METABOLIC PHENOTYPING OF MURINE HEARTS OVEREXPRESSING CONSTITUTIVELY ACTIVE SOLUBLE GUANYLATE CYCLASE

Ramzi Khairallah

Department of Experimental Medicine

Faculty of Medicine

McGill University, Montreal

Quebec, Canada

A thesis submitted to McGill University in

partial fulfillment of the requirements of

the degree of Master's in Science

August 2007

Table of Contents

Abstract

Although enhanced cGMP signaling can prevent hypertrophy, mechanisms underlying this cardioprotective effect are not well understood. In this study, we assessed the potential involvement of alterations in myocardial energy substrate metabolism, a parameter known to be determinant in the development of hypertrophy. We used mice overexpressing a constitutively active soluble guanylate cyclase in a cardiomyocyte-specific manner ($GC^{+/0}$) and *ex vivo* heart perfusion at physiological workload with ^{13}C-labeled substrates. Compared to controls, hearts from $GC^{+/0}$ mice displayed a 38±9% lower contribution of exogenous fatty acids to acetyl-CoA formation, while that of carbohydrates remained unchanged despite a two-fold increase in glycolysis. The lower contribution of exogenous fatty acids to energy production was not associated with changes in energy demand or supply (contractile function, oxygen consumption, tissue acetyl-CoA or CoA levels, citric acid cycle flux rate) or the regulation of β-oxidation (acetyl-CoA carboxylase activity, tissue malonyl-CoA levels). However, $GC^{+/0}$ hearts showed a two-fold increase in the incorporation of exogenous oleate into triglycerides. Furthermore, a concomitant increase in triglyceride hydrolysis is consistent with our findings of a greater abundance of hormone sensitive lipase (HSL) protein (46±6%) and mRNA (22±4%) as well as a 37±13% decrease in its phosphorylation level at Ser-565. The latter covalent modification inhibits HSL and is regulated by AMP-activated protein kinase (AMPK), whose phosphorylation at its activating site Thr-172 was also reduced by 37±13%. These changes in exogenous fatty acid trafficking in $GC^{+/0}$ hearts appear to be functionally relevant, as demonstrated by their resistance to fasting-induced myocardial triglyceride accumulation. This raises the possibility that enhanced cGMP signaling in cardiomyocytes may protect the heart from fatty acid-induced toxic effects, either as part of its anti-hypertrophic effect or as an additional cardioprotective mechanism.

Résumé

Une plus grande utilisation des glucides au dépend des acides gras (AG) pour la production d'énergie a été documentée dans le cœur hypertrophique, mais il n'est toujours pas clair si ces changements métaboliques sont adaptatifs ou maladaptatifs. Étant donné que la voie du cGMP a des propriétés anti-hypertrophiques, nous avons émis l'hypothèse que des changements dans la sélection de substrats énergétiques peuvent être à l'origine de l'effet cardioprotecteur de cette voie. Des cœurs de souris qui surexpriment la guanylate cyclase spécifiquement dans les cardiomyocytes (Tg) ont été perfusés *ex vivo* au travail avec des substrats marqués au carbone-13. L'activité des voies métaboliques impliquées dans la production d'énergie tel que le cycle de Krebs a été corrélée à des paramètres fonctionnels et physiologiques. Comparativement aux souris témoins, les cœurs Tg maintiennent mieux leur intégrité membranaire, tel qu'indiqué par la baisse de la quantité de lactate déshydrogénase relâché par le cœur, tout en maintenant leur travail cardiaque. Au niveau métabolique, les cœurs Tg ne montrent pas de différence dans la contribution des glucides à la formation de l'acétyl-CoA malgré un flux glycolytique augmenté de 127±21% ($p<0.01$), alors que l'utilisation des acides gras (AG) est diminuée de 40±4% ($p<0.05$). Selon les résultats obtenus, cette diminution n'est pas attribuable à des changements: (i) des niveaux tissulaires du malonyl-CoA et de l'acétyl-CoA ou (ii) de l'activité du cycle de Krebs, suggérant que le statut énergétique du cœur n'est pas altéré, ou (iii) de l'expression des gènes métaboliques. Plutôt, il semblerait que les cœurs de souris $GC^{+/0}$ compensent pour la baisse de la contribution des AG par une utilisation accrue des acides gras endogènes provenant des triglycérides. En effet, la quantité totale de lipase hormone-sensible est augmentée de 46±6% et son ARNm de 22±4%. De plus, sa phosphorylation inhibitrice au site Ser-565 est diminuée de 37±13%. Cependant, cette augmentation de la lipolyse n'est pas accompagnée par une diminution des triglycérides et la synthèse de ceux-ci à partir d'acides gras exogènes est doublée par rapport aux cœurs contrôles. Ces changements dans l'utilisation des acides gras apparaissent aussi *in vivo.* En effet, après un jeûne de 24h, les souris $GC^{+/0}$ n'accumulent pas de triglycérides, contrairement aux contrôles. Il est donc possible que l'activation chronique de la voie de signalisation du cGMP est cardioprotectrice par le biais d'un mécanisme qui diminue la lipotoxicité associée avec les acides gras libres.

Acknowledgments

I was truly blessed to be supervised by an incredible scientist and outstanding mentor, Dr Des Rosiers. I am forever grateful for her support, motivation, trust and guidance. Dr Des Rosiers led by example and taught me a thorough, critical approach to scientific research. I also had the great opportunity to be co-supervised by a remarkable scientist, Dr Deschepper. I would like to express my profound gratitude for his tutelage, advice, expertise and support.

I would also like to thank my wonderful lab mates for their assistance and friendship and for making my years in the lab forever memorable. I wish to particularly thank Roselle Gélinas, Caroline Asselin, Janie McDuff, and Isabelle Robillard-Frene, as well as Bertrand Bouchard for his invaluable help in daily problems and Guy Charron for his incredible expertise in molecular biology. Not in the lab, but always by my side helping was Marie-Pierre Mathieu. Her support helped through those long evenings at the lab and her dedication saved me more than once. I am also grateful to Sylvie Picard and Sonia Bélanger for their untiring assistance with the animals and their genotyping.

I also wish to thank Dr Young and Dr Lopaschuk for their invaluable collaboration and advice. Also providing me with a great collaboration and amazing technical help is Dr Allen.

Last, but certainly not least, I would like to express a special thank you to Maya Khairallah, not only for being an incredible sister but also for assisting me in the lab and teaching me everything I know about research today. Your help and support, technical, intellectual and emotional was invaluable. I will never forget your support and will be forever indebted to you. Finally, I wish to thank my parents for their constant encouragements, precious advice and unconditional love. They have inspired me, guided me and believed in me. To them, I dedicate this thesis.

Chapter I - Introduction

Heart disease is the number one cause of mortality in the world, responsible for one in every three deaths and affecting women and men equally, irrespectively of geographic location or economic status. Surprisingly, despite numerous advances in therapies and surgical procedures, the rate of mortality and morbidity has not decreased considerably. Heart disease comprises multiple disorders that can either be inherited, such as familial hypertrophic cardiomyopathies, dilated cardiomyopathies and other genetic disorders, or, more commonly acquired, such as hypertensive cardiomyopathy, valvular disease, myocarditis and coronary heart disease (5-7).

Given the prevalence of heart disease, it is of utmost importance to better understand the mechanisms responsible for these disorders and what may protect against it. Therefore, this Master's project was aimed at characterizing the metabolic outcomes of a cardioprotective anti-hypertrophic pathway, namely the cyclic guanosine monophosphate (cGMP) signaling pathway. These findings will be presented in chapter II, and discussed in chapter III. However, at first, an overview of notions related to this project is mandatory. We will start by introducing the concepts of myocardial remodeling and hypertrophy, then go on to review the major molecular pathways involved in the pathogenesis of hypertrophy. We will then discuss anti-hypertrophic pathways and more specifically, the nitric oxide (NO)/cGMP pathway, and how all of this relates to myocardial energy metabolism, a determining factor in the development of heart disease and progression to heart failure (8;9).

Cardiac hypertrophy and its progression to failure

Faced with an increased workload, the heart undergoes a stage of remodeling that requires a rearrangement of the normal structure of the heart. Although tissue remodeling does not necessarily define a pathological condition, myocardial remodeling is most often found in diseased conditions (6) and is generally triggered by increased mechanical stretch due to hypertension (10) or other causes including ischemia, hormonal and vasoactive peptide stimulation and myocardial scarring (11;12). However, most cases arise from a complex association of all the above underlined causes. Other types of non-pathological remodeling also occur, such as gestational and developmental hypertrophy. For practical purposes, such cases of so-called "physiologic hypertrophy" will not be considered further in the present discussion. It is noteworthy that in contrast to developmental growth of the heart, which occurs through hyperplasia of cardiac muscle cells, growth of the heart after birth occurs primarily through hypertrophy—an increase in the size of cardiac muscle cells. Normal growth of the heart postnatally or in conditioned athletes—so called 'physiological' hypertrophy— enhances cardiac output to meet increased metabolic demands and is molecularly distinct from pathological hypertrophy in response to stress signals and injury.

Multiple alterations are implied by the term remodeling, both at the anatomical and cellular level. The most obvious anatomical differences are the increase in ventricular mass and volume and the change of shape of the ventricle, all of which are components of what is known as ventricular hypertrophy (13;14).

One can distinguish several forms of pathological hypertrophy. Firstly, hypertrophy can be either: (i) symmetric, originating from a general process in which there is no infarct, and (ii) asymmetric, triggered by a myocardial infarct and associated with infarct expansion. In asymmetric hypertrophy, remodeling is the result of volume-overload hypertrophy of non-infarcted segments. These are faced with an increased workload due to the loss of tissue in the ischemic area. Additionally, there is chamber volume enlargement, lengthening of ventricular perimeters, eventually leading to decreased stroke volume and impaired diastolic filling (15). Symmetric hypertrophy can be further be divided into concentric or dilated hypertrophy. Concentric hypertrophy involves the whole ventricle, and is characterized by a thick ventricular wall and septum, a normal internal volume and wall stress, and a high mass-to-volume ratio (16). Dilated hypertrophy presents itself as a gradual increase in left ventricular end-diastolic and end-systolic volumes, wall thinning, and a change in chamber geometry to a more spherical, less elongated shape. This process is usually associated with a continuous decline in ejection fraction (17). In all cases of hypertrophy, there is a significant increase in cardiomyocyte cell size with no change in number, whereas interstitial cells may undergo significant hyperplasia, leading to fibrosis.

Pathological remodeling and hypertrophy is also associated with myocyte loss, myocardial damage, and multinucleation (18;19). At the cellular level, hypertrophied cardiomyocytes show evidence of increased DNA damage and repair (20), increased protein synthesis (21;22) and a reemergence of a fetal molecular phenotype (23-25). Indeed, in response to an increase in workload, hypertrophied hearts express genes usually associated with the developing heart.

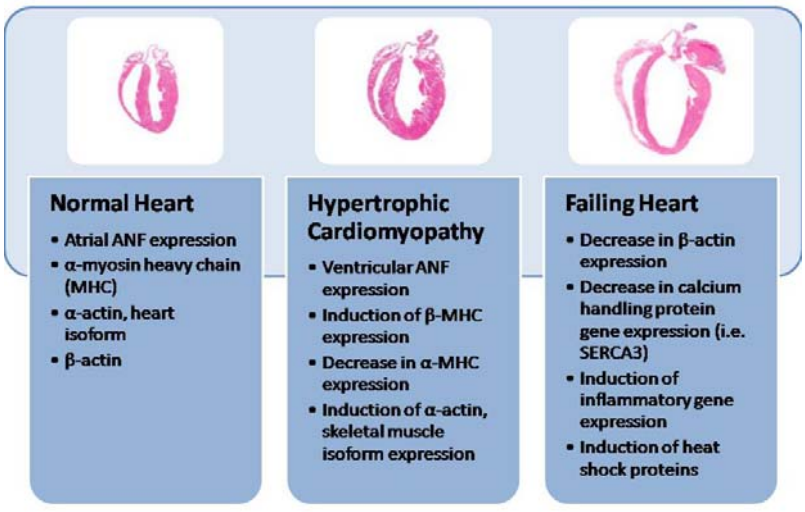


Figure 1. **Changes in gene expression in the hypertrophic and failing heart**. ANF, Atrial natriuretic factor. MHC, myosin heavy chain. SERCA3, sarcoplasmic reticulum calcium ATPase. (Adapted from (2))

This fetal gene program includes a switching of structural proteins from the 'adult' to 'fetal' isoforms (i.e. α-myosin heavy chain (MHC) to β-MHC) (26;27), return of ventricular expression of the atrial natriuretic factor (ANF) gene (28), and induction of growth factors such as transforming growth factor β (TGF-β) (29) (Figure 1). Other hallmark changes in phenotype include a switch from fatty acids to carbohydrates as energy substrates (30), and re-expression of proto-oncogenes (such as c-fos) (31). This dynamic adaptation is initially seen as beneficial and required to sustain cardiac output in the face of stress. However, a prolonged hypertrophic response to stimuli is maladaptive and associated with a significant increase in the risk of progression to heart failure (32-34).

Systolic heart failure, a complex clinical syndrome characterized by impaired cardiac output and circulatory congestion, is the primary cause of human morbidity and mortality, affecting an estimated 5 million Americans with a 5-year mortality rate of about 50%. Over the past decade, there have been major advances in the identification of genes and signaling pathways underlying these cardiac abnormalities, many of which are triggered by the mishandling of ions by cardiac muscle cells.

While some stress signals result in cardiac hypertrophy, which can progress to left ventricular dilatation and heart failure in which the heart is unable to pump sufficient blood to meet the metabolic demands of the body, others can cause cardiac dilatation and failure without an intermediate hypertrophic stage.

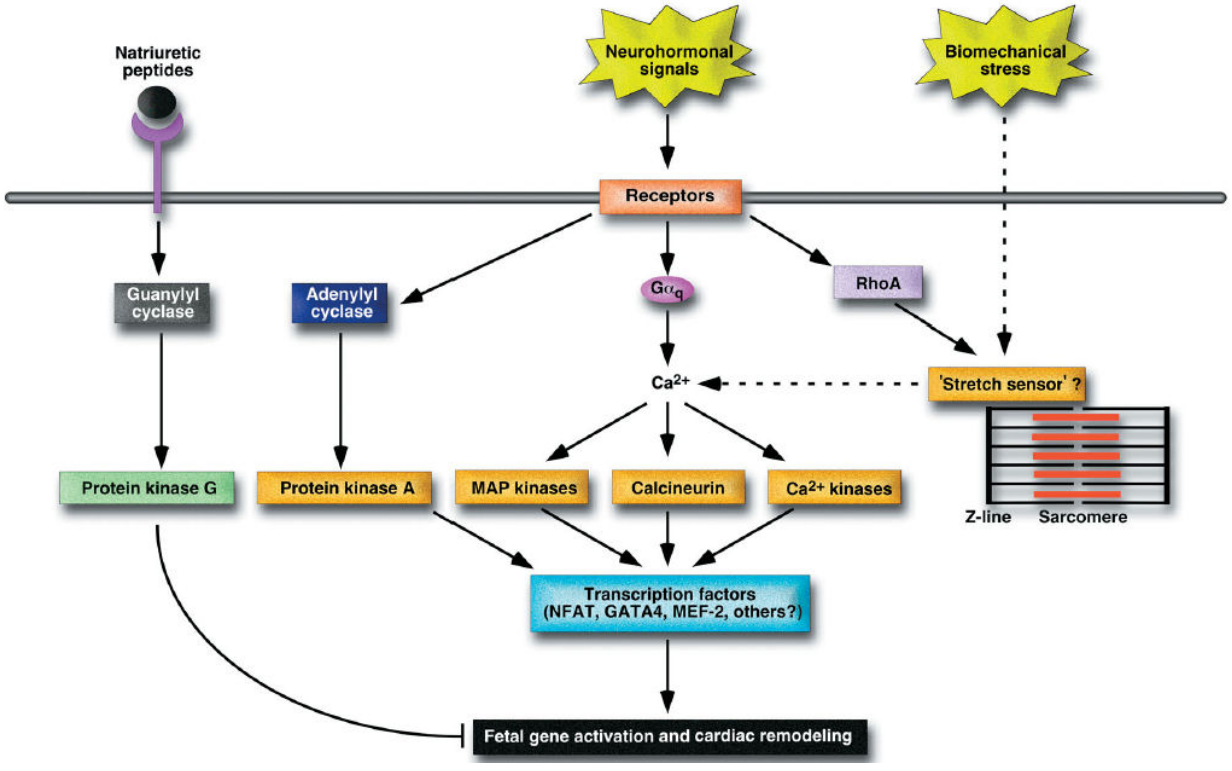


Figure 2. Hypertrophic signaling pathways that influence the growth of the adult heart. Neurohormonal signals or biomechanical stress can cause pathological cardiac hypertrophy, characterized by fetal gene activation and remodeling. Multiple interacting signaling pathways have been shown to connect stress signals with cardiac gene expression through a set of signal-dependent transcription factors. (Adapted from (4))

The balance between cell survival and apoptotic pathways appears to be a major determinant of the transition from hypertrophy to ventricular dilatation.

Molecular pathways leading to cardiomyocyte hypertrophy

Numerous cardiomyocyte autonomous and endocrine/paracrine pathways have been implicated in the heart's molecular response to increased wall stress and the development of hypertrophy (Figure 2). We will first overview some of the most relevant pro-hypertrophic pathways, then continue with a description of anti-growth signaling pathways including the cGMP pathway, a major theme of this Master's project.

Calcineurin- Nuclear Factor of Activated T-cells (NFAT) signaling

Calcineurin is a serine-threonine phosphatase expressed in a variety of tissues. It consists of two subunits, A and B and is activated by calmodulin in response to elevations in cytoplasmic calcium.

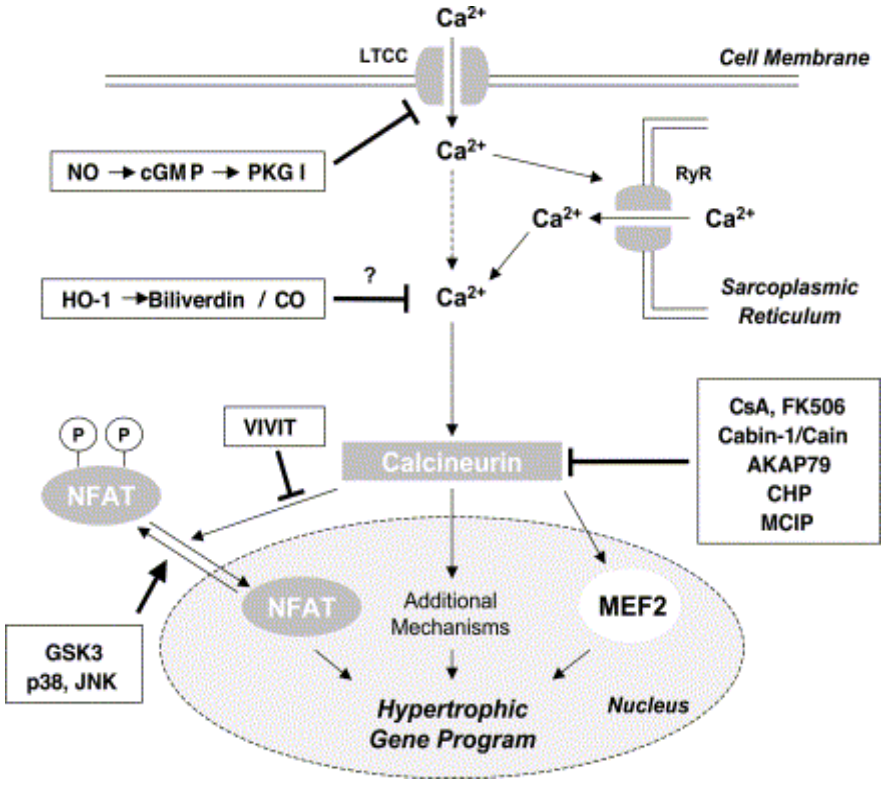


Figure 3: **The pro-hypertrophic Ca^{2+}–calcineurin–NFAT signaling pathway and its regulation.** NO, nitric oxide; PKG I, cGMP-dependent protein kinase type I; HO-1, heme oxygenase-1; CO, carbon monoxide; CsA, cyclosporine A; Cabin-1, calcineurin-binding protein-1 (also known as calcineurin-inhibitory protein, Cain), AKAP79, A-kinase-anchoring protein-79; CHP, calcineurin B homology protein; MCIP, modulatory calcineurin-interacting proteins; GSK3, glycogen synthase kinase-3; JNK, c-Jun N-terminal kinases; p38, p38 mitogen-activated protein kinase; →: stimulation; ⊣: inhibition. Adapted from (3).

Calcineurin dephosphorylates factors of the NFAT family, unmasking their nuclear localization signals and promoting their translocation to the nucleus where they enhance transcription of immune response genes (35) (Figure 3).

Recently, it has been shown that constitutive activation of calcineurin in transgenic mouse hearts is sufficient to induce massive cardiac enlargement (36). Constitutive translocation of NFAT to the nucleus by mutating its phosphorylation site produces a similar result, suggesting that NFAT is the main mediator of the hypertrophic effects of calcineurin.

Several endogenous inhibitors of calcineurin, such as AKAP79, cabin/cain, myocyte-enriched calcineurin-interacting proteins (MCIP), have been discovered and are subject to intense study due to their potential therapeutic effects. Overexpression of any of these inhibitors blunts the increase in calcineurin activity seen in hypertrophy, as well as the hypertrophic response to stimuli (37-40). Interestingly, induction of ventricular expression of ANF was not impaired in calcineurin-null mice (41), suggesting that the pathways leading to cardiac hypertrophy and to initiation of the fetal gene program are, at least in part, dissociated. The multiple studies undertaken over the years to elucidate the role of the NFAT-calcineurin pathway provide strong evidence for its involvement in the development of hypertrophy. Moreover, it has emerged that this signaling pathway is intimately intertwined with other important hypertrophic pathways, such as those controlled by the phosphoinositide-3-kinase (PI3K)/Akt/ glycogen synthase kinase 3 (GSK-3) pathway and by mitogen-activated protein kinases (MAPK) (40).

PI3K/Akt/GSK-3-dependent signaling

PI3K belongs to a family of protein and lipid kinases, responsible for cell growth, survival and proliferation. They are activated by a variety of receptor tyrosine kinases, such as insulin-like growth factor receptor 1 as well as G-protein coupled receptors (GPCR) (42). It is activated by pressure-overload hypertrophy and its constitutive activation results in significant organ enlargement (43;44). Evidence suggests that the hypertrophic response due to PI3K activation is solely due to cell enlargement rather than cell proliferation, which is consistent with the definition of true hypertrophy. Downstream of PI3K is the serine/threonine kinase Akt, also known as protein kinase B. Akt is activated by binding to phosphoinositides phosphorylated by PI3K, and is translocated to the membrane. Again, Akt has been demonstrated to control cell-size and its chronic activation is sufficient to induce cardiac hypertrophy without affecting systolic function (45-48). Akt acts through two main downstream targets: GSK-3 and mTor (Figure 4).

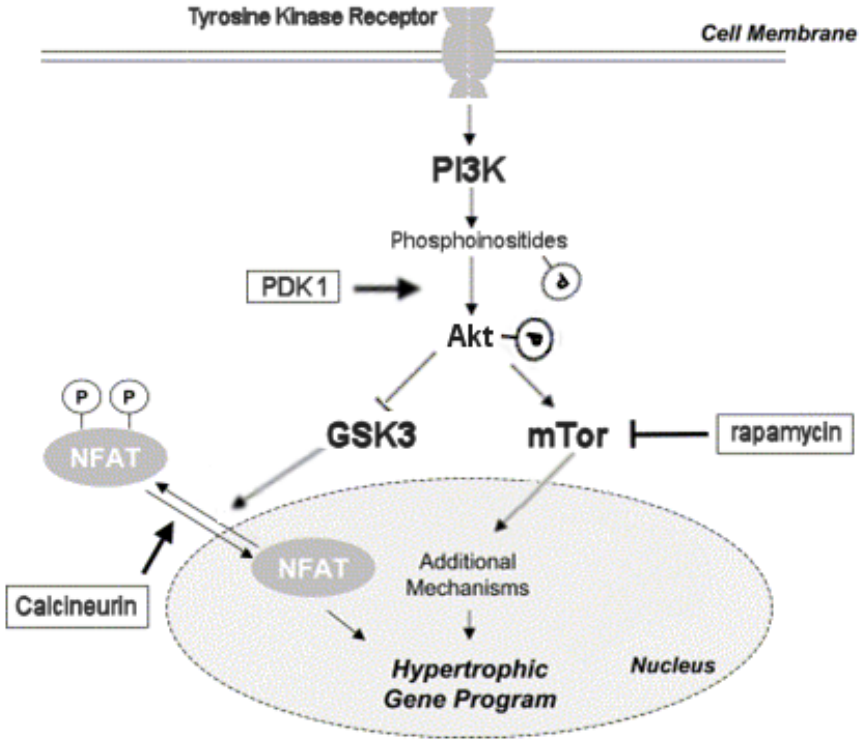


Figure 4: **The pro-hypertrophic PI3K/Akt pathway and its regulation**. PI3K, phosphoinositide-3-kinases; PDK 1, phosphoinositide-dependent kinase 1; GSK3, glycogen synthase kinase 3; mTor, mammalian target of rapamycin; NFAT, nuclear factor of activated T-cells →: stimulation; ⊣ : inhibition. (Adapted from (3))

The first important target of Akt is GSK-3. Conversely to calcineurin, GSK-3 phosphorylates a variety of NFAT proteins and inhibits their translocation to the nucleus (49). When Akt phosphorylates GSK-3, it renders it inactive thereby enabling NFAT activation through other pathways, such as seen by calcineurin. Evidence suggests that inactivation of GSK-3 is a crucial step for the development of hypertrophy (50). However, it seems that Akt phosphorylation is necessary but not sufficient to completely inactivate GSK-3 (45;51). Many of the transcriptional factors inhibited by GSK-3 have been implicated, directly or indirectly, in the development of cardiac hypertrophy. These include, GATA4, c-Jun, c-myc and eukaryotic initiation factor 2B (52-55). Not surprisingly, constitutively active GSK-3 blunts the hypertrophic response to pressure-overload and adrenergic stimulation (56). Again, overexpression of GSK-3 simultaneously with calcineurin lead to inhibition of the development of hypertrophy but does not affect the initiation of the fetal gene program (56), suggesting a dissociation between the two phenomena. In addition, GSK-3 activation upregulates ANF expression while suppressing cardiac growth, suggesting that the effects of this kinase might be mediated in part by ANF (44;57).

Another well-defined downstream target of Akt is mammalian target of rapamycin (mTor). This large serine/threonine kinase is implicated in the regulation of protein translation and is inhibited by a complex formed of rapamycin and fructose 1,2-bisphosphate kinase. Inhibition of mTor results in impaired protein synthesis via inhibition of ribosome assembly and protein translation (58). Not surprisingly, rapamycin is also capable of attenuating cardiac hypertrophy secondary to constitutive

activation of Akt (45), adrenergic stimulation (59), angiotensin II stimulation (60) or pressure-overload (61). Again, inhibition of mTor does not affect the expression of fetal genes (59).

Taken together, it is evident that activation of the PI3K/Akt promotes hypertrophy through two distinct pathways, namely activation of pro-hypertrophic signaling through mTor and inhibition of anti-hypertrophic signaling through GSK-3. Furthermore, it appears that there is significant crosstalk between PI3K/Akt/GSK-3 and other hypertrophic pathways, most importantly with calcineurin/NFAT and GPCRs (62). With the former having been discussed previously, we will now review the mechanisms by which GPCRs promote pathological growth.

