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AMP-Activated Protein Kinase Activation Preconditions the Heart against  
Ischemic Injury

A Thesis Submitted to the  
Yale University School of Medicine  
In Partial Fulfillment of the Requirements for the  
Degree of Doctor of Medicine

By

Tracy M. Wright

2009

## Abstract

### AMP-ACTIVATED PROTEIN KINASE ACTIVATION PRECONDITIONS THE HEART AGAINST ISCHEMIC INJURY.

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AMP-activated protein kinase (AMPK) is a well-established regulator of cellular energy status and metabolic function, and is a vital molecule during the acute response to ischemic stress in the heart. However, its role in preconditioning against ischemic injury is still not clearly defined. Using a novel and specific AMPK activator, A-769662, we wanted to determine if pharmacologic, pre-ischemic activation of AMPK is sufficient to protect the heart against subsequent ischemia-reperfusion injury. Using two mouse models of ischemia: 1) the Langendorff perfused heart and 2) *in vivo* coronary occlusion, we investigated whether A-769662 treatment would activate the AMPK pathway and if pre-ischemic AMPK activation was cardioprotective. In these models, wild type C57BL/6 and transgenic AMPK kinase dead (KD) mice hearts were subjected to ischemia for 25 minutes (perfused heart) or 20 minutes (*in vivo*), followed by reperfusion. A-769662 or vehicle control was administered in the perfusion buffer (100uM, perfused heart) or by intra-peritoneal injection (6mg/kg, *in vivo*), prior to ischemia. A-769662 treatment resulted in AMPK activation in the perfused heart and in the intact heart *in vivo* in the absence of ischemia, and decreased myocardial injury when administered prior to ischemia in both models. These cardioprotective effects were abolished in the AMPK deficient AMPK KD hearts. In the wild type perfused heart, we found evidence that pre-ischemic A-769662 treatment leads to increased end-ischemia adenosine triphosphate (ATP) content, increased end-ischemia phosphorylation of eukaryotic elongation factor 2 (eEF2) at threonine 56, and increased endothelial nitric oxide synthase (eNOS) phosphorylation at serine 1177 residue. These findings show that A-769662 treatment leads to myocardial AMPK activation, and preconditions the heart against ischemia in an AMPK dependent manner, possibly through an AMPK-eEF2 or AMPK-eNOS signaling pathway.

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## **I. Introduction**

Ischemic heart disease is the leading cause of death in developed countries including the United States (1). Presentations of coronary artery disease span the spectrum from angina pectoris to acute myocardial infarction. When a coronary artery becomes occluded and myocardium becomes ischemic, several molecular signaling pathways work to protect the hypoxic cells from injury and death. AMP-activated protein kinase (AMPK) is a regulator of cellular energy balance having multiple metabolic and non-metabolic functions central to energetic stability (2, 3). Sensing energy depletion in the cell, AMPK becomes active and promotes energy producing pathways such as glycolysis, while turning off energy consuming pathways such as fatty acid and cholesterol synthesis (2). The cardioprotective role of AMPK activation during acute ischemia has been well defined (3, 4). Additional studies have suggested a possible role of AMPK in preemptively preconditioning the myocardium against ischemic injury (5-8). Recently, a potent and direct activator of AMPK, known as A-769662, has been identified (9). Using A-769662 in experimental models of myocardial ischemia, the focus of this work is on the pharmacological activation of AMPK, and its potential role in protecting against ischemia-reperfusion injury.

### **A. Ischemic Heart Disease**

Ischemic heart disease is the most common cause of death in developed countries (1) and is also responsible for a large proportion of deaths globally (10). Ischemia occurs when blood supplied to the myocardium by the coronary arteries is not sufficient to meet the heart's energy demands. It is most commonly caused by atherosclerotic narrowing of

the lumen in the coronary arteries. When the lumen is more than 50-60% stenosed, classic angina occurs due to insufficient increase in coronary blood flow and the inability to meet the increase in energy demand. As the stenosis worsens or the artery becomes occluded, permanent myocardial damage may ensue leading to arrhythmias or heart failure. Plaque rupture, coronary artery thrombosis, or coronary vasospasm can lead to acute coronary occlusion (11, 12).

Ischemic heart disease may manifest in patients as one of several clinical syndromes, including chronic angina pectoris, acute coronary syndrome (unstable angina, myocardial infarction, and sudden cardiac death), and chronic ischemic cardiomyopathy with congestive heart failure. Clinical manifestations of ischemic heart disease usually develop in older adults, with a peak incidence after 60 years of age in men, and after 70 years of age in women. Men are more commonly affected, except after the age of 90, when both sexes are equally affected (11). Factors that promote coronary atherosclerosis include hypertension, diabetes mellitus, hyperlipidemia, smoking, and genetics (11, 12). The increase in incidence and prevalence of many of these risk factors for ischemic heart disease (1) is worrisome and highlights the importance of research focused on its treatment and prevention.

Current treatments for ischemic heart disease are aimed at risk factor modification, relief of symptomatic angina, and coronary revascularization. Changes in lifestyle such as diet modification and increased physical activity help in the prevention of ischemic heart disease. Medically targeted risk factors include hypertension and hyperlipidemia. ACE inhibitors and beta-blockers are commonly used to control blood pressure, while statins reduce hypercholesterolemia. These interventions decrease

mortality in chronic ischemic disease (13), while platelet-inhibiting medication and percutaneous and surgical revascularization have been shown to decrease the mortality of acute coronary syndromes (14). Despite efforts to prevent and treat ischemic heart disease, myocardial injury remains a serious clinical problem, and more research is needed to develop new therapies aimed at preventing injury. Current experimental models of ischemia have been very useful in this regard.

### ***Experimental Models of Ischemic Heart Disease***

There are several experimental models of myocardial ischemia that have been used to study the pathophysiology of myocardial damage, and to test treatments to reduce injury. The choice of model for studying ischemia depends on the question being tested (15). Two popular models of myocardial ischemia are the isolated Langendorff-perfused heart and *in vivo* coronary artery occlusion in the anesthetized animal.

First established in 1897 by Oscar Langendorff, the isolated perfused mammalian heart preparation has been a productive model for studying cardiac pathology, physiology, and pharmacology for more than 100 years (16, 17). In this model, the heart is removed from the chest and the aorta is cannulated and perfused with buffer in a retrograde manner. This leads to perfusion of the coronaries with buffer and closure of the aortic valve due to the perfusate pressure. The Langendorff model offers several advantages. One advantage is that many models of ischemia can easily be performed. Examples include global ischemia, created by adjusting the retrograde flow through the aorta, and hypoxia, which is induced by lowering the oxygen content of the buffer. Regional ischemia may also be produced by the placement of a ligature around a

coronary artery, although this model is not commonly used (17). Other advantages include the model's simplicity, and easy control of perfusate content, coronary perfusion, and metabolic content at any time during the perfusion (15). Despite some shortcomings, such as lack of neuronal and humoral regulators found *in vivo*, the Langendorff model's advantages are numerous and it remains one of the most popular models in cardiovascular research (15).

The *in vivo* models of ischemia resemble what might occur in a clinical situation. Regional ischemia due to coronary ligation closely correlates with acute coronary syndrome. This method has been used in canines, pigs, rabbits, rats, and mice (15). In the anesthetized animal, the chest is opened, allowing for occlusion and reperfusion of coronary arteries. In mice, regional ischemia is achieved by occluding the left coronary artery (LCA). The LCA may be occluded proximally or more distally, resulting in different areas at risk (AAR) for ischemia injury. The volume of the AAR is an important determinant of the infarct size (18), and can be revealed using dyes (Evans blue). Injection of blue dyes into the aorta with the coronary artery occluded, demarcates the unperfused tissue. *In vivo* regional ischemia in an anesthetized animal model is also a proven model in studying ischemic preconditioning (a process in which brief periods of ischemia and reperfusion protect against subsequent, more severe ischemia) (15, 19, 20) and tissue remodeling (scarring) following myocardial infarction (21).

### ***Molecular Consequences of Ischemia***

Ischemia causes both oxygen and substrate deprivation, resulting in decreased aerobic energy generation. This leads to a fall in the content of adenosine triphosphate

(ATP), which has several consequences. One important consequence is decreased activity of the ATP-dependent sodium/potassium pump, which is responsible for maintaining cellular sodium and potassium gradients. The accumulation of intracellular sodium leads to increased sodium/calcium exchange causing an increased intracellular calcium concentration, resulting in cellular injury. A second consequence of ischemia is increased anaerobic glycolysis. This is in part due to the increased adenosine monophosphate (AMP):ATP ratio. Increased AMP levels stimulate phosphofructokinase, an important enzyme in the glycolytic pathway, as well as glycogen phosphorylase, in an effort to meet the cell's energy demands. Increased glycolysis leads to the accumulation of lactic acid, resulting in an acidic intracellular pH. This change in cellular environment leads to a third important consequence of ischemia—decreased protein synthesis. This occurs because of ribosome dissociation in the presence of an acidic pH and decreased ATP. Ischemia is reversible if blood flow is restored, but may lead to irreversible membrane damage and cellular injury if prolonged (11).

In addition to profound repercussions of cell injury and death during ischemia, cellular injury may also occur during reperfusion. This is known as ischemia-reperfusion injury. Ischemia-reperfusion injury results from 1) calcium overload in ischemic tissue leading to increased protease, phospholipase, and, endonuclease activities and subsequent cellular damage, and 2) the production of free radicals such as reactive oxygen species, released from damaged mitochondria (22, 23). Free radicals can cause DNA fragmentation, lipid peroxidation of membranes, and protein cross-linking, resulting in cellular injury. The end result may be changes in myocardial structure and function

including, reduced myocardial contractility, cardiac hypertrophy, and cardiac apoptosis (11, 23).

As discussed earlier, current treatments of ischemic heart disease are aimed at preventing ischemia and achieving revascularization as quickly as possible. However, pharmacologic manipulation to alter the metabolic consequences of ischemia may provide a new strategy to decrease myocardial damage during acute ischemia that occurs in the setting of acute myocardial infarction.

### **B. Myocardial Preconditioning**

Myocardial preconditioning is a process in which tissue is made resistant to ischemic injury. Ischemic preconditioning is a process in which brief periods of ischemia and reperfusion prevent injury during subsequent episodes of more sustained, severe ischemia. Ischemic myocardial preconditioning was first demonstrated by Murry et al. (24) in dogs exposed to 4 short 5-minute cycles of coronary artery occlusion, followed by 5 minutes of reperfusion, and subsequently subjected to 40 minutes of occlusion and 4 days of reperfusion. These hearts had significantly smaller infarct size compared with control hearts which did not receive the short periods of ischemia-reperfusion prior to the 40 minute occlusions. Since this work, ischemic preconditioning has been observed in several animal models. It has also become evident that pharmacologic treatment can protect against ischemic injury. Several agents including adenosine agonists, nitric oxide donors, phosphodiesterase inhibitors, bradykinin, and noxious stimuli (i.e. reactive oxygen species (ROS), endotoxin), have been shown to bring about pharmacologic preconditioning (25, 26).

Preconditioning, whether induced by ischemia or pharmacologic agents, has two phases: an early phase and a late phase (25). Both phases have intricate mechanisms for inducing protection. The early phase arises within minutes of the stimulus and lasts 1-2 hours. The late phase of preconditioning develops within 12-24 hours of the stimulus and lasts 3-4 days. Ischemia causes the release of endogenous molecules such as adenosine, opioids, bradykinin, prostaglandins, and ROS. These molecules can initiate the activation of signaling cascades involving protein kinase C (PKC) (27), Src family of tyrosine kinases (28), and mitogen-activated protein (MAP) kinases, including p38MAPK (29). Activation of these pathways leads to modifications of preexisting proteins (early phase) and changes in gene expression and the synthesis of new cardioprotective proteins (late phase), including heat shock proteins, NO synthase (NOS), COX-2, and manganese superoxide dismutase (25).

Endogenous molecules and new protective proteins may exert cardioprotection through effects in the mitochondria. One important mitochondrial change is the inhibition of the opening of the mitochondrial permeability transition pore (mPTP) that usually occurs during early reperfusion (30). The normally impermeable inner mitochondrial membranes become leaky during reperfusion due to mPTP opening from ROS bursts and increases in intracellular  $\text{Ca}^{2+}$ . Increased membrane permeability leads to mitochondrial matrix swelling and irreversible damage and cell death. Therefore, minimizing the permeability of these mitochondrial membranes during ischemia-reperfusion increases the chance for cell survival (25, 30, 31). Several of the before mentioned pathways may ultimately precondition through this mechanism. One specific

study showed that activation of endothelial nitric oxide synthase (eNOS), which increases NO production, inhibits mPTP opening during reperfusion (32).

