substrate use in the development of cardiac hypertrophy. The manipulation of other cardiac enriched metabolic enzyme encoding genes, including that of a fatty acid metabolism[92] are ongoing and should expand our understanding of the role of substrate use in the development of cardiac hypertrophy. Moreover, the manipulation of the transcriptional pathways directing these fatty acid enzyme encoding genes[93, 94] are ongoing and should give us further insight into the role of the regulation of metabolic enzymes and the development of cardiac hypertrophy and heart failure. These studies are however beyond the scope of this dissertation and are, in part, an extension of this work.[93, 94]

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Project Aims

The objectives of this body of work were to:

i) Determine the expression patterns of genes encoding key rate-controlling enzymes in the major cardiac energy catabolic pathways, including fatty acid β-oxidation and glycolysis during the cardiac development and with the onset of cardiac hypertrophy.

ii) Delineate and characterize the molecular programme directing a target metabolic gene (medium chain acyl-CoA dehydrogenase) during cardiac development, with the onset of pressure-overload induced cardiac hypertrophy and with the progression to decompensated heart failure.

The future objectives of this work include the genetic manipulation of the regulatory mechanisms identified, to begin to understand the role of altered fuel substrates in the development of cardiac hypertrophy and heart failure.

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Materials and Methods

Cardiac Tissue Samples

In order to evaluate metabolic gene regulation during cardiac development, rats were sacrificed at various time points of development during the foetal and postnatal periods. To evaluate the regulation of the metabolic genes with development of cardiac hypertrophy a mouse model of right ventricular hypertrophy was used. Thereafter, to evaluate the transcriptional regulation of a candidate gene in-vivo, we utilized the same mouse model of cardiac hypertrophy, however, in this instance the experiments were performed in transgenic mouse harbouring our gene promoter of interest. Subsequently, neonatal rat hearts were extracted to prepare primary cardiac cell cultures for gene promoter transfection studies. To study the temporal regulation of these metabolic genes with the development of cardiac hypertrophy and heart failure, numerous genetic rat strains of pressure overload were studied (described below). In an attempt to extrapolate these data to the human heart, we did evaluate the regulation of some of these metabolic genes in explanted hearts from recipients of heart transplants and from age-matched controls. These various heart preparations are described in greater detail below.

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Cardiac Developmental Time Points

To evaluate the mRNA expression patterns of genes encoding representative metabolic enzymes during cardiac development, we extracted heart tissue from Wistar Furth Rats at various time points during cardiogenesis. The rat foetus develops over 21 days, and an established, contracting four chambered heart is evident after 9.5 - 10.5 days of embryologic development (e9.5 - e10.5)[95]. Embryonic heart tissue was studied at days 16-19 (e16 – e19), and postnatally (p) at days p1, p7, p14, p21 and p70. The rats were sacrificed in a CO_2 chamber, and the hearts excised and stored at –80°C, for subsequent extraction of RNA and protein.

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Models of Cardiac Hypertrophy - Heart Failure

The Pulmonary Artery Banded Mouse Model

Current molecular genetics technology allows researchers to genetically manipulate the mouse genome, facilitating the subsequent functional characterization of the role of proteins of interest in physiology and pathophysiology in-vivo. To exploit this technology in the assessment of

fatty acid metabolic gene regulation in the heart, we initially characterized the regulation of fatty acid oxidation enzyme encoding genes in mice (C57BL/6 x SJL/J mice) with induced right or left ventricular hypertrophy. The right and left ventricular hypertrophy was induced via Pulmonary artery banding (PAB) or thoracic aortic arch banding (TAC) surgery respectively, in the laboratory of Howard Rockman, MD (University of California, San Diego).[96, 97] The PAB was performed as follows: mice underwent induction of anesthesia and intubation, the main pulmonary artery was isolated by blunt dissection and a suture was placed around the vessel and tied against a 25-gauge blunt needles to produce a stenosis. Sham-operated animals (n = 24) underwent the identical surgical procedure except that the constriction was not placed. After 7 days, animals from the PAB and sham groups were sacrificed and ventricular and atrial chambers were dissected out and snap frozen. Seven days was deemed to be a reasonable time point to assess hypertrophic indices, as the hearts had hypertrophied to a significant degree by this time point.[96, 97] The mean age of the mice was 9 months with a similar distribution of male and female animals between the PAB and control groups. The TAC procedure was performed in a similar way.[97] The differences included a smaller blunt needle for the constriction size (26- gauge) and the experiments lasted 14 instead of 7 days. The heart tissue was stored at –70°C for subsequent RNA, DNA and Protein

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analysis work. FAO enzyme gene expression patterns were compared in the PAB and sham operated littermate control mice and between the TAC operated mice and their respective controls.

The right ventricular hypertrophy induced by pulmonary artery banding was more significant, with a greater degree of regulation of FAO enzyme gene expression (discussed in results). We thus used the PAB approach to evaluate the role of gene transcription of medium chain acyl-CoA dehydrogenase gene expression with the development of cardiac hypertrophy. Prior to my arrival in Dan Kelly's laboratory, extensive studies had been performed to clone, sequence and characterize the human medium chain acyl-CoA dehydrogenase gene promoter. The human medium chain acyl-CoA dehydrogenase promoter had being characterized by: footprinting experiments; transfection into transformed cell lines and via the generation of transgenic mouse lines with the incorporation of different length promoter constructs downstream of the chloramphenicol acetyltransferase gene into their genome.[76-79] The promoter elements required for appropriate tissue and developmental regulation of the medium chain acyl-CoA dehydrogenase gene expression, under normal physiologic conditions, had thus been identified. The promoter region inclusive of all these elements lay within 371 base pairs of the transcription start site. The transgenic mice lines harbouring this construct were called MCADCAT.371. A deletion construct with

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removal of the most distal regulatory element was called MCADCAT.317. The regulatory elements present in this 371 base pair promoter region are illustrated in Figures 4 and 5. The pulmonary artery banding procedure and parallel sham control operations were performed in genetically modified mice with the incorporation of the human medium chain acyl-CoA dehydrogenase gene promoter/reporter construct into their genome. The promoter region is described in further detail in Figures 4 and 5.

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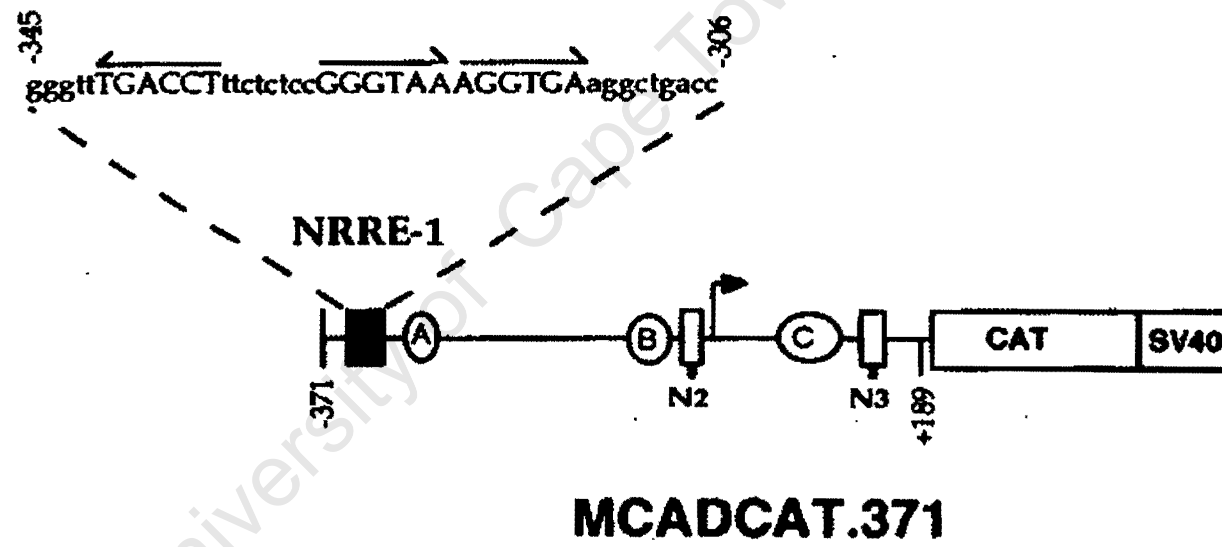

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Figure 5

Figure 5. Schematic representation of the full-length human medium chain acyl-CoA dehydrogenase promoter reporter construct used for subsequent analysis of the transcriptional regulation of medium chain acyl-CoA dehydrogenase in response to pressure-overload. The full-length human medium chain acyl-CoA dehydrogenase promoter-CAT reporter construct - MCADCAT.371 is shown schematically. MCADCAT.371 includes a human medium chain acyl-CoA dehydrogenase gene promoter fragment (horizontal line) containing 371 bp upstream to 189 bp downstream of the transcription start site (arrow) fused to a bacterial CAT gene-simian virus 40 (SV40) intron-polyadenylation signal sequence. The positions of the three known transcriptional regulatory units in the medium chain acyl-CoA dehydrogenase gene promoter are represented. Each unit is composed of a nuclear receptor element (NRRE-1, N2 or N3) and a GC-rich box (A, B, or C). The sequence of potential nuclear receptor binding sites (arrows) within NRRE-1 is also shown. The deletion construct with removal of the most distal regulatory element is called MCADCAT.317 and is schematized in Figure 9.

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The Spontaneously Hypertensive/Heart Failure Rat Model

The SHHF/Mcc^{facp} rat developed by S.A. McCune (The Ohio State University), is a genetically inbred strain that develops hypertension, LV hypertrophy and congestive heart failure.[98, 99] This strain originated from a cross of the Koletsky rat and the spontaneously hypertensive rat (SHR) from the NIH colony. Offspring are lean (75%) or obese (25%). All lean male SHHF animals develop hypertension and left ventricular hypertrophy (LVH) by 3-5 months of age and eventually develop overt congestive HF by 16-20 months of age. Only lean, normoglycemic, male SHHF rats were used for this study at different stages in the natural progression to heart failure. Two month old SHHF males that had not yet developed hypertension were used as control rats (C, n = 6). A second group of 9-12 month old animals with established hypertension and concentric LVH without dilatation served as the compensated LVH group (n = 5). The third group consisted of animals at 16-20 months of age with overt congestive HF (n = 6). Previous characterization of the SHHF male lean rats with Doppler and M-mode echocardiographic studies of lean male SHHF rats[98] have demonstrated that between 13 and 17 months of age, shortening fraction decreases, LV diastolic and end-systolic diameters increase, and aortic velocity time integral x heart rate decrease

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significantly in male lean SHHF rats compared to animals in the LVH group (ages 9-12 months). All older SHHF rats used for this study exhibited the external clinical signs of heart failure, including: dyspnoea, cyanosis, piloerection, lethargy and cold tails; and, internally they had ascites, pulmonary edema, and pleural effusion. The heart failure hearts were enlarged with thickened left and right ventricular walls, dilated lumina, left and right atrial dilatation and frequently contained left atrial thrombi. At the ages indicated, the rats were weighed, anesthetized with pentobarbital, and sacrificed. The whole heart was removed, weighed and ventricles dissected out, snap frozen. To control for the effect of age on gene expression, LV tissue was obtained from a related rat strain [Wistar-Furth (WF)] at 2 and 17 months. The WF rats do not develop hypertension, LVH or HF.

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The Spontaneously Hypertensive Rat Model and the Attenuation of LVH Development in the SHR's Using Carvedilol

To further evaluate the mRNA expression pattern of metabolic genes with the onset of pressure overload induced-cardiac hypertrophy[9] we studied metabolic gene expression patterns in left ventricular tissue extracted from adult (24 week) SHR rats (Harlan, UK) (n=10) compared to age matched WF controls (n=10). Previous characterization of the SHR rats have

demonstrated hypertension in 100% of the rats by the age of 10 weeks and increased LV weight to body weight ratios are characteristic after 13 weeks of age.[100] At 24 weeks the rats were weighed, anesthetized with pentobarbital, and euthanized. The whole heart was removed, weighed and ventricles dissected out, weighed and snap frozen. To control for the effect of age on gene expression, LV tissue was obtained from the related age-matched normotensive Wistar Furth rat strain.

To evaluate the effect of attenuation of hypertrophic growth on metabolic enzyme gene expression in the SHR's, a groups of these rats were fed a diet supplemented by carvedilol [(at 2400 ppm) (n=9)] or placebo supplemented diet (n=7) for four weeks from the age of 20 weeks. This experimental protocol has previously been administered to SHR's, and has demonstrated a reduction in blood pressure and a concomitant attenuation in the advancement of LVH.[101] In addition, a group of WF rats were also fed a diet supplemented by carvedilol over the same 4 week period (n=5). The rats were again sacrificed at 24 weeks of age, and the LV tissue handled as described before for subsequent RNA extraction.

Human Heart Failure

The heart failure group was comprised of 8 heart transplant recipients enrolled in the Heart Transplantation Programme at Barnes Hospital, St

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Louis, Missouri, USA. Inclusion criteria for the heart failure samples included patients who experienced heart failure symptoms with minimal to no exertion (New York Heart Association Functional Class III to IV) and an ejection fraction < 35% as determined by radionuclide ventricular angiography and confirmed by left ventriculography at the time of cardiac catheterization. Subjects were excluded if heart failure was the result of an acute insult (e.g. cardiogenic shock from an acute myocardial infarction), if the subject had an implanted left ventricular assist device, or if the patient remained on bypass for more than 30 minutes prior to explantation of the heart. The control group included 5 postmortem samples from hearts of individuals who had died from non-cardiac causes. The control hearts were rejected as transplant donor organs due to positive cytomegalovirus serology in 2 subjects, age of patient in one, and for unknown reasons in the final two controls (gross cardiac pathology was excluded by palpation of the coronary arteries, viewing cardiac wall thickness and a normal heart weight to body weight ratio at the time of harvest). Left ventricular apical tissue obtained immediately after the heart was removed from the subject's chest was snap frozen in liquid N_2 and stored at –80°C until total RNA and protein were isolated.

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RNA Analysis

RNA Extraction and Quantitation

Total RNA was extracted from 25-100 mg of frozen heart tissue. All samples were kept on ice during extraction to prevent RNA degradation. The tissue was completely homogenized in 2 ml of RNAzol B (Biogenesis, Poole, UK) to prevent enzymatic degradation of the RNA. Fatty acids and proteins were removed by extraction with chloroform as follows: The homogenate was vortexed for 15 seconds with 200 μl of chloroform and left to incubate, on ice, for five minutes. The aqueous and organic layers were separated by centrifugation (11,500 g for 15 minutes at 4 °C) and the top aqueous layer removed to a fresh sterile tube. The chloroform extraction was repeated, using 1 ml of chloroform, and the top aqueous layer again removed to a fresh sterile RNAse free tube. One ml isopropanol was added, the samples vortexed and then stored overnight at –20 °C to precipitate the RNA. The following day, samples were centrifuged at 11,500 g for 15 minutes at 4 °C, to pellet the RNA, and the supernatant was decanted. The pellet was re-suspended in 1 ml 75% v/v ethanol, to wash away salts precipitated by the isopropanolol and then reformed by centrifugation (11,500 g for 10 minutes at 4°C). The ethanol was decanted, the last few drops carefully removed using a pipette, and

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the pellet allowed to air-dry for 30 seconds. The RNA was re-suspended in 20 μl 0.1% v/v DEPC-treated water and stored at –80°C.

Two μl of the prepared RNA was diluted 1:500 in sterile distilled water and the absorbance at 260 nm (A_{260nm}) measured using a spectrophotometer. The total RNA concentration ([RNA]) was determined using the following equation:

$$\text{Total [RNA] } (\mu g/ml) = A_{260nm} \times \text{dilution factor } (500) \times 40\ \mu g/ml$$

The values obtained were divided by 1000 to arrive at a concentration in units of μg/μl. The absorbance of the diluted RNA was subsequently measured at 280nm (A_{280nm}). The ratio of A_{260nm}:A_{280nm} was calculated to determine the protein contamination within the RNA samples. Pure mRNA will have a A_{260nm}:A_{280nm} ratio of 2. Using total RNA, the RNA was considered of sufficient quality for Northern blot analysis if the ratio were > 1.7.

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RNA Gel Electrophoresis and Capillary Transfer to Nylon Membranes

A denaturing 1.2% agarose gel for RNA separation was prepared as follows. Agarose (2.4g) was dissolved, by heating (100 °C), in 146 ml of sterile distilled water. A pre-warmed solution (60 °C) of 37% v/v

formaldehyde (16.2 mls) and 20 mls of 10x MOPS, were added to the warmed agarose and poured into a gel casting apparatus (BIORAD, USA).

The total RNA samples were prepared for gel electrophoresis as follows: A 20 μg sample of total RNA was diluted in 0.1% DEPC water to a volume of 6 μl. The RNA samples were suspended in 19.3 μl of buffer containing; 12.5 μl of de-ionized formamide; 4 μl of 37% v/v formaldehyde; 2.5 μl of 10x MOPS solution and 0.3 μl of 0.5 mg/ml ethidium bromide. Samples were incubated at 60°C for 15 minutes and then placed on ice for two minutes. 2.5 μl of loading dye was added to each sample, which were then mixed and quickly spun in a micro-centrifuge to bring the sample to the bottom of the tube. A previously poured and set gel was immersed in 1x MOPS and the samples carefully loaded into the gel wells. Total RNA was separated electrophoretically at 80 V for four hours. The equivalence of RNA loading was visually confirmed by ultra-violet light, as the intensity of the orange fluorescence produced by the binding of ethidium bromide to RNA is proportional to the RNA concentration. In addition, the integrity of the RNA was confirmed by visualizing the ratio of the 28s to 18s bands (>2) and by the lack of additional smaller degradation banding / smearing.

