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ACTIVATION OF 5'AMP-ACTIVATED PROTEIN KINASE KINASE IN THE ISCHEMIC MYOCARDIUM.

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The 5'-AMP-activated Protein Kinase (AMPK) is a heterotrimeric serine-threonine protein kinase that becomes activated during physiological stress and acts to conserve ATP by modulating a variety of cellular energy pathways. The activation of AMPK has been directly linked with phosphorylation by AMP-activated protein kinase kinase (AMPKK) at a site deemed Threonine 172 (Thr^{172}) on the α catalytic subunit of the protein. Nevertheless, the role that AMPKK plays in regulating AMPK activity has remained unclear as recent research has suggested that AMPKK may be constitutively active. Therefore, we isolated AMPKK in ischemic myocardial tissue induced by either *in vivo* regional ischemia or by *in vitro* low-flow ischemia in isolated working hearts and evaluated AMPKK activity, as measured by phosphorylation of Thr^{172} on synthetic AMPK α_1 subunits or on recombinant heterotrimeric AMPK proteins. We found that levels of phosphorylated Thr^{172} on endogenous AMPK were increased 2-fold ($p < 0.03$) during *in vivo* ischemia and 2.6-fold ($p < 0.01$) during *in vitro* ischemia when compared to control conditions. Furthermore, Thr^{172} phosphorylation of recombinant AMPK proteins was increased after incubation with AMPKK isolated from ischemic tissue ($p < 0.15$ for *in vivo* ischemia and $p < 0.04$ for *in vitro* ischemia with recombinant AMPK α_1 subunits; $p < 0.04$ for *in vivo* ischemia and $p < 0.01$ for *in vitro* ischemia with recombinant heterotrimeric AMPK). These results demonstrate that ischemia increases cardiac AMPKK activity, thereby suggesting that Thr^{172} phosphorylation and AMPK activity is modulated by upstream kinases as opposed to phosphatase actions.

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Introduction

Energetic stress on the heart can result from different physiologic and pathologic situations, including exercise, hypertension and coronary artery disease. As a person exercises, the skeletal muscle requires more oxygen and nutrients, thereby requiring the heart to increase cardiac output, both by increasing stroke volume and by increasing heart rate. Hypertension causes an increase in the afterload on the heart, thereby leading to an increase in the work that the heart must do to continue meeting the metabolic needs of the body. Myocardial oxygen delivery is decreased in patients suffering from coronary artery disease and the heart responds by operating in such a way so as to utilize the limited oxygen in the most efficient way possible.

When cardiac stress continues over a long period of time, the heart responds with anatomical and metabolic renovation. The heart responds to ischemia through a phenomenon termed hibernation. When oxygen delivery to the heart is compromised as occurs in coronary artery disease, the heart has been found to demonstrate decreased contractility along with decreased energy metabolism (1). Researchers have found that the ischemic, hibernating myocardium has a higher chance of recovery when normal coronary blood flow is restored (2), indicating that the heart acts to protect itself by modulating cardiac activity during ischemia.

Hypertrophy of the heart is a common response to both exercise and hypertension. The increase in cardiac work, caused by exercise and hypertension, results in an increase in the tension across the ventricular wall. A formula derived from the law of Laplace suggests that the myocardial wall tension is inversely proportional to the thickness of the ventricular wall (3). Accordingly, several studies have found that there is an increase in

myocardial cell size within hours of an increased myocardial workload (4) (5). By increasing the size of the myocardial cell and thereby the thickness of the ventricular wall, the heart is attempting to decrease the myocardial wall tension and thus compensate for the increased cardiac workload induced by exercise and hypertension.

Signaling Pathways in the Heart

Receptor-mediated signaling pathways

On a molecular level, the heart responds to energetic stress through many, different signal transduction pathways. Some of these signaling pathways, of which two primary ones are the β_1-adrenergic pathway and the Angiotensin II pathway, are mediated via the binding of ligands to receptors in the plasma membrane. When the body senses physiologic stress, catecholamines are released and produce effects by binding to adrenergic receptors, of which there are 4 classes (α_1, α_2, β_1, β_2). β_1-adrenergic receptors are the adrenergic receptors that are predominant in the heart, located specifically in the sinoatrial node, the atrioventricular node and the ventricular tissue. When a stimulatory ligand (catecholamines in this case), binds to the β_1-adrenergic receptor, a G protein, G_s, is activated. G_s subsequently activates adenylate cyclase, and increasing levels of the second messenger, cyclic AMP (cAMP). Increased levels of cAMP result in the activation of protein kinase A (PKA). PKA directly phosphorylates the L-type Ca^{2+} channel located on the plasma membrane, thereby increasing the entry of calcium into the myocardial cell (6). This increase in calcium current activates the release of calcium from the sarcoplasmic reticulum, resulting in greater myocardial contractility (7). Besides activating PKA, cAMP has also been implicated in directly activating ion