GPCR signaling

It has long been known that norepinephrine (63), angiotensin II (64) and endothelin (65) all result in cellular hypertrophy, characteristic changes in cardiac gene expression, and activation of specific kinase signaling pathways. Interestingly, all these molecules transmit their signal to the cytosol in a similar fashion, through GPCRs. GPCRs play an important role in the regulation of cardiac function and adaptation to changes in hemodynamic burden (66). Also known as heptahelical receptors, they form a large protein family of transmembrane receptors whose cellular signaling actions are mediated by G proteins. Upon activation of the receptor by its ligand, the heterotrimeric G protein subunits from either one of the three principle classes: G_q/G_{11}, G_s, or G_i, dissociate and initiate signal transduction pathways. Not surprisingly, receptors for many hypertrophic ligands signal through the same type of G protein, namely G_q/G_{11}, including the aforementioned norepinephrine, angiotensin II and endothelin (Figure 5). When receptors associated with G_q/G_{11} are overexpressed in cardiomyocytes, it is sufficient to mediate cardiac hypertrophy (67). Similarly, overexpression of the G protein by itself promotes organ growth and leads to a cardiomyopathy with depressed contractile functions (68). Conversely, G_{11} null mice with a heart-specific conditional knockout of G_q resulted in an almost complete lack of all features of pressure-overload hypertrophy including activation of the fetal gene program (69). However, overexpression of a dominant-negative mutant of G_q attenuated the hypertrophic response while preserving cardiac contractility (70), thereby shedding doubt on whether organ growth is adaptive. Finally, indirect evidence for an important role of G_q/G_{11} ligands stems from clinical observations in which patients suffering from cardiomyopathy and treated with either angiotensin II receptor blockers and angiotensin converting enzyme inhibitors showed signs of beneficial effects, such as inhibition of cardiac remodeling that exceeded their antihypertensive properties (71).

Another highly expressed receptor in cardiac tissue is the β1-adrenergic receptor. This GPCR is coupled to G_s, which in turn activates adenylyl cyclase (AC) (Figure 5). AC produces cAMP, an important second messenger and mediator of multiple inotropic, chronotropic and lusitropic effects on the heart (72). In contrast to the G_q/G_{11} coupled receptors, overexpression of the β1-adrenergic receptor, while initially providing beneficial effects on the contractile function, rapidly degenerates into cardiomyocyte hypertrophy, fibrosis and deterioration of cardiac performance (73). A similar phenotype was achieved through heart specific overexpression of the G_s protein, but surprisingly, this result was not dependant on AC activation (74). This is supported by the observation that overexpression of an AC isoform has no adverse effects on cardiac function and might even be beneficial in preventing cardiomyopathic changes

in multiple models of hypertrophy (75;76). Peculiarly, protein kinase A, a major effector downstream of AC and a target of cAMP, has been shown to promote the development of hypertrophy, dilated cardiomyopathy and fibrosis (77), explaining the hypertrophic effects of G_s signaling but contradicting the beneficial effect of AC. Another target of G_s signaling, the pro-hypertrophic Ras/Raf signaling pathway, also supports a role for this type of GPCR in hypertrophy (1). Given the critical role of β-adrenergic receptors in the development of the pathology, β-blocker administration (which antagonizes β-adrenergic receptors) is now a very common therapy in patients with heart disease and has been shown to: 1) improve survival in heart failure, and 2) induce a regression of fetal gene expression, as evidenced by increased α-MHC and sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) 2a transcription (78).

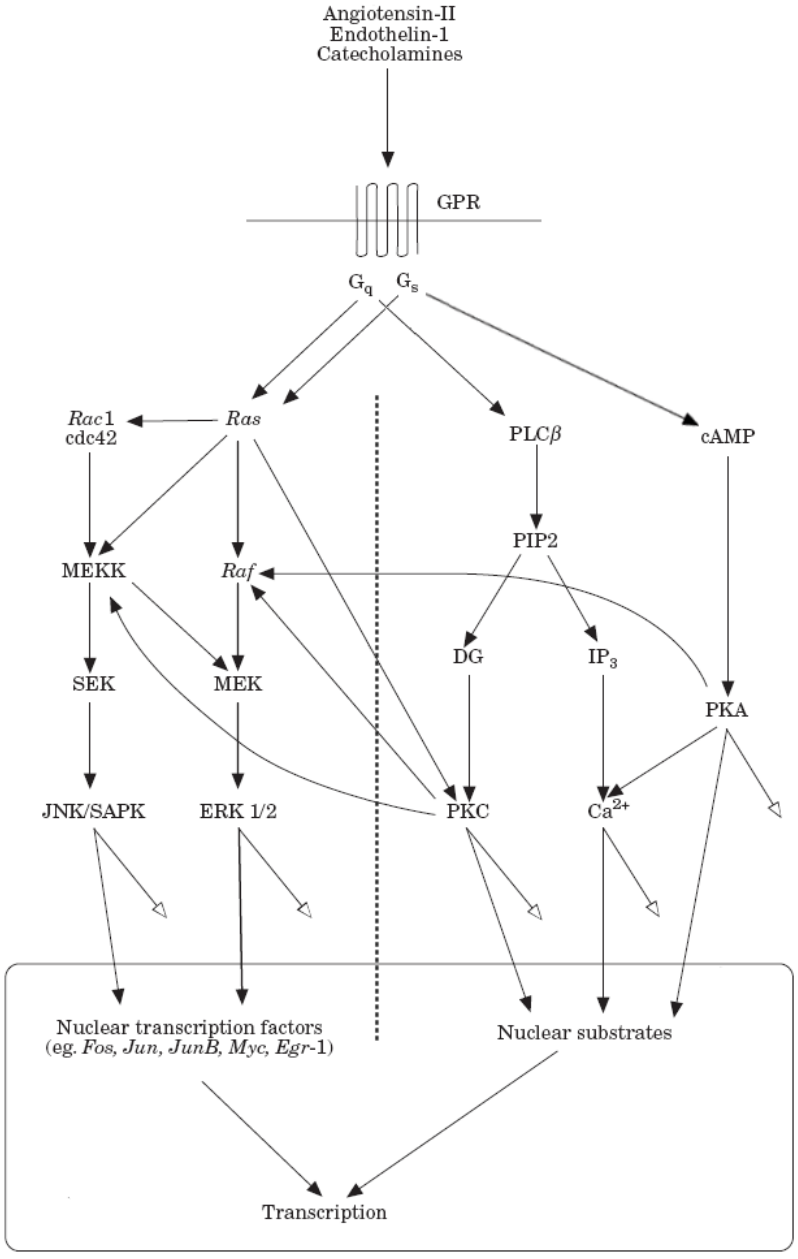


Figure 5: GPCR signaling in the hypertrophied heart. Adapted from (1).

As previously stated, most GPCRs, both those coupled to G_s or to G_q/G_{11}, will activate one or more MAPK pathways, which will be discussed below.

MAPK Pathways

MAPK pathways provide an important link between external stimuli and the nucleus via phosphorylation and regulation of multiple transcription factors. Generally, these are separated into three distinct subfamilies: extracellular responsive kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 MAPK (Figure 6). Contrarily to the ERKs, which only respond to anabolic stimuli and GPCRs, JNKs and p38 MAPKs can be also activated by pathological stress, one of them being ischemia (79). Interestingly, inhibition of the MAPK phosphatase 1, which blocks all three major branches of the pathway, prevents the development of pressure-overload hypertrophy (80;81), thus demonstrating the importance of these pathways in hypertrophic signaling.

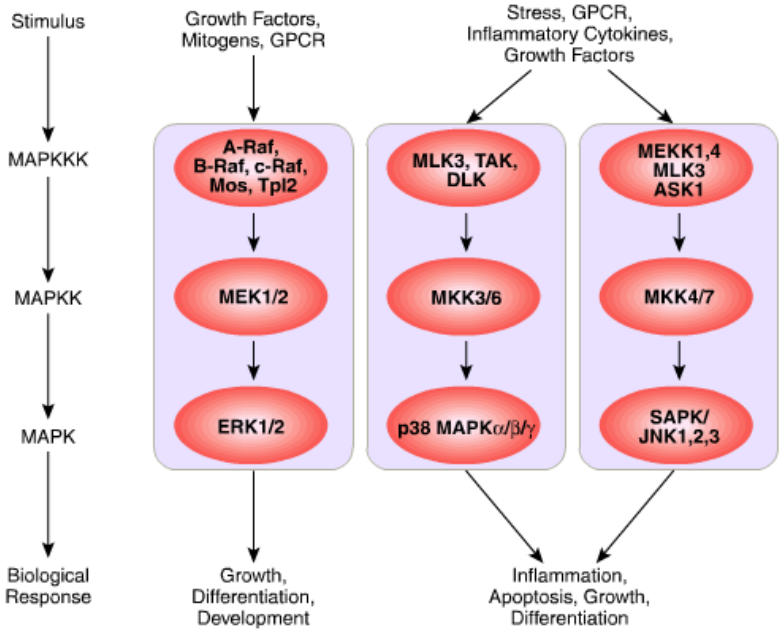


Figure 6. Mitogen activated protein kinase (MAPK) signaling cascades. GPCR, G-protein coupled receptor. MAPKKK, MAPK kinase kinase. MAPKK, MPAK kinase. ERK, extracellular responsive kinase. JNK, c-Jun N-terminal kinases. (From www.cellsignal.com)

ERK signaling in hypertrophy

Significant controversy surrounds the involvement of the ERK pathway in hypertrophy and whether it constitutes a pro- or anti-hypertrophic pathway. Indeed, while one study reports that inhibition of the pathway, either directly by anti-sense ERK 1/2 oligonucleotides, or upstream by inhibition of the MAP/ERK kinase (MEK) 1/2 (82), attenuates the hypertrophic response to agonist stimulation in cultured cells, others fail to document the same effect (83). Moreover, ERK1/2 activation is not observed in transgenic hearts overexpressing G_q (84), but transgenic overexpression of MEK1, a MAPK kinase that activates ERK1/2, but not JNKs or p38 MAPKs, results in considerable cardiac hypertrophy (85). Surprisingly, it was also found that ERK 1/2 activation was necessary for ANF-induced cardioprotection (86). Thus, the exact role of this pathway is still unclear and further research is needed.

JNK signaling in hypertrophy

JNKs are activated through phosphorylation by either MAPK kinase kinase (MKK) 4 or MKK7, which in turn are regulated by MEK kinase (MEKK) 1 phosphorylation. In cardiomyocytes, mechanical stretching or agonist stimulation by endothelin-1, phenylephrine, or angiotensin II results in rapid phosphorylation of JNK (87-89). Furthermore, evidence for an important role of JNK signaling in cardiac hypertrophy stems from studies in mice with a targeted disruption of the MEKK1 gene, which results in selective attenuation of JNK activity. Indeed, this model is resistant to G_q-induced hypertrophy (90) but not to aortic banding-induced hypertrophy. On the contrary, inhibition of JNK seemed to accelerate the development of heart failure (91). Thus, it is apparent that JNK inhibition is not a suitable therapeutic approach (or strategy).

p38 signaling in hypertrophy

There are multiple isoforms of p38 that are present in different amounts in the heart. Similar to the other MAPK branches, p38 is activated in neurohumoral and pressure-overload hypertrophy (92). Furthermore, it was shown that activation of the upstream kinases of p38, MKK3 and MKK6, is sufficient to induce cardiomyocyte hypertrophy and ANF-induction *in vitro*. In addition, TAK1, which is upstream of MKK3/6, is upregulated and activated *in vivo* after aortic banding and its constitutive activation results in cardiac hypertrophy and subsequently failure in transgenic mice, further implicating this branch of MAPK signaling in pathological growth of the myocardium (93). Interestingly, p38 phosphorylates several transcription factors involved in hypertrophic gene expression, including myocyte enhancer factor 2 (94) and NFAT3 (95).

Antihypertrophic pathways

After having reviewed the causes and consequences of myocardial remodeling, we will now look at the possible anti-hypertrophic pathways and therapeutic potential of each one. As outlined above, multiple endogenous and exogenous can inhibit hypertrophy by disrupting hypertrophic pathways, either by pharmacological means or molecular interactions. However, as common in biological systems, there are several counter-regulatory signaling pathways that oppose the actions of pro-hypertrophic pathways.

The Nitric Oxide (NO) signaling pathway

Nitric oxide is a free radical gas with a very short half-life lasting only seconds and is readily diffusible. It acts as a second messenger, an autocrine and a paracrine factor, to mediate many physiological processes such as vascular tone, proliferation, apoptosis, neurotransmission, platelet aggregation, and immune reactions. However, at high concentrations, NO becomes cytotoxic and is thought to play a role in the pathology of several inflammatory diseases such as arthritis, myocarditis, colitis, nephritis, and other conditions such as cancer, diabetes, and neurodegenerative diseases (96). NO is synthesized from L-arginine by the catalytic reaction of different isoforms of NO synthases, including the neuronal type 1 isoform (nNOS), the inducible type 2 isoform (iNOS), and the endothelial, type 3 isoform (eNOS), all three of which are expressed in the cardiomyocyte (97). nNOS and eNOS are constitutively expressed and produce low amounts of NO when activated by Ca^{2+}. iNOS, by contrast, is expressed only in response to appropriate stimuli (e.g. cytokines) and can produce large amounts of NO independently of Ca^{2+}. The distribution of the several isoforms in the cells allows for a highly compartmentalized signaling and sometimes opposing effects. Indeed, (i) nNOS is localized to the sarcoplasmic reticulum in close proximity to the Ca^{2+}-release channel SERCA, thereby promoting enhanced contractility, (ii) eNOS is localized in caveolae at the cell membrane near L-type Ca^{2+} channels (LTCC) through which it can reduce contractility and (iii) iNOS is localized in the cytosol (97). Furthermore, given that NO is readily diffusible, production of this molecule in other cell types in close proximity to the cardiomyocyte, such as endothelial cells, sympathetic varicosities and postganglionic parasympathetic fibers will also affect cell function (98).

NO can act in two specific manners. While most of its action is mediated by activating a soluble guanylate cyclase (sGC) to produce cGMP, its free radical nature also allows it to have direct effects on multiple enzymes, such as the complex 3 of the mitochondrial respiration chain. Furthermore, NO can react with the superoxide free radical producing peroxynitrite, a very strong oxidant with the capability to bind proteins and DNA and cause irreversible damage (99).

While its implication in the fine-tuning of cardiac function has long been known, more recent observations found that it also exerts potent anti-hypertrophic effects. Indeed, it has been shown that both nNOS and eNOS deficient mice spontaneously develop cardiac hypertrophy and a double knockout produces an even more severe phenotype (100). A study in spontaneously hypertensive rats (SHR) provided the first evidence that NO can directly promote anti-hypertrophic effects in the heart (101). As shown in this study, chronic treatment with the NO precursor L-arginine attenuates cardiac hypertrophy in SHR. Importantly, L-arginine administration suppressed cardiac hypertrophy without changes in blood pressure, suggesting a direct cardiac effect, rather than a peripheral vasculature effect. The direct anti-hypertrophic properties of NO were later confirmed in cultured cardiac myocytes, where NO donors and endogenously formed NO were shown to blunt the hypertrophic response to growth factor stimulation (57) and then *in vivo*, where overexpression of eNOS blunted the hypertrophy induced by isoproterenol infusion (102).

The natriuretic peptide signaling pathway

Natriuretic peptides are a family of structurally similar endogenous peptides with complex and distinct functional roles in maintaining normal homeostasis and responding to pathological circumstances. The first identified member of this family, atrial natriuretic peptide (ANP), is mainly synthesized and secreted in the cardiac atria under normal conditions. However, the ventricular myocardium will also produce ANP during fetal development, hypertrophy, or heart failure. Brain natriuretic peptide (BNP) was first isolated from the porcine brain. Surprisingly, it turns out that despite its name, it is preferentially synthesized and secreted by ventricular cardiac myocytes and, like ANP, exhibits increased expression during hypertrophy and heart failure. In contrast, C-type natriuretic peptide (CNP) is mainly produced by vascular endothelial cells and neurons, whereas urodilatin, the latest natriuretic peptide to have been discovered (103), is synthesized and secreted by renal cells (104).

Actions of natriuretic peptides are mediated through binding to 3 distinct natriuretic peptide receptors (NPRs) that are located on the cell surface and bind endogenous ligands with varying specificities and affinities. Two of the receptors, NPR-A and NPR-B, have an extracellular ligand binding domain linked to an intracellular catalytic domain with guanylate cyclase activity. Consequently, binding to NPR-A and NPR-B activates the guanylate cyclase and increases intracellular cGMP (103). The third natriuretic peptide receptor, NPR-C, has no catalytic domain or guanylate cyclase activity, and contributes to the clearance of natriuretic peptides from the circulation trough endocytosis. NPR-A receptors exhibit high affinity for ANP and BNP but have relatively low affinity for CNP. In contrast, NPR-B receptors bind with high affinity to CNP, but not ANP or BNP. The NPR-C receptor binds ANP, BNP, and CNP with approximately equal affinity (104). Differences in the tissue distributions of NPR subtypes and the potential for disease-related alterations further complicate the biology of endogenous natriuretic peptides (103).

Early work examining the functional biology of ANP and BNP supported the notion that endogenous natriuretic peptides had no direct effects on the myocardium, although their anti-growth properties were demonstrated in other tissues such as in the kidneys, neurons, thymus and vasculature (104). However, evidence later emerged that (i) cardiac mass and ventricular expression of ANP were both associated (in an inverse fashion) with a naturally occurring allele of natriuretic peptide precursor A (the gene coding for ANP) (105), (ii) heart-specific inactivation of natriuretic peptide receptor A leads to cardiac hypertrophy (106), and finally, (iii) ANP prevented the hypertrophy in cultured cells (57).

Similarly to NO, natriuretic peptides also cause increases in intracellular cGMP and it was shown that both signaling pathways derive their anti-hypertrophic effects from the activation of downstream cGMP mediated signaling processes.

The cGMP signaling pathway

cGMP, discovered not long after cAMP, is present in almost all cells and is known as a second messenger that regulates many cell signaling events, such as the activation of protein kinases, ion channels, and phosphodiesterases (PDEs) (107). It is synthesized from intracellular GTP by either cytoplasmic soluble guanylate cyclases, which are activated by NO, or by membrane receptors with intrinsic guanylate

cyclase activity, which are activated by natriuretic factors/peptides (ANF and BNF). One major target of cGMP is the cGMP-dependent protein kinase (PKG). When activated, PKG acts as a serine/threonine kinase phosphorylating many substrates, including the inositol-1,4,5-triphosphate (IP_3) receptor, phospholamban, the phosphatase inhibitor G substrate, and subunits of myosin light chain phosphatase. Another target for cGMP is the cyclic nucleotide-gated channels, which regulate the influx of Na^+ and Ca^{2+} into cells, and are opened by cGMP binding. Finally, cGMP also plays a role in the activation of cGMP-regulated phosphodiesterase (PDE). PDEs are crucial for the hydrolysis and modulation of the intracellular concentrations of cyclic nucleotides. There are multiple families of PDEs that differ in their specificity for cGMP and/or cAMP hydrolysis, as well as tissue specific expression. PDE5, PDE6 and PDE9 are specific for cGMP. Details about the anti-hypertrophic effects of cGMP as well as mechanisms will be discussed briefly in chapter 2 and further in chapter 3.

Cardiac energy metabolism

Given the importance of energy metabolism in the context of this Masters project, the following sections will provide a literature review on the heart's metabolic pathways and regulation based on several references (108-130).

In order to continuously support its crucial contractile activity, the heart has constant high-energy demands. Simultaneously, it also has to maintain its specialized cellular processes, including ion transport, sarcomeric function, and intracellular Ca^{2+} homeostasis. Not surprisingly, several kilograms of ATP are synthesized and hydrolyzed every day. The healthy heart's energy requirements are met by the oxidation of exogenous substrates, predominantly carbohydrates, such as glucose, lactate and pyruvate, and free fatty acids, mainly oleate and palmitate. The heart is also considered as a metabolic omnivore. Ketone bodies and amino acids can also be utilized as an energy substrate, but the latter is not a major contributor under normal conditions. The heart is also able to quickly switch from one substrate to another in response to multiple stimuli, including substrate availability, oxygen supply, hormone levels and workload. The regulation of substrate switching can be either acute or chronic in response to short-term or long-term changes in energy demand. Since 1895, many studies have shown that metabolic regulation is inextricably linked with cardiac function and that metabolic alterations can be a cause and a consequence of heart failure, hence our particular interest in energy metabolism in the context of the hypertrophic cardiomyopathy.

Cardiac energy substrate utilization pathways and its acute regulation

General overview

Under normal conditions of substrate and oxygen availability, fatty acids (FAs) are the preferred substrate in the adult myocardium, supplying 60 to 80% of total ATP. They are oxidized to acetyl-CoA in the mitochondrial matrix and to a lesser extent in peroxysomes, by the process of FA β-oxidation, whereas pyruvate derived from glucose, glycogen, lactate, exogenous pyruvate and certain amino acids, is oxidized to acetyl-CoA by the pyruvate dehydrogenase (PDH) complex, localized within the inner mitochondrial membrane. This acetyl-CoA derived from both pathways, fuels the citric acid cycle (CAC).

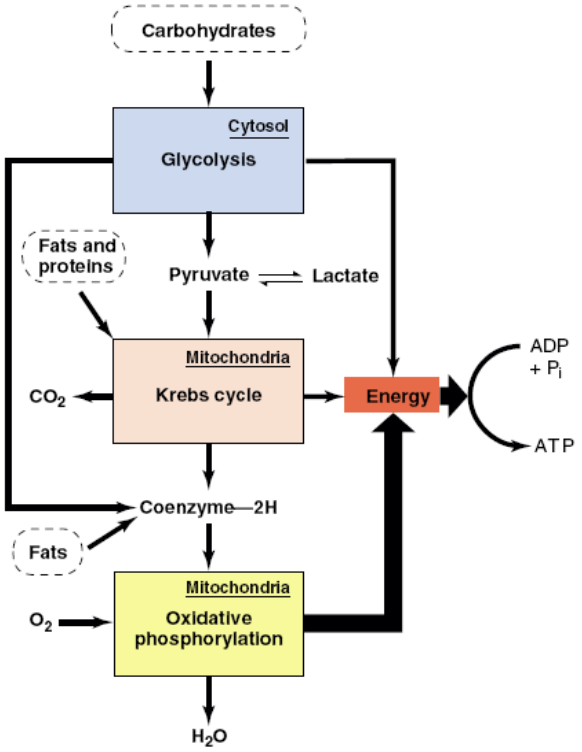


Figure 7. Pathways linking the energy released from the catabolism of fuel molecules to the formation of ATP. From Human Physiology: The Mechanisms of Body Function, Vander 8th Ed.

Reduced flavin adenine dinucleotide ($FADH_2$) and reduced nicotinamide adenine dinucleotide (NADH) are generated by glycolysis. The oxidation of lactate and pyruvate, FA β-oxidation, as well as the CAC, provide electrons to the electron transport chain, resulting in ATP synthesis in the presence of molecular oxygen. This process is referred to as oxidative phosphorylation. Almost all of ATP formation (>90%) in the heart comes from oxidative phosphorylation in the mitochondria, with the remainder derived from glycolysis and guanosine triphosphate (GTP) formation in the CAC. Approximately 60-70% of ATP hydrolysis fuels contractile function, and the remaining 30-40% is primarily used for the ion pumps. Numerous studies suggest that glycolytically generated ATP is preferentially used by ion channels such as SERCA and that glycolytic enzymes form complexes with these channels on the sarcoplasmic membrane (131).

Carbohydrate metabolism

In the heart, glucose is derived either from the bloodstream or from intracellular stores of glycogen. Oxidation of glucose can be divided into two major pathways: anaerobic glycolysis (Figure 8), resulting in the production of lactate and aerobic oxidation resulting in the production of acetyl-CoA for oxidation in the CAC. Intracellular pyruvate constitutes the branch point molecule where glucose commits to either pathway. These reactions as well as their regulation will be discussed below.

Glycolysis

Glycolysis can be seen as consisting of two separate phases. The first is the chemical priming phase requiring energy, and the second is considered the energy-yielding phase. In the first phase, two ATPs are used to convert glucose to fructose-1,6-bisphosphate, which is degraded in the second phase to pyruvate, with the production of four ATP and two NADH. Overall, the net production of the anaerobic breakdown of glucose is two molecules of pyruvate as well as two ATPs and two NADH that are either shuttled into the mitochondria for ATP synthesis via oxidative phosphorylation or used for the conversion of pyruvate to lactate in the cytosol. Glycolysis is regulated at several levels, but has two major control points, namely glucose uptake and the phosphofructokinase-1 (PFK-1) reaction.

Glucose transport into cardiomyocytes is regulated by the transmembrane glucose gradient and the content of glucose transporters in the sarcolemma. The glucose transporter that is predominantly expressed at the surface of adult cardiomyocytes is the insulin-sensitive GLUT-4 isoform, also expressed in adipose tissues and skeletal muscles. The GLUT-1 transporter, which is presumably independent of insulin action and predominant in the fetal myocardium, is also present in adult cardiomyocytes, although it is about five times less abundant than GLUT-4. The normal heart also expresses low amounts of GLUT-3, which has a K_m below the normal plasma glucose concentration and therefore thought to contribute only marginally to glucose uptake in the heart.

The second regulatory step is PFK-1. This enzyme catalyzes the first irreversible glycolytic step and major rate-determining reaction. PFK-1 utilizes ATP to produce fructose-1,6-bisphosphate. Citrate is an important negative allosteric regulator of PFK-1 and links changes in mitochondrial oxidative metabolism to glycolysis. This enzyme is also inhibited by high levels ATP. On the other hand, positive regulators of PFK-1 include ADP, AMP, and P_i. The most important allosteric activator of PKF-1 is fructose-2,6-bisphosphate (F2,6BP). Its synthesis is catalyzed by the bifunctional enzyme phosphofructokinase-2/fructose-2,6-bisphosphatase (PFK-2) from fructose-6-phosphate. The production of F2,6BP itself is highly regulated. PFK-2 is allosterically inhibited by citrate, which results in decreased F2,6BP levels, this latter phenomenon constituting a second mechanism by which citrate can inhibit PFK-1 activity. A number of hormones that activate glycolysis, including insulin, epinephrine, and norepinephrine exert phosphorylation control on PFK-2.

Another regulatory step is the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reaction, which catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate and produces a NADH (Figure 8). This is a major regulatory step since the accumulation of NADH within the cytoplasm of cells inhibits the GAPDH reaction. Furthermore, oxidative stress leads to translocation of GAPDH to the nucleus where it can undergo poly-ADP-ribosylation, which in turn can inhibit the enzyme. This mechanism has been shown to be present in hyperglycemia and following activation of NOS.

The final reaction of glycolysis leads to the production of pyruvate. This pyruvate molecule becomes a substrate for further metabolic pathways presented in the next section.