The electrophoretically separated RNA was transferred onto a nylon membrane (Hybond-N; Amersham Life Sciences, USA and UK), via capillary transfer.

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Northern Blot Analysis

The cDNA fragments described below were used for Northern blot analysis. The cDNA fragments were radiolabelled with α-P^{32}-dCTP, using the Boerhinger Mannheim, random primer labelling kit and the Klenow enzyme. Two μl of the final labeled probe was used for Cherenkov counting on a Beckman Scintillation counter. The final volume of probe used for labelling was calculated to include a final count of 3 -5 x 10^6 cpm in the hybridization solution. Stratagene quickhyb solution was used for the prehybridization and hybridization steps. To reduce non-specific background hybridization of the radiolabelled cDNA probe, adjuvant pre-boiled sonicated salmon sperm cDNA was added to the hybridization solution.

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cDNA Probes used for Northern Blot Analysis:

LCAS The rat gene encoding *long-chain acyl-CoA synthetase*. This cytosolic enzyme catalyzes the formation of fatty acyl-coenzyme A thioesters from free fatty acids in the cytosol.

M-CPT I — the muscle isoform of *carnitine palmitoyltransferase I*, the integral outer mitochondrial membrane protein that catalyzes the initial step in mitochondrial import of long-chain acyl-CoA.

MCAD — The rat gene encoding *medium chain acyl Co-A dehydrogenase*, a nuclear encoded enzyme that catalyzes the first step of medium chain fatty acid catabolism in the mitochondrial β-oxidation cycle.

LCAD — The rat gene encoding *long chain acyl CoA dehydrogenase*, a nuclear encoded enzyme that catalyzes the first step of long chain fatty acid catabolism in the mitochondrial β-oxidation cycle.

LCHAD — the C-terminal portion of the human α subunit of mitochondrial trifunctional protein which catalyzes the third step of long chain fatty acid catabolism in the mitochondrial β-oxidation cycle.

GLUT4 — The rat gene encoding the cardiac adult-enriched glucose transporter.

PFK-M — The rat gene encoding the muscle isozyme of *phosphofructokinase*. This enzyme phosphorylates D-fructose 6-phosphate to D-fructose 1,6-diphosphate, committing the glucose byproduct to the glycolytic pathway

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GAPDH	The human gene encoding the ubiquitous glycolytic enzyme gene, *glyceraldehyde-3-phosphate dehydrogenase*.[102] This enzyme catabolizes the first step in glycolysis which generates ATP production. Interestingly, due to the relative abundance of this enzyme GAPDH is often considered as a "housekeeping gene", and used by investigators to evaluate loading on RNA electrophoretic gels.
ATP_e	A partial cDNA coding and 3' untranslated region sequence (164 bp) of a nuclear-encoded mitochondrial protein, mouse ATP synthase subunit e was obtained by reverse-transcription polymerase chain reaction amplification.[103]
ANF	The rat gene encoding *atrial natriuretic factor* (ANF). This gene is predominantly expressed in the fetal heart and the adult atria. Re-expression in the ventricles are known to occur, following pressure -overload.
cTNI	The rat gene encoding the contractile protein - cardiac troponin I. This protein is considered not to be regulated at the gene expression level in the adult cardiac ventricle.
β-actin	The rat gene encoding a myocyte cytoskeletal protein, used to control for standardising RNA loading
18s	Ribosomal cDNA probe to control for minor differences in RNA loading or RNA integrity.

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Total RNA isolation and Northern blot analyses were performed as described above[104] using the cDNA probes discussed above. Mouse ATP synthase subunit e[103] was obtained by reverse-transcription polymerase chain reaction amplification. The signals were quantified by laser densitometric analysis within the linear range of film sensitivity. The densitometric values shown were normalized to the signal obtained with an 18s ribosomal cDNA probe to control for minor differences in RNA loading or RNA integrity.

Total Protein Extraction

Total cellular protein was prepared from the human and rat left ventricle for immunoblot analysis. In brief, 25 to 50 mg of fresh tissue was sonicated in lysis buffer solution (10 mM Tris, 5 mM EGTA, 0.1 mM DTT at pH 7.2). The following protease inhibitors including 2mM PMSF and 0.125 μg/ml Leupeptin and SDS to a final concentration of 0.5% were added fresh to the lysis buffer prior to use. Once sonicated and in solution, the samples were spun at 5 000 rpm for 5 minutes. The supernatant was placed in a fresh eppendorf tube and quantitated using the Lowry assay for subsequent use for Western Blot analysis.

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A modification of the protein immunoblot (Western) analysis described previously[104] was performed using the Enhanced Chemiluminescence detection system (Amersham). Primary antibodies used include: a polyclonal rabbit antibody to porcine medium chain acyl-CoA dehydrogenase[75] and a polyclonal antibody to the C-terminal actin fragment [universal actin antibody (Sigma Immunochemicals, St. Louis, MO.)]. A horseradish peroxidase-conjugated secondary antibody to rabbit IgG was used on all blots.

Nuclear Protein Extraction

The nuclear protein extraction technique was specifically adapted for the preparation of DNA-binding proteins from animal solid tissues.[105] In brief, between 100 and 500 mg of heart tissue was ground to powder using a mortar and pestle under liquid N_2. All subsequent steps were performed in a walk in +4°C laboratory. The thawed powder was homogenized using a glass dounce homogenizer in 5 mls of solution A (0.6% Nonidet P-40, 150 mM NaCl, 10 mM HEPES pH 7.9, 1 mM EDTA, 0.5 mM PMSF). This solution was then incubated on ice for 5 minutes and then centrifuged for 5 minutes at 5000rpm. The pelleted nuclei were then resuspended in 100-500 μl 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 [DTT], 0.5 mM PMSF,

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2 mM benzamidine, 5 μg/mL of each of the three protease inhibitors: pepstatin, leupeptin and aprotinin) and then incubated on ice for 20 minutes for high salt extraction. The lysed nuclei were transferred to a microcentrifuge tube and cellular debris pelleted by a 15 second centrifugation. The supernatant containing the DNA-binding proteins was then aliquoted into small fractions, frozen in liquid N_2 and stored for subsequent use at -80°C.

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Protein Quantitation

To ensure accurate reproducible measurements of protein content in the various heart tissues, a modified Lowry method was adopted as described by Peterson et al.[106] Using distilled deionised water, the protein samples, blanks and bovine serum albumin standards (between 5 and 100 μg's) were brought up to a volume of 1.0 ml. One ml of reagent A (see appendix) was added to each sample, the sample was vortexed and allowed to stand for 10 minutes to allow complete dissolution of the precipitate. An additional 0.5 ml of the reagent B (see appendix) was added. The reaction was vortexed immediately as the $t_{1/2}$ of the phosphomolybdate regent is only 8 seconds. The tubes were then allowed to stand at room temperature for 30 minutes. Absorbance was then

measured spectrophotometrically using visible light at a λ of 750nm. A standard curve was established using the known bovine serum albumin samples. Thereafter, using linear regression analysis and the known slope of the standard curve (m), the log values of the proteins samples could be calculated from the log A_{750} intercept of the standard curve (b) using the following formula:

$$\text{Log}\ (\mu g\ \text{protein}) = m \times \log(A_{750}) + b$$

or

$$\mu g\ \text{protein} = 10^{(m \times \log(A_{750}) + b)}$$

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Western Blot Analysis

Western blot analysis was performed following standard polyacrylamide gel electophoresis (10 -15%), electrophoretic transfer to a nitrocellulose membrane (Overnight at 25 mA) and immunoblotting using the Enhanced Chemiluminescence detection system (Amersham). Reducing buffers, running buffers and transfer buffers are described in the appendix.

The primary antibodies used include:

1. Polyclonal antibody to porcine medium chain acyl-CoA dehydrogenase.[75]

2. Polyclonal antibody to the C-terminal actin fragment [universal actin antibody (Sigma Immunochemicals, St. Louis, MO, USA)].

3. Polyclonal antibody to long-chain acyl-CoA dehydrogenase (LCAD; a gift from Dr. Arnold Strauss)

4. Polyclonal antibody to the Sp1 transcription factor (Santa Cruz Biotechnology, Santa Cruz, CA, USA)

5. Polyclonal antibody to the Sp3 transcription factor (Santa Cruz Biotechnology, Santa Cruz, CA)

6. Anti-COUP-TF [a gift from A.J. Butler], The anti-COUP-TF antibody recognizes both COUP-TFI and COUP-TFII isoforms.[107]

7. Anti-peroxisome proliferator activated receptor α [PPARα; a gift from Dr. Michael Arand].[108]

The species specific horseradish peroxidase-conjugated IgG antibodies were used as secondary antibodies at a dilution of 1:3000 in all Western immunoblot experiments.

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Transgenic Mouse Studies

Production and characterization of the MCADCAT.371 transgenic mice has been described.[80] These mice have previously been found to have appropriate developmental stage specific expression of the medium chain acyl-CoA dehydrogenase gene.[80] Furthermore, mutant medium chain acyl-CoA dehydrogenase promoter transgenic mice lines were also studied. These mice have a deletion of the NRRE-1 medium chain acyl-CoA dehydrogenase promoter region and the promoter construct is described as MCADCAT.317. Although the mutant promoter is active, the reporter gene does not have the appropriate developmental stage-specific expression in the heart.[80] Pulmonary artery banding and sham procedures were performed on mice from two independent full-length transgenic lines and in two MCADCAT.317 mice lines. CAT enzyme activities were determined on tissue extracts by use of an assay described below.

Choloramphenicol Acetyl-Transferase Assays

Protein extracts for CAT assays were prepared by sonication of frozen tissue in 0.25M Tris buffer. The suspension was cleared by centrifugation and total protein content was determined on the supernatant by the Lowry method. CAT assays were performed on 100 mg of total protein, as

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described previously, with n-butyryl CoA and 14C chloramphenicol substrates. Butyrylated chloramphenicol was separated from free chloramphenicol by xylene extraction. Samples were counted in duplicate on a Beckman LS 6000IC scintillation counter.

Electrophoretic Mobility Shift Assays

EMSAs were performed with crude nuclear extracts prepared from pooled embryonic (day 16.5 and 19.5) mouse hearts and adult control and hypertrophied RVs, as described.[109] The following sense strand nucleotide sequences represent the probes used in the EMSAs: 371/255 (the combined NRRE-1/site A sequence) 5'-ttgaatccgccaagcagacacgatctgggtttgacctttctctccgggtaaaggtgaaggctgaccacg ggggccgctctccctccaggccccagccacgccctctaacccaggttc-3'; NRRE-1, 5'-gatccgggtttgacctttctctccggg-3' and site A, 5'-gatccggccccagcc acgccctctaacccag-3'. Antibody "supershift" experiments were performed with the antibodies described above except that a different anti-COUP-TF antibody was used (a gift from Dr. Ming-Jer Tsai, Baylor University).

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Primary Cardiocyte Extraction and Cell Culture Gene Reporter Transfection Studies.

Cardiomyocytes were prepared from 1-day-old rats as described.[80] Transient transfections were performed with DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate) (Boehringer Mannheim) in 12-well collagen-coated dishes. For each transfection, four μg of reporter DNA and two μg of a plasmid containing a β-galactosidase gene downstream of the Rous sarcoma virus promoter (RSV-βgal) were added to fifteen μl of DOTAP-HEPES solution and incubated for 15 min at room temperature. In some dishes one μg of pCDM.COUP, a mammalian expression vector containing a human COUP-TF I cDNA downstream of a CMV promoter[77] was added. One μg of pCDM(-), the expression plasmid backbone lacking the COUP-TF I cDNA, was added to dishes not containing pCDM.COUP. The mixture was subsequently added to the primary cardiocytes prior to initial plating. The cells were washed and refed the following morning. The cells were harvested 48 h after plating. Luciferase activity was measured by the standard luciferin-ATP assay, and β-galactosidase activity was measured by the Galacto-Light chemiluminescence assay (Tropix) in an Analytical Luminescence Monolight 2010 luminometer. The reporter plasmid used for the

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experiments (MCADLuc.371) contains the identical medium chain acyl-CoA dehydrogenase promoter fragment present in the MCADCAT.371 construct fused to a luciferase reporter in the pGL2-basic plasmid (Promega).

Enzyme Activity Studies

Medium chain acyl-CoA dehydrogenase activity was determined by following the decrease in ferricenium ion absorbance at 300 nM, as described.[104] Activity is shown as μmol octanoyl-CoA oxidized per minute per gram wet weight.

Southern Blot Analysis

Genomic DNA was isolated from human left ventricle using a standard protocol.[110] The mitochondrial DNA probe was obtained by PCR amplification of a region of the human mitochondrial genome (nucleotides 3108 to 3717) using the following primers: sense-5'-TTCAAATTCCTCCCTGTACG-3' and antisense-5'-GGCTACTGCTCGCA GTG-3'. A full-length human GAPDH cDNA probe was also employed in these studies to control for loading and for standardization to a nuclear gene signal. Fifteen μg of total genomic DNA, digested with BamHI restriction enzyme at 37°C overnight was loaded to each lane. A standard

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Southern blotting protocol was used. The final washing solution concentration was 0.1 x SSC/1% SDS for the mitochondrial DNA probe and 1 x SSC/1% SDS for the GAPDH probe at a temperature of 65°C.

Ethical Approval

Animal experiments were conducted in strict accordance with the US National Institutes of Health (NIH) and UK Home Office guidelines regarding humane treatment for the care and use of laboratory animals. All animal experiments were reviewed and approved by the Animal Care Committees of Washington University, The University of California at San Diego, The Ohio State University and University College London, UK.

Statistical Analysis

Differences between values for mRNA, protein levels, and enzyme activity were determined using unpaired Student t-test analyses (mouse and human studies) and one-way analysis of variance (ANOVA) for the rat studies. A statistically significant difference was defined as a p value < 0.05. All values shown represent the mean ± standard error of the mean (SE).

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Results

Coordinate cardiac developmental mRNA expression patterns of rate-controlling fatty acid and glucose utilization enzyme encoding genes

To evaluate whether the expression of genes encoding representative fatty acid and glucose catabolic enzymes, parallel the known increase in fuel substrate utilization during cardiac development, mRNA levels were analyzed by Northern blot analysis of total RNA isolated from foetal, neonatal and adult Wistar Furth (WF) rat ventricles. The mRNA expression patterns of the rate-controlling catabolic enzyme encoding genes including the fatty acid utilization enzyme genes LCAS and medium chain acyl-CoA dehydrogenase and the gene encoding the glucose transporter GLUT4 and the glycolytic enzyme encoding gene PFK-M were significantly upregulated at adulthood by 75 ± 3%, 95 ± 4%, 97 ± 5%, and 70 ± 2% respectively [All p values < 0.01], compared to foetal expression patterns (Figure 6). The rate-controlling adult enriched metabolic enzyme encoding genes therefore do seem to parallel the enhanced energy requirements of the developing heart. Interestingly, the mRNA expression pattern of a "housekeeping" glycolytic enzyme encoding

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gene, GAPDH, was not significantly regulated during cardiac development (Figure 6). The mRNA expression of a gene known to be more highly expressed in the fetal ventricle, ANF, demonstrates expected regulation with high expression in the foetal heart, with reducing levels into the adult period ($p = 0.005$ comparing foetal to adult expression).

