AMP-ACTIVATED PROTEIN KINASE AND HYPERTROPHIC REMODELING OF HEART MUSCLE CELLS

by

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Abstract

Introduction: Cardiac hypertrophy is an adaptive response to increased myocardial workload that becomes maladaptive when hypertrophied hearts are exposed to an acute metabolic stress, such as ischemia/reperfusion. Acceleration of glycolysis occurs as part of the hypertrophic response and may be maladaptive because it enhances glycolytic metabolite accumulation and proton production. Activation of AMP-activated protein kinase (AMPK), a kinase involved in the regulation of energy metabolism, is proposed as a mechanism for the acceleration of glycolysis in hypertrophied hearts. However, this concept has not yet been proven conclusively. Additionally, several studies suggest that AMPK is involved in hypertrophic remodeling of the heart by influencing cardiac myocyte growth, a suggestion that remains controversial.

Hypothesis: AMPK mediates hypertrophic remodeling in response to pressure overload. Specifically, AMPK activation is a cellular signal responsible for accelerated rates of glycolysis in hypertrophied hearts. Additionally, AMPK influences myocardial structural remodeling and gene expression by limiting hypertrophic growth.

Experimental Approach: To test this hypothesis, H9c2 cells, derived from embryonic rat hearts, were treated with (1 μM) arginine vasopressin (AVP) to induce hypertrophy. Substrate utilization was measured and the effects of AMPK inhibition by either Compound C or by adenovirus-mediated transfer of dominant negative AMPK were determined. Subsequently, adenovirus-mediated transfer of constitutively active form of AMPK (CA-AMPK) was expressed in H9c2 to specifically increase AMPK activity and, thereby, further characterize the role of AMPK in hypertrophic remodeling.

Results: AVP induced a metabolic profile in hypertrophied H9c2 cells similar to that in intact hypertrophied hearts. Glycolysis was accelerated and palmitate oxidation was reduced with no significant alteration in glucose oxidation. These changes were associated with AMPK activation, and inhibition of AMPK ameliorated but did not normalize the hypertrophy-associated increase in glycolysis. CA-AMPK stimulated

both glycolysis and fatty acid oxidation, and also increased protein synthesis and content. Howver, CA-AMPK did not induce a pathological hypertrophic phenotype as assessed by atrial natriuretic peptide expression.

Conclusion: Acceleration of glycolysis in AVP-treated hypertrophied heart muscle cells is partially dependent on AMPK. AMPK is a positive regulator of cell growth in these cells, but does not induce pathological hypertrophy when acting alone.

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Abbreviations

2-APB	2-aminoethoxydiphenyl borate
4E-BP	4E-binding protein1
ACC	Acetyl-CoA carboxylase
ADP	Adenosine diphosphate
Ag II	Angiotensin II
AICAr	5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside
AICAR	5-aminoimidazole-4-carboxamide ribonucleoside
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
BCA	Bicinchoninic acid
CaMKK	Ca^{2+}/calmodulin-dependent protein kinase kinase
CA-AMPK	Constitutively active form of AMPK
CBS	Cystathionine β synthase
CCE	Capacitative calcium channel
CPT-1	Carnitine palmitoyl transferase 1
CPT-2	Carnitine palmitoyl transferase 2
DAG	Diacylglycerol
DCA	Dichloroacetate
DMEM	Dulbecco's Modified Eagle's Medium

DMSO	Dimethyl sulphoxide
DN-AMPK	Dominant negative form of AMPK
eEF2	Eukaryotic elongation factor-2
elF2B	eukaryotic translation initiation factor
EGTA	O,O'-bis(2-aminoethyl)ethyleneglycol-N,N,N',N'-tetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
ETC	Electron transport chain
ET-1	Endothelin-1
F1,6P2	Fructose 1,6-bisphosphate
F6K	Fructose-6-phosphate
FABP	Fatty acid binding proteins
FATP	Fatty acid transport protein
FAT/CD36	Fatty acid translocase
FBS	Fetal bovine serum
G6P	Glucose-6-phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBD	Glycogen binding domain
GFP	Green fluorescent protein
GqCR	Gq-coupled receptor
GLUT	Glucose transporter
GSK-3β	Glycogen synthase kinase-3β
HK	Hexokinase
HS	Horse serum
IP3	Inositol 1,4,5-trisphosphate

KH	Krebs-Henseleit
KOH	Potassium hydroxide
LCAD	Long-chain acyl-CoA dehydrogenase
LCFA	Long-chain fatty acids
LDH	Lactate dehydrogenase
LPL	Lipoprotein lipase
mTOR	Mammalian target of rapamycin
MAPK	Mitogen-activated protein kinase
MAS	Malate-aspartate shuttle
MCAD	Medium chain acyl-CoA dehydrogenase
MCD	Malonyl-CoA decarboxylase
MO25	Mouse protein 25
NAD	Nicotinamide adenine dinucleotide
$NADH_2$	Reduced nicotinamide adenine dinucleotide
NOG	Non oxidative glycolysis
P70S6K	P70 ribosomal protein S6 kinase
PBS	Phosphate-buffered saline
PCr	Phospho-creatinine
PDC	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PDP	Pyruvate dehydrogenase phosphatase
PFK-1	Phosphofructokinase-1
PFK-2	Phosphofructokinase-2
PGC-1α	Peroxisome proliferator-activator receptor γ coactivator 1α

PhE	Phenylephrine
PI3K	Phosphoinositol-3-phosphate Kinase
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLCβ	Phospholipase Cβ
PPARα	Peroxisome proliferator-activator receptor α
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide electrophoresis
SHR	Spontaneously hypertensive rats
SR	Sarcoplasmic reticulum
STRAD	STE20-related adaptor protein
TAK1	Transforming growth factor-β-activated kinase-1
TBS	Tris buffered saline
TCA	Tricarboxylic acid
TMZ	Trimetazidine
VDAC	Voltage-dependent anion channels
VLCAD	Very long-chain acyl-CoA dehydrogenase
WPW	Wolff-Parkinson-White

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CHAPTER 1 - INTRODUCTION

1.1 BACKGROUND

Cardiac hypertrophy is an adaptive response of the heart to increased hemodynamic workload [1-3]. Alterations in mechanical function and structure which are accompanied with changes in metabolism as well as gene expression occur in cardiac hypertrophy [4, 5]. Two distinct forms of cardiac hypertrophy exist: pathological cardiac hypertrophy due to pressure or volume overload (i.e., hypertension or aortic stenosis); and physiological cardiac hypertrophy results from endurance exercise training and normal growth [2, 3, 6-8]. These two forms of cardiac hypertrophy demonstrate distinct patterns of substrate utilization with opposite changes in glycolysis and in oxidation of fatty acids and glucose (Figure 1-1) [20, 27]. The focus of the current study is mainly on the pathologic form of cardiac hypertrophy.

Pressure-induced cardiac hypertrophy is very common in our society and is an independent risk factor for cardiac morbidity and mortality [2, 9-15]. For instance, it is associated with a substantially increased risk of myocardial ischemia, myocardial infarction, heart failure, and sudden death [2]. Cardiac hypertrophy occurs in response to increased hemodynamic workload produced by situations such as hypertension, valvular heart disease, and myocardial infarction [2, 6-8]. Hypertrophy is believed to be

an adaptive or compensatory response to increased hemodynamic workload which normalizes myocardial wall stress and maintains cardiac output [2, 7, 16]. However, this response becomes maladaptive if hypertrophied hearts are exposed to metabolic stresses, such as ischemia, or the increased workload on these hearts persists [2, 17-20]. This finding is particularly important since cardiac hypertrophy and ischemic heart disease commonly co-exist [21].

At the cellular and molecular levels, cardiac hypertrophy is characterized by an increase in cell size and protein content of cardiac myocyte without cell division that is accompanied by changes in gene expression and energy metabolism (Figure 1-1) [1, 4, 5, 20, 22-24]. The alterations in energy metabolism observed in hypertrophied hearts are believed to contribute, at least in part, to the adaptive or maladaptive nature of the hypertrophic response [5, 17-20, 24-29]. An acceleration of glycolysis occurs as part of the hypertrophic response, which might contribute to the maladaptation. Accelerated glycolysis increases glycolytic metabolite (lactate, NADH) accumulation and proton production, both of which can interfere with contractile efficiency [18, 20, 30-33]. The exact mechanisms responsible for the acceleration of glycolysis in these hearts are not known.

Activation of AMP-activated protein kinase (AMPK), a regulatory kinase involved in the regulation of energy metabolism of the whole body including the heart, has been proposed to be a key mediator of accelerated glycolysis in hypertrophied hearts [29, 34, 35]. However, a role for AMPK has never been definitively demonstrated. Additionally, several studies suggest that, in addition to hypertrophic metabolic remodeling, AMPK might have a role in structural remodeling in hypertrophied hearts; in other words, AMPK may also inhibit hypertrophic growth, although this idea is controversial [29, 36, 37].

1.2 OVERVIEW OF SUBSTRATE UTILIZATION BY THE NORMAL HEART

Under normal physiologic conditions, the heart utilizes a variety of exogenous substrates, including free fatty acids, carbohydrates, amino acids and ketone bodies, as well as endogenous substrates, including glycogen and triglycerides (Figure 1-2) [38-41]. The flux through metabolic pathways is determined by several factors including arterial carbon substrate concentration, hormone concentrations (such as catecholamines and insulin), coronary flow, inotropic state, and the nutritional status [42-45]. In the normal heart, up to 50-70 % of the heart's energy is derived from fatty acid oxidation. Glucose and lactate oxidation provide most of the remaining energy requirement by the heart, with glycolysis accounting for small additional production of adenosine triphosphate (ATP) by the heart [5, 20, 38, 41, 46, 47].

Triglycerides and glycogen are two endogenous sources for cardiac energy production. Triglycerides can produce fatty acid and account for 10-50 % of ATP produced in isolated perfused rat hearts [39]. Under aerobic conditions, the contribution of glycogen to energy production by the heart is significant, accounting for up to 40 % of ATP produced from catabolism of glucose; however, the contribution of glycogen to energy metabolism increases in situations such as increased work or hypoxia [48, 49].

1.3 FATTY ACID METABOLISM

Fatty acids are hydrophobic molecules; therefore, they are carried in the circulation by albumin or incorporated into lipoproteins [41, 50, 51]. The concentrations of free fatty acids and activities of lipoprotein lipase (LPL) and transporter proteins (CD36, etc.) determine fatty acid uptake by the heart. Upon entering the cells, long-chain fatty acids (LCFA), such as oleic and palmitic acids, which are the major fatty acids used by the heart, are modified before entering into mitochondria. The majority of the LCFA taken up by the heart are oxidized and the rest converted to triglyceride stores or used for other complex lipid synthesis [28, 41, 50, 51]. The transport of fatty acids through the

plasma membrane occurs by both passive diffusion and a facilitated protein membrane mechanism [51, 52]. The uptake and transport of fatty acids by the heart is facilitated by fatty acid transport protein (FATP), fatty acid translocase (FAT/CD36) and fatty acid binding proteins (FABP) (Figure 1-3) [51, 53, 54].

Upon entering the cell, the LCFA are transported inside the cytoplasm by FABPs and are esterified with coenzyme A by the enzyme fatty acyl-CoA synthetase to form fatty acyl-CoA. From here, fatty acyl-CoA is either incorporated into triacylglycerols or other lipids or transported into the mitochondrial matrix for oxidation by means of the β-oxidation spiral and the tricarboxylic acid cycle (Figure 1-3) [50]. Long-chain fatty acyl-CoA transport into the mitochondria occurs through a complex process involving three carnitine-dependent enzymes, carnitine palmitoyl transferase 1 (CPT-1), carnitine-acylcarnitine translocase (CT), and carnitine palmitoyl transferase 2 (CPT-2) (Figure 1-3) [50]**.** Once inside the matrix of mitochondria, long-chain fatty acyl-CoA undergoes β-oxidation [41], a series of four reactions which cleaves two carbons in the form of acetyl-CoA from the parent acyl-CoA molecule per cycle, generating $NADH_2$ and $FADH_2$. Acetyl-CoA in turn enters the tricarboxylic acid (TCA) cycle to produce further reducing equivalents ($NADH_2$). $FADH_2$ and $NADH_2$ are oxidized in the electron transport chain and produce ATP. One molecule of palmitate, which contains 18 carbons, yields 129 molecules of ATP.

The rate of transport into mitochondria and, therefore, oxidation of LCFA is controlled largely by CPT-1 [50]. CPT-1 is inhibited allosterically by malonyl-coA formed by acetyl-CoA carboxylase (ACC) from acetyl-CoA (Figure 1-4) [42]. Malonyl-CoA is therefore an important regulator of fatty acid oxidation. High concentrations of citrate or insulin treatment increase malonyl-CoA production thereby inhibiting fatty acid oxidation. In fact, malonyl-CoA reduces influx of long-chain fatty acyl-CoA into the mitochondria, reducing fatty acid oxidation. Malonyl-CoA can be converted to acetyl-CoA by malonyl-CoA decarboxylase (MCD) [55, 56]. The heart contains an active malonyl-CoA decarboxylase that decarboxylates malonyl-CoA to acetyl-CoA (Figure

1-4) [57]. AMPK activation leads to stimulation of fatty acid oxidation because AMPK phosphorylates and inactivates ACC and, possibly, by phosphorylating and activating MCD [57-61].

There are two isoforms of ACC, ACC-1 (265 KDa) and ACC-2 (280 KDa), which each plays distinct roles [62]. They share approximately 80 % homology; the major difference is that ACC-2 has a unique N-terminal extension of hydrophobic amino acids directing its mitochondrial membrane insertion [63]. ACC-1 localizes mostly in the cytosol and is primarily responsible for fatty acid synthesis; it is abundant in lipogenic tissues such as liver and adipose tissue [64]. ACC-2, on the other hand, is present in the outer leaflet of mitochondria and is the predominant isoform in the heart and muscle [42, 65]. ACC-2 controls malonyl-CoA production and therefore regulates CPT-1 activity and fatty acid oxidation. ACC activity is regulated allosterically by long-chain fatty acyl-CoA and citrate, and covalently by kinases such as protein kinase A (PKA) and AMPK [29, 66-70]. ACC phosphorylation generally leads to inhibition of enzyme activity. Both PKA and AMPK directly phosphorylate and inactivate both isoforms.

1.4 CARBOHYDRATE METABOLISM

Glucose, lactate, and pyruvate are the three major carbohydrate substrates of the heart [41]. Glucose uptake into the cardiac myocyte is regulated by the transmembrane glucose gradient and the concentration and activity of glucose transporters (GLUTs) located at the sarcolemma [47, 71]. GLUT-4 and GLUT-1, are the major GLUT isoforms expressed in the heart, with GLUT-4 predominating [44, 47, 71-73]. Both transporters are present in sarcolemma and in intracellular vesicles. Basal glucose uptake is largely accounted for by the presence of GLUT-1 in the sarcolemma of cardiac myocytes [44]. On the other hand, stimulation of glucose uptake is primarily due to translocation of glucose transporters, especially GLUT-4, from intracellular sites to the sarcolemma [44, 73]. Stimulation of glucose uptake results from translocation of

glucose transporters into the sarcolemma and increasing the membrane capacity for glucose uptake. GLUT4 translocation is stimulated by insulin in the fed state, and by increases in cardiac work and metabolic stresses such as ischemia and hypoxia [44, 71, 73]. AMPK is believed to have an important role in the translocation of GLUTs during metabolic stresses [29, 70, 74-76].

When inside the cell, intracellular glucose is rapidly phosphorylated by the unidirectional enzyme hexokinase (HK) to form glucose-6-phosphate (G6P) [77]. G6P can be either incorporated into glycogen for storage or catabolized directly (Figure 1-5) [38, 44, 72]. When catabolized directly, glucose passes through the glycolytic pathway to pyruvate which is then transported to be oxidized to CO_2 in mitochondria or alternatively reduced to lactate or transaminated to alanine [38, 71].

1.4.1 Glycolysis

Glycolysis is the common pathway for both glucose and glycogen breakdown. It is the major metabolic pathway of G6P in which one molecule of glucose is broken down to two molecules of pyruvate by a sequential series of reactions in the heart (Figure 1-6) [44, 72]. Glycolysis also produces two NADH and 2 ATP per glucose molecule. The NADH and pyruvate produced in the glycolytic pathway are either shuttled into mitochondria to be oxidized or converted to lactate in the cytosol (non oxidative glycolysis (NOG)). The ATP produced in the glycolytic pathway is utilized for ion homeostasis and is important for diastolic relaxation [78-80]. Glycolysis can occur in both aerobic and anaerobic conditions, without the presence of oxygen in the latter case; therefore, its importance is greater during myocardial ischemia when oxidative metabolism is reduced.

The starting point of glycolysis is production of G6P from either glucose through HK or glycogen (glycogen → G1P → G6P). In the heart, HK has two isoforms I and II, with HKII being more abundant. The voltage-dependent anion channels (VDACs), which are pore-forming proteins in the outer mitochondrial membrane, are known to

bind to many molecules including HK [81, 82]. Binding of HK to VDAC leads to reversible translocation of HK from cytosol to the outer mitochondrial membrane [83]. Binding of HK to VDACs is viewed as a key factor responsible for insulin-induced glucose uptake in skeletal muscle and acceleration of glycolysis in malignant neoplasms by providing HK preferential access to mitochondrial-derived ATP and reducing allosteric inhibition by the product of its reaction, G6P [83].

Among the enzymes participating in the glycolytic pathway, phosphofructokinase-1 (PFK-1) is one that most strongly regulates flux (Figure 1-7) [44, 84]. It catalyzes the conversion of fructose-6-phosphate (F6P) to fructose 1,6-bisphosphate (F1,6P2) and hydrolyses one molecule of ATP. PFK-1 is allosterically inhibited by ATP, phospho-creatinine (PCr), citrate, and H^+ and is allosterically activated by AMP, ADP, alkalosis, and very importantly, fructose 2,6-bisphosphate (F2,6BP). In the absence of physiologic concentrations of F2,6BP, PFK-1 activity is almost completely inhibited. F2,6BP is produced from F6P by the enzyme 6-phophofructo-2-kinase (PFK-2) (Figure 1-7). PFK-2 is a bifunctional enzyme which controls both the synthesis and degradation of F2,6BP. PFK-2 is allosterically inhibited by ATP and citrate, and stimulated by AMP and F2,6BP. PFK-2 is also phosphorylated by PKB in response to insulin signaling. Additionally, AMPK can phosphorylate and activate PFK-2 [85, 86].

1.4.2 Glucose Oxidation

Under aerobic conditions, approximately 10-20 % of the pyruvate generated by glycolysis is transported into the mitochondria, where it is decarboxylated and oxidized [84]. The extent of pyruvate oxidation, which in addition to glucose may also be derived from glycogen or lactate, is controlled mainly by the mitochondrial pyruvate dehydrogenase complex (PDC) [44]. The PDC, a multi-enzyme complex in the mitochondrial matrix, is the key irreversible step in carbohydrate oxidation. It consists of several catalytic subunits which includes E1α/β, E2, and E3 components, an E3 – binding protein, as well as regulatory kinases and a phosphatase [44, 87-90]. The PDC oxidatively decarboxylates pyruvate to form CO_2, $NADH_2$ and acetyl-CoA [44]. PDC

activity is inhibited by phosphorylation mediated by pyruvate dehydrogenase kinase (PDK); dephosphorylation by pyruvate dehydrogenase phosphatase (PDP) reverses this inhibition (Figure 1-8) [91, 92]. The activities of PDK and PDP are determined by the intramitochondrial concentrations of metabolites and ions. High concentrations of acetyl-CoA and NADH, as occur during fatty acid oxidation, activate PDK, while pyruvate or its analogue dichloroacetate (DCA), CoA, NAD^+, or inhibition of fatty acid oxidation inhibits PDK [44, 50, 87, 90, 93-98]. On the other hand, Mg^{2+} and Ca^{2+} stimulate PDP [44, 90, 92, 99-101]. Once formed by PDC, acetyl-CoA can enter the TCA cycle and undergoes further series of oxidation reactions to produce substrates for the electron transport chain [5, 44]. For every glucose molecule enters TCA cycle, 36 ATP molecules are formed.

1.5 GLYCOGEN METABOLISM

Glycogen is the storage form of glucose found in the cytoplasm of the heart and other tissues and makes a significant contribution to energy metabolism of the myocardium, accounting for approximately 40 % of ATP generated from glucose catabolism [20, 38, 40]. Under non-ischemic conditions, glycogen turnover; i.e., simultaneous synthesis and degradation, occurs in the heart [40]. In addition, under normal physiologic conditions, glucose-6-phosphate derived from glycogen is preferentially oxidized as compared to its exogenous counterpart [20, 40]. Glycogen synthesis is influenced by the G6P concentration and the activity of glycogen synthase. Under conditions such as hyperinsulinemia and high concentrations of exogenous glucose, increased glycogen synthesis occurs [40, 102]. On the other hand, adrenergic stimulation or a fall in tissue ATP content stimulates glycogen degradation [44, 103]. Glycogenolysis is regulated by glycogen phosphorylase activity.

1.6 SUBSTRATE UTILIZATION IN THE HYPERTROPHIED HEART

Several *ex vivo* or *in vivo* studies in hypertrophied hearts have shown that oxidation of LCFA, the major energy source of these hearts, is reduced by 30-40 % as compared to non-hypertrophied hearts (Figure 1-9) [5, 20, 23, 24, 28, 35, 104-112]. Surprisingly, the oxidation of octanoate, a medium chain fatty acid which can freely diffuse into the mitochondria, is not reduced in these hearts, suggesting that the β-oxidation pathway is not impaired in these hearts; at least, non-failing hypertrophied hearts [5, 106, 113].

In contrast to fatty acid oxidation, glucose utilization is enhanced in hypertrophied hearts presumably as a compensatory response to reduced rates of fatty acid oxidation and/or reduced energy reserve (Figure 1-9) [19, 31, 33, 35, 89, 114, 115]. In fact, both experimental and clinical studies have documented that, in the hypertrophied heart, basal glucose uptake is accelerated, while insulin-dependent glucose uptake is decreased [116-118]. Additionally, several studies in mammalian species, including human, have documented that rates of glycolysis are accelerated in hypertrophied hearts [19, 20, 23, 30, 33-35, 104, 105, 107, 114, 119-123].

However, flux through different pathways responsible for glucose metabolism is not uniformly increased in hypertrophied hearts. The observed acceleration of glycolysis in these hearts is not accompanied by increases in glucose oxidation and, in fact, glucose oxidation is either unchanged or decreased in hypertrophied hearts as compared to non-hypertrophied hearts [19, 20, 24, 31, 33, 35, 89]. As a result, a substantial proportion of pyruvate is converted into lactate instead of being oxidized which leads to a low fractional oxidation rate of glucose (approximately 10-15 % as compared to 14-25% in non-hypertrophied hearts [111]) or increased NOG (NOG, the proportion of glucose passing through glycolysis that is not oxidized) [20, 124]. The low fractional oxidation observed is surprising, especially considering that LCFA oxidation is also low in these hearts [20, 106]. Based on the Randle cycle, a reduction in LCFA oxidation is usually associated with a compensatory acceleration of glucose oxidation [87]. Additionally, as

a result of increased glycolysis and pyruvate production in hypertrophied hearts, one would expect that pyruvate-induced activation of PDC would occur, thereby stimulating glucose oxidation [87, 90]. In hypertrophied hearts, rates of lactate oxidation are either unchanged or decreased as compared to non-hypertrophied hearts [20, 109]. Additionally, consistent with the fact that NOG is increased in these hearts, net release of lactate is increased [20, 109].

1.7 CONSEQUENCES OF ALTERATIONS IN SUBSTRATE UTILIZATION ON FUNCTION OF HYPERTROPHIED HEARTS

The metabolic profile observed in hypertrophied hearts may be a beneficial adaptation by which cardiac output is preserved [25, 26, 28]. In support of this idea, Young et al have shown that reversing this metabolic profile leads to decreased contractile function of hypertrophied hearts [26]. However, while this metabolic profile may be considered an adaptive response in non-stressful conditions, it can become maladaptive when hypertrophied hearts are exposed to metabolic stress, as happens during ischemia-reperfusion [2, 17-20, 35]. Functional recovery after ischemia/reperfusion is lower in hypertrophied hearts as compared to non-hypertrophied hearts [17-19]. Alterations in substrate utilization, particularly glucose, may be responsible for this impaired recovery in hypertrophied hearts [25].

Support for the concept that alterations in glucose metabolism influence function of hypertrophied hearts arises from both clinical and experimental studies in which a decrease in NOG, i.e. by stimulating glucose oxidation and/or by reducing glycolysis, improve functional recovery of ischemic-reperfused hypertrophied hearts (Figure 1-10) [19, 20, 24, 44, 104, 111, 125-127]. For example, several studies have documented that dichloroacetate (DCA), an inhibitor of PDC kinase, and trimetazidine (TMZ), a partial 3-ketoacyl coenzyme A thiolase inhibitor, improve post-ischemic contractile function and efficiency of hypertrophied hearts by reducing NOG (Figure 1-10) [24, 44, 126, 127]. As discussed below, this protective effect might be due to a reduction in H^+

production during glucose catabolism leading to recovery of intracellular pH, which then in turn limits calcium overload and therefore reduces the energetic cost associated with the maintenance of ion homeostasis [44, 124, 127].

In non-hypertrophied hearts, lower rates of glucose oxidation and therefore increased NOG are considered to be, in part, detrimental to heart function during ischemia-and repefusion [8, 124]. During ischemia, oxidative metabolism is decreased and anaerobic glycolysis becomes a more important source of myocardial energy production [38, 44] resulting in increased lactate, NADH, and H^+ production [124]. For each molecule of glucose passing through glycolysis that is not oxidized, two H^+ protons are produced by hydrolysis of adenosine triphosphate (ATP) [124, 128]. Excess H^+ produced from hydrolysis of glycolytically derived ATP during ischemia contributes to acidosis in the myocardium [129], and may therefore lead to development of cardiac arrhythmias [130], decreases in cardiac contractility [130], and increases in sarcolemmal Na^+/H^+ exchanger activity [131]. Upon reperfusion, extracellular pH quickly recovers leading to a large pH gradient across the membrane that activates the Na^+/H^+ exchanger and causes intramyocyte Na^+ overload. This Na^+ overload and membrane depolarization in turn activates the Na^+/Ca^{2+} exchanger resulting in Ca^{2+} influx [44]. The subsequent Ca^{2+} is thought to be a major contributor to ischemia-reperfusion myocyte injury by activating proteases and phosphatases that might lead to myocardium arrhythmias and necrosis [44, 131, 132]. Ca^{2+} overload might also lead to an inappropriate use of ATP since a greater amount of ATP must be used for cellular Ca^{2+} transport rather than contractile function [133]. It should be noted that, during ischemia, circulating free fatty acid concentrations and fatty acid metabolites rise as a result of increased endogenous catecholamine production and therapeutic administration of heparin [44, 134, 135]. During reperfusion, fatty acid oxidation is the prominent source of energy production due not only to its increased circulation level, but also to stimulation of fatty acid oxidation as a result of changes in enzymes and metabolites responsible for the regulation of fatty acid oxidation [44, 134, 136]. Therefore, based on the Randle cycle, this stimulation of fatty acid oxidation is accompanied by attenuation of glucose

utilization with suppression of glucose oxidation to a gtreater extent than glycolysis, leading to further increases NOG and H^+ production [44, 50, 124, 127, 137-139]. Therefore, during reperfusion continued H^+ production contributes to exacerbate the injury [124, 127].

In hypertrophied hearts, the greater NOG might contribute to the increased post-ischemic dysfunction as compared to the non-hypertrophied hearts [19, 20, 113]. In these hearts, even though oxidative metabolism of key exogenous substrates returns to pre-ischemic values as with non-hypertrophied hearts, contractile efficiency and myocardial function do not return to pre-ischemic values [19, 20, 30, 31]. During ischemia and reperfusion, the excess H^+ is carried over and contributes to the severity of acidosis, which leads to greater left ventricular dysfunction in hypertrophied hearts than in normal hearts. It has been known that intracellular accumulation of Na^+ and Ca^{2+} are greater in hypertrophied hearts than in non-hypertrophied hearts due to the greater H^+ production during ischemia [33] and reperfusion [19, 30, 31], and alterations in expression of Na^+/H^+ and Na^+/Ca^{2+} exchangers observed in these hearts [113]. In fact, impaired recovery of hypertrophied hearts during reperfusion is, in part, the result of Ca^{2+} overload, because more ATP has to be used for maintenance of ion balancing than contraction; this explains the discrepancy that exists between oxidative metabolism and function [20].

1.8 METABOLIC REMODELING: (UNDERLYING MECHANISM)

The mechanisms underlying the changes in metabolism in hypertrophied hearts are not fully understood. In the heart, the flux through different metabolic pathways is controlled by extracellular factors (substrate supply, hormones, and myocardial workload) as well as intracellular factors (covalent modulation, allosteric modulation, changes in subcellular distribution, and changes in gene expression) [40, 53, 84]. The reasons for the reduction in fatty acid oxidation rates in hypertrophied hearts might be due to the degree of cardiac hypertrophy, the concentration of fatty acid in blood or

perfusate, the severity of carnitine deficiency, the myocardial workload as well as reduced expression or activity of oxidative enzymes and FABPs [5, 11, 106, 113]. Studies on spontaneously hypertensive rats (SHR) have shown that a FAT/CD36 transport protein was reduced or not detectable at protein and mRNA levels [53, 112, 140]. The other factor that might limit myocardial fatty acid and mitochondrial oxidation is the expression of proteins involved in fatty acid oxidation, namely medium chain acyl-CoA dehydrogenase (MCAD), long-chain acyl-CoA dehydrogenase (LCAD) and very long-chain acyl-CoA dehydrogenase (VLCAD) which together form a family of acetyl-CoA dehydrogenases that start the initial step in the oxidative spiral [28, 141-143]. While we have shown that the protein expression of MCAD and LCAD is not changed in pressure-induced cardiac hypertrophy [24], other studies on human and animal models of heart failure have documented that the activity or expression of MCAD, LCAD, at the mRNA or protein level are downregulated [28, 72, 144, 145, 146 , 147, 148]. In fact, some studies have suggested that this transcriptional downregulation of fatty acid oxidation genes is not associated with a corresponding downregulation of fatty acid oxidation enzymes unless heart failure is also present [25, 146, 149]. The other factor that might limit myocardial fatty acid oxidation in these hearts is low tissue carnitine levels. To address this matter, several experimental and clinical studies have documented that carnitine levels are reduced in hypertrophied hearts, leading to a decrease in the import of fatty acids to the mitochondria [106, 125, 150]. However, after restoring carnitine, fatty acid oxidation was still lower in hypertrophied hearts, indicating that, although carnitine deficiency in hypertrophied hearts is associated with lower rates of fatty acid oxidation in the heart, it is not a principal factor [106, 125, 150].

The underlying mechanism responsible for the enhancement of glucose uptake and glycolysis in hypertrophied hearts is not fully understood. Several studies have indicated that, in cardiac hypertrophy, GLUT-4 expression may be normal [24, 151] or reduced [72, 116], but expression of GLUT-1 is either unchanged or increased [151]. Therefore, the increased glucose uptake by hypertrophied hearts is not simply due to

changes in expression of GLUTs. Other factors such as alterations in activity or subcellular distribution of GLUTs might have a role in this setting. To support the latter, Tian et al [33] have documented that, in hypertrophied hearts, enhanced translocation of GLUT-4 to plasma membrane occurs under non-stressful conditions, which may partly explain enhanced glucose uptake in these hearts. We have shown that, in hypertrophied hearts, the accelerated glycolytic rate was not accompanied by changes in the expression of key metabolic enzymes and proteins [24]; however, others, albeit in different species, have shown that the activity of a number of glycolytic enzymes is enhanced in these hearts [114, 120]. Also, the activity of LDH, the enzyme responsible for reversible production of lactate from pyruvate, is increased in some hearts exposed to a pressure-overload. Moreover, several studies have indicated that, in pressure-induced cardiac hypertrophy, isoenzymes of LDH and enolase shift toward the anaerobic and fetal form [114, 152].

The increased NOG in hypertrophied hearts cannot be explained by alterations in PDC expression or activity. We and others have revealed that, in hypertrophied hearts, the expression of PDC subunits as well as expression of the PDK or PDP does not change [24, 89, 153]. More interestingly, Lydell et al [153] have demonstrated that, in hypertrophied hearts, the activation state of PDC, calculated by expressing active PDC as a percent of total PDC, is higher as compared to non-hypertrophied hearts which contrasts with the lower fractional oxidation of glucose in these hearts. Additionally, the increased NOG observed in hypertrophied hearts cannot be explained by alterations in intramitocondrial concentrations of metabolites which might influence activity of the PDC by allosteric/or product feedback effects. Support for this notion comes from a study in which perfusion of hypertrophied and non-hypertrophied hearts in the absence of exogenous LCFA resulted in decreased intramitochondrial acetyl-CoA/CoA ratio in both groups, but reduced NOG in only non-hypertrophied hearts [153]. Therefore, while changes in the expression and activity of the PDC or defects in metabolite feedback and/or allosteric mechanisms are not responsible for increased NOG in hypertrophied hearts, other mechanisms such as impaired pyruvate or`$NADH_2$ transport

from cytosol to mitochondria or lactate dehydrogenase (LDH) catabolism might have a role in this setting (Figure 1-11). In this regard we have shown that, in isolated mitochondria from hypertrophied hearts, both the sensitivity to pyruvate as well as the maximal oxygen consumption was not significantly altered as compared with non-hypertrophied hearts, indicating that pyruvate transport inside the mitochondria is not restricted in these hearts [89]. In the glycolytic pathway, pyruvate production from glucose leads to the production of cytosolic $NADH_2$. In order to be oxidized, pyruvate needs an equivalent oxidation of cytosolic $NADH_2$ in the mitochondria. $NADH_2$ enters into cardiac mitochondria mainly by the malate-aspartate shuttle (MAS). If MAS were restricted, this would lead to an increase in lactate production and a decrease in glucose oxidation, resulting in an increase in NOG. The contribution of limitations in MAS activity remains to be clarified. Several studies provided evidence that, in hypertrophied hearts, LDH isoenzyme activity is shifted towards the fetal/anaerobic form [114, 154, 155] which favours the production of lactate and an increase in NOG [156].

AMPK is proposed to be centrally involved in the regulation of a number of metabolic enzymes and is therefore considered a major regulator of cardiac energy metabolism. AMPK might be involved in the shift toward glucose utilization in the setting of cardiac hypertrophy.

1.9 5′ AMP-ACTIVATED PROTEIN KINASE

AMP-activated protein kinase (AMPK) acts as a master regulator of metabolism [157] at the cellular and at the whole body level [158], being conserved throughout all eukaryotes [159] and widely expressed in all mammalian tissues including the heart [160]. AMPK functions as a "low fuel warning system" [157]. Upon activation, it switches on catabolic pathways that generate ATP, such as the uptake and metabolism of fatty acid and glucose, and switches off anabolic pathways that consume ATP, such as synthesis of fatty acid, cholesterol and protein thereby maintaining energy homeostasis [159, 161-164]. More recently, AMPK has also emerged as a regulator of

appetite by playing a role in hypothalamic glucose and nutrient sensing. Several studies have shown that pharmacological or genetic activation or inhibition of AMPK result in increased or reduced food intake, respectively [165, 166].

1.9.1 Structure of AMPK

Mammalian AMPK is a heterotrimeric complex containing one α catalytic subunit and two β and γ regulatory subunits (Figure 1-12) [159 , 161, 163]. For full AMPK activity, heterotrimeric complex formation is necessary [167]. Each AMPK subunit exists as several isoforms which have varying tissue expression patterns and each is encoded by distinct genes (α1, α2; β1, β2; γ1, γ2, γ3) [159, 168]. At least 12 heterotrimeric combinations exist, along with alternative splicing and/or alternative transcription sites, so that a diverse collection of αβγ heterotrimers can exist [159, 161, 163]. While subunits of α1, β1, γ1 are expressed in almost all tissues and are responsible for the activity, in the heart subunits α2, β2, γ2 are highly expressed [160]. Interestingly, these different isoforms of AMPK largely influence its activity and subcellular distribution [169-171]. For example, the α2- and β1-subunits contain a putative nuclear localization sequence, and they are therefore enriched in nucleus, whereas the α1-subunit is largely cytoplasmic and appears to be excluded from the nucleus [171-174]. Among the γ-subunits, the γ2-subunit has the highest sensitivity to AMP [29]. The N-terminus of the α-subunits contains a kinase domain [175] and phosphorylation site (Thr-172) and the C-terminal domain is responsible for binding to β- and γ-subunits [176]. Truncation of the α-subunit from 1-548 to 1-392 results in loss of both catalytic activity and βγ binding. Also, truncation to 1-312 results in a constitutively active form of the kinase which indicates that the sequence of 312-392 contains an autoregulatory domain [177]. The C-terminal region of the β-subunits acts as a scaffolding protein which binds to α- and γ-subunits and is regulated by AMP [178, 179]. The N-terminal region of the β-subunits is myristoylated and has glycogen binding domains (GBD) that target binding of the complex to glycogen [178, 180, 181]. The GBD is found in enzymes that metabolize the α1→6 branches in α1→4-linked glucagans such as starch and glycogen.

The γ-subunits contain a variable N-terminal region followed by four tandem repeats of cystathionine β synthase (CBS) motifs. Each pair of CBS motifs forms a nucleotide-binding site called a Bateman domain. Each of these domains binds to one molecule of AMP or ATP in a competitive manner [182, 183]. AMPK is inhibited at high ATP concentrations by ATP binding to the γ-subunit [184]. Mutations in the N- or C-terminal domain of the γ-subunit lead to a hereditary human heart disease, i.e., Wolff-Parkinson-White (WPW) syndrome [182, 185]. The mutations are believed to decrease AMPK activation by AMP; however, there is evidence that some mutations also increase basal phosphorylation and activation of AMPK [186].

1.9.2 Regulation of AMPK

AMPK is activated both allosterically and by phosphorylation through upstream AMPK kinases (AMPKKs). AMPK is activated in response to any physiological (such as endurance exercise) or pathological (such as ischemia, oxidative stress) metabolic stress that leads to a rise in the AMP/ATP ratio [58, 158, 159, 161, 163]. AMP can activate AMPK in three distinct ways (Figure 1-13). First, AMP binding to the γ-subunit of AMPK causes allosteric activation of AMPK [169, 182, 187]. The magnitude of this action depends on AMPK isoforms but it is typically up to 5-fold [159]. Second, AMP binding to the γ-subunit of AMPK makes it a better substrate for the AMPKK [188]. AMPKK phosphorylates Thr-172 of the α-subunit activating the kinase 50- to 100-fold. However, recent studies question the idea that increased AMP concentration makes AMPK a better substrate for phosphorylation by AMPKK [189]. Third, AMP binding to the γ-subunit of AMPK inhibits dephosphorylation of AMPK by protein phosphatases [190]. All these activating effects of AMP on AMPK are antagonized by high ATP concentrations, confirming that AMPK essentially monitors changes in the cellular AMP/ATP. Even though AMPK is dephosphorylated and inactivated by both protein phosphatases 2A and 2C, inactivation by protein phosphatase 2C is more important in intact cells [191].

While the AMP/ATP ratio seems to be responsible for AMPK activation, AMPK can also be activated by a decrease in phosphocreatine/creatine (PCr/Cr) ratio in skeletal muscle [192] but not in the heart [193]. In addition to Thr-172 on AMPK, other phosphorylation sites such as Thr-258 and Ser 485/491 have been identified [194]. Even though these sites are not involved in AMPK activity, they prevent the AMPKK from phosphorylating AMPK at its primary activation site, Thr (172) [195, 196]. In fact, Hurely et al have suggested that these additional phosphorylation sites are controlled by the cAMP signaling pathway to attenuate AMPK activity [197]. In addition to classical mechanisms responsible for AMPK activation, several studies have shown that AMPK can also be activated by other stimuli independent of changes in the AMP/ATP ratio [198, 199]. For example, several studies have reported that leptin, hyperosmotic stresses, metformin, and long-chain fatty acid can activate AMPK in an AMP-independent manner [199-203]. The latter is very interesting and in fact, several studies have shown that perfusion of hearts in the presence of physiologic concentration of LCFA results in AMPK activation [201, 202]. This finding is very important and further supports the concept that AMPK has a role in substrate utilization by the heart.

1.9.3 Upstream Kinases in the AMPK Cascade

To date several AMPKKs have been identified: the mammalian kinase LKB1, which is dependent on elevation in the AMP/ATP ratio [194, 205]; the Ca^{2+}/calmodulin-dependent protein kinase kinases (CaMKK), which is dependent on changes in intracellular calcium [206-210]; and the transforming growth factor-β-activated kinase-1 (TAK1), a member of the mitogen-activated protein kinase kinase kinase family also known as MAPKK kinase-7 (MAP3K7) [211, 212]. The serine/threonine kinase LKB-1 shares significant sequence homology with *S. Cerevisiae* AMPKK [205, 213]. LKB-1 is ubiquitously expressed in all mammalian tissues including the heart [164, 214]. It was originally recognized as a gene mutated in human Peutz-Jegers syndrome [215, 216]. LKB-1, like AMPK, exists as a heterotrimer, with two other proteins: mouse protein 25 (MO25) and STE20-related adaptor protein (STRAD), all of which are

required for full kinase activity [205]. LKB-1 activity is not regulated by the stimuli that activate AMPK, and is not activated directly by AMP but rather appears to be constitutively active. Elevations in AMP levels cause AMPK to become phosphorylated by upstream kinases but also prevent its dephosphorylation by protein phosphatases [194]. Gq-coupled receptor (GqCR) analogues also activate AMPK *in vitro* and *in vivo* through Ca^{2+}/calmodulin-dependent protein kinase kinases (CaMKKα and CaMKKβ); this is a calcium dependent pathway independent of cellular energy status [158, 188, 206, 207, 209]. Increased cytosolic calcium concentration can trigger ATP-consuming pathways. Additionally, this calcium has to be pumped out of cytosol through ATP-pumps. Therefore, AMPK activation by CaMKKs could represent a response to the increased demand for ATP which accompanies calcium release [164]. In the heart, LKB-1 is highly expressed while CaMKK is less abundant [214, 217]. Interestingly, unlike LKB-1, CaMKKs expression is restricted. Both isoforms are abundant in neural tissues; however, CaMKKβ is also expressed in the heart and endothelial cells [164]. We have shown that the level of expression of CaMKKβ does not differ in hypertrophied as compared to non-hypertrophied hearts [35].

Several GqCR agents have been known to activate AMPK including bradykinin, epinephrine, and thrombin [218, 219]. We also found that arginine vasopressin (AVP), a neuropeptide hormone that also acts through a GqCR, activates AMPK. TAK-1, another AMPK activator, has been shown to act downstream of a variety of signaling molecules, including TGF-β1 [220]. TAK1 is widely expressed during mammalian development [221]. Zhang et al have shown that expression of constitutively active TAK1 in the heart induces cardiac hypertrophy [222]. However, Xie et al have shown that inhibition of TAK1 by a cardiac-specific dominant-negative mutation evoked electrophysiological and biochemical properties reminiscent of human WPW syndrome. WPW syndrome arises from mutations in AMPK, most notably, accelerated atrioventricular conduction and impaired AMPK activation [212]. This raises the possibility that the effects of TAK-1 inhibition could be attributed to decreased AMPK activation.