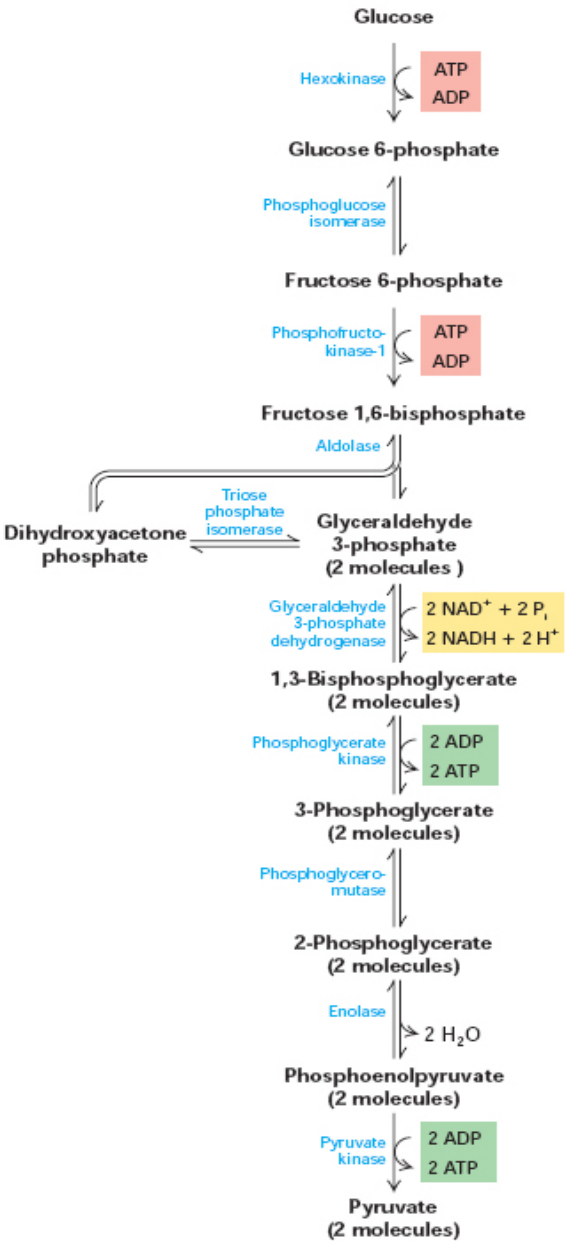


Figure 8. Glycolysis and anaerobic metabolism of glucose. Two reactions consume ATP, forming ADP and phosphorylated sugars (red); two generate ATP from ADP by substrate-level phosphorylation (green); and one yields NADH by reduction of NAD^+ (yellow). Note that all the intermediates between glucose and pyruvate are phosphorylated compounds. Reactions 1, 3, and 10, with single arrows, are essentially irreversible (large negative ΔG values) under conditions ordinarily obtaining in cells. (Adapted from Molecular Cell Biology, Lodish. 5th Ed.)

The pyruvate branch point

Pyruvate is either generated from glycolysis, taken up from the extracellular milieu through monocarboxylic acid transporter-1 (MCT-1) or result from the deamination of certain amino acids. Its three main fates are: 1) conversion to lactate, 2) decarboxylation to acetyl-CoA, and 3) carboxylation to oxaloacetate or malate, (132-135).

The reduction of pyruvate to lactate with the associated oxidation of cytosolic NADH into NAD^+ involves an abundant cytosolic enzyme, lactate dehydrogenase (LDH), which catalyzes the reversible near-equilibrium redox reaction. LDH-H (also referred to as LDH-1) is the most abundant LDH isoenzyme in the heart. Release of lactate into the blood stream is facilitated by the MCT-1. Interestingly, the healthy nonischemic heart is also a consumer of lactate (136).

Under aerobic conditions, most of the pyruvate is transported to the mitochondria where it can be decarboxylated and oxidized into acetyl-CoA by PDH, a multienzyme complex located in the mitochondrial matrix. Its activity in the heart is tightly controlled by work, substrate availability and hormones. The maximal rate of pyruvate oxidation at any given time is dependent on the degree of phosphorylation of PDH, determined by the activity of two enzymes of the complex, a specific PDH kinase (PDK) and a PDH phosphatase. Phosphorylation of PDH by PDK inactivates the enzyme. PDK is inhibited by pyruvate and by decreases in the acetyl-CoA-to-free CoA and NADH-to-NAD^+ ratios, i.e. changes in the energy status of the cell. There are four isoforms of PDK of which PDK4 is the predominant form in heart. The PDH complex also contains a PDH phosphatase that dephosphorylates and activates PDH.

The acetyl-CoA formed from pyruvate decarboxylation generates three additional NADH and one $FADH_2$ in the CAC. It is noteworthy that the heart has a tight concerted regulation between carbohydrate and FA metabolism. The “glucose-FA cycle” was first described by Philip Randle and colleagues in the 1960s. This interrelationship states that the primary physiological regulator of flux through PDH and the rate of glucose oxidation in the heart is the rate of FA oxidation. In fact, high rates of FA oxidation inhibit PDH activity via an increase in mitochondrial acetyl-CoA-to-free CoA and NADH-to-NAD^+, which activates PDH kinase. Conversely, inhibition of FA oxidation increases glucose and lactate uptake and oxidation by (i) decreasing citrate levels and thereby, inhibition of PFK, and (ii) lowering acetyl-CoA and/or NADH levels in the mitochondrial matrix, thereby relieving the inhibition of PDH.

In addition to lactate formation and oxidation by PDH, pyruvate enters the CAC via carboxylation to either malate or oxaloacetate. This anaplerotic reaction acts to maintain the pool size of CAC intermediates as the latter can be depleted via efflux of citrate (and to a lower extent, of succinate, α-ketoglutarate and fumarate) from the mitochondria, a process that has been referred to as “cataplerosis” (137). Both anaplerosis and cataplerosis have been shown to be modulated by substrate abundance, workload and oxygen availability (138;139). Although pyruvate carboxylation accounts for only ~2–6% of the CAC flux under normoxic conditions, the integrity of this pathway is believed to be important for contractile function. However, a recent case-report shows that pyruvate carboxylase deficiency is lethal although no cardiac involvement was documented (140) but another study showed that deficiency in propionyl-CoA carboxylase is associated with a cardiomyopathy (141). Other

anaplerotic pathways include the formation of succinyl-CoA from propionyl-CoA generated from odd-chain FAs such as heptanoate. Pyruvate can also contribute to anaplerosis by transamination with glutamate to form alanine and α-ketoglutarate.

Fatty acid metabolism

Another crucial pathway for energy production is FA metabolism. Free FAs are highly hydrophobic and are never truly free *in vivo* but rather are associated with proteins or bound to coenzyme A or carnitine. They are transported in the plasma either in the non-esterified form attached to albumin, or as triglycerides in chylomicrons or very-low-density lipoproteins. FAs are released from these chylomicrons and lipoproteins via hydrolysis by a lipoprotein lipase bound to the outside of capillary endothelial cells and cardiomyocytes.

The uptake of long chain FAs are facilitated by a FA translocase (FAT) and a plasma membrane FA binding protein (FABP). A specific FAT protein called CD36 is abundantly expressed in skeletal and cardiac muscles and appears to be the predominant form of FAT in the heart (142). Cytosolic FABPs appear to be the primary intracellular carrier of non-esterified FAs. Once transported across the sarcolemma, the non-esterified FAs bind to FABPs and are then activated by esterification to fatty acyl-CoA by fatty acyl-CoA synthase (FACS).

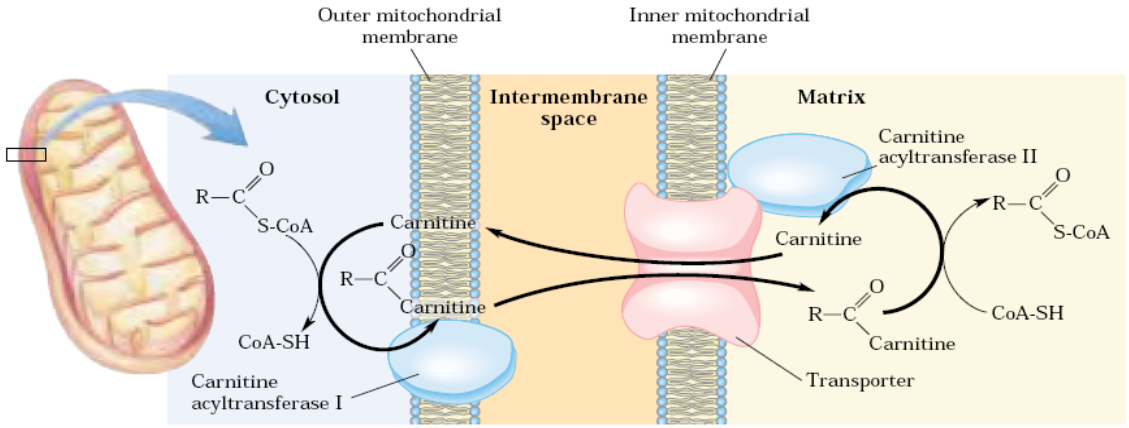


Figure 9. Fatty transport complex in the heart mitochondria. (From Biochemistry, Lehninger 2005)

The cytoplasmic long-chain fatty acyl-CoA destined for β-oxidation must be transported into the mitochondrial matrix. Because the inner mitochondrial membrane is not permeable to long-chain acyl-CoA, the long-chain fatty acyl moiety is transferred from the cytosol into the matrix by a carnitine-dependent transport system (Figure 9). First, carnitine palmitoyltransferase I (CPT-I) catalyzes the formation of long-chain acylcarnitine from long-chain fatty acyl-CoA in the compartment between the inner and outer mitochondrial membranes. Next, carnitine acyltranslocase transports this long-chain acylcarnitine across the inner mitochondrial membrane in exchange for free carnitine. Lastly, carnitine palmitoyltransferase II (CPT-II) regenerates long-chain fatty acyl-CoA in the mitochondrial matrix. Of the three enzymes involved in the transmitochondrial membrane transport, CPT-I serves the key regulatory role in controlling the rate of FA uptake by the mitochondria. The activity of CPT-I is strongly inhibited by

malonyl-CoA, which binds to CPT-I on the cytosolic side of the enzyme. There are two isoforms of CPT-I: CPT-Iα predominates in the liver, and CPT-Iβ is the main isoform in the heart and the more sensitive to malonyl-CoA inhibition (143). Malonyl-CoA is formed from the carboxylation of cytosolic acetyl-CoA by acetyl-CoA carboxylase (ACC). Evidence suggest that extramitochondrial acetyl-CoA can be derived from (i) citrate, shuttled out of the mitochondria (cataplerosis) regenerating oxaloacetate and acetyl-CoA via the ATP-citrate lyase reaction (144), (ii) the export of mitochondrial acetyl-CoA as acylcarnitine or (iii) peroxisomal β-oxidation of long-chain FAs (145). On the other hand, the degradation of malonyl-CoA is regulated by the activity of malonyl-CoA decarboxylase (MCD), which converts malonyl-CoA back to acetyl-CoA and CO_2 in the cytosol and mitochondria (146).

Once taken up by the mitochondria, FAs undergo β-oxidation, a process that repeatedly cleaves off two carbon acetyl-CoA units, generating one NADH and one $FADH_2$ in the process. The β-oxidation process involves four reactions, with specific enzymes for each step, and each reaction has specific enzymes for long-, medium-, and short-chain length FAs. The first step is catalyzed by acyl-CoA dehydrogenase, followed by 2-enoyl-CoA hydratase, and then 3-hydroxyacyl-CoA dehydrogenase. The final step is 3-ketoacyl-CoA thiolase, which regenerates acyl-CoA for another round of β-oxidation and releases acetyl-CoA for the CAC. Acyl-CoA dehydrogenase and 3-hydroxyacyl-CoA dehydrogenase seem to be regulated by the acetyl CoA-to-free CoA ratio. In fact, an increased workload has been shown to decrease this ratio and thereby activate those two enzymes. The acetyl-CoA formed from β-oxidation generates three additional NADH and one $FADH_2$ in the CAC.

Role of triglycerides in fatty acid metabolism

Long-chain fatty acyl-CoA can either be converted to acylcarnitine and oxidized by a mitochondrial FA β-oxidation, as outlined above, or esterified to triglycerides. In the healthy normal heart, it is estimated that 70–90% of the fatty acids entering the cell are immediately oxidized, and only 10–30% enter the intracardiac triglyceride pool. The myocardial triglyceride pool is an important source of FAs, with the rate of lipolysis and its contribution to overall myocardial ATP production being inversely related to the concentration of exogenous FAs (147). Triglyceride hydrolysis and turnover can be rapidly accelerated by adrenergic stimulation, diabetes and during reperfusion of ischemic hearts.

Several enzymes control triglyceride metabolism in the heart and are divided in two groups, namely lipases and acyltransferases. Lipases hydrolyze triglycerides into free FA and glycerol. The main lipase in the heart is adipose triglyceride lipase (ATGL), which converts triglycerides into diglycerides and a free fatty acid. Indeed, it has been shown that ATGL knockout mice accumulated large amounts of neutral lipids (triglycerides and cholesterol esters) in the heart, causing cardiac dysfunction and premature death (148). ATGL is said to account for up to 85% of triglyceride lipolysis (149). Another important player, accounting for the remaining 15% and is more tightly regulated is hormone sensitive lipase (HSL) (149). HSL is the principle lipase active in brown adipocytes and a major lipase in white adipocytes. Triglycerides are not the only substrate for HSL. Indeed, this enzyme can hydrolyse also di- and monoglycerides, as well as cholesteryl esters and other fatty acid esters (150). Although the principal stimuli activating HSL is adrenergic stimulation, other pathways have been uncovered, including a ANP-mediated activation of lipolysis through a cGMP-dependant mechanism (151).

The enzyme responsible for most of the triglyceride biosynthesis is acyl-CoA:diacylglycerol acyltransferase (DGAT). Not only does it participate in lipid metabolism but it also influences metabolic pathways of other fuel molecules. DGAT is also thought to play a mediating or preventive role in the development of ectopic lipotoxicity in the heart (152).

The citric acid cycle

The CAC was first described by Krebs and Johnson in 1937. The principal function of the CAC (also referred to as the Krebs cycle or the tricarboxylic acid cycle) in the heart and skeletal muscle is to oxidize the acetyl group of acetyl-CoA, through a series of eight enzymatic reactions, and to generate NADH and $FADH_2$. The reduced equivalents are used by the electron transport chain to establish a proton gradient that when coupled to oxidative phosphorylation drives ATP formation. The rate at which the CAC operates is a major factor controlling the rate of production of ATP by the heart. An important mechanism regulating the activity of the CAC is the mitochondrial NAD^+-to-NADH or ADP-to-ATP ratios, which depend on the energy status/demand of the cell (153). In fact, the activity of certain key enzymes (isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, malate dehydrogenase) of the CAC is directly stimulated by the change in the mitochondrial redox state (154-156). Although the eight CAC intermediates are neither formed nor consumed during the CAC's net operation, five of them also take part in ancillary reactions such as, for example, cytosolic regulatory pathways involving citrate. Thus, total concentration of CAC intermediates depends on carbon fluxes into and out of the cycle, which are modulated by factors such as workload, external stimuli, substrate abundance, and oxygen supply.

Control of cardiac metabolic phenotype through signaling pathways

Among the many potential kinase signaling pathways regulating energy substrate metabolism, one emerging crucial serine-threonine kinase is of particular interest in the context of this thesis, namely AMPK.

AMPK is now recognized as a "fuel gauge" in mammalian cells and the "guardian of energy status" in the heart (157). The physiologic role of AMPK in maintaining cellular energy stores has emerged over the last decade. AMPK is activated when nutrient supply is limited, ATP production impaired, or energy demand increased. AMPK responds by activating ATP-generating pathways and down-regulating ATP-consuming pathways. AMPK modulates the activity of metabolic enzymes and ion channels, as well as gene expression and protein synthesis. AMPK stimulates glucose transport and PFK-2 to increase glycolysis and phosphorylates glycogen synthase to inhibit glucose storage. Similarly, AMPK inhibits ACC and activates malonyl-CoA decarboxylase, thereby decreasing malonyl-CoA concentrations and favoring FA oxidation. AMPK also inhibits triglyceride synthesis by phosphorylating glycerol-3-phosphate acyltransferase and is involved in the activation of hormone-sensitive lipase and triglyceride breakdown.

Therefore, when the metabolic rate of the heart is increased, activation of AMPK can induce an increase in both carbohydrate and FA metabolism to increase acetyl-CoA production and thereby ensure an adequate replenishment of ATP pool in the heart (158). In addition, AMPK activation regulates transcription factors and their coactivators, to modulate the expression of a number of specific metabolism-related genes. In fact, it has been shown that activation of AMPK leads to stimulation of

various transcription factors, including: (i) PPARα, which upregulates the levels of enzymes of β-oxidation, (ii) mitochondrial transcription factor A, which activates expression of the mitochondrial genome, and (iii) myocyte-enhancing factor 2A, the transcription factor that regulates GLUT4 expression (159). Furthermore, because protein translation is an energy-consuming process, AMPK-mediated down-regulation of protein synthesis may function to conserve energy during stress. In fact, AMPK inhibits protein synthesis by reducing cytoplasmic mRNA-binding proteins and thus, decreasing the stability of several mRNA species as well as by phosphorylating several key modulators of protein translation. Interest in AMPK as a potential cardiovascular modulator has been driven by recent evidence that AMPK has a cardioprotective role in myocardial injury and apoptosis in the ischemic heart (160). AMPK activation was found to mediate an important role in the vasculature where it may exert anti-atherosclerotic effects. However, constitutively active AMPK was shown to be associated with glycogen accumulation and hypertrophic cardiomyopathy (161). Despite the major advances, there is still much to be understood about AMPK's actions in the cardiovascular system.

Metabolic remodeling in the healthy and diseased heart

The heart employs acute and/or chronic mechanisms to adapt to sustained changes in energy demand. Immediate adaptation involves reactions for which the organ has ready-made “allosteric-type” mechanisms in place. This type of acute regulation was known for a long time. More recently, chronic regulation of metabolic processes has only been described since the beginning of the 90s and this type of long-term adaptation involves transcriptional regulation. This property allows the heart to choose the most efficient substrate for a given physiologic environment and is referred to as metabolic plasticity (162). As presented in this section, myocardial fuel selection is highly influenced by developmental stage of the heart as well as pathological conditions such as hypertrophy, the disease of interest in this project.

The normal metabolic switch from fetal to adult state

In the low oxygen environment of the fetal period, the heart is very reliant on glycolysis as a source of energy due to fetal gene expression patterns and in response to oxygen availability (163). While glycolytic rates are high in the fetal heart, glucose oxidation is very low and not a major source of ATP. The capacity to oxidize glucose increases as the heart matures. FA oxidation rates are also low in the fetal heart and provide only a small proportion of overall ATP production. However, after birth, there is a dramatic 10-fold increase in FA oxidation, which is accompanied by a parallel decrease in glycolytic rates and a marked switch from the fetal gene expression pattern to the adult program (164). Therefore, this metabolic switch can be considered as an adaptive response to oxygen availability.

Metabolic remodeling in disease

Metabolic remodeling also occurs in several disease states such as diabetes, ischemia and hypertrophy. Whether these metabolic alterations are adaptive or maladaptive changes in the pathological state remains unclear. Due to space limitations, only metabolic remodeling occurring in hypertrophy and heart failure will be discussed.

Hypertrophy

Several studies using animal models of ventricular hypertrophy due to hypertension or imposed pressure overload have demonstrated a myocardial shift from FA oxidation toward glucose oxidation (165). In rodent models of pressure overload-induced hypertrophy, expression of PPARα and PGC-1α is reduced in the hypertrophied heart (166). PPARα activity is also inhibited post-translationally in the hypertrophied cardiomyocytes in culture through ERK-MAPK-dependent phosphorylation (167). These results suggest that one key mechanism involved in the energy substrate switch in the hypertrophied heart involves deactivation of the PGC-1α/PPARα complex at both transcriptional and post-transcriptional levels, a shift referred to as a return to the metabolic gene fetal program. In addition to the downregulation of PPARα-regulated gene expression, evidence suggests that pressure-overload hypertrophy also induces hypoxia-inducible factor-1α (HIF-1α) mediated transcriptional regulation (168). HIF-1α response elements have been found in many genes, including GLUT-1 and several glycolytic enzymes, as well as LDH (169).

Whether the aforementioned metabolic remodeling during cardiac hypertrophy should be considered adaptive or maladaptive is controversial and remains a subject of intense investigations. In fact, Taegtmeyer's group showed that reactivation of PPARα in rats with pressure-overload induced hypertrophy is associated with contractile dysfunction, while overexpression of PPARα in these hearts results in functional and metabolic changes resembling those of diabetic cardiomyopathy (170;171). However, the cardiac-specific overexpression of GLUT-1 attenuated the development of heart failure in mice with pressure overload (172) and improved the resistance of PPARα knock-out hearts to a high calcium challenge (173). As previously mentioned, several lines of evidence indicate that ATP produced in the cytosol via glycolysis is used preferentially by ion pumps, including SERCA. Thus, the increased rate of glycolysis might be considered an adaptive response that helps attenuate the disturbances in Ca^{2+} homeostasis associated with cardiac hypertrophy. Accordingly, it is tempting to speculate that in the hypertrophied heart, this shift towards glucose metabolism is beneficial. However, the potential detrimental consequences of this shift include a reduced overall ATP production, an adverse effect that might dominate in the end. Furthermore, the decrease in FA oxidation may also limit the capacity of the heart to withstand an increased workload (174). Indeed, subjects with long-chain FA oxidation defects develop cardiomyopathies and have a lower resistance to stress (175).

Heart failure

The progression to heart failure is associated with a gradual diminished capacity for ATP production. This reduced capacity for energy production leads to secondary deregulation of cellular processes critical for cardiac pump function and of contractile function, which results in an increased energy demand and diminished function (176;177). The results of studies on energy substrate shifts in the failing heart, particularly in humans, have not led to a clear conclusion. Some investigators have shown that expression of FA oxidation genes and corresponding enzymatic activities are reduced in the failing rodent and human heart (for review, see Ref. (178)). Consistent with these findings, recent PET studies in humans with idiopathic cardiomyopathy have shown a shift away from FA utilization and a greater glucose uptake (179). In fact, Karbowska et al. found a 54% reduction in PPARα protein level in ventricular biopsies from five patients with compensated end-stage heart failure (180). Others have

demonstrated the opposite metabolic profile or no change in substrate utilization in heart failure of mild to moderate severity (181;182). Animal models of failure induced by pacing or myocardial infarction show that earlier stages are in fact not associated with metabolic remodeling, but a sharp switch away from FA towards carbohydrate oxidation occurs in end-stage heart failure (e.g. left ventricular end-diastolic pressure ≥ 25 mmHg) (183). These apparent discrepancies have been attributed to both the severity of failure and temporal differences during the progressive remodeling that characterizes the transition to heart failure. The specific etiology of the myocardial disease may also play an important role.

In summary, evidence suggests that alterations in myocardial fuel selection and energetics are linked to the development and progression of heart failure. Accordingly, metabolic pathways involved in cardiac FA and glucose utilization or ATP generation are attractive targets of novel therapeutic strategies aimed at the prevention or early treatment of heart failure. For example, multiple metabolic modulators have been developed for the treatment of heart failure and are currently in clinical trials (184-187).

Role of the NO/cGMP pathway in metabolic remodeling

Several lines of evidence suggest that NO may also play a role in the regulation of myocardial substrate metabolism. NO was initially described as an inhibitor of mitochondrial respiration by competing with oxygen for cytochrome c oxidase. In fact, administering NO precursors to isolated hearts lowers oxygen consumption, whereas inhibitors of NO synthesis stimulate oxygen consumption (188). However, the role of NO in the control of metabolism is not limited to the inhibition of oxygen utilization, but is also extended to the modulation of substrate utilization. In fact, it was first shown that NO inhibits the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase through S-nitrosylation and auto-ADP-ribosylation and downregulates GLUT4, thereby limiting glucose utilization by the heart (189). It was then documented that pharmacological inhibition of NO synthesis is associated with an increase in cardiac glucose utilization (190). The enhanced uptake of glucose by *ex vivo* Langendorff perfused hearts from eNOS knock-out mice corroborates this notion (191). The observation that administration of cGMP analogues or NO-donors depressed cardiac glucose uptake by hearts from eNOS knock-out mice suggests that the effect of NO is cGMP-dependent (192). Other studies showed that cardiac glucose uptake and oxidation are increased after systemic NOS inhibition in conscious dogs, and FA uptake and oxidation were concomitantly reduced (193). This action of NO would explain the cardioprotective effects of NOS blockade during myocardial ischemia, but is less consistent with the increase in myocardial oxygen consumption and decrease in cardiac efficiency, given that glucose is more “oxygen-efficient” than FAs (194). NO has also recently emerged as novel signaling molecule involved in regulating mitochondrial biogenesis. It has been shown that NO induces mitochondrial proliferation through a cGMP-dependent mechanism (195). The effects of NO/cGMP appear to be mediated, at least in part, by transcriptional activation of PGC-1α expression.

A couple of interesting findings are worth mentioning. First, one particularly elegant study in a canine model of pacing-induced heart failure showed that end-stage heart failure (decompensation) is associated with a fall in NO production and a concomitant decrease in FA uptake and increase in glucose uptake (196). This was the first evidence for a tight association between reduced NO production and

metabolic changes in the decompensated heart. Furthermore, it seems that the effect of NO/cGMP on glucose transport and metabolism may be opposite in skeletal muscle to that in the heart, since activation of NO/cGMP in skeletal muscles increases glucose uptake, carbohydrate and FA oxidation (197). Additional research is therefore needed to elucidate the mechanisms of action of NO in the regulation of myocardial substrate metabolism. Finally, drugs such as angiotensin converting enzyme inhibitors are recognized as potent stimulators of NO release (198). Others, such as the Ca^{2+} antagonist amlodipine, utilized in the treatment of hypertension, and the cholesterol-lowering statins, also seem to display some pharmacological properties attributable to the release of NO (199). Thus, in light of the previous considerations, it would be overly simplified to conclude that the beneficial effects of NO released in response to these pharmacological agents are limited to the modulation of vascular tone. In fact, some of the drugs utilized for the treatment of heart failure may act as direct modulators of myocardial metabolism through the mediation of NO.

Chapter II - Manuscript

Rationale, objective, hypothesis and experimental strategy

There is currently great interest in deciphering the mechanisms that couple stress signaling to the fetal gene program in order to identify new potential anti-hypertrophic therapeutic strategies. Both pharmacological and genetic approaches have been used in order to modulate a given specific cardiac signaling pathway and thereby demonstrate its potential as a target for an anti-hypertrophic therapeutic strategy (98;200-202). In this regard, our group has recently developed a mouse model overexpressing a cytosolic catalytic fragment of constitutively active guanylate cyclase domain of the atrial natriuretic peptide receptor in a cardiomyocyte-specific manner (α- myosin heavy chain gene promoter), and helped confirm the role of cGMP as a negative intrinsic modulator of cell growth and cardiac hypertrophy (203). It is noteworthy that to avoid the possible artifactual influence of gene inactivation by insertion, all TG animals used for experiments were heterozygous for the transgene. This thesis project was aimed at further understanding the mechanisms by which cGMP confer protection to the heart.

Given that alterations in cardiac energy metabolism have been repeatedly linked to cardiac dysfunction, and the importance of the cGMP signaling pathway in modulating aspects of metabolism and growth, the specific objective of this study, which is presented in this chapter, was to characterize the cardiac metabolic and functional profile of mice constitutively expressing cytosolic guanylate cyclase in a cardiomyocyte-specific manner and, hence, to delineate the metabolic role of cGMP signaling on specifically one cardiac cell type namely, cardiomyocytes. Our working hypothesis proposes that chronic activation of the cGMP signaling pathway in a cardiomyocyte specific manner modifies cardiac substrate energy metabolism.

To achieve our objective, we used our previously described methodology that combines the working mouse heart perfusion system to a ^{13}C-labeling methodology of substrates enabling the simultaneous assessment of various hemodynamic and metabolic parameters. In addition to assessing the contribution of various substrates for energy production, we also provide data on (i) triglyceride turnover, (ii) signaling mechanisms regulating lipolysis, (iii) mitochondrial CAC-related parameters, and (iv) mRNA levels for selected metabolic genes. The importance and implication of these results will be discussed.

Overall, we were able to (i) identify changes in energy metabolism by documenting the contribution of exogenous and endogenous substrates to the formation of acetyl-CoA, namely an increase in glycolysis and a decrease in exogenous fatty acid oxidation, (ii) relate these changes to metabolic signaling pathways regulating lipid metabolism by measuring acetyl-CoA and malonyl-CoA levels, acetyl-CoA carboxylase (ACC) activation and AMPK activation and (iii) identify a cGMP-mediated increase in lipid compartmentalization, namely through increases in both lipolysis through hormone sensitive lipase (HSL) activation and triglyceride synthesis.