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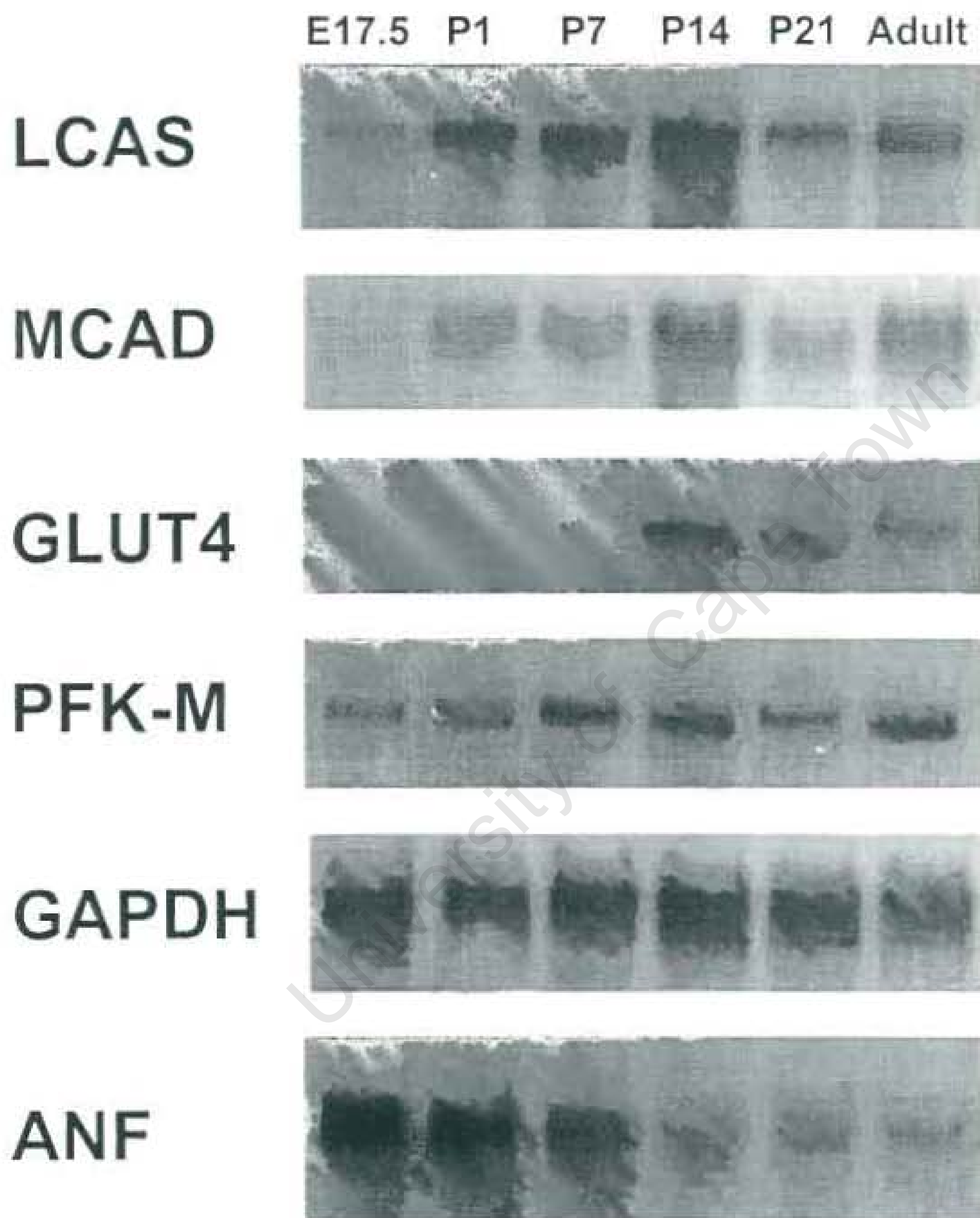

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Figure 6

Figure 6. mRNA levels of metabolic enzyme encoding genes in the developing rat heart.

Representative Northern blot analysis performed with total RNA isolated from foetal (e17.5), neonate (p1, p7, p14, p21) and adult WF rats as described in the text. Each lane contains 20 μg total RNA. Probe abbreviations are described in the Methods section and under abbreviations.

Effects of pressure-overload induced cardiac hypertrophy on the expression of fatty acid oxidation and glycolytic enzymes encoding genes.

To characterize the expression of genes encoding enzymes involved in myocardial fatty acid and glucose utilization pathways with the development of cardiac hypertrophy, Northern blot studies were performed with RNA isolated either from the right ventricles (RV) of mice subjected to pulmonary artery banding (PAB) or ventricles of sham-operated controls. After 7 days of pressure overload, a significant increase ($p < 0.01$) was observed in mean absolute RV weight (from 29.8 ± 0.7 mg to 62.0 ± 3 mg), RV/total body weight ratio (from 96 ± 2 to $202 \pm 12 \times 10^{-5}$), and RV/LV weight ratio (from 0.41 ± 0.01 to 0.94 ± 0.07) in the PAB group

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compared with values in controls. Levels of mRNA encoding the following enzymes involved in the myocyte fatty acid utilization pathway were evaluated: *long-chain acyl-CoA synthetase (LCAS)*, the cytosolic enzyme that catalyzes the formation of fatty acyl-coenzyme A thioesters from free fatty acids entering the cell; the muscle isoform of *carnitine palmitoyltransferase I (M-CPT I)*, the integral outer mitochondrial membrane protein that catalyzes the initial step in mitochondrial import of long-chain acyl-CoA; and enzymes that catalyze the first *(LCAD and MCAD)* and third *(long-chain 3-OH acyl-CoA dehydrogenase or LCHAD)* steps of the mitochondrial β-oxidation cycle. These enzymes are shown in figure 2. For comparison, mRNA encoding the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was also examined. The expression of all FAO enzyme mRNAs' evaluated were significantly lower in the hypertrophied RV than in ventricles from control mice (Figure 7A). Mean LCAS and M-CPT I mRNA levels were reduced to 21 ± 2% ($p < 0.001$) and 60 ± 2% ($p = 0.004$) of control levels, respectively (Figure 7B). Mitochondrial β-oxidation cycle enzyme mRNA levels were markedly lower in the hypertrophied ventricles (LCAD mRNA levels were 40 ± 5% of control levels, $p < 0.001$; medium chain acyl-CoA dehydrogenase mRNA levels 15 ± 2% of control, $p < 0.001$; and LCHAD mRNA levels 23 ± 3% of control, $p < 0.001$). In contrast, mean GAPDH mRNA levels were 75 ± 10% higher in the pressure-overloaded RVs than

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in controls ($p = 0.01$; Figures 7A and B), consistent with the known increase in glucose utilization in the hypertrophied heart. As expected, expression of atrial natriuretic factor (ANF) mRNA, a known marker for ventricular hypertrophy, was markedly induced in samples from hypertrophied RV. β-actin mRNA levels were modestly increased in the hypertrophied RVs compared with those in controls. These results indicate that the expression of genes encoding cardiac fatty acid utilization enzymes are coordinately repressed at the pretranslational level, in parallel with the known reduction in myocardial fatty acid utilization during pressure overload-induced cardiac hypertrophy. Furthermore, these data suggest that this pretranslational regulation may play a role in directing the known metabolic remodelling which occurs with the development of cardiac hypertrophy.

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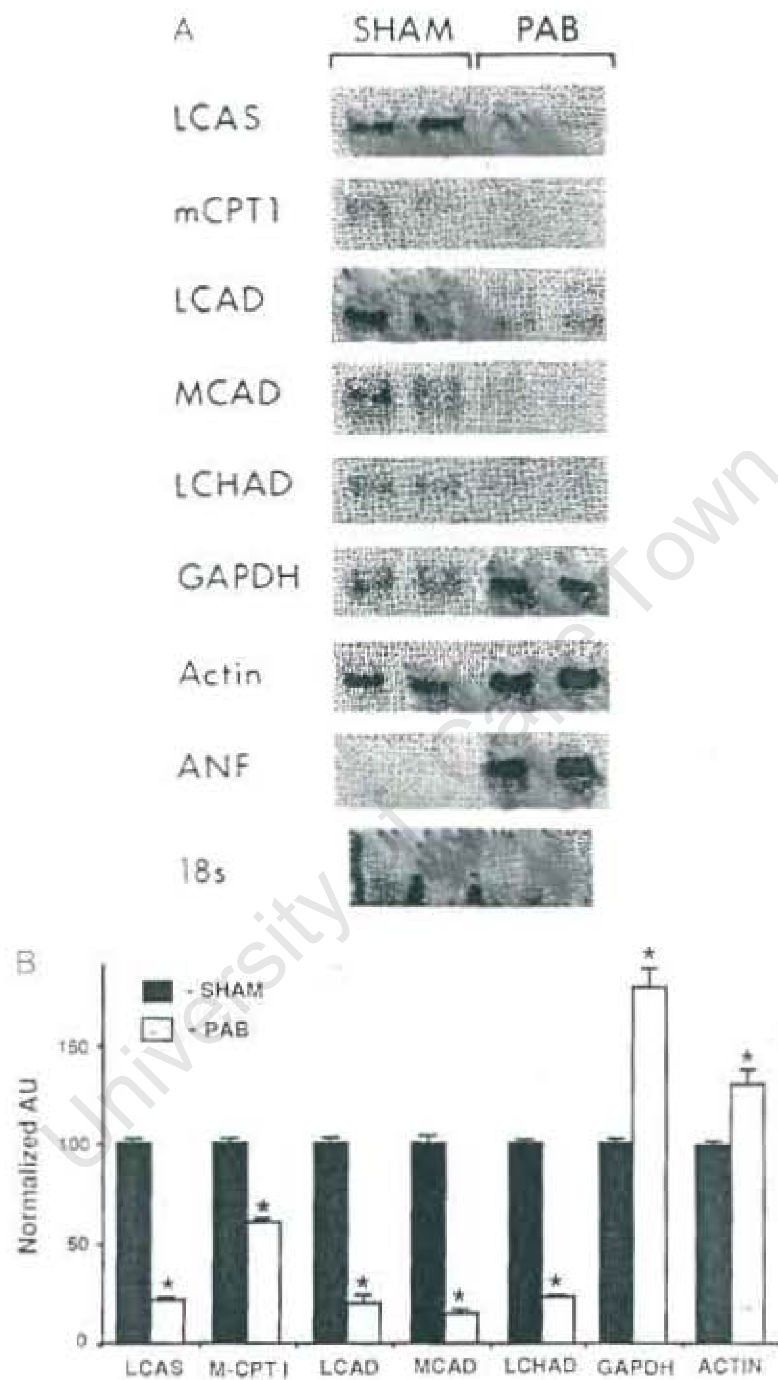

Figure 7

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Figure 7. Expression of genes involved in myocardial fatty acid utilization and glycolysis parallels known changes in substrate preferences in the hypertrophied heart.

A. Representative Northern blot analysis obtained with total RNA isolated from the RV of two mice subjected to pulmonary artery banding (PAB) or from sham-operated controls (SHAM). Each lane contains 15 μg of total RNA. cDNA probe abbreviations are defined in the text. The 18s rRNA (stained with ethidium bromide) within each lane is shown at the bottom. B. Densitometric analysis of Northern blots obtained with RNA isolated from the RV of six PAB or six sham-operated control mice. The bars represent mean (± SEM) steady-state mRNA levels (in arbitrary units or AU) normalized to sham-operated control values (mean control value set at 100). The asterisks denote $p < 0.01$ compared with the corresponding control value. Abbreviations are described in the text and in the abbreviations section on page 19.

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Repression of medium chain acyl-CoA dehydrogenase gene expression during cardiac hypertrophy occurs at the transcriptional level through cis-acting sequences within the proximal promoter region.

To determine whether the repression of medium chain acyl-CoA dehydrogenase gene expression during pressure overload-induced cardiac hypertrophy occurs at the level of transcription, PAB studies were repeated on mice transgenic for a 560 bp human medium chain acyl-CoA dehydrogenase promoter fragment fused to a chloramphenicol acetyltransferase reporter (MCADCAT.371). Previous studies have shown that the expression of MCADCAT.371 in the developing murine heart parallels that of the endogenous medium chain acyl-CoA dehydrogenase gene.[80] Specifically, expression of the MCADCAT.371 transgene is induced markedly after birth in parallel with increased postnatal myocardial fatty acid utilization rates. Medium chain acyl-CoA dehydrogenase and CAT mRNA levels were coordinately downregulated in the pressure-overloaded RV compared with levels in the control group and compared with those in the contralateral LV within the same animal (representative Northern blot shown in Figure 8A). In the right ventricular hypertrophy group, mean right ventricular medium chain acyl-CoA

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dehydrogenase and CAT mRNA levels were 14 ± 2% ($p < 0.001$) and 22 ± 5% ($p < 0.001$) of control levels, respectively (Figure 8B). Mean right ventricular CAT enzyme activity in the pulmonary artery banded group was 22 ± 10% ($p < 0.001$) of corresponding control values (Figure 8B). As expected, RV GAPDH mRNA levels were upregulated by 73 ± 8% ($p = 0.01$) in the PAB group. In the LV, mean MCAD, CAT, and GAPDH mRNA levels were not significantly different in the PAB and control groups (Figure 8A). Similar but less pronounced results were observed in the hypertrophied LV after aortic banding (parallel experiments, data not shown). These results indicate that repression of medium chain acyl-CoA dehydrogenase expression during cardiac hypertrophy occurs solely or in large part at the level of gene transcription, that the cis-acting elements involved in this transcriptional regulatory mechanism are located within the proximal medium chain acyl-CoA dehydrogenase promoter, and that the upstream signalling pathway involved in this energy metabolic response is triggered by the direct effect of the pressure overload stimulus.

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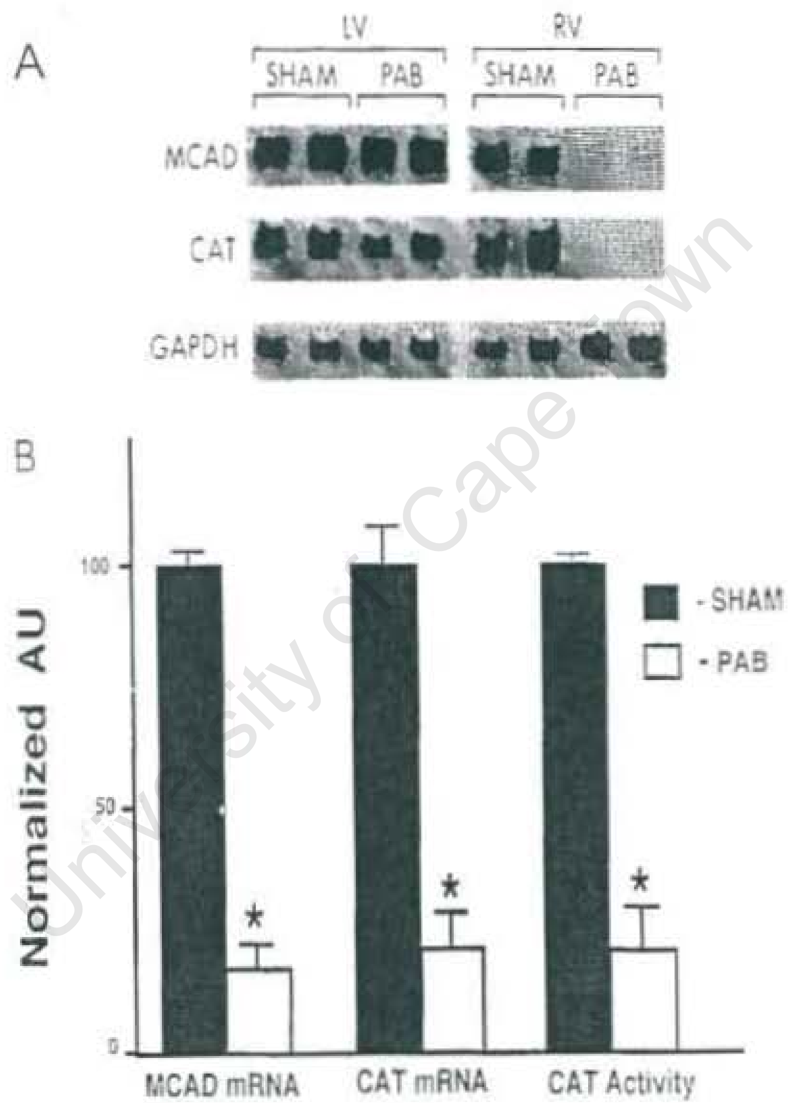

Figure 8

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Figure 8. Expression of the MCADCAT.371 transgene is repressed in the hypertrophied right ventricle.

A. The Northern blot autoradiogram is representative of the results of PAB experiments performed on mice transgenic for the MCAD promoter-CAT reporter construct MCADCAT.371 (shown schematically in Figure 5). The Northern blot was obtained with total RNA isolated from LV and RV tissue of PAB or sham-operated control (SHAM) MCADCAT.371 mice. The blot was sequentially hybridized with cDNA probes encoding mouse MCAD, bacterial CAT, and human GAPDH. B. Expression of the MCADCAT.371 transgene (CAT mRNA and enzymatic activity) and endogenous mouse MCAD gene (MCAD mRNA) in RV of PAB or sham-operated controls. mRNA values represent mean (±SEM) levels as determined by densitometric analysis of RNA blots obtained with total RNA from eight PAB and ten control MCADCAT.371 mice from two independent transgenic lines [lines 10-1 (transgene copy number = 55) and 10-4 (transgene copy number = 14) described[80]. All values are shown relative to mean control values set arbitrarily at 100. Mean RV CAT enzymatic activities (normalized to mean control levels = 100) are also shown. The asterisks denote a statistically significant difference ($p < 0.05$) compared with controls.

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Characterization of the role of the NRRE-1/Site A regulatory region in hypertrophy induced regulation of the medium chain acyl-CoA dehydrogenase gene using promoter deletional analysis.

To explore the role of the NRRE-1/site A regulatory unit in the transcriptional repression of medium chain acyl-CoA dehydrogenase in response to ventricular pressure overload, the pulmonary artery banding experiments were repeated with the mice transgenic for a mutant medium chain acyl-CoA dehydrogenase promoter in which NRRE-1 was deleted (MCADCAT.317; see Figure 9). In striking contrast to the results with the MCADCAT.371 transgenic lines, the MCADCAT.317 transgene was not repressed in response to the pressure overload stimulus (Figure 9). However, because cardiac expression of MCADCAT.317 was markedly lower compared to MCADCAT.371, these data do not definatively establish a role for NRRE-1/site A in the hypertrophic response. Nonetheless, these results do strongly suggest that the NRRE-1/Site A element is required for repression of medium chain acyl-CoA dehydrogenase gene transcription in the pressure overloaded ventricle.