1.9.4 Function of AMPK

AMPK has a central role in the regulation of energy metabolism. In the heart, AMPK activation leads to activation of ATP-producing pathways and is therefore, accompanied by stimulation of fatty acid oxidation, glucose uptake, and glycolysis [56, 58, 68, 223]. In muscle tissue, AMPK regulates lipid and carbohydrate metabolism by controlling uptake and catabolism for energy generation [175, 224]. AMPK achieves these effects both by acute, direct phosphorylation of metabolic enzymes and proteins controlling key metabolic pathways and long-term stimulation of mitochondrial biogenesis and expression of oxidative metabolism enzymes and proteins involved especially in fatty acid metabolism [29, 36, 37, 158, 159, 225-231].

Fatty acid oxidation: In the heart, AMPK is able to mediate the uptake and utilization of fatty acids as shown in Figure 1-14. AMPK activation in the heart is accompanied by increases in both protein expression and plasma membrane translocation of CD36 and FABP [232, 233]. Given the importance of lipoprotein lipase (LPL) in providing hearts with fatty acids, An et al have shown that activation of AMPK augments synthesis and recruitment of lipoprotein lipase (LPL) to the coronary lumen [234]. AMPK activation, also, stimulates fatty acid oxidation by phosphorylation and inactivation of ACC [35, 68] and, possibly, by phosphorylating and activating malonyl-CoA decarboxylase (MCD), which degrades malonyl-CoA [57, 61, 235]. The latter does not appear to be a direct effect of AMPK since AMPK does not phosphorylate MCD directly [236]. Sambandam et al have recently demonstrated that AMPK might increase MCD association with mitochondria [237].

Glucose Utilization: AMPK activation in the heart mediates content of glucose uptake, glycolysis, and glycogen metabolism (Figure 1-14). AMPK stimulation of glucose uptake occurs independent of the insulin signaling pathway and, in fact, AMPK appears to control a different intracellular pool of glucose transporters from that of insulin [29, 238, 239]. AMPK activation increases both the expression and sarcolemmal translocation of GLUT-4, and, therefore; stimulates glucose uptake [199, 240, 241]. For

example, several studies have shown that AMPK activation stimulates glucose uptake in the heart during ischemia or oxidative stress [76, 239, 242], in muscles in response to 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAr) [239, 243], in skeletal muscle during increased contractile activity [243-245], and in pathological hypertrophied hearts [34, 35, 246]. AICAr is an adenosine analog and cell-permeable activator of AMPK [247]. Incubation of cells with AICAr leads to accumulation of the monophosphorylated derivative 5-aminoimidazole-4-carboxamide ribonucleoside (ZMP or AICAR) inside the cell. ZMP activates AMPK by mimicking all activating effects of AMP; thereby activating AMPK by direct allosteric activation and promoting the phosphorylation by AMPK kinases. The molecular mechanisms responsible for these effects of AMPK are proposed to be mediated through protein kinase C (PKC), endothelial nitric oxide synthase, and the p38 mitogen activated protein kinase/transforming growth factor β-activated protein kinase -1 binding protein (TAB1) complex [74, 242, 248]. In addition to stimulation of glucose uptake, AMPK activation causes stimulation of glycolysis by phosphorylation and activation of PFK-2 to produce the PFK-1 activator, F2,6BP [86].

Gene expression and Mitochondrial Biogenesis: Chronic AMPK activation mediates the transcription of genes involved in lipid and glucose metabolism [164]. For example, chronic muscle AMPK stimulation leads to upregulation of GLUT-4 and HKII expression, mitochondrial biogenesis in muscle, and downregulation of genes encoding enzymes involved in fatty acid synthesis and gluconeogenesis in liver [199, 226, 228, 230, 240, 249]. These effects of AMPK are believed to be mediated through activation of transcription factors and co-activators [225, 228]. Recently, Narkar et al [250] have shown that oral treatment of mice with AICAr results in increased activation of metabolic genes and enhancement of oxidative metabolism.

Ischemia/Reperfusion: AMPK has an essential role during myocardial ischemia. In hearts exposed to ischemia, AMPK activation is accompanied by an increase in AMP levels and stimulation of glucose uptake, glycolysis and fatty acid oxidation [68, 239,

241]. During reperfusion, AMPK activity remains high, accompanied by acceleration of fatty acid oxidation. These metabolic effects of AMPK can be both beneficial and detrimental during ischemia and during reperfusion following ischemia. Transgenic mice lacking AMPK activity have provided useful information regarding the role of AMPK during ischemia/reperfusion. In AMPKα2 deficient mice, hearts exposed to ischemia/reperfusion failed to increase glucose uptake [241, 251].

Protein Synthesis: Several studies have shown that AMPK activation inhibits protein synthesis by inhibition of the target of rapamycin (TOR) pathway that stimulates translational initiation, and by activation of elongation factor-2 kinase which inhibits the elongation step [252-255]. Another mechanism by which AMPK might inhibit protein synthesis is through glycogen synthase kinase-3β (GSK-3β). When GSK-3β is phosphorylated it becomes inactivated diminishing its inhibitory effect on the eukaryotic translation initiation factor 2B (eLF2B) and promoting protein synthesis [256-260]. To support this, several studies have shown that AMPK activation results in phosphorylation and inactivation of GSK-3β or decrease GSK-3β protein expression [261-263]. However, others have shown that AMPK activation might activate GSK-3β [419-421].

1.9.5 Pharmacological Manipulation of AMPK Activity

The pharmacological agent AICAr enters cells by adenosine transporters and is phosphorylated by adenosine kinase to AICAR or ZMP [247, 264-266]. ZMP, a normal intermediate in purine-nucleotide biosynthesis, accumulates in different cell types, including muscle and cardiac cells, and activates AMPK by mimicking AMP. AMPK activation by AICAr in both skeletal muscle and the heart is accompanied by fatty acid uptake [233, 267]. In muscle cells, AMPK activation by AICAr is accompanied by decreases in both ACC activity and malonyl-CoA levels; and thereby stimulation of fatty acid oxidation [268]. In addition to activating fatty acid oxidation, AICAr treatment of muscle leads to increase in glucose uptake and stimulation of glycolysis by increasing the translocation of GLUT-4 to plasma membrane and activation of PFK-2,

leading to increases fructose 2,6-bisphosphate production and activation of PFK-1 [86, 268-271]. However, ZMP is not fully specific for AMPK and can modulate other AMP-sensitive enzymes, such as glycogen phosphorylase, and fructose 1,6-bisphosphate, independent of any effects on AMPK [204, 265]. Additionally, AICAr can compete with adenosine for uptake by the nucleoside transporter leading to accumulation of adenosine in the extracellular environment and subsequent activation of adenosine receptors [264].

Metformin, an insulin-sensitizing biguanide widely used to treat type 2 diabetes mellitus, [272] has metabolic actions that are believed to be mediated by AMPK in intact cells and/or *in vivo* [199, 273-275]. Metformin exerts its action by increasing the cytosolic AMP concentration inside the cytosol possibly through inhibiting complex 1 of the respiratory chain, thereby activating AMPK [207, 275-277]. However, an adenine nucleotide-independent activation of AMPK by metformin has been proposed in other cell types including heart and liver [273]

Compound C, 6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrrazolo[1,5-a]-pyrimidine, an inhibitor of AMPK, was originally developed by Zhou et al [273]. It is a cell-permeable pyrrazolopyrimidine compound acting as a potent, reversible, selective, and ATP-competitive inhibitor of AMPK. It apparently has no effect on the activities of other protein kinases, which have been examined including PKCθ, PKA, or JAK3.

1.10 POTENTIAL ROLE OF AMPK IN HYPERTROPHIED HEARTS

Metabolic remodelling: Several studies of moderate forms of cardiac hypertrophy have shown that the activity and expression of AMPK is increased in association with an increase in glucose uptake and PFK-2 activity, and a decrease in energy status of the cardiac myocytes [34, 119]. We have shown that in mildly hypertrophied hearts the activity of AMPK is increased, accompanied by acceleration of glycolysis and a decrease in rates of fatty acid oxidation, but without any measurable changes in the

energy status of cells [35]. Additionally, in the same study we documented that, by increasing rates of fatty acid oxidation, both accelerated glycolysis and elevated AMPK activity were normalized to the same value seen in non-hypertrophied hearts, suggesting that AMPK has a role in the acceleration of glycolysis in these hearts [35]. AMPK activation in mild forms of cardiac hypertrophy might be a compensatory response to pressure-overload without any changes in energy status of cells possibly through Gq-coupled receptors; however, in moderate forms of cardiac hypertrophy, it might be a maladaptive response which is dependent on energy status of cells.

Structural remodelling: The exact role of AMPK in myocardial growth in response to pressure-overload is not fully understood [29]. Several studies have shown that AMPK activation results in prevention of cardiac hypertrophy by inhibiting protein synthesis [246, 278-283]. Additionally, Liao et al have shown that in mice with low AMPK activity, transverse aortic constriction leads to progressive cardiac remodeling [284]. However, several other studies have shown that transgenic mice lacking the AMPK α-subunits do not demonstrate increases in cardiac mass and hypertrophy indicating that AMPK does not restrict normal cardiac growth [214, 241, 251, 285-287]. Additionally, mutations in the human γ2-subunit encoded by PRKAG2 lead to the human cardiomyopathy WPW Syndrome, which is characterized by hypertrophy and abnormal glycogen accumulation and conduction system disease [29, 36, 186, 288-291]. Interestingly, mutations in the γ-subunit in mice models lead to cardiac hypertrophy; however, it is not obvious if these mutations cause activation or inhibition of AMPK [29, 36].

Taken together, AMPK seems to have a role in metabolic as well as structural remodelling in pressure-overload induced cardiac hypertrophy. However, the exact role of AMPK in these settings needs to be characterized. The following hypothesis and specific aims, which correspond to the experimental chapters of this thesis, have been designed to address some of the questions regarding the role of AMPK in metabolic and structural remodeling of hypertrophied hearts.

1.11 HYPOTHESIS

AMPK mediates hypertrophic remodeling in response to pressure-overload. Specifically, AMPK activation is a cellular signal responsible for accelerated rates of glycolysis in hypertrophied hearts. Additionally, AMPK influences myocardial structural remodeling and gene expression by limiting hypertrophic growth.

1.12 EXPERIMENTAL APPROACH

1.12.1 Effect of Metformin and AICAr on AMPK Activity

To investigate the role of AMPK in hypertrophic remodeling in the heart, pharmacological activators of AMPK, such as metformin and AICAr were administrated. The hypothesis that metformin and AICAr activate AMPK and alter metabolism in H9c2 cells, a line of cells derived from embryonic rat ventricular myocytes, was investigated. If proven correct, metformin and AICAr would be useful agents to use in characterizing the effects of AMPK activation. Energy metabolism as well as AMPK activity in response to AICAr and metformin treatment was measured. Also, the effects of inhibition of AMPK activity on normalization of the metabolic effects induced by metformin were investigated. The role of phosphoinositol-3-phosphate kinase (PI3K), MAP kinases, and PKC pathways in metabolic actions induced by metformin were characterized.

1.12.2 Cellular Model of Cardiac Hypertrophy

Here, it was determined if AMPK plays a role in metabolic remodeling in response to pressure overload-induced cardiac hypertrophy. Specifically, it was investigated if increased expression and activity of AMPK in hypertrophied hearts is responsible for the acceleration of glycolysis. A model of hypertrophy in which H9c2 cells were made hypertrophic by exposure to arginine vasopression (AVP) was developed. The activation state of AMPK in hypertrophied H9c2 cells was investigated and it was

determined if hypertrophied cells recapitulated the hypertrophic remodeling characteristic of the intact heart. Pharmacological agents (Compound C) as well as molecular approaches, employing adenoviral gene transduction, were then used in hypertrophied myocytes to specifically decrease AMPK activity and thereby further establish the role of AMPK in the control of glycolysis in the setting of myocyte hypertrophy.

1.12.3 Acute Effects of AVP on Glucose Utilization in H9c2 Cells

The acute effect of AVP on AMPK activity and glucose utilization in H9c2 cells was investigated. By inhibiting the AMPK activity, it was determined if the accelerated rates of glycolysis induced by acute treatment with AVP were dependent on AMPK. Then the mechanism by which AVP accelerated glucose utilization was determined. Specifically, the role of PI3K and/or calcium-dependent signaling pathways on AVP-induced acceleration of glycolysis was investigated.

1.12.4 Effect of Constitutively Active AMPK in H9c2 Cells

Here, the role of AMPK on structural remodeling in cardiac hypertrophy was investigated by using a constitutively active form of AMPK (CA-AMPK) expressed in H9c2 cells by means of adenoviral gene transduction. Specifically, it was determined if AMPK activation led to structural and metabolic remodeling in the setting of cardiac hypertrophy. Also, the role of eEF2 and GSK-3β signaling pathways in AMPK-induced protein synthesis in H9c2 cells was investigated.

1.13 Using H9c2 Cells to Investigate the Role of AMPK in Hypertrophic Remodeling

Among many different approaches, one popular approach to investigate the energy metabolism and signaling transduction pathway is the use of cultured cell models [292, 293, 347]. In the current study, H9c2 cells, a cell line derived from embryonic rat

ventricular myocytes, was used. The cultured cells have a number of advantages. First, they provide a homogeneous population of the cells so that the data collected using them will be more specific and accurate. Second, in cultured cells, cellular-molecular approaches can be performed easly. It should be recognized that, although H9c2 cells are derived from the rat heart and show some characteristics of adult cardiac myocytes, they cannot be considered to be identical to cardiac myocytes from adult or neonatal rats. H9c2 cells were used in the current investigation for several very practical reasons. Firstly, long-term culture of these cells is feasible whereas it is very difficult in primary cardiac myocytes, and this was important to establish new methodologies such as measurement of substrate use and adenoviral gene transfer. For the same reasons, the scope of investigation which could be undertaken to unravel potential mechanisms was wider. Second, H9c2 cells do resemble cardiac myocytes and although results must be extrapolated with caution, they can provide important and relevant insights.

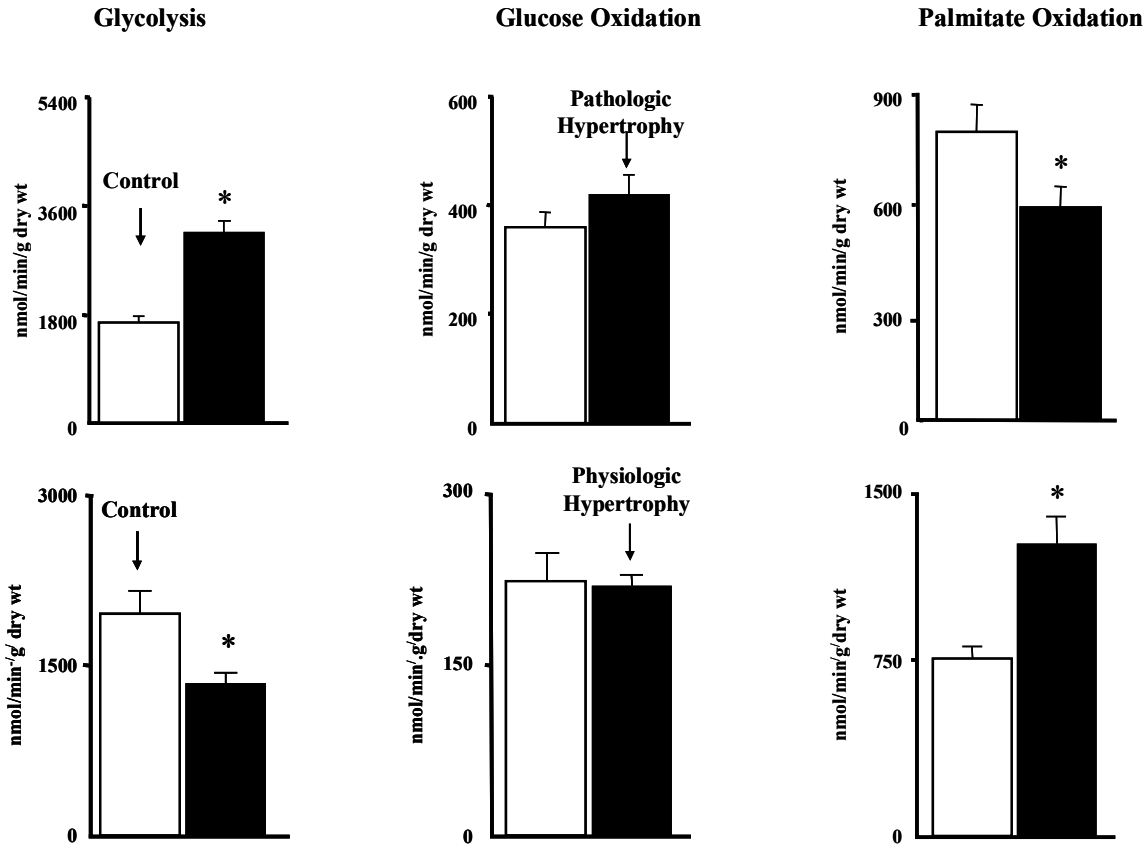

Figure 1-1: Substrate utilization profile in pathologic and physiologic cardiac hypertrophy.

Rates of glycolysis and oxidations of glucose and palmitate were measured in isolated working hearts from Sham-operated and abdominal aortic constricted rats (Top panel) and in isolated working hearts from sedentary Control and treadmill trained rats (Below panel). Values are Mean ± SEM. * vs. corresponding non-hypertrophied rat hearts; $p < 0.05$. N=8-14 per each group.

Allard et al, Am J Physiol 1994, 267(2 pt2):H742-750.

Burelle et al, Am J Physiol HeartCirc Phsiol 2004, 287(3):H1055-1063.

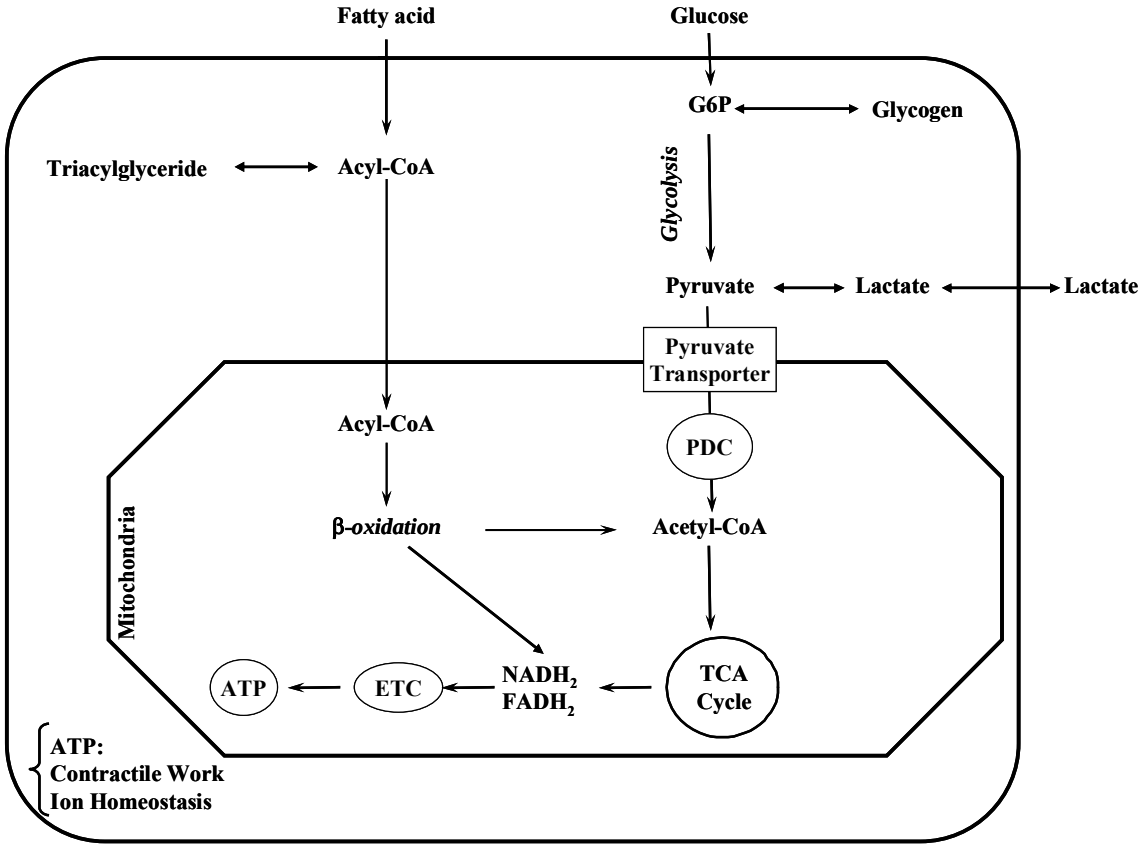

Figure 1-2: Overview of energy metabolism in the myocardium.

Heart utilizes a variety of substrates to meet its energy demand including exogenous as well as endogenous substrates. Glucose taken up is converted to G6P which can be converted to either glycogen or pyruvate. Pyruvate enters mitochondria to be oxidized. Fatty acids also enter mitochondria, undergo β-oxidation, and enter TCA cycle to generate reducing equivalents. These pass electrons to ETC which generates ATP.

Tricarboxylic acid (TCA) cycle, Electron transport chain (ETC), Adenosine triphosphate (ATP), Glucose-6-phosphate (G6P), Reduced nicotinamide adenine dinucleotide ($NADH_2$), Pyruvate dehydrogenase (PDC).

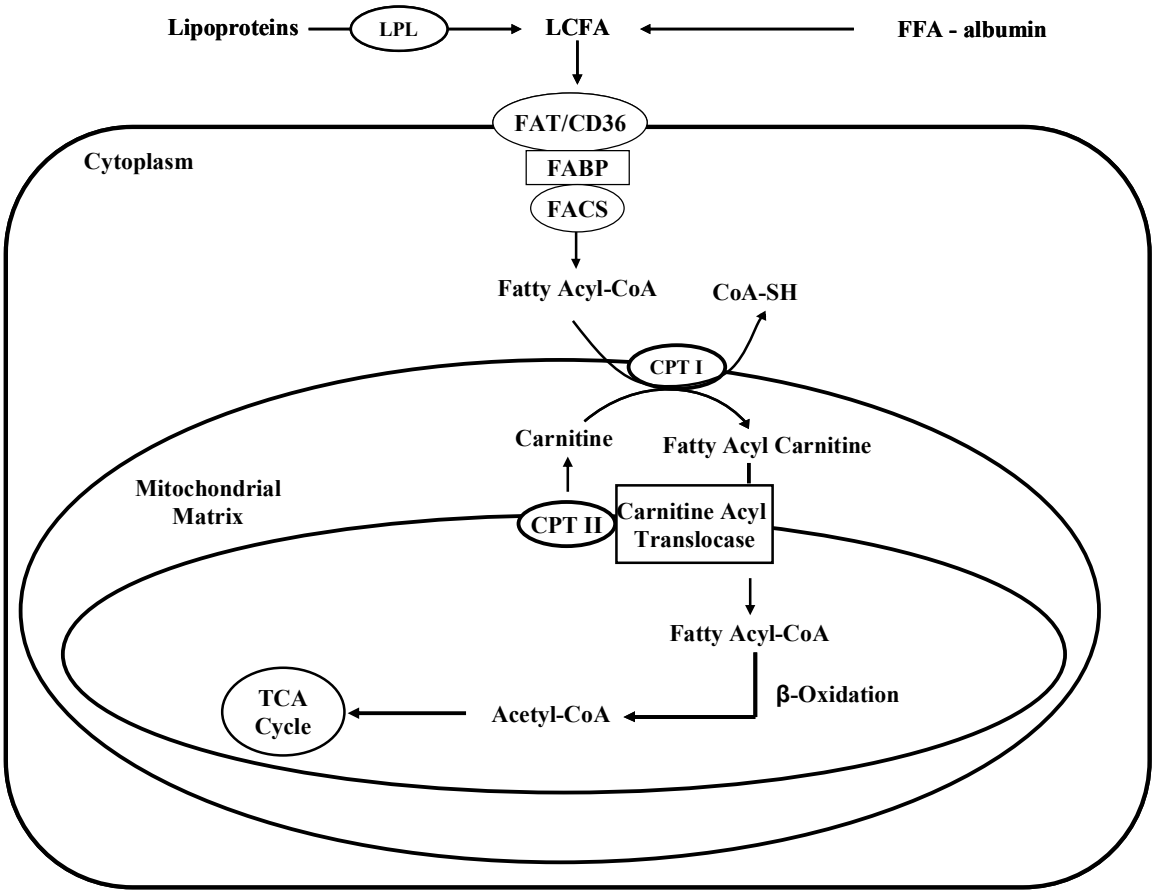

Figure 1-3: Myocardial fatty acid metabolism.

Fatty acids are taken up by CD36 and FABPs. Fatty acyl-CoA is taken up by the carnitine shuttle system to the mitochondria. The fatty acyl-CoA undergoes β-oxidation generating acetyl-CoA which further enters TCA cycle.

Lipoprotein lipase (LPL), Long-chain fatty acid (LCFA), Fatty acyl-CoA synthatase (FACS), Fatty acid translocase (FAT/CD36), Fatty acid binding proteins (FABP), Carnitine palmitoyl transferase 1 (CPT-1), Carnitine palmitoyl transferase 2 (CPT-2), Tricarboxylic acid (TCA); Adenosine monophosphate (AMP), Adenosine triphosphate (ATP).

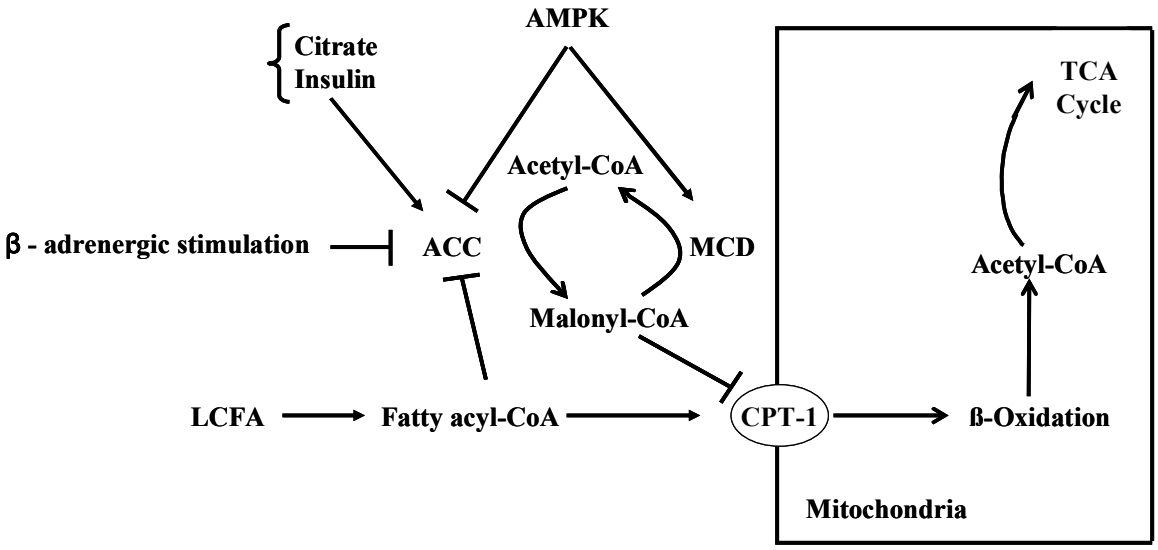

Figure 1-4: Regulation of myocardial fatty acid oxidation.

Malonyl-CoA is produced from cytosolic acetyl-CoA by ACC. Both AMPK and β-adrenergic stimulation phosphorylate and inhibit ACC leading to a decrease in malonyl-CoA level and therefore relieving inhibition of CPT-1. This results in stimulation of fatty acid oxidation.

Long-chain fatty acid (LCFA), Acetyl-CoA carboxylase (ACC), Malonyl-CoA decarboxylase (MCD), Carnitine palmitoyl transferase 1 (CPT-1), AMP-activated protein kinase (AMPK), Protein kinase A (PKA), Tricarboxylic acid (TCA) cycle.

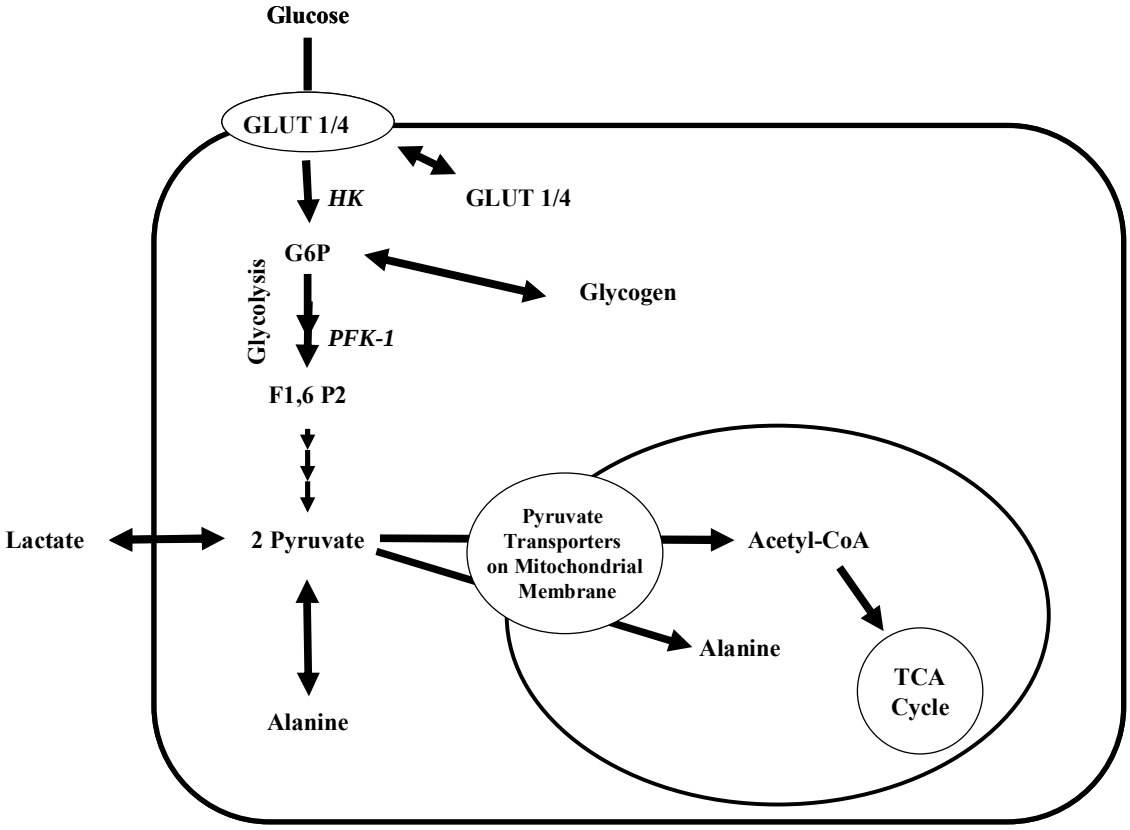

Figure 1-5: Myocardial glucose metabolism.

Glucose is taken up by GLUT1/4 and converted to G6P which then can enter the glycolytic pathway to produce pyruvate. PFK-1 is a key enzyme in glycolytic pathway. Pyruvate enters mitochondria through pyruvate transporters to be oxidized.

Glucose transporter (GLUT), Hexokinase (HK), Glucose-6-phosphate (G6P), Phosphofructokinase-1 (PFK-1), Fructose 1,6- bisphosphate (F1,6P2), Tricarboxylic acid (TCA) cycle.

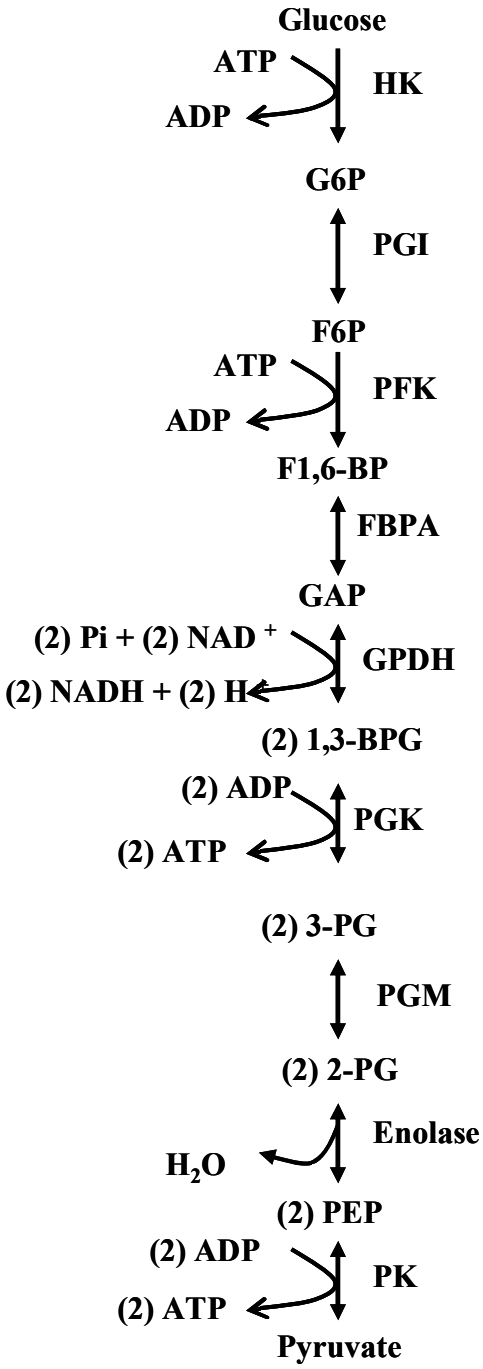

Figure 1-6: Glycolytic pathway.

Hexokinase (HK), Glucose-6-phosphate (G6P), Fructose-6-phosphate (F6P), Phosphofructokinase (PFK), Fructose 1,6- bisphosphate (F1,6P2), 1,3

Bisphosphoglycerate (1,3-BPG), 2-Phosphoglycerate (2-PG), 3-Phosphoglycerate (3-PG), Phosphoenolpyruvate (PEP), Glyceraldehyde-3-phosphate (GAP), Glycerol-3-phosphate dehydrogenase (GPDH), Phosphoglycerate kinase (PGK), Fructose-1,6-bisphosphate aldolases (FBPA), Phosphoglycerate mutase (PGM), Pyruvate kinase (PK), Tricarboxylic acid (TCA) cycle.

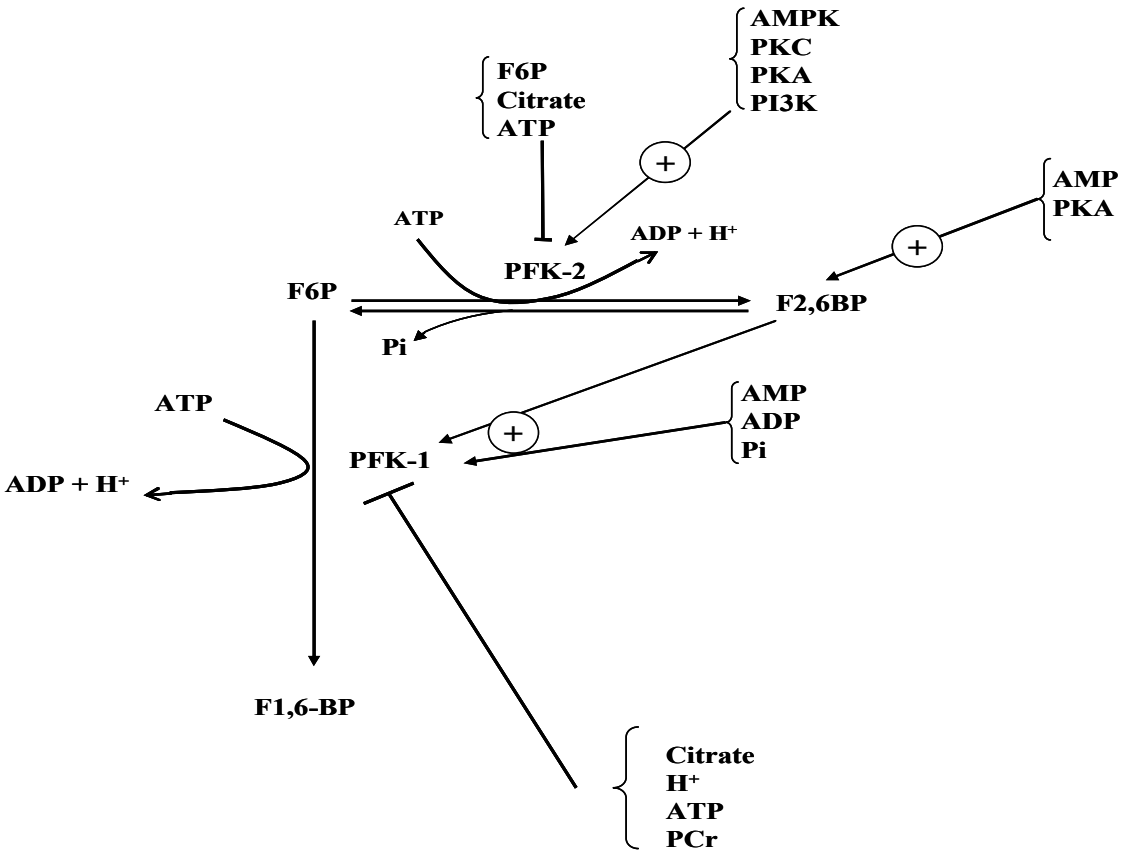

Figure 1-7: Regulation of phosphofructokinase-1.

PFK-1 is a key regulatory enzyme in the glycolytic pathway and catalyzes the first irreversible step. PFK-1 uses ATP to produce F1,6-BP and is activated by ADP, AMP, and Pi and is inhibited by ATP, citrate and by a fall in pH. F2,6BP is a potent stimulator of PFK-1 and is formed by PFK-2. PKC, PKA, PI3K, and AMPK can activate PFK-2 therefore stimulate glycolysis.

Fructose-6-phosphate (F6P), Phosphofructokinase-1 (PFK-1), Phosphofructokinase-2 (PFK-2), Fructose-1,6- bisphosphate (F1,6P2), AMP-activated protein kinase (AMPK), Protein kinase A (PKA), Adenosine monophosphate (AMP), Adenosine triphosphates (ATP), Adenosine diphosphates (ADP), Protein kinase C (PKC), Phosphoinositol-3-phosphate Kinase (PI3K), Phospho creatinine (PCr).

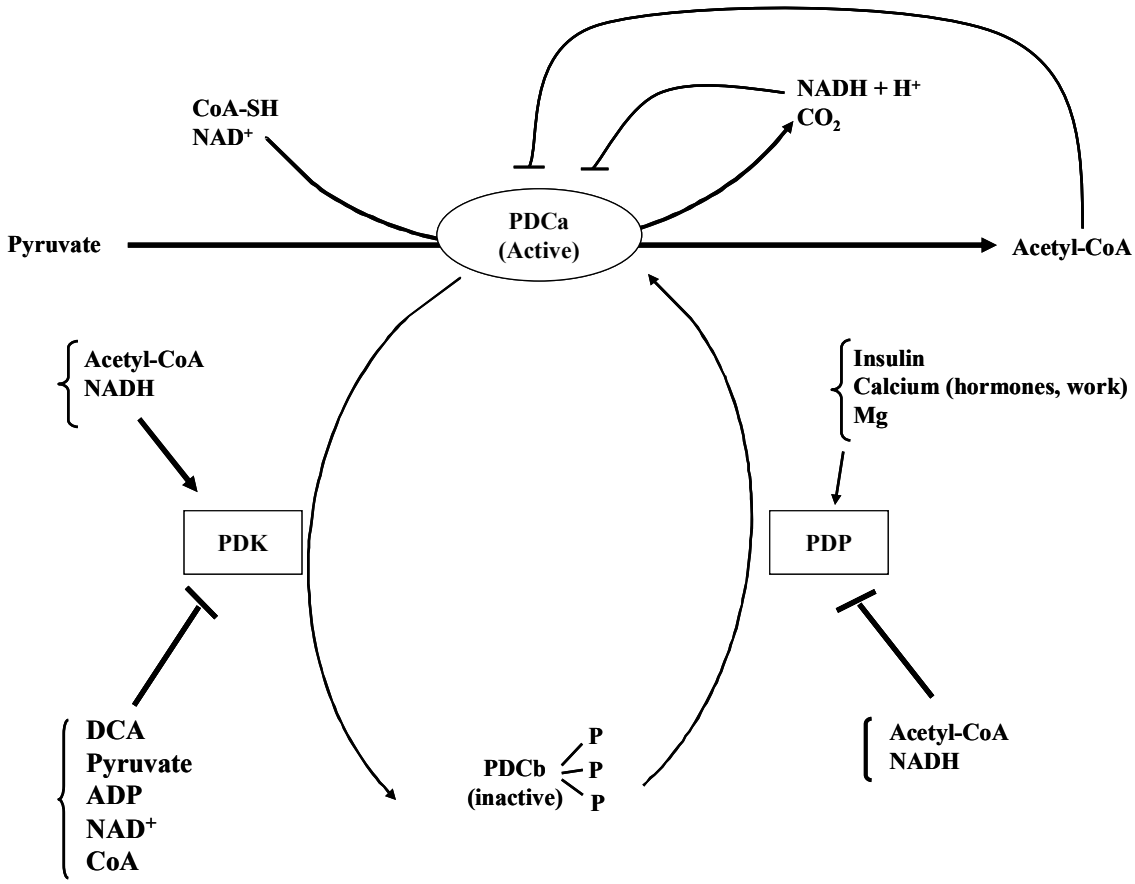

Figure 1-8: Pyruvate dehydrogenase complex (PDC) regulation.

The PDC oxidatively decarboxylates pyruvate to form acetyl-CoA. PDK phosphorylates and inhibits PDC activity; by contrast, PDP dephosphorylates and activates PDC. The activities of PDP and PDK are determined by intramitochondrial concentration of ions and metabolites.

Pyruvate dehydrogenase kinase (PDK), Pyruvate dehydrogenase phosphatase (PDP), Pyruvate dehydrogenase (PDC), Dichloroacetate (DCA), Oxidized nicotinamide adenine dinucleotide (NAD), Adenosine diphosphate (ADP), Coenzyme A (CoA), Reduced nicotinamide adenine dinucleotide (NADH2).