Cyclic GMP signaling in cardiomyocytes modulates fatty acid trafficking and prevents triglyceride accumulation

Ramzi Khairallah, Maya Khairallah, Martin E. Young, Bruce G. Allen, Gary D. Lopaschuk

Christian F. Deschepper, and Christine Des Rosiers

Submitted to Journal of Biological Chemistry on November the 5th, 2007. Under revision as of November the 23rd, 2007.

Running title: Metabolic effects of chronic activation of cGMP signaling

From the Montreal Heart Institute (R.K., M.K., B.G.A., C.D.R.), and the Clinical Research Institute of Montreal (C.F.D.), University of Montreal, Montreal, Quebec, H1T 1C8, Canada; USDA/ARS Children's Nutrition Research Center, Baylor College of Medicine, Houston, Texas, 77030, USA (M.E.Y.); Departments of Pediatrics and Pharmacology, University of Alberta, Edmonton, Alberta, T6G 2S2, Canada (G.D.L.); and McGill University, Montreal, Quebec, H2W 1R7, Canada (R.K., C.F.D., C.D.R.)
Correspondence to C.F. Deschepper, MD, Institut de Recherche Clinique de Montréal, 110 Pine Ave West, Montreal, Quebec, H2W 1R7, Canada. Tel.: (514) 987-5759; Fax: (514) 987-5585. E-mail: christian.deschepper@ircm.qc.ca

Abstract

Although enhanced cGMP signaling can prevent hypertrophy, mechanisms underlying this cardioprotective effect are not well understood. In this study, we assessed the potential involvement of alterations in myocardial energy substrate metabolism, a parameter known to be determinant in the development of hypertrophy. We used mice overexpressing a constitutively active soluble guanylate cyclase in a cardiomyocyte-specific manner ($GC^{+/0}$) and *ex vivo* heart perfusion at physiological workload with ^{13}C-labeled substrates. Compared to controls, hearts from $GC^{+/0}$ mice displayed a 38±9% lower contribution of exogenous fatty acids to acetyl-CoA formation, while that of carbohydrates remained unchanged despite a two-fold increase in glycolysis. The lower contribution of exogenous fatty acids to energy production was not associated with changes in energy demand or supply (contractile function, oxygen consumption, tissue acetyl-CoA or CoA levels, citric acid cycle flux rate) or the regulation of β-oxidation (acetyl-CoA carboxylase activity, tissue malonyl-CoA levels). However, $GC^{+/0}$ hearts showed a two-fold increase in the incorporation of exogenous oleate into triglycerides. Furthermore, a concomitant increase in triglyceride hydrolysis is consistent with our findings of a greater abundance of hormone sensitive lipase (HSL) protein (46±6%) and mRNA (22±4%) as well as a 37±13% decrease in its phosphorylation level at Ser-565. The latter covalent modification inhibits HSL and is regulated by AMP-activated protein kinase (AMPK), whose phosphorylation at its activating site Thr-172 was also reduced by 37±13%. These changes in exogenous fatty acid trafficking in $GC^{+/0}$ hearts appear to be functionally relevant, as demonstrated by their resistance to fasting-induced myocardial triglyceride accumulation. This raises the possibility that enhanced cGMP signaling in cardiomyocytes may protect the heart from fatty acid-induced toxic effects, either as part of its anti-hypertrophic effect or as an additional cardioprotective mechanism.

Keywords: cGMP • metabolism • fatty acids • perfusion • isotopes

Introduction

Left ventricular hypertrophy is an adaptive process by which the myocardium increases its size to face increases in workload and wall stress. However, upon sustained hemodynamic overload, the cellular, molecular and morphological changes often become maladaptive, leading to decompensation and heart failure. Currently, modulation of hypertrophic growth without adversely affecting contractile function is recognized as a potentially auspicious approach in the prevention and treatment of heart failure (1). In this regard, in the last decade, multiple studies have highlighted the anti-hypertrophic effects of the nitric oxide (NO) and atrial natriuretic factor pathways as well as their common downstream effector, cyclic guanosine monophosphate (cGMP) (for review, see (2-5)). However, the mechanisms responsible for this cardioprotective effect are still the subject of active research.

Recently, our group developed a mouse model overexpressing a catalytic fragment of the constitutively active guanylate cyclase domain of the atrial natriuretic peptide receptor in a cardiomyocyte-specific manner (6). These mice were shown to be resistant to the hypertrophic effects of either the pharmacological agent isoproterenol or abdominal aortic constriction, thereby providing evidence that the cGMP pathway prevents left ventricular hypertrophy *in vivo* via a direct local action on cardiomyocytes. Based on the following considerations, we hypothesized that modulation of myocardial energy substrate metabolism might underlie at least partly this cardioprotective effect. In fact, alterations in cardiac substrate metabolism are currently considered as an independent determining factor contributing to contractile dysfunction and to the heart's susceptibility to injury and, more recently, to disease progression from left ventricular hypertrophy to heart failure (for review, see (7;8)). Furthermore, a number of studies have reported that NO or cGMP mimetics modulate energy metabolism in various tissues by influencing substrate selection for ATP production, expression of metabolic genes as well as genes of the nutrient signaling pathways (9-13). However, there appears to be a complex relationship between NO, the cGMP pathway and energy metabolism in the heart, which differs from that in the skeletal muscle and depends on many factors such as the level of myocardial activation of AMP-dependent kinase (AMPK) or contractility. For example, myocardial glucose uptake or utilization has been reported to be (i) enhanced following addition of NO synthase inhibitors (11) or in eNOS null mouse (14)), and, conversely, (ii) decreased with addition of the cGMP analog, 8-bromo-cGMP or of NO donors (12). In contrast, a recent study shows that activation of the cGMP pathway contributes to the AMPK stimulation of glucose uptake in left ventricular papillary muscle (15).

In this study, we assessed the impact of enhanced cGMP signaling on cardiac metabolism using our transgenic mice that constitutively overexpress soluble guanylate cyclase in cardiomyocytes ($GC^{+/0}$), and our previously described methodology of *ex vivo* working heart perfusion with ^{13}C-labeled substrates, which enables the simultaneous assessment of various hemodynamic and metabolic flux parameters (16). Our results demonstrate substantial differences in substrate selection for energy production as well as in lipid partitioning between β-oxidation and esterification between control and $GC^{+/0}$ mice hearts.

Experimental Procedures

Materials and animal model

Sources of chemicals, biological products, and ^{13}C-substrates have been reported previously (16-20). Phospho-HSL antibodies were purchased from Cell Signaling Technologies (Danvers, USA), and total HSL antibody from Cayman Chemicals (Ann Arbor, USA).

All procedures on the animals were approved by the local ethics committee in agreement with the guidelines of the Canadian Council on Animal Care. Twelve to thirteen week old male transgenic ($GC^{+/0}$) mice overexpressing constitutively active guanylate cyclase in cardiac specific manner (6), backcrossed for at least 12 generations into the C57Bl/6 mouse strain, were compared to age-matched non-transgenic (WT) littermates of similar body weight (30.0 ± 0.1 vs 29.0 ± 0.1 g). To avoid the possible artefactual influence of gene inactivation by insertion, all TG animals used for experiments were heterozygous for the transgene.

Working mouse heart perfusion

Mice were anesthetized (1 µL/g, i.p.) with a mixture of ketamine (100 mg/mL) and xylazine (20 mg/mL) and heparinized (5000 U/kg, i.p.) 15 min before surgery. The procedure for heart isolation and its *ex vivo* perfusion in the working mode has been previously described in detail (21). The composition of the Krebs-Henseleit buffer (110 mM NaCl, 4.7 mM KCl, 2.1 mM $CaCl_2$, 0.24 mM KH_2PO_4, 0.48 mM K_2HPO_4, 0.48 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 0.1 mM EDTA) was modified to adjust free calcium levels (1.55 ± 0.02 mM) and sodium concentration to a physiological value. The preload and afterload pressures were set at 15 and 50 mmHg, respectively. Myocardial oxygen consumption (MVO_2; µmol/min), intracellular pH, rate pressure product (mmHg·beats·min^{-1}·10^{-3}), cardiac power (mW), and cardiac efficiency (mW·µmol^{-1}·min^{-1}) were calculated from previously reported equations (16).

Working mouse hearts were perfused for 30 min with a semi-recirculating modified Krebs-Henseleit solution containing physiological concentrations of substrates (11 mM glucose, 0.8 nM insulin, 50 µM carnitine, 5 nM epinephrine, 1.5 mM lactate, 0.2 mM pyruvate, and 0.4 mM oleate bound to 3% albumin). For any given perfusion, one of the unlabeled substrates was replaced by its corresponding labeled substrate, i.e. either: [U-$^{13}C_{18}$]oleate (25% initial molar percent enrichment (MPE)), [U-$^{13}C_6$]glucose (25% initial MPE), and [U-$^{13}C_3$]lactate/[U-$^{13}C_3$]pyruvate (100% initial MPE).

Throughout the perfusion, influent and effluent perfusates were collected at regular intervals to document lactate dehydrogenase (LDH) release rates (every 5 min), the oxygen and carbon dioxide partial pressures (at 10 and 20 min) and the lactate and pyruvate efflux rates (at 30 min). Subsequent to each perfusion period, hearts were freeze-clamped with metal tongs chilled in liquid nitrogen and weighed. All samples were stored at -80°C until further analysis.

Tissue processing

Flux measurements. Our previously published studies (16;22) provide (i) definitions of the ^{13}C terminology and detailed descriptions for the measurements by gas chromatography-mass spectrometry (GCMS; Hewlett-Packard 6890N gas chromatograph coupled to a 5973N mass spectrometer) of (i) the ^{13}C-enrichment of citric acid cycle (CAC) intermediates (citrate, fumarate, and OAA moiety of citrate) necessary for calculations of flux ratios relevant to substrate selection for citrate synthesis, and (ii) other metabolites (lactate and pyruvate) used to determine the glycolytic flux, as well as for (ii) the calculation of the absolute CAC flux rates from oxygen consumption rates and the stoichiometric relationship between oxygen consumption and citrate formation from β-oxidation and pyruvate decarboxylation. Briefly, GCMS data are expressed as MPE. Mass isotopomers of metabolites containing 1 to *n* ^{13}C-atoms are identified as M*i* with *i* = 1, 2, … *n*, and the absolute MPE of individual ^{13}C-labeled mass isotopomers (M*i*) of a given metabolite are calculated as follows: MPE (M*i*) = % A_{Mi} / [A_M + ΣA_{Mi}], where A_M and A_{Mi} represent the peak areas from ion chromatograms corrected for natural abundance, corresponding to unlabeled (M) and ^{13}C-labeled (M*i*) mass isotopomers, respectively. Metabolic flux ratios, which reflect the contribution exogenous fatty acids (oleate) and of carbohydrates (lactate, pyruvate and glucose) to acetyl-CoA and or oxaloacetate (OAA) formation for citrate synthesis, are calculated from the molar percent enrichment (MPE) in M*i* isotopomers of the acetyl (AC^{CIT}) and oxaloacetate (OAA^{CIT}; corrected for the formation of M3 OAA from CAC metabolism of citrate isotopomers) moiety of citrate, and expressed relative to CS: (i) pyruvate decarboxylation (PDC) = M2 AC^{CIT} / M3 pyruvate, (ii) oleate oxidation (OLE) = M2 AC^{CIT} / M18 oleate, and (iii) pyruvate carboxylase (PC) = M3 OAA^{CIT}/M3 pyruvate.

Quantification and ^{13}C-enrichment of triglycerides. Fatty acids from heart tissue triglycerides (TG) were analyzed by GCMS as their methyl ester (FAME) derivative. Briefly, tissue was pulverized under liquid nitrogen and a labeled external standard ([$^2H_{33}$]heptadecanoic acid) added. Total tissue lipids were extracted by a modified Folch method (23), in 20-fold weight-to-volume ratio of chloroform/methanol (2:1), containing 0.1% butylated hydroxytoluene (BHT), overnight at 4˚C (24). Homogenates were then filtered through gauze, dried under nitrogen gas and resuspended in a detergent for quantification. Triglycerides were quantified enzymatically with a commercial kit (GPO Trinder; Sigma, USA). Triolein, dissolved in chloroform/methanol and processed similarly to samples, was used as a standard (25). For ^{13}C-enrichment, lipids were resuspended in 100 µl of hexane/chloroform/methanol (95:3:2). Lipid classes were separated using a solid phase extraction aminopropyl column (Varian, Harbor City, USA) according to the method of Ruiz (26). Samples were applied to a column previously activated with 7.5 ml of hexane and triglycerides eluted with 5 ml of chloroform stabilized with pentene. The triglyceride fraction was dried, resuspended in 2 ml methanol/hexane (4:1) containing 0.004% BHT, 200 µl acetyl chloride was added and then samples were heated to 80˚C for one hour to yield FAMEs according to a modification of the method described by Lepage and Roy (27). The remaining acetyl chloride was neutralized with 5 ml 6% potassium carbonate and the upper hexane phase, containing FAMEs, was collected and analyzed by GCMS on an Agilent Technologies HP-5 column of 50 m, 0.2 mm ID, 0.5 µm film thickness. Oleate was analyzed in selected ion monitoring mode and the molecular and major ions

were quantified for both unlabelled (ions 296 and 264) and [U-$^{13}C_{18}$]oleate (ions 314 and 282) at a retention time of 20.66 minutes.

Levels and activity of factors involved in triglyceride hydrolysis

Immunoblotting**.** Heart extracts were resolved by SDS-PAGE. AMPK phosphorylation was assessed as described previously (28) using either anti-phospho-αAMPK (Thr-172) or anti-αAMPK (18). To quantify HSL expression and phosphorylation, tissue extraction, electrophoresis, and immunoblotting were performed as previously described (20) with minor modifications. Nitrocellulose membranes (0.22 μm) were probed with antisera to either phospho-HSL (Ser-565), phospho-HSL (Ser-660), total HSL, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Immunoreactive bands were quantified by digitizing the ECL signal using a VersaDoc 4000 Gel Imaging System and analyzed using Quantity One software (Bio-Rad, Hercules, USA).

ACC activity**.** Powdered heart tissue was homogenized in 0.05 M Tris, 0.25 M mannitol, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM Na pyrophosphate, 10% glycerol, pH7.5 and protease inhibitor cocktail (4:1 buffer-to-tissue ratio), then centrifuged for 10 min at 800 *g*. ACC assays were based on acetyl-CoA-dependent $^{14}CO_2$ fixation as described previously (29;30).

Malonyl-CoA and CoASH quantification. Malonyl-CoA was measured as previously described (31). Briefly, [^{3}H]acetyl-CoA was converted by fatty acid synthase into petroleum ether-soluble material in proportion to the malonyl-CoA content of the sample. Assays were calibrated using exogenous malonyl-CoA as an internal standard. CoASH was measured by a recycling assay using CoASH internal standards (32).

Gene expression analysis. Analyses of changes in gene expression were carried out in hearts freeze-clamped from mice sacrificed in the afternoon (light phase). RNA was extracted using standard methods and analyzed using reverse transcription followed by real-time quantitative polymerase chain reaction, as described previously (33). The following genes were studied: (i) enzymes involved in fatty acid metabolism, namely medium-chain acyl-CoA dehydrogenase (*mcad*), adipose triglyceride lipase (*atgl*), diacylglycerol acyltransferase-2 (*dgat*2) and hormone sensitive lipase (*hsl*), and (ii) enzymes involved in glucose metabolism, namely glucose transporter 4 (*glut*4), pyruvate dehydrogenase kinase-4 (*pdk*4) and pyruvate carboxylase (*pcx*).

Primer and probe sequences for *hsl* for the Taqman assay were as follows: forward: 5′-GCGCTGGAGGAGTGTTTTT-3′ reverse: 5′-TGTCCCCTGCAAGGCATAT-3′ probe: 5′-FAM-TCTCCAGTTGAACCAAGCAGGTCACA-TAMRA-3′. The remaining primer and probe sequences have been

previously published (17;19). Transcript levels were normalized to total RNA content (as measured by UV spectrophotometry).

Statistical Analysis

Data are expressed as means ± SEM. Statistical significance was reached at $P \leq 0.05$ using an unpaired *t*-test, a one-way or a two-way ANOVA followed by a Bonferroni selected-comparison test.

Results

Perfused GC$^{+/0}$ hearts show decreased contribution of exogenous oleate to acetyl-CoA and enhanced glycolysis

We used our previously established isolated working heart model and ^{13}C-methodology to evaluate functional and metabolic differences between hearts from GC$^{+/0}$ mice and control non-transgenic littermates. Upon *ex vivo* perfusion at a physiological afterload of 50 mmHg with a buffer containing a mixture of substrates and hormones mimicking the *in vivo* milieu in the fed state (11 mM glucose, 1.5 mM lactate, 0.2 mM pyruvate, 0.4 mM oleate bound to 3% albumin, and 0.8 nM insulin), hearts from both groups maintained similar values for the various functional and physiological parameters over the entire 30-min perfusion period (Table 1). However, LDH release rate (an index of membrane integrity) was significantly decreased in the GC$^{+/0}$ hearts (Figure 1).

In these hearts, the contribution of exogenous substrates to mitochondrial acetyl-CoA formation for citrate synthesis was evaluated using various ^{13}C-labeled substrates. Compared to controls, GC$^{+/0}$ hearts perfused with [U-$^{13}C_{18}$]oleate (initial MPE=25%) showed lower values for (i) the MPE M2 of the acetyl moiety of citrate (9.4 ± 2.0 vs 12.4 ± 1, P<0.05) and, accordingly, (ii) the flux ratio OLE/CS (Table 2), thus demonstrating a significantly decreased formation of acetyl-CoA formation for citrate synthesis from exogenous β-oxidation (Table 2). In contrast, perfusions with [U-$^{13}C_6$]glucose (initial MPE=25%) or [U-$^{13}C_3$]lactate plus [U-$^{13}C_3$]pyruvate (initial MPE=100%) demonstrate a similar percent contribution of individual or total carbohydrates to acetyl-CoA formation for citrate synthesis via mitochondrial pyruvate decarboxylation. This is evidenced from the similar values for the MPE M2 of the acetyl moiety of citrate and the PDC/CS flux ratio in GC$^{+/0}$ and control hearts (Table 2).

We also assessed rates of cytosolic glycolysis from the release rates of [U-$^{13}C_3$]lactate and [U-$^{13}C_3$]pyruvate in hearts perfused with [U-$^{13}C_6$]glucose. Both lactate and pyruvate release rates were significantly increased in GC$^{+/0}$ hearts when compared to wild-type (Figure 2A), although their production ratio, which reflects the cytosolic redox [NAD$^+$]-to-[NADH] ratio, was unchanged (Figure 2B).

The observed alterations in exogenous fatty acid β-oxidation and glycolysis in GC$^{+/0}$ hearts cannot be readily explained by changes in gene expression since the mRNA levels of key metabolic enzymes, namely *glut4*, *pdk4*, *pcx* and *mcad*, were similar in both groups (as assessed by real-time RT-PCR; see Suppl. Table 1).

Lack of alteration in mechanisms regulating β-oxidation for perfused GC$^{+/0}$ hearts

Given the observed decrease in the contribution of exogenous oleate to acetyl-CoA formation in perfused GC$^{+/0}$ hearts, we investigated several mechanisms involved in β-oxidation regulation. These include: (i) concentration of malonyl-CoA, which potently inhibits carnitine palmitoyltransferase I (CPT-I), an enzyme required for entry of long chain fatty acyl groups into mitochondria; (ii) the activity of ACC, the enzyme responsible for malonyl-CoA synthesis, and (iii) the [acetyl-CoA]-to-[CoA] ratio (34). As

reported in Table 2, none of the measured values for the metabolic parameters were different between $GC^{+/0}$ and WT hearts. Furthermore, the fact that similar values were obtained in both groups for (i) the [acetyl-CoA]-to-[CoA] ratio, and (ii) the citric acid cycle (CAC) flux rate, indicates that energy supply from substrate oxidation is not affected by the expression of the transgene.

Perfused $GC^{+/0}$ hearts shuttle more exogenous ^{13}C-labeled oleate into triglycerides

Next, we evaluated whether the decreased contribution of exogenous [U-$^{13}C_{18}$]oleate to acetyl-CoA formation observed in perfused $GC^{+/0}$ hearts could result from its preferential partitioning of fatty acids into triglyceride synthesis. While there were no differences in triglyceride content before and after perfusion of $GC^{+/0}$ hearts (4.92 ± 1.5 vs 4.20 ± 1.76 μmol/gww; NS), we found a significant 2.5-fold increase in the MPE of the oleate moiety of triglycerides in $GC^{+/0}$ when compared to WT hearts (Figure 3A). As reflected by a labeling ratio of ~ 1.0 between the MPE of oleate from triglycerides and that of the acetyl moiety of citrate (Figure 3B) in perfused $GC^{+/0}$ hearts, there is an equal partitioning of [U-$^{13}C_{18}$]oleate between its two primary metabolic fates, namely esterification and β-oxidation, respectively. This contrasts with wild-type hearts, for which less than one third of the exogenous oleate was shuttled into triglyceride stores (Figure 3B).

$GC^{+/0}$ hearts exhibit alterations in factors influencing triglyceride lipolysis

Given that exogenous oleate incorporation into triglycerides is increased in $GC^{+/0}$ hearts, yet total triglyceride content was not altered, we investigated the possibility that $GC^{+/0}$ hearts exhibit changes in triglyceride hydrolysis. As such, factors known to influence triglyceride turnover, namely HSL and AMPK, were investigated (35;36). Firstly, compared to controls, $GC^{+/0}$ hearts had greater levels of HSL protein (Figure 4B) and mRNA (244 ± 9 vs 200 ± 11 transcripts/ng RNA; $P<0.01$). However, we found no difference in the transcript level of other enzymes involved in triglyceride hydrolysis, namely *atgl* and *dgat*2 (see Suppl. Table 1). Secondly, we detected differences in the phosphorylation status of HSL, namely a 46.4 ± 5.9 % decrease at Ser-565 (Figure 4A), without any changes at Ser-660, for $GC^{+/0}$ versus wild-type hearts (data not shown). Phosphorylation of Ser-565 inhibits HSL, whereas phosphorylation of Ser-660 activates its enzymatic activity. Finally, phosphorylation of AMPK at Thr-172 (which results in its activation) was significantly decreased by 36.7 ± 13.4 % in $GC^{+/0}$ hearts compared to WT hearts (Figure 4C), a finding that is consistent with the observed decrease in phosphorylation of HSL at Ser-565, a known AMPK site.

$GC^{+/0}$hearts are resistant to fasting-induced triglyceride accumulation

To assess the significance of the documented changes in exogenous long chain fatty acid handling by $GC^{+/0}$ hearts, wild-type and $GC^{+/0}$ mice were subjected to a 24-h fast, a condition that has been shown to increase myocardial triglyceride levels. In contrast to wild-type hearts, hearts from fasted $GC^{+/0}$ mice did not show the expected increase in triglycerides in response to fasting (Figure 5).

Discussion

While a number of previous studies have shown that activation of cGMP signaling may prevent or reverse the development of hypertrophy (6;37;38), little is known regarding the cardiomyocyte-specific effects of cGMP signaling on energy substrate metabolism, a factor that plays critical roles in the development of hypertrophy (7;8). In this study, we used our model of *ex vivo* working heart perfusion with ^{13}C-labeled substrates to simultaneously assess various hemodynamic and metabolic parameters of our cardiomyocyte-specific model of constitutive cGMP signaling pathway activation. Collectively, our results demonstrate that enhanced cGMP signaling within cardiomyocytes modifies exogenous substrate utilization, including fatty acid partitioning, in the absence of changes in cardiac function.

The most striking metabolic effect of chronic activation of the cGMP signaling pathway that was observed is the difference in the partitioning of exogenous long chain fatty acids between β-oxidation (for energy production) and esterification to triglycerides. Specifically, we found that the contribution of exogenous oleate to acetyl-CoA formation was significantly decreased in $GC^{+/0}$ mouse hearts, while that of carbohydrate was unchanged. The decreased exogenous oleate contribution was not explained by changes in the tissue levels of malonyl-CoA, a known inhibitor of β-oxidation (39). Furthermore, it did not appear to affect myocardial energy status, given that perfused $GC^{+/0}$ and wild-type mouse hearts maintained similar levels of acetyl-CoA and free CoA, calculated CAC flux rate and contractile function. However, we found that the incorporation of exogenous ^{13}C-labeled oleate into triglycerides was increased almost 3-fold in perfused $GC^{+/0}$ mouse hearts. Given that the heart's triglyceride content remained constant during perfusion in these hearts, we reasoned that the increased incorporation of exogenous oleate into triglycerides was compensated by a matching increase in the mobilization of unlabeled long chain fatty acid from endogenous triglyceride stores (i.e. turnover), which could subsequently undergo β-oxidation to acetyl-CoA. In support of this interpretation are our findings of: 1) a greater abundance of HSL protein and mRNA in the hearts of $GC^{+/0}$ mice, and 2) a decreased phosphorylation of HSL at the Ser-565 site. The latter event should activate HSL, as phosphorylation of Ser-565 is the mechanism by which AMPK (a major regulator of lipolysis (40)) inhibits HSL activity (41). In fact, consistent with our findings on HSL phosphorylation, phosphorylation of AMPK at Thr-172 (an event that activates AMPK activity) was significantly lower in the hearts of $GC^{+/0}$ mice. Our findings are also similar to those reported in human adipose tissue, where atrial natriuretic factor-induced cGMP-dependant lipolysis occurs via AMPK inactivation and HSL activation (13;42). Of note, we found no changes in phosphorylation of HSL at Ser-660, a positive regulatory site at which protein kinase A (PKA) acts. Ser-600, another important positive regulatory site, is phosphorylated by the extracellular signal-regulated protein kinase 1/2 (ERK 1/2) (41), and ERK1/2 are known to be strongly activated by the cGMP signaling pathway (42;43). Unfortunately, antibodies against phosphorylated Ser-600 are not currently available.

Collectively, our isotopic and molecular data support the notion that enhanced cGMP signaling in cardiomyocytes promotes both triglyceride synthesis and hydrolysis. Consistent with this metabolic effect is an absence of fasting-induced myocardial TG accumulation in $GC^{+/0}$ mice (Figure 5). Interestingly, such a response to fasting was also observed in transgenic mice with heart-specific HSL overexpression (43). The importance of lipolysis for normal cardiac homeostasis has recently been

emphasized by studies in transgenic mice lacking enzymes involved in this process. For example, hearts lacking adipose triglyceride lipase develop severe cardiac dysfunction and altered energy metabolism (44). One of the potential benefits of increased triglyceride synthesis/hydrolysis appears to be increased compartmentalization of lipids in order to reduce their cardiotoxic effects (45). Proposed mechanisms explaining the deleterious effects of fatty acids (also referred to as lipotoxicity) include direct effect of neutral droplets or fatty acids on myofibrillar function, fatty acid-induced apoptosis, generation of reactive oxygen species as toxic by-products of lipid oxidation, and activation of signaling pathways via protein kinase C or ceramide-mediated processes (46-48).