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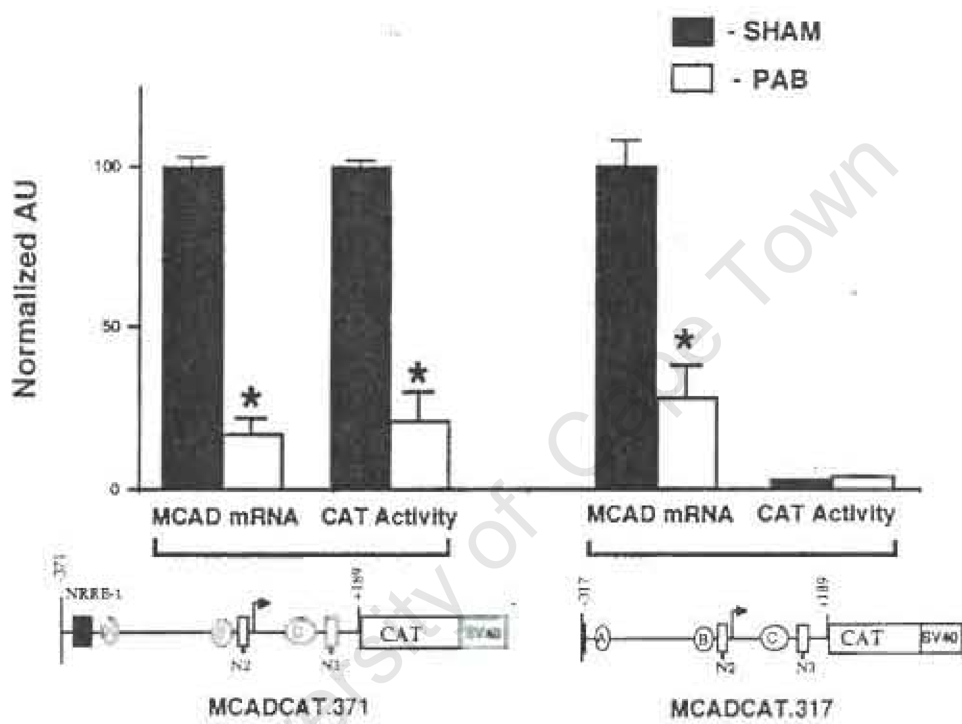

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Figure 9

Figure 9. Localisation of the regulatory unit (NRRE-1/Site A) required for transcriptional repression of the medium chain acyl-CoA dehydrogenase gene in response to ventricular pressure-overload.

Expression of the MCADCAT.371 and MCADCAT.317 transgenes (CAT enzymatic activity) and endogenous mouse medium chain acyl-CoA dehydrogenase gene (medium chain acyl-CoA dehydrogenase mRNA) in right ventricular hypertrophy of the pulmonary artery banded mice or sham-operated controls. MCADCAT.371 and MCADCAT.317 constructs in the transgenic mice are shown schematically under the respective data. MCADCAT.371 includes a human medium chain acyl-CoA dehydrogenase gene promoter fragment (horizontal line) containing 371 base pairs upstream to 189 base pairs downstream of the transcription start site (arrow) fused to a bacterial CAT gene-simian virus 40 (SV40) intron-polyadenylation signal sequence. The positions of the three known transcriptional regulatory units in the medium chain acyl-CoA dehydrogenase gene promoter are represented. Each unit is composed of a nuclear receptor element (NRRE-1, N2 or N3) and a GC-rich box (A, B, or C). The MCADCAT.317 construct is similar except for a deletion disruption of the NRRE-1/site A unit. mRNA values represent mean (± SEM) levels as determined by densitometric analysis of RNA blots obtained with total RNA from eight PAB and ten control MCADCAT.371

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mice and from seven PAB and seven control MCADCAT.317 mice. The CAT activities were measured as described in the methods section, and were quantitated as mean (± SEM) counts per min. All values are shown as arbitrary units (AU) relative to mean control values set at 100. The asterisk denote a statistically significant difference ($p<0.05$) compared to controls.

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The foetal pattern of protein/DNA interactions is reactivated at a pressure overload-responsive medium chain acyl-CoA dehydrogenase promoter unit (NRRE-1/site A).

Previous studies have shown that the proximal medium chain acyl-CoA dehydrogenase promoter region consists of three transcriptional regulatory units, each composed of a nuclear hormone receptor response element (NRRE 1, 2, or 3) juxtaposed to a GC-rich sequence (sites A,B,or C) (elements shown schematically in Figure 5;[79]). As an initial step in the localization of the medium chain acyl-CoA dehydrogenase gene promoter pressure overload-responsive regions, electrophoretic mobility shift assays (EMSA) were performed with a series of probes derived from overlapping DNA fragments spanning the entire proximal medium chain acyl-CoA dehydrogenase promoter region from bp -371 to bp +189 (relative to the transcription start site = +1). EMSA performed with a probe spanning the NRRE-1/site A unit (probe 371/255 which includes human medium chain acyl-CoA dehydrogenase gene promoter sequence from bp -371 to bp -255, see Methods) demonstrated a significantly increased formation of DNA/protein complexes with crude nuclear protein extract prepared from hypertrophied mouse RV samples compared to sham-operated control samples (data not shown). We have shown previously that the NRRE-1/site A unit is required for appropriate cardiac

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developmental stage-specific expression of the MCADCAT.371 transgene.[80] To further characterize the protein/DNA interactions at the NRRE-1/site A unit, separate probes containing either the NRRE-1 or site A sequence were used in electromobility shift assays. As Dr. Kelly's group had observed previously,[80] the NRRE-1 probe formed two specific complexes (I and II) with cardiac nuclear proteins. Formation of complex I, but not complex II, was increased with the nuclear extracts prepared from the hypertrophied ventricle (Figure 10A, lanes 2,3). With the site A probe, formation of the three specific complexes (I, II, III) with the extracts derived from hypertrophied RV was markedly increased (Figure 10A, lanes 5-7). Parallel competition studies with unlabeled specific and unrelated double-standed oligonucleotides confirmed that complexes I and II with the NRRE-1 probe and complexes I to III with the site A probe represented specific DNA/protein interactions.

The electromobility shift assays were also performed with cardiac nuclear proteins prepared from normal embryonic day 16.5 (E16.5) mouse heart to determine whether the hypertrophy-induced protein/DNA interactions involved reactivation of the foetal DNA binding patterns. The DNA/protein interaction patterns observed with the E16.5 extracts were nearly identical to those observed with the samples from hypertrophied ventricle with either probe; bands representing complex I (with the NRRE-1 probe) and those representing complexes I, II, and III (with site A)

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were significantly more intense than the corresponding bands seen with the control samples from adult mice (Figure 10A, lanes 3,4,7,8). These data suggest that repression of medium chain acyl-CoA dehydrogenase gene expression during cardiac hypertrophic growth involves reactivation of foetal transcriptional control mechanisms at NRRE-1 and site A.

Members of the nuclear hormone receptor and Sp families of transcription factors bind the medium chain acyl-CoA dehydrogenase gene hypertrophy-responsive unit.

To identify the proteins involved in the pressure overload-induced interactions at the NRRE-1/site A unit, antibody "supershift" studies were performed. Previous studies performed *in vitro* have shown that NRRE-1 is a pleiotropic nuclear receptor response element that confers bidirectional transcriptional regulation via activator (e.g., retinoid X receptor α or RXRα) and repressor (COUP-TF) transcription factors.[77, 78] The actual nuclear receptors involved in the control of medium chain acyl-CoA dehydrogenase gene expression *in vivo* are unknown, although the results of recent antibody supershift studies with normal adult mouse heart nuclear protein extract have identified COUP-TF or a closely related protein within complex I of the NRRE-1/protein interaction.[80] The putative activator(s) in complex II remain unidentified. EMSAs performed with

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anti-COUP-TF antibody and the NRRE-1 probe revealed that complex I, but not complex II, was supershifted by the antibody (Figure 10B). Thus, the known transcriptional repressor COUP-TF or an antigenically related protein is indeed present within the induced complex I.

Previous *in vitro* studies have shown that Sp1 is capable of binding medium chain acyl-CoA dehydrogenase promoter site A.[79] Accordingly, antibodies to multiple members of the Sp transcription factor family were used in supershift studies in an attempt to identify the proteins involved in the pressure overload-induced interactions at site A. Complex I was partially supershifted with an anti-Sp1 antibody but not by pre-immune serum (Figure 10B, lane 6). When an anti-Sp3 antibody was used, formation of complex I was diminished and complex II was abolished (Figure 10B, lane 7). Addition of both anti-Sp1 and anti-Sp3 to the sample completely supershifted complex I and abolished complex II (Figure 10B, lane 8). These results indicate that complex I contains both Sp1 and Sp3, whereas complex II contains Sp3 only. The Sp antibodies did not recognize complex III. Supershift studies with anti-Sp4 did not recognize any of the complexes (data not shown).

The identification of COUP-TF as one of the candidate factors responsible for the downregulation of medium chain acyl-CoA dehydrogenase gene transcription in response to pressure overload is consistent with its known role as a repressor. Dr. Kelly's group had shown

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previously that COUP-TF represses the transcriptional activity of homologous or heterologous promoters via NRRE-1 in cotransfection studies performed in several mammalian cell lines, including hepatoma G2, CV-1, and NIH 3T3 cells.[77, 79] Medium chain acyl-CoA dehydrogenase promoter elements NRRE-2 and NRRE-3 (labeled N2 and N3 in Figure 5) also confer modest transcriptional repression by COUP-TF.[79] To confirm that COUP-TF does act to repress medium chain acyl-CoA dehydrogenase gene transcription in the cardiomyocyte, cotransfection experiments were performed in which primary rat neonatal cardiomyocytes were transfected with a plasmid containing the same 560 bp promoter fragment present in MCADCAT.371 fused to a luciferase reporter (MCADLuc.371) in the presence or absence of a mammalian expression vector containing a COUP-TF I cDNA (pCDM.COUP). As shown in Figure 11, cotransfection of pCDM.COUP markedly reduced MCADLuc.371 activity (to 28 ± 4% of control). These results are consistent with those of the electromobility shift assays in the *in vivo* pressure overload studies described above and taken together, implicate COUP-TF as a repressor of medium chain acyl-CoA dehydrogenase gene expression during pressure overload-induced cardiac hypertrophy.

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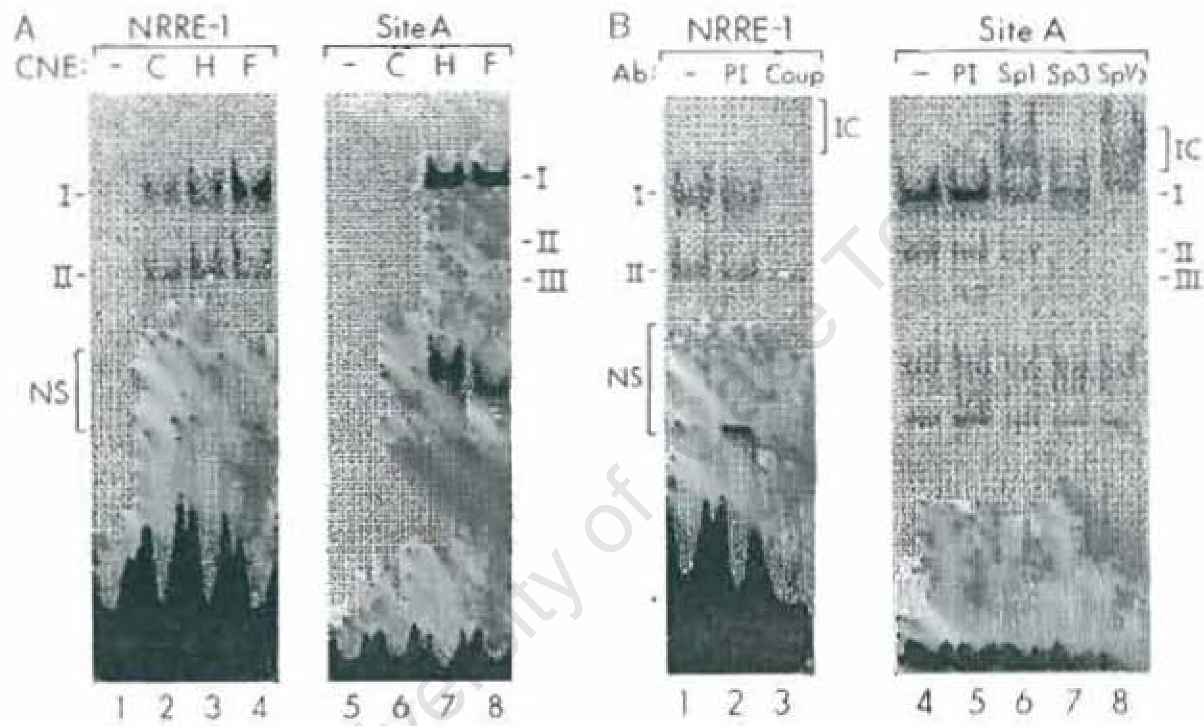

Figure 10

Figure 10. Binding of Sp proteins and COUP-TF to the medium chain acyl-CoA dehydrogenase promoter unit NRRE-1/Site A is induced in response to pressure overload.

(Panel A) Representative electromobility shift assays performed with site A and NRRE-1 probes with nuclear protein extract (CNE) prepared from RV of sham-operated control mice (C), RV subjected to PAB for 7 days (H), and normal fetal (E16.5) mouse heart (F). Specific complexes are denoted by roman numerals and nonspecific interactions by "NS" (as defined by competition studies with unlabeled DNA fragments). (Panel B) Antibody supershift studies performed with NRRE-1 and site A probes and nuclear protein extracts prepared from the RV of the PAB mice. Antibodies (Ab) to COUP-TF (COUP), Sp1, and Sp3 were added to the samples in lanes 3, 6, 7, and 8. Preimmune serum (PI) was added to lanes 2 and 5 as a control. Supershifted immune complexes (IC) are denoted by brackets at the right margins.

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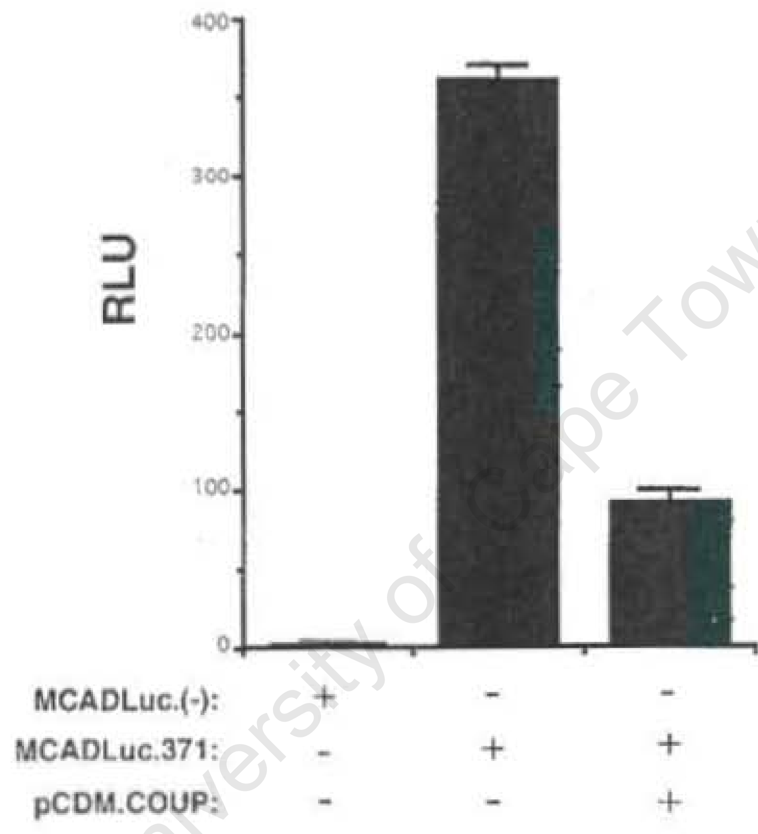

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Figure 11

Figure 11. COUP-TF represses medium chain acyl-CoA dehydrogenase gene promoter activity in cardiomyocytes.

The results of transient cardiomyocyte cotransfections (see Methods) are shown. The transcriptional activity of the medium chain acyl-CoA dehydrogenase promoter-luciferase reporter (MCADLuc.371) or luciferase reporter plasmid lacking the medium chain acyl-CoA dehydrogenase promoter [MCADLuc.(-)] is indicated by relative luciferase units (RLU). The bars represent mean RLU x 10^{-3} following correction for transfection efficiency based on the activity of cotransfected RSV βGal (see Methods). Addition of the COUP-TF expression vector pCDM.COUP is indicated. The values are representative of three separate experiments.

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Nuclear levels of Sp 1/3 and COUP-TF parallel medium chain acyl-CoA dehydrogenase promoter binding activities in developing and hypertrophied mouse heart.

To determine whether expression of Sp1, Sp3, and COUP-TF parallel medium chain acyl-CoA dehydrogenase promoter binding activities during cardiac hypertrophy and in the foetal heart, steady-state nuclear levels of these transcription factors were delineated by Western blot analysis. As shown in Figure 12, steady-state nuclear levels of Sp1, Sp3, and COUP-TF are significantly higher in hypertrophied RV and fetal heart nuclear protein extracts compared to normal adult control RV samples. In contrast, levels of PPARα, an orphan nuclear receptor known to induce expression of FAO enzyme genes including medium chain acyl-CoA dehydrogenase,[78], followed a reciprocal pattern. Given that the foetal samples represented both RV and LV tissue, the immunoblot studies were also performed with nuclear proteins prepared from right and left ventricles of normal adult mice to address the possibility that the observed results were due to chamber-specific expression of these factors. However, the nuclear levels of Sp1 and COUP-TF were identical in right and left ventricles. These data indicate that the nuclear expression of several Sp

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and nuclear receptor transcription factors is induced to foetal levels in response to pressure overload of the ventricle.