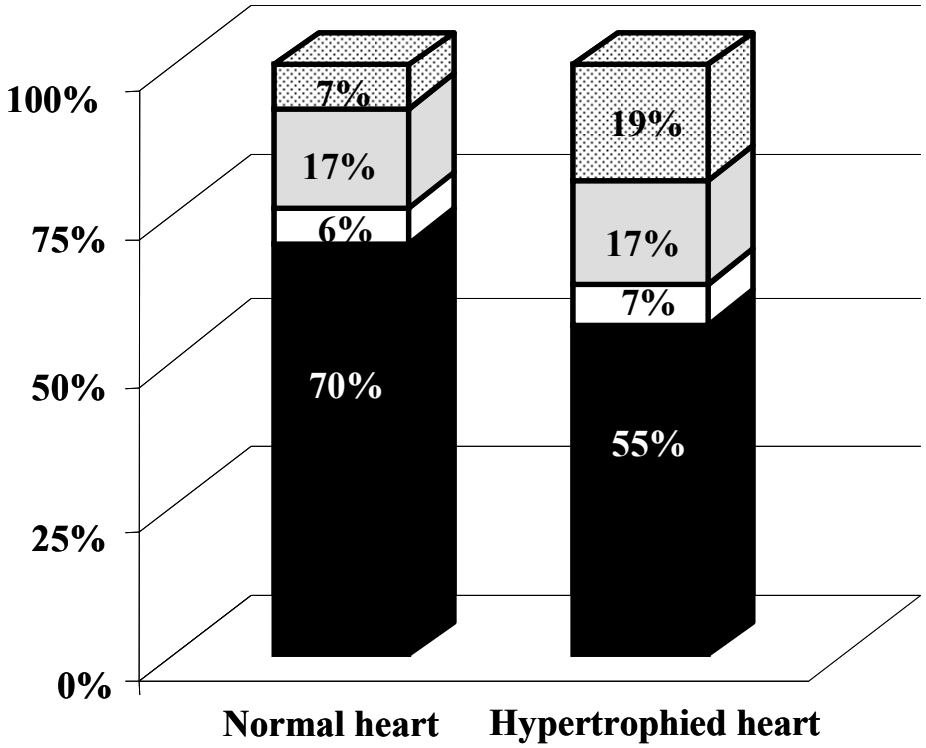

Figure 1-9: Myocardial energy production in hypertrophied and non-hypertrophied hearts when hearts utilizes exogenous substrate.

Isolated working hearts from sham-operated and abdominal aortic-constricted rats were perfused with Krebs-Henseleit solution containing 5.5 mM [5-^{3}H/U-^{14}C]-glucose, 1.2 mM [1-^{14}C]-palmitate, 0.5 mM [U-^{14}C]-lactate, and 100 mU/l insulin to measure glycolysis and glucose oxidation in one series and oxidation of palmitate and lactate in the second.

Black represents fatty acid oxidation, white represents lactate oxidation, grey represents glucose oxidation and stippled represents glycolysis.

Allard et al, Am J Physiol 1994, 267(2 pt2):H742-750.

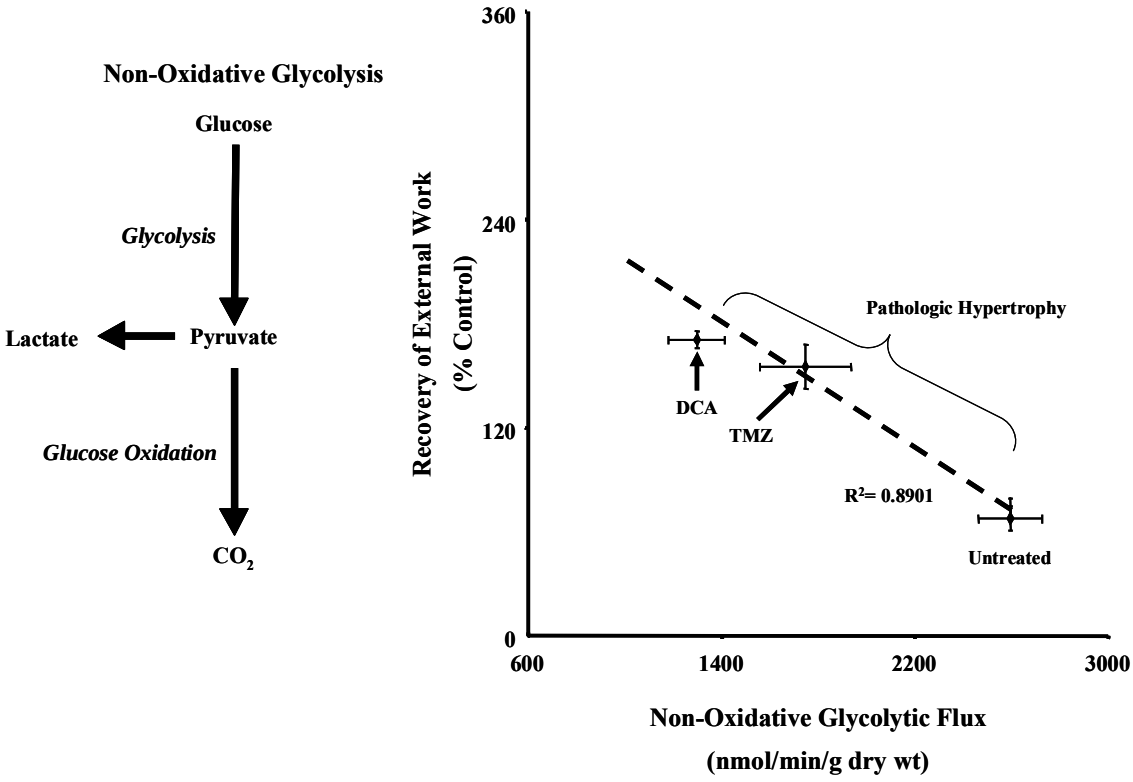

Figure 1-10: Non oxidative glycolysis (NOG).

This figure shows NOG and its relationship with functional recovery during reperfusion after ischemia in rodent model of pathologic cardiac hypertrophy. NOG is the proportion of glucose passing through glycolysis that is not oxidized. Reduction in NOG leads to improvement of post-ischemic contractile function and efficiency of hypertrophied hearts. Pharmacological agents that decrease glycolysis (Trimetazidine) and/or stimulate glucose oxidation (Dichloroacetate, Trimetazidine) lead to improvement of post-ischemic recovery of hypertrophied hearts by decreasing NOG.

Dichloroacetate (DCA), Trimetazidine (TMZ).

Lydell et al, Cardiovasc Res 2002, 53(4):841-51

Saeedi et al, J Pharmacol Exp Ther 2005, 314(1):446-54.

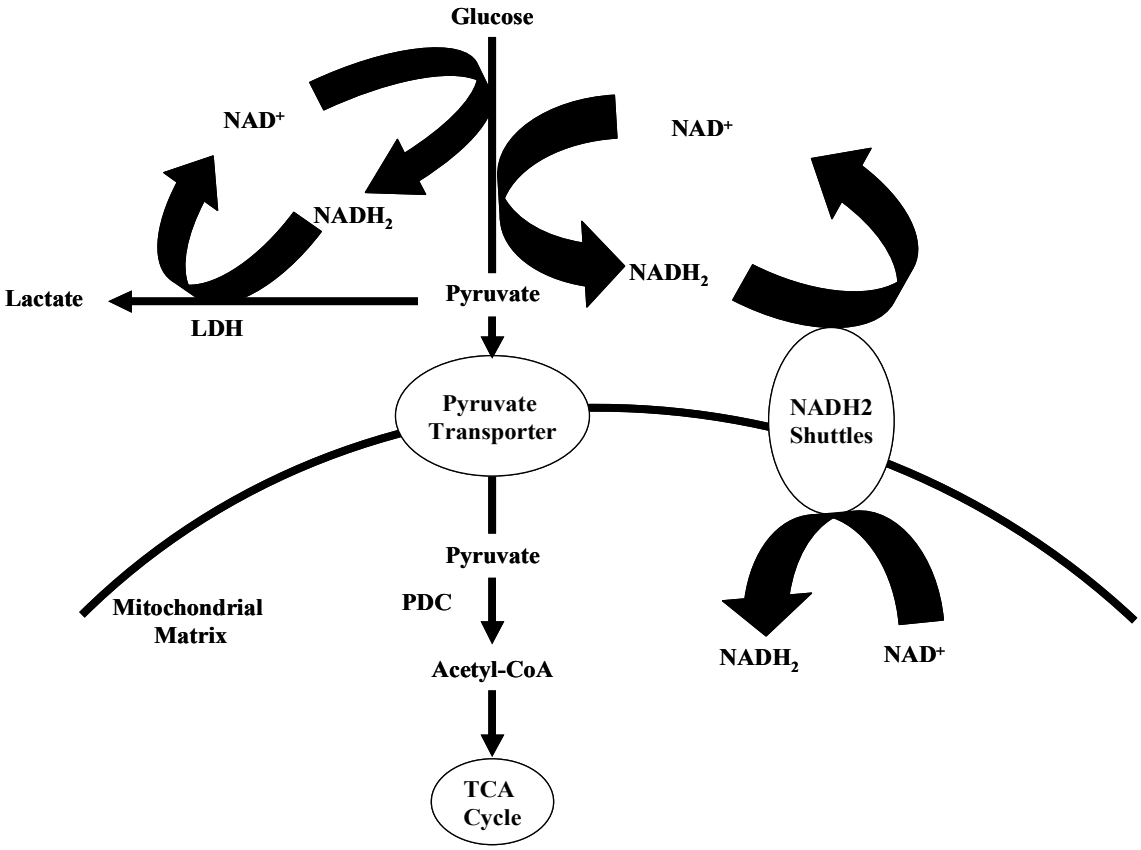

Figure 1-11: A possible mechanism for limitation in glucose oxidation in hypertrophied hearts.

In order to be oxidized, pyruvate needs an equivalent oxidation of cytosolic $NADH_2$ in the mitochondria. $NADH_2$ enters into cardiac mitochondria mainly by the $NADH_2$ shuttles. If this shuttle is restricted, this leads to an increase in lactate production and a decrease in glucose oxidation.

Oxidized nicotinamide adenine dinucleotide (NAD), Reduced nicotinamide adenine dinucleotide ($NADH_2$), Lactate dehydrogenase (LDH), Tricarboxylic acid (TCA) cycle, Pyruvate dehydrogenase complex (PDC).

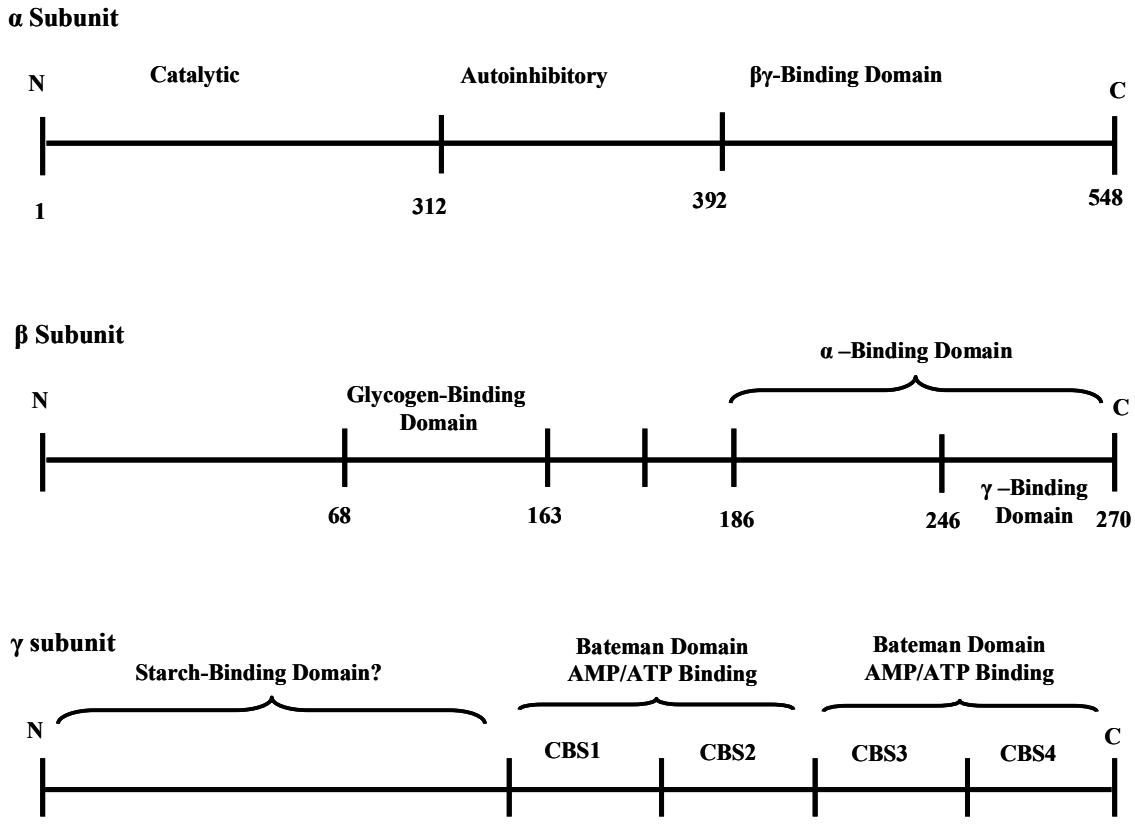

Figure 1-12: Structure of AMPK.

AMPK contains one catalytic subunit (α subunit) and two regulatory subunits (β and γ).

The N-terminus of the α-subunits contains a kinase domain and the C-terminal domain is responsible for binding to the β- and γ-subunits. Each bateman domain of γ-subunit binds to one molecule of AMP or ATP.

Glycogen binding domains (GBD), Cystathionine β synthase (CBS), Adenosine monophosphate (AMP), Adenosine triphosphate (ATP).

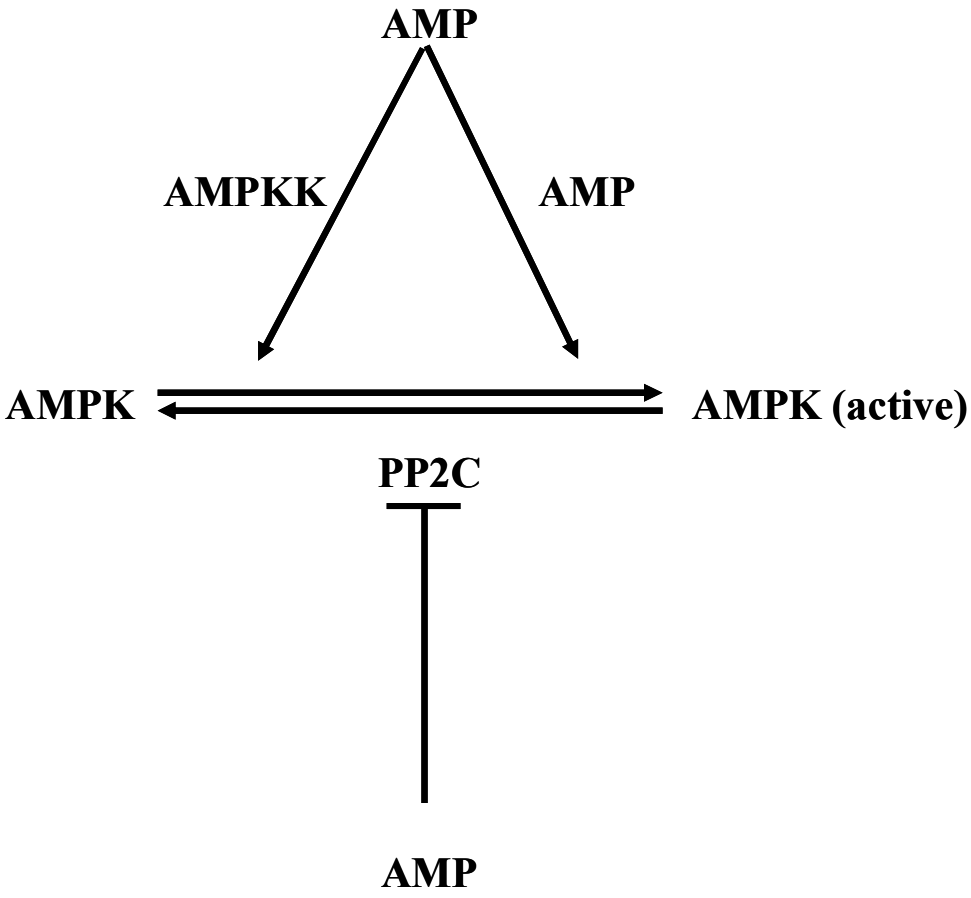

Figure 1-13: AMPK activation by AMP.

AMP binding to AMPK makes AMPK a better substrate for the upstream kinase, AMPKK.

Second, AMP binding to AMPK inhibits dephosphorylation of AMPK by protein phosphatases. Third, AMP binding to AMPK results in allosteric activation of AMPK.

Adenosine monophosphate (AMP), AMP-activated protein kinase (AMPK), AMP kinase kinase (AMPKK), Protein phosphatase-2C (PP2C).

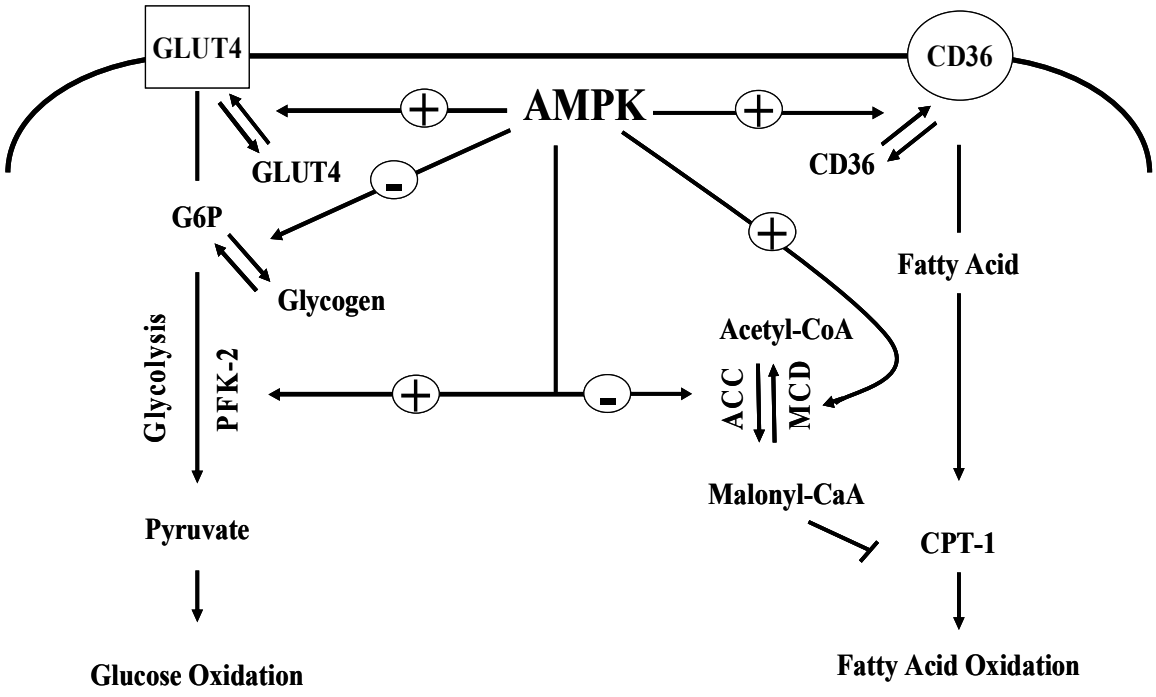

Figure 1-14: Effects of AMPK on metabolism of fatty acid and glucose.

AMPK can stimulate glucose utilization by stimulating glucose uptake and by activation of PFK-2. Also, it stimulates fatty acid oxidation by increasing uptake of fatty acids and by phosphorylation and inhibition of ACC activity.

AMP-activated protein kinase (AMPK), Fatty acid translocase (FAT/CD36), Acetyl-CoA caarboxylase (ACC), Malonyl-CoA decarboxylase (MCD), Carnitine palmitoyl transferase 1 (CPT-1), Glucose transporters (GLUT), Glucose-6-phosphate (G6P), Phosphofructokinase-2 (PFK-2).

CHAPTER 2 - MATERIALS AND METHODS

2.1 CELL CULTURE

H9c2 (2-1) cells (passage number 12), CRL-1446, lot number 2056335, embryonic rat ventricular myocytes, were obtained from American Type Culture Collection (ATCC) (Manassas, VA). The cell stocks were designated passage 1 and cultured. Aliquots of cultured cells from different passages were set aside and kept frozen in liquid nitrogen. In all experiments, H9c2 cell cultures were maintained through a maximum of 12 passages. Cells were cultured in a water-jacketed cell culture incubator (Model 3326, Forma Scientific, Marietta, Ohio) under standard culture conditions (at 37 ºC in a humified atmosphere of 95 % O_2 / 5 % CO_2). All culture material used, such as 6 well plates, 25 cm^2 and 75 cm^2 flasks, and 60 mm dishes were purchased from Sarstedt (Quebec, Canada). The medium used in cell cultures was based on Dulbecco's Modified Eagle's Medium (DMEM) from Invitrogen (Carlsbad, California) containing 5.5 mM (1000 mg/l) D-glucose, 4 mM L-glutamine, 0.02 mM pyridoxine hydrochloride (4 mg/l), and 1 mM sodium pyruvate (110 mg/l). 25 mM Sodium bicarbonate (2.2 g/l; Sigma, St.Louis, Missouri) was added to the DMEM for pH balancing as indicated by the supplier. When indicated, DMEM/F12, a 1:1 mixture of DMEM and Ham's F-12 nutrient mixture was used. It contains 17.51 mM (3.151 g/l) D-glucose, L-glutamine, and essential amino acids and vitamins as well as sodium bicarbonate at a final

concentration of 50 mM (2.4 g/l) to balance the pH. All media were supplemented with antibiotics, including penicillin (100 IU/ml) and streptomycin (100 IU/ml) (Invitrogen, Carlsbad, California). Phosphate-buffered saline (PBS) was purchased from Invitrogen, Carlsbad, California. In selected experiments, Krebs-Henseleit (KH) solution containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 0.12 mM $MgSO_47H_2O$, 2.5 mM $CaCl_22H_2O$, 0.025 mM $NaHCO_3$ was used. Arginine vasopressin (AVP), all-trans retinoic acid, 2-aminoethoxydiphenyl borate (2-APB), O,O'-bis(2-aminoethyl)ethyleneglycol-N,N,N',N'-tetraacetic acid (EGTA), SP600125, SB203580, PD98059, Wortmannin, and protease inhibitor cocktail containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), E-64, bestatin, leupeptin, aprotinin, and sodium EDTA were purchased from Sigma (St.Louis, Missouri). Compound C, (6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyyrazolo [1,5-a] pyrimidine), was obtained from Merck (San Diego, California). LY-294002, [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one], was obtained from Cell Signaling Technology Inc. (Danvers, Massachusetts).

2.1.1 H9c2 Cell Culture

Stock H9c2 cells were cultured in 75 cm^2 culture flasks and were grown in the DMEM supplemented with 10 % fetal bovine serum (FBS) and 0.1 mM L-Carnitine (Sigma, St. Louis, Missouri), as previously described [292, 293]. At 70-80 % confluency, H9c2 cultures were split in ratio of 1: 2 to 1: 4 using a PBS wash and trypsin to detach the cells. For experiments, cells were plated into 25 cm^2 culture flasks, 60 mm culture dishes or 6 well plates with a density of 4 x 10^4 cells /cm^2. Cultured cells were grown in 10 % FBS DMEM supplemented with 0.1 mM L-Carnitine until they reached 70-80 % confluency and before fusion into myotubes occured. At this stage, differentiation toward a cardiac phenotype was induced by culturing cells in DMEM containing 1 % horse serum (HS) (Invitrogen, Carlsbad, California) and 100 nM all-trans-retinoic acid (Sigma, St. Louis, Missouri) for four days [292, 293].

In the absence of retinoic acid, H9c2 cells will undergo myogenic transdifferentiation to acquire a skeletal muscle phenotype [292, 293]. However, chronic treatment of these cells with low concentrations of retinoic acid has been shown to favour development of a cardiac muscle phenotype [292, 293]. Retinoic acid was prepared in the dark in dimethyl sulphoxide (DMSO) and aliquots were stored at –20 ºC until use. The concentration of DMSO in the culture medium was less than 0.2 %. Medium was changed every day.

2.2 RETINOIC ACID AND CARDIAC PHENOTYPE OF H9C2 CELLS

Exposure of H9c2 cells to medium containing retinoic acid for four days resulted in increased expression of the cardiac isoform of troponin-T as compared to untreated cells (Figure 2-1). Such findings are in keeping with the view that the culture conditions used in these experiments favour development of a cardiac-like phenotype in H9c2 cells [292, 293].

2.3 TREATMENT OF H9C2 CELLS WITH METFORMIN OR AICAr

For metformin/AICAr experiments, differentiated H9c2 cells were treated with or without metformin (2 mM), or 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAr, 1.2 mM) in KH solution containing 0.4 mM [U-^{14}C]-palmitate pre-bound to 3 % bovine serum albumin (BSA), 5.5 mM glucose, and 10^{-7} M insulin for 8 hours. It had previously been found that 8 hours was required for metformin to achieve maximal metabolic effects (data not shown), in keeping with the view that metformin is poorly taken up by cells and prolonged treatment is needed for maximal effects [294]. In the above medium, tracer amounts of labeled [5-^{3}H]-glucose (1.0 μCi/ml), and [U-^{14}C]-palmitate (0.4 μCi/ml) were added during the last hour of study to measure glycolysis and fatty acid oxidation, respectively.

The concentration of AICAr used in the current study (1.2 mM) is based on the previous studies of AMPK activation in the heart [204]. Higher concentrations of AICAr were not used because we have previously shown that it adversely affected heart function. In selected experiments, AMPK activity was reduced by either treating cells with Compound C or adenovirus over expressing dominant negative form of AMPK (DN-AMPK) by means of adenoviral transduction.

Differentiated H9c2 cells were treated with Compound C (40 μM) in KH solution for 30 minutes before addition of 2 mM metformin [273]. For viral mediated transducing of DN-AMPK, differentiated H9c2 cells were transduced with adenoviruses containing either green fluorescent protein (GFP) or DN-AMPK at a multiplicity of infection (moi) of 150 virus particles per cell and left in culture for 24 hours prior to addition of metformin.

2.3.1 Investigation of the Role of MAP Kinases, PKC, and PI3-Kinase Pathways on Metabolic Actions of Metformin

Modulation of several signaling pathways, including mitogen-activated protein kinase (MAPK), protein kinase C (PKC) and phosphoinositol-3-phosphate kinase (PI3K)-PKB pathways, have been implicated in the cellular effects of metformin [295-298]. As such, we evaluated the role of these pathways in mediating the metabolic response of H9c2 cells to metformin using pharmacological inhibitors. Differentiated H9c2 cells were pretreated with 10 μM SB203580, a reportedly selective p38 MAPK inhibitor, 10 μM SP600125 and 50 μM PD98059, specific inhibitors of JNK and ERK pathways, respectively, 10 nM calphostin C, a potent and specific inhibitor of conventional and novel isoforms of PKC [299, 300], or 10 μM LY-294002, a selective PI3K inhibitor [301], before addition of metformin. Studies were conducted in KH solution supplemented with fatty acid, glucose, and insulin as above.

The concentrations of MAP kinase inhibitors as well as PKC inhibitor used in this study did not influence basal glucose utilization by the cells. However, higher concentrations affected basal glucose utilization.

2.4 CHRONIC TREATMENT OF H9C2 CELLS WITH AVP

Hypertrophy of H9c2 cells was induced by exposure of differentiated H9c2 cells to DMEM/F12 containing 0.5 % horse serum with or without 1μM arginine vasopressin (AVP) for 48 hours, as previously described [292]. After 48 hours, AVP-containing medium was replaced with serum-free DMEM. A trace amount of [5-^{3}H]-glucose (1.0 μCi/ml) was added to the medium 7 hours later to determine rates of glycolysis over a subsequent 1 hour period. In order to more closely reproduce the *in vivo* milieu in terms of energy substrates and hormones, additional studies were performed as above in either DMEM supplemented with 0.25 mM oleic acid pre-bound to 3 % bovine serum albumin, 5.5 mM glucose, 10^{-7} M insulin or in Krebs-Henseleit (KH) solution containing 0.4 mM palmitate pre-bound to 3 % bovine serum albumin, 5.5 mM glucose, and 10^{-7} M insulin for 8 hours. In the latter medium, glycolysis and oxidations of glucose and fatty acid in hypertrophied H9c2 cells were studied 8 hours after withdrawal of the AVP. Tracer amount of labeled [5-^{3}H]-glucose (1.0 μCi/ml), [U-^{14}C]-glucose (1.0 μCi/ml), and [U-^{14}C]-palmitate (0.4 μCi/ml) were added during the last hour of study to measure glycolysis, glucose oxidation, and fatty acid oxidation, respectively.

Regardless of the medium used, glycolytic rates in hypertrophied H9c2 cells were measured over a period of 2-3 days after withdrawal of the AVP in order to determine how long the metabolic alterations persisted after removal of AVP. In these studies, AVP containing medium was removed and cells were cultured in DMEM/F12 without AVP for up to 2-3 days. Every 24 hours a subset of H9c2 cells were treated as above with removal of DMEM/F12 and exposure to KH solution or DMEM for 8 hours.

Tracer amounts of labeled [5-^{3}H]-glucose (1.0 μCi/ml) were added during the last hour of study to measure rates of glycolysis.

To suppress AMPK activity, hypertrophied and non-hypertrophied cells were treated with 40 μM Compound C for 8 hours in KH solution containing fatty acid, glucose, and insulin as above. As an alternative to pharmacological inhibition by Compound C, experiments were carried out with DN-AMPK [321, 322]. For these experiments, hypertrophied and non-hypertrophied H9c2 cells were transduced with adenovirus containing GFP (moi 150) or DN-AMPK plus GFP (moi 150) by incubation in DMEM containing 1 % HS for 24 hours after removal of AVP. This medium was replaced with KH solution containing 0.4 mM palmitate pre-bound to 3 % bovine serum albumin, 5.5 mM glucose, and 10^{-7} M insulin and rates of glycolysis were measured by addition of tracer amounts of [5-^{3}H]-glucose 7 hours later, as described above.

2.5 ACUTE TREATMENT OF H9C2 CELLS WITH AVP

To investigate the acute metabolic actions of AVP, differentiated H9c2 cells were treated with or without AVP (1μM) for 2 hours and rates of glycolysis were measured in KH solution containing 0.4 mM palmitate pre-bound to 3 % fatty acid free BSA, [5-^{3}H] 5.5mM glucose (1.0 μCi/ml) and 10^{-7} M insulin. In selected experiments, cells were incubated for two hours in serum-free DMEM containing [5-^{3}H] 5.5 mM (1.0 μCi/ml) glucose with or without 1 μM AVP. The lowest concentration of AVP capable of stimulating glycolysis was assessed by exposing H9c2 cells to a range of AVP concentrations, including concentrations observed in the setting of heart failure in humans and in rodents [302-304].

The potential role of AMPK in the acute metabolic actions of AVP was assessed by using pharmacological and molecular means to inhibit AMPK in H9c2 cells. Specifically, H9c2 cells were exposed to 40 μM Compound C for 30 minutes prior to the switch to KH solution containing fatty acid, glucose and insulin with or without 1

μM AVP. For inhibition by DN-AMPK, cells were exposed to GFP (moi 150) or DN-AMPK (moi 150) for 24 hours prior to acute exposure to AVP.

2.5.1 Determination of the Role of Calcium in the Acute Metabolic Actions of AVP

Binding of AVP to the V1a receptor, the receptor subtype shown to be involved with the hypertrophic response to AVP [305], leads to activation of phospholipase C, production of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) and the mobilization of intracellular calcium and influx of extracellular calcium (Figure 2-2) [292, 306]. Due to the unavailability of calcium free DMEM commercially, DMEM/F12, which has a glucose concentration of 17.5 mM, was used as a substitute to investigate the effects of different signaling pathways on glucose utilization induced by AVP. The role of calcium in mediating the metabolic response of H9c2 cells to AVP was evaluated by selectively modulating intracellular calcium mobilization and extracellular calcium influx.

In one series of experiments, H9c2 cells were pretreated for 30 min with 25 μM dantrolene, an inhibitor of Ca^{2+} release from sarcoplasmic reticulum (SR) via the ryanodine receptor [307]. To evaluate the involvement of the IP3 receptor, whose activation also leads to calcium release from the SR, cells were pretreated for 5 minutes with 100 μM 2-amino-ethoxydiphenyl borate (2-APB), a non-competitive IP3 receptor antagonist [308]. The role of extracellular Ca^{2+} was assessed by preincubating H9c2 cells with 3 mM EGTA (to chelate extracellular calcium) for 5 minutes before a challenge with AVP.

The concentrations of all above inhibitors used in the study did not influence basal glucose utilization by the cells. However, higher concentrations affected basal glucose utilization.

2.5.2 Investigating the Role of Phosphoinositol-3-Phosphate Kinase (PI3K) in the Acute Metabolic Action of AVP

Considering the fact that AVP stimulates PI3K pathway (Figure 2-2) [310] and that PI3K is known to accelerate glucose use [298, 311], a pharmacological approach was used to assess the role of PI3K in the observed AVP- induced acceleration of glucose utilization. H9c2 cells were studied with and without 30 minutes of pretreatment with 300 nM wortmannin, a well known inhibitor of PI3K [312], before addition of AVP.

2.6 TREATMENT OF H9C2 CELLS WITH CA-AMPK

To assess the role of AMPK in structural remodeling, differentiated H9c2 cells were transduced with an adenovirus containing constitutively active form of AMPK (CA-AMPK) (moi 25 and 150) or GFP (moi 150) by incubation in DMEM containing 1 % HS for 48 hours. The recombinant CA-AMPK adenovirus vectors contain a reporter gene of green fluorescent protein (GFP), which serves as a marker of successful viral transduction and protein over expression. Additionally, the recombinant CA-AMPK protein contains a short sequence derived from c-myc, which could be detected by means of a monoclonal anti-c-myc antibody. After 48 hours, the DMEM was replaced with Krebs-Henseleit solution containing 0.4 mM palmitate pre-bound to 3 % bovine serum albumin, 5.5 mM glucose, and 10^{-7} M insulin for 8 hours. In this medium, glycolysis and oxidation of glucose and fatty acids were studied over 8 hours. Tracer amount of labeled [5-^{3}H]-glucose (1.0 μCi/ml), [U-^{14}C]-glucose (1.0 μCi/ml), and [U-^{14}C]-palmitate (0.4 μCi/ml) were added during the last hour of study to measure glycolysis, glucose oxidation, and fatty acid oxidation, respectively.

2.7 ANIMALS

All the animal-related procedures were conducted by experienced technicians in our laboratory.

Male Sprague-Dawley rats weighing 350-450 g were housed in a temperature-controlled (22 ± 1ºC) and light-controlled (12:12-hours light-dark cycle) room. Rats had free access to food and water. Animal care and experimentation were approved by the institutional animal care committee and were in accordance with guidelines set forth by the Canadian Council on Animal Care and with the American Physiological Society's "Guiding Principles for Research Involving Animals and Human Beings".

2.7.1 Isolated Heart Preparation and Perfusion Protocol

Hearts from halothane (3-4 %)-anesthetized rats were isolated and perfused in working preparations with Krebs-Henseleit (KH) solution under normoxic non-ischemic conditions at a left atrial preload of 11.5 mmHg and an aortic afterload of 80 mmHg, as described [20]. The KH solution contained 1.2 mM [1-^{14}C]-palmitate prebound to fatty acid-free albumin (3 %), 5.5 mM [5-^{3}H]-glucose, 0.5 mM lactate, and 100 mU/l insulin. A higher concentration of insulin was used to ensure that insulin-dependent glucose uptake was not limiting, while a high concentration of palmitate was used to overcome possible inhibitory effects of insulin on AMPK and to prevent the net loss of endogenous substrates [39, 202, 274, 313]. The KH solution was oxygenated with 95 % O_2-5 % CO_2 and maintained at 37 ºC. Hearts were exposed to metformin (2 mM) or vehicle (distilled H_2O, dH_2O) throughout the perfusion period. A pressure transducer (Viggo-Spectramed, Oxnard, CA) inserted in the afterload line was used to measure heart rate and peak systolic pressure. Cardiac output and aortic flow were measured via external flow probes (Transonic Systems, Ithaca, NY) on the left atrial preload and aortic afterload lines, respectively. Rate-pressure product, the product of heart rate and peak systolic pressure, and hydraulic work, the product of cardiac output and peak systolic pressure, were used to measure external work performed by the heart. Hearts were perfused for either 15 or 30 minutes at which time they were rapidly frozen in liquid nitrogen for biochemical analysis or determination of wet-dry tissue weight ratio.

2.8 EXOGENOUS SUBSTRATE METABOLISM

2.8.1 Cellular Glycolysis

Glycolytic rates were determined by measuring the rate of production of ^3H_2O from [5-^{3}H]-glucose (1.0 μCi/ml) included in the experimental medium [20, 314, 315]. Tritiated glucose that enters the glycolytic pathway is completely detririated at the enolase and triosephosphate isomerase steps to produce ^3H_2O. ^3H_2O was separated from [^{3}H] glucose by use of screening columns (Fisher Scientific) containing Dowex 1-X4 anion exchange resin (200-400 mesh) suspended in potassium tetraborate. Potassium tetraborate (0.4 mM; 122.196 g/l ddH_2O) was dissolved in ddH_2O before addition of 90 g/l Dowex anion exchanger and was slowly stirred overnight in room temperature. On the day of the experiment, 2 ml of the Borate/Dowex mixture was added to each column and extensively rinsed with ddH_2O (3 times). After double distilled H_2O (ddH_2O) was drained thoroughly, the columns were placed over 7 ml-scintillation vials. The cell medium (200 μl) were added slowly in duplicates to each column followed by 2 aliquots of 400 μl ddH_2O for washing purpose. Following elution of $3H_2O$ into the scintillation vial, Ecolite scintillant (4 ml) (ICN, Costa Mesa, CA) was added to each vial. Vials were vortexed and left overnight in room temperature before subjection to isotope counting procedure. The specific activity was calculated by measuring the radioactivity of [^{3}H]-labeled experimental medium. Glycolytic rates were expressed as nanomoles of glucose catabolized per minute per milligram protein.

2.8.2 Cellular Glucose Uptake

2-Deoxyglucose uptake was measured in H9c2 cells as previously described [307]. To measure glucose uptake, trace amounts of 2-deoxy-D-[1-^{14}C] glucose (0.2 μCi/ml) were added to the experimental medium and incubated for 1 hour with the cells. Upon the removal of labeled medium, the cells were rinsed twice with ice-cold PBS and then dissolved in 0.1 M potassium hydroxide (KOH) (500 μl). Cell lysates were collected

and transferred to eppendorf tubes. Aliquots of dissolved cell lysate (in duplicate) were transferred into scintillation vials containing Ecolite scintillant (4 ml). Vials were vortexed and left overnight at room temperature before subjection to isotope counting procedure. The specific activity was calculated by measuring the radioactivity of [1-^{14}C]-labeled experimental medium. Glucose uptake was expressed as nanomoles glucose catabolized per minute per milligram protein.

2.8.3 Cellular Fatty Acid and Glucose Oxidation

Rates of glucose and fatty acid oxidation were measured by treating cells with [U-^{14}C]-glucose and [U-^{14}C]-palmitate, respectively, as previously described [20, 315, 316]. Rates of glucose and fatty acid oxidation were measured by quantitative collection of $^{14}CO_2$ released as a gas and dissolved in the cell medium as [^{14}C]-bicarbonate. H9c2 cells cultured in T25 flasks were incubated in preoxygenated medium containing [U-^{14}C]-palmitate (0.4 μCi/μmol) complexed to 3 % bovine serum albumin (BSA) or [U-^{14}C]-glucose (1.0 μCi/ml) of a final volume of 2 ml. The flasks were sealed with a rubber stopper fitted with a scintillation vial and parafilm containing pieces of Whatman #1 filter paper soaked with 0.3 ml of 1 M hyamine hydroxide that acted as a $^{14}CO_2$ trap. After incubation for 1 hour at 37 ºC, the reaction was stopped by injection of 5 M H_2SO_4 (1 ml) through the rubber stopper into the flask, which caused the liberation of $^{14}CO_2$ dissolved in the medium. The $^{14}CO_2$ entered the vials and was trapped in the hyamine-saturated filter paper. The filter papers were taken for scintillation counting after 2 hours of gentle shaking at room temperature. Both glucose and fatty acid oxidation rates were expressed as nanomoles per minute per milligram protein.

2.8.4 Myocardial Fatty Acid and Glucose Oxidation, Glycolysis, and Glucose Uptake

In hearts, palmitate oxidation was determined by quantitative collection of $^{14}CO_2$ released from [U-^{14}C]-palmitate as a gas and dissolved in the perfusate as [^{14}C]-

bicarbonate [20]. Rates of glycolysis were determined by quantitatively measuring the rate of $^{3}H2O$ production released into the perfusate from [5-^{3}H]-glucose [20]. Perfusate and gas samples were taken every 10 minutes during the perfusion period. Metabolic rates are as expressed as nmole per minute per gram dry weight. Rates of glucose uptake by the heart were determined using a previously described method [317].

2.9 GLYCOGEN METABOLISM

2.9.1 Total Glycogen Content

Total glycogen content of cells was determined by enzymatic degredation of total glycogen into glucose as described prevoiusly [318]. Upon removal of experimental medium, cell cultures were washed with ice-cold PBS (pH 7.4) and subjected to alkaline extraction of glycogen from exogenous glucose by the addition of 0.5 ml of 0.4 M KOH and incubation at room temperature for 15 minutes. The cell lysates were boiled at 95 ºC for 10 minutes to remove existing exogenous glucose in the samples [318]. Then, duplicate samples of the above heat-exposed cell lysate (150 µl) were spotted onto 2 cm x 2 cm 31ET filter papers (Whatman, Kent, UK) in duplicate. This was followed by ethanol precipitation of the glycogen by washing the filter papers in ice-cold 66 % ethanol (3 washes of 15 minutes each). The filter papers were air-dried over night and then each filter paper was cut into 8 pieces and placed into 2 ml centrifuge tubes. To release endogenous glucose from precipated glycogen, 500 µl of glycogen buffer consisting of 0.2 M acetate buffer, pH 4.7, and 0.625 mg/ml amyloglucosidase (Sigma, St. Louis, Missouri) was added to each tube, and the tubes were incubated at 37 ºC for 2 hours with shaking. After this incubation, an aliquot of 200 µl of glycogen buffer containing glucose product from the complete degradation of the glycogen was transferred into a standard 1.5 ml cuvette (Sarstedt, Quebec, Canada). Glucose released from glycogen was assayed colorimetrically. 1 ml of glucose-detecting reagent (500 ml ddH_2O/capsule containing glucose oxidase (500 U/calsule and peroxidase (100 U/capsule); Sigma Diagnostics, St. Louis, Missouri) was added to

each cuvette and incubated at 37 ºC for 30 minutes with gentle shaking. The absorbance at 475 nm was read by a spectrophotometer and the data was compared to a glucose standard curve created with glucose standard solution (Sigma, St. Louis, Missouri). Glycogen content was expressed as nanomoles of glucose per milligram protein.