The opening of mitochondrial ATP dependent potassium ( $K_{ATP}$ ) channels has also been studied as a mechanism of preconditioning (25). The role of  $K_{ATP}$  channels in preconditioning is supported by the observation that hydrogen peroxide-induced preconditioning is prevented by the use of  $K_{ATP}$  channel inhibitors such as glybenclamide (25). A second study showed that patients with stable angina had decreased mortality with the use of nicorandil, a  $K_{ATP}$  channel opener, suggesting clinical relevance for this pathway (33). While the mechanism is not completely understood, mitochondrial  $K_{ATP}$  channels may contribute to myocardial preconditioning by inhibiting intracellular calcium overload or by generating ROS (34). ROS, though often injurious to the cell, can actually lead to the activation of protective redox signaling molecules like thioredoxin (35). In addition, the opening of mitochondrial  $K_{ATP}$  channels has been shown to secondarily activate the PKC pathway (36), a pathway implicated in preconditioning (27).

### **C. AMP-Activated Protein Kinase**

AMP-activated protein kinase (AMPK) is a key regulator of several protective cellular pathways during periods of energetic stress, including ischemia (37). AMPK regulates these changes both acutely, through phosphorylation of key enzymes, and in the long term, by regulating gene expression. AMPK increases ATP generation by stimulating catabolic processes such as glycolysis during ischemia and fatty acid oxidation during reperfusion, leading to energy production. AMPK has been shown to

increase glucose uptake in muscle (38), and to improve insulin resistance in cells (39). Conversely, AMPK also inhibits anabolic pathways that consume energy (2), therefore safeguarding the cellular energy state. AMPK is a key metabolic player globally and is found in several organs including heart, liver, and skeletal muscle.

### ***Structure***

AMPK is a heterotrimeric complex: a protein containing three subunits. These subunits include one catalytic  $\alpha$  subunit, and two regulatory subunits, the  $\beta$  and  $\gamma$  subunits. Each subunit has two or more isoforms found in various combinations in different tissues. The heart contains the  $\alpha 1$  and  $\alpha 2$ ,  $\beta 1$  and  $\beta 2$ , and  $\gamma 1$  and  $\gamma 2$  isoforms, notably missing the  $\gamma 3$  isoform that is found predominantly in skeletal muscle (3).

### ***Mechanisms of Activation***

AMPK's  $\alpha$  subunit contains its catalytic domain which is a serine-threonine kinase. It is phosphorylated by upstream kinases, such as the tumor suppressor kinase LKB1. The threonine-172 residue in the activation loop of the  $\alpha$  subunit is phosphorylated by upstream kinases, causing AMPK to be activated. The  $\beta$  subunit is an important AMPK structural unit, binding both the  $\alpha$  and  $\gamma$  subunits. A glycogen-binding domain is present on the  $\beta$  subunit, allowing AMPK to associate with glycogen in intact cells. AMPK's  $\gamma$  subunit contains regions known as Bateman domains, which contain the AMP binding domain (3), important for sensing the energy status of the cell.

In the presence of an increased AMP:ATP ratio, greater binding of AMP to the  $\gamma$  subunit's Bateman domains leads to AMPK activation following a conformational

change of the molecule. This allosteric activation of the  $\alpha$  subunit together with enhanced phosphorylation of the threonine-172 residue by upstream kinases leads to increased activity of AMPK (40). AMP binding inhibits protein phosphatase action on AMPK, preventing dephosphorylation of threonine 172. AMPK can also be phosphorylated by calmodulin-dependent protein kinase kinase (CaMKK) (41, 42), although the extent to which this occurs in the heart is still under investigation.

### ***Cardioprotective Role during Acute Ischemia***

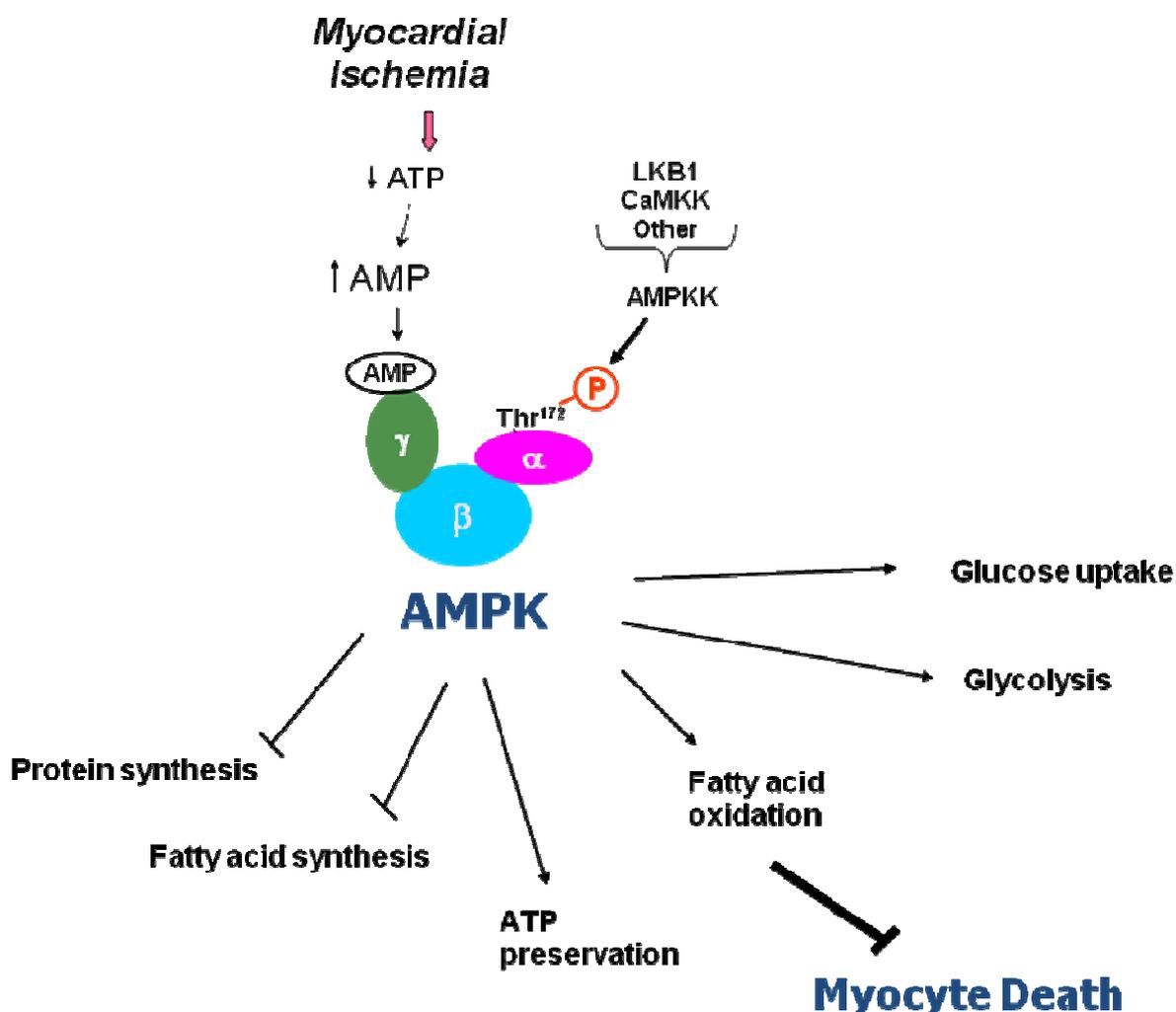
AMPK has been shown to be a significant cardioprotective molecule during acute ischemia. Our lab previously demonstrated that hearts expressing a mutated AMPK  $\alpha 2$  subunit (K45R mutation), resulting in lack of AMPK activity (AMPK kinase dead (KD)), had greater myocardial necrosis and poorer recovery of function following global ischemia compared with wild type hearts (4), strongly suggesting AMPK's vital metabolic role during episodes of acute ischemia.

During ischemia, intracellular ATP is utilized, while ADP, AMP, creatine, and inorganic phosphate concentrations increase. An increased AMP:ATP ratio activates AMPK, leading to several metabolic consequences. One of the important AMPK-mediated metabolic events in the ischemic heart is an increase in glucose uptake by cardiac myocytes. To stimulate increased glucose transport, GLUT4 glucose transporters must move to the cell surface from their storage sites in intracellular membrane vesicles, a process called translocation. AMPK has been shown to mediate GLUT4 translocation during ischemia (4). Once glucose enters the cell, AMPK promotes its utilization in the glycolytic pathway by stimulating the phosphorylation of 6-phosphofructo-2-kinase

(PFK2), an important enzyme in glycolysis (43). PFK2 produces  $\beta$ -D-fructose 2, 6-bisphosphate (F-2, 6-BP), a molecule which activates phosphofructokinase 1 (PFK1), a catalyst of the conversion of fructose 6-phosphate and ATP to fructose 1,6-bisphosphate and ADP, one of the main rate-limiting steps of glycolysis.

AMPK activation in the heart also increases myocardial fatty acid oxidation during reperfusion. AMPK phosphorylates and inhibits acetyl-CoA carboxylase (ACC), inhibiting the production of malonyl-CoA, and ultimately relieving inhibition of fatty acid transport into the mitochondria via carnitine palmitoyltransferase-1 (CPT-1). Therefore, increased fatty acid oxidation occurs during reperfusion, generating more ATP for the stressed cell (44). In the AMPK KD mice, fatty acid oxidation has been shown to decrease, further pointing to AMPK's role in ischemia-reperfusion (4). Though fatty acid oxidation is important for ATP generation, a detrimental effect of increased fatty acid oxidation during reperfusion is the inhibition of pyruvate dehydrogenase, an important enzyme in glucose oxidation (45). This can potentially increase intracellular acidosis by the accumulation of lactic acid. Therefore, it is still debated as to whether AMPK activation is a beneficial or injurious during ischemia-reperfusion (46).

In addition to stimulating the creation of energy, AMPK switches off metabolic cellular processes that consume energy. AMPK has been shown to inhibit fatty acid synthesis by inactivating ACC in the liver (47), glycogen synthesis through the inhibition of muscle glycogen synthase (48), and cholesterol synthesis through the inhibition of HMG-CoA reductase in liver (47). All of these functions consume ATP, so their inhibition preserves more energy during times energetic stress.



**Figure 1. AMPK activation in the ischemic heart—mechanism and downstream effects.** When the intracellular AMP:ATP ratio increases during myocardial ischemia, there is increased AMP binding of AMPK's  $\gamma$  subunit, leading to a conformational change in the molecule. This leads to enhanced threonine-172 phosphorylation of AMPK's  $\alpha$  subunit by upstream kinases (ie. LKB1 and CaMKK) and greater AMPK activity. Activated AMPK increases ATP generation by stimulating catabolic processes such as glucose uptake and glycolysis during ischemia and fatty acid oxidation during reperfusion. Activated AMPK also decreases ATP consumption by inhibiting anabolic processes such as protein and fatty acid synthesis. These actions result in ATP preservation and decreased myocardial death following acute ischemia (3, 4).

AMPK also conserves ATP through the inhibition of protein synthesis and cellular proliferation. AMPK has been shown to play a role in the inhibition of cell proliferation in normal and malignant cells (49). The discovery that the tumor suppressor kinase LKB1 lies upstream of AMPK (50) and that the key tumor suppressors, such as the mammalian target of rapamycin (mTOR), tuberous sclerosis complexes (TSC1 and TSC2), and p53, lie downstream of AMPK, suggests that this molecule conserves energy by regulating cellular proliferation (49).

AMPK also slows protein synthesis in non-dividing cells such as those found in the heart. Specifically, AMPK inhibits mTOR either by direct phosphorylation or by phosphorylation and activation of TSC2. Increased TSC2 activation leads to greater TSC1-TSC2 complex activity. This complex inhibits mTOR. mTOR inhibition leads to decreased p70S6 kinase activation (S6 kinase), an important enzyme in protein translation (49). AMPK also prevents protein synthesis, through the activation of eukaryotic elongation factor 2 (eEF2) kinase (51). Once activated, eEF2 kinase inhibits eEF2, a molecule important for the elongation step in protein translation.

### ***Role in Myocardial Preconditioning***

Though intrinsic AMPK activation has been shown to be protective in the cellular response to ischemia, its role in preconditioning prior to ischemia is still controversial. Data from our lab has shown that ischemic preconditioning is possible in both wild type and AMPK KD mice, suggesting that AMPK is not necessary for this process (52). However, pharmacological activation of AMPK prior to ischemia may be sufficient to decrease myocardial injury during ischemia-reperfusion.