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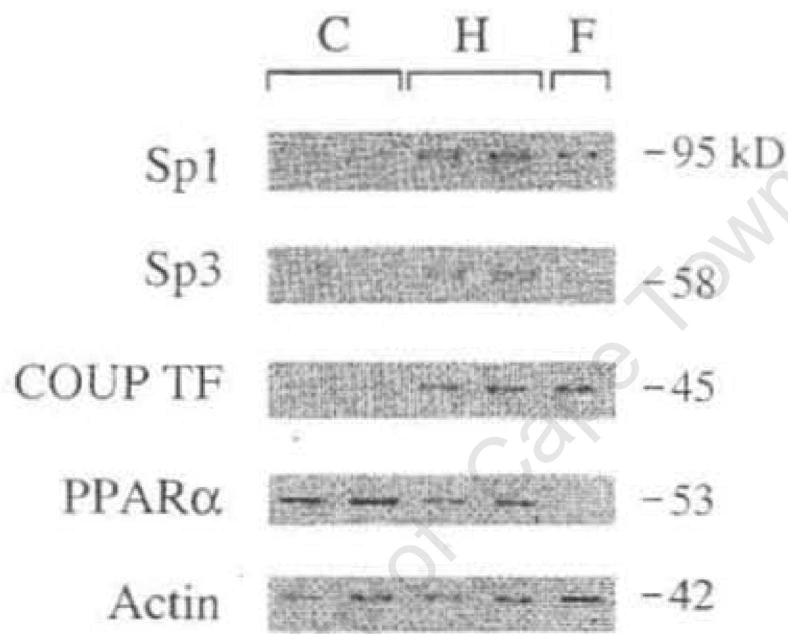

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Figure 12

Figure 12. Nuclear levels of Sp1, Sp3, and COUP-TF parallel DNA binding activities in hypertrophied, foetal, and adult heart.

A representative autoradiograph of a Western blot performed with 8 μg of crude nuclear protein extract per lane. C, sham-operated control RV; H, RV hypertrophy sample; F, embryonic day 16.5 mouse heart. Protein mass markers are displayed on the right in kilodaltons (kD). Purified Sp1 migrates on SDS-polyacrylamide gels as two polypeptide species with molecular masses of 95 and 105 kD due to differential phosphorylation of a single polypeptide.[111] The anti-Sp3 antiserum recognizes 97, 60, and 58 kD polypeptides. Only the 60 and 58 kD Sp3 signals are shown, although the expression of the 97 kD Sp3 protein was identical to the pattern shown for the smaller isoforms (data not shown). Results with anti-PPARα and anti-actin antibodies are shown for comparison.

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Characterization of the Temporal Patterns of Fatty Acid Oxidation mRNA Levels in the Hypertrophied and Failing SHHF Rat LV.

The data shown above indicate that the medium chain acyl-CoA dehydrogenase gene is regulated at the transcriptional level with the development of pressure overload-induced cardiac hypertrophy. Moreover, these data suggest that this transcriptional repression is due to the reactivation of a foetal gene regulatory programme. To determine if this alteration in metabolic gene expression occurred in a separate model of cardiac hypertrophy and with the onset of cardiac failure we have delineated the temporal patterns of FAO enzyme mRNA and protein expression during the development of pressure overload-induced cardiac hypertrophy and heart failure in the SHHF rat strain. The SHHF rat strain is an established model for progressive LV hypertrophy and heart failure (Methods; refs.[98, 112]). Three age groups of SHHF rats were studied i) a control group of 2 month old animals without evidence of significant ventricular hypertrophy; ii) a group of animals with compensated LV hypertrophy (LVH) between ages 9-12 months; and iii) a group of rats with overt heart failure (HF), aged 16-20 months. Mean absolute heart weights and heart-to-brain weight ratios were significantly increased in both the LVH and HF groups compared to controls (Table 2). To control for age-

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related effects, normotensive, non-failing 2 month old and 17 month old Wistar-Furth (WF) rats were also studied.

The levels of mRNA encoding medium chain acyl-CoA dehydrogenase, LCAD, and LCHAD were all markedly lower in LV samples from the LVH and HF rats compared to controls (Figure 13A). As expected, expression of ANF mRNA, was markedly induced in the LVH and HF groups (Figure 13A). Mean steady-state medium chain acyl-CoA dehydrogenase mRNA levels were reduced in the LVH and HF groups by 79 ± 5% ($p<0.01$) and 89 ± 3% ($p<0.001$), respectively (Figure 13B). Mean LCAD mRNA levels were repressed to a similar degree in the LVH and HF groups (Figure 13B). LCHAD mRNA levels were downregulated by 72 ± 9% in LVH ($p<0.01$) and 85 ± 4% in HF ($p<0.001$) (Figure 12B). Although mean mRNA levels for each enzyme were lower in the HF group compared to the LVH group, the difference was not statistically significant. Levels of mRNA encoding the glycolytic enzyme GAPDH were not significantly different among the three groups (Figures 13A and B). No significant difference was found in medium chain acyl-CoA dehydrogenase and GAPDH mRNA levels in the LV of 2 month old (n=4) and 17 month old (n=4) control WF rats, indicating that age-related factors do not account for the reduced fatty acid β-oxidation enzyme mRNA expression.

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Table 2. Characteristics of the SHHF/Mcc-fa^{cp} rats.

	C	LVH	HF
number	6	5	6
Age (months)	2	11±0.7	19±0.7
BW (grams)	221±15	434±8*	415±9*
HW (grams)	0.96±0.06	1.78±0.7*	2.93±0.1*,§
BrW (grams)	1.75±0.03	2.13±0.04*	2.04±0.03*
HW/BW ($x10^{-3}$)	4.4±1.8	4.0±1.2	7.0±3.1‡,§
HW/BrW	0.55±0.03	0.84±0.03*	1.43±0.06‡,§

Abbreviations: C - control; LVH - left ventricular hypertrophy; HF- heart failure; BW - body weight; HW - heart weight; BrW - brain weight. * $p < 0.01$ vs control. ‡ $p < 0.001$ vs control. § $p < 0.05$ vs. LVH

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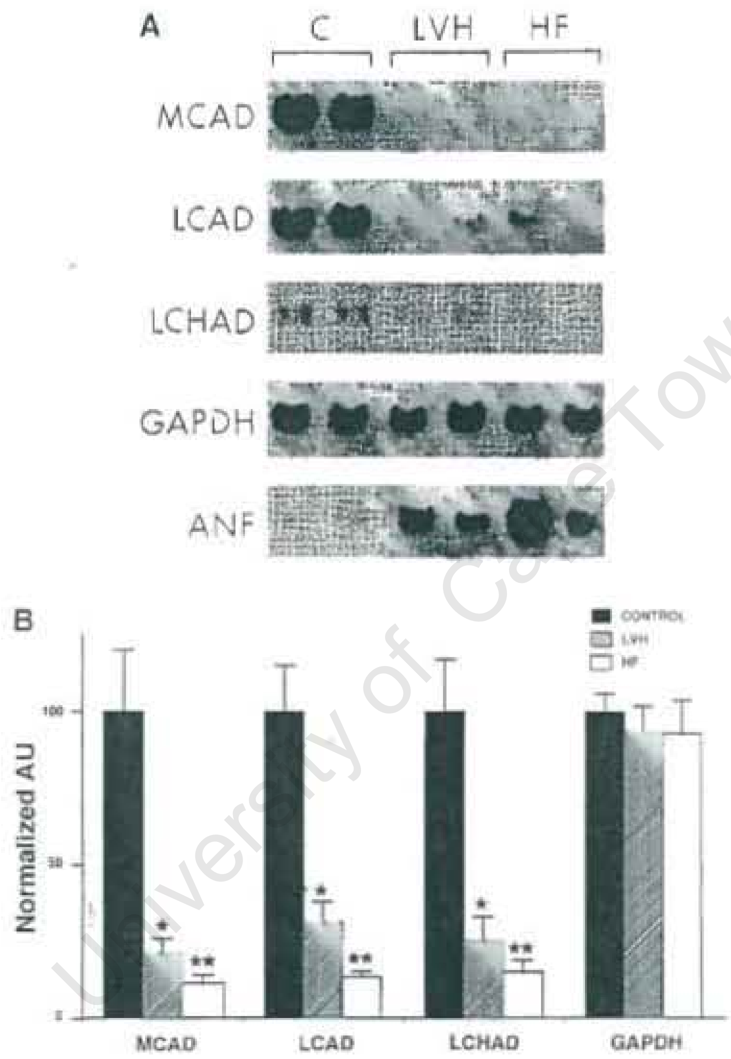

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Figure 13

Figure 13. Fatty acid β-oxidation enzyme mRNA levels in the hypertrophied and failing left ventricle in lean male SHHF rats.

(Panel A) Representative Northern blot analysis performed with total RNA isolated from the LV of SHHF rats in the control (C), LVH, or HF groups as described in the text. Each lane contains 18 μg total RNA. The mean age and number of animals in each group is described in Table 2. Probe abbreviations are described in Methods. (Panel B) The bars represent mean (± SE) steady-state mRNA levels, as determined by densitometric analysis of Northern blots, in LV samples obtained from the control, LVH, and HF stages of male SHHF rats. Values shown are arbitrary units (AU) normalized to control values (=100). All values were first normalized to the signal obtained with an 18s rRNA probe to control for loading differences. * denotes $p < 0.01$ vs control value; ** denotes $p < 0.001$ vs control.

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Characterization of the Temporal Patterns of Fatty Acid Oxidation Enzyme Levels in the Hypertrophied and Failing SHHF Rat LV.

Immunoblot analyses were performed with anti-medium chain acyl-CoA dehydrogenase antibody to determine whether the downregulated expression of medium chain acyl-CoA dehydrogenase mRNA in the LVH and HF stages of the SHHF rats was reflected at the protein level. In surprising contrast to medium chain acyl-CoA dehydrogenase mRNA levels, mean steady-state medium chain acyl-CoA dehydrogenase protein levels were not significantly different in the LVH samples compared to controls (Figure 14). However, medium chain acyl-CoA dehydrogenase mRNA and protein levels were coordinately downregulated to a similar degree in the HF group (Figure 14). Compared to controls, steady-state medium chain acyl-CoA dehydrogenase protein and mRNA levels in the HF rats were reduced by 82 ± 3% and 89 ± 3%, respectively. β-actin protein levels were not significantly different among the three groups. As observed with mRNA levels, medium chain acyl-CoA dehydrogenase protein levels were not significantly different in the 2 month old and 17 month old male WF control rats (data not shown). Medium chain acyl-CoA dehydrogenase enzymatic activities paralleled protein levels during the transition from LVH to HF (Figure 14). These findings identify a discordance between medium chain acyl-CoA dehydrogenase mRNA and

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protein levels during the LVH stage of this rat model and suggest that translational or post-translational regulatory mechanisms are involved in the maintenance of medium chain acyl-CoA dehydrogenase protein levels in compensated ventricular hypertrophy.

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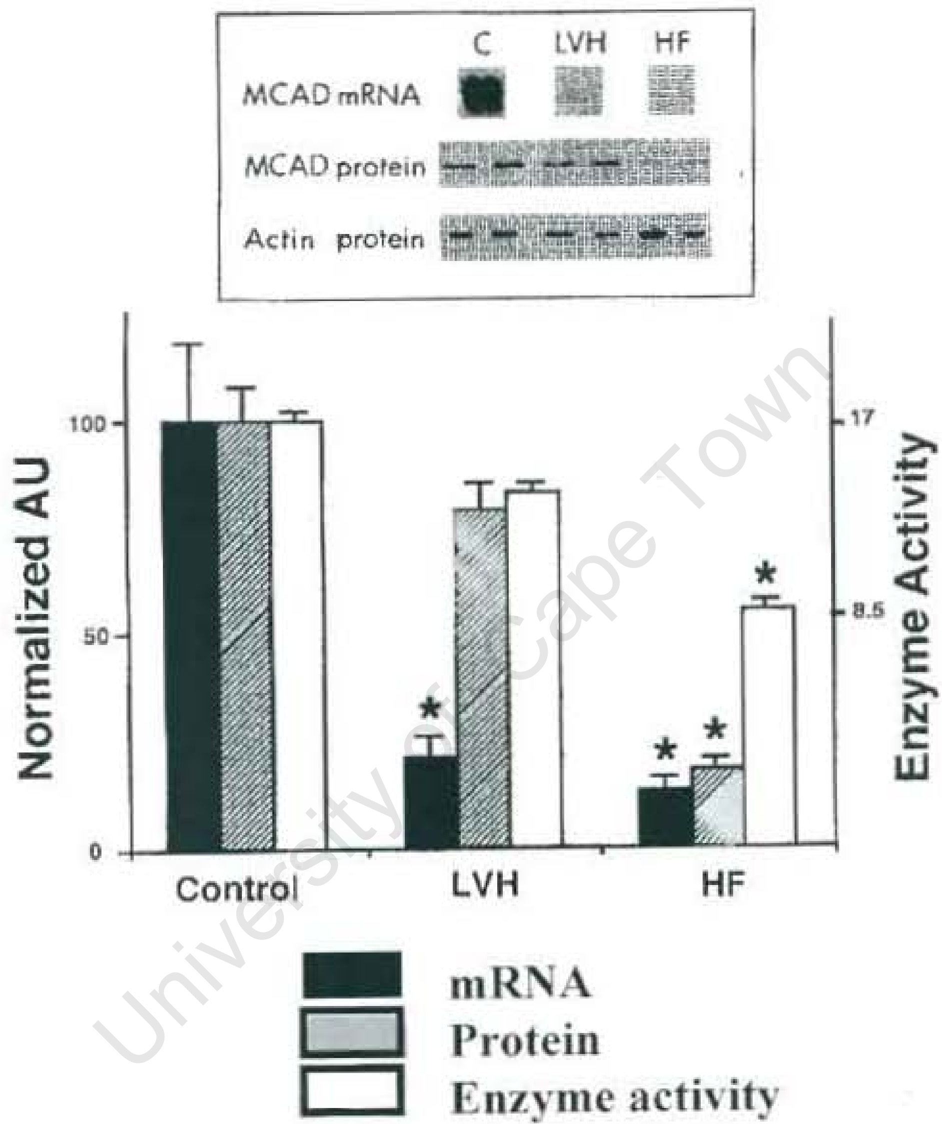

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Figure 14

Figure 14. Comparison of medium chain acyl-CoA dehydrogenase mRNA, protein and enzymatic activity levels in control, hypertrophied and failing SHHF rat hearts.

Solid bars indicate steady-state MCAD mRNA levels, hatched bars MCAD immunodetectable protein levels and open bars MCAD enzymatic activity. MCAD mRNA and immunodetectable protein values are normalized (=100) to corresponding control values. Steady-state MCAD protein levels were determined by immunoblot analysis of protein extracts prepared from the same ventricular samples used for isolation of total RNA. MACD mRNA and protein values represent densitometric analysis of the blots and are expressed as mean (± SE) arbitrary units (AU; left ordinate). The open bars represent mean (± SE) MCAD enzyme activity expressed as μmol of octanoyl-CoA oxidized per minute per gram of wet weight (right ordinate; see Methods). The asterisks represents a p value < 0.05 compared to the corresponding control values. The inset contains representative Northern and Western blot autoradiographs performed with an MCAD cDNA probe and an anti-MCAD and anti-actin antibody, respectively.

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Attenuation of the Downregulation of Medium Chain Acyl-CoA Dehydrogenase mRNA Following Anti-Hypertensive Therapy in Spontaneously Hypertensive Rats.

To evaluate whether treatment of pressure-overload induced hypertrophy could attenuate the downregulation of adult enriched fuel substrate catabolic enzyme encoding genes, we treated spontaneously hypertensive rats with the vasodilating α- and β-adrenoreceptor antagonist carvedilol or vehicle alone supplemented diets for four weeks. The left ventricular (LV) to body weight ratio of the groups are shown in Figure 15. The LV mass in the carvedilol treated spontaneously hypertensive rats were 16 $\pm$ 2% less than the vehicle alone supplemented spontaneously hypertensive rats ($p = 0.09$). Of note in the carvedilol treated group, the adult enriched metabolic enzyme gene expression patterns of LCAS, medium chain acyl-CoA dehydrogenase, GLUT4, and PFK-M were maintained at levels closer to the control WF's than to the placebo treated SHR group (Figure 16). Treatment of the WF control group with carvedilol did not significantly alter LV mass nor gene expression compared to placebo treated controls (data not shown).