Beyond the aforementioned changes in lipid trafficking in $GC^{+/0}$ mouse hearts, our finding of enhanced lactate and pyruvate production suggests that glucose uptake and glycolysis are also increased by enhanced cGMP signaling in these hearts. This finding concurs with that of Li *et al.* (15) who reported a stimulation of glucose uptake by activation of cGMP signaling in heart papillary muscle. However, it contrasts with other studies reporting that glucose uptake is (i) increased following inhibition of NO synthase by either pharmacologic inhibitors or gene inactivation (11;14) and, conversely, it is (ii) decreased using cGMP agonists or NO donors (12) (which, respectively, decreases and increases the cardiac concentration of cGMP). Several factors may explain these differences. Specifically, in our model, cGMP signaling is increased chronically and specifically in cardiomyocytes, which excludes possible effects mediated by (i) cells other than cardiomyocytes and by (ii) NO, which exerts cGMP-independent metabolic effects such as inhibition of glycolytic glyceraldehydes 3-phosphate dehydrogenase through enhanced ADP-ribosylation (49) and of phosphofructokinase (50). While the mechanism underlying the observed enhanced glycolysis in $GC^{+/0}$ mouse hearts remains to be clarified, it appears possible that this may be linked to the aforementioned changes in lipid trafficking by providing the extra cytosolic ATP necessary to support triglyceride synthesis and hydrolysis. In fact, triglyceride hydrolysis will release free fatty acids, which need to be subsequently activated to fatty acyl-CoA by the ATP-requiring acyl-CoA synthases before entering mitochondrial β-oxidation (51).

The metabolic profile of $GC^{+/0}$ mouse hearts (namely decreased β-oxidation of exogenous fatty acids, no change in carbohydrate oxidation and increased glycolysis) is partly reminiscent of that seen in hypertrophied hearts (52-54). While it is still controversial whether a shift in exogenous substrate selection from fatty acid to carbohydrates is a compensatory or maladaptive mechanism, our finding of a similar shift in $GC^{+/0}$ mouse hearts suggests that it is beneficial and cardioprotective. It is also noteworthy that, despite a 2-fold increase in lactate plus pyruvate production rates, $GC^{+/0}$ mouse hearts maintained a normal cytosolic [NAD^+]-to-[NADH] ratio, as indicated by the lactate-to-pyruvate production ratio. This contrasts with other situations (such as ischemia and hypertrophy) where enhanced glycolysis is uncoupled from pyruvate oxidation, a metabolic situation that has postulated to be detrimental (52-54). Other anti-hypertrophic models, such as the calcineurin-knockout mouse (55), or the glycogen synthase kinase-3 (GSK-3) (56), display a shift in substrate selection from fatty acids to carbohydrates in the presence of increased expression of atrial natriuretic factor and in the absence of organ enlargement. Likewise, mechanical unloading of heart (which constitutes another model of cardioprotection) displays a similar metabolic shift along with improvement of contractility (57). However, two striking and distinctive features that we documented in our model are that: (i) triglyceride

synthesis is enhanced concomitantly with lipolysis; and (ii) the cytosolic redox state of the cells is conserved. To the best of our knowledge (and in contrast to the present study), previous reports have not assessed directly whether partitioning of exogenous fatty acids between β-oxidation and esterification is altered in the hypertrophied heart. Nonetheless, we had previously found (using a stable isotope approach in *ex vivo* perfusion) that endogenous sources (postulated at the time to be triglycerides) accounted for up to 20% of acetyl-CoA formation in hypertrophied hearts from spontaneously hypertensive rats, while it was 9 ±1% and negligible in control WKY and Wistar rat hearts, respectively (58).

With regards to the potential cardioprotective effects of cGMP signaling, an interesting peripheral observation of this study was that the expression of the transgene resulted in decreased release of LDH during *ex vivo* perfusion. While providing what are generally accepted to be physiological levels of workload, nutrients and calcium, the *ex vivo* working heart perfusion still constitutes a mild stress (59). It is noteworthy, however, that the magnitude of the LDH release in hearts perfused under normoxia is marginal compared to that observed following reperfusion after ischemia or anoxia (60). The cGMP-dependent reduction of LDH release may be independent of the aforementioned metabolic effects, since cGMP prevents cell death by combined effects on the mitochondrial transition pore, intracellular calcium regulation and stress-activated signaling (61-64). Alternatively, increased availability of cytosolic glycolytically-derived ATP might improve ionic homeostasis, as multiple membrane-associated channels preferentially use glycolytically-derived ATP (65-67) (68;69), and thus contribute in this fashion to improved membrane integrity.

In summary, this study expands on our previous findings that chronic activation of cGMP signaling protects the heart against pressure overload-induced or pharmacological stresses. We provide direct evidence that cGMP signaling modulates cardiac energy metabolism in a cardiomyocyte-specific manner. Through an HSL-dependent activation of lipolysis, cGMP specifically acts on lipid compartmentalization. This may mediate some of its beneficial effects by favoring triglyceride cycling and thereby preventing triglyceride accumulation in a cardiomyocyte-specific manner, especially under conditions such as fasting where circulating long chain fatty acids are increased. Further investigations appear warranted to link the lipid compartmentalization, herein described, to cGMP-mediated cardioprotection not only in models of hypertrophy but also in models of high fat feeding and diabetes where fatty acid-induced toxic effects has been reported (47;70;71).

Acknowledgements

This work was presented at the at the Society Heart and Vascular Metabolism held in Semihamoo, in September 2006, and in Maastricht in June 2007, and at the World Congress of the International Society of Heart Research held in Bologna in June 2007. This study was supported by the Canadian Institutes of Health Research (grants #74460 to C.F.D. and C.D.R. #77791 to B.G.A. and #10865 to G.D.L.), by the Heart and Stroke Foundation of Canada (studentship to M.K.), and by the National Heart, Lung, and Blood Institute (grants #HL-074259 to MEY). B.G.A. is a senior scientist of the FRSQ.

Table 1. Functional and physiological parameters of isolated working heart from control WT and $GC^{+/0}$ mice

Parameters	WT	$GC^{+/0}$
Heart rate (beats/min)	447 ± 1	429 ± 2
LVSP (mmHg)	87 ± 1	92 ± 1
LVEDP (mmHg)	14.8 ± 0.1	14.9 ±0 .1
+dP/dt ($mmHg \cdot s^{-1}$)	4302 ± 35	4579 ± 32
-dP/dt ($mmHg \cdot s^{-1}$)	3582 ± 25	3470 ± 31
Rate pressure product ($mmHg \cdot beats \cdot min^{-1} \cdot 10^{-3}$)	31819 ± 226	32480 ±167
Cardiac output (ml/min)	8.01 ± 0.05	7.82 ± 0.05
Aortic flow (ml/min)	4.31 ± 0.10	4.57 ± 0.07
Coronary flow (ml/min)	3.33 ± 0.04	3.24 ± 0.02
Cardiac power (mWatts)	1.66 ± 0.10	1.67 ± 0.05
MVO_2 (µmol/min)	1.93 ± 0.11	1.71 ± 0.10
Cardiac efficiency ($mWatts \cdot \mu mol_{O2}^{-1} \cdot min^{-1}$)	1.01 ± 0.04	1.13 ± 0.06
pHi	7.37 ± 0.00	7.38 ± 0.01

Data are means ± SEM of 13-17 heart perfusion experiments. Values shown represent averages for the entire perfusion period. MVO_2 and pHi were calculated from pO_2 and pCO_2 values determined in influent and effluent perfusate collected between 15 and 20 min. LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; MVO_2, oxygen consumption; pHi, intracellular pH.

Table 2. Metabolic parameters of $GC^{+/0}$ hearts and their WT littermates.

Parameters	WT	$GC^{+/0}$
OLE/CS	0.49 ± 0.03	0.31 ± 0.05*
PDC/CS	0.59 ± 0.07	0.52 ± 0.04
PC/CS	0.05 ± 0.01	0.04 ± 0.01
Contribution of carbohydrates to tissue pyruvate formation (%)		
Glucose	32 ± 4 %	33 ± 2 %
Pyruvate + Lactate	54 ± 3 %	54 ± 1 %
Others (glycogen)	18 ± 4 %	13 ± 3 %
Malonyl-CoA concentration (nmol/gww)	1.16 ± 0.04	1.23 ± 0.07
ACC activity ($\mu mol.min.^{-1}.gww^{-1}$)	0.76 ± 0.12	0.83 ± 0.09
[Acetyl-CoA]-to-[CoA] ratio (arbitrary units)	0.0559 ± 0.0054	0.0580 ± 0.0073
Citric Acid Cycle flux rate (in $\mu mol.min^{-1}.gww^{-1}$)	2.85 ± 0.20	3.11 ± 0.08

Data are means ± SEM of 3-8 heart perfusion experiments. The Experimental Procedures provide details about the (i) determinations of flux ratios, which reflect the contribution exogenous fatty acids (oleate) and of carbohydrates (lactate, pyruvate and glucose) to acetyl-CoA and or oxaloacetate (OAA) formation for citrate synthesis (CS), and (ii) calculation of citric acid cycle flux rate, and (iii) determinations of levels of CoA derivatives and ACC activity. OLE: oleate oxidation; PDC: pyruvate decarboxylation; PC: pryuvate carboxylation. *P<0.05

Supplemental Table 1. mRNA expression of enzymes implicated in energy and triglyceride metabolism in $GC^{+/0}$ hearts and their WT littermates.

Gene mRNA (in transcript number/per ng total RNA)	WT	$GC^{+/0}$
Energy metabolism and regulatory enzymes		
glut4	20460 ± 2518	18510 ± 1859
pdk4	32380 ± 4937	30160 ± 4980
pcx	484.0 ± 41.9	499.4 ± 31.0
mcad	14150 ± 663	15190 ± 796
Triglyceride metabolism enzymes		
atgl	13750 ± 995	12960 ± 683
dgat2	9344 ± 1137	8209 ± 800
hsl	200.2 ± 10.7	244.2 ± 8.6**

Data are means ± SEM of 6-9 freeze-clamped hearts collected in the middle of the light phase. *glut4,* glucose transporter 4; *pdk4,* pyruvate decarboxylase kinase 4; *pcx,* pyruvate carboxylase; *mcad,* medium-chain acyl-CoA dehydrogenase; *atgl,* adipose triglyceride lipase; *dgat2,* diacylglyerol O-acyltransferase 2; *hsl*, hormone sensitive lipase. ***P*<0.01

Figure Legends

Figure 1. Index of membrane integrity in isolated hearts from control WT and $GC^{+/0}$ mice

Data are means ± SEM of 13-15 heart perfusion experiments. Values shown represent averages over the entire perfusion period. Lactate dehydrogenase release rates of control (WT) mice (solid bars) and hearts from mice overexpressing guanylate cyclase in a cardiomyocyte-specific manner ($GC^{+/0}$) mice (open bars) were determined enzymatically by spectrophotometric method in effluent perfusates collected every 5 minutes. $^{**}P<0.01$ $GC^{+/0}$ vs control WT mouse hearts.

Figure 2. Lactate and pyruvate production rate (A) and the lactate-to-pyruvate production ratio (B) assessed in isolated working hearts from control WT and $GC^{+/0}$ mice

Data are means ± SEM of 4 heart perfusion experiments. **A.** Lactate and pyruvate release rates were calculated from the product of coronary flow rates and concentration differences in the influent and effluent perfusates of [U-$^{13}C_3$]lactate (open bars) and [U-$^{13}C_3$]pyruvate (solid bars) in hearts perfused with [U-$^{13}C_6$]glucose as determined by gas chromatography coupled to mass spectrometry (GCMS) and enzymatic assays. **B.** Lactate-to-pyruvate ratio, in arbitrary units, is expressed as the release rate of lactate divided by the release rate of pyruvate in WT hearts (solid bars) and $GC^{+/0}$ hearts (open bars). $^{*}P<0.05$ and $^{**}P<0.01$ $GC^{+/0}$ vs WT mouse hearts.

Figure 3. Incorporation of exogenous oleate into triglyceride stores of isolated working hearts from control WT and $GC^{+/0}$ mice

Data are means ± SEM of 5 heart perfusion experiments. The ^{13}C-enrichment of oleate in triglycerides was assessed in hearts following a 30-min perfusion in the working mode with [U-$^{13}C_{18}$]oleate by GCMS analysis of fatty acid methyl esters. Data are expressed as (A) absolute molar percent enrichment (MPE) or (B) relative to the MPE of the acetyl moiety of citrate. $^{*}P<0.05$ $GC^{+/0}$ vs WT hearts.

Figure 4. Mechanisms regulating lipolysis: phosphorylation status of hormone sensitive lipase (HSL; A and B) and AMP-activated kinase (AMPK; C) in WT and $GC^{+/0}$ mouse hearts

Data are means ± SEM of 7 (A & B) and 13 (C) freeze-clamped hearts of WT and $GC^{+/0}$ mouse hearts. Representative immunoblots using: (**A**) anti-phospho-HSL (Ser-565) with anti-HSL serving as loading controls for total HSL protein, (**B**) total HSL protein normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and (**C**) anti-phospho-αAMPK (Thr-172) antibodies with anti-αAMPK antibodies serving as loading controls for total αAMPK protein. Densitometry of phosphorylated protein to total protein ratios from experiments performed on tissue homogenates extracts is shown. $^{*}P<0.05$ and $^{***}P<0.001$ $GC^{+/0}$ vs WT hearts.

Figure 5. Effect of fasting on WT and $GC^{+/0}$ mouse heart triglyceride levels

Data are means ± SEM of 4-5 freeze-clamped hearts. Triglycerides were quantified spectrophotometrically using a commercial kit (Sigma). *P<0.05 WT vs $GC^{+/0}$ hearts and fasted vs fed hearts.

References

1. Frey, N. and Olson, E. N. (2003) *Annu. Rev. Physiol.* **65,** 45-79

2. Nishikimi, T., Maeda, N., and Matsuoka, H. (2006) *Cardiovasc. Res.* **69,** 318-328

3. Booz, G. W. (2005) *Hypertension* **45,** 341-346

4. Calderone, A. (2003) *Heart Fail. Rev.* **8,** 55-70

5. Deschepper, C. F. (2005) *Vasc. Dis. Prevention* **2,** 151-157

6. Zahabi, A., Picard, S., Fortin, N., Reudelhuber, T. L., and Deschepper, C. F. (2003) *J. Biol. Chem.* **278,** 47694-47699

7. Stanley, W. C., Recchia, F. A., and Lopaschuk, G. D. (2005) *Physiol. Rev.* **85,** 1093-1129

8. Neubauer, S. (2007) *N. Engl. J. Med.* **356,** 1140-1151

9. Recchia, F. A., McConnell, P. I., Loke, K. E., Xu, X., Ochoa, M., and Hintze, T. H. (1999) *Cardiovasc. Res.* **44,** 325-332

10. Recchia, F. A., Osorio, J. C., Chandler, M. P., Xu, X., Panchal, A. R., Lopaschuk, G. D., Hintze, T. H., and Stanley, W. C. (2002) *Am. J. Physiol.* **282,** E197-E206

11. Depre, C., Vanoverschelde, J. L., Goudemant, J. F., Mottet, I., and Hue, L. (1995) *Circulation* **92,** 1911-1918

12. Depre, C., Gaussin, V., Ponchaut, S., Fischer, Y., Vanoverschelde, J. L., and Hue, L. (1998) *Am. J. Physiol.* **274,** H1443-H1449

13. Sengenes, C., Bouloumie, A., Hauner, H., Berlan, M., Busse, R., Lafontan, M., and Galitzky, J. (2003) *J. Biol. Chem.* **278,** 48617-48626

14. Tada, H., Thompson, C. I., Recchia, F. A., Loke, K. E., Ochoa, M., Smith, C. J., Shesely, E. G., Kaley, G., and Hintze, T. H. (2000) *Circ. Res.* **86,** 270-274

15. Li, J., Hu, X., Selvakumar, P., Russell, R. R., III, Cushman, S. W., Holman, G. D., and Young, L. H. (2004) *Am. J. Physiol.* **287,** E834-E841

16. Khairallah, M., Labarthe, F., Bouchard, B., Danialou, G., Petrof, B. J., and Des Rosiers, C. (2004) *Am. J. Physiol.* **286,** H1461-H1470

17. Young, M. E., Razeghi, P., Cedars, A. M., Guthrie, P. H., and Taegtmeyer, H. (2001) *Circ. Res.* **89,** 1199-1208

18. Chan, A. Y., Soltys, C. L., Young, M. E., Proud, C. G., and Dyck, J. R. (2004) *J. Biol. Chem.* **279,** 32771-32779

19. Durgan, D. J., Trexler, N. A., Egbejimi, O., McElfresh, T. A., Suk, H. Y., Petterson, L. E., Shaw, C. A., Hardin, P. E., Bray, M. S., Chandler, M. P., Chow, C. W., and Young, M. E. (2006) *J. Biol. Chem.* **281,** 24254-24269

20. Boivin, B., Villeneuve, L. R., Farhat, N., Chevalier, D., and Allen, B. G. (2005) *J. Mol. Cell. Cardiol.* **38,** 665-676

21. Taigen, T., De Windt, L. J., Lim, H. W., and Molkentin, J. D. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 1196-1201

22. Vincent, G., Bouchard, B., Khairallah, M., and Des Rosiers, C. (2004) *Am. J. Physiol.* **286,** H257-H266

23. Folch, J., Lees, M., and Sloane Stanley, G. H. (1957) *J. Biol. Chem.* **226,** 497-509

24. Ametaj, B. N., Bobe, G., Lu, Y., Young, J. W., and Beitz, D. C. (2003) *J. Agric. Food Chem.* **51,** 2105-2110

25. Roduit, R., Masiello, P., Wang, S. P., Li, H., Mitchell, G. A., and Prentki, M. (2001) *Diabetes* **50,** 1970-1975

26. Ruiz, J., Antequera, T., Andres, A. I., Petron, M., and Muriel, E. (2004) *Analytica Chimica Acta* **520,** 201-205

27. Lepage, G., Levy, E., Ronco, N., Smith, L., Galeano, N., and Roy, C. C. (1989) *J. Lipid Res.* **30,** 1483-1490

28. De Windt, L. J., Lim, H. W., Bueno, O. F., Liang, Q., Delling, U., Braz, J. C., Glascock, B. J., Kimball, T. F., del, M. F., Hajjar, R. J., and Molkentin, J. D. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 3322-3327

29. Vavvas, D., Apazidis, A., Saha, A. K., Gamble, J., Patel, A., Kemp, B. E., Witters, L. A., and Ruderman, N. B. (1997) *J. Biol. Chem.* **272,** 13255-13261

30. Goodwin, G. W. and Taegtmeyer, H. (1999) *Am. J. Physiol.* **277,** E772-E777

31. McGarry, J. D., Stark, M. J., and Foster, D. W. (1978) *J. Biol. Chem.* **253,** 8291-8293

32. Allred, J. B. and Guy, D. G. (1969) *Anal. Biochem.* **29,** 293-299

33. Lei, B., Lionetti, V., Young, M. E., Chandler, M. P., d'Agostino, C., Kang, E., Altarejos, M., Matsuo, K., Hintze, T. H., Stanley, W. C., and Recchia, F. A. (2004) *J. Mol. Cell. Cardiol.* **36,** 567-576

34. Kerbey, A. L., Randle, P. J., Cooper, R. H., Whitehouse, S., Pask, H. T., and Denton, R. M. (1976) *Biochem. J.* **154,** 327-348

35. Small, C. A., Garton, A. J., and Yeaman, S. J. (1989) *Biochem. J.* **258,** 67-72

36. Schweiger, M., Schreiber, R., Haemmerle, G., Lass, A., Fledelius, C., Jacobsen, P., Tornqvist, H., Zechner, R., and Zimmermann, R. (2006) *J. Biol. Chem.* **281,** 40236-40241

37. Calderone, A., Thaik, C. M., Takahashi, N., Chang, D. L., and Colucci, W. S. (1998) *J. Clin. Invest.* **101,** 812-818

38. Takimoto, E., Champion, H. C., Li, M., Belardi, D., Ren, S., Rodriguez, E. R., Bedja, D., Gabrielson, K. L., Wang, Y., and Kass, D. A. (2005) *Nat. Med.* **11,** 214-222

39. Cuthbert, K. D. and Dyck, J. R. (2005) *Curr. Hypertens. Rep.* **7,** 407-411

40. Yeaman, S. J. (2004) *Biochem. J.* **379,** 11-22

41. Garton, A. J., Campbell, D. G., Carling, D., Hardie, D. G., Colbran, R. J., and Yeaman, S. J. (1989) *Eur. J. Biochem.* **179,** 249-254

42. Holm, C. (2003) *Biochem. Soc. Trans.* **31,** 1120-1124

43. Suzuki, J., Shen, W. J., Nelson, B. D., Patel, S., Veerkamp, J. H., Selwood, S. P., Murphy, G. M., Jr., Reaven, E., and Kraemer, F. B. (2001) *Am. J. Physiol.* **281,** E857-E866

44. Haemmerle, G., Lass, A., Zimmermann, R., Gorkiewicz, G., Meyer, C., Rozman, J., Heldmaier, G., Maier, R., Theussl, C., Eder, S., Kratky, D., Wagner, E. F., Klingenspor, M., Hoefler, G., and Zechner, R. (2006) *Science* **312,** 734-737

45. Listenberger, L. L., Han, X., Lewis, S. E., Cases, S., Farese, R. V., Jr., Ory, D. S., and Schaffer, J. E. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 3077-3082

46. Dyntar, D., Eppenberger-Eberhardt, M., Maedler, K., Pruschy, M., Eppenberger, H. M., Spinas, G. A., and Donath, M. Y. (2001) *Diabetes* **50,** 2105-2113

47. Zhou, Y. T., Grayburn, P., Karim, A., Shimabukuro, M., Higa, M., Baetens, D., Orci, L., and Unger, R. H. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 1784-1789

48. Sharma, S., Adrogue, J. V., Golfman, L., Uray, I., Lemm, J., Youker, K., Noon, G. P., Frazier, O. H., and Taegtmeyer, H. (2004) *FASEB J.* **18,** 1692-1700

49. Dimmeler, S., Lottspeich, F., and Brune, B. (1992) *J. Biol. Chem.* **267,** 16771-16774

50. Tsuura, Y., Ishida, H., Shinomura, T., Nishimura, M., and Seino, Y. (1998) *Biochem. Biophys. Res. Commun.* **252,** 34-38

51. Lewin, T. M. and Coleman, R. A. (2003) *Biochim. Biophys. Acta* **1634,** 63-75

52. Allard, M. F., Wambolt, R. B., Longnus, S. L., Grist, M., Lydell, C. P., Parsons, H. L., Rodrigues, B., Hall, J. L., Stanley, W. C., and Bondy, G. P. (2000) *Am. J. Physiol.* **279,** E487-E493

53. Lydell, C. P., Chan, A., Wambolt, R. B., Sambandam, N., Parsons, H., Bondy, G. P., Rodrigues, B., Popov, K. M., Harris, R. A., Brownsey, R. W., and Allard, M. F. (2002) *Cardiovasc. Res.* **53,** 841-851

54. Nascimben, L., Ingwall, J. S., Lorell, B. H., Pinz, I., Schultz, V., Tornheim, K., and Tian, R. (2004) *Hypertension* **44,** 662-667

55. Bueno, O. F., Wilkins, B. J., Tymitz, K. M., Glascock, B. J., Kimball, T. F., Lorenz, J. N., and Molkentin, J. D. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 4586-4591

56. Antos, C. L., McKinsey, T. A., Frey, N., Kutschke, W., McAnally, J., Shelton, J. M., Richardson, J. A., Hill, J. A., and Olson, E. N. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 907-912

57. Depre, C., Shipley, G. L., Chen, W., Han, Q., Doenst, T., Moore, M. L., Stepkowski, S., Davies, P. J., and Taegtmeyer, H. (1998) *Nat. Med.* **4,** 1269-1275

58. Labarthe, F., Khairallah, M., Bouchard, B., Stanley, W. C., and Des Rosiers, C. (2005) *Am. J. Physiol.* **288,** H1425-H1436

59. Khairallah, M., Khairallah, R., Young, M. E., Dyck, J. R., Petrof, B. J., and Des Rosiers, C. (2007) *J. Mol. Cell. Cardiol.* **43,** 119-129

60. Marshall, T., Williams, J., and Williams, K. M. (1994) *Biochem. Soc. Trans.* **22,** 312S

61. Abdallah, Y., Gkatzoflia, A., Pieper, H., Zoga, E., Walther, S., Kasseckert, S., Schafer, M., Schluter, K. D., Piper, H. M., and Schafer, C. (2005) *Cardiovasc. Res.* **66,** 123-131

62. Das, A., Smolenski, A., Lohmann, S. M., and Kukreja, R. C. (2006) *J. Biol. Chem.* **281,** 38644-38652

63. Monastyrskaya, E., Folarin, N., Malyshev, I., Green, C., and Andreeva, L. (2002) *Nitric Oxide* **7,** 127-131

64. Fiedler, B., Feil, R., Hofmann, F., Willenbockel, C., Drexler, H., Smolenski, A., Lohmann, S. M., and Wollert, K. C. (2006) *J. Biol. Chem.* **281,** 32831-32840

65. Weiss, J. N. and Lamp, S. T. (1989) *J. Gen. Physiol* **94,** 911-935

66. Aasum, E., Lathrop, D. A., Henden, T., Sundset, R., and Larsen, T. S. (1998) *J. Mol. Cell. Cardiol.* **30,** 1703-1712

67. Xu, K. Y., Zweier, J. L., and Becker, L. C. (1995) *Circ. Res.* **77,** 88-97

68. Glitsch, H. G. and Tappe, A. (1993) *Pflugers Arch.* **422,** 380-385

69. Paul, R. J., Hardin, C. D., Raeymaekers, L., Wuytack, F., and Casteels, R. (1989) *FASEB J.* **3,** 2298-2301

70. Finck, B. N., Lehman, J. J., Leone, T. C., Welch, M. J., Bennett, M. J., Kovacs, A., Han, X., Gross, R. W., Kozak, R., Lopaschuk, G. D., and Kelly, D. P. (2002) *J. Clin. Invest.* **109,** 121-130

71. Unger, R. H. and Orci, L. (2000) *Int. J. Obes. Relat. Metab. Disord.* **24 Suppl 4,** S28-S32

Figure 1.

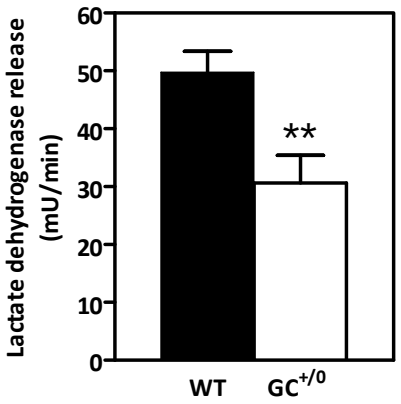

Lactate dehydrogenase release (mU/min)
60
50
40
30
20
10
0
**
WT
GC+/0

Figure 2.

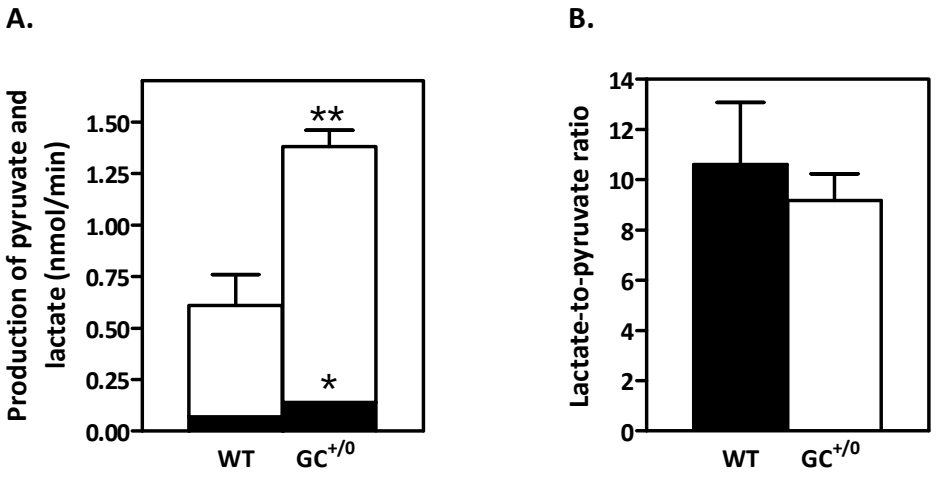

A.
Production of pyruvate and lactate (nmol/min)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
**
*
WT
GC+/0
B.
Lactate-to-pyruvate ratio
14
12
10
8
6
4
2
0
WT
GC+/0

Figure 3.