AMPK appears to play a role in preconditioning experimentally. Treatment with 5-aminoimidazole-4-carboxamide 1- $\beta$ -D-ribofuranoside (AICAR), a nonspecific AMPK activator, in tandem with ischemic preconditioning led to smaller infarct size in rabbits during coronary artery ligation (53). However, neither AICAR, nor ischemic preconditioning alone, led to any significant change in infarct size following coronary ligation in this study. AICAR has also been shown to extend the duration of the window of protection by ischemic preconditioning in rabbits (5). In addition, AICAR preconditions rat livers against ischemia-reperfusion injury through the activation of AMPK (6). Ischemic preconditioning in rabbits was also associated with AMPK activation and increased GLUT4 mRNA expression (7). Finally, AMPK has been shown to mediate preconditioning against ischemia-reperfusion injury in mice treated with metformin, possibly through its activation of eNOS (8). AMPK phosphorylates eNOS (54, 55) on serine 1177 and eNOS is a molecule known to be involved in preconditioning (8, 32).

There are several possible mechanisms by which AMPK may lead to preconditioning. One study showed that ischemic preconditioning leads to AMPK activation and GLUT4 up-regulation (7). The authors hypothesized that GLUT4 up-regulation may precondition against myocardial stunning, a lingering contractile dysfunction that occurs after brief ischemia, potentially by increasing the energy available during ischemia. In addition, other functions of AMPK as a metabolic regulator (including protein synthesis inhibition through eEF2 inactivation) could potentially be involved (56).

AMPK is also involved in known preconditioning pathways. AMPK has been observed to mediate the recruitment and activation of mitochondrial  $K_{ATP}$  channels in cardiomyocytes, leading to preconditioning against hypoxic injury (57). AMPK also activates p38MAPK (58) and eNOS (54, 55), both of which are involved in preconditioning (8, 32, 58).

#### **D. A-769662 General History and Mechanism for its Activation of AMPK**

Given AMPK's important role in metabolic homeostasis and possible involvement in preconditioning, research aimed at pharmacologically activating this molecule could ultimately lead to important advances in the clinical intervention and treatment of coronary artery disease and organ ischemia. Three pharmacologic agents have previously been used as activators of AMPK: 1) AICAR, 2) biguanides, (i.e. metformin), and 3) thiazolidinediones, (i.e. rosiglitazone) (59). These agents all have additional effects and are nonspecific activators of AMPK. AICAR undergoes intracellular conversion to ZMP, an AMP analog, leading to ZMP's binding of AMP binding sites and AMPK activation. However, AICAR also increases extracellular adenosine concentrations. Metformin is an inhibitor of complex I of the mitochondrial respiratory chain (60), and may activate AMPK by increasing the cellular AMP:ATP ratio. Treatment with thiazolidinediones causes adipocyte expression and release of adiponectin, a molecule which stimulates glucose uptake and fatty acid oxidation through the activation of AMPK (61). In addition, these agents are peroxisome proliferator-activator receptor (PPAR) agonists, nuclear receptors involved in metabolism and cellular differentiation. Unwanted side effects such as hypoglycemia and heart failure occur with

intravenous AICAR (3). Gastrointestinal changes are seen with metformin (59), while weight gain is observed with thiazolidinediones (59). Thus, a compound that directly activates the AMPK pathway may have metabolic and non-metabolic benefits and avoid some of the side effects seen with the administration of other agents.

A-769662, a specific and potent AMPK activator, was developed following a screen of >700,000 compounds. In the original study, this molecule was shown to activate AMPK, independent of AMP or ZMP concentration, distinguishing it from AICAR (9). A-769662 has been proposed to allosterically activate AMPK, without actively increasing the phosphorylation of threonine-172 (62). AMPK phosphorylation was only slightly increased in isolated cells treated with A-769662, while ACC, a direct target of AMPK alone and a measure of AMPK's overall activity, showed significant increases in phosphorylation (62). Further studies revealed that direct binding between A-769662 and AMPK may lead to a conformational change in AMPK, rendering it resistant to dephosphorylation at the threonine-172 residue (62, 63), maintaining AMPK's active, phosphorylated state. This conformation has been observed to require the phosphorylation of the Serine-108 residue in the glycogen-binding domain of the AMPK's  $\beta$  subunit, leading to steric hinderance of threonine-172 to protein phosphatases by the  $\beta$  subunit, and ultimately preventing its dephosphorylation. The authors of this study propose that the phospho-Serine-108 may interact with another region of the heterotrimeric AMPK, and that this interaction is promoted by A-769662 (63).

Therefore, it seems that A-769662 works both as an allosteric activator of AMPK and as an inhibitor of its dephosphorylation (62, 63). Though advances have been made to elucidate the mechanism by which A-769662 activates AMPK, the interaction is still

not completely understood and is the focus of ongoing research. However, given that this molecule has already been shown to specifically activate AMPK, it is a valuable tool for studying the role of AMPK in preconditioning.

### **E. Importance of Pharmacologic Activation of AMPK and Myocardial Preconditioning**

AMPK is a key regulator of several pathways that maintain the cell's energy balance. AMPK's central role in metabolic pathways and cellular development has already led to its being studied as a drug target for the treatment of diabetes (64), atherosclerosis (49), and cancer (49). Given recent studies looking at AMPK's role in preconditioning (5-8), this molecule could potentially have clinical use in treating patients at risk for myocardial ischemia. This includes patients with unstable angina or those going for solid organ transplant and high risk coronary interventions with either angioplasty or coronary artery bypass graft placement, in which organ ischemia is expected. Further work uncovering AMPK's role in preconditioning may be key in clinical advancement, and the development of A-769662, a specific and potent AMPK activator, provides the means for novel advances.

## **II. Statement of Purpose, Hypothesis, and Specific Aims**

### **Purpose**

The purpose of this work was to determine whether activation of the AMPK pathway protects the myocardium against ischemia-reperfusion injury. In order to address this possibility, we used a specific and potent AMPK activator to pre-activate AMPK prior to myocardial ischemia. We pursued this work in order to provide insight into the potential role of AMPK as a cardiovascular drug target in ischemic heart disease. Given that ischemic heart disease is a leading cause of morbidity and mortality throughout the world, the purpose of this research is to develop new potential methods for treating this devastating clinical disease.

### **Hypothesis**

Pre-ischemic activation of AMPK with A-769662 is sufficient to protect the heart against ischemia-reperfusion injury.

### **Specific Aims**

**Specific Aim 1.** *To determine whether pretreatment with A-769662 activates AMPK in the isolated perfused heart and in the intact heart in vivo.*

A-769662 has been shown to activate AMPK in isolated skeletal muscle, primary hepatocytes and mouse embryonic fibroblasts *in vitro* (62, 63) and in the liver *in vivo* following intra-peritoneal (IP) injection (9). However, the activation of AMPK by A-769662 has not been studied in the heart. Our goal was to determine whether A-769662 activates AMPK in the isolated perfused heart and in the intact heart *in vivo*, and to

uncover the optimal time and dose of A-769662 to achieve this activation in these models. This information allowed us to plan our preconditioning experiments more precisely.

**Specific Aim 2.** *To determine whether pre-ischemic activation of AMPK leads to myocardial protection against ischemia.*

Following the completion of aim 1, we wanted to evaluate the effects of pre-ischemic activation of AMPK on myocardial injury. We planned to measure several parameters to assess myocardial injury, including myocardial necrosis, creatine kinase (CK) release, and heart function. This was done in both in C57BL/6 wild type mice and in AMPK deficient KD mice. These experiments determined if 1) pre-ischemic A-769662 treatment was protective against ischemic injury and 2) if the effects of A-769662 treatment were dependent on AMPK activation.

**Specific Aim 3.** *To explore potential downstream mechanisms by which pre-ischemic AMPK activation leads to preconditioning.*

We wanted to explore the effect of pre-activation of AMPK on downstream targets that might be involved in myocardial preconditioning. eEF2 kinase, which play a role in energy conservation in the cell by phosphorylating eEF2 and inhibiting protein synthesis, is an AMPK target(51), and has previously studied for its possible involvement in ischemic preconditioning (56). As described earlier, eNOS is also an AMPK target (54, 55) that has been shown to be involved in preconditioning (8, 32). We planned to look both of these molecules to see if their phosphorylation corresponds to A-769662

treatment and AMPK activation in our experiments as a preliminary probe into the mechanism of A-769662's preconditioning effects.

### III. Materials and Methods

**Animals.** All animal studies were approved by the Yale Animal Care and Use Committee. Wild type C57BL/6 (WT C57BL/6) male mice were ordered from Charles River Laboratory and stored in Yale's facilities. The AMPK KD mice (4), expressing the K45R mutation on AMPK's  $\alpha 2$  subunit, were bred and kept in Yale's facilities. Male mice (10-12 weeks of age) were used for our studies.

**A-769662.** A-769662 was received from Dr. D.G. Hardie, a collaborator from the University of Dundee in Scotland. The compound was dissolved using 100% dimethyl sulfoxide (DMSO) solvent to 278 mM stock aliquots and stored at  $-20^{\circ}\text{C}$  until ready for use. Stock A-769662 was then diluted in modified Krebs-Henseleit (KH) (to 0.04 % DMSO) for isolated perfused heart experiments and in saline (to 1% DMSO) for *in vivo* experiments.

**Antibodies.** Primary antibodies used for western blotting were phospho-AMPK-threonine 172 (Cell Signaling), total AMPK  $\alpha$  subunit (pan  $\alpha$ ) (Cell Signaling), phospho-ACC-serine 79 (Upstate), total ACC (Cell Signaling), phospho-eEF2-threonine 56 (Cell Signaling), total eEF2 (Cell Signaling), phospho-eNOS-serine 1177 (Cell Signaling), and total eNOS (Cell Signaling).

**AMPK activation by A-769662 in the isolated perfused mouse heart.** Hearts from 10-12 week old C57BL/6 male mice were perfused using the Langendorff method with modified oxygenated Krebs-Henseleit (KH) buffer with 1% bovine serum albumin (BSA)

containing 7 mM glucose, 0.4 mM oleate, insulin 10 uU/ml, at 37°C (4, 48). A small latex balloon connected to a pressure transducer was inserted into the left ventricle through a small left atrial incision and was used to measure left ventricular developed pressure (LVDP), LV dp/dt, and heart rate (HR) throughout the study.

Initial experiments were performed to determine the dose response of AMPK activation following A-769662 treatment in the isolated perfused hearts. In this set of experiments, following 25 minutes of baseline perfusion at 4/ml/min with KH buffer, hearts were perfused with 50uM, 100uM, and 200uM concentrations of A-769662 for 30 minutes to determine the minimum concentration necessary for AMPK activation. A-769662 or DMSO (vehicle control) containing buffer was added via a two-way stopcock with one tube in a beaker containing KH buffer and the second tube in a beaker containing KH buffer with A-769662 or DMSO. Following the perfusions, hearts were freeze-clamped and stored in liquid nitrogen for later processing.

For these experiments, I assisted in gathering and organizing hemodynamic data recorded during the perfusions. All other steps in isolated heart perfusions were done by Dr. Agnes Kim, a cardiology fellow and a PhD candidate in our laboratory.

**Pretreatment with A-769662 prior to global ischemia in the isolated perfused mouse heart.** After determining the optimal dose (100uM) of A-769662 in the isolated perfused heart, our next set of experiments was designed to determine if pre-activation of AMPK is cardioprotective during ischemia. Both C57BL/6 and AMPK KD mice were used in these studies. Following 15 minutes of baseline perfusion at 4 ml/min, hearts were perfused with A-769662 (100 uM) or DMSO vehicle control for 30 minutes. Because

our preliminary perfusions showed decreases in heart rate for A-769662 treated hearts (**Figure 3**), all hearts were paced using an electrical stimulator with electrodes connected to the atria, to keep the HR at or above 450 beats per minute (bpm) during the baseline and treatment periods. This ensured that nearly equal work was done by the hearts pre-ischemia. Following treatment with A-769662 or DMSO vehicle, hearts underwent 25 minutes of no-flow ischemia, after which they were reperfused at a coronary flow rate of 4 ml/min for 30 minutes. Pacing was not restarted at this time. During reperfusion, coronary effluent was collected for measurement of CK release. At the end of reperfusion, hearts were stained with triphenyltetrazolium chloride (TTC) for assessment of infarct size (as described below) or freeze-clamped and stored in liquid nitrogen for later processing.

For these experiments, I assisted in collecting coronary effluent during reperfusion and in gathering hemodynamic data recorded during the perfusions. All other steps in the isolated heart perfusions with global ischemia were done by Dr. Kim.

**AMPK activation by A-769662 *in vivo*.** C57BL/6 male mice were anesthetized using pentobarbital (100mg/kg, Abbott Pharmaceuticals). After successful anesthesia, mice were weighed, secured on a heating pad, placed under a heating lamp, and monitored with a rectal thermometer (AD Instruments) to ensure a core temperature of 37°C. The heating lamp was adjusted according to the core body temperature of the animal. In addition, surface ECGs were monitored using needle electrodes and a bioamplifier (AD Instruments). Following 10 minutes of stabilization, mice received an IP injection of A-769662 at concentrations of 6 mg/kg, 8 mg/kg, and 10 mg/kg (dissolved in DMSO,

diluted to 1% in saline) or vehicle control (1% DMSO in saline). Following 20 minutes of monitoring, hearts were harvested, immediately freeze-clamped, and stored in liquid nitrogen for later processing.