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The LV mass / body weight ratio of the groups are shown in Figure 15. The absolute increase in LV weight / body weight ratio of the 24 week old vehicle treated spontaneously hypertensive rats was 50.1±4% greater than the age-matched WF controls ($p=0.01$). In parallel with the low expression pattern demonstrated during neonatal cardiac development (Figure 5) the mRNA expression of the fatty acid utilization enzyme encoding genes LCAS and MCAD; the glucose transporter GLUT4 and glycolytic enzyme encoding gene PFK-M were significantly reduced by ≥ 40% ($p<0.05$) (Figure 16) respectively in the SHR rats compared to normal adult WF control. In addition, the mRNA levels of atrial natriuretic factor (ANF), the well characterized marker of cardiac pressure overload, were profoundly upregulated in the SHR's vs. the age-matched WF controls (data not shown). Consistent with the lack of cardiac developmental regulation of GAPDH, the expression pattern of this gene was not significantly different between the normal control WF rat and hypertrophic SHR ventricles.

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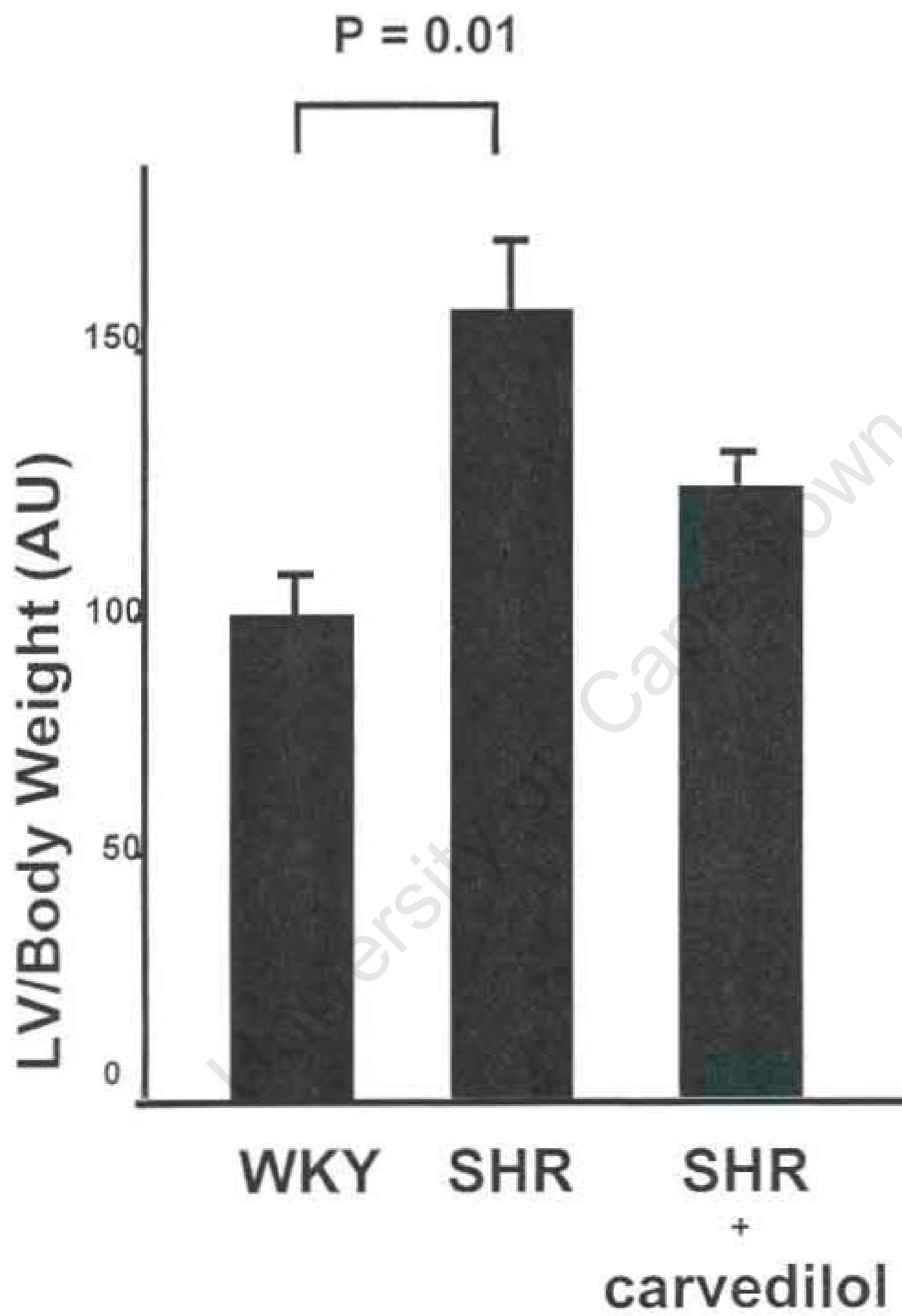

Figure 15

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Figure 15. Left ventricular to body weight ratio's in the control, vehicle treated and carvedilol treated SHR rats.

The bars represent mean (± SEM) of the left ventricle to body weight ratio of the groups determined by wet weight analysis at 24 weeks of age. Values shown are arbitrary units (AU) normalized to control values (=100).

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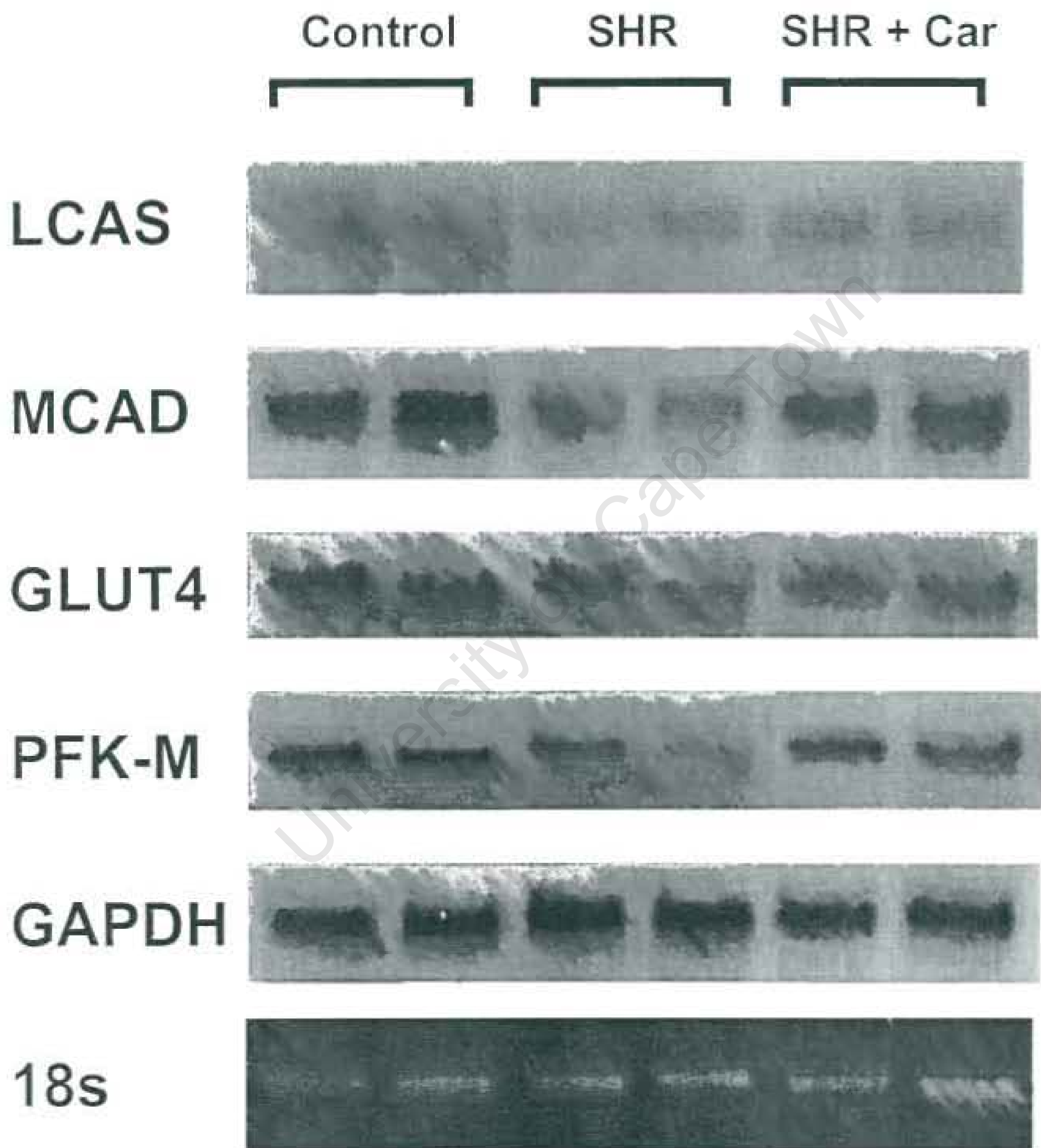

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Figure 16 A

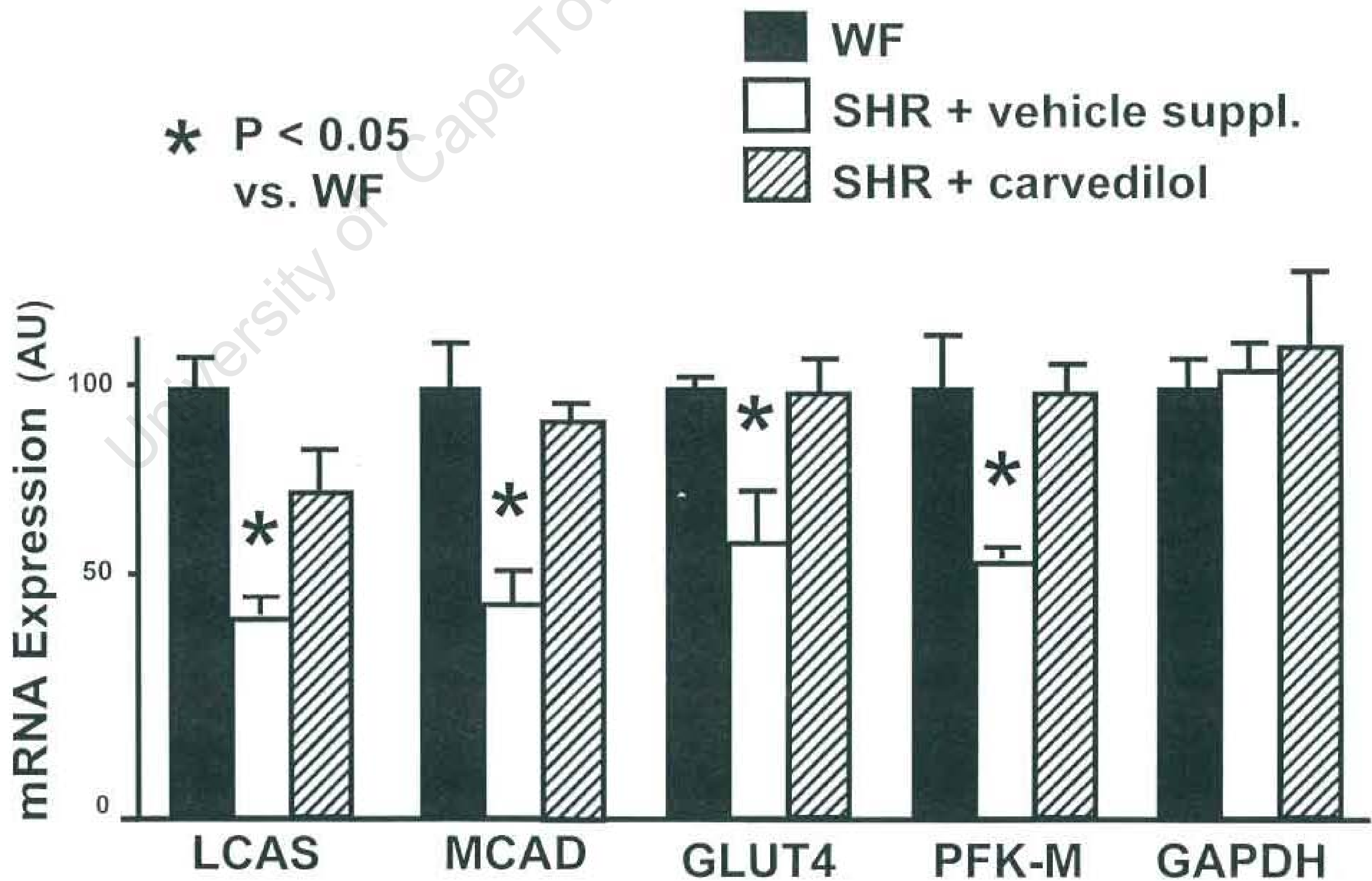

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Figure 16 B

Figure 16. Metabolic enzyme mRNA levels in the normal WKY and hypertrophied SHR rat left ventricles.

(Panel A) Representative Northern blot analysis performed with total RNA isolated from the LV of control WF rats and from the hypertrophied LV of the SHR's. Each lane contains 20 μg total RNA. Probe abbreviations are described in Methods. **(Panel B)** The bars represent mean (± SEM) steady-state mRNA levels, as determined by densitometric analysis of Northern blots, in LV samples obtained from the WF (control) and SHR rats. Values shown are arbitrary units (AU) normalized to control values (=100). All values were first normalized to the signal obtained with 18s to control for loading differences.

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Characterization of Fatty Acid β-oxidation Enzyme Gene Expression in the Failing Human Ventricle.

We then determined whether the expression of genes encoding fatty acid β-oxidation cycle enzymes was regulated in the failing human heart. MCAD and LCHAD mRNA levels were analyzed by Northern blot analysis using total RNA isolated from the LV of human cardiac transplant recipients with severe heart failure (idiopathic cardiomyopathy, n = 6; ischemic heart disease, n = 2) compared to age-matched normal control hearts (n = 5) obtained at postmortem. The clinical characteristics of the two groups are shown in Table 3. MCAD and LCHAD mRNA levels were significantly reduced in the heart failure group compared to controls (representative Northern blot autoradiograph, Figure 17A). Compared to controls, mean (± SE) MCAD and LCHAD mRNA levels were reduced by 56 ± 9% (p=0.03) and 64 ± 6% (p=0.03), respectively (Figure 17B). LV MCAD and LCHAD mRNA levels in the subgroup of patients with idiopathic cardiomyopathy (n = 6) were also significantly reduced compared to controls (data not shown). Levels of mRNA encoding atrial natriuretic factor (ANF), a known marker for ventricular hypertrophy and heart failure (22), were markedly upregulated in the heart failure group compared to the controls (Figure 17A). As determined by immunoblot

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studies, LV steady-state MCAD protein levels paralleled MCAD mRNA levels (decreased by 42±4%; p=0.01) in the heart failure group compared to controls (Figures 17A and 1B).

To exclude the possibility that the downregulated expression of MCAD and LCHAD mRNA in the heart failure samples was due to cardiomyocyte dropout or to a generalized reduction in cardiac gene transcription, several control mRNAs were analyzed. Levels of mRNA encoding the cardiac-specific protein, cardiac troponin I, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were not different in the heart failure group compared to controls (Figures 16A and 16B). Similarly, the levels of mRNA encoding ATP synthase subunit e, a nuclear-encoded mitochondrial protein were unchanged between the two groups.

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Table 3. **Characteristics of human control subjects and cardiac transplant recipients.**

CONTROLS			HEART FAILURE				
AGE	SEX	COD	DIAGNOSIS	AGE	SEX	NYFC	EF
33	F	T	IDC	42	M	IV	35%
49	M	T	IDC	50	M	IV	12%
55	M	CVA	IDC	51	M	III	10%
59	M	CVA	IDC	59	M	III	22%
65	F	CVA	IDC	65	M	III	20%
			IDC	23	M	III	25%
			IHD	58	M	IV	16%
			IHD	59	M	III	27%

Age is in years. Abbreviations : COD - cause of death; NYFC - New York Heart Association Functional Class; EF - left ventricular ejection fraction; T - trauma; CVA - cerebrovascular accident; IDC - idiopathic dilated cardiomyopathy; IHD - ischaemic heart disease

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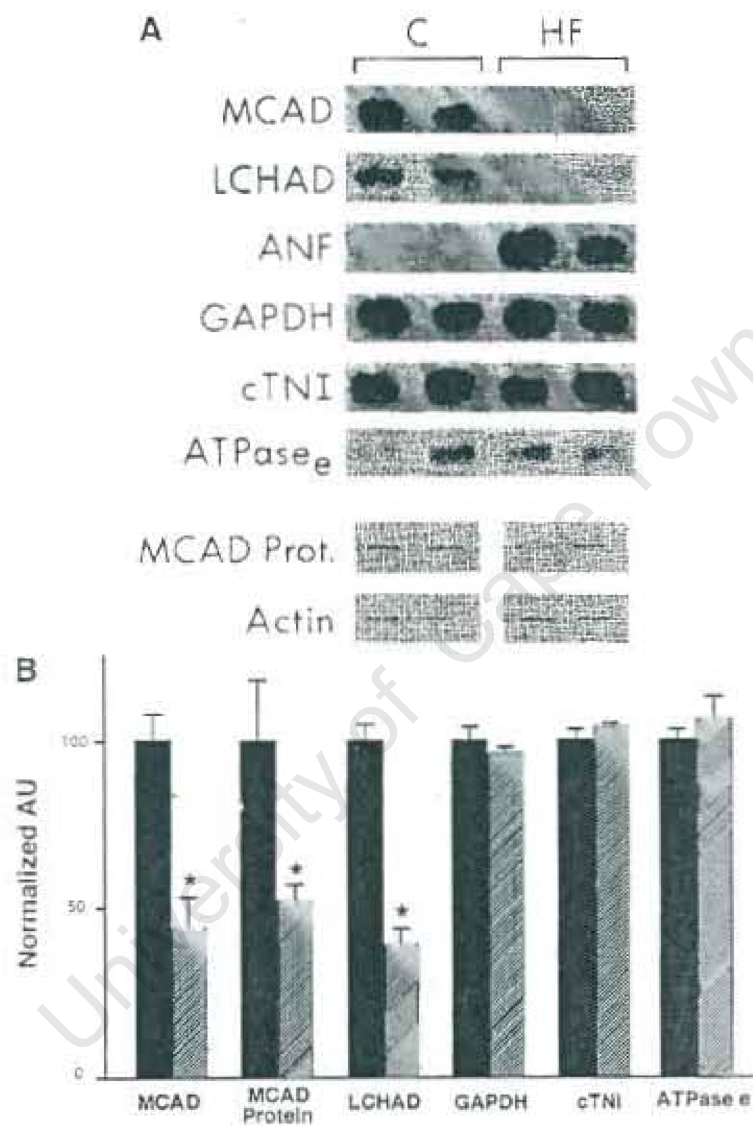

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Figure 17

Figure 17. FAO enzyme mRNA and protein levels in LV from failing human hearts.