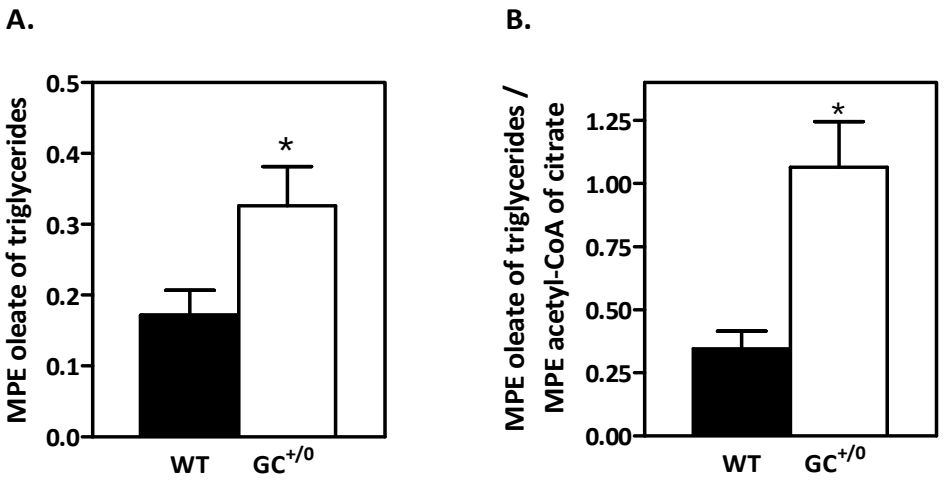

A.
MPE oleate of triglycerides
0.5
0.4
0.3
0.2
0.1
0.0
*
WT
GC+/0
B.
MPE oleate of triglycerides / MPE acetyl-CoA of citrate
1.25
1.00
0.75
0.50
0.25
0.00
*
WT
GC+/0

Figure 4.

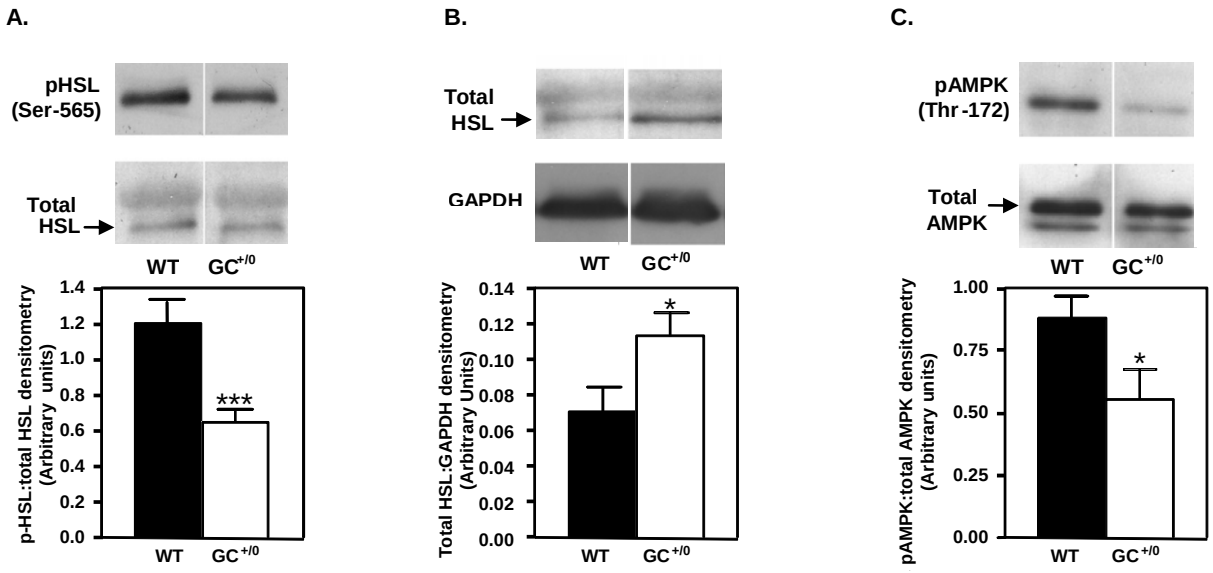
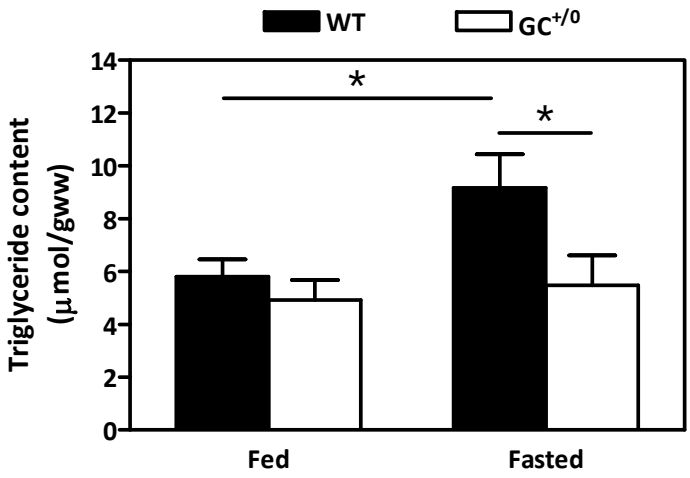

A.
pHSL
(Ser-565)
Total
HSL
WT
GC+/0
p-HSL:total HSL densitometry
(Arbitrary units)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
WT
GC+/0
B.
Total
HSL
GAPDH
WT
GC+/0
Total HSL:GAPDH densitometry
(Arbitrary Units)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
WT
GC+/0
C.
pAMPK
(Thr-172)
Total
AMPK
WT
GC+/0
pAMPK:total AMPK densitometry
(Arbitrary units)
1.00
0.75
0.50
0.25
0.00
*
WT
GC+/0

Figure 5.


WT
GC+/0
Triglyceride content (μmol/gww)
14
12
10
8
6
4
2
0
*
*
Fed
Fasted

Chapter III - Discussion

While cardiac energy metabolism has been a topic of interest for many years, interest has been surging in the last decade in the light of recent findings linking metabolism to cardiac function. For instance, it has been found that the adult heart switches its metabolic genes in response to pathologial stimuli (such as for instance pressure overload (204)), and that the metabolism of energy substrates have pleiotropic actions that go beyond the mere production of ATP, including signals for cardiac growth, programmed cell death and survival, as well as formation of reactive oxygen species (9). In fact, metabolic perturbations are currently considered as one of many mechanisms that contribute to cardiopathological conditions such as ischemia/reperfusion injury and hypertrophy (5). Accordingly, metabolic therapies are now being considered as new adjuncts or alternatives to current treatments for heart diseases (9;205). However, the modalities of these therapies remain to be defined on the basis of a better understanding of the role of the various components governing optimal cardiac metabolism, which include signaling pathways. In this regard, results from this project provide for the first time evidence that chronic activation of the cardioprotective cGMP signaling pathway in a cardiomyocyte specific manner modifies cardiac substrate energy metabolism. Specifically, we showed that activation of this pathway modulates FA trafficking and prevents triglyceride accumulation, which could significantly contribute to the protection against pressure-overload hypertrophy. This section will first discuss methodological considerations that ought to be considered in data interpretation, then further elaborate on some findings and, finally, present the broader (patho)physiological relevance of our studies.

Methodological Considerations

Working heart perfusion system

In order to fulfill the goals set by this study, we have utilized the *ex vivo* working heart perfusion system. This widely used tool was combined with the use of ^{13}C-labeled substrates and GCMS. This approach allows for the continuous monitoring of cardiac mechanics and metabolism and, hence, is particularly useful for clarifying the link between substrate choice for energy production and cardiac function within the whole intact beating organ. The measurements of dynamic fluxes can then be integrated with static measurements of metabolite concentrations, protein levels and gene expression. Perfusion conditions were chosen in an attempt to mimic as closely as possible the *in vivo* milieu, with respect to workload, concentrations of calcium, substrates, hormones and co-factors. In fact, this *ex vivo* study model combines the advantages of (i) flexibility in terms of experimental protocols and conditions, and (ii) relevance to the *in vivo* situation in terms of work load, substrate selection, and metabolism (9). However, this experimental model remains nevertheless an *ex vivo* system with some inherent limitations. For example, beyond being denervated, these hearts were perfused with oleate as the sole exogenous fatty acid source. While oleate is among the preferred FA substrates taken up by the heart, it only accounts for about 60% of ATP derived from FAs *in vivo*. Other major contributors include, in order of importance, palmitate, stearate and other FAs used to a lesser extent. Furthermore, multiple studies

have highlighted the different effects of saturated and unsaturated FAs on signaling pathways and on triglyceride synthesis (206;207). On the same note, we extrapolate FA oxidation on the assumption that albumin-bound free FAs are the only contributors to energy synthesis in the heart. However, this is not the case *in vivo*, where lipoproteins and chylomicrons also contribute to the transport and supply of FAs to the heart. Indeed, lipoprotein lipase knockout mice develop cardiomyopathies (208-210), supporting the notion that FAs derived from lipoprotein lipolysis are important for cardiac lipid metabolism. Furthermore, our buffer lacks amino acids crucial to the heart such as glutamine, leucine, and alanine as well as other hormones, namely glucagon. However, due to the short perfusion time, i.e. 30 minutes, the lack of these amino acids and hormones should have limited consequences. Finally, another limitation of the *ex vivo* heart perfusion system is the lack of an efficient oxygen transport system. While *in vivo*, the heart can rely on hemoglobin for oxygen transport, very few studies have used washed red blood cells in their perfusions due to technical difficulties. Consequently, our perfusate needs to be saturated in oxygen, yielding O_2 partial pressures much higher than physiological values and possibly submitting the heart to higher levels of oxidative stress.

While the use of an intact whole organ preparation is essential to study the intricate pathways involved in cardiac energy metabolism, it does not discriminate the specific contribution of the organ's various cell types to the measured metabolic processes. In fact, although the heart is composed predominantly of cardiomyocytes (on a weight basis >85%), it includes also other cell types with different metabolic profiles. Indeed, while cardiomyocytes (which constitute the contractile unit of the heart) have an oxidative metabolism, the metabolism of fibroblasts and endothelial cells (which constitute the structural unit of the heart) is mostly glycolytic. This is of particular importance in the diseased heart, which undergoes structural remodeling. Pathological states such as hypertrophy are associated with fibroblast proliferation (211). In the intact heart, although the metabolic activity of cardiomyocytes outweighs by far that of other cell types, the observed metabolic status reflects that of the organ as a whole rather than cardiomyocytes specifically.

Regional metabolic variability might also occur in the isolated intact heart. In fact, substrate concentration differences at various regions of the heart can be caused by the diffusive capacity of the capillary wall and the permeating substrates metabolized by the different cell types. With colloid-free perfusion, concentration differences can be underestimated since the lack of plasma proteins will increase capillary permeability (212). Regional differences in the concentration of energy substrates and/or metabolites (expressed as percentages of interstitial to vascular values) can be important, as glucose concentration may decrease by as much as 50%, lactate by as much as 700%, and glutathione, uric acid, adenosine, and catecholamines (when released by the myocardium) may increase by as much as 200-600% (213). The magnitude of these gradients has to be taken into consideration. This is of further importance in most *ex vivo* perfusion systems that operate in the circulating mode, meaning that the buffer released by the heart is pumped back into the coronaries. We circumvented this major technical flaw by collecting the coronary perfusate instead of recirculating it back to the heart. The use of a non-recirculating buffer for heart perfusion is also a crucial prerequisite when using ^{13}C-labeled substrates to avoid recycling of ^{13}C-labeled metabolites released by the heart. This would prevent

achievement of an isotopic steady-state, which is an assumption of the equations that are used to calculate metabolic flux ratios.

Measurements of myocardial triglyceride turnover and lipolysis

Although endogenous triglyceride stores may be a potentially substantial source of FAs for β-oxidation in the healthy or diseased heart, only few studies have measured triglyceride turnover in working hearts (214;215). These studies have used the traditional radiolabeled experiments with carbon-14 and tritium labeling in a pulse-chase protocol. Triglyceride turnover was extrapolated from a two-point linear analysis of data, by measuring endogenous and exogenous FA oxidation through $^{14}CO_2$ and $^{3}H_2O$ release in the heart effluents at different time points. While this is a powerful approach, it is an indirect measurement of triglyceride turnover and oxidation, and it cannot measure the enrichment of triglyceride pools. More recently, a new method was developed using nuclear magnetic resonance (NMR). NMR provided the opportunity for the development of the first kinetic model of triglyceride turnover from ^{13}C isotopic enrichment data assessed in the *ex vivo* Langendorff-perfused mouse heart (216). Our study, for the first time, allowed for the direct measurement of exogenous FA incorporation into endogenous triglyceride stores of *ex vivo* perfused mice hearts using GCMS. One of the major strength of our approach is that it enables the direct and simultaneous assessment of the incorporation of exogenous oleate into myocardial triglycerides and acetyl-CoA, which reflects the partitioning of this FA into esterification (storage) and β-oxidation (energy), respectively. However, our method does not enable us to measure triglyceride turnover precisely, because this calculation would require measurements of pre- and post-perfusion triglyceride content as well as measurements of the ^{13}-C enrichment of this pool at several time points during the perfusion period. In fact, the extrapolation of a turnover rate from the ^{13}C-enrichment of triglycerides that is assessed solely in hearts freeze-clamped at the end of the 30-minute perfusion period assumes that the rate of label incorporation would be linear with time. However, this assumption does not appear to be valid given that one study has shown that shorter enrichment periods yielded greater turnover rates when based on end-point linear enrichment (215). Thus, measurements in the change in turnover are not possible if it is assessed for a single pool under steady-state conditions.

There is another aspect by which our work differs from that of most other studies using ^{13}C-labeled FAs to perfuse ex vivo hearts, since previous published heart perfusion studies have all used palmitate (instead of oleate in the present study). Both oleate and palmitate constitute the most abundant circulating free FAs and are preferred substrates for the heart, but they appear to be handled differently by the hearts, at least with respect to triglyceride metabolism. In fact, it has been previously shown that oleate prevents palmitate-induced apoptosis, possibly by promoting triglyceride synthesis (206;207). Furthermore, the propensity of palmitate to enter neutral lipid stores in significantly lower than that of oleate (206). Incidentally, the triglyceride turnover rate that is estimated from our ^{13}C-enrichment values for the oleate moiety of triglycerides measured in perfused control mouse hearts is approximately three-times greater than that reported in the literature using palmitate (216) (457 ± 63 vs 160 ± 30 $nmol.min^{-1}.gdw^{-1}$). Hence, to clarify these aspects and further substantiate our conclusion that chronic cGMP signaling increases triglyceride turnover, additional experiments could have been undertaken

using a pulse-chase protocol, whereby enrichments of triglyceride pools and direct endogenous FA oxidation would be assessed after a 15- or 30-min "chase" of unlabeled oleate or palmitate.

Based on experiments and results presented in chapter II, we concluded that hearts from $GC^{+/0}$ mice display an increased lipolysis. However, it is noteworthy that this conclusion is supported by our finding of a decrease in the phosphorylation status of an inhibitory site of HSL. However, one other site whose phosphorylation contributes to activation of HSL activating did not show any changes in its phosphorylation status, and we did not have antibodies to test the phosphorylation status of other possible important sites. Despite these limitations, our finding that triglyceride content following a 24h-fast did not increase in hearts from $GC^{+/0}$ mice - an effect that is similar to that seen in mice overexpressing HSL (217) - further support the possibility that activation of HSL increases lipolysis. However, we cannot exclude, though it appears unlikely, that triglyceride content did not increase due to decreased synthesis rather than increased breakdown. In fact, previous experiments conducted with isolated cardiomyocytes from $GC^{+/0}$ mice showed an increased glycerol release compared to controls (data from A. Zahabi, not shown), supporting an accelerated lipolysis in these transgenic cells. However, the amount of glycerol measured in these experiments was very close to the limit of detection of the assay used and precluded the formulation of a definitive conclusion. Nevertheless, to further support and substantiate our conclusion, we could have assessed lipolytic activity in heart extracts incubated with radiolabeled triolein or tripalmitin. However, this would have given us the activity of both ATGL and HSL. There are no current methods to discriminate between these lipases.

Having discussed the methodological considerations of our study, the following sections will further elaborate on the results and discussions presented in Chapters II, III and IV.

Conceptual considerations

In addition to our finding that chronic activation of cGMP signaling affects lipid trafficking and glycolysis, we made an additional observation that deserves further comments. In fact, during *ex vivo* perfusion in the working mode, we found that hearts from $GC^{+/0}$ mice displayed a lower release of LDH, which was used as a surrogate marker of membrane integrity. This finding may also be relevant to the cardioprotective mechanism associated with enhanced cGMP signaling. Indeed, while providing what are generally accepted to be physiological levels of workload, nutrients and calcium, the *ex vivo* working heart perfusion still constitutes a mild stress on the organ resulting in minimal tissue damage (218), as noted by baseline levels of LDH release. The observation that the transgene improves this surrogate marker of membrane integrity highlights the importance of the cGMP pathway in cardioprotection. Interestingly, the decreased LDH release rate in perfused $GC^{+/0}$ hearts was not associated with changes in cardiac power or efficiency. This contrasts with other models in which membrane integrity is maintained through downregulation of contractility, such as the hibernating myocardium (219). Furthermore, these findings emphasize the importance of cGMP signaling in counteracting not only chronic stresses, such as those induced by pressure-overload or isoproterenol stimulation (203), but also acute stresses, such as 30 min isolated working heart perfusions.

Initially, we attempted to investigate the relationship between the lower LDH release and the enhanced glycolysis, postulating that by partially inhibiting glycolysis in $GC^{+/0}$ mouse hearts, we would be able to block the decrease in LDH without affecting function and mimic the control phenotype. This working hypothesis was based on the following reasoning. Multiple ion channels form complexes with glycolytic enzymes, which appear to be associated with the sarcolemmal and sarcoplasmic reticulum (220-222). Furthermore, some ion channels, including sarcoplasmic reticulum calcium ATPase (SERCA) and the Na^+/K^+ pump preferentially, use glycolytically-derived ATP (223;224). Hence, we reasoned that the enhanced glycolysis observed in our transgenic animal model may be beneficial, improving ion homeostasis by providing readily available cytosolic ATP *in situ*. While other authors have proposed that increases in glycolysis are detrimental in the context of hypertrophy (225;226), increased glycolysis in the latter studies was generally accompanied by alterations in the redox state of the cell, more specifically, increases in lactate production that are not matched by increases in pyruvate production. However, the $GC^{+/0}$ mouse heart increases its release rate of pyruvate to the same extent of that of lactate, which means that the $[NAD^+]$-to-[NADH] ratio remains unaltered and that the cell's redox state is therefore unchanged. The following section will discuss additional mechanisms that may possibly explain the lower LDH release of perfused $GC^{+/0}$ mouse hearts compared to control, and that remain to be explored.

The mitochondrial permeability transition pore (MPTP) and PKCε

Several pathways leading to cell necrosis do so by opening the MPTP, thereby abolishing the mitochondrial membrane potential essential for ATP production and releasing the caspase activator and apoptosis signalling molecule cytochrome c. Opening of the MPTP eventually leads to cytochrome c release, caspase activation, and cell death. One of the common causes of MPTP opening is an ischemic insult. For more than two decades, it has been known that several brief periods of ischemia prior to a prolonged lethal ischemic insult protects the myocardium against ischemia-induced infarction (227). This phenomenon (referred to as "ischemic preconditioning") was found to inhibit opening of the MPTP through a cGMP dependent signalling pathway (228;229). While in our experimental model of *ex vivo* heart perfusion in the working mode used normoxic conditions, we cannot exclude the possibility that a transient ischemic episode may occur during heart isolation (although we took care to constantly rinse heart with cold buffer during the dissection). Given that chronic activation of the cGMP pathway partially mimics preconditioning, $GC^{+/0}$ mouse hearts could possibly be more resistant to the mild ischemic stress associated with isolation before *ex vivo* perfusion. In this regard, we found that control mouse hearts displayed a LDH release of 50±4 mU/min in the first 5 minutes, which decreased by 12 % to 44±4 mU/min at the end of the perfusion ($p<0.05$). This contrasts with $GC^{+/0}$ mouse hearts, which showed no difference in the LDH release throughout the perfusion, on average 29.7±0.1 mU/min.

More recently, Costa and colleagues demonstrated that the addition of exogenous PKG and cGMP to isolated mitochondria resulted in the opening of mitochondrial ATP-sensitive potassium ($mitoK_{ATP}$) channels, which subsequently prevents opening of the MPTP. PKG-dependent MPTP channel activation could be blocked by 5-hydroxydecanoate, glibenclamide, and tetraphenylphosphonium, which block

mitoK_{ATP} opening (230;231). The mitoK_{ATP} are located on the inner mitochondrial membrane, and cytosolic PKG would not be able to penetrate the outer membrane. That implies that intermediate steps exist to transmit the signal between the two membranes. Indeed, Costa showed that channel opening was dependent on the PKCε isoform. It is uncertain how many other steps within the mitochondria might be involved. Additional data from these authors supported the involvement of PKG in mediating the cardioprotective signal from the cytosol to the mitochondria (231). Additionally, PKCε appears to be a target for multiple signaling pathways and is thought to somehow provide a memory of the protection that persists after the trigger is withdrawn and which continues during prolonged periods of stress (232). While we have made attempts to assess PKCε phosphorylation in hearts from control and $GC^{+/0}$ mice, we found no difference in the protein content of PKCε in whole heart or mitochondrial extracts between the two groups. We were unable to quantify the phosphorylation level of PKCε because of the poor quality of phospho-peptide antibodies recognizing phoshorylated PKCε.

Other mechanisms - potential involvement of calcium, calcineurin-NFAT, lipotoxicity and MAPK signalling

One factor that is likely to be involved in mediating an acute cardioprotective effect of enhancing cGMP signaling is improved calcium handling. Indeed, it is widely accepted that cGMP, through protein kinase G (PKG), regulates several aspects of ion homeostasis, and more importantly, calcium release and uptake (233;234). It was recently demonstrated that a cGMP analogue reduces peak intracellular [Ca^{2+}] and intracellular [Ca^{2+}] oscillation (235) after ischemia-reperfusion, thereby attenuating the excessive activation of the contractile machinery that otherwise leads to the development of hypercontracture and cell damage. Indeed, cGMP/PKG I-dependent inhibition of the L-type Ca^{2+} channel current has been implicated in the negative inotropic effects of NO in cardiac myocytes (236) and to inhibit SR calcium release through the ryanodine receptor channel (237). Simultaneously, PKG-1 also relieves SERCA inhibition through phosphorylation and inactivation of phospholamban (238). Taken together, cGMP appears to be able to decrease calcium influx in the cytosol as well as increase the efficiency by which it is pumped back into the SR, i.e. regulating the main channels responsible for calcium homeostasis in cardiomyocytes. This may thus affect contraction, but also the other events that are Ca^{2+}-dependent in cardiomyocytes. Beyond the mere regulation of the concentration of calcium, the cGMP pathway can also modulate the ability of that ion to trigger the opening of the MPTP pathway as mentioned above, but also to act as a second messenger activating the calcineurin-NFAT signaling pathway. As described in Chapter I, calcineurin becomes activated by sustained elevations of intracellular Ca^{2+} concentration. Once activated, calcineurin dephosphorylates its primary downstream effector NFAT, leading to its nuclear translocation (35). However, in cell culture models, NO has been shown to promote antihypertrophic effects via cGMP-dependent PKG activation (239). Although the actual source of Ca^{2+} that activates calcineurin in cardiac myocytes is incompletely understood (240), it has been proposed that calcineurin activation in cardiac myocytes depends on Ca^{2+} entry via the L-type Ca^{2+} channel (39). Along this line, it has been shown that the growth-inhibitory effects of the NO–cGMP–PKG pathway upstream from calcineurin are mediated by inhibition of the L-type Ca^{2+} channel current (239;241). More

acutely, calcineurin has also been shown to be involved in isoproterenol-mediated cardiomyocyte apoptosis, and this effect was abrogated by the calcineurin inhibitors Cyclosporine A and FK506 (242). Similarly, transgenic mice expressing dominant-negative calcineurin in the heart were refractory to isoproterenol-induced TUNEL reactivity *in vivo* (243). However, the role of calcineurin is controversial, as it has also been shown to protect against ischemia-reperfusion injury and other acute assaults. Indeed, transgenic mice expressing a constitutively active mutant of calcineurin are significantly protected from ischemia-reperfusion-induced DNA laddering (244) and in cultured cardiomyocytes adenoviral-mediated gene transfer of activated calcineurin reduced 2-deoxyglucose-induced TUNEL, while calcineurin inhibition with a Cain expressing adenovirus increased TUNEL (244). While more investigation is necessary to further clarify the role of calcineurin in mediating both chronic and acute cardioprotection, the effects of cGMP of calcium transients can possibly enable better regulation of the calcineurin signaling pathway and promote an anti-apoptotic effect.

Another potential benefit of increase cGMP signaling is the decrease in lipid signaling and lipotoxicity. Indeed, it is widely accepted that the sphingolipid ceramide is a ubiquitous regulator of cellular stress and contributes to insulin resistance in diabetic patients (245) Moreover, studies have shown that ceramide and/or its derivatives, such as ganglioside GM3 and sphingosine, induce oxidative stress, apoptosis, inhibit glucose uptake and storage and antagonize insulin signaling (245). Through increased cellular compartmentation of lipid storage and oxidation, cGMP may therefore prevent free FAs from taking part in other pathways such as the synthesis of ceramides, or activation of PKC through increased diacyglycerol. While we did not measure ceramides or PKC activation, it is appears likely that these factors are decreased in our model and contribute to the increased cell survival during working heart perfusion.

There are at least two other potential mechanisms by which cGMP signalling can promote cell survival and increased membrane integrity, as seen in the $GC^{+/0}$ mouse hearts. Both of these mechanisms involve interference with MAPK signaling. As detailed in Chapter I, MAPK signalling is upregulated in heart failure and is generally considered to be a major player in the development of the disease. However, its actions are generally considered to be chronic and cannot readily explain the difference seen during an acute stress such as a working heart perfusion. Given the chronic activation of the cGMP pathway noted in our model, it is nevertheless a possibility that the heart is better prepared to an acute assault through increased regulation of pro-inflammatory or pro-apoptotic factors, such as TNFα (245), iNOS (246) and Mcl-1 (247).

One mechanism involving MAPK that could protect the myocardium would involve the potential interference of cGMP signaling with the pro-apoptotic TAB-1-p38 MAPK signaling pathway. Cardiac myocyte death by apoptosis is tightly controlled by a complex network of stress-responsive signalling pathways. One pro-apoptotic pathway involves the interaction of the scaffold protein TAB1 with p38 mitogen-activated protein kinase (p38 MAPK) leading to the autophosphorylation and activation of p38 MAPK. This process is generally activated during ischemia and independent of upstream p38 kinases MKK3 and MKK6 (248). It has been shown that activation of p38 leads to hypertrophy while suppression of p38α, the predominant isoform of p38 MAPK in the mammalian heart, by transgenic overexpression of a dominant-negative mutant reduces cardiac myocyte apoptosis and myocardial infarct sizes during

I/R (249). Recently, one study showed that the NO/cGMP downstream target PKG protects cardiac myocytes from ischemia/reperfusion-induced injury by interfering with the pro-apoptotic TAB1-p38 MAPK signaling pathway. Binding of cGMP-activated PKG to p38 MAPK and inhibition of TAB1-induced p38 MAPK autophosphorylation appeared to be the molecular mechanism of p38 MAPK inhibition by PKG (250). Similarly, cGMP was also shown to protect from ROS induced apoptosis in cultured macrophage cells through inhibition of p38 activation (251).