2.9.2 Glycogen Synthesis

Glycogen synthesis was determined by the incorporation of [5-^{3}H]-glucose into glycogen [40]. When indicated, 1.0 µCi/ml of labeled [5-^{3}H]-glucose was added to the cell medium to allow the measurement of glycogen synthesis. Glycogen was collected and quantified as described above. To determine the amount of [5-^{3}H]-labeled glycogen, 200 ul of cell lysate were spotted onto 3IET filter papers and washed in ice-cold ethanol as desribed above. After the filter papers were dried, they were inserted into scinitillation vials and 4 ml of scintillant was added to each vial. The vials were vortexed and left over night at room temperature before they were subjected to counting. The specific activity of medium was determined by measuring unused [^{3}H]-labeled experimental medium aliquots. Glycogen synthesis was expressed as nanomoles of glucose incorporated per milligram protein.

2.10 Enzyme-linked Immunosorbent Assay (ELISA) and Atrial Natriuretic Factor/ Peptide (ANF/ANP) Expression

ANF expression was measured in H9c2 cell lysates using a commercially available colorimetric ELISA kit (R & D systems; Mississauga, Ontario, Canada). Cultured cells were rinsed with ice-cold PBS and lysed on ice by addition of 300 µl of total protein extraction buffer (pH 7.4) containing 20 mM HEPES, 250 mM sucrose, 1 mM EDTA, phosphatase inhibitors (100 mM sodium fluoride, NaF, 100 mM sodium pyrophosphate, $Na_4P_2O_7$, and 10 mM sodium orthovanadate, Na_3O_4V), and protease inhibitor cocktail. The cell lysates were scraped, collected, sonicated and centrifuged for 10 minutes at 13000 g at 4 ºC. Protein concentration was measured using the

bicinchoninic acid method. Samples of supernatant containing 10 µg of total protein were added to the 96-well polystyrene plates precoated with polyclonal sheep anti-proANP (1-98) provided in the kit. The N terminal part of ANP, proANP (1-98), has a longer half life (1-2 hours) and thus is a better indicator for measurement of ANF in the circulation. The ANF in the cell lysate acted as an antigen. This antigen was complexed with a polyclonal anti-proANP antibody conjugated to a horseradish peroxidase that produces a coloured product in the presence of substrates. The absorbance at 620 nm was read in a spectrophotometer (Perkin Elmer, Shelton, Connecticut). The change in absorbance at 620 nm was directly proportional to the ANF concentration in the sample. A standard curve of known ANF concentrations ranging from 0-10 fM was generated using synthetic human proANP standard solution provided with the kit. ANF production was expressed as fmole per microgram protein.

2.11 AMPK ACTIVITY ASSAY

Measurement of isoform-specific AMPK activity was determined in H9c2 cell lysates, as described [35, 202, 315] with minor modifications. H9c2 cells were washed in ice-cold PBS and 300 µl of AMPK homogenization buffer (pH 7.5) containing 50 mM Tris-HCl, 0.25 M mannitol, 1 mM EDTA, 1 mM EGTA, 50 mM sodium flouride (NaF), 5 mM sodium pyrophosphate ($Na_4P_2O_7$), protease inhibitor cocktail and 1 mM dithiothreitol (DTT) was added (on ice). After scraping the cells, the homogenates were collected, sonicated and centrifuged for 10 minutes at 13000 g at 4 ºC. Protein concentration was measured using the bicinchoninic acid method. The supernatant containing approximately 150 µg total protein was incubated with the isoform-specific anti-α1 or anti- α2 AMPK antibodies (1:500 dilutions, Upstate, Charlottesville, VA) in 150 µl of AMPK homogenization buffer overnight at 4 ºC on a rotator. Then 20 µl of 50 % protein A-sepharose slurry (Amersham) was added to the mixture and incubated for 1 hour at 4 ºC on a rotator. The supernatant was removed followed by three washing/recentrifugation steps at 4 ºC with AMPK resuspension buffer containing 100 mM Tris-base, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM $Na_4P_2O_7$, 10 %

glycerol, 0.02 % Na azide, protease inhibitor cocktail, 1 mM DTT and 0.12 % Triton. After the final centrifugation the supernatant was removed so that a total volume of 50 µl was left. Five µl of the above samples were added to 12.5 µl of AMPK assay buffer (pH 7.5) containing 40 mM HEPES, 80 mM NaCl, 8 % glycerol, and 0.8 mM EDTA. A timed reaction to measure AMPK activity of the immunoprecipitated samples was performed in the presence of 200 µM synthetic AMARA (AMARAASAAALARRR) peptide [319], 0.8 mM DTT, 200 µM AMP at a total volume of 25 µl of assay mixture was made. After addition of 17.5 µl of assay mixture to 2.5 µl of ATP mix containing 1 µCi [^{32}P- γ]ATP, 200 µM ATP, 5 mM $MgCl_2$, cells were incubated for 10 min at 30 ºC. This incubation causes incorporation of ^{32}P into the AMARA peptide. At the end of 10 minutes, 17 µl of the incubation mixture was blotted onto a 1 cm^2 square of phosphocellulose paper. The paper was washed three times each for 10 minutes in 150 mM phosphoric acid, air dried and placed in scintillation vials. 4 ml of scintillation fluid (EcoLite™, ICN, CA, USA) was added to each vial, and the samples counted in a scintillation counter. A standard was created by counting of 2 µl of untreated ATP mix. AMPK activity was expressed as picomoles of ^{32}P incorporated into AMARA peptide per minute per milligram protein.

Total AMPK activity was also measured from homogenized H9c2 cells by using a saturated ammonium sulfate (AS) precipitation assay to purify AMPK, followed by measurement of AMPK activity using AMARA peptide. To make super saturated ammonium sulfate solution, 76.1 g of AS was added to 100 ml ddH_2O and allowed to stir overnight, after which some remained undissolved. Aliquots of 250 µl of cell homogenate prepared as above were added to 250 µl of AS solution. After incubation of the mixture for 20 minutes on ice, they were centrifuged at maximum RPM for 20 minutes at 4 ºC. The supernatant was removed and the pellet was resuspended in 15 µl of AMPK lysis buffer containing 1:100 phosphotase inhibitor, 50 mM NAF, 1:1000 protease inhibitor cocktail, 1 % TritonX-100, 1:1000 DTT. Protein quantification was performed on the resuspended lysate. Finally, 5 µg of protein was used to measure AMPK activity using AMARA peptide as described above.

2.12 IMMUNOBLOT ANALYSIS

Selected proteins in H9c2 cells were assessed by immunoblot analysis and quantified by densitometry of ECL images as described previously [24]. Cultured cells were lysed in the above described total extraction buffer. Cellular homogenates were centrifuged for 10 minutes at 13000 g at 4 ºC and the supernatant was used for immunoblot analysis. Protein concentration was measured using the bicinchoninic acid method.

Samples of supernatant containing 30 μg total protein or immunoprecipitated protein (150 μg of total protein) were solubilized by boiling samples for 10 minutes in reducing sample buffer containing 6 % sodium dodecyl (SDS), 185 mM Tris-HCl, 30 % glycerol, 14 % mercaptoethanol, 0.7 % bromophenol blue. The proteins were separated by electrophoresis on 5-10 % polyacrylamide gels using SDS-polyacrylamide electrophoresis (SDS-PAGE), and transferred by electroblotting to a nitrocellulose membrane. The membranes were blocked with 100 mM tris buffered saline (TBS) containing 5 % non-fat dry milk or BSA (low free fatty acid) and 0.1 % Tween 20 for 60 minutes at room temperature with constant agitation. After blocking, the blots were probed overnight in blocking buffer plus primary antibodies with constant agitation at 4 ºC. The primary antibodies used were: those against the cardiac troponin-T (0.1 μg/ml, NeoMarker, CA), GLUT-4 (1:3000 dilution, Cell Signalling Technology, Missisauga, Ontario), HKII (1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), PDC E1α (1:100000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), phosphoAMPK (1:1000 dilution, Upstate Cell Signalling Solutions, Lake Placid, NY), phosphoACC (1:500 dilution, Upstate Cell Signalling Solutions, Lake Placid, NY), PKB (1:1000 dilution, Upstate Cell Signalling Solutions, Lake Placid, NY), PKC (1:750 dilution, Upstate Cell Signalling Solutions, Lake Placid, NY), α-pan-AMPK (1:1000 dilution, Upstate Biotechnologies, Lake Placid, NY), phosphoeEF2 (1:1000 dilution, Upstate Biotechnologies, Lake Placid, NY), total eEF2 (1:1000 dilution, Upstate Biotechnologies, Lake Placid, NY), GAPDH (1:15,000 dilution, Molecular Probes, Eugene, Oregon), GSK-3β (4 μg/ml dilution, Upstate Biotechnologies, Lake Placid,

NY). After removal of the primary antibody solution, the nitrocellulose membranes were rinsed twice briefly with TBS followed by 3 washes with TBS for 5 minutes at room temperature. The membranes were incubated with blocking buffer plus secondary antibody (e.g. 1:4000 Goat anti-Rabbit conjugated with horse radish peroxidase) for 1 hour at 4 ºC. The membranes were rinsed twice briefly with TBS followed by 3 washes with TBS for 5 minutes at room temperature. The membranes were incubated with enhanced chemiluminescent substrates and the subsequent signals were detected with a Syngene Chemigenius (Cambridge, UK). Densitometry of bands was measured using the Image J program (NIH).

2.13 MEASUREMENT OF ADENINE NUCLEOTIDES AND CREATINE PHOSPHATE

Adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, creatine and creatine phosphate were measured in Dr. Roger Brownsey's laboratory at the University of British Columbia by perchloric acid extraction of frozen ventricular tissue [204]. Since HPLC measures the total amount of AMP in tissue extracts with the functionally relevant free AMP representing a small fraction of the total, concentrations of free AMP were determined using the creatine kinase and adenylate kinase equilibrium reactions, as previously described [35].

2.14 [^{14}C]-PHENYLALANINE INCORPORATION

Phenylalanine incorporation into the cells was assessed by exposure of cultures to [^{14}C]-phenylalanine (1 μCi/ml) provided in the experimental medium. The incorporation of radioactivity into the acid insoluble cell mass was measured. After treatment of cells with medium containing [^{14}C]-phenylalanine for 24 hours, cells were washed with ice-cold PBS, and 500 μl of ice-cold 10 % trichloroacetic acid was added and incubated overnight at 4 ºC [320] to precipitate the proteins. The precipitates were

washed with ice-cold PBS and dissolved in 500 µl of 1 M NaOH-0.01 % sodium dodecyl sulfate by incubation for 2 hours at 37 ºC. 100 µl of this solution was added to scintillation vials containing 4 ml of Ecolite scintillant (ICN, Costa Mesa, CA). Vials were vortexed and left overnight in room temperature before being subjected to an isotope counting procedure. The standard was generated by counting an aliquot of unused medium containing [^{14}C]-phenylalanine in scintillation counting machine.

2.15 MEASUREMENT OF TOTAL PROTEIN CONTENT

All metabolite concentrations and enzyme activities were expressed relative to the protein content of the sample, determined using a commercial Bicinchoninic Acid (BCA) protein assay kit from Sigma (St. Louis, Missouri). A standard curve of BSA from Sigma (St. Louis, Missouri) ranging from 0 to 15 µg/µl was made that included an appropriate amount of the buffer used to homogenize the sample. Then aliquots of 25 µl of suitably diluted cell samples (5–30X) were added to a 96-well plate (BD Biosciences, Lexington, Kentucky) in duplicates. After the addition of 150 µl of assay reagent consisting of of 95 % BCA: 5 % $CuSO_4$, the plate was incubated at 37 ºC for 30 minutes with gentle shaking. The plate was cooled to room temperature before the absorbance at 562 nm was measured in a spectrophotometer (Perkin Elmer, Shelton, Connecticut). The protein content was expressed in milligrams of protein.

2.16 CELL COUNTING

After washing the cells with ice-cold PBS, cells were trypsinized and the cell cultures were incubated at room temperature for 5-10 minutes until the cells were fully dissociated from the cell surface. After adding DMEM to the cells (the volume was estimated from the cell surface area of the dish or well), the cells were resuspended thoroughly. The cell number in the samples was determined by adding 18 µl of the cell suspension to a hemocytometer and counting four quadrants under a light microscope.

The cell number is determined using a hemocytometer and adjusted to approximately 20-100 cells/mm^2 with medium.

2.17 GENERATION OF RECOMBINANT ADENOVIRAL VECTORS

Recombinant, replication-deficient adenoviral vectors were obtained from Dr. Jason Dyck at the University of Alberta. Adenoviral vectors containing a dominant negative form of AMPK (DN-AMPK) were produced by administration of cDNA encoding α1-AMPK with a mutation in which aspartic acid 157 was changed to alanine [321, 322]. Adenoviral vectors containing constitutively active form of AMPK (CA-AMPK) were produced by administration of cDNA encoding residues of 1-312 of α1-AMPK with a mutation in which threonine 172 was changed to aspartic acid. The recombinant adenovirus vectors contain a reporter gene of green fluorescent protein (GFP), which serves as a marker of successful viral transduction and protein over expression. The same protocol was used to construct adenoviruses encoding a c-myc tagged CA-AMPK, DN-AMPK, as well as an adenovirus encoding GFP alone.

2.17.1 Generation of Recombinant Adenoviral Plasmids by Homologous Recombination in Escherichia Coli

The cDNA of interest was subcloned into the pAdTrack-CMV shuttle vector. The resultant plasmid-containing insert was linearized by digesting with restriction endonuclease PmeI, purified by phenol/chloroform, and precipitated with sodium acetate and ethanol. The dry DNA was resuspended in sterile water and quantified. The resultant PmeI digested shuttle vector (100-300 ng) containing insert (100 ng/µl) was inserted by pAdEasy-1 system into 20 µl of the electrocompetent Escherichia coli BJ5183 cells (stratagene Cat. # 200157) which contains the adenoviral backbone vector pADEasy-1. This mixture was placed in a pre-chilled electroporation cuvette and electroporation was performed at 2500 V, 200 ohm, and 25 µF in a Bio-Rad Gene Pulser electroporator. The cells then were placed in 500 µl of L-Broth (LB) (Life

Technology) and incubated at 37 ºC for 1 hour with rotation. An increased stepwise amount of the cell suspension (from 50 to 200 µl) was inoculated into 100 mm petri dishes containing L-agar supplemented with 25 µg/ml of kanamycin and incubated at 37 ºC overnight. Around 10-20 of the smallest colonies were picked and grown in 2.5 ml LB containing kanamycin (25 µg/ml) in a 15 ml conical falcon tubes with vigorous shaking in a 37 ºC orbital shaker overnight. Clones were first screened with extensive restriction digestion. The digestion patterns were compared with the pAdEasy stock DNA made in strains incapable of homologous recombination. Then recombinants were selected for kanamycin resistance, and recombination was further confirmed by digestion with restriction endonucleases, generally PacI, speI, and BamHI. The resultant digested recombinant adenoviral construct was transfected into the packaging cell line HEK293 to propagate.

2.17.2 Production of Adenovirus in HEK293 Packaging Cells

HEK293 cells were grown at density of 2 X 10^6 cells per T75 flask in DMEM supplemented with 10 % FBS less than 24 hours prior to transfection. At the time of transfection, cells were 50-70 % confluent. During transfection, no antibacterial agent was used. After washing the cells with serum free OPTI-MEM, 3 ml of OPTI-MEM was added to the flasks and the flasks were incubated in the incubator for 15-30 minutes before transfection. Meanwhile a transfection mix was prepared by adding 8 µg of PacI digested recombinant and 40 µl of Lipfectamine to 200 µl of serum-free OPTI-MEM and incubating at room temperature for 15-30 minutes. After incubation at room temperature, the transfection mixture was added to the cells and incubated for 4-6 hours at 37 ºC incubator. Then, the medium containing the transfection mix was removed and 6 ml of DMEM supplemented with 5 % HS and 10 µg/ml was added; cells were then incubated for a further 48 hours. Then a final medium change was made and the transfected cells were monitored for GFP expression and collected 7-10 days after transfection during which viral lysis occured. The cells then were collected by scraping and centrifugation at 500 g for 10 minues. After three cycles of freezing in a

liquid nitrogen and rapid thawing at 37 ºC, 1 ml of viral lysate was used to infect 3-5 X 10^6 cells and the efficiency of transduction was assessed by GFP expression. After 3-4 days viruses were harvested as described above.

In order to generate higher titer viral stocks, packaging cells were infected at a multiplicity of infection (moi) of 25 to 300 and grown for 3-4 days when viruses were harvested as described above.

The recombinant adenoviruses were purified by ultracentrifugation on a cesium chloride gradient at 604000 g for 15 minutes at 4º C and tangential flow filtration as described previously [323, 324]. The purification caused removal of all cell debris and cytokines.

2.17.3 Titration of the Adenovirus

Titration of the adenovirus was performed as described previously [323, 324]. The 293 cells were cultured in 6-well culture dishes till they reached about 50-60 % confluency. In a final of 1 ml MEM, 5 % horse serum, 10 μg/ml gentamicin and serial dilutions of adenovirus (10^3 to 10^{12}) were made and added to the wells. One well was preserved as a Control without having adenovirus. Cells were incubated with the virus for 4 hours in the incubator. Meanwhile 1 % agar (Difco 214530 (0145-17)) was made in ddH_2O (autoclaved) and heated in the microwave. Once it was dissolved, it was placed in an incubator at 42 ^oC. After 4 hours of incubation of cells with the adenovirus, the adenovirus was removed and 2 ml of 1 X MEM alpha (Gibco) supplemented with 5 % horse serum and 0.5 % agar was added and cells were incubated. Every 4 to 5 days 1.5 ml of 1 X MEM alpha with 5 % horse serum and 0.5 % agar was added on top of the existing agar. Generally it takes 6 or more days before the plaques are obvious. The plaques were counted everyday and were maintained for 2 weeks. For the fluorescent titer all the fluorescing cells were counted 4 days after infection.

2.18 STATISTICAL ANALYSIS

Results were expressed as means ± SEM. Differences among groups were compared by means of Analysis of Variance (ANOVA) using NCSS, version 2000 (Statistical Solutions, Saugus, MA). Newman-Keuls was used as a post-hoc test to identify which groups were significantly different when the ANOVA yielded a significant F-ratio. A P value <0.05 was considered significant.

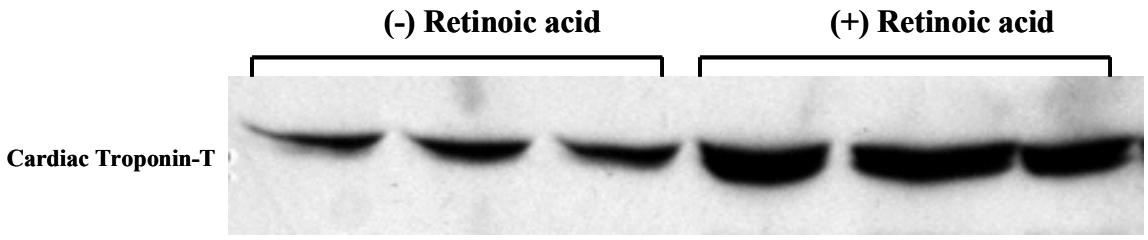

Figure 2-1: Cardiac troponin-T expression in H9c2 cells.

Representative immunoblot of cardiac troponin-T expression in H9c2 cells differentiated in 1 % horse serum in DMEM in the presence or absence of 100 nM retinoic acid for 4 days. Each lane represents a different sample. N=3 per each group.

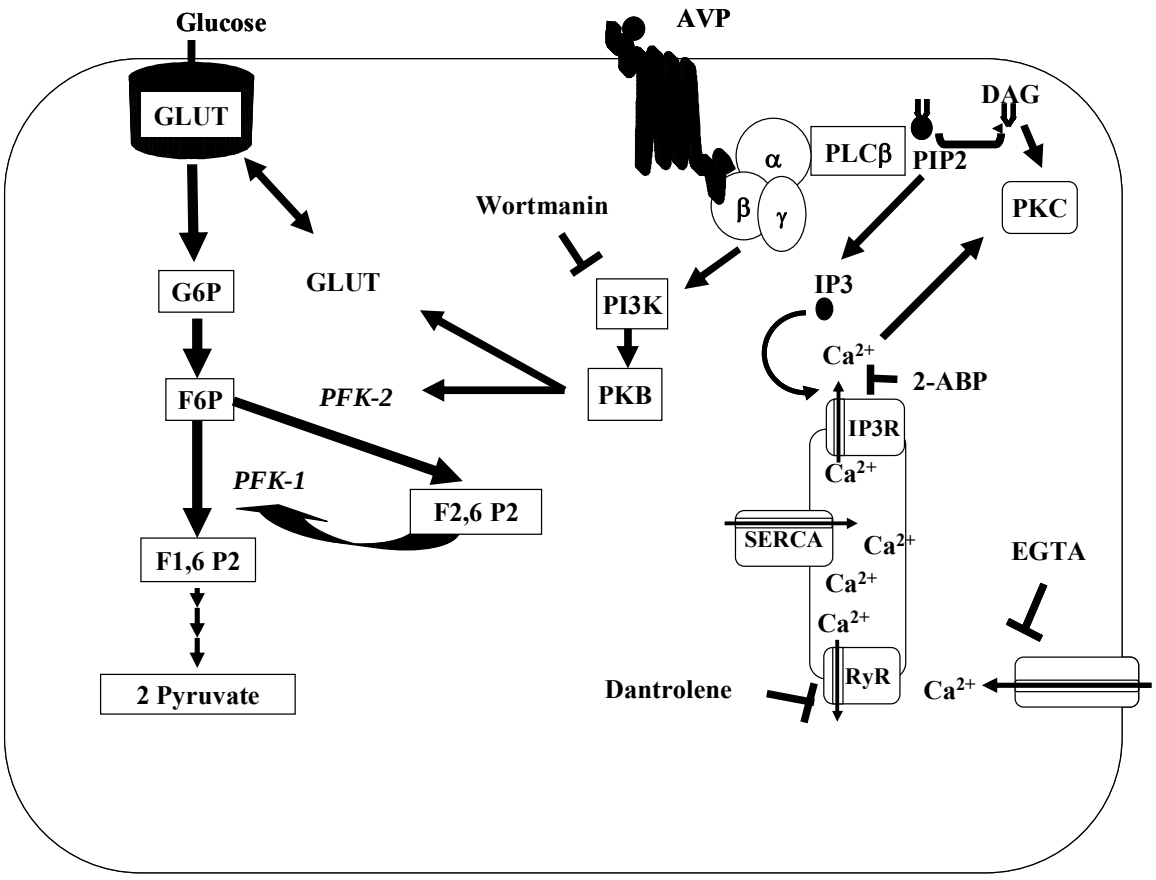

Figure 2-2: Potential signaling pathways responsible for stimulation of glucose use in H9c2 by AVP.

Binding of AVP to V1a receptor promotes the dissociation of Gq-coupled receptors. The α subunit of Gq stimulates phospholipase Cβ (PLCβ) leading to hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PIP2), increasing intracellular levels of diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (IP3). IP3 promotes release of calcium from intracellular stores via the IP3 receptor and evokes calcium entry across the plasma membrane via the capacitative calcium channel (CCE). In addition, the β-subunit of Gq activates phosphatidylinositol-3-phosphate kinase (PI3K) leading to activation of protein kinase B (PKB). A rise in intracellular calcium or activation of PKB involves the recruitment of the glucose transporter GLUT to the plasma membrane and the activation of phosphofructo kinase-2 (PFK-2), which in turn

increases the concentration of fructose 2,6-bisphosphate, a well-known stimulator of PFK-1 and therefore of glycolysis.

CHAPTER 3 - METABOLIC ACTIONS OF METFORMIN IN THE HEART CAN OCCUR BY AMPK-INDEPENDENT MECHANISMS

3.1 INTRODUCTION

AMP-activated protein kinase (AMPK) is considered to be a "metabolic fuel gauge" that is activated by several mechanisms including allosteric activation during periods of decreased cellular energy state, phosphorylation activation of the α catalytic subunit by one or more upstream kinases (AMPK kinase, AMPKK), and/or activation by inhibition of phosphatase [157, 158, 161]. Additionally, AMPK can be activated in cells without changes in energy state by a calcium-dependent pathway involving calcium/calmodulin-dependent protein kinase kinases as upstream AMPK kinases [158, 206, 207, 209] and by long-chain fatty acids [202, 325]. In the heart, AMPK is involved in the control of fatty acid oxidation, glucose uptake, and glycolysis [223]. 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAr), a cell-permeable adenosine analog [247], has been shown to pharmacologically activate myocardial AMPK resulting in stimulation of fatty acid oxidation [69, 204] as well as translocation of glucose transporters to the sarcolemma and increased glucose uptake [239].

Metformin, an insulin-sensitizing biguanide widely used to treat type 2 diabetes mellitus [272], has metabolic actions that are believed to be mediated by AMPK activation as observed through studies in liver and skeletal muscle [199, 273]. Metformin also has been used to activate AMPK in isolated cardiac myocytes [274, 298, 326] and in intact hearts [275]. Consequently, metformin is now widely used as a means to activate AMPK in experimental model systems. The concentration of metformin used in the experiments with isolated cardiac myocytes [274] and intact hearts [275] was sufficiently high; however, to cause a reduction in high energy phosphates leading to an elevation of AMP [275, 327], presumably because metformin inhibits complex 1 of the respiratory chain [161]. Such findings indicate that metformin at these concentrations, not surprisingly, causes activation of myocardial AMPK via an adenine nucleotide-dependent mechanism. However, an adenine nucleotide-independent activation of AMPK by metformin has been proposed in other cell types [198], including liver, where metformin may act in liver to prevent alcohol-induced injury via an AMPK-independent pathway [328].

Of additional importance is the fact that clinically-relevant and metabolically-active concentrations of metformin are substantially lower than those typically used experimentally [294] raising the distinct possibility that the metabolic effects of metformin occur without alterations in cellular energy status. Moreover, the effects of metformin on metabolism have been characterized in tissues such as liver and skeletal muscle [273, 329, 330] and cardiac myocytes [298, 326], whereas the metabolic effects of metformin on the intact working heart are less well understood.

Thus, we set out to test the hypothesis that metformin has AMPK-independent effects on energy metabolism in heart muscle using isolated working rat hearts and cultured heart-derived H9c2 cells as experimenta model systems. Further, we also investigated selected signal transduction pathways potentially responsible for the metabolic actions of metformin. The effects of metformin on AMPK activation and energy metabolism to those of AICAr, a well-recognized activator of AMPK in the heart, were compared.

Also, the roles of selected signal transduction pathways that are potentially responsible for the metabolic actions of metformin were investigated.

3.2 METHODS

Details of methods used for this investigation are described in the Materials and Methods chapter.

3.3 RESULTS

3.3.1 Isolated Rat Heart

3.3.1.1 Heart Function

Cardiac output and hydraulic work of metformin-treated hearts were significantly higher than in vehicle-treated hearts while heart rate was slightly but significantly decreased in hearts exposed to metformin (Table 3-1). Function was stable throughout the perfusion in all groups (Figure 3-1).

3.3.1.2 Substrate Utilization and Content of Triglyceride and Glycogen

Metformin treatment significantly increased palmitate oxidation (Figures 3-2A) and significantly reduced triglyceride content as compared to Control (Control, 14.09 ± 0.59; Metformin, 11.38 ± 0.75 mg/g dry wt; N=8 per each group; $p<0.05$). This difference in rates of fatty acid oxidation was not simply due to an increase in cardiac work in response to metformin because rates of fatty acid oxidation remained elevated in metformin-treated hearts when rates were normalized to cardiac work (Control, 1.03 ± 0.05; Metformin, 1.55 ± 0.05 nmol/min/cardiac work; N=8 per each group; $p<0.05$).

Glycolysis (Figure 3-2B) and glucose uptake (Table 3-2) were significantly reduced in response to metformin in hearts perfused with 1.2 mM palmitate. Compared to vehicle-treated hearts, overall glycogen content was not different, although synthesis of glycogen was significantly increased by metformin (Table 3-2).

3.3.1.3 Activity of AMPK and PKB Phosphorylation

Myocardial isoform-specific activity of AMPK was not significantly increased by metformin (Table 3-3). Metformin also failed to increase total AMPK activity as measured in ammonium sulfate (AS) precipitated samples from myocardial homogenates (Control, 787 ± 52; Metformin, 761 ± 37 pmol/min/mg protein; N=6-8 per each group. Total AMPK activity determined in ischemic myocardial tissue was 1586 pmol/min/mg protein; N=2). Finally, western blot analysis of the hearts indicated that neither phosphorylation of Thr-172 of the α-catalytic subunit of AMPK (Figure 3-3A) nor phosphorylation of ACC differed from that in vehicle-treated Control hearts (Figure 3-3B). Phosphorylation state of PKB in hearts exposed to metformin was measured as metformin has been shown to enhance insulin-induced phosphorylation of PKB in cardiac myocytes [298]; however, metformin did not significantly alter PKB phosphorylation (Figure 3-3C).

3.3.1.4 Myocardial High Energy Phosphates

Metformin treatment did not significantly alter the content of adenine nucleotides (Table 3-4). Myocardial content of phosphocreatine was significantly elevated in metformin-treated hearts as compared to Control hearts (Table 3-4). Free AMP was not elevated in metformin-treated hearts and, in fact, there were no significant differences among groups (Control, 0.38 ± 0.08 vs. Metformin, 0.20 ± 0.09 μmol/l, N=5 per each group, P=NS). Taken together, these results indicate that, if anything, energy state was improved in the heart by this concentration of metformin.

3.3.2 H9c2 Cells

3.3.2.1 Effects of AICAR and Metformin on Metabolism

Metformin dose-dependently increased rates of glycolysis in cultured heart muscle cells from concentrations as low as 10 μM up to 5 mM, the highest concentration assessed (Figure 3-4). A metformin concentration of 5 mM reduced the energy state of intact hearts [275]. In contrast, 2 mM did not lead to a reduction of in high energy phosphates

in a rat hepatoma cell line, H4IIE [198]. Thus, a concentration of 2 mM was chosen for use in the current experiments because it had the greatest metabolic effect without any reported impact on cellular energy status.

Metformin (2 mM) significantly increased rates of glycolysis as compared to Control values (Control 18.7 ± 1.3 vs. Metformin 35.0 ± 2.2; nmol/hr/mg protein, N=10-12 per each group, $P<0.05$). In contrast, AICAr did not affect glycolysis as compared to Control values (Control 18.7 ± 1.3 vs. AICAr 21.6 ± 0.5; nmol/hr/mg protein, N=10-12 per each group, P=NS). Of note, as shown in Figure 3-3, 10 μM metformin, a clinically relevant concentration, also significantly increased glycolytic rates compared to Control rates (Control 18.7 ± 1.3 vs. Metformin 27.4 ± 2.6; nmol/hr/mg protein, $P<0.05$). Rates of glucose uptake were also increased significantly by 2 mM metformin in H9c2 cells (Control, 24.5 ± 2.4 vs. Metformin, 34.9 ± 1.2 nmol/hr/mg protein, N=6 per group, $p<0.05$). Fatty acid oxidation rates were significantly lower in H9c2 cells treated with 2 mM metformin as compared to Control (Control 4.6 ± 0.14 vs. metformin 3.7 ± 0.15; nmol/hr/mg protein, N=5 per each group, $P<0.05$). Rates of fatty acid oxidation did not alter in response to AICAr treatment as compared to controls (Control 4.6 ± 0.14 vs. AICAr 5.1 ± 0.24; nmol/hr/mg protein, N=5 per each group, P=NS). Metformin at 2 mM concentration did not have any significant effect on total glycogen content (Control 412.6 ± 26.8 vs. metformin 442.1 ± 44 nmol/mg protein, N=6 per each group, P=NS).

3.3.2.2 AMPK Activity

Activity of AMPK did not differ between Control and metformin treated H9c2 cells (Control 205.87 ± 22.14 vs. Metformin 244.24 ± 34.71, pmol/min/mg protein, N=18 per each group, P=NS). Similarly, phosphorylation state of both AMPK and ACC was not altered by either AICAr or metformin (Figure 3-5). Collectively, these data indicate that AMPK is not activated by either AICAr (1.2 mM) or metformin (2 mM) in heart-derived H9c2 cells. The latter is in keeping with data obtained in the heart.

3.3.2.3 Inhibition of AMPK Fails to Alter the Metabolic Actions Induced by Metformin in H9c2 Cells

In order to more clearly determine the role of AMPK in metformin-induced changes in metabolism in H9c2 cells, AMPK was inhibited by molecular and pharmacologic means, prior to treatment with 2 mM metformin. Twenty-four hours after transduction of cells with adenovirus containing a dominant-negative form of AMPK α2 (DN-AMPK) (moi 150) nearly 100 % of cells showed GFP (Figure 3-6A), indicative of successful viral-mediated gene transfer and protein expression. Importantly, AMPK activity was significantly reduced in H9c2 cells infected with DN-AMPK α2 as compared to Control cells or cells exposed to adenovirus containing GFP alone (Figure 3-6B).

Rates of glycolysis were stimulated by metformin to the same degree, regardless of the presence or absence of DN-AMPK (Figure 3-7). Inhibition of AMPK by 40 μM Compound C, a pharmacological inhibitor of AMPK, also significantly reduced AMPK activity (Figure 3-8A) but failed to reduce the acceleration of glycolysis induced by metformin (Figure 3-8B). Taken together, these findings indicate that acceleration of glycolysis by metformin at a concentration of 2 mM and under these experimental conditions occurs independently of AMPK activation.

3.3.2.4 Metabolic Actions of Metformin in H9c2 Cells Occur by p38-MAP Kinase- and PKC-Dependent Pathways

Modulation of other signaling pathways, including mitogen-activated protein kinase (MAPK), PKC and phosphoinositol-3-phosphate kinase (PI3K)-PKB pathways, have been implicated in cellular effects of metformin [295-298]. As such, the role of these pathways in mediating the metabolic response of H9c2 cells to metformin was investigated by use of pharmacological inhibitors. It was found that metformin elevated measured activity of p38 MAPK (Control, 12.4 ± 1.6 vs. Metformin, 20.0 ± 2.7 pmol/min/mg protein, N=9-12 per each group, $p=<0.05$) and increased phosphorylation of PKC (Figure 3-9) in H9c2 cells. Treatment of cells with 10 μM SB203580, a reportedly selective p38 MAPK inhibitor, significantly reduced but did not abolish the

metabolic effects of metformin (Figure 3-10A). In contrast, pre-treatment of cells with SP600125 (Figure 3-10B) and PD98059 (Figure 3-10C), specific inhibitors of JNK and ERK, respectively, had no effect on metformin induced acceleration of glycolysis in H9c2 cells. Pretreatment of cells with 10 nM calphostin C, a potent and specific inhibitor of conventional and novel isoforms of protein kinase C (PKC) [299, 300], completely abolished the metabolic actions of metformin (Figure 3-10D), while a selective PI3K inhibitor, LY-294002 [301] (10 μM), did not significantly alter metformin-induced changes in glycolysis (Figure 3-10E). Interestingly, insulin did not increase rates of glycolysis in H9c2 cells (Insulin, 19.05 ± 0.75 vs. No insulin, 20.56 ± 0.91 nmol/hr/mg protein, N=9-19 per each group, P=NS).

3.4 DISCUSSION

The current study demonstrated that metformin has AMPK independent effects on energy metabolism in heart muscle cells. Metformin (2 mM) altered fatty acid oxidation and glucose utilization in the intact heart and isolated heart muscle cells. The metabolic changes induced by metformin in the heart occurred in the absence of a reduction in energy status and without measurable activation of AMPK. Here, it was confirmed that the metabolic effects of metformin occurred independently of AMPK by showing that inhibition of AMPK activity by either over expression of a dominant negative form of AMPK or by administration of the AMPK inhibitor, Compound C, failed to prevent the metabolic effects observed. These findings were extended by demonstration that the metabolic effect of metformin is abrogated by pharmacological inhibition of p38 MAPK and PKC. Additionally, AICAr (1.2 mM) failed to activate AMPK or alter energy metabolism in H9c2 cells.

Several investigations in tissues and cells [198, 199, 273, 275, 298, 326 , 331] have provided strong support for the concept that metformin activates AMPK. The current study, however, showed that in intact hearts and heart-derived H9c2 cells, the activity and phosphorylation state of AMPK were not significantly altered by metformin, even

though the concentration used yielded metabolic effects. It is important to recognize that failure to detect measurable changes in AMPK activity or phosphorylation in tissue extracts does not rule out allosteric activation of AMPK [204], an effect that can be assessed by measuring phosphorylation of ACC, a downstream target of AMPK. In the current study, ACC phosphorylation, at Serine-79 which is an AMPK specific phosphorylation site, did not differ between metformin treated and untreated hearts and H9c2 cells, in keeping with measured activity and phosphorylation of AMPK. Thus, these findings indicate that AMPK is not activated in the intact heart and H9c2 cells by metformin at the concentration used and under these experimental conditions.

In the current study, specifically a concentration and time of exposure to metformin was chosen that did not alter energy state. To support this, cellular energy status, including absence of elevation in free AMP concentration, was not impaired by 2 mM metformin (Table 3-4). This finding contrasts with the finding of other researchers that have used higher concentrations of metformin (5 to 10 mM) and impaired cellular energy status in cardiac myocytes and intact hearts [275, 327] that resulted in increase in AMPK activity in the intact heart [275]. The reduction in cellular energy status likely occurred because metformin inhibits complex 1 of the respiratory chain when used at high concentrations [161]. The fact that cellular energy status was not impaired by metformin in the current experiments (Table 3-4) indicates that no significant inhibition of complex 1 of the respiratory chain occurred and provides a good explanation as to why AMPK was not activated.

Metformin has been reported to reduce [332], stimulate [273], or not significantly alter [333] rates of fatty acid oxidation in a variety of tissues and cells. This variability likely reflects underlying cell- or tissue-specific characteristics as well as different concentration used that determine the nature of the metabolic response to metformin. The current study showed that metformin stimulates myocardial fatty acid oxidation (Figure 3-2A) in intact heart. This finding serves to emphasize that cell - or tissue-specific characteristics are key determinants of the metabolic response produced by

metformin. The exact mechanisms responsible for the cell and tissue-specific metabolic response are not yet known.

Metformin led to net degradation of endogenous triglycerides in isolated perfused hearts. Endogenous triglycerides have been shown to contribute substantially to energy production in the heart and to be simultaneously synthesized and degraded [39]. With the data available, it is not possible to determine if metformin-induced changes in total myocardial triglyceride content were due to reduced synthesis or enhanced degradation of triglycerides.

The suppression of glucose use by metformin in intact hearts is not surprising as the suppression of glucose catabolism likely is a reflection of the well-recognized glucose-fatty acid cycle in which elevated fatty acid oxidation rates lead to a reduction in glucose uptake, glycolysis, and glucose oxidation [334]. Metformin reportedly enhances both glucose and fatty acid catabolism in non-cardiac tissues [273, 333]. Our data indicate that the stimulatory effect of metformin on fatty acid oxidation (Figure 3-1A), when present, is the predominant effect in the heart, accompanied by a compensatory reduction in glucose use (Figure 3-2B and Table 3-2).

In contrast to the intact heart, metformin decreased fatty oxidation rates in cultured H9c2 cells, presumably as a consequence of increased rates of glucose use and the well recognized reciprocal relationship between fatty acid and glucose use [334]. The discrepancy between the intact heart and cultured H9c2 cells may possibly be due to fact that cultured cells are quiescent with substantially lower energy requirements and that the fate of fatty acid taken up by the cell is directed predominantly toward storage rather than oxidation [335]. An additional factor that may have contributed to the discrepant responses of H9c2 cells and hearts to metformin is the fact that H9c2 cells might be resistant to the effects of insulin. As such, others have shown that exposure of cardiac myocytes to insulin for a prolonged period is known to cause insulin resistance [298]. The current study showed that the incubation of H9c2 cells with insulin (10^{-7}M) did not significantly increase glycolysis as compared to cells incubated without insulin.

This indicates that the H9c2 cells are resistant to the metabolic actions of insulin with respect to glycolysis, under the conditions of our study. Additionally, the presence or absence of insulin did not influence rates of glycolysis in cells exposed to metformin. Therefore, it is conceivable that this finding might account for differential responses of intact hearts and H9c2 cells to metformin.

The absence of effects of DN-AMPK and Compound C on metformin-induced changes in metabolism (Figures 3-7 and 3-8) confirms that activation of AMPK is not necessary for the metabolic effects of metformin to occur in heart muscle, at least without changes in cellular energy status. This result is consistent with the observation that metabolic effect of metformin in liver occurs independently of AMPK [336]. The finding that exposure of H9c2 cells to Compound C caused a reduction in measurable AMPK activity is interesting because Compound C is considered an allosteric inhibitor of AMPK [273] whose effects should disappear during the purification procedure [199]. Detection of a measurable reduction in AMPK activity by Compound C, which has also been observed by others in neural tissue [337], indicates that phosphorylation state of AMPK has been decreased and suggests that Compound C has other as yet, uncharacterized actions on AMPK.

A relationship between metformin-induced AMPK activation and a metabolic effect has been reported previously in adult cardiac myocytes [298, 326]. Importantly, those studies also highlight the concentration- and time-dependent nature of the effects of metformin on metabolism. For example, in the latter study, a lower concentration of metformin (1 mM) activated AMPK and increased glucose uptake in cardiac myocytes, but the duration of exposure to metformin was very prolonged (18 hours) [326]. A 4 hour duration of exposure to the same concentration of metformin increased glucose uptake but did not activate AMPK significantly [298], Bertrand et al have documented that exposure to substantially higher concentrations of metformin (5 and 10 mM) for 4 hours caused both activation of AMPK and stimulation of glucose uptake in cardiac myocytes [298]. In neither study was the energy status measured, so it is possible that

higher concentrations of metformin or longer exposures to metformin led to sufficiently high intracellular concentrations of metformin that had an inhibitory effect on the respiratory chain and caused AMPK activation. Notably, in the same study by Bertrand et al, 4 hour metformin (1 mM) incubation resulted in stimulation of glucose uptake, but no significant alteration in AMPK activity, a result in keeping with our data. These results highlight the fact that the metabolic actions of metformin are concentration and time dependent.

The absence of a role for AMPK indicates that other signaling pathways are responsible for the metabolic effects of lower concentrations of metformin in heart muscle. Investigations in other tissues suggest that PI3K [298, 338], PKC [242], p38 MAPK [297], JNK MAPK [339], and ERK MAPK [242] pathways are potential mediators of the effects of metformin in heart muscle. By use of selective inhibitors of each above pathways in H9c2 cells, metformin-induced acceleration of glycolysis in H9c2 cells was partially abrogated by inhibition of p38 MAPK and completely abrogated by inhibition of PKC. The metabolic effect of metformin on H9c2 cells is independent of PI3K, ERK, and JNK pathways. All above data suggest that the metabolic effects of metformin are related to p38 MAPK and PKC pathways. It is important to recognize that even though this study showed that p38 MAPK activity was increased in response to metformin, SB203580, the p38 MAPK inhibitor used in these experiments, has been shown to influence glucose use independently of p38 MAPK [340] indicating that an alternative means of inhibiting p38 MAPK is required to fully characterize its role.