(Panel A) Representative autoradiographs of Northern (top) and Western (bottom two panels) blot analysis performed with total RNA (18 μg) or protein (1 μg total protein) prepared from LV of two controls (C) and two subjects with heart failure (HF). Probe abbreviations are described in Methods. The Western blot analysis was performed with a polyclonal anti-MCAD antibody and actin control antibody as described in Methods.

(Panel B) The bars represent mean steady-state mRNA or protein levels shown as arbitrary units (AU), normalized (= 100) to controls and standardized to the signal obtained with an 18s rRNA probe or to the total protein (MCAD protein). Asterisks denote a significant difference ($p < 0.05$) compared to control values.

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The coordinate downregulation of fatty acid β-oxidation enzyme gene expression in human heart failure could reflect a generalized decrease in expression of all mitochondrial protein genes, such as might occur with reduction of cellular mitochondrial number due to alterations in mitochondrial biogenesis or turnover. As an indicator of mitochondrial number, a semi-quantitative analysis of mitochondrial DNA was performed using Southern blot analysis. For these studies, total genomic DNA, isolated from the LV samples, was digested with the restriction endonuclease BamHI which cleaves the human mitochondrial genome at a single site. Age-matched samples from non-failing ventricle were used to control for the known accumulation of mitochondrial DNA deletions with aging.[113] The blot was sequentially hybridized with a mitochondrial DNA-specific probe and a second probe that hybridizes with the nuclear gene encoding GAPDH. The mitochondrial DNA signal, normalized to the GAPDH signal in three control samples, was not significantly different than that in three age-matched heart failure samples (Figure 18). These results and the observed lack of regulation of ATP synthase subunit e mRNA expression strongly suggest that the altered expression of FAO enzymes in the failing heart is not due to a decrease in cellular mitochondrial number, but rather reflects a pathway-specific regulatory phenomenon.

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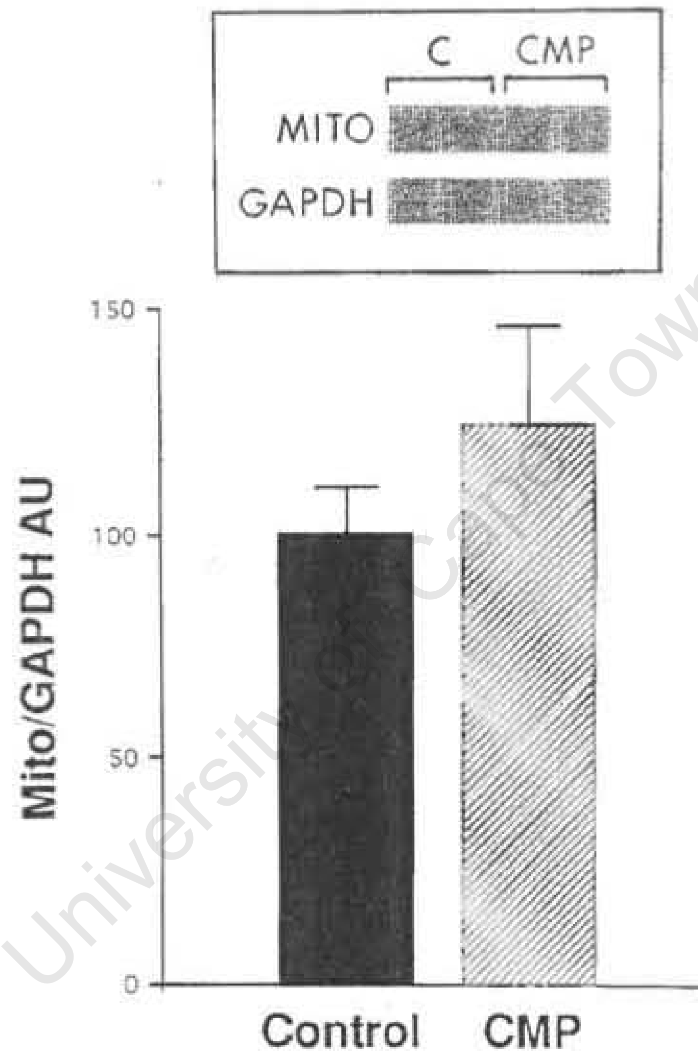

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Figure 18

Figure 18. Quantification of mitochondrial DNA in LV samples from humans with heart failure compared to controls using Southern blot analysis.

The inset shows a representative autoradiograph of a Southern blot analysis performed with total cellular DNA isolated from LV tissue of two patients with idiopathic cardiomyopathy (CMP) compared to controls. The blots were sequentially hybridized with a mitochondrial genome probe (MITO) and with a human GAPDH probe (see Methods). The bars represent the mean signal intensities of the Southern blot analyses performed with the MITO probe, normalized to the signal obtained with GAPDH probe for three CMP samples (ages 51, 59 and 65 years, Table 4) and three age-similar human control samples (ages 55, 59 and 65 years, Table 3). The signals were quantified by laser densitometry and are shown as a ratio of the mitochondrial DNA signal/GAPDH DNA signal in arbitrary units (AU). The difference in mean values was not statistically significant.

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Discussion

The major findings from this body of work include:

1. The identification of a transcriptional regulatory mechanism involved in the reinduction of a foetal metabolic programme during pressure overload-induced cardiac hypertrophy.
2. The finding of discordant regulation of MCAD at the gene and protein levels during cardiac hypertrophy with a more concordant regulation with the development of heart failure. These data suggest a post-translational regulatory control programme directing the steady-state enzyme levels of MCAD during the transition from cardiac hypertrophy to heart failure.
3. The advancement of the paradigm of the control of cardiac metabolism to include modulation at the level of metabolic enzyme gene regulation.

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Reactivation of the foetal metabolic gene regulatory programmes with metabolic remodelling

The reversion to the foetal programme with the development of cardiac hypertrophy is emerging as a common motif in the regulation of multiple cardiac enriched genes and gene products. More specifically, the re-expression of foetal isoforms of a variety of myocardial ATP utilising pathway molecules, including cardiac enriched contractile and ion regulatory proteins has been demonstrated in cardiac hypertrophy (reviewed[72-74]). These findings suggest that activation of foetal gene regulatory programmes are likely adaptive structural and metabolic responses to pressure and/or volume overload in the terminally differentiated cardiomyocyte. The contribution of this reactivation of foetal gene regulation to the subsequent maladaptive cardiac failure is currently poorly understood.

To begin to understand the role of metabolic remodelling in the development of cardiac hypertrophy and in the transition to heart failure numerous investigators have begun to explore cardiac metabolism at the molecular level. This includes the study of the regulation of metabolic enzyme encoding genes during cardiac development and with the onset of cardiac hypertrophy and heart failure. Firstly, the perinatal developmental expression pattern of fatty acid oxidation enzyme encoding genes

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including the medium chain acyl-CoA dehydrogenase gene were characterized in the heart.[75] The expression patterns of these genes were shown to parallel the postnatal biogenesis of mitochondria[20] and the known developmental energy substrate switch from glucose to fatty acids in the heart. The ontogenesis of GLUT4 and Hexokinase II (HK II - a rate-limiting adult enriched glycolytic enzyme, which commits glucose to intracellular metabolism) gene expression have been similarly studied in the rat.[52] These cardiac enriched glucose transport and glycolytic enzyme encoding genes show a similar developmental pattern to medium chain acyl-CoA dehydrogenase gene expression with low expression in the foetal and neonatal periods, and maximal expression in the adult heart. In the pilot part of this study, we demonstrated that the expression pattern of the adult enriched PFK-M parallels the expression patterns of GLUT4 and HK II. Moreover, in this study we demonstrate that the developmental expression pattern of LCAS parallels that of medium chain acyl-CoA dehydrogenase. The lower expression levels of LCAS and MCAD at postnatal day 21 (p21) are consistent with the weaning period and the transition from the consumption of milk - a high-fat, low carbohydrate diet in the suckling period to the normal chow diet. The increased expression patterns of GLUT4, and PFK-M in the adult heart correlate with the known utilization of carbohydrates by the adult hearts, especially in the post-

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prandial period.[14, 114] The lack of developmental regulation of GAPDH at the level of gene expression implies that this enzyme functions as a true "housekeeping gene" during cardiac development. These data suggest that during cardiac development, GAPDH gene expression may be regulated at the pre-translational level via a distinct gene regulatory programme compared to that of the other metabolic enzyme encoding genes studied.

The developmental regulation of the enzymes controlling the major fuel substrates utilized by the heart, may be directed coordinately with the regulation of contractile and ion regulatory molecule encoding genes during cardiac development. These metabolic gene regulatory programmes therefore, putatively, match energy production in the heart with low expression during development correlating with the reduced heart work per cell volume required by the foetal contractile machinery.[64] Recently, Taegtmeyer and colleagues[64] demonstrated that unloading the rat heart, with a subsequent reduction in energy requirement, results in the upregulation of foetal contractile genes (βMHC), and a reduction in the levels of the adult enriched cardiac glucose transporter (GLUT4) and in the adult enriched fatty acid mitochondrial import enzyme [muscle carnitine palmitoyltransferase I (CPT I)]. This unloaded heart model could equate to a "foetal-like" heart with respect to energy requirements and contractile load. Taken together, these data support the hypothesis that

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this coordinate reactivation of the foetal gene expression pattern with respect to the adult enriched fatty acid and glycolytic encoding genes may be directed by integral and/or overlapping gene regulatory programmes.

Transcriptional Control of Medium Chain Acyl-CoA Dehydrogenase Gene Regulation During Pressure Overload-Induced Cardiac Hypertrophy

To begin to explore this metabolic remodelling at the gene regulatory level we focussed on the regulatory control of the gene encoding medium chain Acyl-CoA dehydrogenase, which catalyzes the first step in the mitochondrial β-oxidation cycle. In these studies we have demonstrated that the medium chain acyl-CoA dehydrogenase gene is repressed in response to pressure overload through reactivation of foetal transcriptional control mechanisms.

Previous studies have shown that expression of the transcription factors c-Fos and c-Jun are induced as an immediate early response to ventricular pressure overload,[71, 115, 116] α_1-adrenergic agonist-induced hypertrophy,[117] or cardiomyocyte stretch.[118] Our data identify two groups of transcription factors involved in the development of the hypertrophic response. To our knowledge this was the first report of a cis-

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acting hypertrophy-responsive element and corresponding trans-acting regulatory proteins in an *in vivo* preparation of ventricular pressure overload.[109] We show that COUP-TF/EAR-3 interacts with the cis-acting element NRRE-1/site A to repress medium chain acyl-CoA dehydrogenase gene transcription during cardiac hypertrophic growth. We also demonstrate that binding of Sp1 and Sp3 to site A is induced during cardiac hypertrophy. Although members of the Sp family may act as transcriptional activators, both Sp1 and Sp3 have also been shown to repress gene expression in a promoter context-dependent manner.[119-121] Recently, we have identified a series of Sp binding sites in the promoter region of the gene encoding M-CPT I which catalyzes a rate-limiting step in mitochondrial fatty acid import.[63] Accordingly, we hypothesize that there could be a general role for Sp proteins in the coordinate regulation of genes involved in cardiac fatty acid oxidation. Future studies will be necessary to determine whether Sp1 or Sp3 actually repress transcription of genes encoding medium chain acyl-CoA dehydrogenase and other fatty acid oxidation enzymes. Interestingly, as discussed below our work and others now suggest a role of the Sp3 zinc-finger transcription factor in the pre-translational downregulation of both fatty acid and glycolytic metabolic enzyme encoding genes. In this study we have demonstrated that the molecular regulatory programme directing

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medium chain acyl-CoA dehydrogenase gene downregulation, during cardiac development and with the onset of pressure overload induced cardiac hypertrophy is directed in part by the upregulation of the foetally induced transcriptional repressor Sp3.[109] Moreover, Discher, et al,[122] has recently demonstrated that the overexpression of Sp3 repressed expression of the glycolytic enzyme encoding gene, β-enolase. Thus, activation of Sp3, may repress genes encoding enzymes in two distinct metabolic pathways. This regulation would suggest that coordinated downregulation of the adult enriched metabolic enzyme encoding genes, during the development of cardiac hypertrophy may be via the reactivation of similar and or overlapping foetal molecular regulatory programmes.

Our findings and the results of recent studies by others suggest that the transcriptional regulatory pathway described here is linked to other genes known to be regulated during cardiac hypertrophy. For example, a Sp1 binding site within the skeletal α-actin gene promoter is required for transactivation of this gene during hypertrophic growth of rat neonatal cardiocytes in cell culture studies.[123] Thus, it is possible that Sp1 may function as an activator (e.g., skeletal α-actin) or repressor (e.g., medium chain acyl-CoA dehydrogenase) in a promoter-specific manner in response to upstream signals that trigger hypertrophic growth. Two recent reports[111, 124] indicate that nuclear receptor signaling pathways are also

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involved in cardiac hypertrophy programmes. In these studies, the retinoid receptor α (RX$R\alpha$) antagonized α-adrenergic agonist- or endothelin-mediated cardiomyocyte hypertrophy in cell culture. Dr. Kelly's group has shown previously that COUP-TF competes with RX$R\alpha$ for binding to NRRE-1 to repress transcription.[77] It is tempting to speculate that COUP-TF, as a known antagonist of RXR, regulates the expression of many genes during cardiac hypertrophy.

Our data do not allow us to delineate the upstream regulatory mechanisms involved in the induction of COUP-TF and Sp1/3 DNA binding activities during cardiac hypertrophy. Our immunoblotting studies do, however, indicate that the nuclear levels of these factors increase during development of hypertrophy. The mechanism involved in the increased nuclear levels of these factors is unknown but could involve a post-translational modification such as phosphorylation. The results of previous studies have implicated mitogen-activated phosphorylation events in the cardiac growth response.[125, 126] Recent reports have also shown that several nuclear hormone receptors including the oestrogen receptor and orphan receptor PPARγ are targets for mitogen-activated kinases.[127] Others have shown that a DNA-dependent kinase phosphorylates members of the Sp family to modify DNA binding activities.[128] We speculate that growth factor signalling pathways play a

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role in the activation of the transcription factors described here. Future studies exploring the potential link between COUP-TF, Sp1/3, and upstream signaling pathways should prove interesting.

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Temporal Regulation of Medium Chain Acyl-CoA Dehydrogenase During Cardiac Hypertrophy and the Progression to Heart Failure.

Comparison of the temporal patterns of medium chain acyl-CoA dehydrogenase mRNA and protein expression during the left ventricular hypertrophy (LVH) and the heart failure (HF) stages in SHHF rats, revealed that regulation occurs at multiple levels. Most notably, medium chain acyl-CoA dehydrogenase mRNA levels are markedly reduced in LVH compared to controls whereas medium chain acyl-CoA dehydrogenase protein levels and enzyme activities are not significantly different until the HF stage. These results suggest that an independent regulatory mechanism increases medium chain acyl-CoA dehydrogenase translation or stabilises MCAD protein in compensated ventricular hypertrophy. The maintenance of MCAD enzyme levels despite reduced mRNA levels in the hypertrophied but non-failing ventricle could be due to the known increase in protein synthesis in the hypertrophied cardiocyte or may involve a gene- or metabolic pathway-specific phenomenon.

Our present results do not distinguish between a primary role of decreased myocardial fatty acid oxidative capacity in promoting the transition from compensated ventricular hypertrophy to heart failure, and a secondary phenomenon. Although MCAD protein levels and enzyme activities are reduced in parallel with the transition to heart failure in SHHF

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rats, these data do not establish a cause-effect relationship. However, our results suggest the intriguing possibility that repression of fatty acid oxidation enzyme expression in the hypertrophied heart becomes maladaptive and therefore contributes to the progression from compensated ventricular hypertrophy to overt heart failure. It is well recognized that genetic defects in almost every enzyme in the fatty acid oxidation pathway, including the acyl-CoA dehydrogenases, cause childhood cardiomyopathy and sudden death.[7, 8] One possible cause of heart failure in the setting of defective FAO is inadequate myocardial energy supply. Alternatively, fatty acid intermediates, which could be injurious to the sarcolemma, may accumulate in the context of diminished myocardial β-oxidative capacity. In support of this latter hypothesis, long-chain acylcarnitines have been shown to cause ventricular arrhythmias and cardiac dysfunction during myocardial ischemia.[129] Moreover, in a recent study Bonnet et al[130] has linked the accumulation of intermediary metabolites of fatty acids, such as long-chain acylcarnitines to sudden death or near-miss sudden death in infants with inherited defects in fatty acid oxidation. Furthermore, postmortem studies have revealed lipid droplet accumulation in the myocardium of humans with dilated cardiomyopathy.[131] Future studies, including delineation of the temporal pattern of the reduction of β-oxidative flux during the transition from

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cardiac hypertrophy to failure, will be useful in determining the role of altered fatty acid oxidation as a causal factor in the development of heart failure.