After the dose response experiments, more experiments were done as above, using 6mg/kg A-769662 for 10, 20, 30, 60, and 90 minutes to establish the time course of maximal AMPK activation. Following these variable time points, hearts were processed as above.

I, along with Dr. Kim, performed the dose response and time course experiments. In the experiments that I performed, Dr. Kim was still responsible for removal of the heart from the chest following the experiment.

**Pretreatment with A-769662 prior to proximal LCA occlusion *in vivo*.** C57BL/6 male mice or AMPK KD mice were anesthetized, weighed, intubated, and temperatures and ECGs were monitored as above. Following intubation, the mice were ventilated using a rodent ventilator set to a respiratory rate of 105 breaths/min, tidal volume of 240 $\mu$ L, and positive end expiratory pressure of 2cm H<sub>2</sub>O, to replicate physiologic acid/base and respiratory status. Following 10 minutes of stabilization, the mice were injected with A-769662 (6 mg/kg) or vehicle control (1% DMSO) intra-peritoneally. After 30 minutes, they underwent left lateral thoractomy, exposing the LCA. A suture was placed proximally on the LCA to occlude the artery. Following 20 minutes of ischemia, the suture was loosened and reperfusion was continued for 4 hours. After reperfusion, hearts were harvested and underwent dual staining for infarct size determination (as described below) or were freeze-clamped for later processing. In one additional set of experiments,

hearts were freeze-clamped immediately following ischemia and stored in liquid nitrogen for later processing.

All *in vivo* occlusion surgical experiments were done by Jennifer Hu, a technician in our lab who is very experienced with the surgical techniques required.

**Creatine Kinase Assay.** A commercial assay (Catachem) was used to analyze creatine kinase content in effluent collected from the isolated perfused hearts. A series of three enzyme-dependent reactions was used in this assay with all reagents except creatine kinase in excess:

- 1) Creatine phosphate and ADP are converted to creatine and ATP by creatine kinase.
- 2) The ATP generated in 1) is used to phosphorylate glucose, generating glucose-6-phosphate. This reaction is catalyzed by hexokinase.
- 3) Glucose 6 phosphate is then oxidized by NADP in the presence of glucose-6-phosphate dehydrogenase to produce NADPH and 6-phosphogluconate.

The NADPH, measured using a spectrophotometer at 340 nm, is proportionate to the creatine kinase activity in the sample.

This assay was done by Gary Cline at NIH Mouse Metabolic Phenotyping Center in the Shulman Laboratory at Yale University.

**Planimetry for infarct size analysis.** Infarct size in the isolated perfused hearts was determined by injecting 5 ml of filtered 1% triphenyltetrazolium chloride (TTC, Sigma)/phosphate buffer into the aorta in a retrograde manner, ultimately filling the

coronary vasculature. The hearts were incubated in TTC for 3 minutes at 37°C. The heart chambers were then filled with Alginate (Quala) to maintain morphology and placed overnight in 4% formalin (Sigma) for tissue fixation. Following fixation, hearts were sectioned using a 1mm mouse heart matrix after being mounted on 5% agarose for stabilization. Photographs of the tissue slices were analyzed using Image J software (NIH).

In the *in vivo* studies, dual staining was used to demarcate both the necrotic areas and the AAR. This was done using a combination of both TTC and Evan's blue dye. Following *in vivo* experiments, hearts were excised from the thorax and placed in cold PBS buffer for cardioplegia. The LCA suture was loosened and 5ml of PBS was injected retrogradely to wash out the vasculature. TTC was then injected as described above. The suture ligature was then replaced on the LCA and 5ml of filtered Evan's Blue dye (Sigma) was injected through the cannula into the aorta. The dye did not reach tissue distal to the suture, staining only the area of the heart not supplied by the sutured vessel, differentiating the AAR. The hearts were then processed as described above.

I was primarily responsible for planimetry analysis with Image J software. The other steps described above were completed by Dr. Kim (isolated perfused hearts) or Jennifer Hu (*in vivo* occlusions).

**ATP Content.** Mouse heart tissue (25-35 mg) was homogenized in 6% perchloric acid. Homogenates were centrifuged and the supernatant was separated and neutralized in  $K_2CO_3$ . The neutralized extracts were then filtered and added to the high performance liquid chromatography (HPLC) vials. HPLC water as a blank and nucleotide standards

(1mM ATP, ADP, and AMP), nucleotide content was determined by reverse phase chromatography with UV detection.

These experiments were primarily done by Kwame Atsina, a Yale Medical Student and a member of our lab.

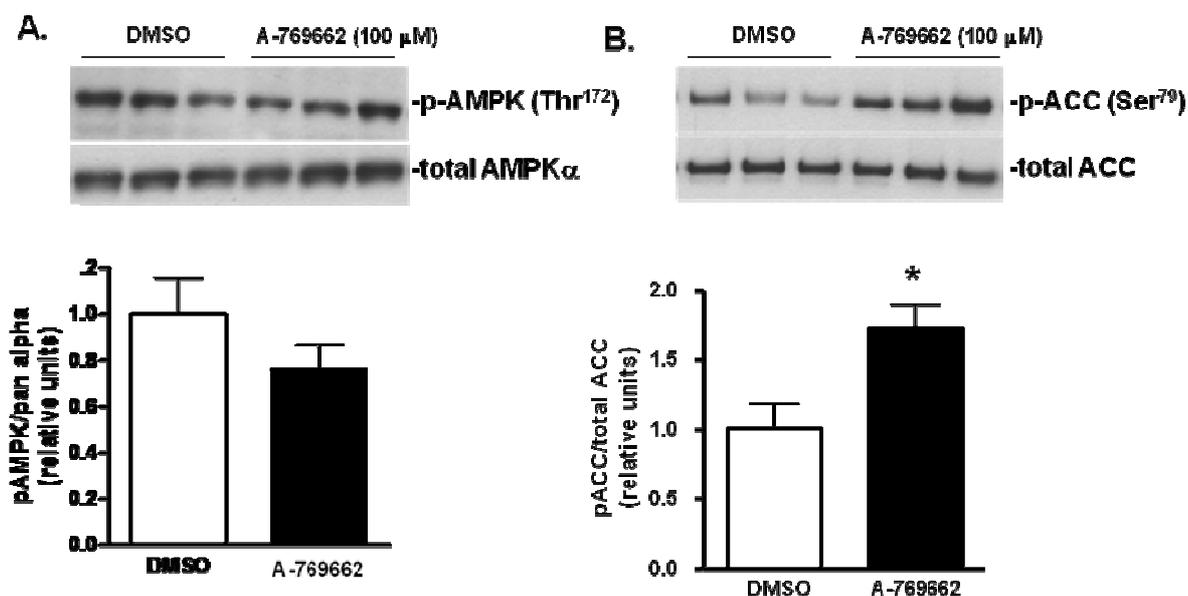
**Western Blot Analysis.** Following experiments, hearts were frozen in liquid nitrogen for storage until they were ready for homogenization. Whole hearts were homogenized on ice using lysis buffer containing 125 mM Tris, 1 mM EDTA, 1 mM MEGTA, 250 mM mannitol, 50 mM NaF, 5 mM NaPPi, 1 mM DTT, 1 mM benzamide, 0.004% trypsin inhibitor, and 3 mM NaN<sub>3</sub> (pH 7.5) (40, 52). Protein concentrations were measured using the Bradford assay (Biorad) and stabilized in 4X loading dye (Laemmli dye) containing 1 M Tris pH 6.8, sodium dodecyl sulfate (SDS), glycerol, 0.02% bromophenol blue,  $\beta$ -mercaptoethanol, and water. Equal amounts of protein were loaded in each well on polyacrylamide-SDS gels and subjected to electrophoresis. The proteins were then transferred to PVDF membranes, which were subsequently blocked in 5% milk and incubated overnight in primary antibody at 4°C. Secondary antibody was then used to detect immune-reactive proteins at room temperature for 45 to 60 minutes. The proteins were detected using enhanced chemiluminescence (ECL, Biorad) against the HRP-bound secondary antibody. The final blots were electronically scanned using HP Precisionscan Pro software and densitometry was done for quantification.

I was primarily responsible for all steps involved in western blot analysis.

**Statistical Analysis.** All data are represented as means,  $\pm$  SEM. A p value  $< 0.05$  was considered significant. Using GraphPad Prism software, all data in this work used Student's t tests to compare 2 means, or one-way ANOVA to compare 3 or more means.

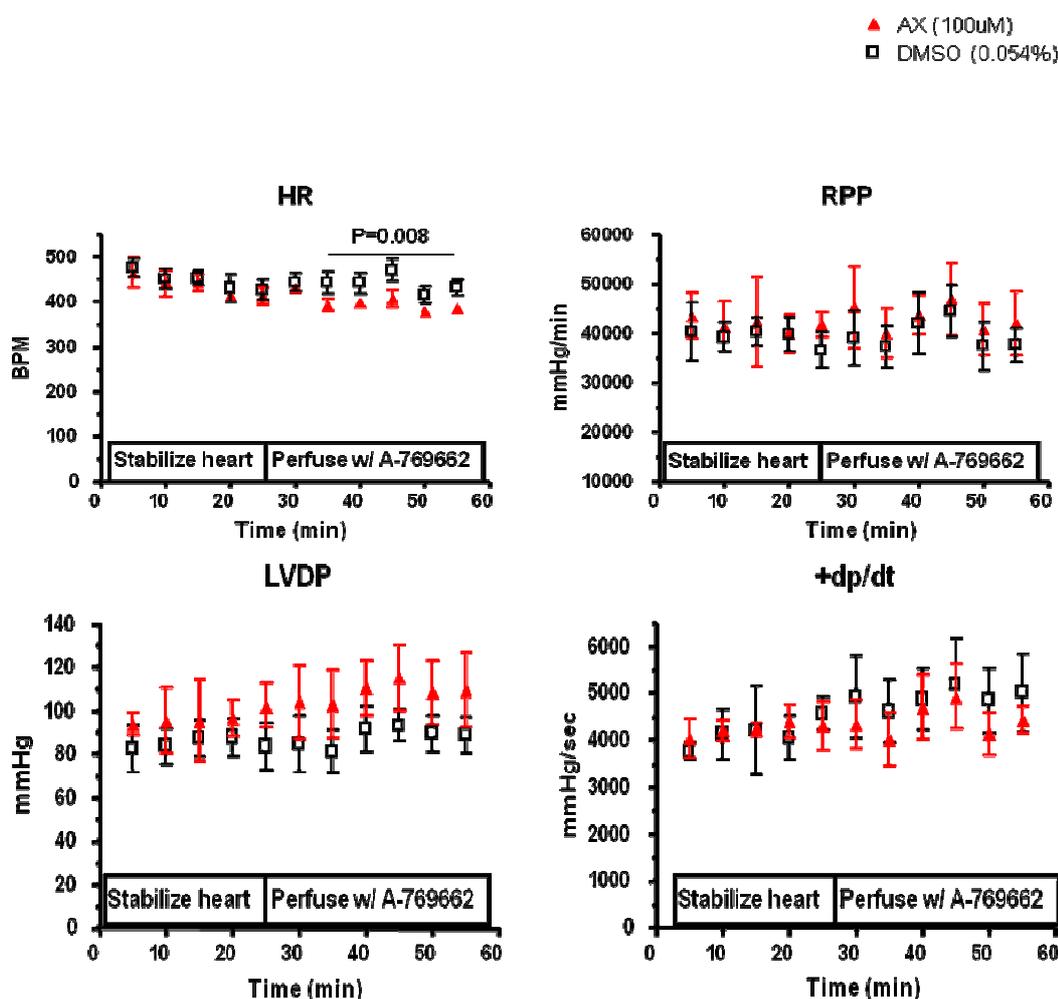
#### **IV. Results**

**AMPK is activated by treatment with A-769662 in the isolated perfused heart.** In preliminary perfusions in the Langendorff model, WT C57BL/6 mouse hearts were stabilized with standard KH buffer for 25 minutes and then perfused with A-769662 concentrations of 50uM, 100uM, and 200uM for 30 minutes. Previous work in our lab showed that 50uM was sufficient for AMPK activation in isolated rat heart papillary muscles (personal communication, Ji Li), so the 50uM concentration was deemed a good starting point for isolated perfused heart studies. We found that 50uM concentration of A-769662 was not sufficient to consistently activate AMPK in the isolated perfused heart (data not shown), but that AMPK was consistently activated at concentrations of 100uM (**Figure 2**) and 200uM (data not shown). AMPK threonine 172 phosphorylation was not significantly different (**Figure 2A**). However, ACC serine 79 phosphorylation was significantly increased in A-769662 treated hearts compared to DMSO controls ( $p=0.03$ ) (**Figure 2B**).