Another interesting finding in the current study is that under these experimental conditions AMPK activity of H9c2 cells also was unchanged by treatment with AICAr. Additionally, AICAr treatment did not influence energy metabolism in H9c2 cells. Upon entering into the cell, AICAr is monophosphorylated by adenosine kinase to form 5-aminoimidazole-4-carboxamide ribonucleoside (ZMP) [341]. ZMP mimics all the effects of AMP on AMPK, causing allosteric activation and also promoting phosphorylation and activation by the upstream kinase, AMPK kinase (AMPKK) [157,

342]. The effects of AICAr on glucose and lipid metabolism have been documented in numerous studies [69, 204, 239, 343-345]. It should be mentioned that the concentration of AICAr used in current study has been shown to be sufficient to increase AMPK activity in intact heart [204]. Thus, in the context of the current experiments, absence of changes in phosphorylation of AMPK and ACC, a downstream target of AMPK whose phosphorylation and activation states are useful indicators of AMPK activity, confirms that AMPK is not activated in H9c2 cells by AICAr.

The reason that AICAr failed to activate AMPK or alter metabolism in H9c2 cells is not immediately apparent. One possible explanation as to why AICAr was unable to alter metabolism or activate AMPK may be that AICAr is unable to enter into the heart muscle cells. Also, a tissue- or cell-specific response to agents such as AICAr has been reported previously and is one possible explanation. For example, AMPK is required for the metabolic response to AICAr in skeletal muscle, but not adipocytes [331]. Consistent with our study, others have also shown that AICAr does not activate AMPK in vascular smooth muscle from rat carotid artery [346] and even within skeletal muscle, the extent to which AMPK is activated by AICAr depends upon the fiber-type of the muscle in question [172]. Such cell type specific responses to these agents are likely a reflection, at least in part, of unique regulation and cell-type specific expression patterns of AMPK and perhaps of the transporter required for AICAr uptake. Functional AMPK exists as a heterotrimeric complex made up of a catalytic α-subunit and two regulatory subunits, β and γ [157]. Each subunit exists in several isoforms, which have variable tissue distributions as well as differing regulatory properties, a situation that may contribute to the cell and tissue specific responses that have been observed.

In conclusion, the most significant and the major outcome of this investigation is that metformin has AMPK independent effects on energy metabolism in heart muscle cells; a finding that provides insights into the mechanism of action of metformin and establishes the basis for future investigations. Such a result may occur because

insufficient metformin entered the heart muscle cells. These data also indicate that metabolic effects of metformin in the heart do not require AMPK activation and that p38 MAPK and PKC signaling pathways are involved.

Table 3-1: Function of hearts perfused with or without 2 mM metformin.

Isolated working rat hearts were perfused in KH solution supplemented with 5.5 mM glucose, 1.2 mM palmitate, 0.5 mM lactate and 100 mU/l insulin in the presence or absence of 2 mM metformin for 30 minutes. Values are Mean ± SEM at the end of perfusion. Metformin, 2 mM. *, significantly different from Control value in group, $p<0.05$.

	Control (n=8)	**Metformin** (n=8)
Heart Rate (bpm)	261 ± 6	238 ± 3*
Peak Systolic Pressure (mmHg)	112.1 ± 1.1	115.6 ± 1.9
Cardiac Output (ml/min)	74.4 ± 2.1	81.5 ± 1.5*
Rate Pressure Product (bpm x mmHg x 10^{-3})	29.2 ± 0.6	27.5 ± 1.1
Hydraulic Work (ml/min x mmHg x 10^{-3})	83.3 ± 2.1	94.3 ± 2.9*

Table 3-2: Myocardial glucose and glycogen metabolism of hearts perfused with or without 2 mM metformin.

Isolated working rat hearts were perfused in KH solution containing 5.5 mM [5-^{3}H]-glucose, 1.2 mM palmitate, 0.5 mM lactate and 100 mU/l insulin to measure glucose uptake and glycogen content in the presence or absence of 2 mM metformin. Values are Mean ± SEM. Metformin, 2 mM. *, significantly different from Control value within group, $p<0.05$.

	Control (n= 4 to 8)	**Metformin** (n= 4 to 8)
Glucose uptake (nmol/min/g dry wt)	2129 ± 138	1535 ± 92*
Glycogen (μmol/g dry wt)	138.4 ± 5.5	130.0 ± 8.2
Glycogen synthesis (nmol/min/g dry wt)	120 ± 6	276 ± 25*

Table 3-3: Isoform-specific AMPK activity of hearts perfused with or without 2 mM metformin.

Values are Mean ± SEM. Metformin, 2 mM. Isoform-specific AMPK activity determined in ischemic myocardial tissue was 54.2 pmol/min/mg protein for the α1-AMPK and 20.3 pmol/min/mg protein for the α2-AMPK, N=2. *, significantly different from Control value within group, $p<0.05$.

	Control (n=8)	**Metformin** (n=8)
α1-AMPK (pmol/min/mg protein)	12.4 ± 2.5	7.8 ± 1.3
α2-AMPK (pmol/min/mg protein)	6.0 ± 0.7	4.3 ± 0.8

Table 3-4: Myocardial adenine nucleotide and creatine phosphate content of hearts perfused with or without 2 mM metformin.

Values are Mean ± SEM. Metformin, 2 mM. Values for 1.2 mM palmitate were obtained at 15 min of perfusion. *, significantly different from Control value within group, $p<0.05$.

	Control (n=8)	**Metformin** (n=8)
ATP (μmol/g dry wt)	9.4 ± 1.0	11.3 ± 0.6
ADP (μmol/g dry wt)	3.5 ± 0.3	4.2 ± 1.0
AMP (μmol/g dry wt)	3.1± 0.8	3.4 ± 0.2
Creatine (Cr) (μmol/g dry wt)	33.1 ± 2.4	37.5 ± 0.6
Phosphocreatine (PCr) (μmol/g dry wt)	13.7 ± 1.1	22.8 ± 3.5*

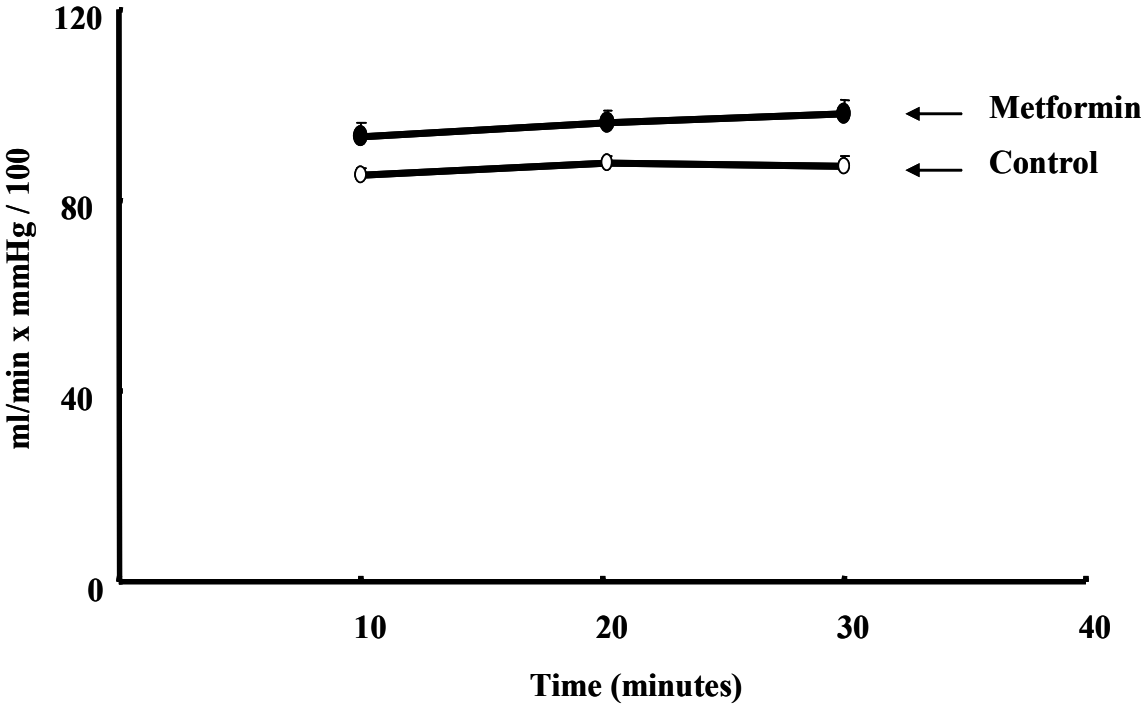

Figure 3-1: Cardiac work in hearts perfused with metformin.

Isolated working rat hearts were perfused in KH solution containing 5.5 mM glucose, 1.2 mM palmitate, 0.5 mM lactate and 100 mU/l insulin for 30 minutes in the presence or absence of 2 mM metformin. Values are Mean ± SEM. N=8 hearts per each group.

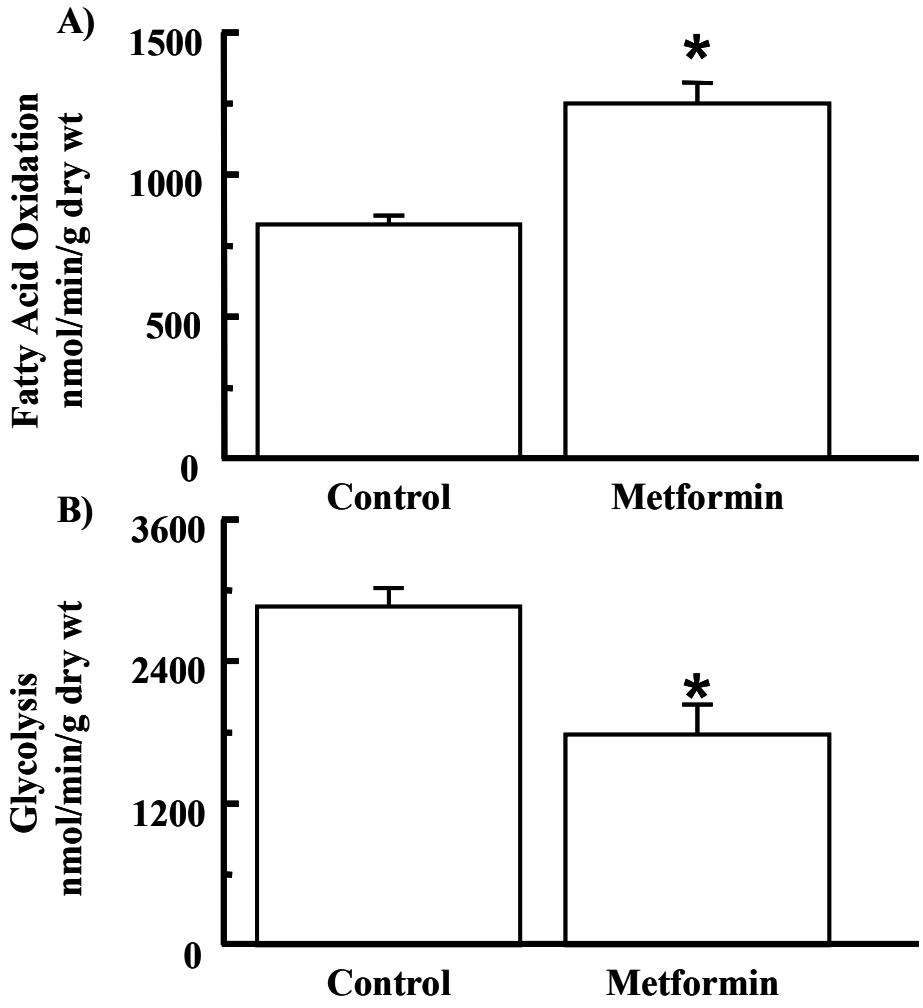

Figure 3-2: Substrate utilization in hearts perfused with metformin.

Isolated working rat hearts were perfused in KH solution containing 5.5 mM [5-^{3}H]-glucose, 1.2 mM [U-^{14}C]-palmitate, 0.5 mM lactate and 100 mU/l insulin to measure palmitate oxidation (A) and glycolysis (B), respectively, in the presence or absence of 2 mM metformin. Values are Mean ± SEM. *, vs. control hearts ($p<0.05$). N=6-8 hearts per each group.

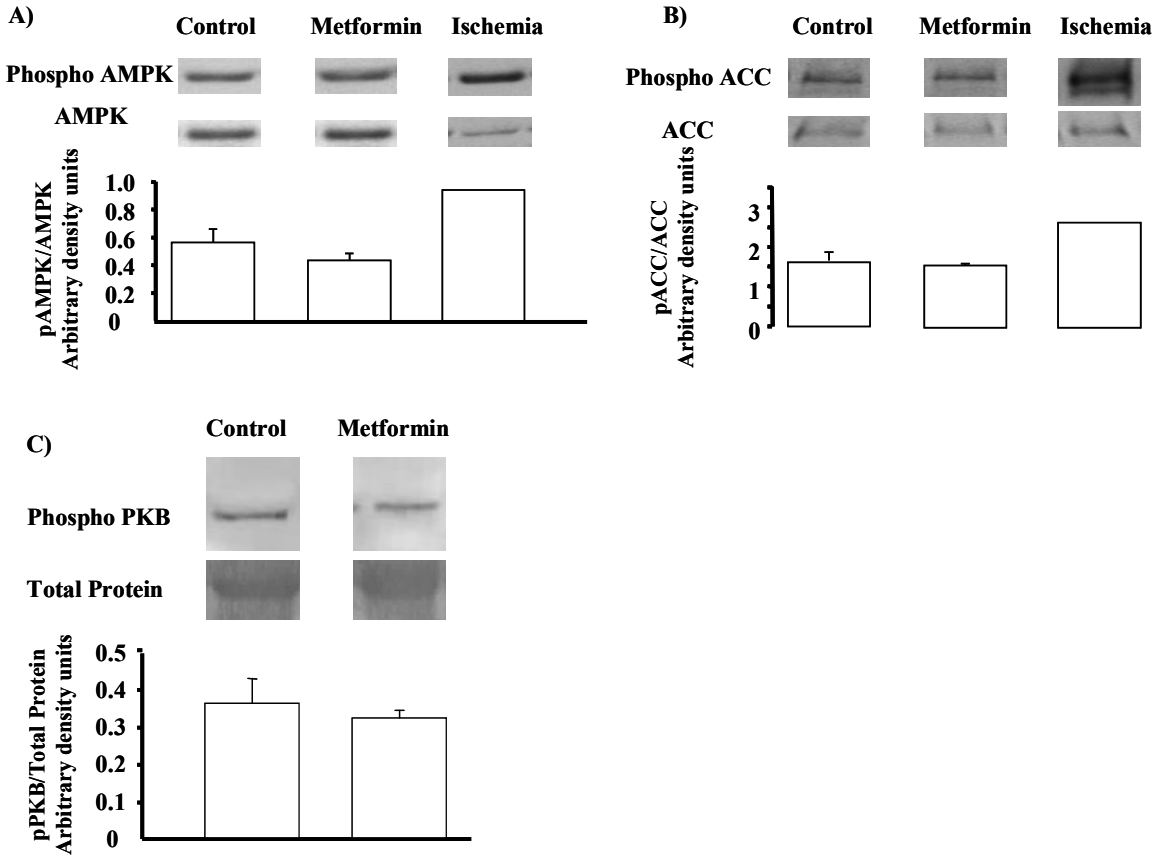

Figure 3-3: Role of AMPK and PKB in metabolic action of metformin in hearts perfused with or without 2 mM metformin.

Phosphorylation state of AMPK (A), ACC (B), and PKB (C) in hearts perfused in KH solution containing 5.5 mM glucose, 1.2 mM palmitate, 0.5 mM lactate and 100 mU/l insulin in the presence or absence of 2 mM metformin. Phosphorylation state of AMPK and ACC was assessed in selected hearts exposed to global no-flow ischemia for 20 min (Ischemia), serving as a positive control for increased phosphorylation. Values are Mean ± SEM and are expressed as arbitrary density units. *, vs. control hearts ($p<0.05$). N=6-8 hearts per group except for Ischemia where N=2.

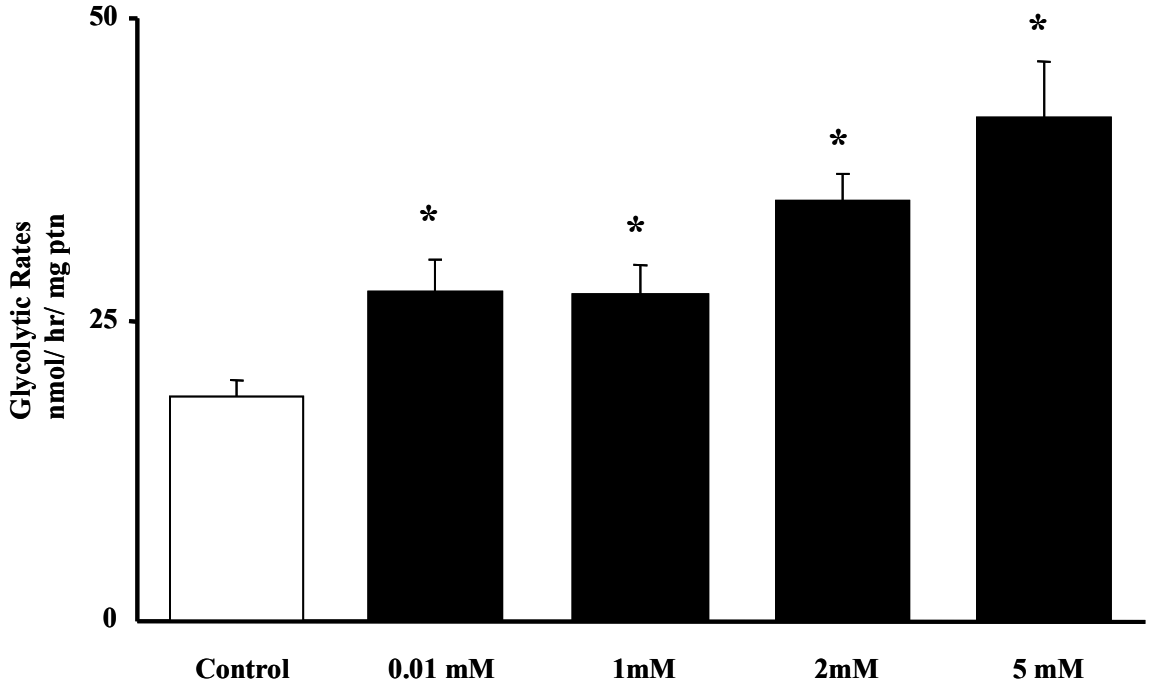

Figure 3-4: Effects of metformin on glycolysis.

Differentiated H9c2 cells were treated with different concentrations of metformin for 8 hours in KH solution containing 5.5 mM [5-^{3}H]-glucose, 0.4 mM palmitic acid pre-bound to albumin and 10^{-7} M insulin in the absence (Control) or in the presence of different concentrations of metformin. Tracer amounts of labeled [5-^{3}H]-glucose (1.0 μCi/ml) was present in the medium for the last hour of study to measure glycolytic rates. Values are Mean ± SEM. *, significantly different from Control ($p<0.05$). N=10-12 per each group.

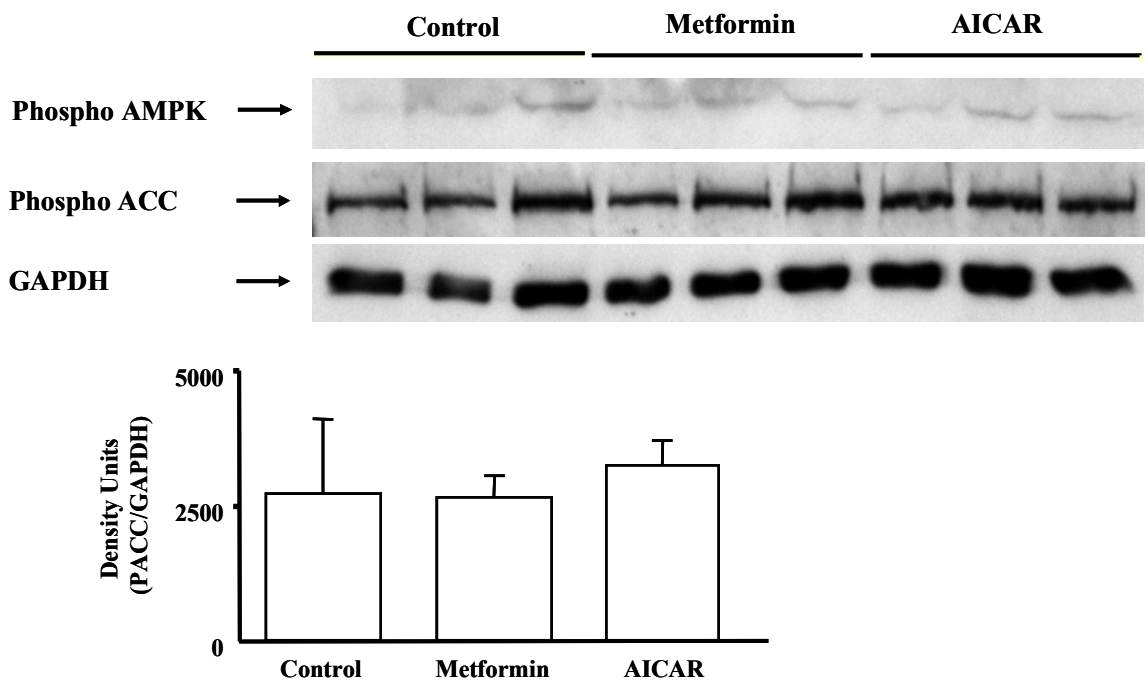

Figure 3-5: AMPK activity in cells treated with metformin or AICAr.

Representative immunoblots and corresponding densitometric analysis of phosphorylation state of AMPK and ACC proteins in differentiated H9c2 cells treated with or without 2 mM metformin or 1.2 mM AICAr for 8 hours in KH solution supplemented 5.5 mM glucose, 0.4 mM palmitic acid pre-bound to albumin and 10^{-7} M. Values are Mean ± SEM and are expressed as arbitrary density units. Each lane represents a different sample. N=3 per each group.

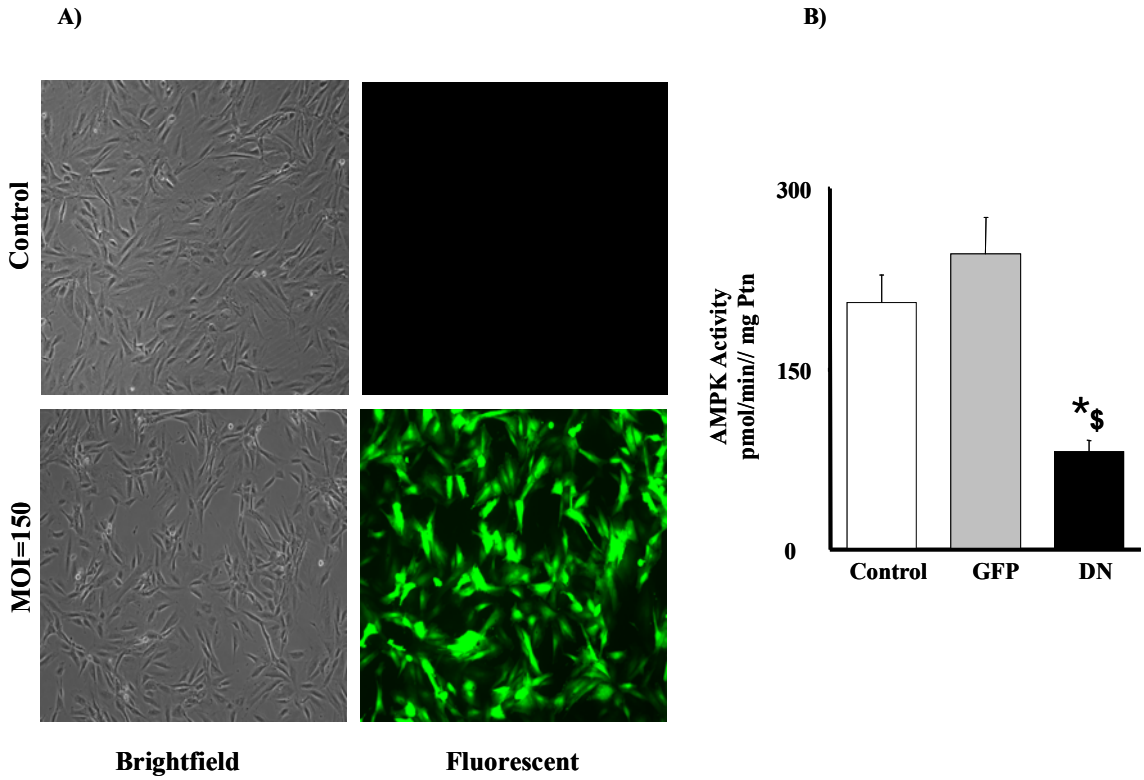

Figure 3-6: GFP expression and AMPK activity in cells treated with DN-AMPK.

(A) Representative images of GFP expression in differentiated H9c2 cells infected with DN-AMPK (moi 150) for 24 hours in 1 % HS DMEM. (B) AMPK activity in differentiated H9c2 cells infected with GFP or DN-AMPK (moi 150) for 24 hours in 1 % HS DMEM prior to treatment with or without 2 mM metformin in KH solution supplemented with fatty acid, glucose, and insulin for 8 hours. Activity values are Mean ± SEM. *, significantly different from Control, $p<0.05$; $, significantly different from GFP, $p<0.05$. N=6-12 per each group.

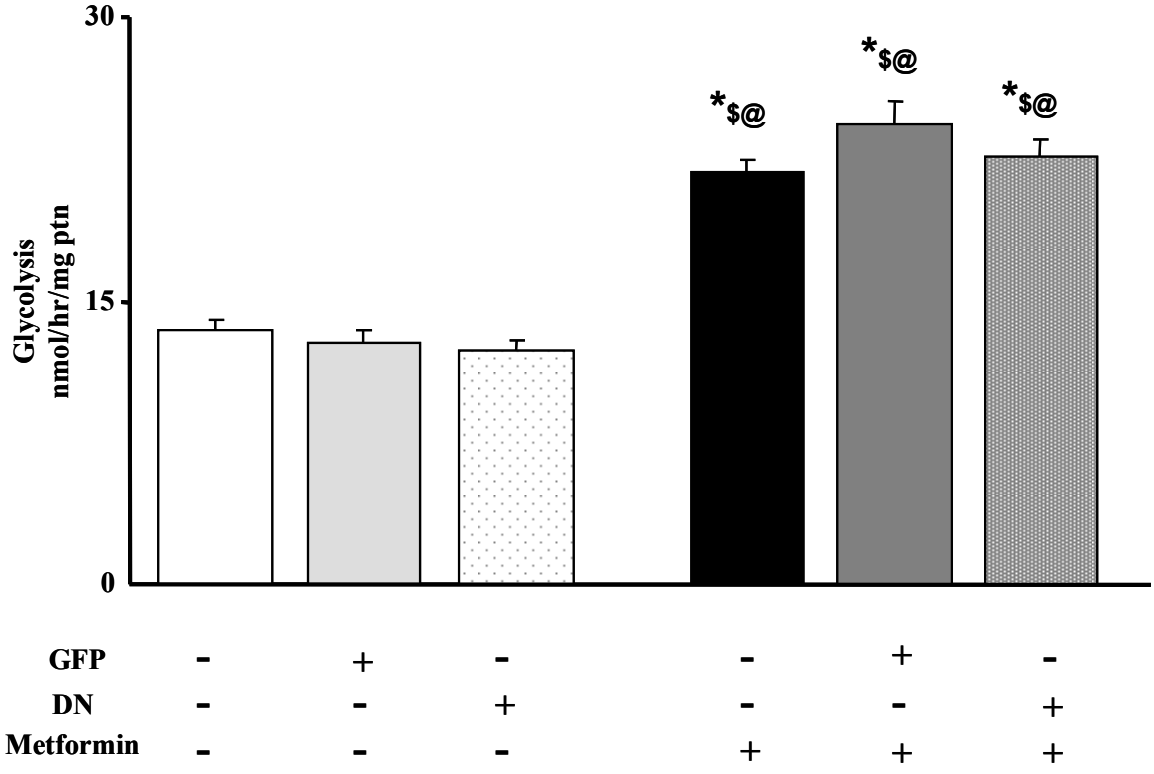

Figure 3-7: Rates of glycolysis in GFP or DN-AMPK treated H9c2 cells.

Differentiated H9c2 cells were treated with or without DN-AMPK (mio 150) or GFP (moi 150) for 24 hours in 1 % HS DMEM. Then cells were treated in presence or absence of 2 mM metformin for 8 hours in KH solution supplemented with 5.5 mM [5-^{3}H]-glucose, 0.4 mM palmitic acid pre-bound to albumin and 10^{-7} M insulin. Tracer amounts of labeled [5-^{3}H]-glucose (1.0 μCi/ml) was present at the last hour of study to measure rates of glycolysis. Values are Mean ± SEM. *, significantly different from Control, $p<0.05$; $, significantly different from, $p<0.05$; GFP; @ significantly different from DN-AMPK, $p<0.05$. N=22-24 per each group.

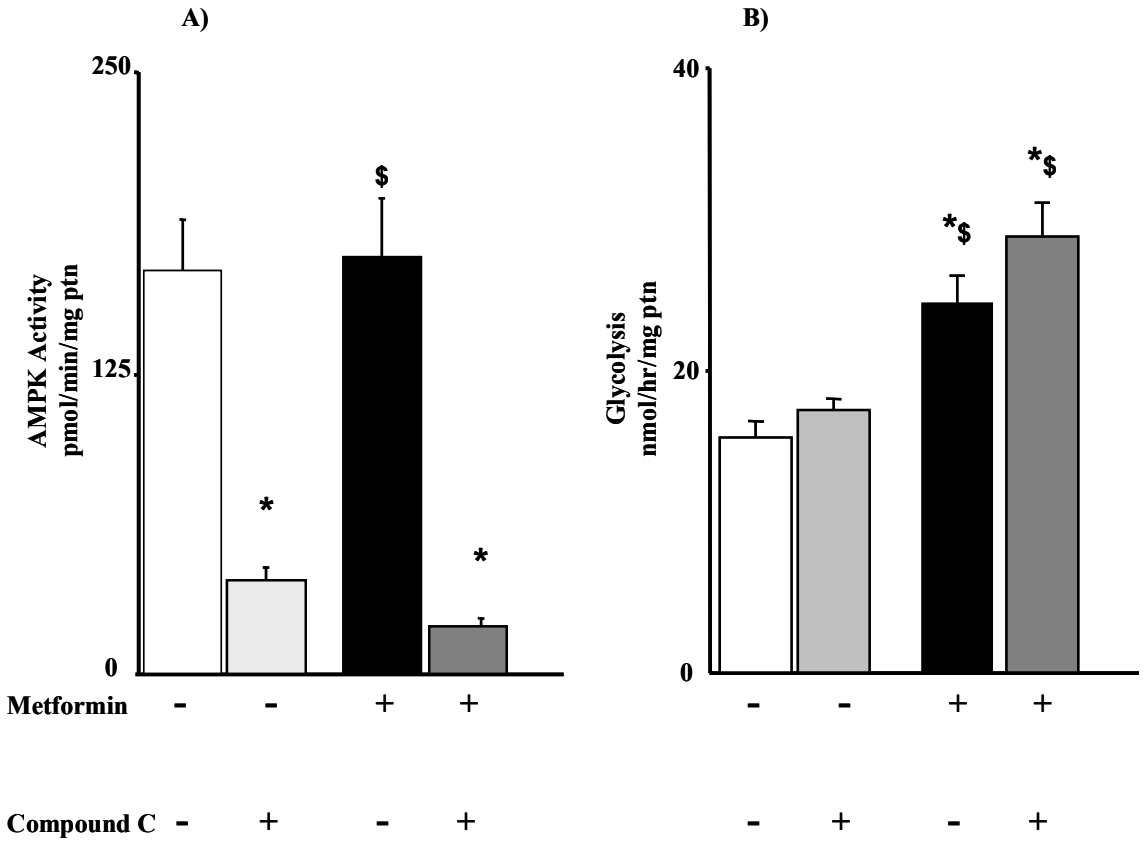

Figure 3-8: AMPK activity (A) and rates of glycolysis (B) in H9c2 cells incubated with or without Compound C.

Differentiated H9c2 cells were preincubated with or without 40 μM Compound C for 30 minutes in KH solution in the presence of 5.5 mM glucose, 0.4 mM palmitic acid pre-bound to albumin and 10^{-7} M insulin before 8 hours study in the presence or absence of 2 mM metformin. Tracer amounts of labeled [5-^{3}H]-glucose (1.0 μCi/ml) was present at the last hour of study to measure rates of glycolysis. White bar, Control treated H9c2 cells; light grey bar, Compound C treated H9c2 cells; black bar, metformin treated H9c2 cells; dark grey bar, Compound C plus metformin treated H9c2 cells. Values are Mean ± SEM. *, significantly different from Control, $p<0.05$; $, significantly different from Compound C, $p<0.05$. N=6 per each group.

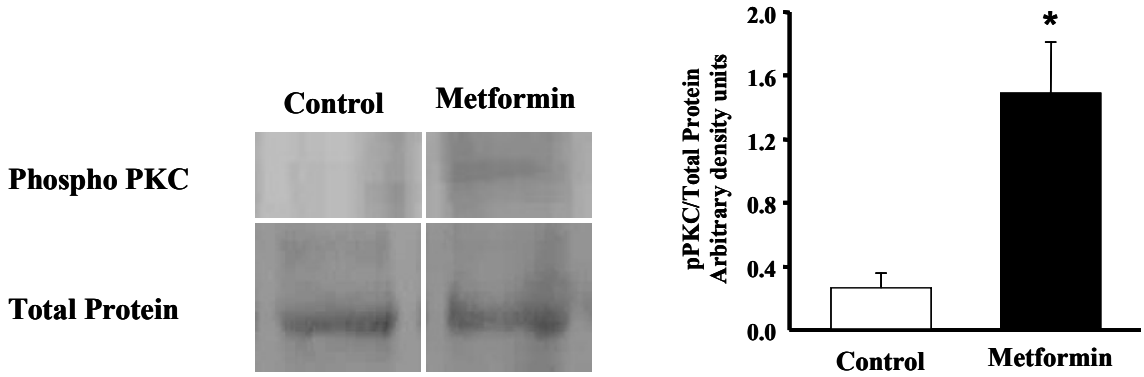

Figure 3-9: PKC expression in H9c2 cells treated with or without metformin.

Representative immunoblots and corresponding densitometric analysis of phosphorylation state of PKC protein in differentiated H9c2 cells treated with or without 2 mM metformin for 8 hours in KH solution containing 5.5 mM glucose, 0.4 mM palmitic acid pre-bound to albumin and 10^{-7} M insulin. Values are Mean ± SEM and are expressed as arbitrary density units. *, significantly different from Control, $p<0.05$. N=3 per each group.

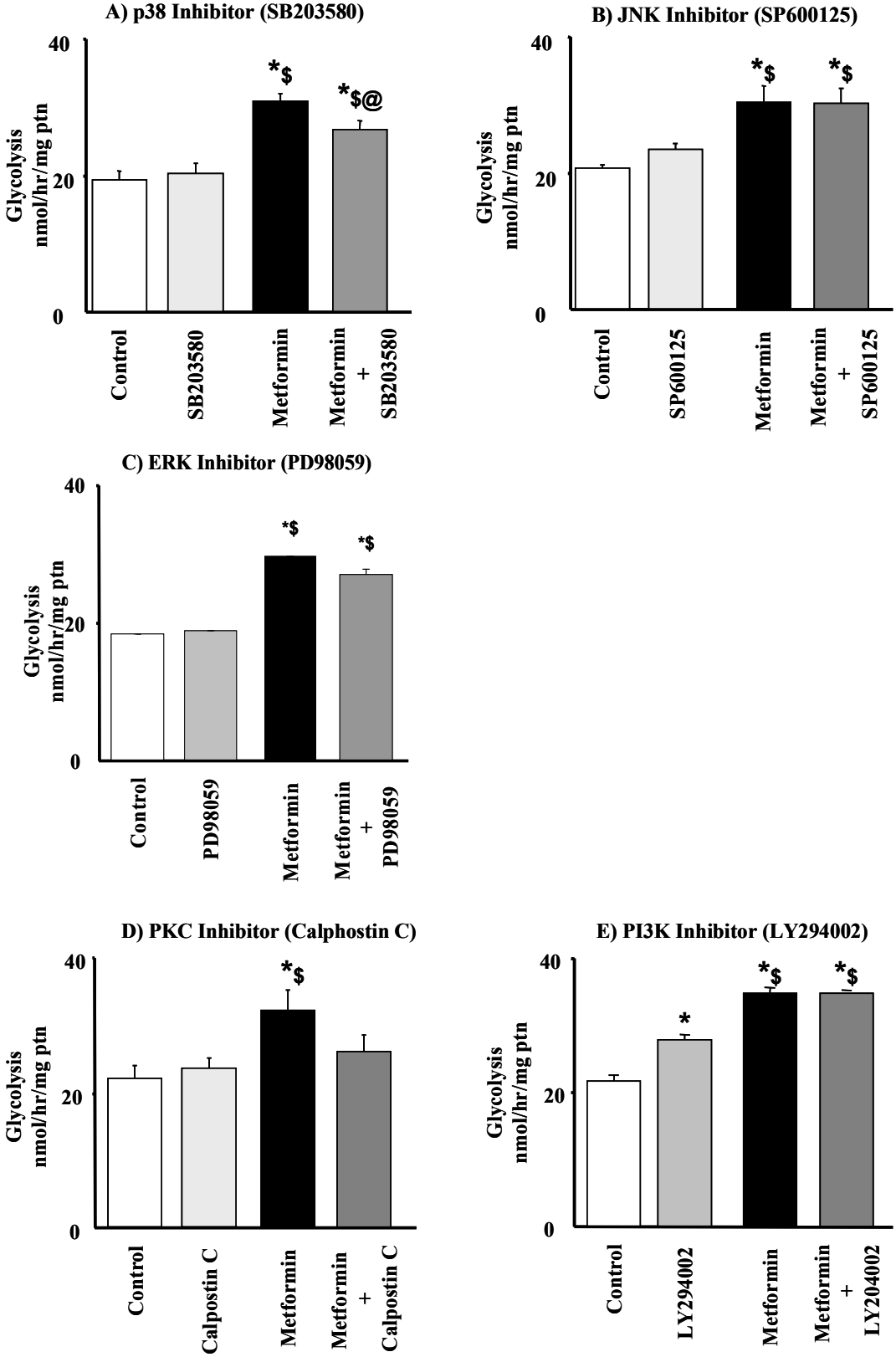
A) p38 Inhibitor (SB203580)
Glycolysis nmol/hr/mg ptn
40
20
0
*$
*$@
Control
SB203580
Metformin
Metformin + SB203580
B) JNK Inhibitor (SP600125)
Glycolysis nmol/hr/mg ptn
40
20
0
*$
*$
Control
SP600125
Metformin
Metformin + SP600125
C) ERK Inhibitor (PD98059)
Glycolysis nmol/hr/mg ptn
40
20
0
*$
*$
Control
PD98059
Metformin
Metformin + PD98059
D) PKC Inhibitor (Calphostin C)
Glycolysis nmol/hr/mg ptn
40
20
0
*$
Control
Calpostin C
Metformin
Metformin + Calpostin C
E) PI3K Inhibitor (LY294002)
Glycolysis nmol/hr/mg ptn
40
20
0
*
*$
*$
Control
LY294002
Metformin
Metformin + LY204002

Figure 3-10: Role of p38-MAPK, PKC, and PKB in the metabolic actions of metformin in H9c2 cells.

Differentiated H9c2 cells were preincubated with 10 μM SB203580 (P38 MAPK inhibitor) (A), 10 μM SP600125 (JNK MAPK inhibitor) (B) and 50 μM PD98059 (ERK MAPK inhibitor) (C), 10 nM Calphostin C (PKC inhibitor) (D), or 10 μM LY294002 (PI3K inhibitor) (E) or vehicle for 30 min before study in the presence or absence of 2 mM metformin. Tracer amounts of labeled [5-^{3}H]-glucose (1.0 μCi/ml) was present at the last hour of study to measure rates of glycolysis. White bar, Control treated H9c2 cells; light grey bar, inhibitor treated H9c2 cells; black bar, metformin treated H9c2 cells; dark grey bar, inhibitor plus metformin treated H9c2 cells. Values are Mean ± SEM. *, significantly different from Control, $p<0.05$; $, significantly different from inhibitor, $p<0.05$; @ significantly different from metformin, $p<0.05$. N=6-15 per each group.

CHAPTER 4 - AMP-ACTIVATED PROTEIN KINASE INFLUENCES METABOLIC REMODELING IN HYPERTROPHIED HEART-DERIVED H9c2 CELLS

4.1 INTRODUCTION

AMP-activated protein kinase (AMPK) has a major role in control and regulation of energy metabolism [29, 36]. AMPK is activated by physiologic or pathologic stresses that deplete cellular high energy phosphate including ATP and phosphocreatine [29, 36]. Additionally, it has been shown that AMPK can be activated by hormones acting through Gq-linked coupled receptors and a calcium-dependent pathway independent of any changes in energy status of the cells [36, 158]. Once activated, AMPK acts on substrates that mediate inhibition of energy consuming pathways and stimulation of energy producing pathways, such as fatty acid oxidation, glucose uptake, and glycolysis [29, 36].

Activation of AMPK stimulates fatty acid oxidation in muscle by increasing uptake of fatty acids across the cell and mitochondrial membranes [29, 36, 50]. The effects of AMPK on glucose metabolism in the heart are accounted for by enhanced translocation

of glucose transport proteins to the sarcolemma [36, 239] and by stimulation of glycolysis through phosphorylation and activation 6-phosphofructo-2-kinase (PFK-2) [86]. The consequent increase in F26P2 leads to allosteric activation of PFK-1, a major determinant of glycolytic rates in the heart and other tissues [86].

Substrate use switches from fatty acids toward glucose in pressure-overload induced cardiac hypertrophy with an acceleration of glycolysis being characteristic [24, 34, 35, 112, 119]. We and others have shown that AMP-activated protein kinase (AMPK) is activated in hypertrophied hearts [34, 35, 119]. Furthermore, we have shown that accelerated rates of glycolysis are normalized when fatty acid oxidation is elevated to rates similar to those in non-hypertrophied hearts [35]. At the same time, elevated activity of AMPK in hypertrophied hearts is correspondingly reduced to values observed in non-hypertrophied hearts. These data provide correlative support for the view that AMPK is responsible for the acceleration of glycolysis in hypertrophied hearts, although this concept is not yet proven conclusively.

The current investigation was designed to directly test the hypothesis that AMPK is responsible for the acceleration of glycolysis in hypertrophied heart muscle cells. Cultured H9c2 cells [347] were used as an *in vitro* model of cardiac myocyte hypertrophy in which AMPK activity was selectively altered by pharmacologic and molecular approaches.

4.2 METHODS

Details of methods used for this investigation are described in the Materials and Methods chapter.