Similarly to the above mechanism, cGMP-dependant signalling can also chronically attenuate MAPK signalling through dual specificity MAPK phosphatases, such as MKP-1, which inactivate MAPK by dephosphorylation of the two critical MAPK residues accountable for their activity and regulate the magnitude and duration of p38, JNK, and ERK activation. MKP-1 is constitutively expressed at a very low level and underlies a tight and rapid transcriptional induction by different stimuli (252). Little is known about the signaling events leading to an MKP-1 induction besides reports describing that ROS (253) and different kinases (MAPK and PKC)(254) might be involved. Recently, it has been shown that constitutive activation of MKP-1 limits the cardiac hypertrophic response (80) and protects the heart from injury and cell death following ischemia-reperfusion *in vivo* (249). This provided a framework for a novel mechanism by which cGMP signaling can be cardioprotective. Indeed, it has been found that ANF activated Rac1, leading to the production of superoxide. Superoxide activates the JNK/AP-1 pathway, resulting in transcriptional upregulation of MKP-1 and overall downregulation of all MAPK pathways (255) and therefore protect the myocardium both acutely and chronically. It has also been hypothesized that the ROS production also serves a pre-conditioning purpose and can account for part of the cardioprotective effect of ANF.

Broader relevance of this study and clinical perspectives

Our finding of an effect of enhanced cGMP signaling on lipid trafficking, reported in chapter II, appears to have specific relevance for preventing cardiac lipotoxicity, a condition that has been linked to the development of diabetic cardiomyopathy. In fact, diabetes is one of the most prevailing causes that lead to cardiac dysfunction (256). It is the single most important risk factor for coronary artery disease and over 30% diabetics in the United-States are diagnosed with diabetic heart disease (257). Furthermore, two-thirds of diabetics will eventually die of some sort of cardiovascular disease (258). In addition to large vessel disease and accelerated atherosclerosis (which are very common in diabetes), diabetic cardiomyopathy (DCM) is a clinical condition diagnosed when ventricular dysfunction develops in patients with diabetes in the absence of coronary atherosclerosis and hypertension (259).

Metabolic perturbations are possibly one of the first alterations that were noticed in diabetic hearts. As early as in the 1950s, it was recognized that cardiac myocytes from a diabetic patient had an abnormal, energy-inefficient metabolic function, with almost no carbohydrate oxidation (260) and a decrease in glucose uptake (261). The changes seen in DCM are not dissimilar to those seen in reperfusion after transient ischemia. Of note, following a myocardial infarction, diabetic patients have almost twice the rate of mortality and 3 times the rate of progression to congestive heart failure when compared to non-diabetic patients (262). The reasons behind this increased susceptibility to ischemic damage are

controversial but are probably related to: 1) the increased FA oxidation, which is further exacerbated during reperfusion (263), and 2) the fact that carbohydrate oxidation (whose capacity is already reduced) is even further compromised by ischemia, thus reducing the capacity to properly utilize glucose for energy production (264). Indeed, diabetes leads to a persistent hyperglycemia accompanied by a hyperlipidemia. This alters the availability of substrates to the diabetic heart, which, combined with altered insulin signaling, alters substrate metabolism. In fact, the diabetic heart is faced with a reduced glucose oxidation rate and a dramatically increased FA β-oxidation, the latter accounting for almost all of the ATP production in this condition (265). Accordingly, there is a dramatic increase of FA transporters, especially CD36, which is postulated to have an important role in the etiology of cardiac disease (266).

Several lines of evidence, both clinical and experimental, suggest that high plasma levels of free FAs and high rates of FA oxidation in the myocardium result in impaired contractile function and more arrhythmias during and after ischemia both in the normal and diabetic heart (267;268). Accumulation of FAs and their toxic intermediates have been associated with mechanical dysfunction and cell damage in diabetic hearts subjected to ischemia (269) and to depressed sarcoplasmic reticulum Ca^{2+} pump and myofibrillar ATPase activities and myosin isozymes (270). The best evidence for a causative link between high FA oxidation and impaired cardiac function comes from studies in isolated rat hearts where either FA oxidation was inhibited (with CPT-I inhibitors) or PDH activity stimulated and contractile recovery from ischemia improved.

Faced with increased lipid accumulation and overload, the diabetic heart incurs cellular dysfunction, cell death, and eventual organ dysfunction. Indeed, it is suggested that the basis of TG accumulation seen in the diabetic heart is due to both inhibition of lipolysis and enhancement of synthesis (271) and accelerated turnover kinetics (216) resulting from high levels of exogenous free FA and glucose. Accordingly, the findings demonstrated in chapter II may provide a novel role for the cGMP pathway in the treatment of DCM. Indeed, increased triglyceride synthesis and breakdown might prove beneficial in preventing lipotoxicity in these cases through activation of HSL.

In addition to its effect on lipid metabolism, it appears likely that cGMP signaling may protect the diabetic heart through its impact on Ca^{2+} homeostasis. Indeed, studies indicate a decrease in the ability of the cell to remove Ca^{2+} through Na^{+}-Ca^{2+} exchange and Ca^{2+}-pump systems in the sarcolemma of diabetic rat hearts (272). More recently, decreased SERCA activity was shown to be a major contributor to the development of cardiac dysfunction in diabetes (273;274) and decreased expression of the channel was also reported (275). These differences are partly explained by altered calcium signaling at the level of the ryanodine receptor, a key regulator of SERCA (276) as well as increases in phospholamban observed in diabetic hearts (277). Originally, these abnormalities were thought to be associated with intracellular calcium overload (278); however, subsequent evidence emphasized altered $[Ca^{2+}]_i$ transients with unchanged basal concentrations (279). As outlined earlier, cGMP, through protein kinase G (PKG), regulates several aspects of ion homeostasis, and more importantly, calcium release and uptake (233;234), and would thus provide a second mechanism by which it could prove beneficial in the treatment of DCM.

Conclusion and future perspectives

In summary, this study describes metabolic alterations in the hearts of mice overexpressing a constitutive guanylate cyclase resulting in chronic activation of the cGMP signaling pathway. We provide direct evidence that cGMP signaling modulates cardiac energy metabolism in a cardiomyocyte-specific manner. Through an HSL-dependent activation of lipolysis, cGMP specifically acts on lipid compartmentalization. This may mediate some of its beneficial effects by favoring triglyceride cycling and thereby preventing triglyceride accumulation in a cardiomyocyte-specific manner; especially under conditions such as fasting where circulating long chain FAs are increased. An interesting follow-up to this study would be to investigate the effects of our model on the appearance and progression of type 2 diabetes. For this purpose, it would be possible to cross $GC^{+/-}$ mice with a mouse model of diabetes, such as ob/ob mice or db/db mice.

We also identified an increased resistance to cell necrosis following an acute stress such as the working heart perfusion. While we did not directly investigate the mechanisms behind this cardioprotection, we speculated on its origin, whether it was mediated by MPTP inhibition, increased calcium homeostasis, or decreased pro-apoptotic signaling. Thus, it appears relevant to investigate these different hypotheses. It is possible to assess pore opening in isolated mitochondrial preparations as well as in the intact heart. These measurements could be coupled to investigations of the various signaling pathways such as MAPK pathways. Finally, it would be interesting to see whether the increased anaerobic glycolysis is necessary to mediate this cardioprotection. Partial inhibition of LDH with iodoacetate would enable titration of anaerobic glycolysis without affecting glucose oxidation and enable assessment of the importance of this metabolic anomaly.

Reference

1. Hefti, M. A., Harder, B. A., Eppenberger, H. M., and Schaub, M. C. (1997) *J. Mol. Cell Cardiol.* **29,** 2873-2892

2. Nanni, L., Romualdi, C., Maseri, A., and Lanfranchi, G. (2006) *J. Mol. Cell Cardiol.* **41,** 934-948

3. Fiedler, B. and Wollert, K. C. (2004) *Cardiovasc. Res.* **63,** 450-457

4. Olson, E. N. (2004) *Nat. Med.* **10,** 467-474

5. Frey, N. and Olson, E. N. (2003) *Annu. Rev. Physiol* **65,** 45-79

6. Swynghedauw, B. (1999) *Physiol Rev.* **79,** 215-262

7. Cowie, M. R., Mosterd, A., Wood, D. A., Deckers, J. W., Poole-Wilson, P. A., Sutton, G. C., and Grobbee, D. E. (1997) *Eur. Heart J.* **18,** 208-225

8. Neubauer, S. (2007) *N. Engl. J. Med.* **356,** 1140-1151

9. Stanley, W. C., Recchia, F. A., and Lopaschuk, G. D. (2005) *Physiol Rev.* **85,** 1093-1129

10. Yamazaki, T., Komuro, I., Nagai, R., and Yazaki, Y. (1996) *Cardiovasc. Res.* **31,** 493-498

11. Klug, D., Robert, V., and Swynghedauw, B. (1993) *Am. J. Cardiol.* **71,** 46A-54A

12. Yamazaki, T., Komuro, I., and Yazaki, Y. (1999) *Am. J. Cardiol.* **83,** 53H-57H

13. Cohn, J. N. (1995) *Clin. Cardiol.* **18,** IV4-12

14. Cohn, J. N. (1995) *Circulation* **91,** 2504-2507

15. Gaudron, P., Eilles, C., Kugler, I., and Ertl, G. (1993) *Circulation* **87,** 755-763

16. Stephens, N. L. and Swynghedauw, B. (1990) *Mol. Cell Biochem.* **93,** 1-6

17. Karkkainen, S. and Peuhkurinen, K. (2007) *Ann. Med.* **39,** 91-107

18. Olivetti, G., Melissari, M., Balbi, T., Quaini, F., Cigola, E., Sonnenblick, E. H., and Anversa, P. (1994) *Cardiovasc. Res.* **28,** 1199-1208

19. Grove, D., Zak, R., Nair, K. G., and Aschenbrenner, V. (1969) *Circ. Res.* **25,** 473-485

20. Quaini, F., Cigola, E., Lagrasta, C., Saccani, G., Quaini, E., Rossi, C., Olivetti, G., and Anversa, P. (1994) *Circ. Res.* **75,** 1050-1063

21. Schreiber, S. S., Oratz, M., Evans, C. D., Gueyikian, I., and Rothschild, M. A. (1970) *Am. J. Physiol* **219,** 481-486

22. Morkin, E. (1974) *Circ. Res.* **35,** suppl-48

23. Swynghedauw, B. (1986) *Physiol Rev.* **66,** 710-771

24. Sadoshima, J. and Izumo, S. (1997) *Annu. Rev. Physiol* **59,** 551-571

25. Parker, T. G. and Schneider, M. D. (1991) *Annu. Rev. Physiol* **53,** 179-200

26. Lompre, A. M., Schwartz, K., d'Albis, A., Lacombe, G., Van, T. N., and Swynghedauw, B. (1979) *Nature* **282,** 105-107

27. Sadoshima, J., Jahn, L., Takahashi, T., Kulik, T. J., and Izumo, S. (1992) *J. Biol. Chem.* **267,** 10551-10560

28. Mercadier, J. J., Samuel, J. L., Michel, J. B., Zongazo, M. A., de la, B. D., Lompre, A. M., Wisnewsky, C., Rappaport, L., Levy, B., and Schwartz, K. (1989) *Am. J. Physiol* **257,** H979-H987

29. Schwartz, K., Boheler, K. R., de la, B. D., Lompre, A. M., and Mercadier, J. J. (1992) *Am. J. Physiol* **262,** R364-R369

30. Taegtmeyer, H. and Overturf, M. L. (1988) *Hypertension* **11,** 416-426

31. Komuro, I., Kaida, T., Shibazaki, Y., Kurabayashi, M., Katoh, Y., Hoh, E., Takaku, F., and Yazaki, Y. (1990) *J. Biol. Chem.* **265,** 3595-3598

32. Vakili, B. A., Okin, P. M., and Devereux, R. B. (2001) *Am. Heart J.* **141,** 334-341

33. Levy, D., Garrison, R. J., Savage, D. D., Kannel, W. B., and Castelli, W. P. (1990) *N. Engl. J. Med.* **322,** 1561-1566

34. Kannel, W. B., Gordon, T., and Offutt, D. (1969) *Ann. Intern. Med.* **71,** 89-105

35. Crabtree, G. R. and Olson, E. N. (2002) *Cell* **109 Suppl,** S67-S79

36. Molkentin, J. D., Lu, J. R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Grant, S. R., and Olson, E. N. (1998) *Cell* **93,** 215-228

37. De Windt, L. J., Lim, H. W., Bueno, O. F., Liang, Q., Delling, U., Braz, J. C., Glascock, B. J., Kimball, T. F., del, M. F., Hajjar, R. J., and Molkentin, J. D. (2001) *Proc. Natl. Acad. Sci. U. S. A* **98,** 3322-3327

38. Hill, J. A., Rothermel, B., Yoo, K. D., Cabuay, B., Demetroulis, E., Weiss, R. M., Kutschke, W., Bassel-Duby, R., and Williams, R. S. (2002) *J. Biol. Chem.* **277,** 10251-10255

39. Taigen, T., De Windt, L. J., Lim, H. W., and Molkentin, J. D. (2000) *Proc. Natl. Acad. Sci. U. S. A* **97,** 1196-1201

40. Vega, R. B., Yang, J., Rothermel, B. A., Bassel-Duby, R., and Williams, R. S. (2002) *J. Biol. Chem.* **277,** 30401-30407

41. Bueno, O. F., Wilkins, B. J., Tymitz, K. M., Glascock, B. J., Kimball, T. F., Lorenz, J. N., and Molkentin, J. D. (2002) *Proc. Natl. Acad. Sci. U. S. A* **99,** 4586-4591

42. Cantley, L. C. (2002) *Science* **296,** 1655-1657

43. Naga Prasad, S. V., Esposito, G., Mao, L., Koch, W. J., and Rockman, H. A. (2000) *J. Biol. Chem.* **275,** 4693-4698

44. Shioi, T., Kang, P. M., Douglas, P. S., Hampe, J., Yballe, C. M., Lawitts, J., Cantley, L. C., and Izumo, S. (2000) *EMBO J.* **19,** 2537-2548

45. Shioi, T., McMullen, J. R., Kang, P. M., Douglas, P. S., Obata, T., Franke, T. F., Cantley, L. C., and Izumo, S. (2002) *Mol. Cell Biol.* **22,** 2799-2809

46. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) *Curr. Biol.* **7,** 261-269

47. Rintelen, F., Stocker, H., Thomas, G., and Hafen, E. (2001) *Proc. Natl. Acad. Sci. U. S. A* **98,** 15020-15025

48. Bodine, S. C., Stitt, T. N., Gonzalez, M., Kline, W. O., Stover, G. L., Bauerlein, R., Zlotchenko, E., Scrimgeour, A., Lawrence, J. C., Glass, D. J., and Yancopoulos, G. D. (2001) *Nat. Cell Biol.* **3,** 1014-1019

49. Zhou, P., Sun, L. J., Dotsch, V., Wagner, G., and Verdine, G. L. (1998) *Cell* **92,** 687-696

50. Haq, S., Choukroun, G., Kang, Z. B., Ranu, H., Matsui, T., Rosenzweig, A., Molkentin, J. D., Alessandrini, A., Woodgett, J., Hajjar, R., Michael, A., and Force, T. (2000) *J. Cell Biol.* **151,** 117-130

51. Dufner, A., Andjelkovic, M., Burgering, B. M., Hemmings, B. A., and Thomas, G. (1999) *Mol. Cell Biol.* **19,** 4525-4534

52. Liang, Q., De Windt, L. J., Witt, S. A., Kimball, T. R., Markham, B. E., and Molkentin, J. D. (2001) *J. Biol. Chem.* **276,** 30245-30253

53. Boyle, W. J., Smeal, T., Defize, L. H., Angel, P., Woodgett, J. R., Karin, M., and Hunter, T. (1991) *Cell* **64,** 573-584

54. Sears, R., Nuckolls, F., Haura, E., Taya, Y., Tamai, K., and Nevins, J. R. (2000) *Genes Dev.* **14,** 2501-2514

55. Cohen, P. and Frame, S. (2001) *Nat. Rev. Mol. Cell Biol.* **2,** 769-776

56. Antos, C. L., McKinsey, T. A., Frey, N., Kutschke, W., McAnally, J., Shelton, J. M., Richardson, J. A., Hill, J. A., and Olson, E. N. (2002) *Proc. Natl. Acad. Sci. U. S. A* **99,** 907-912

57. Calderone, A., Thaik, C. M., Takahashi, N., Chang, D. L., and Colucci, W. S. (1998) *J. Clin. Invest* **101,** 812-818

58. Fingar, D. C., Salama, S., Tsou, C., Harlow, E., and Blenis, J. (2002) *Genes Dev.* **16,** 1472-1487

59. Boluyt, M. O., Zheng, J. S., Younes, A., Long, X., O'Neill, L., Silverman, H., Lakatta, E. G., and Crow, M. T. (1997) *Circ. Res.* **81,** 176-186

60. Sadoshima, J. and Izumo, S. (1995) *Circ. Res.* **77,** 1040-1052

61. McMullen, J. R., Sherwood, M. C., Tarnavski, O., Zhang, L., Dorfman, A. L., Shioi, T., and Izumo, S. (2004) *Circulation* **109,** 3050-3055

62. Dorn, G. W. and Force, T. (2005) *J. Clin. Invest* **115,** 527-537

63. Simpson, P. (1983) *J. Clin. Invest* **72,** 732-738

64. Sadoshima, J. and Izumo, S. (1993) *Circ. Res.* **73,** 413-423

65. Shubeita, H. E., McDonough, P. M., Harris, A. N., Knowlton, K. U., Glembotski, C. C., Brown, J. H., and Chien, K. R. (1990) *J. Biol. Chem.* **265,** 20555-20562

66. Rockman, H. A., Koch, W. J., and Lefkowitz, R. J. (2002) *Nature* **415,** 206-212

67. Koch, W. J., Lefkowitz, R. J., and Rockman, H. A. (2000) *Annu. Rev. Physiol* **62,** 237-260

68. Sakata, Y., Hoit, B. D., Liggett, S. B., Walsh, R. A., and Dorn, G. W. (1998) *Circulation* **97,** 1488-1495

69. Wettschureck, N., Rutten, H., Zywietz, A., Gehring, D., Wilkie, T. M., Chen, J., Chien, K. R., and Offermanns, S. (2001) *Nat. Med.* **7,** 1236-1240

70. Akhter, S. A., Luttrell, L. M., Rockman, H. A., Iaccarino, G., Lefkowitz, R. J., and Koch, W. J. (1998) *Science* **280,** 574-577

71. Yusuf, S., Sleight, P., Pogue, J., Bosch, J., Davies, R., and Dagenais, G. (2000) *N. Engl. J. Med.* **342,** 145-153

72. Ishikawa, Y. (2003) *J. Cardiovasc. Pharmacol.* **41 Suppl 1,** S1-S4

73. Bisognano, J. D., Weinberger, H. D., Bohlmeyer, T. J., Pende, A., Raynolds, M. V., Sastravaha, A., Roden, R., Asano, K., Blaxall, B. C., Wu, S. C., Communal, C., Singh, K., Colucci, W., Bristow, M. R., and Port, D. J. (2000) *J. Mol. Cell Cardiol.* **32,** 817-830

74. Gaudin, C., Ishikawa, Y., Wight, D. C., Mahdavi, V., Nadal-Ginard, B., Wagner, T. E., Vatner, D. E., and Homcy, C. J. (1995) *J. Clin. Invest* **95,** 1676-1683

75. Roth, D. M., Bayat, H., Drumm, J. D., Gao, M. H., Swaney, J. S., Ander, A., and Hammond, H. K. (2002) *Circulation* **105,** 1989-1994

76. Roth, D. M., Gao, M. H., Lai, N. C., Drumm, J., Dalton, N., Zhou, J. Y., Zhu, J., Entrikin, D., and Hammond, H. K. (1999) *Circulation* **99,** 3099-3102

77. Antos, C. L., Frey, N., Marx, S. O., Reiken, S., Gaburjakova, M., Richardson, J. A., Marks, A. R., and Olson, E. N. (2001) *Circ. Res.* **89,** 997-1004

78. Lowes, B. D., Gilbert, E. M., Abraham, W. T., Minobe, W. A., Larrabee, P., Ferguson, D., Wolfel, E. E., Lindenfeld, J., Tsvetkova, T., Robertson, A. D., Quaife, R. A., and Bristow, M. R. (2002) *N. Engl. J. Med.* **346,** 1357-1365

79. Sugden, P. H. and Clerk, A. (1998) *Circ. Res.* **83,** 345-352

80. Bueno, O. F., De Windt, L. J., Lim, H. W., Tymitz, K. M., Witt, S. A., Kimball, T. R., and Molkentin, J. D. (2001) *Circ. Res.* **88,** 88-96

81. Fuller, S. J., Davies, E. L., Gillespie-Brown, J., Sun, H., and Tonks, N. K. (1997) *Biochem. J.* **323 (Pt 2),** 313-319

82. Glennon, P. E., Kaddoura, S., Sale, E. M., Sale, G. J., Fuller, S. J., and Sugden, P. H. (1996) *Circ. Res.* **78,** 954-961

83. Post, G. R., Goldstein, D., Thuerauf, D. J., Glembotski, C. C., and Brown, J. H. (1996) *J. Biol. Chem.* **271,** 8452-8457

84. D'Angelo, D. D., Sakata, Y., Lorenz, J. N., Boivin, G. P., Walsh, R. A., Liggett, S. B., and Dorn, G. W. (1997) *Proc. Natl. Acad. Sci. U. S. A* **94,** 8121-8126

85. Bueno, O. F., De Windt, L. J., Tymitz, K. M., Witt, S. A., Kimball, T. R., Klevitsky, R., Hewett, T. E., Jones, S. P., Lefer, D. J., Peng, C. F., Kitsis, R. N., and Molkentin, J. D. (2000) *EMBO J.* **19,** 6341-6350

86. Silberbach, M., Gorenc, T., Hershberger, R. E., Stork, P. J., Steyger, P. S., and Roberts, C. T., Jr. (1999) *J. Biol. Chem.* **274,** 24858-24864

87. Komuro, I., Kudo, S., Yamazaki, T., Zou, Y., Shiojima, I., and Yazaki, Y. (1996) *FASEB J.* **10,** 631-636

88. Choukroun, G., Hajjar, R., Kyriakis, J. M., Bonventre, J. V., Rosenzweig, A., and Force, T. (1998) *J. Clin. Invest* **102,** 1311-1320

89. Yano, M., Kim, S., Izumi, Y., Yamanaka, S., and Iwao, H. (1998) *Circ. Res.* **83,** 752-760

90. Minamino, T., Yujiri, T., Terada, N., Taffet, G. E., Michael, L. H., Johnson, G. L., and Schneider, M. D. (2002) *Proc. Natl. Acad. Sci. U. S. A* **99,** 3866-3871

91. Sadoshima, J., Montagne, O., Wang, Q., Yang, G., Warden, J., Liu, J., Takagi, G., Karoor, V., Hong, C., Johnson, G. L., Vatner, D. E., and Vatner, S. F. (2002) *J. Clin. Invest* **110,** 271-279

92. Wang, Y., Huang, S., Sah, V. P., Ross, J., Jr., Brown, J. H., Han, J., and Chien, K. R. (1998) *J. Biol. Chem.* **273,** 2161-2168

93. Zhang, D., Gaussin, V., Taffet, G. E., Belaguli, N. S., Yamada, M., Schwartz, R. J., Michael, L. H., Overbeek, P. A., and Schneider, M. D. (2000) *Nat. Med.* **6,** 556-563

94. Han, J., Jiang, Y., Li, Z., Kravchenko, V. V., and Ulevitch, R. J. (1997) *Nature* **386,** 296-299

95. Yang, T. T., Xiong, Q., Enslen, H., Davis, R. J., and Chow, C. W. (2002) *Mol. Cell Biol.* **22,** 3892-3904

96. Jobgen, W. S., Fried, S. K., Fu, W. J., Meininger, C. J., and Wu, G. (2006) *J. Nutr. Biochem.*

97. Wollert, K. C. and Drexler, H. (2002) *Heart Fail. Rev.* **7,** 317-325

98. Booz, G. W. (2005) *Hypertension* **45,** 341-346

99. Uppu, R. M., Nossaman, B. D., Greco, A. J., Fokin, A., Murthy, S. N., Fonseca, V. A., and Kadowitz, P. J. (2007) *Clin. Exp. Pharmacol. Physiol* **34,** 933-937

100. Barouch, L. A., Harrison, R. W., Skaf, M. W., Rosas, G. O., Cappola, T. P., Kobeissi, Z. A., Hobai, I. A., Lemmon, C. A., Burnett, A. L., O'Rourke, B., Rodriguez, E. R., Huang, P. L., Lima, J. A., Berkowitz, D. E., and Hare, J. M. (2002) *Nature* **416,** 337-339

101. Matsuoka, H., Nakata, M., Kohno, K., Koga, Y., Nomura, G., Toshima, H., and Imaizumi, T. (1996) *Hypertension* **27,** 14-18

102. Ozaki, M., Kawashima, S., Yamashita, T., Hirase, T., Ohashi, Y., Inoue, N., Hirata, K., and Yokoyama, M. (2002) *Circ. J.* **66,** 851-856

103. Lee, C. Y. and Burnett, J. C., Jr. (2007) *Heart Fail. Rev.* **12,** 131-142

104. Pandey, K. N. (2005) *Peptides* **26,** 901-932

105. Deschepper, C. F., Masciotra, S., Zahabi, A., Boutin-Ganache, I., Picard, S., and Reudelhuber, T. L. (2001) *Circ. Res.* **88,** 223-228

106. Holtwick, R., van, E. M., Skryabin, B. V., Baba, H. A., Bubikat, A., Begrow, F., Schneider, M. D., Garbers, D. L., and Kuhn, M. (2003) *J. Clin. Invest* **111,** 1399-1407

107. Schlossmann, J., Feil, R., and Hofmann, F. (2003) *Ann. Med* **35,** 21-27

108. Stanley, W. C., Recchia, F. A., and Lopaschuk, G. D. (2005) *Physiol Rev.* **85,** 1093-1129

109. Opie, L. H. (1998) *The heart: Physiology, from cell to circulation*, 3rd Ed., 3rd Edition; New York: Lippincott-Raven Publisher.,

110. Lopaschuk, G. D. (2004) *Curr. Opin. Investig. Drugs* **5,** 290-294

111. Luiken, J. J., Coort, S. L., Koonen, D. P., van der Horst, D. J., Bonen, A., Zorzano, A., and Glatz, J. F. (2004) *Pflugers Arch.* **448,** 1-15

112. Abel, E. D. (2004) *Front Biosci.* **9,** 201-215

113. Luiken, J. J., Coort, S. L., Koonen, D. P., Bonen, A., and Glatz, J. F. (2004) *Proc. Nutr. Soc.* **63,** 251-258

114. Carvajal, K. and Moreno-Sanchez, R. (2003) *Arch Med Res.* **34,** 89-99

115. Davila-Roman, V. G., Vedala, G., Herrero, P., de las, F. L., Rogers, J. G., Kelly, D. P., and Gropler, R. J. (2002) *J. Am. Coll. Cardiol.* **40,** 271-277

116. Depre, C., Rider, M. H., and Hue, L. (1998) *Eur. J. Biochem.* **258,** 277-290

117. Depre, C., Vanoverschelde, J. L., and Taegtmeyer, H. (1999) *Circulation* **99,** 578-588

118. Hardie, D. G. and Pan, D. A. (2002) *Biochem. Soc. Trans.* **30,** 1064-1070

119. Kahn, B. B., Alquier, T., Carling, D., and Hardie, D. G. (2005) *Cell Metab* **1,** 15-25

120. Khairallah, M., Labarthe, F., Bouchard, B., Danialou, G., Petrof, B. J., and Des, R. C. (2004) *Am. J. Physiol Heart Circ. Physiol* **286,** H1461-H1470