**Figure 2. A-769662(100uM) activates the AMPK pathway in the isolated perfused heart** **A:** Representative western blot of phosphorylated AMPK at threonine 172 (p-AMPK-Thr<sup>172</sup>) and total AMPK  $\alpha$  subunit following 25 minutes of baseline perfusion and 30 minutes with A-769662 or DMSO vehicle control. Blots are quantified by densitometry representing the ratio of p-AMPK-Thr<sup>172</sup> to total AMPK  $\alpha$ . **B:** Representative western blot of phosphorylated ACC at serine 79 (p-ACC-Ser<sup>79</sup>) and total ACC following 25 minutes of baseline perfusion and 30 minutes with A-769662 or DMSO vehicle control. Blots are quantified by densitometry representing the ratio of p-ACC-Ser<sup>79</sup> to total ACC. \* $p=0.03$  compared with DMSO control.  $N=3-4$  hearts per group. Data are shown as means,  $\pm$  SEM.

**A-769662 treatment decreases heart rate in the isolated perfused heart.** A balloon pressure transducer inserted into the left ventricle during perfusions provided the hemodynamic measurements seen in **Figure 3**. This data shows that following the addition of A-769662 to the perfusions, heart rate (HR) was significantly decreased compared with DMSO control hearts ( $p=0.008$ ), while left ventricular developed pressure (LVDP), the first derivative of LVDP (dp/dt, signifying contractility), and the rate-pressure product (RPP) were not significantly affected. These findings led us to pace the

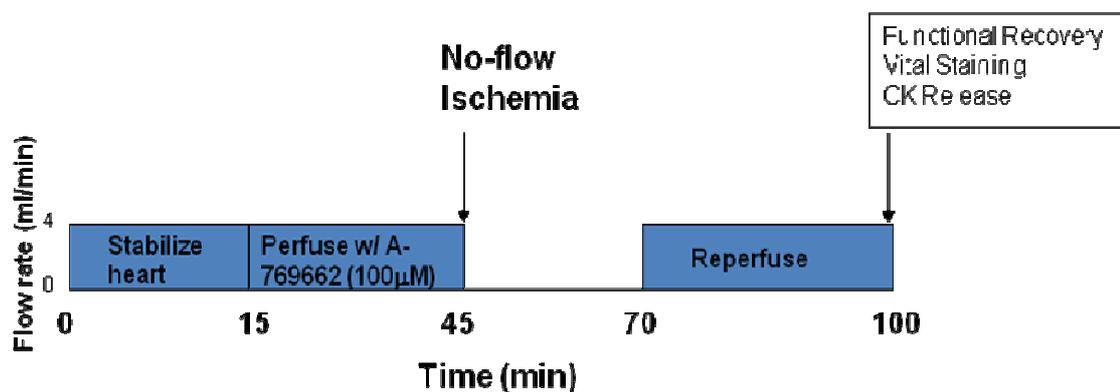


**Figure 3. Treatment with A-769662 decreases heart rate (HR), but does not affect LVDP, dp/dt, or rate pressure product (RPP) in the isolated perfused heart.**

Mouse hearts were stabilized with standard KH buffer for 25 minutes and then perfused with A-769662 (100uM) for 30 minutes. A balloon pressure transducer was inserted into the left ventricle for hemodynamic measurements. Measurements of heart rate (HR), left ventricular developed (LVDP), rate-pressure product (RPP), and the first derivative of LVDP (dp/dt), are shown for all portions of the experiment. N= 3-4 hearts per group.

hearts throughout the stabilization and treatment periods in our subsequent experiments.

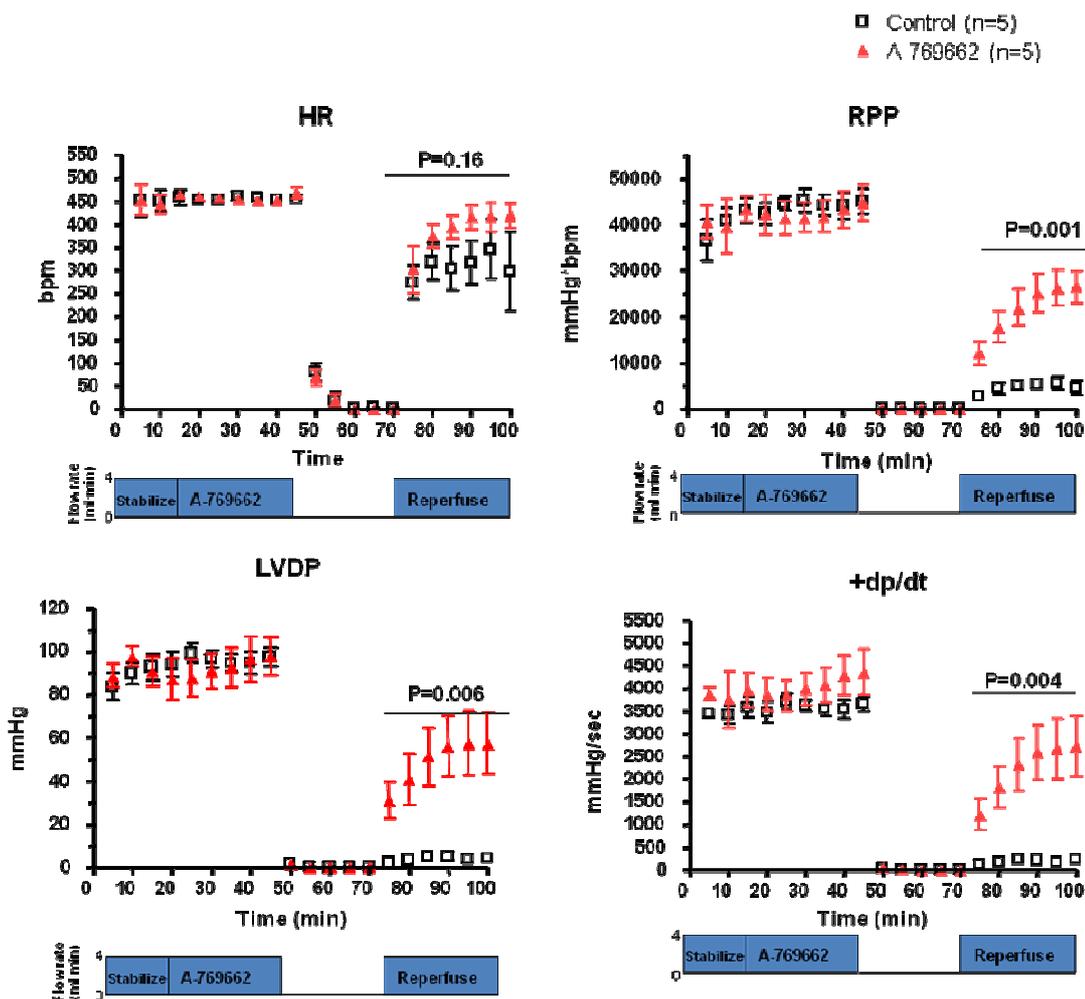
Though relative cardiac work (RPP) was not different between the groups, we paced the hearts to ensure that pre-ischemic cardiac work was equal among hearts.



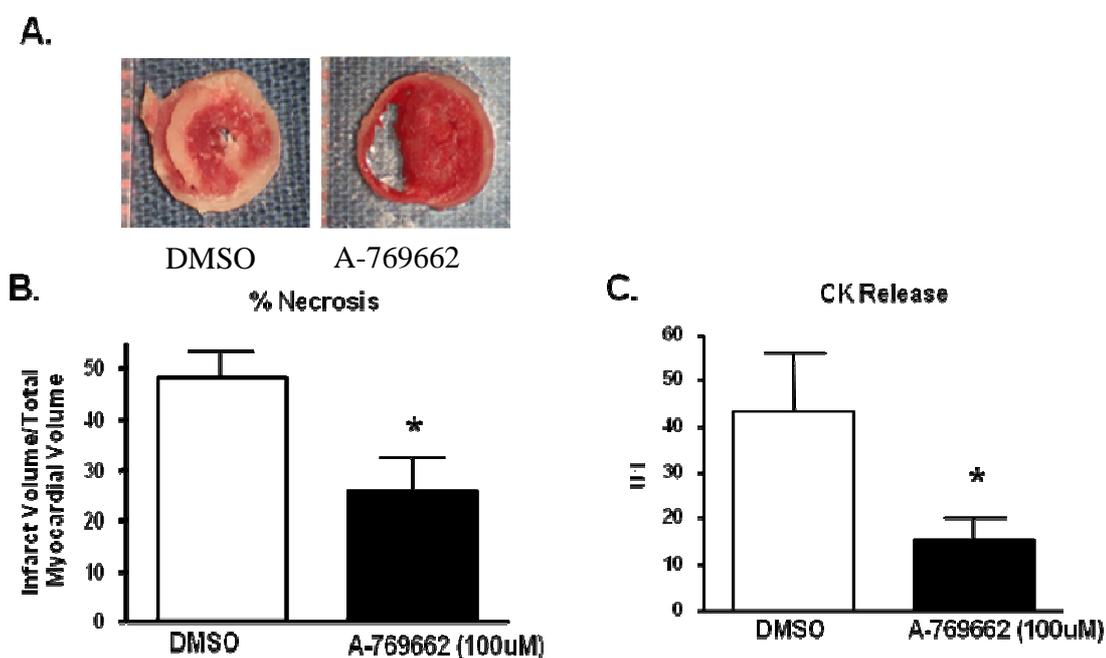
**Figure 4. A-769662 preconditioning protocol in the isolated perfused heart.**

As described in the methods, hearts from 10-12 week old C57BL/6 wild type male mice were isolated and perfused in Langendorff mode with modified K-H buffer. After 15 minutes of baseline perfusion at 4 ml/min, hearts were perfused with A-769662 (100 uM) or vehicle control (DMSO) for 30 minutes. The hearts were then subjected to 25 minutes of no-flow global ischemia, after which they were reperfused for 30 minutes at a coronary flow rate of 4 ml/min. During reperfusion, effluent was collected for measurement of CK release. Following reperfusion the hearts were stained with TTC for assessment of infarct size or freeze clamped and stored in liquid nitrogen.

**Pre-ischemic AMPK activation with A-769662 increases functional recovery and diminishes myocardial injury following ischemia-reperfusion in the isolated perfused heart.** The protocol (**Figure 4**) for preconditioning experiments in the isolated perfused hearts was designed on the basis of our preliminary findings. We used the minimum A-769662 concentration needed in this model to consistently activate AMPK (100uM). In these experiments, WT C57BL/6 hearts were subjected to 25 minutes of no-flow ischemia, and subsequently reperfused for 30 minutes. **Figure 5** shows that LVDP ( $p=0.006$ ),  $dp/dt$  ( $p=0.004$ ), and RPP ( $p=0.001$ ) were all significantly greater during reperfusion in hearts pretreated with A-769662 compared to DMSO. HR recovery was greater as well in the A-769662 treated mice, though this difference was not significant



**Figure 5. Pretreatment with A-769662 (100uM) improves myocardial functional recovery following ischemia in the isolated perfused heart.** Mouse hearts were perfused as per the preconditioning protocol in the isolated perfused heart (Figure 4). Measurements of heart rate (HR), left ventricular developed (LVDP), rate-pressure product (RPP), and the first derivative of LVDP (dp/dt) are shown for DMSO control and A-769662 treated hearts during all portions of the experiment. N= 5 hearts per group.