4.3 RESULTS

4.3.1 AVP Causes Hypertrophy and Metabolic Remodelling of H9c2 Cells

Cellular hypertrophy was evaluated by measuring changes in protein synthesis and content as well as the expression of atrial natriuretic factor (ANF), a well-recognized marker of pathologic cardiac hypertrophy [348, 349]. Exposure of H9c2 cells to AVP for 48 hours significantly increased protein content (Figure 4-1A), [^{14}C]-phenylalanine incorporation (Figure 4-1B), and ANF expression (Figure 4-1C) without changes in overall cell number (51.5 x 10^4 ± 0.8 vs. 52.3 x 10^4 ± 0.5 cells /60 mm dish, N=12 per group, p=NS), indicating that H9c2 cells exhibited several major characteristics of hypertrophy following treatment with AVP. Furthermore, rates of glycolysis were accelerated approximately 50 % in H9c2 cells following AVP treatment for 48 hours (Figure 4-2A). There were no significant differences in glucose oxidation between hypertrophied H9c2 cells and non-hypertrophied cells (Figure 4-2B). However, rates of fatty acid oxidation were significantly decreased in hypertrophied H9c2 cells as compared to non-hypertrophied cells (Figure 4-2C). Notably, when H9c2 cells were treated with AVP for 48 hours and then the AVP was removed, the accelerated rates of glycolysis persisted for at least a further 48 hours after AVP removal (Figure 4-3). Rates of glycolysis were accelerated in H9c2 cells hypertrophied by AVP, regardless of the presence or absence of fatty acid and insulin as it is summarized in Table 4-1. And these changes persisted for two days after removal of AVP.

4.3.2 Cellular Content of Selected Metabolic Proteins

Accompanying the changes in substrate use, expression of several key proteins involved in the control of glucose metabolism was altered in hypertrophied H9c2 cells. Specifically, expression of HKII was significantly increased, while that of GLUT-4 and PDC E1α was slightly but significantly decreased (Figure 4-4).

4.3.3 Inhibition of AMPK Reduces Glycolysis in Hypertrophied H9c2 Cells

AMPK activity was significantly elevated in hypertrophied H9c2 cells (Figure 4-5A), while expression of the catalytic subunits of AMPK did not differ significantly between the two groups (Figure 4-5B).

In order to determine if AMPK is responsible for the accelerated rates of glycolysis observed in H9c2 cells hypertrophied by AVP, two approaches were used to suppress AMPK activity. In one approach, AMPK was inhibited by expression of a dominant negative construct (DN-AMPK) and in the alternate pharmacological approach, AMPK was inhibited by incubating cells with Compound C. Exposure of H9c2 cells to adenovirus containing DN-AMPK (moi 150) for 24 hours resulted in nearly 100 % gene transfer as demonstrated by expression of GFP (Figure 4-6A). Compared to cells that were untreated or that were treated with the control GFP construct, AMPK activity was significantly reduced in H9c2 cells infected with DN-AMPK (Figure 4-6B). The effectiveness of inhibition of AMPK was confirmed by the substantial abrogation of the acceleration of glycolysis caused by mannitol and oligomycyin, two potent activators of AMPK (Table 4-2) [206, 350]. Likewise, Compound C resulted in a substantial reduction in AMPK activity (Figure 4-6B), a reduction that was significantly greater than that caused by DN-AMPK.

Inhibition of AMPK after induction of hypertrophy in H9c2 cells by AVP caused a partial suppression of glycolysis in hypertrophied H9c2 cells by both DN-AMPK (Figure 4-7A) and Compound C (Figure 4-7B) such that rates of glycolysis were still 30 to 50 % higher than non-hypertrophied H9c2 cells. Interestingly, suppression of glycolytic rates was relatively greater for Compound C (26 ± 0.02 % reduction) than for DN-AMPK (16 ± 0.04 % reduction), a finding that likely reflects the greater reduction in AMPK activity caused by Compound C (Figure 4-6B).

4.3.4 Myocardial High Energy Phosphates

The content of ADP in H9c2 cells hypertrophied with AVP was significantly elevated as compared to Controls (Table 4-3). There was no significant difference in free AMP between hypertrophied and Control groups (Control, 2.09 ± 0.33 vs. hypertrophied, 2.43 ± 0.56 μmol/l, N=7, P=NS).

4.3.5 Other Hypertrophic Agents

Treatment of H9c2 cells with other GqCR hypertrophic agents, which are shown to induce hypertrophy in adult and neonatal cardiac myocytes, such as endothelin-I (ET-1) [351-354] and phenylephrine (PhE) [280, 355, 356], and angiotensin II (AgII) [351, 353, 357] under different conditions as mentioned in table legends were unable to induce cardiac hypertrophy (Table 4-4 & 5).

4.4 DISCUSSION

In the present study, arginine vasopressin (AVP) induced cellular hypertrophy in H9c2 cells with parallel metabolic remodeling of heart muscle cells characterized by an acceleration of glycolysis and a reduction in long-chain fatty acid oxidation. The metabolic changes observed in hypertrophied heart muscle cells resembled those observed in intact hypertrophied hearts and included alterations in the expression of metabolic proteins and activation of AMPK. For the first time it was shown that the acceleration of glycolysis in hypertrophied heart muscle cells was only partially dependent on AMPK, a finding that indicates factors in addition to AMPK are involved. Of interest, these data demonstrated that AMPK was activated in hypertrophied cells without any measurable changes in the energy status of the cells, indicating that AMPK was activated by energy-independent mechanisms.

The present study showed that AVP caused metabolic remodeling of heart muscle cells, in addition to causing cellular hypertrophy. AVP is well recognized for its ability to

cause hypertrophy of heart muscle cells [292, 305, 358] by way of the V1a receptor [358]. Here it was demonstrated that prolonged exposure to AVP for 48 hours induced hypertrophy which was accompanied by an increase in protein content and synthesis as well as elevation of ANP expression (Figure 4-1), without changes in overall cell number. AVP is released from the posterior pituitary and affects body fluid balance and vascular tone [359, 360]. Levels of AVP are significantly and chronically elevated in the plasma of patients and experimental animals with heart failure [302-304]. Moreover, the heart possesses a local AVP system that is activated in response to pressure-overload and may act in a paracrine or autocrine fashion [361]. Systemic and myocardial AVP may therefore contribute to structural remodeling of the failing heart.

Of interest, this study showed that, besides causing hypertrophy, AVP led to metabolic remodeling of H9c2 cells. Specifically, glycolysis was accelerated and long-chain fatty acid oxidation was reduced in hypertrophied H9c2 cells with no significant alteration in glucose oxidation (Figure 4-2 & Table 4-1). That metabolic alteration in hypertrophied H9c2 cells persisted for at least 2 days after removal of AVP (Figure 4-3 & Table 4-1) indicate that the changes are related to a chronic remodeling process. The metabolic alterations observed in hypertrophied H9c2 cells recapitulate those seen in intact hearts from experimental animal models of cardiac hypertrophy induced by pressure-overload [24, 34, 35, 119].

In animal models of pressure-induced cardiac hypertrophy, acceleration in rates of glycolysis has been consistently observed [20, 23, 35, 110, 119]. Currently, the mechanisms responsible for the acceleration of glycolysis in hypertrophied hearts are not yet fully understood. Here it was shown that expression of selected proteins involved in the control of glucose catabolism in the heart and other tissues was altered in hypertrophied H9c2 cells, including an increase HKII expression and a slight decrease in expression of GLUT-4 (Figure 4-4). Similar changes in activity or expression of these proteins have been observed in some experimental models of hypertrophy in the intact heart [154] and heart failure [120], but not all studies have

given consistent results [24, 117, 362]. For instance, hexokinase activity is elevated in pressure-overload models of right [154] and left [120] ventricular hypertrophy in ferrets and rabbits but decreased or unchanged in hypertrophied hearts from rats [24, 362, 363]. GLUT-4 expression has also been reported not to change [24, 362] or to decrease [119, 34]. Therefore, accelerated rates of glycolysis in hypertrophied hearts cannot easily be explained by changes in expression of relevant glycolytic enzymes, suggesting that other mechanisms such as sarcolemmal translocation of glucose transporters and activation of relevant enzymes by allosteric or covalent modification are responsible for acceleration of glycolysis observed in these hearts.

By way of effects on glucose transporter translocation and the reaction catalyzed by phosphofructokinase, increased activity of AMPK in hypertrophied H9c2 cells provides a potential explanation for the acceleration of glycolysis observed. AMPK activity has been shown to increase in intact hearts hypertrophied by pressure-overload [34-35, 119] and to correlate to rates of glycolysis [35]. Additionally, we have found that accelerated rates of glycolysis in hypertrophied rat hearts were normalized by a combination of long-chain (palmitate) and medium-chain (octanoate) fatty acids as substrates, a combination that elevates fatty acid oxidation and results in overall fatty acid oxidation rates in hypertrophied hearts similar to those in non-hypertrophied hearts [35]. Interestingly, elevated activity of AMPK in hypertrophied hearts is also reduced to values observed in non-hypertrophied hearts, an outcome that provides strong support for the concept that AMPK is involved in alterations in glucose use in cardiac hypertrophy.

However, inhibition of glycolysis in hypertrophied H9c2 cells by complementary pharmacological and molecular approaches (Figures 4-6 and 4-7), which were used to inhibit AMPK activity, unequivocally demonstrate that AMPK contributes to the acceleration of glycolysis in hypertrophied heart muscle cells. Of significance, accelerated rates of glycolysis induced by administration of oligomycin and mannitol, two potent activators of AMPK, were completely blocked in cells treated with DN-

AMPK (Table 4-2). That inhibition of AMPK failed to completely normalize rates of glycolysis (Figure 4-7) suggests other factor(s) contribute to the increased glycolysis in this setting.

As discussed earlier, AMPK, a key intracellular energy sensor, is activated by metabolic stresses which deplete cellular high energy phosphate including ATP and phosphocreatine [29, 36, 157, 163]. LKB1 is recognized as a major upstream kinase involved in activation of AMPK in response to increased in AMP/ATP ratio in the heart [214] and other tissues [161]. Additionally, AMPK can also be activated by hormones acting through Gq-coupled receptors (GqCR) and a calcium-dependent pathway through calcium/calmodulin-dependent protein kinase kinases (CaMKK) [36, 158, 163, 206, 207] and long-chain fatty acids [202, 325], without any measurable changes in energy status of the cell.

The current study showed that AMPK was activated in hypertrtrophied H9c2 cells without any measurable changes in the energy status of the cells (Table 4-3), including no significant changes in creatine phosphate, ATP, or free AMP among groups. This indicates that AMPK is activated independent of changes in energy status of cells, consistent with our previous study [35] in which we have demonstrated that, in pressure-overarload induced cardiac hypertrophy, myocardial AMPK is activated by energy-independent mechanisms; however, it contradicts other studies [34] showing that AMPK is activated in hypertrophied hearts by energy-dependent mechanisms. This discrepancy between our finding and others is likely due to the different experimental models used as well as the severity of hypertrophy. In the current study and our previous study, in which abdominal aortic constriction was used as a model of pressure-overload induced cardiac hypertrophy [35], we observed a mild form of cardiac hypertrophy (~ 20-25 % increase in total protein or mass) without any signs of decompensation. However, the thoracic aortic constriction model used by others [34, 119] resulted in a moderate form of cardiac hypertrophy (~50 % increase in total heart mass) with signs of ventricular dysfunction, and a reduced myocardial energy status.

These findings indicate that, in hypertrophied hearts, AMPK is activated irrespective of the energy status of the cell. The exact mechanisms of activation of AMPK in hypertrophied hearts remain unclear. However, it could be speculated that the energy status-dependent activation of AMPK is possibly mediated by LKB-1; while the energy status-independent activation of AMPK is possibly mediated by a calcium and CaMKK-dependent pathway downstream of GqCRs [158, 364]. The latter also has a role in the development of cardiac hypertrophy [72]. In this regard, the agent used in the current study, AVP, belongs to the GqCR protein superfamily and is well known to induce cardiac hypertrophy [292, 305]. While the role of CaMKK in the control of AMPK is not investigated in the current study, we have previously shown that there are no significant changes in expression of CaMKK in hypertrophied hearts as compared to non-hypertrophied hearts [35]. This indicates that if CaMKK is involved in AMPK activation in hypertrophied hearts, it does so independent of any changes in protein expression. Future studies will be required to investigate the potential role of CaMKK in this setting.

In addition to CaMKK, another mechanism that might modulate AMPK activity is LCFA. While several studies have indicated that LCFA activates AMPK [202, 325], others have shown that it inhibits AMPK activity [365]. Since LCFA oxidation is significantly reduced and glycolysis is accelerated in hypertrophied hearts [23, 24, 33, 35, 104, 110-112, 125, 148, 313, 366] and the fact that enhancement of fatty acid oxidation normalized AMPK activity and glycolysis [35], it is conceivable that LCFA might have a role in activation of AMPK in hypertrophied hearts. However, future studies need to confirm this idea.

Consistent with previous studies, the current study showed that rates of fatty acid oxidation were reduced in hypertrophied H9c2 cells as compared to controls (Figure 4-2). The exact mechanism responsible for this reduction in LCFA oxidation is unknown. However, a reduction in the expression of enzymes and proteins involved in myocardial fatty acid uptake/transport [53, 140] and fatty acid oxidation [146, 149] as well as low

levels of myocardial carnitine [5, 104, 106, 125] have been described as being responsible. Therefore, unlike non-hypertrophied hearts in which AMPK activation results in increased in rates of fatty acid oxidation, in hypertrophied hearts these changes restrict oxidation of LCFA.

The present study also demonstrated that rates of glucose oxidation in hypertrophied H9c2 cells did not differ from Controls (Figure 4-2). This finding is consistent with previous studies [19, 20, 30, 31, 33, 35, 104, 125] indicating that in hypertrophied hearts rates of glucose oxidation are similar to or even lower than of non-hypertrophied hearts. In fact, those studies have suggested that glucose oxidation is limited in hypertrophied hearts. This study also demonstrated that in hypertrophied H9c2 cells, expression of pyruvate dehydrogenase complex (PDC E1α) was slightly but significantly decreased (Figure 4-4). Regulation of PDC is crucial in determining the rates of glucose oxidation in the heart. However, in contrast with current study, significant differences in expression of PDC isoforms have not been observed in the intact hypertrophied heart [24, 89, 153]. Of interest, those studies have documented that while total PDC expression is unchanged in hypertrophied hearts, the fraction of PDC active was higher in these hearts, which contrasts with the lower fractional oxidation of glucose. Collectively, these findings indicate that while changes commonly occur in expression of proteins and enzymes controlling glucose use in hypertrophied heart muscle, the direction and extent of change that occurs is highly model dependent.

Treatment of cells with other GqCR agonists, including ET-1, AgII, and PhE, under the same conditions in which AVP caused hypertrophy, were unable to induce hypertrophy (Tables 4-4 and 4-5). Several studies have shown that treatment of cardiac myocytes with retinoic acid inhibits GqCR agene-induced cardic hypertrophy [357, 367]. Therefore, we performed additional studies in which H9c2 cells were differentiated in the absence of retinoic acid; however, under these conditions, those agents were also unable to induce hypertrophy in H9c2 cells (Tables 4-4 and 4-5). The reasons that these agents failed to induce hypertrophy is not known. It is possible that H9c2 cells lack

some post-receptor signaling transduction pathways that ultimately result in cardiac hypertrophy. Alternatively, longer incubation times may be needed for these agents to cause hypertrophy in H9c2 cells.

In conclusion, a cellular model of cardiac hypertrophy was established with altered metabolic phenotype resembling that observed in intact hypertrophied hearts and that includes alterations in the expression of metabolic proteins and activation of AMPK without changes in energy status. In this model, AMPK has a partial role in hypertrophic metabolic remodeling. Additionally, administration of several other GqCR agonists which are well known to induce cardiac hypertrophy, were unable to induce hypertrophy in H9c2 cells.

Table 4-1: Rates of glycolysis in H9c2 cells hypertrophied by exposure to AVP.

Differentiated H9c2 cells were treated with or without AVP (1 μM) for 48 hours in DMEM/F12 containing 0.5 % HS. Rates of glycolysis were measured over 2 days after removal of AVP as described in Materials and Methods chapter. Tracer amounts of labeled [5-^{3}H]-glucose (1.0 μCi/ml) was present for the last hour of study to measure rates of glycolysis. Values are Mean ± SEM and are expressed as nmol glucose/hr/mg protein. N=5 per group. *, significantly different from Controls, $p<0.05$.

	Glycolysis (nmol/hr/mg ptn) (Day 1)	**Glycolysis** (nmol/hr/mg ptn) (Day 2)
DMEM		
Control	3.74 ± 0.38	5.03 ± 0.54
AVP	7.65 ± 0.95 *	7.52 ± 0.72 *
DMEM + Oleic acid + Insulin		
Control	2.24 ± 0.61	2.44 ± 0.31
AVP	7.98 ± 0.68 *	6.01 ± 0.46 *

Table 4-2: Effect of DN-AMPK on glycolysis stimulated by oligomycin and mannitol.

Differentiated H9c2 cells treated with DN-AMPK (moi 150) for 24 hours before challenging with oligomycin or mannitol for 25 minutes. Values are Mean ± SEM and are expressed as nmol glucose/hr/mg protein. Numbers are 3-14 per group. DN-AMPK, Adenoviral-mediated transfer of dominant-negative AMP-activated protein kinase. *, significantly different from corresponding no treatment group; $ significantly different from corresponding no DN-AMPK $p<0.05$.

Treatment	No DN-AMPK	DN-AMPK
None	4.23 ± 0.29 (N=14)	4.72 ± 0.33 (N=13)
Oligomycin (0.5 µM)	9.65 ± 2.22* (N=3)	6.10 ± 0.11*$ (N=3)
Mannitol (0.5 mM)	10.08 ± 1.02* (N=3)	4.94 ± 1.35$ (N=3)

Table 4-3: Myocardial adenine nucleotide and creatine phosphate content in H9c2 cells.

Differentiated H9c2 cells were treated with AVP (1 μM) for 48 hours in DMEM/F12. Then cells were treated for 8 hours in KH solution supplemented with 5.5mM glucose, 0.4 mM palmitic acid pre-bound to albumin and 10^{-7} M insulin. Values are Mean ± S.E.M. *, significantly different from Control value within Group, $p<0.05$.

	Control (n=7)	**Hypertrophied** (n=8)
ATP (μmol/mg protein)	2.78 ± 0.19	2.88 ± 0.08
ADP (μmol/mg protein)	0.32 ± 0.02	0.42 ± 0.03 *
AMP (μmol mg protein)	0.07 ± 0.01	0.08 ± 0.01
Creatine (Cr) (μmol/mg protein)	28.43 ± 1.21	22.01 ± 3.22
Phosphocreatine (PCr) (μmol/mg protein)	8.35 ± 0.43	6.89 ± 0.59

Table 4-4: Effects of other hypertrophic agents on total protein content in H9c2 cells. H9c2 cells were differentiated with or without retinoic acid. Then cells were treated with different concentrations of hypertrophic agents (phenylephrine (PhE), angiotensin (AgII), and endothelin-1 (ET-1)) in DMEM/F12 for 48 hours. Values are normalized to 100 % Control (in total mg protein). Values are Mean ± SEM. N=3-6 per group.

	(+) Retinoic acid			(-) Retinoic acid		
Concentration	PhE	Ag II	ET I	PhE	Ag II	ET I
Control	100.1±3.3	100.1±1.6	99.9±2.7	99.9±6.1	100.1±2.7	100.7±4.0
10^{-4}	105.7±0.6			84.5±2.4		
10^{-5}	99.4±7.3	109.2±0.4		80.3±2.3	116.6±3.8	
10^{-6}	104.0±1.2				99.2±3.4	
10^{-7}		99.4±1.9	103.0±1.9		106.0±4.1	103.1±5.5
10^{-8}			100.2±3.0		97.8±1.7	104.6±7.1
10^{-9}		97.1±2.3	104.7±1.7		107.0±2.2	110.6±0.8
10^{-10}					99.6±0.5	
10^{-11}					105.4±1.8	
10^{-12}					104.1±2.5	

Table 4-5: Effects of other hypertrophic agents on total protein content in H9c2 cells. H9c2 cells were differentiated with or without retinoic acid. Then cells were treated with different concentrations of hypertrophic agents (phenylephrine (PhE), angiotensin (AgII), and endothelin-1 (ET-1)) in DMEM for 48 hours. Values are normalized to 100 % Control (in total mg protein). Values are Mean ± SEM. N=3-6 per group.

	(+) Retinoic acid		(-) Retinoic acid	
Concentration	PhE	Ag II	PhE	Ag II
Control	100.0±0.4	100.0±4.4	100.1±.7	99.9±0.4
10^{-4}	103.0±1.6		108.9±2.7	
10^{-5}	107.8±2.4	100.7±1.8	109.8±4.4	95.4±4.2
10^{-6}	93.8±1.9	109.6±2.7	102.7±2.2	96.0±1.7
10^{-7}		102.8±5.6		102.6±1.9
10^{-8}		103.4±1.9		81.7±23.0
10^{-9}		106.8±1.9		97.4±5.3
10^{-10}		103.1±0.7		98.6±1.8
10^{-11}		104.8±0.4		101.8±1.8
10^{-12}		98.5±2.0		98.3±0.7

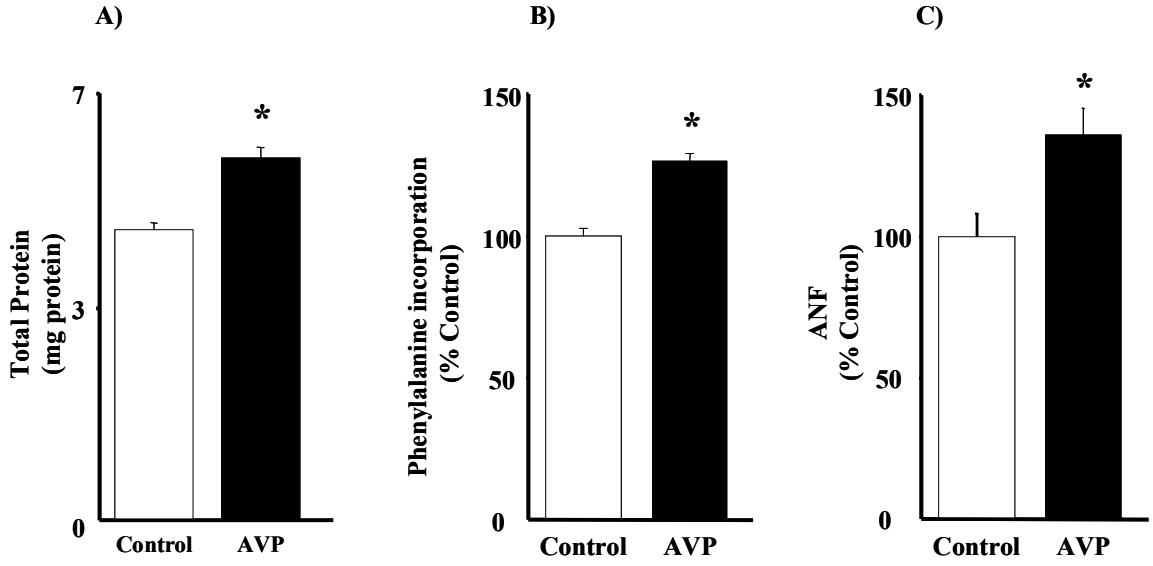

Figure 4-1: Hypertrophic characteristic in response to chronic treatment with AVP.

Total protein content (Panel A), [^{14}C]-phenylalanine incorporation (Panel B), and ANF expression (Panel C) were measured in differentiated H9c2 cells treated with to AVP (1 μM) for 24-48 hours in DMEM/F12 supplemented with 0.5 % HS. Values are normalized to 100 % Control. Values are given as Mean ± SEM. *, significantly different from Control ($p<0.05$). N=7-22 per group.

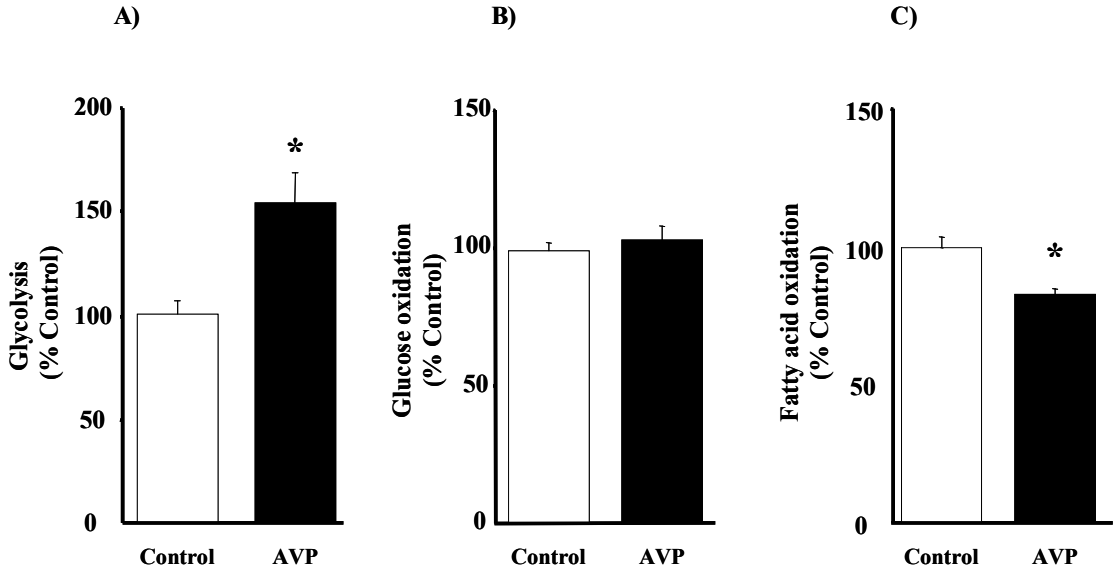

Figure 4-2: Effects of AVP on H9c2 cells metabolism.

Differentiated H9c2 cells were treated for 48 hours with (black bar) or without (white bar) AVP (1 μM) in DMEM/F12 supplemented with 0.5 % HS. Then cells were treated for 8 hours in KH solution supplemented with 5.5mM [5-^{3}H/U-^{14}C]-glucose, 0.4 mM [U-^{14}C]-palmitic acid pre-bound to albumin and 10^{-7} M insulin to measure rates of glycolysis (Panel A), glucose oxidation (Panel B), and fatty acid oxidation (Panel C), respectively. All the labeled mterials were added at the last hour of study. Values are normalized to 100 % Control (in nmol/hr/mg ptn). Values are Mean ± SEM. *, significantly different from Control ($p<0.05$). N=4-14 per group.

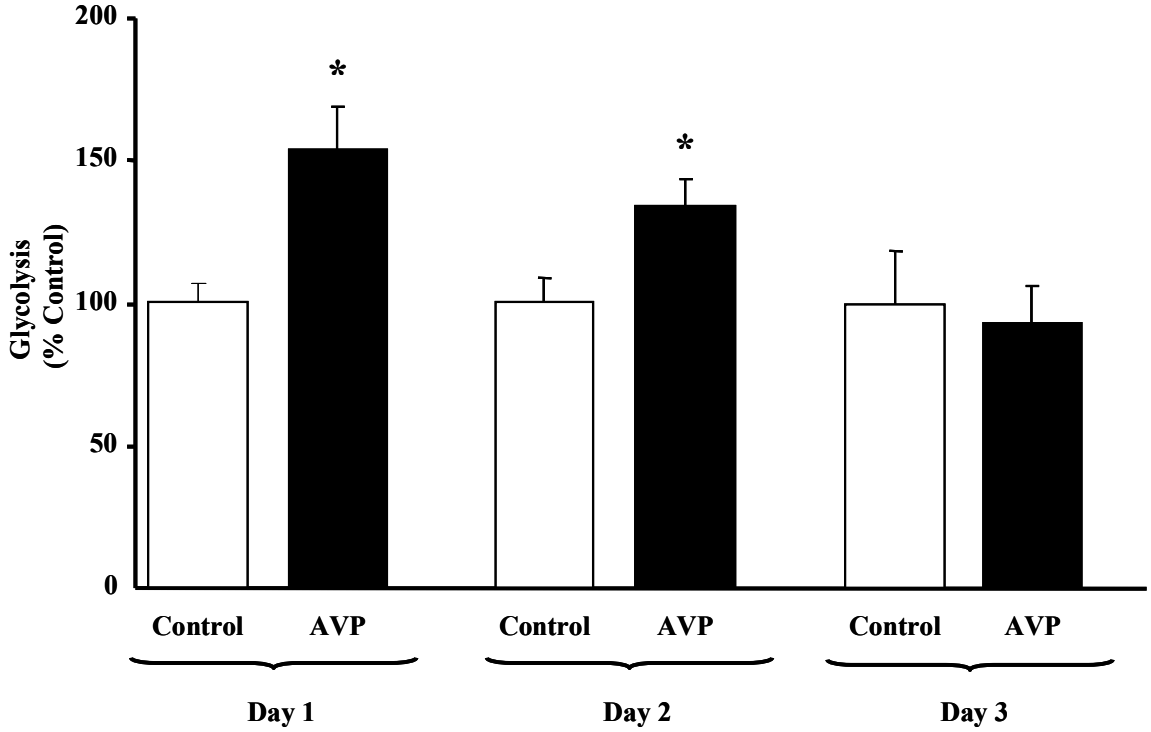

Figure 4-3: Time course of changes in glycolysis in H9c2 cells after removal of AVP.

Differentiated H9c2 cells were treated with to AVP (1 μM) for 48 hours in 0.5 % HS DMEM/F12. Rates of glycolysis were measure over 3 days in in KH solution supplemented with 5.5 mM glucose, 0.4 mM palmitic acid pre-bound to albumin and 10^{-7} M insulin. Tracer amounts of labeled [5-^{3}H]-glucose (1.0 μCi/ml) was present at the last hour of study to measure rates of glycolysis. Values are normalized to 100 % Control (in nmol/hr/mg ptn). Values are Mean ± SEM. *, significantly different from Control ($p<0.05$). N=7-14 per group.

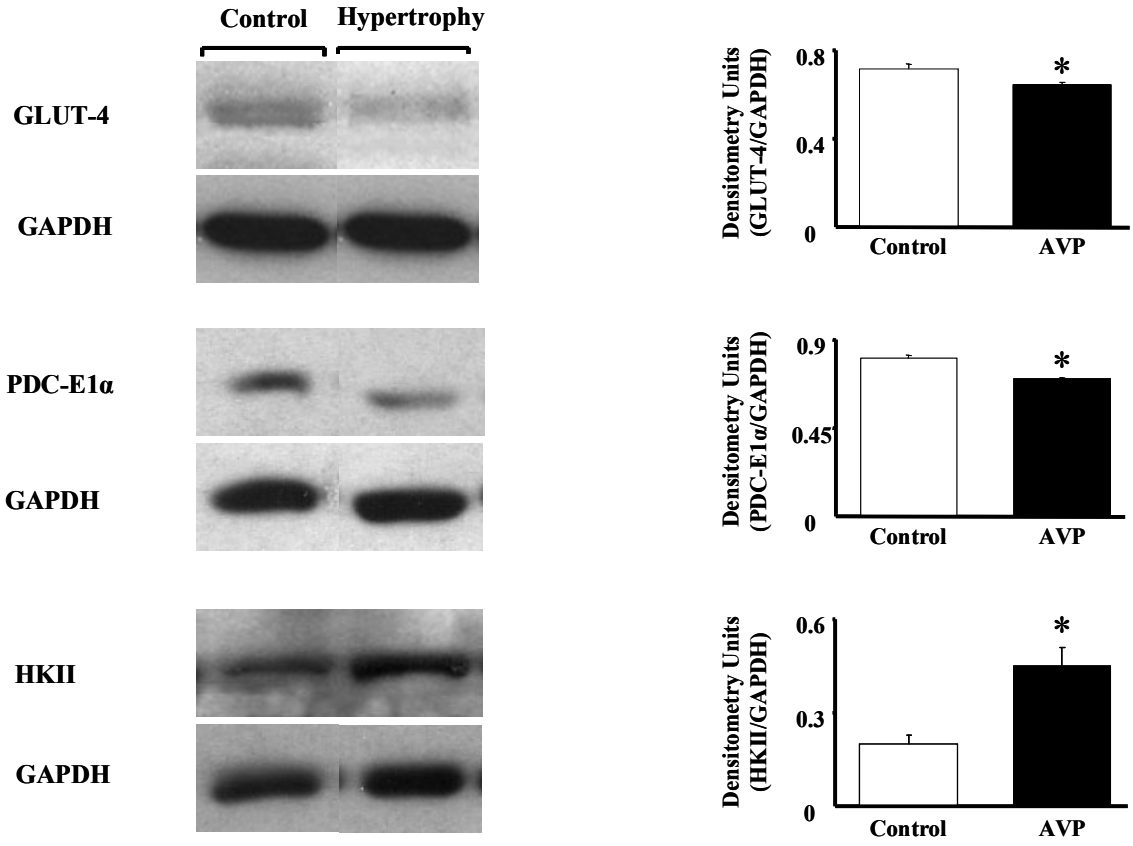

Figure 4-4: Effects of AVP on expression of proteins involved in glucose metabolism in H9c2 cells.

Differentiated H9c2 cells were treated with AVP (1 μM) for 48 hours in 0.5 % HS DMEM/F12. Expression of several enzymes in glycolytic pathways were determined. Glucose transporter-4 (GLUT-4). HexokinaseII, (HKII). E1-alpha subunit of pyruvate dehydrogenase, (PDC-E1-a). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard to calculate relative expressions. Each lane represents a separate dish of cells. Values are Mean ± SEM. N=3 per each group. *, significantly different from Control ($p<0.05$).

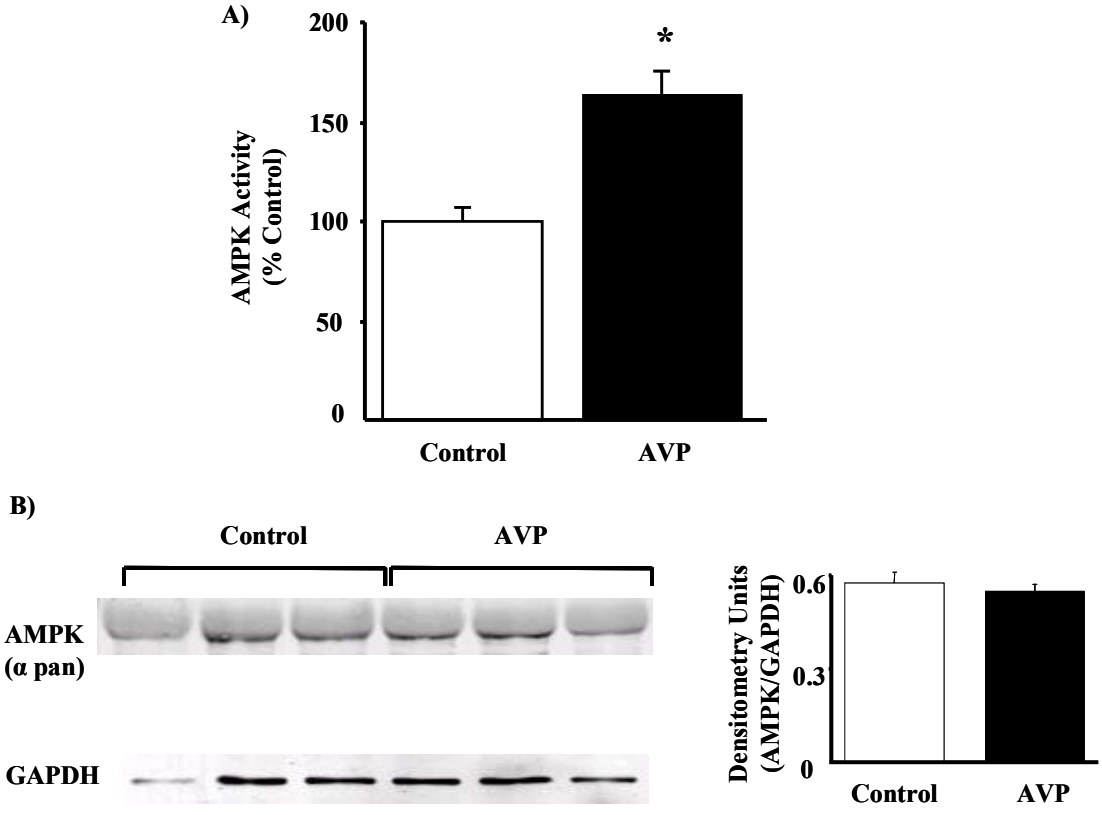

Figure 4-5: Effects of AVP on activity and expression of AMPK.

Differentiated H9c2 cells treated with (Black Bar) or without (White Bar) AVP (1 μM) for 48 hours in 0.5 % HS DMEM/F12. Then cells were treated with KH solution containing 5.5mM glucose, 0.4 mM palmitic acid pre-bound to albumin and 10^{-7} M insulin before measuring AMPK activity (Panel A) or expression (Panel B). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard to calculate relative expression. Values are normalized to 100 % Control (in pmol/hr/mg ptn). Values are Mean ± SEM. *, significantly different from Control ($p<0.05$). N=16-28 per group.

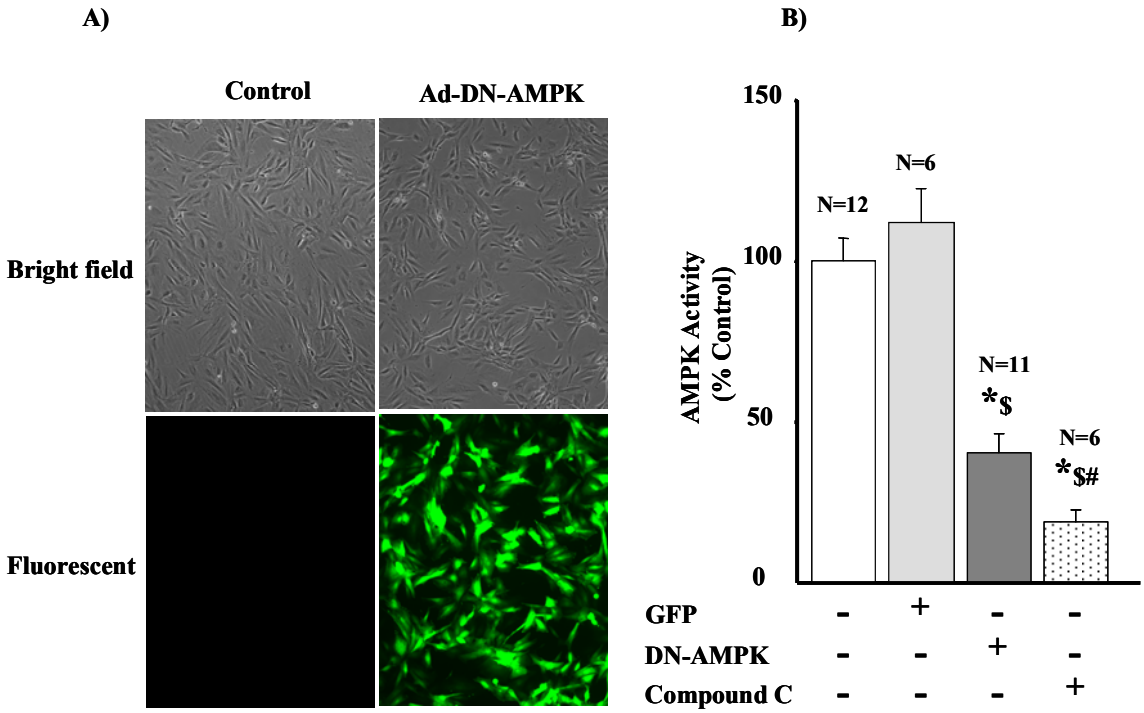

Figure 4-6: Effect of AMPK inhibition on AMPK activity in H9c2 cells.

AMPK activity was removed in differentiated H9c2 cells by treated with DN-AMPK (moi 150) for 24 hours or with Compound C (40 μM). Panel A illustrates extent of expression of the DN-AMPK (moi 150) construct, based on co-expressed GFP. Panel B illustrates the effects of inhibition of AMPK activity. Values are normalized to 100 % Control (in pmol/hr/mg ptn). Values are expressed as Mean ± SEM. *, significantly different from Control ($p<0.05$); $, significantly different from GFP ($p<0.05$); # significantly different from DN-AMPK ($p<0.05$). N=6-12 per group.

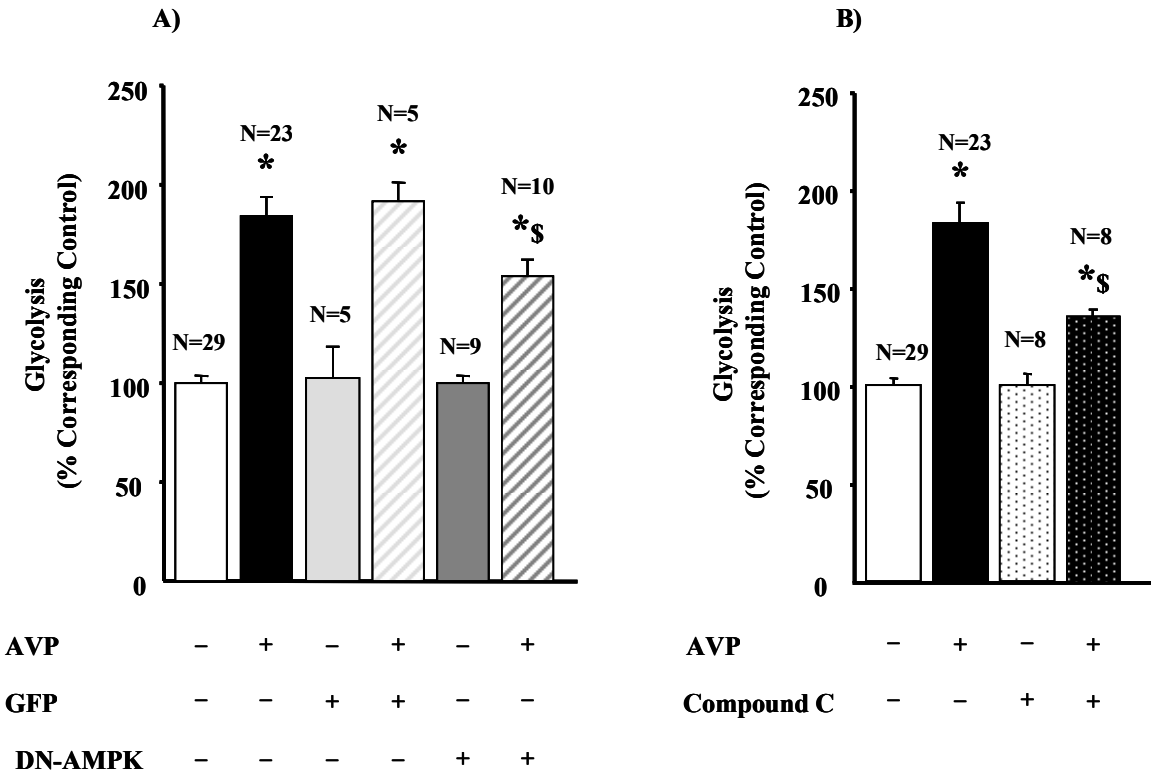

Figure 4-7: Effects of AMPK inhibition on glycolysis in H9c2 cells.