Attenuation of Left Ventricular Hypertrophy with Carvedilol Treatment Diminishes the Downregulation of Metabolic Enzyme Gene Expression Patterns

Carvedilol treated spontaneously hypertensive rats have a reduction in blood pressure and an attenuation in the development of cardiac hypertrophy.[101] We therefore used these rats to evaluate whether the prevention of the development of cardiac hypertrophy could attenuate metabolic remodelling at the level of gene expression. Using the same dosing and formulation of carvedilol, our data confirms a modest reduction in LV hypertrophy in carvedilol treated SHR rats compared to placebo treated controls. The relatively modest attenuation in LV hypertrophy in our SHR group treated with carvedilol compared to that found by Ohlstein et al,[101] suggest to us that the blood pressure reduction we obtained was less pronounced. However, we did demonstrate that in the carvedilol treated SHR rats, the expression pattern of the adult-enriched metabolic enzyme encoding genes is maintained at near control levels, compared to their downregulation in the placebo treated hypertrophic control group.

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Therefore, at a purely speculative level, a molecular mechanism whereby carvedilol may confer cardioprotective effects in heart failure[132] may be, in part, via preservation of the adult metabolic gene regulatory program. The concept of maintaining the adult gene expression pattern has been previously demonstrated with another class of cardioprotective drugs, i.e. angiotensin converting enzyme inhibitors in post-infarction myocardial remodeling.[133]

Some reservations have been raised regarding the use of the SHR strain for metabolic studies as perturbations in fatty acid and glucose metabolism are thought to be present in the SHR rats due to the known insulin resistant phenotype in this rat strain.[134, 135] Moreover, recent data suggest that the SHR rats have a genetic defect in the fatty acid translocator gene (also know as Cd36), which may be associated with a characteristic metabolic phenotype inthis strain.[136] Our data, however, suggest that at the level of metabolic enzyme encoding gene expression, the levels of the enzymes we studied rather reflect the degree of hypertrophy in the spontaneously hypertensive rats as opposed to the genetic background. It is also unlikely that the expression patterns of the metabolic enzyme encoding genes in the carvedilol treated rats are due to the metabolic effects of β-adrenoreceptor blockade as no regulation is seen comparing the treated and control WF rats. Moreover, in a study of

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normal volunteers, no change in the serum levels of glucose or free fatty acids were found after treatment with carvedilol for 14 days compared to placebo treated controls.[137]

GAPDH gene regulation during cardiac development and hypertrophy

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the first energy harvesting enzyme in the glycolytic pathway and is often used as a "constitutively expressed control" in different tissues and cell types, including the heart. In this study, we demonstrate that GAPDH is not significantly regulated during rat cardiac development nor with the development of pressure-overload induced hypertrophy in the SHHF and SHR rats. These data suggest that the GAPDH gene regulatory programme may be distinct form that of the rate controlling glycolytic and fatty acid utilization enzyme encoding genes evaluated in this study. Of note, in the SHHF and SHR rats, the degree of hypertrophy was approximately a 50% increase in left ventricular mass, compared to age and sex matched controls. On the contrary, in the mouse pulmonary artery banding study where we induced a 100% increase in right ventricular weight, the gene expression pattern of GAPDH was significantly upregulated compared to sham operated controls.[109] Thus, we postulate

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that the upregulation of the GAPDH gene expression pattern with profound right ventricular hypertrophy, may be the result of this 'supraphysiologic' hypertrophy, resulting in a much greater energy demand in these hearts compared to their sham-operated control group and compared to the SHHF and SHR rats. An alternate but less likely explanation may be that there is a ventricular specific regulatory programme. In the mouse study we were evaluating gene regulation in right ventricular hypertrophy, whereas in both the rat studies we evaluated gene expression in left ventricular hypertrophy. A species difference is unlikely as we have demonstrated similar patterns of regulation of MCAD in left ventricular hypertrophy in mice, rats and rabbits.[138]

Coordinate Downregulation of Fatty Acid Oxidation Enzyme Expression in Human Cardiomyopathy.

The ability to study the molecular regulatory programmes of metabolic remodelling in human disease is limited by the availability of human tissue during cardiogenesis and during the development of cardiac disease. We were however fortunate to be able to obtain tissue from end stage cardiomyopathic patients with some control samples from the Heart Transplant Program at Barnes Hospital, Washington University School of Medicine in St. Louis, USA. RNA and protein analysis of these tissues has

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allowed us to evaluate whether this pattern of metabolic remodelling is applicable to humans at the molecular level.

Our studies of failing human heart samples indicate that expression of fatty acid oxidation enzymes is coordinately downregulated at the pre-translational level in parallel with known alterations in myocardial energy substrate in failing human ventricles. The reduced MCAD protein levels and enzyme activity in the cardiomyopathy subjects compared to controls parallel those found in the SHHF rat. It appears that similar translational or post-translational regulatory programme are activated in both humans and in this rat model of cardiomyopathy.

A model of fatty acid oxidation enzyme gene regulation during the development of cardiac hypertrophy and the progression to heart failure.

In this dissertation, I have described our recent studies in which we have identified a metabolic gene regulatory programme that is initially activated during cardiac hypertrophic growth. Transcription of the medium chain acyl-CoA dehydrogenase gene is repressed during the development of pressure-overload hypertrophy. This transcriptional repression involves re-activation of foetal transcriptional control by the transcription factors COUP-TF, Sp1 and Sp3. However, as demonstrated by the studies of the

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SHHF rat, during the early stages of hypertrophy, the levels of MCAD protein are maintained despite a drop in mRNA levels. The mechanisms of this post-transcripitonal control are unclear but likely involve control at the translational or post-translational levels. Moreover, in the carvedilol treated SHR rats we have demonstrated that this downregulation of the medium chain acyl-CoA dehydrogenase gene expression may be attenuated by blocking the development of pressure overload-induced hypertrophy. Finally, during the transition to overt heart failure, medium chain acyl-CoA dehydrogenase enzyme levels drop and, as demonstrated by others, myocardial fatty acid utilization may fall. This scheme is summarised in Figure 19. The studies included in this disseratation have not established a causal relationship between the reduction of cardiac fatty acid oxidation rates and the development of heart failure. However, the occurrence of heart failure in individuals with inborn errors in fatty acid oxidation enzymes suggests that this reduced capacity for myocardial fatty acid utilisation may indeed cause cardiac dysfunction. Accordingly, this metabolic regulatory pathway represents a useful target for future experimental studies aimed at the characterisation of the role of alterations in cellular lipid metabolism in the genesis of heart failure. Studies in which this gene regulatory pathway is altered by transgenesis or targeted gene disruption are presently underway.

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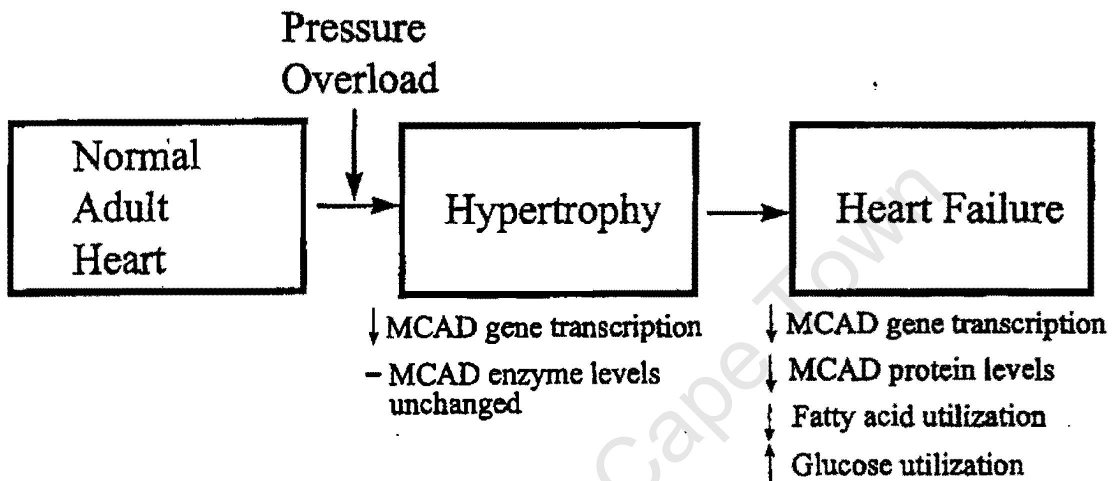

Figure 19. Scheme for the regulation of MCAD expression and energy substrate switches during development of pressure overload-induced heart failure.

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Molecular regulation - expanding the paradigm of metabolic control in the heart during health and disease.

Historically, interest in glucose metabolism dates back to 1907 when Locke and Rosenheim[139] described glucose uptake in the Langendorff isolated heart preparation. In the following decade numerous investigators discovered that fatty acids and glucose constitute the major cardiac substrates (Reviewed[140]). In the early 1950's Bing suggested that energy substrate used by the heart was dependent on the blood levels of these major fuels and on the corresponding levels of insulin.[13] The biochemical concept of rate-limiting enzymes in energy metabolism was introduced at the end of the fifth decade and extensively studied in the heart by the likes of Randle, Morgan, Neely and others (Reviewed[140]). Thus during the last few decades the intricate biochemical regulation of cardiac metabolism has been delineated (Reviewed[14, 59, 114]). The regulation of metabolism at the genomic level is, however, a more recent advance. This advance has followed on the sequencing of metabolic enzyme encoding genes during the last ten to fifteen years. In this latter era, we and others have begun to describe the differential expression patterns of these genes during in the normal and diseased heart (e.g.[35, 75, 104, 122]). In this body of work, we have taken this concept a step further, in that we have

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identified a pressure-overload responsive regulatory unit within the promoter region of a metabolic gene. These data demonstrate a direct link between the genomic regulation of a metabolic enzyme encoding gene with a pathophysiologic stress on the heart. Future work in this arena will enable investigators to increase our understanding of how disease states can dictate substrate metabolism in the heart.

Conclusion

In conclusion, we have demonstrated coordinate and parallel pre-translational regulation of genes encoding enzymes directing the two dominant metabolic fuel substrate utilization pathways during cardiac development and with the onset of cardiac hypertrophy. We have identified a gene regulatory mechanism involved in the energy substrate switch using the regulation of the medium chain acyl-CoA dehydrogenase gene as the model system. This mechanism involves, in part, the reactivation of foetal transcriptional control via members of the Sp and COUP-TF/EAR-3 families of transcription factors. These data and the results of previous studies suggest that this regulatory pathway is linked to other genes regulated during hypertrophic growth of the cardiac myocyte.

Futhermore, in this study we have identified a putative post-translational regulatory programme which may direct the switch in energy

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metabolism between the 'compensated' hypertrophied heart and the 'decompensated' failing heart. However, this concept requires additional investigation to clarify the mechanims of this control.

Finally, we believe that the data obtained from these studies has played a significant role in advancing the concept of genomic control in the regulation of metabolism in the heart.

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Scientific Acknowledgements

The studies performed to complete this PhD were undertaken at Washington University Medical Center, St. Louis, USA, University College London Medical School, London, UK and the University of Cape Town Medical School, Cape Town South Africa. I would like to acknowledge the support I obtained from multiple people at these three medical schools during this research period. Moreover, as a Howard Hughes Physician Postdoctoral Research Fellow, while in St. Louis, I was funded by the HHMI. Additional funding was obtained from the Clinical Research and Development Committee, University College London, UK (G.86) and in part by an equipment grant from the Wellcome Trust (055852/Z/98Z). The study drug carvedilol and an educational grant was provided by SmithKline Beecham, King of Prussia, PA, USA (Dr. Giora Feuerstein).

I would like to thank Jeffrey Saffitz (Department of Pathology) and the Washington University School of Medicine Cardiac Transplant Group for providing us with human heart tissue.

The following individuals need to be thanked for donated as gifts cDNA fragments. These were used to evaluate gene expression patterns in the

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numerous cardiac developmental and pathophysiologic states discussed in this dissertation:

LCAS - a gift from Jean Schaffer, Washington University, St Louis, USA

LCAD - a gift from Bryan E. Hainline, Indiana University; USA

LCHAD - gift from Arnold Strauss, Washington University, St Louis, USA

GLUT4 - a gift from Mike Meuckler, Washington University, St Louis, USA

PFK-M - a gift from Hiromu Nakajima, Osaka University, Osaka Japan

ANF - a gift from James E. Greenwald, Washington Univ., St. Louis, USA

cTNI - a gift from Anne M. Murphy, Johns Hopkins Univ., Baltimore, USA

The following individuals need to be thanked for donated as gifts antibodies for Western blot analysis. These were used to evaluate gene expression patterns in the numerous cardiac developmental and pathophysiologic states discussed in this dissertation:

1. anti-COUP-TF Ab. - a gift from Dr. Ming-Jer Tsai, Baylor University, Houston, USA
2. anti - long-chain acyl-CoA dehydrogenase - a gift from Dr. Arnold Strauss, Washington University, St. Louis, MO, USA
3. anti-COUP-TF - a gift from A.J. Butler. The anti-COUP-TF antibody recognizes both COUP-TFI and COUP-TFII isoforms.
4. anti-peroxisome proliferator activated receptor α - a gift from Dr. Michael Arand.

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Appendix

Modified Lowry Reagents

CTC reagent (Copper-Tartarate-Carbonate)

20 gms Na_2CO_3 dissolved in 100 ml's DW (distilled water)

0.2 gms $CuSO_4.5H_2O$ and 0.4 gms K_2 tartarate in 100 ml's DW.

Reagent A:

Equal volumes of CTC, 10% (w/v) Sodium Lauryl Sulfate, 0.8 N NaOH and DW.

Reagent B:

0.33 N Folin reagent (20mls of 2 N Folin-Ciocalteu reagent (Sigma chemicals)

Western Blot Reagents/Solutions

For use on the mini-protean electrophoresis rig - BioRad

Separating Gel Solution (e.g of 7.5% gel)

5 ml 30% Acrylamide/ 0.8% Bis

5 ml 1.5M Tris-HCl, 0.4%SDS, pH 8.8

10 ml deionised DW

100 μl 105 APS

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20 μl TEMED

Stacking Gel

3 ml 0.5M Tris-HCl, 0.4% SDS, pH 6.8

1.6mls 30% Acrylamide / 0.8% Bis

7.2 ml Deionised DW

60 μl 10% APS

12 μl TEMED

Electrophoresis Gel Running Buffer

10 X Buffer

0.25M Tris-HCl, 1.9M Glycine, 1%SDS, pH 8.6.

Protein Reducing Buffer

1ml 0.5M Tris-HCL pH 6.8

1ml 10% SDS

1ml Glycerol

250μl 0.025 Bromophenol Blue

0.1 ml β-mercaptoethanol

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Academic outputs resulting from this work

Manuscripts:

1. 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 1996;94:2837-2842.

2. Sack MN, Disch DD, Rockman HA, Kelly DP. A role for Sp and nuclear receptor transcription factors in a cardiac hypertrophic growth program. Proc. Natl. Acad. Sci. USA, 1997;94:6438-6443.

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1. Sack MN, Johnson CM, Pogwizd SM, Kelly DP. Regulation of mitochondrial fatty acid oxidation enzyme gene expression in the failing heart. J. Mol. Cell. Cardiol.1995;27:A58.

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Invited Seminar Presentations of this Work:

1. Transcriptional regulation of medium chain acyl-CoA dehydrogenase in pressure overload-induced cardiac hypertrophy. - Center for Cardiovascular Research, Washington University Medical School, St.Louis, MO, USA. - November 1996.

2. Reactivation of a Metabolic Fetal Gene Regulatory Program with the Induction of Pressure Overload-Induced Cardiac Hypertrophy - Hatter Institute for Cardiovascular Research, University College London Medical School. - March 1997.

3. Understanding the Role of Genes Involved in the Metabolic Control of the Failing Heart - Cardiology at the Limits 2. - University of Cape Town Medical School April 18, 1998.

4. Metabolic Gene Regulatory Programmes During Cardiac Hypertrophy - XXth Congress of the European Society of Cardiology. - Vienna - Austria, August 24, 1998.

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5. Differentail regulation of GLUT4 gene expression in pressure-overload and hypoxia induced ventricular hypertrophy. The University of Texas - Houston. Symposium on Metabolic Regulation of Cardiac Gene Expression. March 4-5 1999.

6. Molecular Control of Fatty Acid Metabolism in the Normal, Hypertrophied and Failing Heart. Circulation 99', Japan. 63rd International Annual Scientific Meeting of the Japanese Circulatory Society. Tokyo, March 28, 1999.

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