121. Latronico, M. V., Costinean, S., Lavitrano, M. L., Peschle, C., and Condorelli, G. (2004) *Ann. N. Y. Acad. Sci.* **1015,** 250-260

122. Lopaschuk, G. D. (2004) *Curr. Opin. Investig. Drugs* **5,** 290-294

123. Luiken, J. J., Coort, S. L., Koonen, D. P., Bonen, A., and Glatz, J. F. (2004) *Proc. Nutr. Soc.* **63,** 251-258

124. Mallet, R. T. (2000) *Proc. Soc. Exp. Biol. Med* **223,** 136-148

125. Plas, D. R. and Thompson, C. B. (2005) *Oncogene* **24,** 7435-7442

126. Razeghi, P., Young, M. E., Alcorn, J. L., Moravec, C. S., Frazier, O. H., and Taegtmeyer, H. (2001) *Circulation* **104,** 2923-2931

127. Sambandam, N. and Lopaschuk, G. D. (2003) *Prog. Lipid Res.* **42,** 238-256

128. Van, B. M. (2004) *Ann. N. Y. Acad. Sci.* **1015,** 238-249

129. van Bilsen, M., Smeets, P. J., Gilde, A. J., and van der Vusse, G. J. (2004) *Cardiovasc. Res.* **61,** 218-226

130. Young, L. H., Li, J., Baron, S. J., and Russell, R. R. (2005) *Trends Cardiovasc. Med.* **15,** 110-118

131. Kusuoka, H. and Marban, E. (1994) *J. Clin. Invest* **93,** 1216-1223

132. Henderson, A. H., Craig, R. J., Gorlin, R., and Sonnenblick, E. H. (1969) *Am. J. Physiol* **217,** 1752-1756

133. Laughlin, M. R., Taylor, J., Chesnick, A. S., DeGroot, M., and Balaban, R. S. (1993) *Am. J. Physiol* **264,** H2068-H2079

134. Mallet, R. T., Sun, J., Knott, E. M., Sharma, A. B., and Olivencia-Yurvati, A. H. (2005) *Exp. Biol. Med (Maywood.)* **230,** 435-443

135. Sharma, N., Okere, I. C., Brunengraber, D. Z., McElfresh, T. A., King, K. L., Sterk, J. P., Huang, H., Chandler, M. P., and Stanley, W. C. (2005) *J. Physiol* **562,** 593-603

136. Chatham, J. C., Des Rosiers, C., and Forder, J. R. (2001) *Am. J. Physiol Endocrinol. Metab* **281,** E794-E802

137. Owen, O. E., Kalhan, S. C., and Hanson, R. W. (2002) *J. Biol. Chem.* **277,** 30409-30412

138. Vincent, G., Khairallah, M., Bouchard, B., and Des Rosiers, C. (2003) *Mol. Cell Biochem.* **242,** 89-99

139. Vincent, G., Bouchard, B., Khairallah, M., and Des Rosiers, C. (2003) *Am. J. Physiol Heart Circ. Physiol*

140. Garcia-Cazorla, A., Rabier, D., Touati, G., Chadefaux-Vekemans, B., Marsac, C., de, L. P., and Saudubray, J. M. (2006) *Ann. Neurol.* **59,** 121-127

141. Baumgartner, D., Scholl-Burgi, S., Sass, J. O., Sperl, W., Schweigmann, U., Stein, J. I., and Karall, D. (2007) *J. Pediatr.* **150,** 192-7, 197

142. van der Vusse, G. J., van Bilsen, M., and Glatz, J. F. (2000) *Cardiovasc. Res.* **45,** 279-293

143. Hopkins, T. A., Dyck, J. R., and Lopaschuk, G. D. (2003) *Biochem. Soc. Trans.* **31,** 207-212

144. Poirier, M., Vincent, G., Reszko, A. E., Bouchard, B., Kelleher, J. K., Brunengraber, H., and Des, R. C. (2002) *Am. J. Physiol Heart Circ. Physiol* **283,** H1379-H1386

145. Reszko, A. E., Kasumov, T., David, F., Jobbins, K. A., Thomas, K. R., Hoppel, C. L., Brunengraber, H., and Des, R. C. (2004) *J. Biol. Chem.* **279,** 19574-19579

146. Hopkins, T. A., Dyck, J. R., and Lopaschuk, G. D. (2003) *Biochem. Soc. Trans.* **31,** 207-212

147. Saddik, M. and Lopaschuk, G. D. (1991) *J. Biol. Chem.* **266,** 8162-8170

148. Haemmerle, G., Lass, A., Zimmermann, R., Gorkiewicz, G., Meyer, C., Rozman, J., Heldmaier, G., Maier, R., Theussl, C., Eder, S., Kratky, D., Wagner, E. F., Klingenspor, M., Hoefler, G., and Zechner, R. (2006) *Science* **312,** 734-737

149. Schweiger, M., Schreiber, R., Haemmerle, G., Lass, A., Fledelius, C., Jacobsen, P., Tornqvist, H., Zechner, R., and Zimmermann, R. (2006) *J. Biol. Chem.* **281,** 40236-40241

150. Carmen, G. Y. and Victor, S. M. (2006) *Cell Signal.* **18,** 401-408

151. Sengenes, C., Bouloumie, A., Hauner, H., Berlan, M., Busse, R., Lafontan, M., and Galitzky, J. (2003) *J. Biol. Chem.* **278,** 48617-48626

152. Yu, Y. H. and Ginsberg, H. N. (2004) *Ann. Med.* **36,** 252-261

153. Krebs, H. A. (1970) *Adv. Enzyme Regul.* **8,** 335-353

154. Owen, O. E., Kalhan, S. C., and Hanson, R. W. (2002) *J. Biol. Chem.* **277,** 30409-30412

155. Peuhkurinen, K. J. (1984) *J. Mol. Cell Cardiol.* **16,** 487-495

156. Wan, B., LaNoue, K. F., Cheung, J. Y., and Scaduto, R. C., Jr. (1989) *J. Biol. Chem.* **264,** 13430-13439

157. Dyck, J. R. and Lopaschuk, G. D. (2006) *J. Physiol* **574,** 95-112

158. Hopkins, T. A., Dyck, J. R., and Lopaschuk, G. D. (2003) *Biochem. Soc. Trans.* **31,** 207-212

159. Lee, W. J., Kim, M., Park, H. S., Kim, H. S., Jeon, M. J., Oh, K. S., Koh, E. H., Won, J. C., Kim, M. S., Oh, G. T., Yoon, M., Lee, K. U., and Park, J. Y. (2006) *Biochem. Biophys. Res. Commun.* **340,** 291-295

160. Young, L. H., Li, J., Baron, S. J., and Russell, R. R. (2005) *Trends Cardiovasc. Med.* **15,** 110-118

161. Arad, M., Benson, D. W., Perez-Atayde, A. R., McKenna, W. J., Sparks, E. A., Kanter, R. J., McGarry, K., Seidman, J. G., and Seidman, C. E. (2002) *J. Clin. Invest* **109,** 357-362

162. Taegtmeyer, H., Golfman, L., Sharma, S., Razeghi, P., and van, A. M. (2004) *Ann. N. Y. Acad. Sci.* **1015,** 202-213

163. Razeghi, P., Young, M. E., Alcorn, J. L., Moravec, C. S., Frazier, O. H., and Taegtmeyer, H. (2001) *Circulation* **104,** 2923-2931

164. Stanley, W. C., Recchia, F. A., and Lopaschuk, G. D. (2005) *Physiol Rev.* **85,** 1093-1129

165. Sambandam, N., Lopaschuk, G. D., Brownsey, R. W., and Allard, M. F. (2002) *Heart Fail. Rev.* **7,** 161-173

166. Lehman, J. J. and Kelly, D. P. (2002) *Heart Fail. Rev.* **7,** 175-185

167. Barger, P. M., Brandt, J. M., Leone, T. C., Weinheimer, C. J., and Kelly, D. P. (2000) *J. Clin. Invest* **105,** 1723-1730

168. Van, B. M. (2004) *Ann. N. Y. Acad. Sci.* **1015,** 238-249

169. Van, B. M. (2004) *Ann. N. Y. Acad. Sci.* **1015,** 238-249

170. Young, M. E., Laws, F. A., Goodwin, G. W., and Taegtmeyer, H. (2001) *J. Biol. Chem.* **276,** 44390-44395

171. Finck, B. N., Lehman, J. J., Leone, T. C., Welch, M. J., Bennett, M. J., Kovacs, A., Han, X., Gross, R. W., Kozak, R., Lopaschuk, G. D., and Kelly, D. P. (2002) *J. Clin. Invest* **109,** 121-130

172. Liao, R., Jain, M., Cui, L., D'Agostino, J., Aiello, F., Luptak, I., Ngoy, S., Mortensen, R. M., and Tian, R. (2002) *Circulation* **106,** 2125-2131

173. Luptak, I., Balschi, J. A., Xing, Y., Leone, T. C., Kelly, D. P., and Tian, R. (2005) *Circulation* **112,** 2339-2346

174. Labarthe, F., Khairallah, M., Bouchard, B., Stanley, W. C., and Des, R. C. (2005) *Am. J. Physiol Heart Circ. Physiol* **288,** H1425-H1436

175. Roe, C. R. and Ding, J. H. (2001) Mitochondrial fatty acid oxidation disorders. In Valle, D., Sly, W. S., Beaudet, A. L., and Scriver, C. R., editors. *The metabolic and molecular bases of inherited disease*, McGraw-Hill, New York, New York, USA

176. Stanley, W. C. and Chandler, M. P. (2002) *Heart Fail. Rev.* **7,** 115-130

177. Stanley, W. C., Recchia, F. A., and Lopaschuk, G. D. (2005) *Physiol Rev.* **85,** 1093-1129

178. Stanley, W. C., Recchia, F. A., and Lopaschuk, G. D. (2005) *Physiol Rev.* **85,** 1093-1129

179. Davila-Roman, V. G., Vedala, G., Herrero, P., de las, F. L., Rogers, J. G., Kelly, D. P., and Gropler, R. J. (2002) *J. Am. Coll. Cardiol.* **40,** 271-277

180. Karbowska, J., Kochan, Z., and Smolenski, R. T. (2003) *Cell Mol. Biol. Lett.* **8,** 49-53

181. Taylor, M., Wallhaus, T. R., Degrado, T. R., Russell, D. C., Stanko, P., Nickles, R. J., and Stone, C. K. (2001) *J. Nucl. Med.* **42,** 55-62

182. Chandler, M. P., Kerner, J., Huang, H., Vazquez, E., Reszko, A., Martini, W. Z., Hoppel, C. L., Imai, M., Rastogi, S., Sabbah, H. N., and Stanley, W. C. (2004) *Am. J. Physiol Heart Circ. Physiol* **287,** H1538-H1543

183. Osorio, J. C., Stanley, W. C., Linke, A., Castellari, M., Diep, Q. N., Panchal, A. R., Hintze, T. H., Lopaschuk, G. D., and Recchia, F. A. (2002) *Circulation* **106,** 606-612

184. Sesti, C., Simkhovich, B. Z., Kalvinsh, I., and Kloner, R. A. (2006) *J. Cardiovasc. Pharmacol.* **47,** 493-499

185. Fragasso, G., Palloshi, A., Puccetti, P., Silipigni, C., Rossodivita, A., Pala, M., Calori, G., Alfieri, O., and Margonato, A. (2006) *J. Am. Coll. Cardiol.* **48,** 992-998

186. Abozguia, K., Clarke, K., Lee, L., and Frenneaux, M. (2006) *Nat. Clin. Pract. Cardiovasc. Med.* **3,** 490-498

187. Lee, L., Campbell, R., Scheuermann-Freestone, M., Taylor, R., Gunaruwan, P., Williams, L., Ashrafian, H., Horowitz, J., Fraser, A. G., Clarke, K., and Frenneaux, M. (2005) *Circulation* **112,** 3280-3288

188. Jobgen, W. S., Fried, S. K., Fu, W. J., Meininger, C. J., and Wu, G. (2006) *J. Nutr. Biochem.*

189. Recchia, F. A. (2002) *Heart Fail. Rev.* **7,** 141-148

190. Li, J., Hu, X., Selvakumar, P., Russell, R. R., III, Cushman, S. W., Holman, G. D., and Young, L. H. (2004) *Am. J. Physiol Endocrinol. Metab* **287,** E834-E841

191. Tada, H., Thompson, C. I., Recchia, F. A., Loke, K. E., Ochoa, M., Smith, C. J., Shesely, E. G., Kaley, G., and Hintze, T. H. (2000) *Circ. Res.* **86,** 270-274

192. Tada, H., Thompson, C. I., Recchia, F. A., Loke, K. E., Ochoa, M., Smith, C. J., Shesely, E. G., Kaley, G., and Hintze, T. H. (2000) *Circ. Res.* **86,** 270-274

193. Recchia, F. A., Osorio, J. C., Chandler, M. P., Xu, X., Panchal, A. R., Lopaschuk, G. D., Hintze, T. H., and Stanley, W. C. (2002) *Am. J. Physiol Endocrinol. Metab* **282,** E197-E206

194. Wolff, A. A., Rotmensch, H. H., Stanley, W. C., and Ferrari, R. (2002) *Heart Fail. Rev.* **7,** 187-203

195. Clementi, E. and Nisoli, E. (2005) *Comp Biochem. Physiol A Mol. Integr. Physiol* **142,** 102-110

196. Recchia, F. A., McConnell, P. I., Bernstein, R. D., Vogel, T. R., Xu, X., and Hintze, T. H. (1998) *Circ. Res.* **83,** 969-979

197. Young, M. E., Radda, G. K., and Leighton, B. (1997) *Biochem. J.* **322 (Pt 1),** 223-228

198. Trochu, J. N., Bouhour, J. B., Kaley, G., and Hintze, T. H. (2000) *Circ. Res.* **87,** 1108-1117

199. Trochu, J. N., Bouhour, J. B., Kaley, G., and Hintze, T. H. (2000) *Circ. Res.* **87,** 1108-1117

200. Nishikimi, T., Maeda, N., and Matsuoka, H. (2006) *Cardiovasc. Res.* **69,** 318-328

201. Calderone, A. (2003) *Heart Fail. Rev.* **8,** 55-70

202. Deschepper, C. F. (2005) The cardiac antihypertrophic effects of cyclic GMP-generating agents: an experimental framework for novel treatments of left ventricular remodeling.

203. Zahabi, A., Picard, S., Fortin, N., Reudelhuber, T. L., and Deschepper, C. F. (2003) *J. Biol. Chem.* **278,** 47694-47699

204. Sack, M. N. and Kelly, D. P. (1998) *Int. J. Mol. Med.* **1,** 17-24

205. Stanley, W. C., Recchia, F. A., and Lopaschuk, G. D. (2005) *Physiol Rev.* **85,** 1093-1129

206. Listenberger, L. L., Han, X., Lewis, S. E., Cases, S., Farese, R. V., Jr., Ory, D. S., and Schaffer, J. E. (2003) *Proc. Natl. Acad. Sci. U. S. A* **100,** 3077-3082

207. Miller, T. A., LeBrasseur, N. K., Cote, G. M., Trucillo, M. P., Pimentel, D. R., Ido, Y., Ruderman, N. B., and Sawyer, D. B. (2005) *Biochem. Biophys. Res. Commun.* **336,** 309-315

208. Augustus, A., Yagyu, H., Haemmerle, G., Bensadoun, A., Vikramadithyan, R. K., Park, S. Y., Kim, J. K., Zechner, R., and Goldberg, I. J. (2004) *J. Biol. Chem.* **279,** 25050-25057

209. Augustus, A. S., Buchanan, J., Park, T. S., Hirata, K., Noh, H. L., Sun, J., Homma, S., D'armiento, J., Abel, E. D., and Goldberg, I. J. (2006) *J. Biol. Chem.* **281,** 8716-8723

210. Noh, H. L., Okajima, K., Molkentin, J. D., Homma, S., and Goldberg, I. J. (2006) *Am. J. Physiol Endocrinol. Metab* **291,** E755-E760

211. Manabe, I., Shindo, T., and Nagai, R. (2002) *Circ. Res.* **91,** 1103-1113

212. Kammermeier, H. (1995) *J. Mol. Cell Cardiol.* **27,** 195-200

213. Kammermeier, H. (1995) *J. Mol. Cell Cardiol.* **27,** 195-200

214. Saddik, M. and Lopaschuk, G. D. (1994) *Can. J. Physiol Pharmacol.* **72,** 1110-1119

215. Saddik, M. and Lopaschuk, G. D. (1991) *J. Biol. Chem.* **266,** 8162-8170

216. O'Donnell, J. M., Zampino, M., Alpert, N. M., Fasano, M. J., Geenen, D. L., and Lewandowski, E. D. (2006) *Am. J. Physiol Endocrinol. Metab* **290,** E448-E455

217. Suzuki, J., Shen, W. J., Nelson, B. D., Patel, S., Veerkamp, J. H., Selwood, S. P., Murphy, G. M., Jr., Reaven, E., and Kraemer, F. B. (2001) *Am. J. Physiol Endocrinol. Metab* **281,** E857-E866

218. Khairallah, M., Khairallah, R., Young, M. E., Dyck, J. R., Petrof, B. J., and Des, R. C. (2007) *J. Mol. Cell Cardiol.*

219. Depre, C. and Vatner, S. F. (2007) *Heart Fail. Rev.* **12,** 307-317

220. Weiss, J. N. and Lamp, S. T. (1989) *J. Gen. Physiol* **94,** 911-935

221. Aasum, E., Lathrop, D. A., Henden, T., Sundset, R., and Larsen, T. S. (1998) *J. Mol. Cell Cardiol.* **30,** 1703-1712

222. Xu, K. Y., Zweier, J. L., and Becker, L. C. (1995) *Circ. Res.* **77,** 88-97

223. Glitsch, H. G. and Tappe, A. (1993) *Pflugers Arch.* **422,** 380-385

224. Paul, R. J., Hardin, C. D., Raeymaekers, L., Wuytack, F., and Casteels, R. (1989) *FASEB J.* **3,** 2298-2301

225. Leong, H. S., Brownsey, R. W., Kulpa, J. E., and Allard, M. F. (2003) Comp Biochem. Physiol A Mol. Integr. Physiol 135, 499-513

226. Nascimben, L., Ingwall, J. S., Lorell, B. H., Pinz, I., Schultz, V., Tornheim, K., and Tian, R. (2004) Hypertension 44, 662-667

227. Murry, C. E., Jennings, R. B., and Reimer, K. A. (1991) Circulation 84, 442-445

228. Bolli, R., Dawn, B., Tang, X. L., Qiu, Y., Ping, P., Xuan, Y. T., Jones, W. K., Takano, H., Guo, Y., and Zhang, J. (1998) Basic Res. Cardiol. 93, 325-338

229. Lochner, A., Marais, E., Genade, S., and Moolman, J. A. (2000) Am. J. Physiol Heart Circ. Physiol 279, H2752-H2765

230. Costa, A. D., Jakob, R., Costa, C. L., Andrukhiv, K., West, I. C., and Garlid, K. D. (2006) J. Biol. Chem. 281, 20801-20808

231. Costa, A. D., Garlid, K. D., West, I. C., Lincoln, T. M., Downey, J. M., Cohen, M. V., and Critz, S. D. (2005) Circ. Res. 97, 329-336

232. Korichneva, I., Hoyos, B., Chua, R., Levi, E., and Hammerling, U. (2002) J. Biol. Chem. 277, 44327-44331

233. Lucas, K. A., Pitari, G. M., Kazerounian, S., Ruiz-Stewart, I., Park, J., Schulz, S., Chepenik, K. P., and Waldman, S. A. (2000) Pharmacol. Rev. 52, 375-414

234. Klein, G., Drexler, H., and Schroder, F. (2000) Cardiovasc. Res. 48, 367-374

235. Abdallah, Y., Gkatzoflia, A., Pieper, H., Zoga, E., Walther, S., Kasseckert, S., Schafer, M., Schluter, K. D., Piper, H. M., and Schafer, C. (2005) Cardiovasc. Res. 66, 123-131

236. Schroder, F., Klein, G., Fiedler, B., Bastein, M., Schnasse, N., Hillmer, A., Ames, S., Gambaryan, S., Drexler, H., Walter, U., Lohmann, S. M., and Wollert, K. C. (2003) Cardiovasc. Res. 60, 268-277

237. Kannan, M. S., Prakash, Y. S., Johnson, D. E., and Sieck, G. C. (1997) Am. J. Physiol 272, L1-L7

238. Zhang, Q., Scholz, P. M., He, Y., Tse, J., and Weiss, H. R. (2005) Cell Calcium 37, 259-266

239. Fiedler, B., Lohmann, S. M., Smolenski, A., Linnemuller, S., Pieske, B., Schroder, F., Molkentin, J. D., Drexler, H., and Wollert, K. C. (2002) Proc. Natl. Acad. Sci. U. S. A 99, 11363-11368

240. Bueno, O. F., van, R. E., Molkentin, J. D., Doevendans, P. A., and De Windt, L. J. (2002) Cardiovasc. Res. 53, 806-821

241. Yang, L., Liu, G., Zakharov, S. I., Bellinger, A. M., Mongillo, M., and Marx, S. O. (2007) Circ. Res. 101, 465-474

242. Saito, S., Hiroi, Y., Zou, Y., Aikawa, R., Toko, H., Shibasaki, F., Yazaki, Y., Nagai, R., and Komuro, I. (2000) J. Biol. Chem. 275, 34528-34533

243. Iwai-Kanai, E. and Hasegawa, K. (2004) Mol. Cell Biochem. 259, 163-168

244. De Windt, L. J., Lim, H. W., Taigen, T., Wencker, D., Condorelli, G., Dorn, G. W., Kitsis, R. N., and Molkentin, J. D. (2000) Circ. Res. 86, 255-263

245. Kalra, D., Baumgarten, G., Dibbs, Z., Seta, Y., Sivasubramanian, N., and Mann, D. L. (2000) Circulation 102, 1302-1307

246. Yamamoto, K., Ikeda, U., and Shimada, K. (1997) J. Mol. Cell Cardiol. 29, 2375-2382

247. Wu, C. F., Bishopric, N. H., and Pratt, R. E. (1997) J. Biol. Chem. 272, 14860-14866

248. Ge, B., Gram, H., Di, P. F., Huang, B., New, L., Ulevitch, R. J., Luo, Y., and Han, J. (2002) Science 295, 1291-1294

249. Kaiser, R. A., Bueno, O. F., Lips, D. J., Doevendans, P. A., Jones, F., Kimball, T. F., and Molkentin, J. D. (2004) J. Biol. Chem. 279, 15524-15530

250. Fiedler, B., Feil, R., Hofmann, F., Willenbockel, C., Drexler, H., Smolenski, A., Lohmann, S. M., and Wollert, K. C. (2006) J. Biol. Chem. 281, 32831-32840

251. Yoshioka, Y., Yamamuro, A., and Maeda, S. (2006) J. Pharmacol. Sci. 101, 126-134

252. Camps, M., Nichols, A., and Arkinstall, S. (2000) FASEB J. 14, 6-16

253. Hannken, T., Schroeder, R., Stahl, R. A., and Wolf, G. (2001) Am. J. Physiol Renal Physiol 281, F81-F90

254. Valledor, A. F., Xaus, J., Marques, L., and Celada, A. (1999) J. Immunol. 163, 2452-2462

255. Hayashi, D., Kudoh, S., Shiojima, I., Zou, Y., Harada, K., Shimoyama, M., Imai, Y., Monzen, K., Yamazaki, T., Yazaki, Y., Nagai, R., and Komuro, I. (2004) Biochem. Biophys. Res. Commun. 322, 310-319

256. Gonzalez, G. L., Manrique, C. M., and Sowers, J. R. (2006) J. Cardiometab. Syndr. 1, 178-183

257. McCarron, P. and Davey, S. G. (2005) *Int. J. Epidemiol.* **34,** 248-250

258. Kannel, W. B. and McGee, D. L. (1979) *JAMA* **241,** 2035-2038

259. Avogaro, A., Vigili, d. K., Negut, C., Tiengo, A., and Scognamiglio, R. (2004) *Am. J. Cardiol.* **93,** 13A-16A

260. Ungar, I., Gilbert, M., Siegel, A., Blain, J. M., and Bing, R. J. (1955) *Am. J. Med* **18,** 385-396

261. Lopaschuk, G. D. and Stanley, W. C. (1997) *Circulation* **95,** 313-315

262. Stone, P. H., Muller, J. E., Hartwell, T., York, B. J., Rutherford, J. D., Parker, C. B., Turi, Z. G., Strauss, H. W., Willerson, J. T., Robertson, T., and . (1989) *J. Am. Coll. Cardiol.* **14,** 49-57

263. Lopaschuk, G. D. (1997) *Am. J. Cardiol.* **80,** 11A-16A

264. Stanley, W. C., Lopaschuk, G. D., and McCormack, J. G. (1997) *Cardiovasc. Res.* **34,** 25-33

265. Oliver, M. F. and Opie, L. H. (1994) *Lancet* **343,** 155-158

266. Finck, B. N., Han, X., Courtois, M., Aimond, F., Nerbonne, J. M., Kovacs, A., Gross, R. W., and Kelly, D. P. (2003) *Proc. Natl. Acad. Sci. U. S. A* **100,** 1226-1231

267. Oliver, M. F., Kurien, V. A., and Greenwood, T. W. (1968) *Lancet* **1,** 710-714

268. Lopaschuk, G. D. (2002) *Heart Fail. Rev.* **7,** 149-159

269. Feuvray, D., Idell-Wenger, J. A., and Neely, J. R. (1979) *Circ. Res.* **44,** 322-329

270. Dhalla, N. S., Elimban, V., and Rupp, H. (1992) *Mol. Cell Biochem.* **116,** 3-9

271. Paulson, D. J. and Crass, M. F., III (1982) *Am. J. Physiol* **242,** H1084-H1094

272. Makino, N., Dhalla, K. S., Elimban, V., and Dhalla, N. S. (1987) *Am. J. Physiol* **253,** E202-E207

273. Zhao, X. Y., Hu, S. J., Li, J., Mou, Y., Chen, B. P., and Xia, Q. (2006) *J. Physiol Biochem.* **62,** 1-8

274. Wold, L. E., Dutta, K., Mason, M. M., Ren, J., Cala, S. E., Schwanke, M. L., and Davidoff, A. J. (2005) *J. Mol. Cell Cardiol.* **39,** 297-307

275. Teshima, Y., Takahashi, N., Saikawa, T., Hara, M., Yasunaga, S., Hidaka, S., and Sakata, T. (2000) *J. Mol. Cell Cardiol.* **32,** 655-664

276. Yaras, N., Ugur, M., Ozdemir, S., Gurdal, H., Purali, N., Lacampagne, A., Vassort, G., and Turan, B. (2005) *Diabetes* **54,** 3082-3088

277. Choi, K. M., Zhong, Y., Hoit, B. D., Grupp, I. L., Hahn, H., Dilly, K. W., Guatimosim, S., Lederer, W. J., and Matlib, M. A. (2002) *Am. J. Physiol Heart Circ. Physiol* **283,** H1398-H1408

278. Allo, S. N., Lincoln, T. M., Wilson, G. L., Green, F. J., Watanabe, A. M., and Schaffer, S. W. (1991) *Am. J. Physiol* **260,** C1165-C1171

279. Pereira, L., Matthes, J., Schuster, I., Valdivia, H. H., Herzig, S., Richard, S., and Gomez, A. M. (2006) *Diabetes* **55,** 608-615