Differentiated H9c2 cells were treated with AVP (1 μM) supplemented with 0.5 % HS for 48 hours. To remove AMPK activity, cells were treated with DN-AMPK (moi 150) for 24 hours or pre-incubated with Compound C (40 μM) for 30 minutes. Rates of glycolysis were measured over 8 hours in KH solution supplemented with 5.5 mM [5-^{3}H]-glucose, 0.4 mM palmitic acid pre-bound to albumin and 10^{-7} M insulin. Panel A illustrates glycolytic rates in hypertrophied and non-hypertrophied GFP or DN-AMPK treated H9c2 cells. Panel B illustrates glycolytic rates in hypertrophied and non-hypertrophied H9c2 cells treated with Compound C. Values are normalized to 100 % corresponding Control (in nmol/hr/mg protein). Values are expressed as Mean ± SEM. *, significantly different from corresponding Control ($p<0.05$); $, significantly different from hypertrophied H9c2 cells ($p<0.05$).

CHAPTER 5 - ARGININE VASOPRESSIN STIMULATES GLUCOSE UTILIZATION IN HEART-DERIVED H9c2 CELLS INDEPENDENT OF AMP-ACTIVATED PROTEIN KINASE ACTIVATION

5.1 INTRODUCTION

Experiments described in the previous chapter showed that chronic treatment of cells with arginine vasopressin (AVP) led to cardiac hypertrophy and AMP activated protein kinase (AMPK) activation. AMPK can be activated by metabolic stresses that deplete cellular high energy phosphate including ATP through upstream kinase LKB1 [57, 155, 156, 158, 160, 189, 200]. AMPK can also be activated by hormones acting through Gq-coupled receptors (GqCR) and a calcium-dependent pathway through calcium/calmodulin-dependent protein kinase kinases (CaMKK) as upstream AMPK kinases [200-205]. Also, others have shown that calcium ionophore treatment of several cell lines lead to AMPK activation through CaMKK-AMPK pathway [206, 207, 209].

AVP is significantly and chronically elevated in the plasma of human patients [359, 360] and experimental animals [303] with heart failure and has effects on a number of processes that may participate in the pathogenesis of heart failure, including effects on

body fluid regulation, vascular tone, and cardiac contractile function and remodelling (See [360] for review). The AVP receptor is one of the GqCR family proteins that stimulate the release of calcium. Binding of AVP to the V1a receptor, the receptor subtype shown to be involved with the hypertrophic response to AVP [305], leads to activation of phospholipase C, production of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) and the mobilization of intracellular calcium and influx of extracellular calcium (Figure 2-2) [292, 306]. In turn, it has been documented that increases in intracellular concentration of calcium can lead to increase in glucose utilization of cells [368, 369].

Alterations in myocardial energy metabolism, especially those related to glucose and fatty acids, are part of the remodeling process in heart failure and are considered to be key contributors to contractile dysfunction and worsening of heart failure [370]. It has been demonstrated that a switch away from utilization of fatty acids toward glucose utilization occurs during decompensated heart failure in dogs [371] and humans [372]. The shift away from fatty acid metabolism observed in heart failure is consistent with the observation that genes related to oxidation of fatty acids are downregulated in failing hearts [146]. It is also in keeping with the fact that inborn errors of fatty acid metabolism are associated with cardiomyopathy and heart failure [373]. Of interest, AVP is known to stimulate glucose (and glycogen) catabolism in a variety cell types, tissues, and organs [374, 375] including isolated perfused rat hearts [376]. However, AVP is a potent vasoconstrictor and, as such, the increased glucose use observed in perfused hearts may be due to regional ischemia caused by vasoconstriction. Since cardiac myocytes are able to respond to AVP directly [377], it is possible that elevations in AVP, of either local or systemic origin, may alter myocardial glucose metabolism and, in doing so, contribute to metabolic remodeling of the failing heart. Moreover, experiments in the chapter 4 have shown that chronic exposure to AVP leads to structural and metabolic remodelling. It is not known; however, if acute exposure to AVP also has metabolic effects in heart muscle cells.

Since AVP causes increases in calcium concentration inside the cell, and calcium can alter glucose metabolism and activate AMPK, the hypothesis that AVP directly activates AMPK and influences glucose metabolism in heart muscle cells was tested. A cell culture model was used in order to avoid confounding effects of AVP on the coronary circulation. The receptor subtype responsible for the effect of AVP was also determined. Additionally, the current study investigated whether the metabolic actions of AVP were mediated by calcium and phosphatidylinositol 3-kinase (PI3K) signaling pathways.

5.2 METHODS

Details of methods used for this investigation are described in the Materials and Methods chapter.

5.3 RESULTS

5.3.1 AVP Acutely Influencess Glycolysis and Glycogen Content in H9c2 Cells

Rates of glycolysis were accelerated in H9c2 cells exposed to DMEM or DMEM/F12 containing 1 μM AVP as is summarized in Table 5-1. Regardless of the medium, glycogen synthesis was also significantly reduced in response to AVP. Total glycogen content in H9c2 cells was decreased significantly by AVP as compared to untreated cells (Table 5-1). The lower total glycogen in AVP-treated cells might be due to AVP-induced acceleration of glycogenosis or AVP-inhibition of glycogen synthesis.

Treatment of H9c2 cells with AVP led to a dose-dependent stimulation of glycolysis, achieving a plateau at approximately 1 μM AVP (Figure 5-1). The lowest effective dose of AVP, 10 nM, is comparable to concentrations of circulating AVP seen in the setting of heart failure in humans [302, 304].

5.3.2 AVP Acutely Accelerates Glycolysis via the V1a Receptor

In order to determine the receptor involved in AVP-induced stimulation of glycolysis, H9c2 cells were pretreated with either d(CH2)5[Tyr(Me)2]AVP, a potent and selective V1a receptor antagonist [378], or d(CH2)5[D-Ile2,Ile4,Tyr-NH(2)9]AVP, a potent and selective V2 receptor antagonist [378]. Cells were exposed to these receptor antagonists at concentrations ranging from 10^{-7} to 10^{-5} M for 30 minutes before addition of 1 μM AVP. These receptor subtypes are known to be present on cardiac myocytes [379]. Pre-treatment of H9c2 cells with the V1a receptor antagonist completely abolished the acceleration of glucose utilization that occurs in response to AVP (Figure 5-2). It is noteworthy that the maximum degree of inhibition was observed for all three concentrations of V1a receptor antagonist used. On the other hand, the V2 receptor antagonist did not significantly abrogate the effect of AVP on glucose utilization.

5.3.3 AMPK and Acute Stimulation of Glycolysis by AVP

Treatment of H9c2 cells for 2 hours with AVP resulted in a significant 1.6-fold increase in measured activity of AMPK (Figure 5-3A). To inhibit AMPK activity, H9c2 cells were exposed to 40 μM Compound C for 30 minutes or DN-AMPK for 24 hours prior to addition of AVP. Both approach resulted in a substantial reduction in AMPK activity (Figure 4-6B), with Compound C caused a reduction that was greater than that caused by DN-AMPK. Inhibition of AMPK by either Compound C or DN-AMPK did not cause a significant reduction in glycolysis in H9c2 cells treated for this short duration with AVP (Figure 5-3B and Table 5-2); a result differing from that seen in H9c2 cells hypertrophied by longer term (48 hours) exposure to AVP (Figures 4-6 and 4-7).

5.3.4 Myocardial High Energy Phosphates

Treatment of H9c2 cells with AVP for 2 hours altered the content of adenine nucleotides (Table 5-3). In AVP treated cells, the content of AMP was significantly higher as compared to Controls. Also, cellular content of PCr was significantly elevated

in AVP-treated cells as compared to Control hearts (Table 5-3). Free AMP; however, was significantly decreased in AVP-treated cells (Control, 10.36 ± 4.53 vs. AVP, 0.63 ± 0.13 μmol/l, N=6-8 per group, $P<0.05$).

5.3.5 Intracellular and Extracellular Calcium and Acute Stimulation of Glycolysis by AVP

Given the well known effects of AVP on cellular calcium [380, 381] and the potential for calcium to accelerate glucose use [382, 383], pharmacological agents that interfere with calcium entry or intracellular release from calcium stores were used to assess the role of calcium in the acute metabolic actions of AVP. In one series of experiments, H9c2 cells were pretreated for 30 min with 25 μM dantrolene, an inhibitor of Ca^{2+} release from sarcoplasmic reticulum (SR) via the ryanodine receptor [307]. To evaluate the involvement of the IP3 receptor, whose activation also leads to calcium release from the SR, cells were pretreated for 5 minutes with 100 μM 2-amino-ethoxydiphenyl borate (2-APB), a non-competitive IP3 receptor antagonist [308, 384]. The role of extracellular Ca^{2+} was assessed by preincubating H9c2 cells with 3 mM EGTA (to chelate extracellular calcium) for 5 minutes before challenge with AVP.

In contrast to AMPK inhibition, treatment of H9c2 cells with 2-APB, completely abolished the effect of AVP on glycolysis (Figure 5-4A), while incubation with dantrolene (Figure 5-4B) or EGTA (Figure 5-4C) partially attenuated the elevation in glycolysis caused by AVP. These data indicate the acute stimulation of glycolysis by AVP is mediated by changes in cellular calcium especially calcium release from the sarcoplasmic reticulum by stimulation of 2-APB receptor.

5.3.6 Phosphoinositol-3-Phosphate Kinase Pathway and Acute Stimulation of Glycolysis by AVP

Knowing that AVP stimulates the PI3K pathway (Figure 2-2) [310] and the known role of PI3K to accelerate glucose use [385], a pharmacological agent that inhibits PI3K was

used to assess the role of PI3K in the acute metabolic actions of AVP. To determine if the PI3K signaling pathway was involved in the observed acceleration of glucose utilization induced by AVP, H9c2 cells were studied with and without 30 minutes of pretreatment with 300 nM wortmannin, a well known inhibitor of PI3K [312, 386], before addition of AVP. As shown in Figure 5-5, pre-treatment of H9c2 cells with Wortmannin did not prevent the acceleration of glucose utilization caused by AVP, even though basal glucose utilization rates were slightly but significantly reduced by wortmannin.

5.4 DISCUSSION

The present study demonstrated that the acute exposure of H9c2 cells to AVP accelerated glycolysis in a dose-dependent manner and through the V1a receptor. This AVP-induced acceleration of glycolysis was not dependent upon AMPK activation, even though AMPK was activated. Rather, AVP acutely stimulated glycolysis by a calcium-dependent mechanism.

This study showed that acute exposure of H9c2 cells to AVP dose-dependently accelerated glycolysis (Figure 5-1). Notably, glycolysis was initially stimulated at concentrations of AVP comparable to those reported in plasma of patients and rodents with congestive heart failure and therefore is potentially clinically relevant [302-304]. A stimulatory effect of AVP on glucose catabolism has been described previously in a variety of cell types, tissues, and organs including isolated perfused rat hearts [374, 376, 387]. However, the latter finding could result from AVP-induced vasoconstriction leading to areas of regional myocardial ischemia and, thereby, stimulates glucose use indirectly. Results from the current investigation clearly demonstrated that acceleration of glycolysis in H9c2 cells is a direct effect of AVP on heart muscle cells. That glycolysis was enhanced at concentrations of AVP observed in patients and experimental animal models with heart failure raises the possibility that AVP may influence glucose use by the heart *in vivo*.

AVP exerts its actions through binding to three specific receptor subtypes, including V1a, V2, and V3 (V1b) all of which belong to the Gq-coupled receptor (GqCR) superfamily [380]. The V1a receptor is expressed in cardiac myocytes and a number of other cell types and tissues, including vascular smooth muscle, liver, testis, spleen, platelets, adrenal cortex, brain, and various immortalized cell types [379, 388, 389]. The the V2 receptor is expressed in the medullary portion of the kidney and cardiac myocytes as well as several cell lines [379, 390], while the V3 the receptor is expressed in pituitary gland and kidney, but is not in liver, myometrium or adrenal glands. The current experiments showed that AVP exerted its acute metabolic effects in H9c2 cells via the V1a receptor (Figure 5-2), the receptor subtype responsible for AVP-induced hypertrophy of heart muscle cells [358].

AVP binding to the V1a receptor activates a complex array of signaling pathways and effector molecules (Figure 2-2), many of which may influence glucose metabolism. Such binding activates phospholipases causing hydrolysis of membrane phosphoinositides leading to the formation of the second messengers, inositoltrisphosphate (IP3) and diacylglycerol (DAG) [388, 391]. Formation of IP3 leads to mobilization of sarcoplasmic reticulum calcium stores via the IP3 receptor and a rapid initial rise in free intracellular calcium [391]. This rise is followed by a sustained increase in intracellular calcium caused by entry of extracellular calcium [392]. At the same time, DAG activates protein kinase C (PKC) by recruiting it to the plasma membrane [381, 391]. V1a receptor activation also leads to activation of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signaling pathway [393]. Importantly, binding of the V1a receptor does not lead to increases in cAMP and activation of protein kinase A [391].

Acute exposure to AVP caused a substantial elevation of AMPK activity (Figure 5-3A), possibly via GqCR induced alterations in calcium. Others have shown that the GqCR superfamily activates AMPK [158, 206, 207, 209]. However, acute AVP-induced acceleration of glycolysis in H9c2 cells was independent of AMPK activity since

inhibition of AMPK by Compound C or DN-AMPK failed to decrease glycolysis in response to AVP (Figure 5-3B and Table 5-2). This finding indicates that mechanisms other than AMPK activation are responsible for the acute acceleration of glycolysis induced by AVP. Moreover, this result indicates that the factors causing increased glycolysis differ between heart muscle cells hypertrophied by chronic treatment with AVP and those exposed acutely to AVP.

The current study demonstrated that AVP-induced AMPK activation caused a significant reduction in free AMP (Table 5-3), indicating that AMPK is activated independent of changes in the energy status of cells. This finding is consistent with the notion that AMPK can be activated through GqCR agonists without any measurable changes in the energy status of cells.

The stimulatory effect of AVP on glucose use observed in the current study might have occurred by several mechanisms (Figure 2-2). There is good evidence to implicate calcium-dependent pathways in the stimulation of glucose and/or glycogen use in response to a variety of agents, including AVP [394-396]. Elevation of intracellular calcium has been shown to play an important role in mediating the stimulation of glucose transport caused by mitochondrial uncoupling [383] and insulin [397]. In addition, results from other studies show that agents which raise intracellular calcium concentrations, such as W-7, caffeine, and Ca^{2+} ionophores, stimulate rates of glucose uptake [368, 369]. Increases in fructose 2,6- bisphosphate content and 6-phosphofructo-2-kinase activity have both been related to a rise in intracellular calcium [398], the effect of which would lead to activation of the key glycolytic enzyme, 6-phosphofructo-1-kinase and stimulation of glycolysis. Given that AVP increases intracellular calcium, it can be speculated that calcium may mediate the AVP-induced increased in glucose use observed in H9c2 cells.

As a consequence, the role of intracellular and extracellular calcium in AVP-induced stimulation of glycolysis was assessed by administration of selective inhibitors of intracellular calcium release or chelation of extracellular calcium. Of significance,

2APB, an inhibitor of the sarcoplasmic reticulum IP3 receptor [308], completely abrogated the AVP-induced acceleration of glucose use in H9c2 cells (Figure 5-4A). At the same time, 2-APB did not significantly alter basal (AVP–free) rates of glucose use. Calcium may also be released from the sarcoplasmic reticulum via the ryanodine receptor, an effect that can be inhibited by dantrolene [307]. Dantrolene, which did not reduce basal rates of glucose use in H9c2 cells slightly but insignificantly, attenuated the response to AVP (Figure 5-4B). Chelation of extracellular calcium with EGTA reduced but did not eliminate the stimulation of glucose use induced by AVP (Figure 5-4C).

Another possible mechanism to account for the metabolic effects of AVP is the activation of the PI3K/protein kinase B (PKB) pathway. This pathway is the well recognized means by which insulin stimulates glucose uptake and glycolysis [399]. In the current study, inhibition of PI3K by wortmanin did not significantly abrogate the response to AVP indicating that PI3K was not involved (Figure 5-5).

Taken together, these data strongly implicate calcium-dependent pathways, especially those related to intracellular calcium associated with IP3 receptors, as key mediators of the AVP-induced stimulation of glucose use in heart-derived H9c2 cells. This interpretation is consistent with that of others, who also found that the metabolic effects of AVP in hepatocytes or liver were calcium-dependent [396]. Of interest and in contrast to results presented here, several investigators found the metabolic effect of AVP in hepatocytes to be more dependent on extracellular calcium [396]. The discrepancy in results may be a reflection of differences in cell type, even though the receptor subtype is presumably similar in H9c2 cells and hepatocytes. Further studies will be necessary to fully clarify this discrepancy. When considering this interpretation, it should be recognized that the agents used in the current study were not necessarily fully selective. For example, 2-APB, while considered an inhibitor of the IP3 receptor, may also influence other aspects of calcium homeostasis such as inhibition of calcium ATPase activity and the store-operated calcium entry channel [384]. Of importance, the

effects of 2-APB all involve intracellular calcium mobilization, in keeping with the primacy of intracellular calcium in control of AVP-induced alterations in glycolysis in H9c2 cells. Further studies will be required to fully define the specific calcium-dependent pathways involved in H9c2 cells.

Total glycogen content was significantly decreased in response to AVP (Table 5-1), indicating that AVP caused glycogenolysis in H9c2 cells. This observation is entirely consistent with those of others who demonstrated that AVP causes glycogen degradation in hepatocytes [374, 387], due to stimulatory effects on glycogen phosphorylase [396] and inhibitory effects on glycogen synthase [400].

The results of the current investigation, coupled with those in the preceding chapter and with those of others, are of potential clinical significance. In heart failure, the outward phenotypic expression of this disorder, characterized clinically by alterations in the size, shape, and function, results from remodeling at cellular, interstitial, molecular, and genomic levels due a variety of factors, including neurohormonal activation. Metabolic remodeling, characterized by a switch in myocardial substrate utilization away from fatty acids toward glucose, occurs in the failing heart by mechanisms that are not yet fully understood. The finding that AVP directly stimulates glucose use in heart-derived muscle cells at concentrations comparable to those in heart failure patients coupled with evidence that it promotes metabolic and structural remodeling of myocyte and that its antagonism is beneficial, raise the distinct possibility that AVP contributes to remodeling of the failing heart and that such remodeling is detrimental.

In summary, it could be concluded that AVP acutely stimulates glucose use in heart-derived H9c2 cells by a mechanism that is dependent on calcium, especially calcium released from intracellular stores via the IP3 receptor, but independent of AMPK or PI3K activation.

Table 5-1: Rates of glycolysis and glycogen content in H9c2 cells treated acutely with AVP.

Differentiated H9c2 cells were treated with AVP (1 μM) AVP for 2 hours. A small amount of [5-^{3}H]-glucose (1.0 μCi/ml) was present in the medium throughout the experiment to measure rates of glycolysis and glycogen synthesis. Values are Mean ± SEM. N=6-18 per group. *, significantly different from Controls, $p<0.05$.

	Glycolysis (nmol/hr/mg ptn)	**Total Glycogen** (nmol/mg ptn)	**Glycogen Synthesis** (nmol/hr/mg ptn)
DMEM			
Control	5.53 ± 0.66	410.57 ± 28.01	19.49 ± 1.07
AVP Treated	9.94 ± 0.46 *	304.74 ± 19.60 *	15.97 ± 0.66 *
DMEM/F12			
Control	4.96 ± 0.09	432.40 ± 24.88	13.05 ± 0.65
AVP Treated	7.55 ± 0.29 *	353.70 ± 26.78 *	9.06 ± 0.55 *

Table 5-2: The effect of AMPK inhibition on glycolysis in H9c2 cells.

Differentiated H9c2 cells were treated with DN-AMPK (moi 150) for 24 hours or preincubated with 40 μM Compound C for 30 minutes prior to incubation with 1 μM AVP. For AVP treatment cells were incubated for 2 hours in KH solution supplemented with 5.5 mM glucose, 0.4 mM palmitic acid pre-bound to albumin and 10^{-7} M insulin. A small amount of [5-^{3}H]-glucose (1.0 μCi/ml) was present throughout the incubation time with AVP to measure rates of glycolysis. Each column shows different day of experiment. Values are are Mean ± SEM. *, significantly different from Control ($p<0.05$). $, significantly different from Corresponding Control ($p<0.05$).

	Experiment Day 1 Glycolysis (nmol/hr/mg ptn)	Experiment Day 2 Glycolysis (nmol/hr/mg ptn)	Experiment Day 3 Glycolysis (nmol/hr/mg ptn)
Control	4.43 ± 0.21 (N=6)	5.28 ± 0.01 (N=6)	7.28 ± 0.83 (N=6)
AVP	6.36 ± 0.45* (N=3)	9.83 ± 0.20* (N=3)	14.52 ± 0.33*$ (N=6)
DN-AMPK		5.70 ± 0.48 (N=3)	
DN-AMPK + AVP		8.01 ± 0.57*$ (N=3)	
Compound C			6.29 ± 0.25 (N=6)
Compound C + AVP			11.98 ± 1.31*$ (N=5)

Table 5-3: Myocardial adenine nucleotide and creatine phosphate content of H9c2 cells.

Differentiated H9c2 cells were treated with or without 1 μM AVP for 2 hours in KH solution supplemented with 5.5 mM glucose, 0.4 mM palmitic acid pre-bound to albumin and 10^{-7} M insulin. Values are Mean ± SEM. *, significantly different from Control value within Group, $p<0.05$.

	Control (n=7-8)	**AVP** (n=6-8)
ATP (μmol/mg protein)	3.02 ± 0.18	3.03 ± 0.18
ADP (μmol/mg protein)	0.38 ± 0.07	0.46 ± 0.04
AMP (μmol/mg protein)	0.10 ± 0.01	0.14 ± 0.02*
Creatine (Cr) (μmol/mg protein)	25.26 ± 2.16	24.63 ± 1.50
Phosphocreatine (PCr) (μmol/mg protein)	5.35 ± 0.94	11.12 ± 0.45*

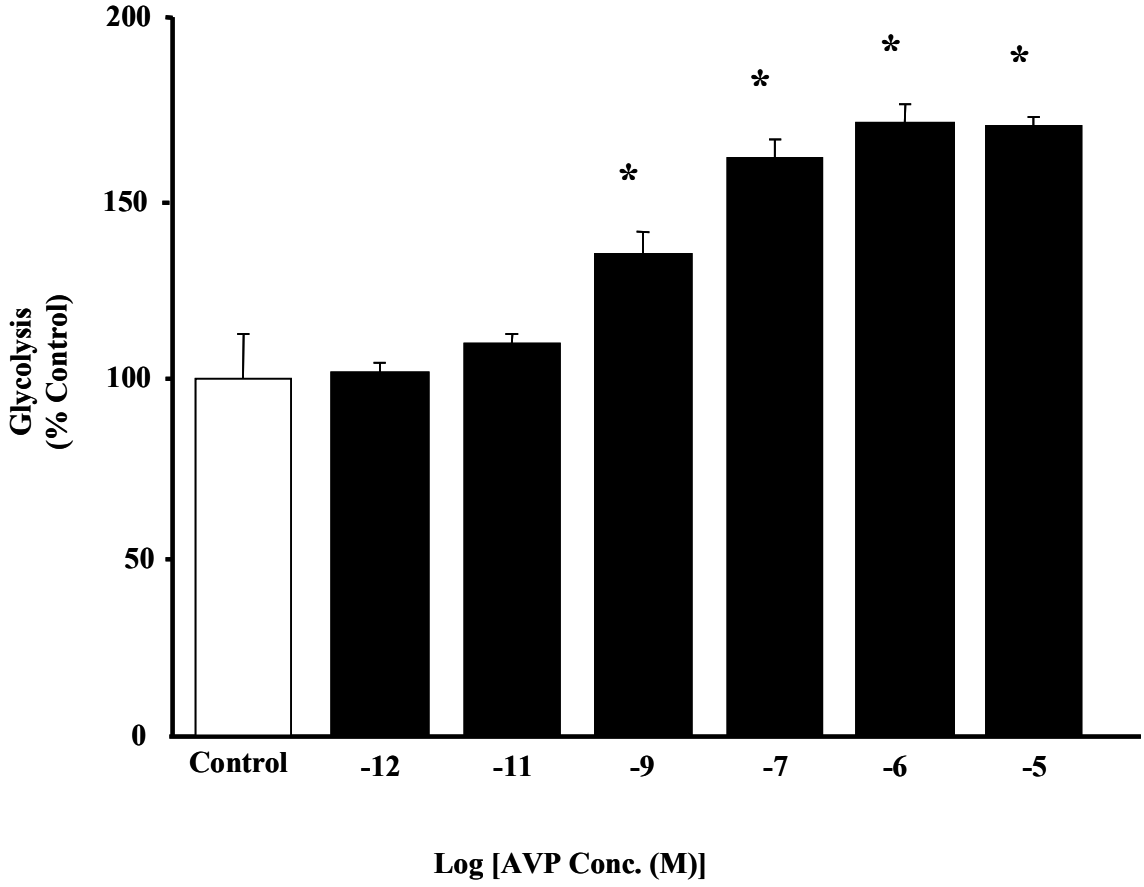

Figure 5-1: Concentration dependency for acute effect of AVP on glycolysis.

Differentiated H9c2 cells were incubated for 2 hours in DMEM/F12 with the indicated concentrations of AVP. A small amount of [5-^{3}H]-glucose (1.0 μCi/ml) was present throughout the incubation time to measure rates of glycolysis. Values are normalized to 100 % Control (in nmol/hr/mg ptn). Values are expressed as Mean ± SEM. *, significantly different from Control ($p<0.05$). N=6-9 per group.

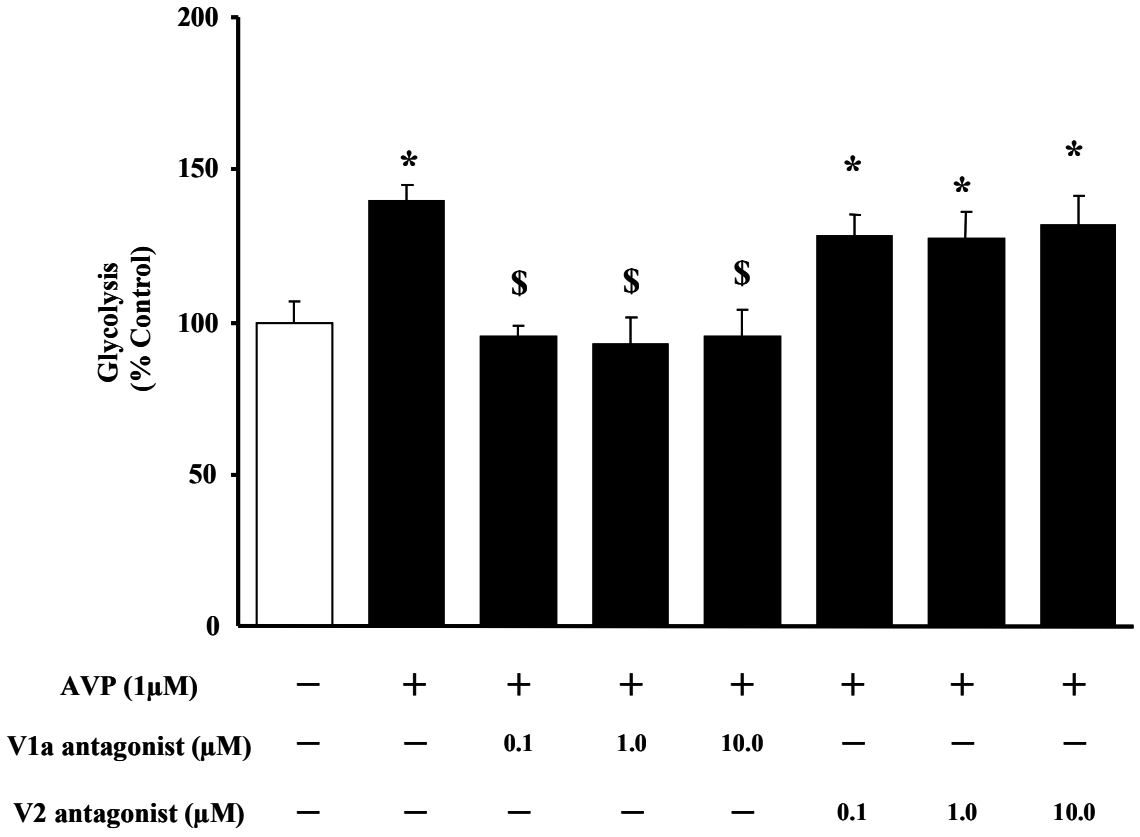

Figure 5-2: Receptor subtype responsible for acute effect of AVP on glycolysis.

To test the role of specific AVP receptors, differentiated cells were treated with the indicated different concentrations of antagonists in DMEM/F12 for 30 minutes before addition of 1 μM AVP. Then H9c2 cells were incubated for additional 2 hours with AVP and rates of glycolysis were determined. Control, no AVP. V1a antagonist, V1a receptor antagonist; V2 antagonist, V2 receptor antagonist. Values are normalized to 100 % Control (in nmol/hr/mg ptn). Values are expressed as Mean ± SEM. *, significantly different from Control ($p<0.05$). $, significantly different from AVP ($p<0.05$). N=6-9 per group.

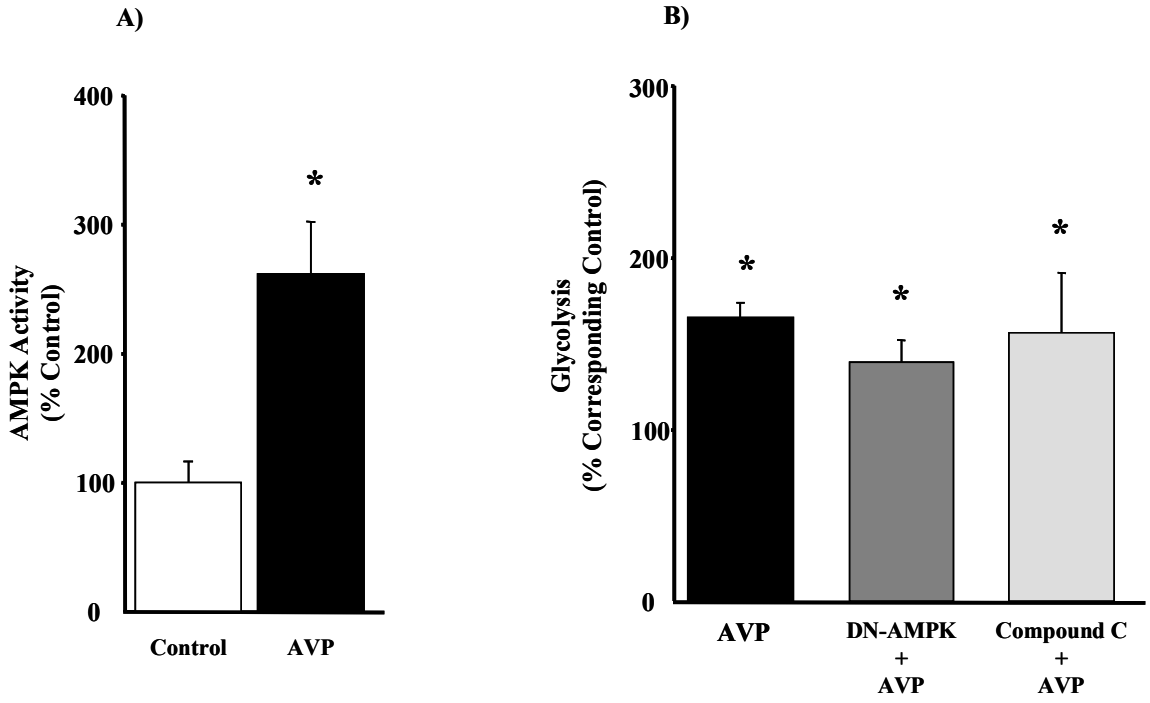

Figure 5-3: AMPK activity in H9c2 cells exposed to AVP acutely and effect of AMPK inhibition on glycolysis.

In Panel A, differentiated H9c2 cells were incubated in KH solution supplemented with 5.5 mM [5-^{3}H]-glucose, 0.4 mM palmitic acid pre-bound to albumin and 10^{-7} M insulin for 2 hours in the absence (Control) or presence (AVP) of 1 μM AVP prior to measurement of AMPK activity. Alternatively, cells were treated with DN-AMPK (moi 150) for 24 hours or preincubated with 40 μM Compound C for 30 minutes prior to incubation with 1 μM AVP for additional two hours and measurement of glycolysis (Panel B). Values are normalized to 100 % corresponding Control (in nmol/hr/mg ptn). Values are are Mean ± SEM. *, significantly different from corresponding Control ($p<0.05$). N=3-15 per group.

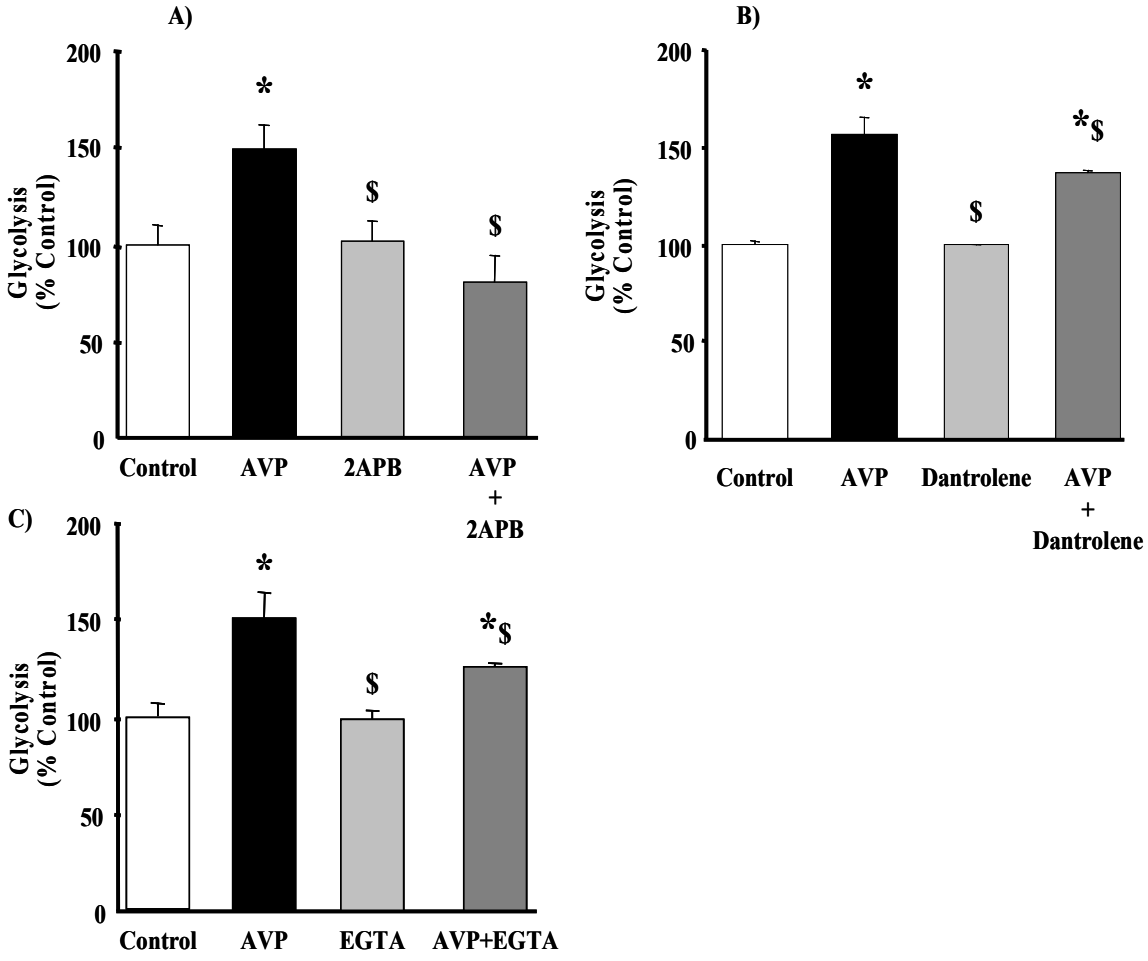

Figure 5-4: Role of calcium in the metabolic actions of AVP in H9c2 cells.

Differentiated H9c2 cells were first incubated in the absence or presence of either 100 μM 2-aminoethoxydiphenyl borate (2-APB) for 5 minutes (Panel A), 25 μM dantrolene for 30 minutes (Panel B), or 3 mM O,O'-bis(2-aminoethyl)ethyleneglycol-N,N,N',N'-tetraacetic acid (EGTA) for 5 minutes (Panel C). All experiments were performed in calcium-free DMEM/F12 and H9c2 cells exposed to the relevant vehicle served as Controls. Following this initial period, control and antagonist-treated cells were further incubated in the absence or presence of 1 μM AVP for 30 minutes (in the case of 2-ABP) or 2 hours to measure rates of glycolysis. A small amount of [5-^{3}H]-glucose (1.0 μCi/ml) was present throughout the incubation time to measure rates of glycolysis. Values are normalized to 100 % Control (in nmol/hr/mg ptn). Values are are Mean ±

SEM. *, significantly different from Control ($p<0.05$). $, significantly different from AVP ($p<0.05$). N=6-8 per each group.

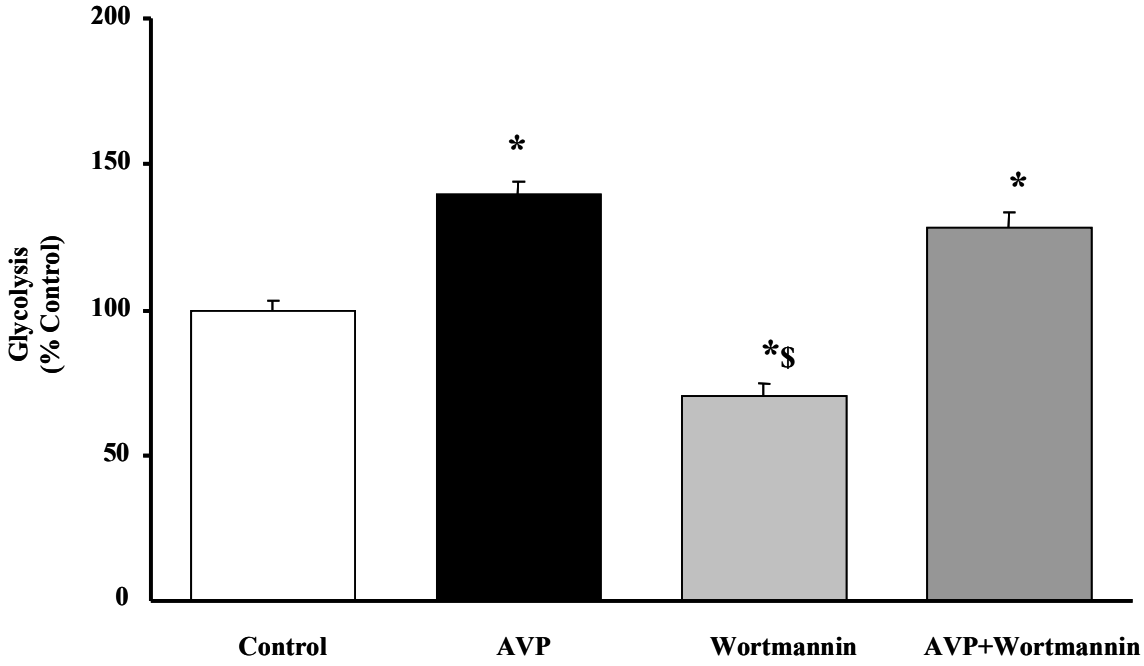

Figure 5-5: Effect of PI3K signaling pathway on AVP-induced acceleration of glucose use in H9c2 cells.

Differentiated H9c2 cells were preincubated with 300 nM wortmannin or vehicle for 30 min in DMEM/F12 before addition of 1 μM AVP. Following this pre treatment, Control and antagonist-treated cells were further incubated in the absence or presence of 1 μM AVP for 2 hours. A small amount of [5-^{3}H]-glucose (1.0 μCi/ml) was present throughout the incubation time to measure rates of glycolysis. Values are normalized to 100 % Control (in nmol/hr/mg ptn). Values are are Mean ± SEM. *, significantly different from control ($p<0.05$). $, significantly different from AVP ($p<0.05$). N=12 per group.

CHAPTER 6 - AMP-ACTIVATED PROTEIN KINASE AND HYPERTROPHIC STRUCTURAL AND METABOLIC REMODELING IN HEART-DERIVED H9c2 CELLS

6.1 INTRODUCTION

As discussed in the introduction, cardiac hypertrophy is an adaptive response to increased hemodynamic workload. Cardiac hypertrophy is characterized by increased cell size, increased protein synthesis, alterations in energy metabolism, alterations in gene expression, and increased myofibrillar assembly [349, 401]. A necessary component of cardiac myocyte enlargement is an increase in protein synthesis, a process which is regulated through both initiation of translation and elongation [401-403]. The mammalian target of rapamycin (mTOR) is known to be an important positive regulator of protein synthesis and cell growth (Figure 6-1) [404, 405]. mTOR activates translation through activation of the p70 ribosomal protein S6 kinase (P70S6K) and an increase in the phosphorylation of 4E-binding protein-1 (4E-BP1), which relieves inhibition of translation initiation [406]. mTOR also activates eukaryotic elongation factor-2 (eEF2) by inhibiting eukaryotic elongation factor-2 kinase (eEF2 Kinase) resulting in stimulation of translation elongation [256]. mTOR activation

results in stimulation of protein synthesis by decreasing eEF2 phosphorylation. eEF2 is involved in the translocation of ribosomes relative to mRNA during the elongation phase [403]. Phosphorylation of eEF2 on Thr-56 by eEF2 kinase prevents its activity by reducing its affinity for ribosomes, thereby preventing translocation [403, 407, 408].

Protein synthesis can be stimulated independently of the mTOR pathway through glycogen synthase kinase-3β (GSK-3β). GSK-3β is a negative regulator of both normal [259, 409] and pathologic stress–induced (i.e. pressure-overload) hypertrophy [410]. In unstimualted cells, GSK-3β is active. However, phosphorylation of GSK-3β at serine 9 results in its inactivation and therefore stimulation of protein synthesis by the activation of transcriptional regulators, such as NFAT, GATA4, etc., and translational regulator eukaryotic translation initiation factor 2B (elF2B) [257, 258, 260, 411].

Among a number of intracellular signaling pathways that have been implicated in regulation of hypertrophic response and cardiac myocyte growth, AMP-activated protein kinase (AMPK) has emerged as a regulator of cardiac myocyte growth [36, 280, 282]. AMPK has been suggested to participate in the regulation of protein synthesis in the heart and other cell types. AMPK turns off energy-consuming pathways such as protein synthesis [159, 226] and numerous studies have revealed that AMPK activation in the heart attenuates cardiac hypertrophy by inhibiting protein synthesis [195, 254, 279-282, 412]. It has been shown that AMPK activation inhibits eEF2 through activation of eEF2 kinase, resulting in inhibition of translation elongation and protein synthesis [195, 281]. Activation of AMPK is also known to downregulate the mTOR pathway, ultimately leading to inhibition of translation initiation [254, 413, 414].

In addition, AMPK might influence protein synthesis through modulation of GSK-3β activity. Several studies have shown that AMPK activation results in phosphorylation or reduced expression of GSK-3β which results in inactivation of GSK-3β [261-263]; whereas, others have shown that AMPK activation might activate GSK-3β [419-421].