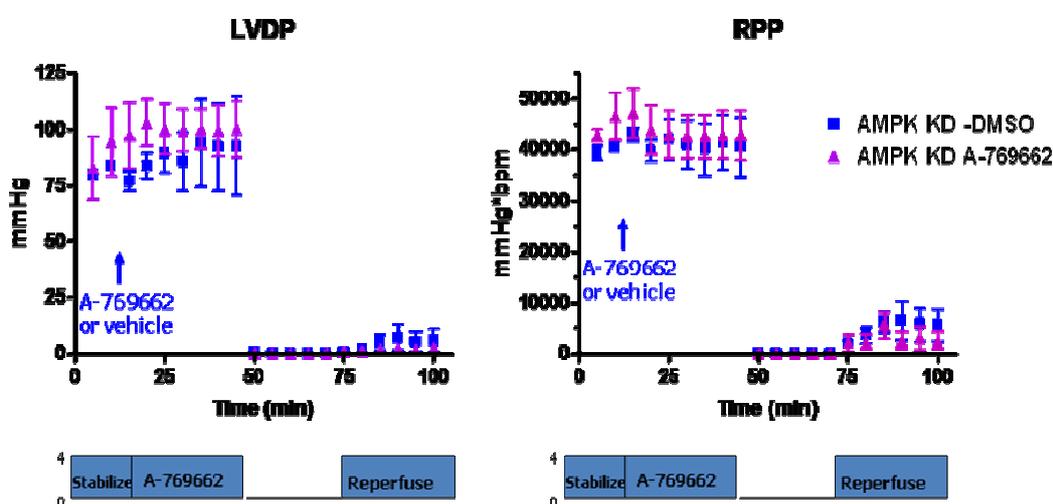


**Figure 6. Pretreatment with A-769662 (100uM) is cardioprotective following ischemia-reperfusion in the isolated perfused heart.**

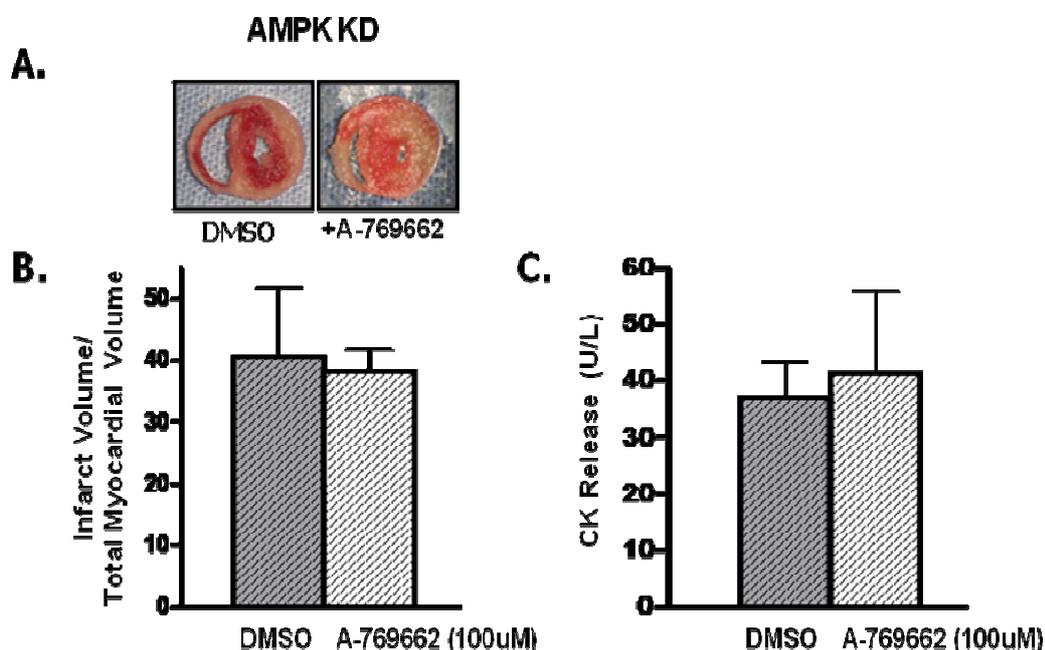
**A:** Representative heart slices of WT hearts treated with DMSO control or A-769662 prior to ischemia and stained with TTC following reperfusion. Myocardial areas stained red (TTC-positive) represent viable tissue. Myocardial areas appearing whitish-yellow (TTC-negative) are necrotic and represent areas of infarction. **B:** Infarct volume with respect to total myocardial area in DMSO control group and A-769662 treated group. \* $p=0.01$  compared to DMSO controls. **C:** Creatine kinase (CK) released into effluent in DMSO control group and A-769662 treated group. \* $p=0.02$  compared to DMSO controls. N=8-13 hearts per group. Data are shown as means,  $\pm$  SEM.

( $p=0.16$ ). In addition to better functional recovery, **Figure 6 (A-C)** shows that significantly less myocardial damage occurred in A-769662 treated hearts following ischemia-reperfusion. A-769662 treated hearts had approximately 50% smaller infarct sizes based on TTC staining ( $p=0.01$ ) and approximately 50% less creatine kinase (CK) released into effluent during reperfusion ( $p=0.02$ ).

**The cardioprotective effects seen with pre-ischemic A-769662 treatment depend on AMPK activity in the isolated perfused heart.** In order to show that AMPK activation was critical for the effects of A-769662 on the improved functional recovery and diminished myocardial injury seen in the A-769662 preconditioning experiments, we repeated the preconditioning experiments in AMPK KD hearts. **Figure 7** shows that there was no recovery of LVDP or RPP (**Figure 7**) during reperfusion in AMPK KD hearts perfused with A-769662 or DMSO control. In addition, we found that KD hearts had similar infarct size (**Figure 8A-B**) and CK release (**Figure 8C**), following ischemia-reperfusion in both A-769662 and DMSO control treated groups. These data suggest that A-769662-induced cardioprotective effects following ischemia-reperfusion are dependent on AMPK activation.



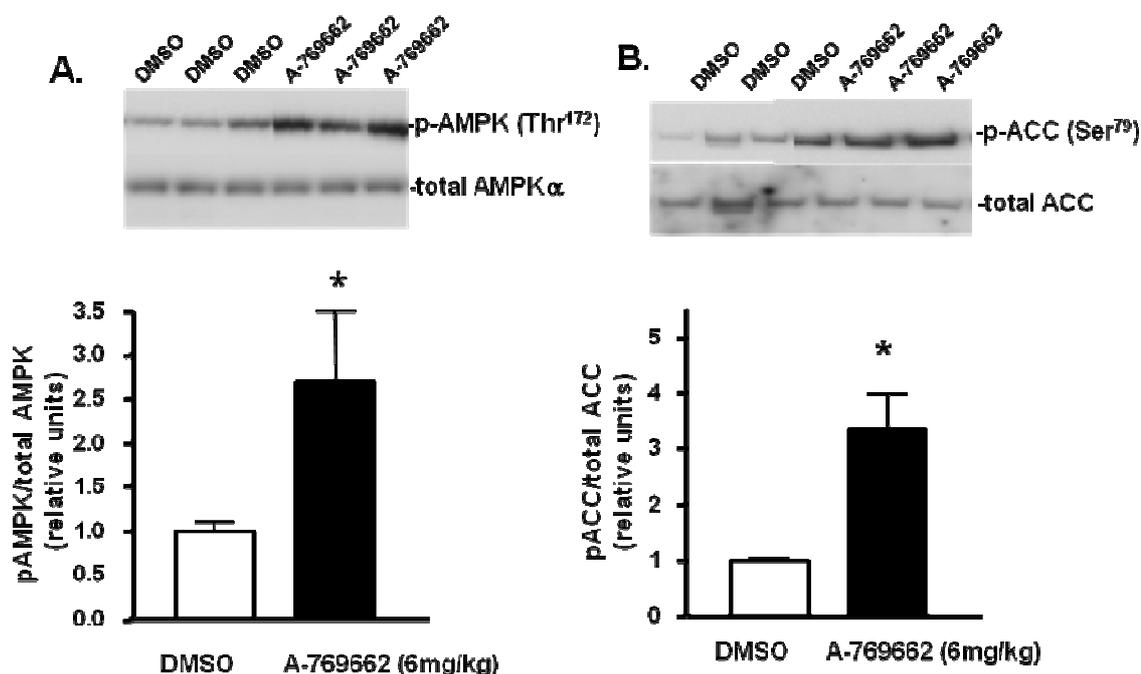
**Figure 7. Loss of AMPK activity (KD hearts) abolishes A-769662-induced functional recovery following ischemia in the isolated-perfused heart.** Mouse hearts were perfused as per the preconditioning protocol in the isolated perfused heart (**Figure 4**). Graphs show LVDP and RPP during all phases of the preconditioning experiment in the isolated perfused heart for DMSO control and A-769662 (100uM) treated KD hearts. N=5 hearts per group.



**Figure 8. Loss of AMPK activity (KD hearts) abolishes cardioprotection in pre-ischemic A-769662 treatment in the isolated perfused heart.**

**A:** Representative heart slices of KD hearts treated with DMSO control or A-769662 prior to ischemia and stained with TTC following reperfusion. Myocardial areas stained red (TTC-positive) represent viable tissue. Myocardial areas appearing whitish-yellow (TTC-negative) are necrotic and represent areas of infarction. **B:** Infarct volume with respect to total myocardial area in DMSO control group and A-769662 treated group. **C:** Creatine kinase (CK) released into effluent in DMSO control group and A-769662 treated group from KD hearts. N=5 hearts per group. Data are shown as means,  $\pm$  SEM.

**A-769662 dose response and time course of AMPK activation *in vivo*.** In order to design our *in vivo* preconditioning experiments, we first wanted to see if IP injections of A-769662 would activate AMPK in the heart. A previous study using IP injections of A-769662 to activate AMPK in the liver (9) was done at a concentration 6mg/kg IP. Therefore, using C57BL/6 male mice, we administered IP injections of A-769662 at

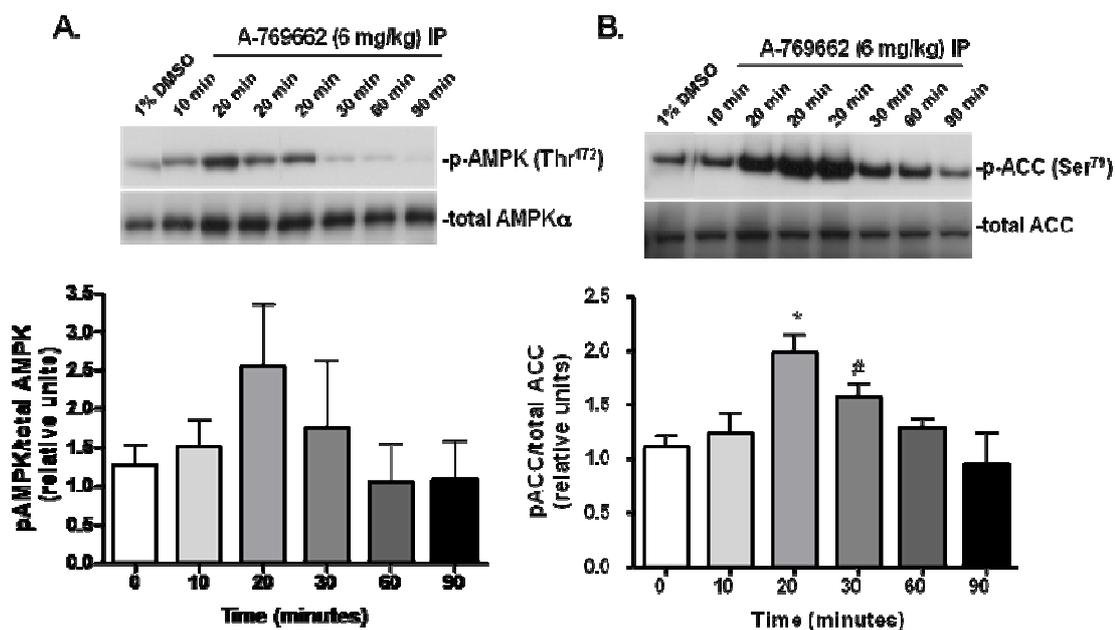


**Figure 9. A-769662(6mg/kg) activates the AMPK pathway following IP injection *in vivo*.**

**A:** Representative western blot of phosphorylated AMPK at threonine 172 (p-AMPK-Thr<sup>172</sup>) and total AMPK  $\alpha$  subunit from C57BL/6 heart lysates 30 minutes after IP injection with A-769662 (6mg/kg) or DMSO vehicle control. Blots are quantified by densitometry representing the ratio of p-AMPK-Thr<sup>172</sup> to total AMPK  $\alpha$ . \*p= 0.08 compared with DMSO control **B:** Representative western blot of phosphorylated ACC at serine 79 (p-ACC-Ser<sup>79</sup>) and total ACC from C57BL/6 heart lysates 30 minutes after IP injection with A-769662(6mg/kg) or DMSO vehicle control. Blots are quantified by densitometry representing the ratio of p-ACC-Ser<sup>79</sup> to total ACC. \*p= 0.008 compared with DMSO control. N= 5-6 hearts per group. Data are shown as means,  $\pm$  SEM.

concentrations of 6mg/kg, 8mg/kg, and 10mg/kg to determine if we could activate the AMPK pathway in the heart. Western blot analysis of lysates taken from WT hearts showed that A-769662, at concentrations of 6mg/kg (**Figure 9**), 8mg/kg (data not shown), and 10mg/kg (data not shown), sufficiently activated AMPK *in vivo*.