Several studies on neonatal cardiac myocytes [255, 278-283, 412] and intact rodent hearts [415] have documented that AMPK activation inhibits protein synthesis and hypertrophy. Also, Liao et al [284] have shown that inhibiting AMPK activity in mice produces progressive cardiac remodeling in pressure overload cardiac hypertrophy.

Although a number of studies have implicated AMPK as a negative regulator of protein synthesis and hypertrophic growth, we and others have shown that the activity and expression of AMPK has been upregulated in hypertrophied hearts and correlates with the development of cardiac hypertrophy [34, 35, 291]. Also, Gq-coupled receptor (GqCR) activation, a key component of hypertrophic growth, is associated with AMPK activation [218]. Additionally, mutations in the γ-subunit of AMPK which activate or inhibit its activity are associated with cardiac hypertrophy [29, 36, 186, 263, 288, 289, 416]. Over expression of a constitutively active form of AMPK in neonatal cardiac myocytes has also been shown to result in increased protein synthesis [263]. More interestingly, transgenic mouse models lacking the AMPK α-subunit fail to demonstrate increased cardiac mass [214, 241, 251, 285-287]; these findings raise questions about the exact role of AMPK in hypertrophic growth. Taken together, although much attention has been given to AMPK and hypertrophic growth, these data indicate that the relationship between AMPK and protein synthesis in the context of cardiac hypertrophy is not yet completely understood.

Therefore, the current study set out to determine if AMPK directly plays a role in the regulation of protein synthesis in heart muscle cells. In the current investigation, cultured H9c2 cells [347] were used as an *in vitro* model system in which AMPK activity was selectively enhanced by molecular means.

6.2 METHODS

Details of methods used for this investigation are described in the Materials and Methods chapter.

6.3 RESULTS

6.3.1 Over expression of AMPK Increased AMPK Activity

Treatment of H9c2 cells with adenovirus containing CA-AMPK for 48 hours led to over expression of GFP and c-myc in a dose response matter as shown in Figure (6-2A and 62-B), indicating successful viral transduction and gene transfer. Also, AMPK activity was significantly increased in cells infected with CA-AMPK (moi 150) as compared to the GFP Controls (Figure 6-2C).

6.3.2 Over expression of AMPK Caused Hypertrophy and Metabolic Remodeling

Exposure of H9c2 cells to CA-AMPK (moi 150) significantly increased protein content (Figure 6-3A), and [^{14}C]-phenylalanine incorporation (Figure 6-3B), indicating that the cells were hypertrophied. This hypertrophy occured without any increase in ANF expression (Figure 6-3C). Furthermore, rates of glycolysis were accelerated by approximately 50 % in H9c2 cells overexpressing CA-AMPK (moi150) (Figure 6-4A). There were no significant differences in glucose oxidation between CA-AMPK treated H9c2 cells and GFP Control cells (Figure 6-4B). Additionally, rates of fatty acid oxidation were also significantly increased in CA-AMPK (moi 150) treated H9c2 cells as compared to GFP Control cells (Figure 6-4C).

6.3.3 Over expression of AMPK Did not Influence eEF2 Phosphorylation

To understand the signaling mechanisms that control protein synthesis in CA-AMPK treated H9c2 cells, cell lysates from cardiac myocytes treated as above were subjected to immunoblot analysis using anti-phospho-eEF2 (Thr 56). Treatment of cells with CA-AMPK (moi 150) did not significantly alter the phosphorylation state of eEF2 at Thr 56 (Figure 6-5).

6.3.4 Over expression of AMPK Decreased GSK-3β Expression

GSK-3β plays a role as a negative regulator of hypertrophic growth by phosphorylating and thereby inhibiting the transcription factor NFAT [259, 410]. Given the role of GSK-3β in protein synthesis, it was determined if over expression of AMPK had any effect on GSK-3β expression. As shown in Figure 6-6, exposure of H9c2 cells to CA-AMPK (moi 150) decreased GSK-3β expression as compared to GFP Controls.

6.4 DISCUSSION

In the current study, adenoviral gene transduction of the constitutively active form of AMPK (CA-AMPK) was used to elucidate the role of AMPK in protein synthesis in heart muscle cells. Here, it has been demonstrated that over expression of AMPK induced a number of responses in H9c2 cells indicating cellular hypertrophy with parallel metabolic remodeling characterized by acceleration of rates of glycolysis and fatty acid oxidation. The AMPK-induced protein synthesis observed in H9c2 cells occured in association with reduced expression of glycogen synthase kinase -3β (GSK-3β) but no changes in signaling via eukaryotic elongation factor-2 (eEF2).

Transfer of CA-AMPK to H9c2 cells increased total protein content and phenylalanine incorporation, two well known markers of hypertrophy. However, the cellular hypertrophy produced by CA-AMPK is different from that of pressure overload-induced cardiac hypertrophy or to that caused by agents such as AVP because expression of ANF was not increased. The finding that CA-AMPK increased protein synthesis is consistent with result reported by Folmes et al [263] who showed that over expression of the γ-subunit of AMPK tended to increase protein synthesis in neonatal cells. Even though they did not observe hypertrophic growth when cells were treated for 48 hours with γ-mutant, they concluded that longer treatment would likely induce hypertrophic growth. Additionally, Arad et al [291] have shown that, in mice overexpressing the γ-subunit of AMPK, heart muscle mass increases as does the

expression of genes associated with hypertrophy. In contrast, several animal studies in which AMPK activity was increased failed to demonstrate cardiac hypertrophy, indicating that AMPK does not restrict the normal growth of heart [36, 70, 263]. Moreover, several studies have shown that mutations in PRKAG2, the gene encoding the γ2-subunit of AMPK, are associated with Wolff Wolfe-Parkinson-White Syndrome and cardiac hypertrophy [288, 416].

In contrast to the finding that AMPK is a direct positive regulator of protein synthesis, several studies have documented that AMPK activation leads to inhibition of protein synthesis and cardiac hypertrophy [254, 280, 282]. To support this, several studies have shown that reduced AMPK activity in mice is accompanied by excessive cardiac hypertrophy after aortic banding [246, 284]. The discrepancy between these findings might be due to differences in model systems, study design, and/or the approach used to modulate AMPK activity.

Cardiac hypertrophy can occur as a result of a number of different signaling pathways; therefore, the role of eEF2 and GSK-3β in AMPK-induced cardiac hypertrophy was investigated. eEF2 is a GTP binding protein, which is involved in translocation of ribosomes relative to mRNA during the elongation phase [403]. Phosphorylation of eEF2 on Thr-56 by eEF2 kinase prevents its activity thereby preventing translocation of ribosomes [403, 417]. Several studies on cardiac myocytes and other cell types have shown that activation of AMPK is accompanied by an increase in eEF2 phosphorylation via eEF2 kinase activation resulting in decreased in protein synthesis [255, 279, 280]. The current study showed that AMPK-induced protein synthesis does not occur through activation of eEF2 since CA-AMPK (moi150) treatment of H9c2 cells did not result in alterations in eEF2-phosphorylation at Thr-56 (Figure 6-5). It is interesting that several hypertrophic agents involved in pressure overload-induced cardiac hypertrophy activate eEF2 leading to stimulation of translation elongation [258, 418].

The current study provided evidence that AMPK-induced stimulation of protein synthesis might, in part, be mediated through decreased expression of GSK-3β (Figure 6-6). This finding might be related to the nature of the mutated α-subunit since pharmacological activation of AMPK is accompanied by activation of GSK-3β and has no effect on its expression [419-421]. Interestingly, and consistent with this finding, Folmes et al [103] have documented that over expression of the γ-subunit of AMPK also results in a decrease in GSK-3β expression.

GSK-3β has been found to be a negative regulator of cardiac myocyte growth in response to pathologic and physiologic stimuli [259, 409, 410]. To support this idea, Sanbe et al [422] have shown that GSK-3β over expression leads to regression of established pressure overload hypertrophy. GSK-3β is unusual in that it is negatively regulated by hypertrophic agonists and growth factors. GSK-3β is active in unstimulated cells until it is turned off by these and other stimuli. GSK-3β negatively regulates most of its substrates and so the inhibiting phosphorylation of GSK-3β at serine-9 therefore relieves its inhibitory effect on the translation initiation factor eIF2B and promotes protein synthesis [256-260]. Phosphorylation of GSK-3β also stimulates the activity of a number of transcription factors that are directly implicated in cardiac growth, including c-myc, NFAT, GATA4, and β-catenin [411, 423, 424] and therefore may be particularly important in the reprogramming of gene expression that characterizes both adaptive and maladaptive hypertrophy. Inactivation of GSK-3β by phosphorylation also induces cardiac hypertrophy by increasing nuclear translocation of NFAT and activation of hypertrophic gene program [259, 410]. Folmes et al [103] have shown that decreased GSK-3β expression induced by over expression of AMPK is accompanied by increased nuclear NFAT activity [261-263]. Consequently, it is possible that in the current study decreased expression of GSK-3β induced by CA-AMPK led to increased protein synthesis by increased nuclear translocation. Further work is needed to elucidate these pathways.

Consistent with previous reports that activation of AMPK results in stimulation of fatty acid oxidation [28, 55, 57, 69, 154, 158, 170, 195], the current study demonstrated that CA-AMPK treatment of H9c2 cells, caused increased in rates of fatty acid oxidation (Figure 6-4C) AMPK activation in hearts and other tissues has been known to increase fatty acid oxidation by inactivating acetyl-CoA carboxylase (ACC) [35, 68] and, possibly, by activating malonyl-CoA decarboxylase (MCD) [57, 61, 235]. Malonyl-CoA is an allosteric inhibitor of carnitine palmitoyltransferase-1 (CPT-1) which controls transfer of long-chain fatty acyl-CoA into the mitochondria. Therefore, AMPK activation results in decreased malonyl-CoA concentrations and thus increased fatty acid oxidation.

In addition to activating fatty acid oxidation, CA-AMPK treatment of H9c2 cells resulted in acceleration of glycolysis (Figure 6-4A). It is well known that AMPK stimulates glycolysis by phosphorylation and activation of phosphofructokinase-2 [86], which produced fructose 2,6-bisphosphate, a potent stimulator of glycolysis pathway.

It should be noted that, in addition to the acute metabolic functions of AMPK, AMPK also regulates the expression of specific genes. Given the critical role of AMPK in regulation of energy metabolism in the body in response to acute energy depletion, it is not surprising that AMPK has a major role in response to chronic energy depletion. Repetitive pharmacological activation of AMPK *in vivo* mimicks some of the effects of exercise training including increases in mitochondrial biogenesis, and increased transcription of genes involved in fatty acid and glucose metabolism [228, 425-427]. Prolonged activation of AMPK by daily injections with AICAr, chronic intake of the creatine analogue β-guanadinopropionic acid as well as exercise-training skeletal muscle promotes expression of glucose transporter-4 (GLUT-4), hexokinase II, mitochondrial biogenesis, and other mitochondrial enzymes [228-231, 240, 249, 428]. An important mediator of many of the above actions is peroxime prolifrator-activator receptor γ coactivator 1α (PGC-1α) [228, 429]. PGC-1α activation increases expression of transcription factors such as nuclear respiratory factors 1 and 2, mitochondrial

transcription factor A, and peroxime prolifrator-activator receptor α (PPARα). The former, which is predominatly expressed in liver, heart, and skeletal muscle, plays a role in the transcriptional control of mitochondrial fatty acid oxidation by upregulation of genes involved in fatty acid oxidation [149, 430-434]. AMPK, therefore, might have a key role in inducing metabolic adaptations of skeletal muscle to exercise-training. Since CA-AMPK treatment of H9c2 cells led to increase in both fatty acid oxidation and glycolysis, it can be speculated that this effect is mediated by PGC-1α and increased mitochondrial biogenesis, in keeping with the proposed adaptive role of AMPK in exercised trained heart.

The other interesting finding in this study is that CA-AMPK led to increases in both glycolysis and fatty acid oxidation which raises the possibility that AMPK activation can bypass the Randle cycle as suggested by others [440]. This finding is also in agreement with the study performed by Merrill et al [435] in which they showed that AICAr-perfused skeletal muscle in rodent was accompanied with stimulation of both glucose uptake and fatty acid oxidation. They speculated that the concurrent stimulation of both glucose uptake and fatty acid oxidatoion in response to AMPK activation might have a role in the overall capacity of cell to meet energy demand. The finding that CA-AMPK treatment of H9c2 cells might bypass the Randle cycle further strengthens the idea that prolonged AMPK activation might have an adaptive role in the heart.

In summary, the current study showed that over expression of AMPK in H9c2 cells resulted in both structural and metabolic hypertrophic remodeling. The structural hypertrophic remodeling induced by over expression of AMPK is different from the pressure overload-induced cardiac hypertrophy due to lack of upregulation of ANF. Additionally, the hypertrophy induced by CA-AMPK was accompanied decreased expression of GSK-3β. Given the fact that GSK-3β is involved in both physiologic and pathologic forms of cardiac hypertrophy, it can be speculated that over expression of AMPK in heart muscle cells leads to a physiologic form of cardiac hypertrophy. Additionally, over expression of AMPK led to metabolic remodeling with acceleration

of both glycolysis and fatty acid oxidation. This action of AMPK to increase both glycolysis and fatty acid oxidation raises the possibility that AMPK can bypass the Randle Cycle and might enable the heart to meet increased energy requirements. Future studies are required to investigate whether AMPK activation in the heart leads to stimulation of mitochondrial biogenesis and expression of specific genes and proteins by PGC-1α and PPARα dependent pathways. Overall, this study adds to the emerging concept that AMPK enables the heart to adapt to changes in demand. Acting alone, AMPK does not induce a pathologic, but a more physiologic hypertrophic phenotype.

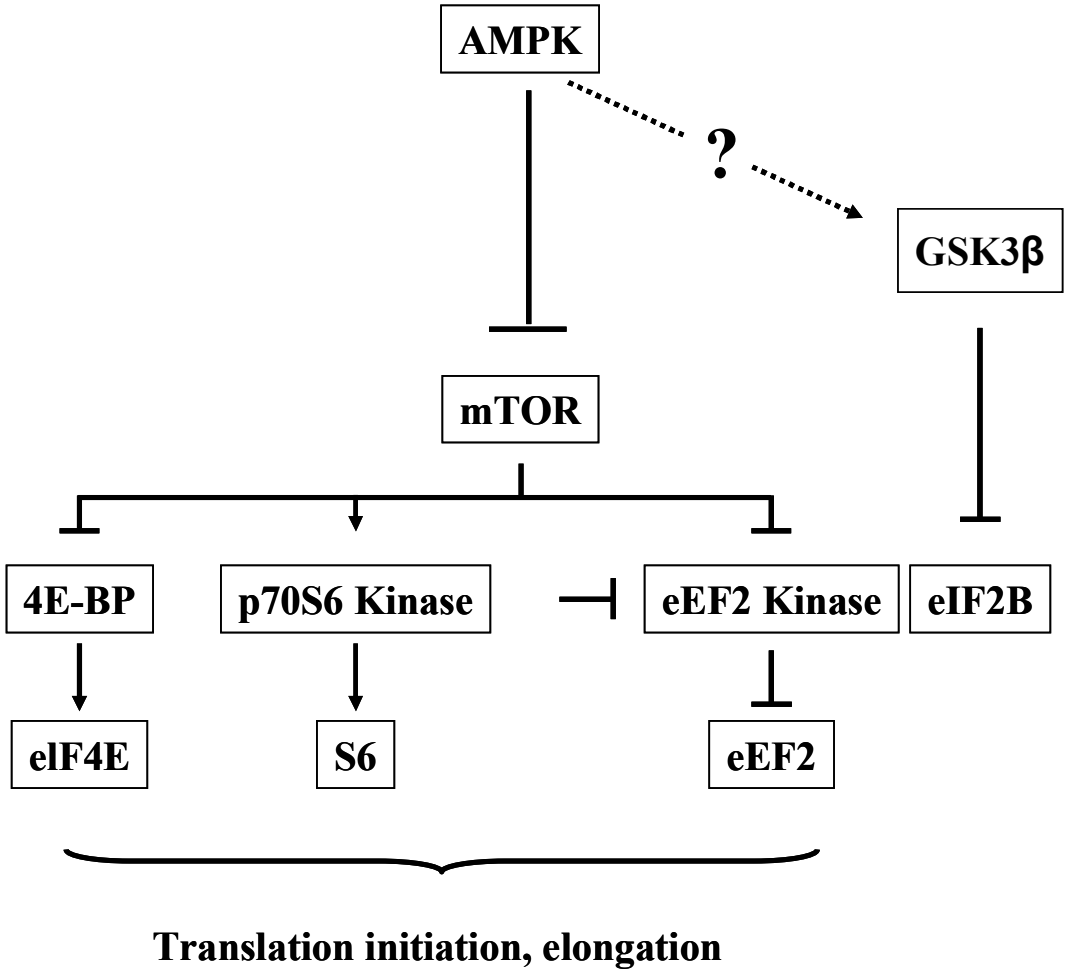

Figure 6-1: Regulation of protein synthesis by AMPK.

AMPK activation inhibits mTOR which this in turn stimulates protein synthesis, and hence cell growth, through P70S6K and 4E-BP1. AMPK activation also increases eEF2 kinase activity thereby inhibits protein synthesis. AMPK activation can also modulate GSK-3β activity.

AMP-activated protein kinase (AMPK), mammalian target of rapamycin (mTOR), P70 ribosomal protein S6 kinase (P70S6K), 4E-binding protein1 (4E-BP1), eukaryotic elongation factor-2 (eEF2), Glycogen synthase kinase-3β (GSK-3β), Protein kinase B (PKB), eukaryotic translation initiation factor (elF2B).

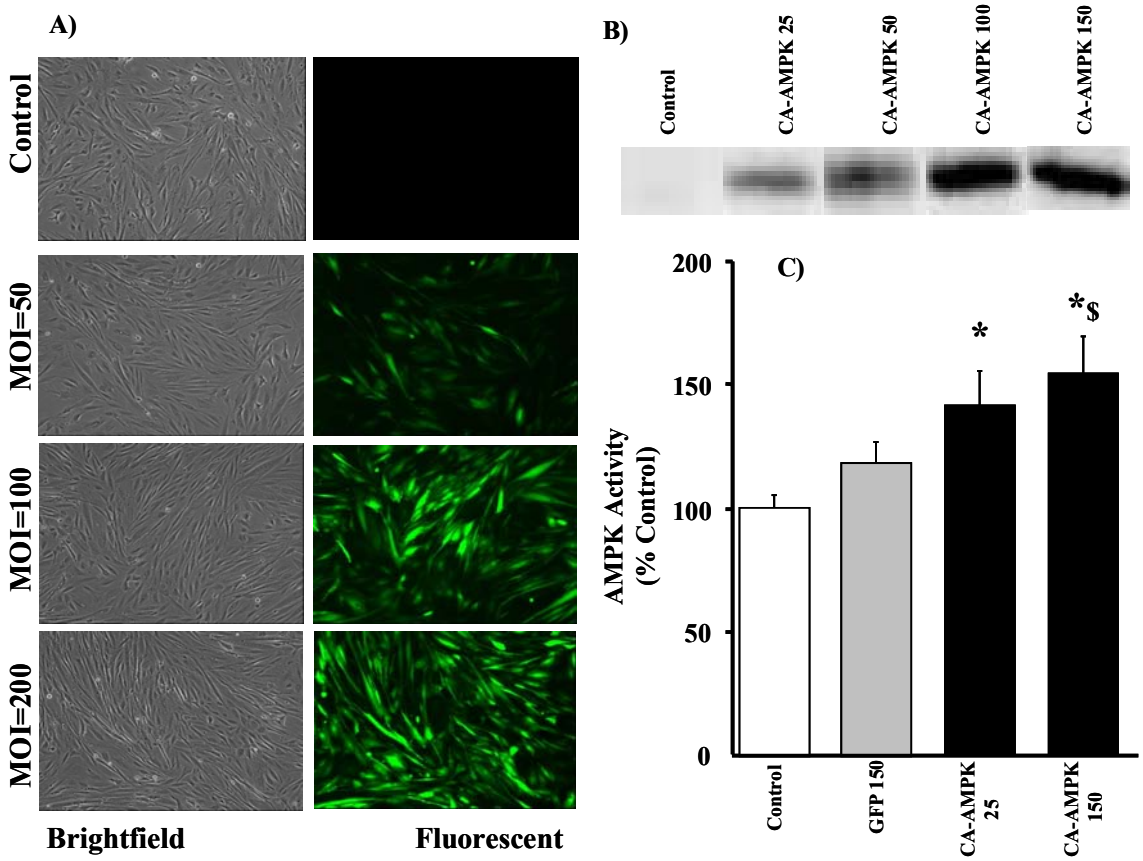

Figure 6-2: Effect of over expression of AMPK on AMPK activity in H9c2 cells.

Representative images of GFP expression (A), western blot showing c-myc expression (B), and AMPK activity (C) in differentiated H9c2 cells treated with CA-AMPK (moi 25 and 150) for 48 hours in DMEM supplemented with 1 % HS. Values are normalized to 100 % Control (in nmol/hr/mg ptn). Values are are Mean ± SEM. *, significantly different from Control ($p<0.05$). $, significantly different from GFP ($p<0.05$). N=26-34 per group.

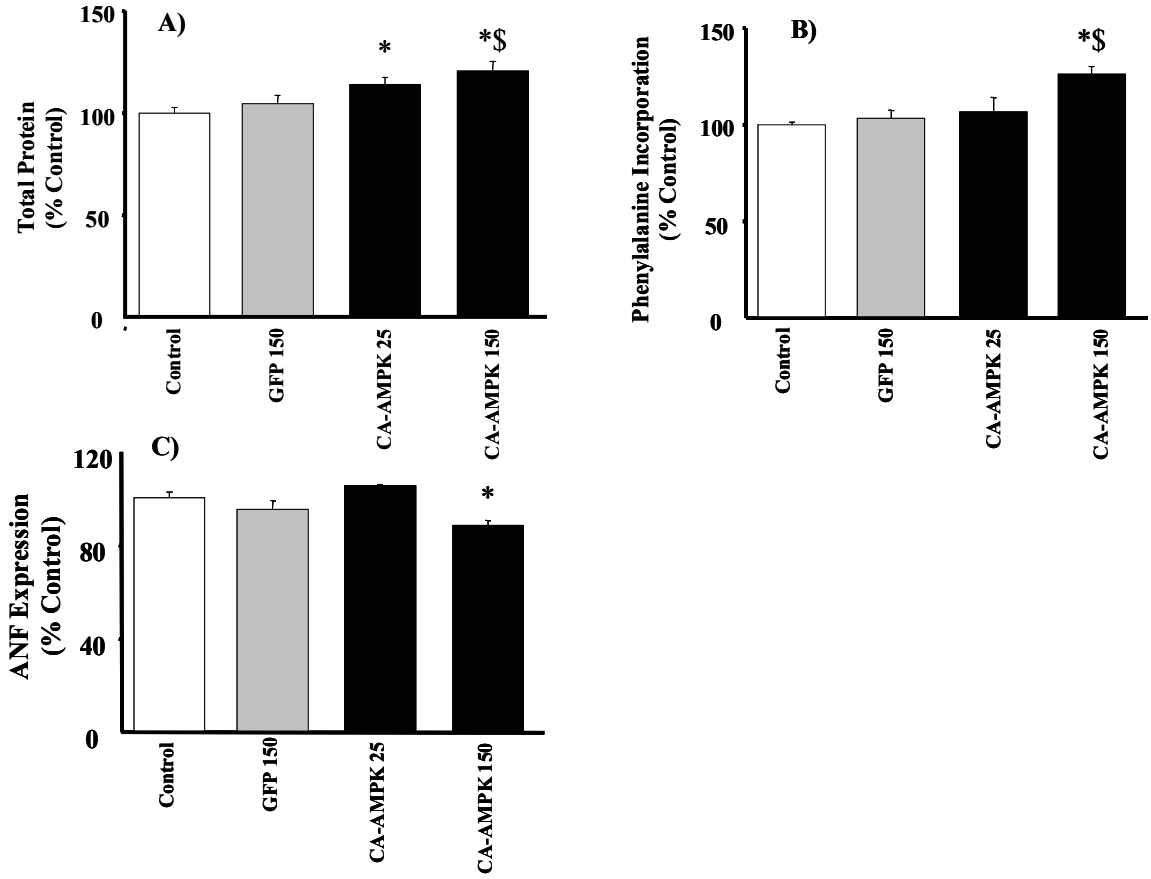

Figure 6-3: Hypertrophic characteristic in response to treatment with CA-AMPK.

Total protein content (A), [^{14}C]-phenylalanine incorporation (B), and ANF expression (C) in differentiated H9c2 cells treated with CA-AMPK (moi 25 and 150) in DMEM supplemented with1 % HS for 48 hours. Values are normalized to 100 % Control (in nmol/hr/mg ptn). Values are Mean ± SEM.*, significantly different from Control ($p<0.05$). $, significantly different from GFP ($p<0.05$). N=4-31 per each group.

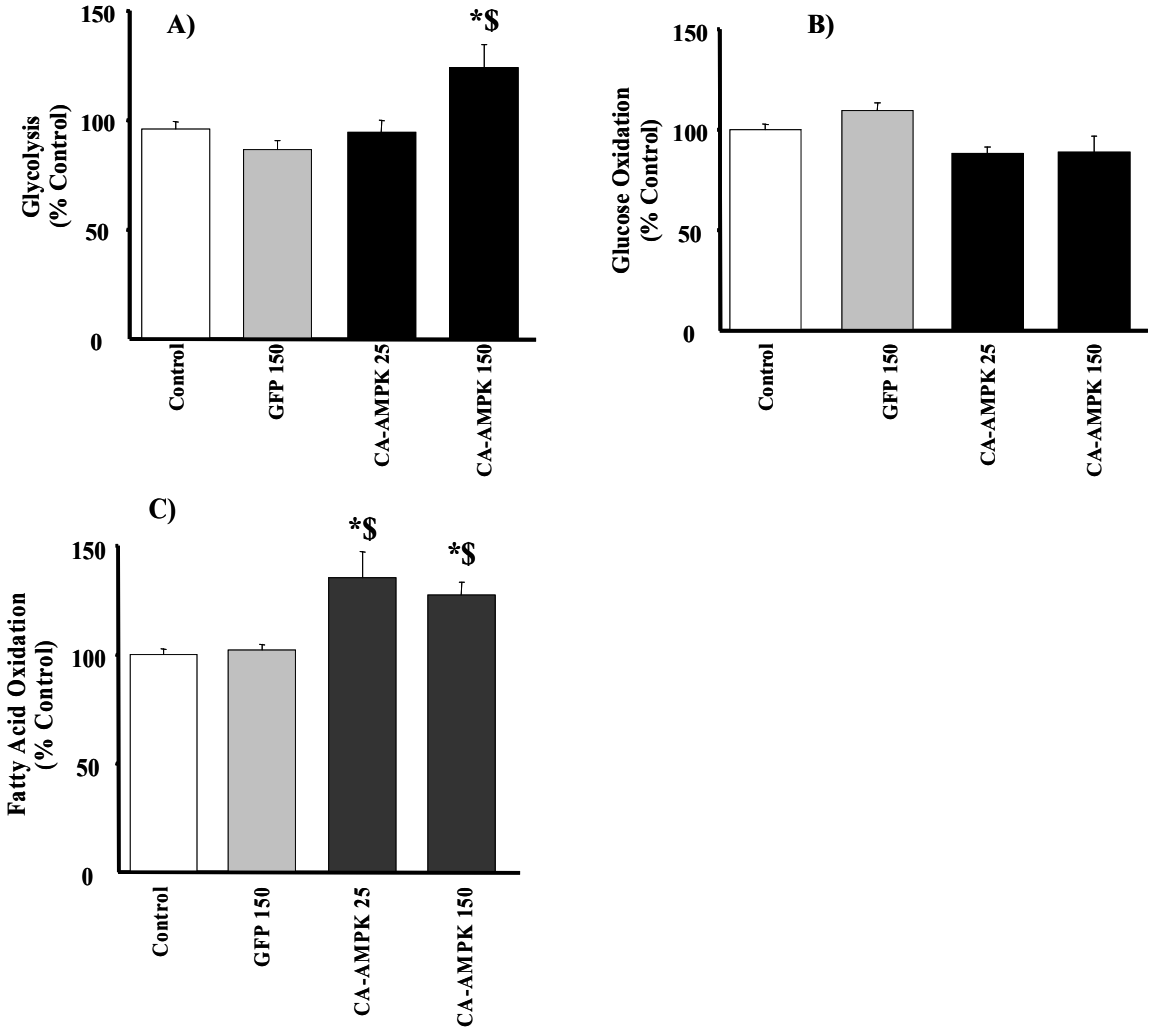

Figure 6-4: Effects of CA-AMPK on metabolism in H9c2 cells.

Differentiated H9c2 cells were treated with CA-AMPK (moi 25 and 150) for 48 hours before switching the KH solution containing 0.4 mM palmitate pre-bound to 3 % bovine serum albumin, 5.5 mM glucose, and 10^{-7} M insulin for 8 hours. Tracer amounts of labeled [5-^{3}H]-glucose (1.0 μCi/ml), [U-^{14}C]-glucose (1.0 μCi/ml), and [U-^{14}C]-palmitate (0.4 μCi/ml) were present for the last hour of study to measure glycolysis (Panel A), glucose oxidation (Panel B), and fatty acid oxidation (Panel C), respectively. Values are normalized to 100 % Control (in nmol/hr/mg ptn). Values are are Mean ±

SEM.*, significantly different from Control ($p<0.05$). $, significantly different from GFP ($p<0.05$). N=12-27 per group.

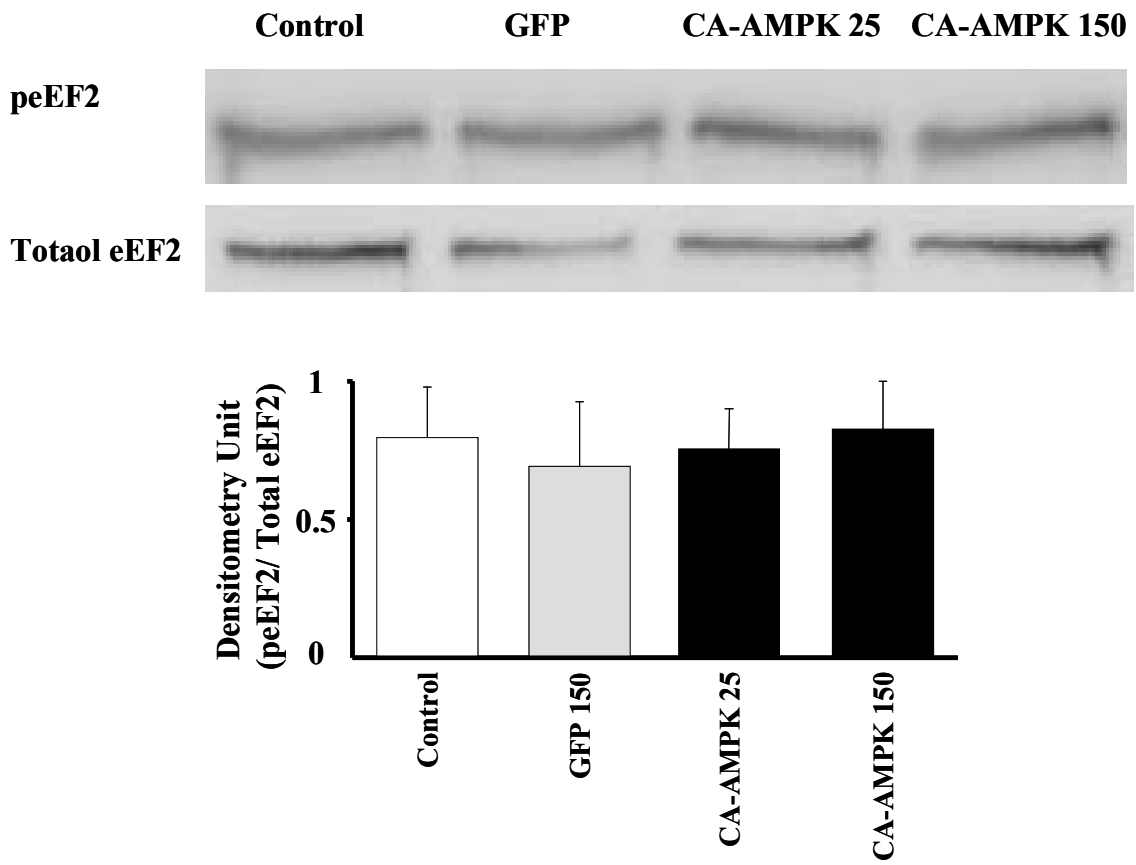

Figure 6-5: Effect of CA-AMPK on eEF2 expression in H9c2 cells.

Representative immunoblots and corresponding densitometric analysis of phosphorylation state of eEF2 protein in differentiated H9c2 cells treated with CA-AMPK (moi 25 and 150) in 1 % HS DMEM for 48 hours. Expression of total eEF2 was used as an internal standard to calculate relative expressions. Values are Mean ± SEM and are expressed as arbitrary density units. N=3 per each group.

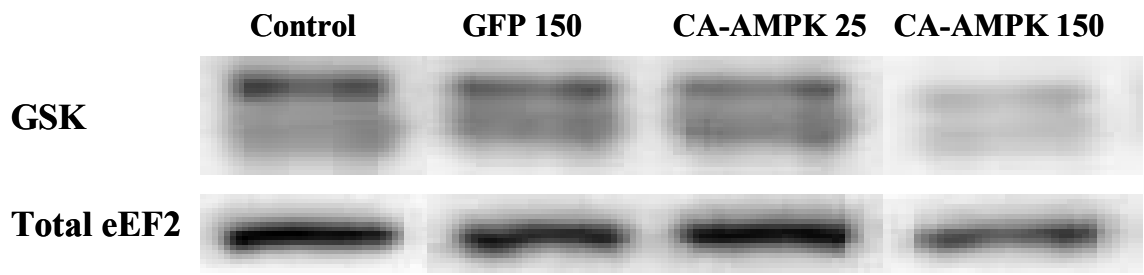

Figure 6-6: Effect of CA-AMPK on GSK3β expression in H9c2 cells.

Representative immunoblots of total GSK3β protein expression in H9c2 cells treated with CA-AMPK (moi 25 and 150) in 1 % HS DMEM for 48 hours. Expression of total eEF2 was used as an internal standard to calculate relative expressions. Values are expressed as arbitrary density units. N=2-3 per each group.

CHAPTER 7 - SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

7.1 SUMMARY

On the basis of the research presented here, several important and novel conclusions can be made regarding AMP-activated protein kinase (AMPK) activation in heart muscle cells and its role in the structural and metabolic remodeling.

First, chronic treatment of H9c2 cells with arginine vasopressin (AVP, 1 μM), induced both hypertrophy and alterations in energy metabolism similar to those of pressure overload-induced cardiac hypertrophy with an acceleration of glycolysis being characteristic. In AVP-treated hypertrophied H9c2 cells, AMPK activity was increased, confirming previous studies which demonstrated that AMPK activity is increased in pressure overload-induced hypertrophied hearts. The increased activity of AMPK in AVP-treated hypertrophied H9c2 cells was independent of any measurable changes in the energy status of cells, which further confirms previous studies that AMPK can be activated by energy state-independent mechanisms in hearts exposed to pressure-overload. Given the positive role of AMPK in glycolysis, activation of AMPK in hypertrophied hearts provides one potential mechanism for the acceleration of glycolysis. Here, it was shown for the first time that inhibition of AMPK by either

Compound C or DN-AMPK (moi 150) partially reduced glycolysis in AVP-treated hypertrophied H9c2 cells but did not normalize it. This finding unequivocally demonstrates a role for AMPK in the control of glycolysis in hypertrophied heart muscle cells and also suggests the involvement of other signaling pathways in acceleration of glycolysis in hypertrophied hearts.

Second, over expression of a constitutively active form of AMPK by means of adenoviral gene transfer (CA-AMPK) (moi 150) in H9c2 cells induced a number of responses including cellular hypertrophy as well as metabolic remodeling. AMPK-induced cardiac hypertrophy was accompanied by reduced expression of glycogen synthase kinase -3β (GSK-3β) but was independent of the eukaryotic elongation factor-2 (eEF2) pathway. Interestingly, the hypertrophy induced by CA-AMPK was not accompanied with increased expression of atrial natriuretic peptide, suggesting that AMPK activation causes a form of hypertrophy in heart muscle cells which is different from that observed in response to pressure overload and maybe more adaptive and physiologic. The metabolic remodeling induced by over expression of AMPK was characterized by acceleration of both glycolysis and fatty acid oxidation. This finding raises the interesting possibility that AMPK activation can bypass the fatty acid-glucose cycle described by Randle, as seen in endurance exercise training or when AMPK is activated for longer time with AICAr.

Third, in addition to its chronic effect, acute treatment of H9c2 cells with AVP (1 μM) caused AMPK activation and accelerated glycolysis. Also, AVP exerted its acute metabolic effects in H9c2 cells via the V1a receptor, the receptor subtype responsible for AVP-induced hypertrophy of heart muscle cells. Interestingly, the elevated rates of glycolysis induced by AVP were not altered by AMPK inhibition (by either Compound C or DN-AMPK), but were blocked by agents that interfere with calcium ion signaling, including extracellular EGTA, dantrolene and 2-aminoethoxydiphenyl borate. It can be concluded that the acute glycolytic effects of AVP are AMPK-independent and at least partially calcium-dependent.

Fourth, treatment of cells with other agents that may be involved in pressure overload-induced hypertrophy, such as ET-1, AgII, and PhE, did not induce hypertrophy when given under the same conditions as AVP. The reasons that these agents failed to induce hypertrophy in H9c2 cells under the conditions used are not known. It is possible that H9c2 cells lack some post-receptor signaling transduction pathways that ultimately result in cardiac hypertrophy. Additionally, a longer incubation time may be needed for these agents to cause hypertrophy in H9c2 cells.

Fifth, AMPK activity was not altered in isolated working rat hearts or H9c2 cells perfused/ or treated with 2 mM metformin in spite of the metabolic changes it induced. The absence of any measurable changes in AMPK activity in response to metformin supports data by others which indicate that activation of AMPK by metformin is due to reduction in the energy status of the cell. In the same study, it was found that inhibition of AMPK activity by either 6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrrazolo [1,5-a]-pyrimidine (Compound C), a well recognized pharmacological inhibitor of AMPK, or by over expression of a dominant negative form of AMPK by means of gene transfer (DN-AMPK) failed to normalize the metabolic effect-induced by metformin in H9c2 cells. Exposure of H9c2 cells to inhibitors of p38 mitogen activated protein kinase (p38 MAPK) or protein kinase C (PKC), partially or completely abrogated metformin-induced alterations in metabolism in these cells, respectively. Thus, it can be concluded that the metabolic actions of metformin in heart muscle can occur independent of changes in AMPK activity and may be mediated by p38 MAPK- and PKC-dependent mechanisms.

Sixth, treatment of H9c2 cells with 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAr), a cell permeable activator of AMPK, was unable to activate AMPK or alter metabolism in H9c2 cells. In fact, in the context of the current study, AICAr did not elicit any measurable changes in phosphorylation of AMPK and ACC, a downstream target of AMPK, whose phosphorylation and activation states are useful indicators of AMPK activity, suggesting that AMPK was not activated in H9c2 cells by

AICAr. The reason that AICAr was unable to activate AMPK or alter metabolism in H9c2 cells is not immediately apparent, but it could be speculated that AICAr was unable to enter the cells. To support this idea, a tissue- or cell-specific response to AICAr has been reported previously.

Overall, these studies indicate that AMPK is not the sole mediator of the metabolic phenotype of pathological hypertrophy. Inhibition of AMPK does not completely reverse the metabolic remodelling of pathological hypertrophy, and stimulation of AMPK does not completely induce it. The view of AMPK as the key mediator of the metabolic phenotype of pathological hypertrophy, especially related to glycolysis, is oversimplified. AMPK accelerates glycolysis, but is not consistently recruited to do so by all stimuli. This study has revealed the existance of other pathways which can stimulate glycolysis in heart muscle cells. AMPK increases protein content by increasing protein synthesis, but does not induce ANF expression suggesting participation in an adaptive, physiological process. These studies have therefore revealed that AMPK produces parallel effects on metabolism and hypertrophy, playing a pivotal role in a wider signaling cascade.

7.2 CONCLUSIONS

- Chronic treatment of H9c2 cells with AVP caused hypertrophy characterized by increased protein content, phenylalanine incorporation, and ANF expression without changes in overall cell number. However, other hypertrophic agents (ET-1, AgII, and PhE) failed to induce hypertrophy in these cells.

- In AVP-hypertrophied H9c2 cells glycolysis was accelerated and palmitate oxidation reduced with no significant alteration in glucose oxidation. The acceleration of glycolysis was associated with AMPK activation; inhibition of AMPK by molecular or pharmacological means ameliorated the increase in

glycolysis but did not prevent it. The increase in AMPK represented a change in activation state, not expression.

- CA-AMPK increased protein content and phenylalanine incorporation but did not increase ANF expression; the effects on protein synthesis were associated with changes in GSK-3β expression, but not eEF2 activation.

- CA-AMPK accelerated both glycolysis and fatty acid oxidation, therefore bypassing the Randle cycle.

- Activation of AMPK alone is insufficient to induce pathological hypertrophy.

- AVP was found to acutely and dose-dependently stimulate glycolysis via V1a receptor. Although AVP also acutely stimulated AMPK, inhibition of AMPK did not affect the stimulation of glycolysis; this response was blocked by agents that interfere with calcium ion signaling.

- Metformin did not activate AMPK in H9c2 cells. The action of metformin to accelerate glycolysis was not prevented by AMPK inhibition, but was prevented by inhibition of p38MAP kinase and and PKC pathways.

7.3 RECOMMENDATIONS AND FUTURE DIRECTIONS

In the present study, H9c2 cells were used as an *in vitro* model system to investigate the effect of AVP on metabolic remodeling in the heart. These cells, which were originally derived from the rat embryonic ventricle, have morphological characteristics similar to those of immature embryonic cardiac myocytes [347, 436]. The H9c2 cells also show biochemical and electrophysiological properties similar to those of adult cardiac cells [347] and express cardiac and skeletal isoforms of L-type Ca^{2+} channels and sarcolemmal ATPase splice variants present in the normal heart [436, 437]. As a consequence, they are widely accepted as a model for the study of cardiac myocytes *in*

vitro [438], including the study of metabolism and signal transduction pathways [439]. Such cells, however, cannot be considered completely representative of adult or neonatal cardiac myocytes. Thus, the findings from the current study must be confirmed using adult cardiac myocytes in culture and, ultimately, in intact hearts. Therefore, cellular and animal models of cardiac hypertrophy in which the activity of AMPK could be altered using molecular approaches in cardiac myocytes before or after induction of hypertrophy should be used. Specifically, future studies should be performed to confirm that AMPK has a partial role in the acceleration of glucose use in hypertrophied cells. If this is true, the additional mechanisms responsible for increased glucose use in hypertrophied hearts should be investigated. Additionaly, the role of over expression of AMPK in hypertrophic growth and metabolism should be determined.

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