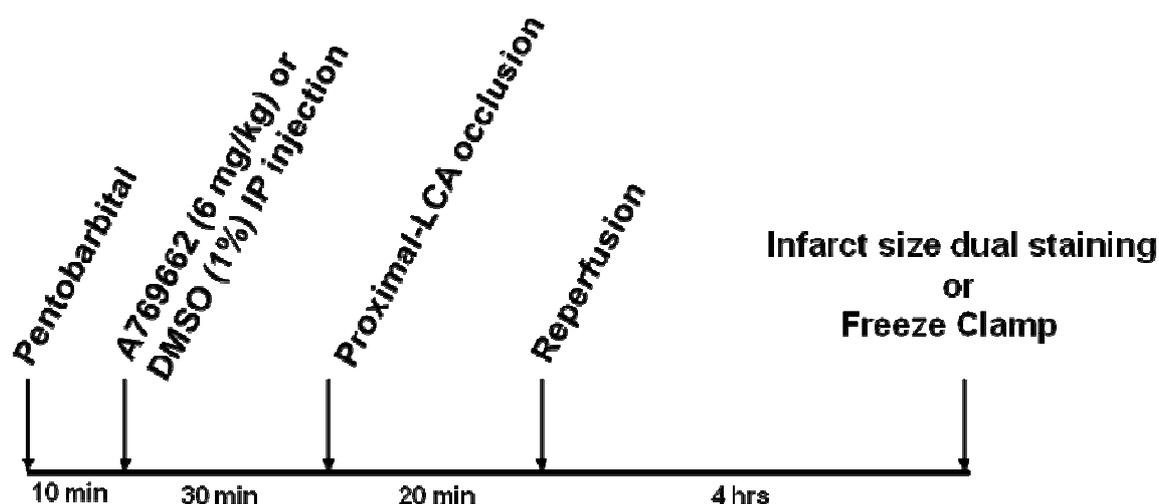
In preparation for our *in vivo* preconditioning experiments, we next wanted to study the pharmacokinetics of A-769662 following IP injection. To obtain a time course



**Figure 10.** IP injection of A-769662 maximally activates the AMPK pathway in the myocardium after 20-30 minutes.

**A:** Western blot analysis of phosphorylated AMPK at threonine 172 (p-AMPK-Thr<sup>172</sup>) and total AMPK  $\alpha$  subunit from C57BL/6 heart lysates at 10, 20, 30, 60, and 90 minutes following IP injection of A-769662 (6mg/kg) or DMSO vehicle control. The “0” time point represents IP administration of 1% DMSO for 20 minutes. Blots are quantified by densitometry representing the ratio of p-AMPK-Thr<sup>172</sup> to total AMPK  $\alpha$ . **B:** Western blot analysis of phosphorylated ACC at serine 79 (p-ACC-Ser<sup>79</sup>) and total ACC from C57BL/6 heart lysates at 10, 20, 30, 60, and 90 minutes following IP injection of A-769662 (6mg/kg) or DMSO vehicle control. The “0” time point represents IP administration of 1% DMSO for 20 minutes. Blots are quantified by densitometry representing the ratio of p-ACC-Ser<sup>79</sup> to total ACC. \*p=0.003 vs. DMSO control. #p=0.03 vs. DMSO control. N=3-6 hearts per group. Data are shown as means,  $\pm$  SEM.

of AMPK activation, we performed western blot analysis of phosphorylated AMPK (threonine 172) and phosphorylated ACC (serine 79) in hearts from C57BL/6 mice taken at 10, 20, 30, 60, and 90 minutes following IP injection of A-769662 (**Figure 10A-B**). We found that p-ACC (serine 79) was significantly increased at 20 minutes (p=0.003) and 30 minutes (p=0.03) following injection (**Figure 10B**), compared to DMSO controls.

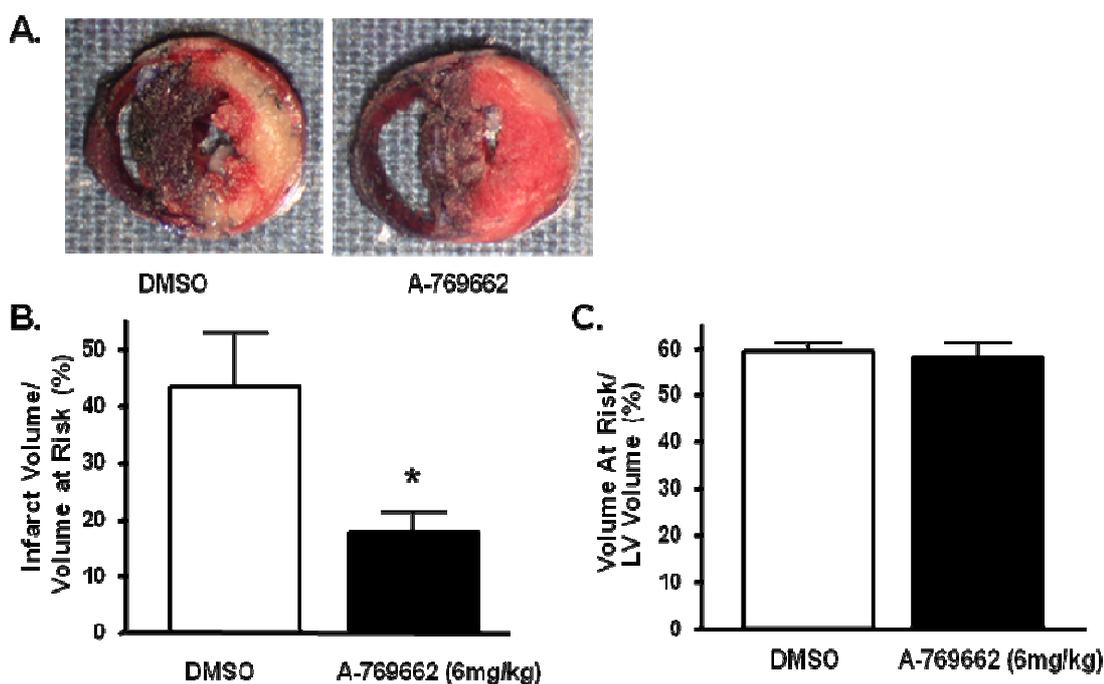


**Figure 11.A-769662 preconditioning protocol *in vivo*.**

For our preconditioning experiments *in vivo*, 10-12 week old C57BL/6 male mice were anesthetized, intubated, and stabilized for 10 minutes. They were injected with A-769662 (6 mg/kg) or 1% DMSO vehicle control, intra-peritoneally. After 30 minutes, the proximal LCA was occluded using a suture. Following 20 minutes of occlusion, the suture was loosened to allow reperfusion. After 4 hours of reperfusion, the hearts were harvested and underwent dual staining for infarct size determination or were freeze clamped for homogenization later.

**A-769662 diminishes myocardial injury following ischemia-reperfusion *in vivo*.**

Following the dose response and time course experiments, we constructed a protocol for preconditioning experiments *in vivo* (**Figure 11**). We used the minimum A-769662 concentration needed in this model to consistently activate AMPK (6mg/kg). In these experiments, 30 minutes after IP injection of A-769662 or DMSO control, WT C57BL/6



**Figure 12. Pre-ischemic treatment with A-769662 (6mg/kg, IP) is cardioprotective following ischemia-reperfusion *in vivo*.**

**A:** Representative heart slices of C57BL/6 hearts treated with DMSO control and A-769662 prior to ischemia and stained with Evans Blue dye and TTC following reperfusion. Myocardial areas that appear blue (Evan's Blue Dye) represent areas not at risk for infarction. Myocardial areas stained red (TTC-positive) represent viable tissue at risk for infarction. Myocardial areas appearing whitish-yellow (TTC-negative) are necrotic and represent areas of infarction. **B:** Infarct volume with respect to total myocardial area in DMSO control group and A-769662 treated group. \*  $p = 0.02$  vs. DMSO control. **C:** Volume at risk with respect to left ventricular volume in DMSO control group and A-769662 treated group.  $N = 7$  hearts per group. Data are shown as means,  $\pm$  SEM.

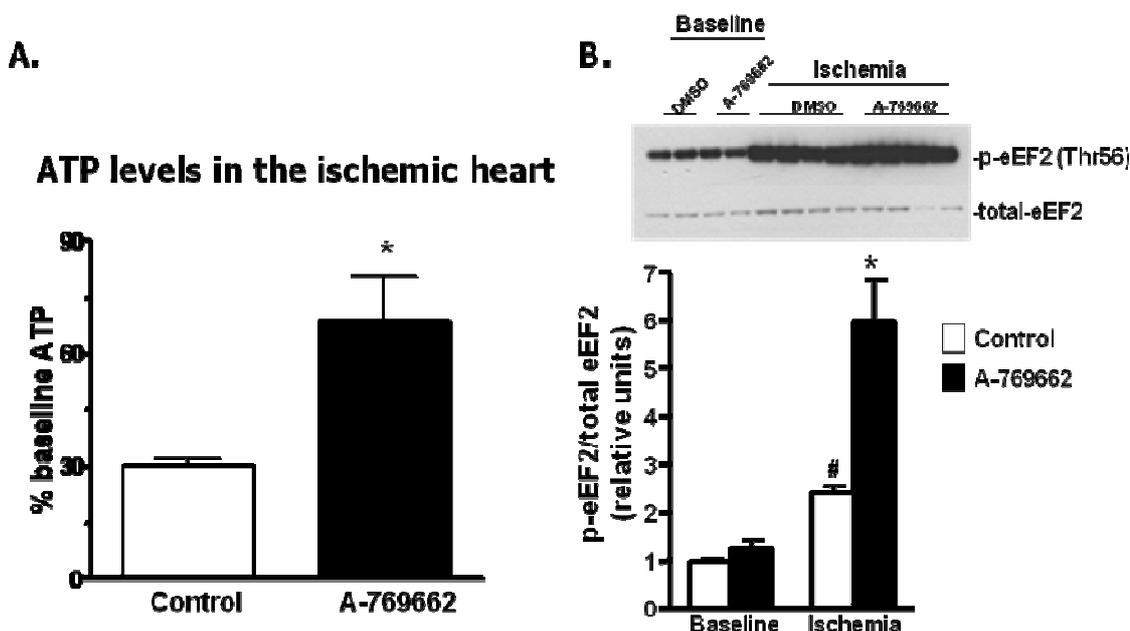
hearts were subjected to 20 minute of proximal LCA occlusion to induce ischemia.

Following ischemia, sutures were released and hearts reperfused for 4 hours. **Figure 12 (A-C)** shows that infarct size was significantly decreased in A-769662 treated hearts compared to DMSO controls ( $p=0.02$ ). The volume at risk (TTC positive red tissue + infarct) over total left ventricular volume (**Figure 12C**) was not significantly different,

indicating that the severity of ischemia administered was similar in both the A-769662 and DMSO control treated groups. These data suggest that IP injection of A-769662 and subsequent AMPK activation prior to ischemia-reperfusion is cardioprotective *in vivo*.

**Pre-ischemic AMPK activation promotes cellular ATP preservation and increased eEF2 phosphorylation.** In considering possible mechanisms of preconditioning by pre-ischemic activation of AMPK, we measured energy content in hearts from our isolated perfused hearts immediately after ischemia. This was done to determine if the preconditioning effects of pre-ischemic A-769662 treatment could result from reduced ATP depletion during ischemia. These hearts were processed immediately after ischemia, and did not undergo reperfusion, to assess ATP content after ischemia alone. Using HPLC, ATP levels were measured and showed a greater than 50% increase in ATP content in hearts treated with A-769662 compared with hearts treated with DMSO control ( $p=0.02$ ) following ischemia (**Figure 13A**).

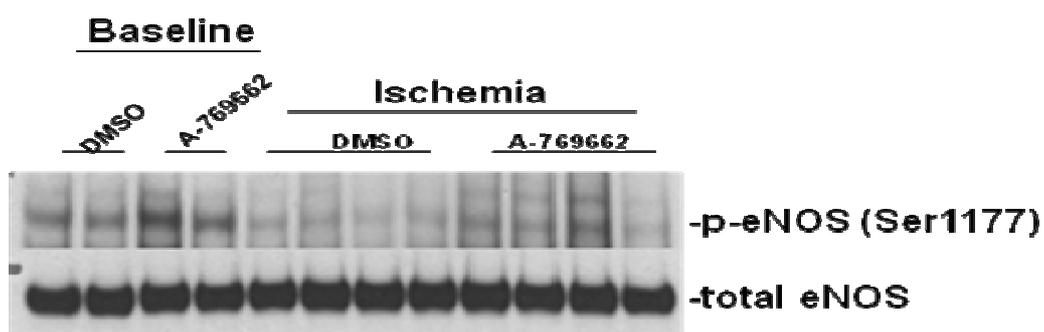
To investigate the potential role of eEF2 inactivation and protein synthesis inhibition in the energy conservation and cardioprotective effects seen with A-769662 treatment, we looked at eEF2 phosphorylation by western blot analysis. **Figure 13B** shows that phosphorylation (inactivation) of eEF2 at threonine 56 was significantly increased in A-769662 treated hearts compared with DMSO hearts following ischemia ( $p=0.008$ ). However, there was no difference in eEF2 phosphorylation in A-769662 or DMSO control treated hearts, prior to ischemia (baseline). These results indicate that A-769662 treatment potentiates eEF2 phosphorylation during ischemia, which may inhibit protein synthesis during ischemia in an effort to conserve cellular energy.



**Figure 13. Pre-ischemic A-769662 treatment promotes end-ischemia ATP preservation and eEF2 phosphorylation at threonine 56 in the isolated perfused heart.** **A:** ATP content in extracts from DMSO control and A-769662 (100uM) treated hearts, in the isolated-perfused heart. \* $p=0.02$  vs. DMSO control. **B:** Representative western blots of phosphorylated eEF2 at threonine 56 (p-eEF2-Thr56) and total eEF2 from C57BL/6 heart lysates following 30 minutes of perfusion with 100uM A-769662 or DMSO vehicle control (baseline) and following 30 minutes of perfusion with A-769662 or vehicle control and 25 minutes of no-flow ischemia (ischemia) in the isolated perfused heart. Blots are quantified by densitometry of phosphorylated eEF2 at the threonine 56 residue representing the ratio of p-eEF2-Thr56 to total EF2. \* $p=0.008$  vs. DMSO control ischemia. # $p=0.003$  vs. DMSO control baseline.  $N=4$  hearts per group. Data are shown as means,  $\pm$  SEM.

**Pre-ischemic AMPK activation leads to increased eNOS phosphorylation.** To explore the possibility that the AMPK-eNOS pathway is a mechanism of pre-ischemic AMPK activation-induced cardioprotection, we did western blot analysis of eNOS phosphorylation at serine 1177 in hearts treated with A-769662 or DMSO controls in the isolated perfused heart. Heart lysates were taken prior to ischemia and following ischemia. **Figure 14** shows preliminary evidence that A-769662 treatment before and

after ischemia increased p-eNOS (serine 1177) compared with DMSO control treated hearts. This suggests that eNOS may also be a downstream target of activated AMPK that leads to its preconditioning effects.



**Figure 14. Pre-ischemic A-769662 treatment promotes end-ischemia eNOS activation in the isolated perfused heart.** Western blot of phosphorylated eNOS at serine 1177 (p-eNOS-Ser1177) and total eENOS from C57BL/6 heart lysates following 30 minutes of perfusion with 100uM A-769662 or DMSO vehicle control (baseline) and following 30 minutes of perfusion with A-769662 or DMSO control and 25 minutes of no-flow ischemia (ischemia) in the isolated perfused heart. N= 4 hearts per group.

## V. Discussion

Our results show that treatment with A-769662 effectively activates AMPK in the isolated perfused heart and in the intact heart *in vivo*. They also demonstrate that pre-ischemic AMPK activation is sufficient for myocardial preconditioning. Isolated hearts treated with A-769662 have improved functional recovery, decreased infarct size and decreased creatine kinase released following ischemia-reperfusion. In addition, the absence of these effects in the isolated perfused AMPK KD hearts demonstrates that these preconditioning effects require AMPK activation. WT mice receiving treatment with A-769662 prior to ischemia had significantly smaller infarcts following proximal LCA occlusion. In essence, we have shown that pre-ischemic activation of AMPK is possible with A-769662 and that this activation is cardioprotective against ischemia-reperfusion injury. We also demonstrate that A-769662 pretreatment decreases cellular ATP depletion during ischemia. Finally, A-769662 treatment leads to eEF2 inactivation and eNOS activation, which may be potential mechanisms for the cardioprotective effects of pre-ischemic A-769662 treatment.

Activators of AMPK are under intense research given their potential in treating conditions such as ischemic heart disease (8), diabetes (64) and cancer (49). A-769662 is a novel, specific AMPK activator that has been studied *in vitro* (62, 63) and *in vivo* (9) in non-cardiac models. However, the efficacy of this compound's use in physiologic studies is still relatively unknown. Our results show that A-769662 is effective in activating myocardial AMPK in two mouse models: the isolated perfused heart (**Figure 2**) and in the intact heart *in vivo* (**Figures 9 and 10**).

After establishing consistent activation of AMPK pharmacologically in our mouse models, we tested the protective effects of pre-ischemic AMPK activation. A few studies have previously shown that pharmacological AMPK activation leads to myocardial preconditioning. However, these studies used non-specific AMPK activators such as AICAR (5, 6, 53) and metformin (8). Our study shows that administration of a specific AMPK activator leads to increased post-ischemic functional recovery (**Figure 5**) and decreased myocardial injury in the isolated perfused heart (**Figure 6A-C**) and in the intact heart *in vivo* (**Figure 12A-C**). In addition, because all cardioprotective effects of A-769662 were abolished in isolated AMPK KD hearts (**Figure 7 and Figure 8A-C**), we have demonstrated that A-769662-induced myocardial preconditioning is dependent on AMPK activation in this system.

More *in vivo* work will be needed to confirm our preliminary findings of myocardial preconditioning in **Figure 12**. Creatine kinase release, measured in the isolated perfused heart, was not used *in vivo*, because it is too nonspecific and would be elevated following surgery due to release from skeletal muscle. Assays looking at troponin release are still under investigation. Also we are pursuing *in vivo* ischemia studies in AMPK KD mice, which could confirm that IP administration of A-769662 leads to cardioprotection in an AMPK-dependent manner.

Though this work shows that AMPK is sufficient for myocardial preconditioning, previous work from our lab has shown that ischemic preconditioning can be induced in the absence of AMPK activity (52). Therefore, the AMPK pathway may not be required for ischemic preconditioning, but rather might be one of many communicating pathways that can result in preconditioning.

AMPK is a well known regulator of several pathways important for cellular energy balance (3, 37). AMPK's involvement in processes such as protein synthesis, cellular proliferation, and known preconditioning pathways, such as p38MAPK (58) and eNOS (54, 55), provide many possible mechanisms by which activation of this molecule may lead to preconditioning. Our preliminary results show that pre-ischemic treatment with A-769662 leads to increased post-ischemic ATP content in myocardial extracts (**Figure 13A**) as well as increased eEF2 threonine 56 phosphorylation (**Figure 13B**). AMPK has been previously shown to phosphorylate eEF2 kinase, leading to the phosphorylation and inhibition of eEF2 (51), a known regulator of the elongation step during translation in protein synthesis. Studies have shown that protein translation is nearly completely inhibited in early post-ischemic reperfusion in the brain (65, 66). One group examined eEF2 phosphorylation as a possible mechanism of this response to ischemia, but found insufficient evidence for its role in post-ischemic protein synthesis inhibition (56). However, its role in ischemic preconditioning is still an interesting topic that has yet to be fully studied. Our finding that eEF2 phosphorylation is significantly increased during ischemia is strengthened by preliminary studies showing increased ATP content in these hearts (**Figure 13 A**). Inhibition of protein synthesis, an ATP consuming process, may be one way to conserve energy. Future studies using a protein synthesis assay to directly measure protein synthesis would be useful in examining the AMPK-eEF2 pathway as a possible mechanism for A769662 preconditioning. In addition, the measurement of creatine phosphate, which regenerates ATP by phosphate transfer from creatine phosphate to ADP, will provide additional insight into the energy status of myocardial cells following A-769662 treatment. Investigating protein synthesis,

creatine phosphate content, ATP content, and eEF2 phosphorylation in AMPK KD hearts could also expound on our preliminary observations in C57BL/6 mice.

We also investigated whether the eNOS pathway is a possible mechanism for A-769662-induced preconditioning. AMPK is a known activator of eNOS (54, 55), a molecule which increases the bioavailability of nitrous oxide (NO), leading to several cardioprotective effects. In the heart, eNOS is expressed in both endothelial cells and in cardiomyocytes, and both cell types are important for this molecule's cardioprotective role (67). In endothelial cells, NO mediates vasodilation, leading to increased blood flow (67). In cardiomyocytes, NO has several protective effects including inhibition of mPTP opening (32), reduction in oxygen demand, inhibition of oxidative stress, and modulation of hypertrophic remodeling and myocardial regeneration (67- 69). An AMPK-eNOS pathway has previously been suggested in response to shear stress (70) and myocardial ischemia (8, 55). Our preliminary results suggest that eNOS activation is increased in A-769662-treated hearts, both prior to and following ischemia (**Figure 14**). Further studies in our model using eNOS deficient mice will determine if A-769662-induced preconditioning is still possible, and provide more insight into the possibility of an AMPK-eNOS mechanism.

Though there is still a great deal to learn in regards to myocardial preconditioning through AMPK activation, our results support a protective role for AMPK activation in the setting of ischemia-reperfusion. It has previously been suggested that AMPK activation may actually be harmful during reperfusion because it promotes increased fatty acid oxidation, resulting in the inhibition of glucose oxidation leading to myocardial injury (46). Work from our lab shows no A-769662-induced AMPK activation following

reperfusion (data not shown), suggesting that AMPK activity is not increased in A-769662 treated hearts during reperfusion per se. Therefore, the theoretical detrimental effect of ACC inhibition during reperfusion should not be an issue in these experiments. In addition, we do show that pre-ischemic activation of the AMPK pathway is protective and sufficient for myocardial preconditioning in our animal models.

This work with A-769662 may shed light on additional AMPK related physiologic effects in the heart. In our preliminary experiments in isolated perfused hearts, we observed that A-769662 treatment significantly lowers heart rate (**Figure 3**). The reason for this observation is still unclear. This may be a direct effect of AMPK activation on the sinus node, given the absence of other neural and humoral regulation in the isolated perfused heart, or it may be an off-target effect of the drug itself. More work will be done in future studies to address these possibilities. In either case, because A-769662 slows heart rate in the isolated perfused heart, we paced the hearts in all subsequent isolated heart experiments, in order to ensure that there was equal pre-ischemia work among hearts. In the future, thorough examination of ECG tracings in the isolated heart and *in vivo*, as well as blood pressure, catecholamine release, and blood glucose measurements *in vivo*, must be done to shed light on A-769662's effects in these animal models.

AMPK is a key player in several metabolic pathways and has already been studied as a drug target for the treatment of diabetes (64), atherosclerosis (49), and cancer (49). Our work builds on previous studies exploring AMPK's role in preconditioning (5-8), and explores a new spectrum of diseases for which AMPK activation may be beneficial clinically. We have found that a specific AMPK activator can

pharmacologically precondition the myocardium against ischemic injury, and therefore AMPK may be a promising drug target for clinical disease in which ischemic injury is expected, including ischemic heart disease. Today, most therapies for ischemic heart disease aim at either increasing the oxygen supply to the heart (i.e. revascularization and antiplatelet therapy) or decreasing the oxygen demand of the heart (i.e. beta-blockers and nitrates). No therapies commonly focus on modifying the cardiac energy metabolism to reduce injury. Theoretically, therapies that increase the efficiency of oxygen utilization and energy production by the heart could benefit the ischemic heart. This idea is beginning to be intensely studied in an attempt to discover new treatments against ischemic injury (71). Our work supports the notion that AMPK is a promising drug target for ischemic heart disease and other diseases involving ischemic injury.

While this work shows that acute pre-ischemic AMPK activation results in preconditioning, the effects of using an AMPK activator in the long term are still unclear. One group showed that 7 weeks of AICAR administration and activation of AMPK inhibits pressure-overload-induced cardiac hypertrophy in rats by blocking protein synthesis and pathways involved in cardiac growth (72). On the other hand, mutations in the gamma subunit lead to high baseline AMPK activity and are associated with cellular glycogen accumulation leading to cardiac hypertrophy (73). The glycogen overload seen in these mice is thought to be a result of an AMPK-induced increase in glucose uptake and the inhibition of glucose oxidation (secondary to increased fatty acid oxidation), resulting in more glucose available for glycogen synthesis (73, 74). Given these studies, there is still much to discover in terms of using AMPK activators clinically. Long-term administration of A-769662 may lead to the detrimental effect of glycogen overload *in*

*vivo*, potentially presenting a problem in its long-term clinical use. However, this work shows that administration of this AMPK activator, especially in the acute setting, is still very promising in the treatment of ischemia.

In summary, our data show that pre-ischemic treatment with an AMPK activator (A-769662) is sufficient to precondition the myocardium against ischemia-reperfusion injury, both in the isolated perfused heart and in the intact heart *in vivo*. In addition, we demonstrate that this preconditioning is mediated through AMPK activation given that A-769662 induced cardioprotection is lost in AMPK deficient mice (KD) hearts. Despite limitations discussed above and potential hurdles down the road, this work is a stepping stone for future experiments to better understand the mechanism of protection by this drug. The potential clinical implications of pharmacologically protecting the heart against ischemia-reperfusion injury are enormous and could lead to innovative treatments and preventions of ischemic heart disease, a leading cause of morbidity and mortality in the world.

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