channels present in pacemaker cells (8), thereby resulting in an increase in the rate of phase 4 depolarization in pacemaker cells and thus an increase in heart rate.

Not only can energetic stress induce the activation of β_1-adrenergic receptors, but stress can also result in the release of Angiotensin II. Acting via Angiotension II receptors (ATRs) present in cardiac tissue (9), Angiotensin II has been associated with hypertrophy and remodeling of the myocardium in response to pathologic stress, such as heart failure and myocardial infarction (10) (11) (12) (13) (14). Like the β_1-adrenergic receptors, the ATRs are also associated with a G protein, G_q in this case. After stimulation of the ATR, G_q activates phospholipase C, which cleaves phosphatidyl inositol bisphosphate (PIP_2) into the second messengers inositol-1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 and DAG act together to activate protein kinase C (PKC) (15). PKC has been implicated in the regulation of gene transcription via the activation of the activator protein-1 complex (AP-1) (7), a protein that enhances the transcription of several genes, including the genes coding for atrial natriuretic factor and for myosin light chain (16) (17). Furthermore, PKC has been implicated in activating c-Raf, which in turn activates the Mitogen-activated-protein-kinase (MAPK) cascade, a protein kinase system that is involved in regulating cell growth (18).

Researchers also have found that some of the effects of Angiotensin II are independent of G_q and PKC. Indeed, recent studies have shown that activation of the ATR results in the stimulation of the STAT (Signal Transducers and Activators of Transcription) pathway, another signaling pathway involved in the regulation of cellular growth as well as with the inflammatory response (19) (20) (21). Although the exact mechanism by which the ATR activates the STAT pathway is still unknown, it has been

theorized that the ATR may interact with soluble protein tyrosine kinases and/or with the Janus Kinase Family (JAKs), both of which have been shown to directly activate the STAT pathway (21) (22) (23). Taken together, it appears that Angiotensin II utilizes multiple mechanisms, including PKC and STAT activation, to exert a significant effect on the growth of the myocardial cell.

Non-receptor-mediated signaling pathways

Unlike the β_1-adrenergic and the Angiotensin II pathway, other stress-activated signaling pathways in the heart are not dependent upon activation via a plasma membrane receptor, but instead exert effects by other sensing mechanisms. Mechanical stretch of a cell, as caused by volume overload, has been determined to be a potent activator of multiple different signaling pathways, all of which are intimately involved with the regulation of cell growth. Indeed, stretch can directly activate specific ion channels (so-called stretch-activated ion channels) (24) as well as activate the G protein, G_q (25), which stimulates PKC and thus affects the transcription of cell growth through the MAPK cascade as detailed above. The G protein Ras, which activates the MAPK cascade, as well as phospholipase C were both found to be activated following stretch of integrin-associated cell adhesions in cardiac fibroblasts (26) (27). Furthermore, researchers have discovered that several humoral molecules involved in cardiac remodelling are released following stretch, including Angiotensin II (see above discussion), (28) and insulin-like growth factor, a molecule involved in the normal growth of the heart (29) (30). Cytokines of the IL-6 family are also released following stretch and act to stimulate the JAK-STAT pathway via gp130 (31) (32). Clearly then,

mechanical stretch serves as a powerful stimulant of several diverse signaling pathways associated with cellular growth.

Studies have convincingly identified hypoxia as another condition, which is capable of activating several different non-receptor mediated signaling pathways in the attempt to maintain function. Hypoxia-inducible factor 1(HIF-1) is a transcription factor that is upregulated in situations of low oxygen pressure (33) (34). Once HIF-1 is activated, it increases the transcription of multiple genes, including erythropoiten (35), a hormone that stimulates erythropoiesis, vascular endothelial growth factor (36), which is involved in angiogenesis, the glucose transporter GLUT1 (37) and lactate dehydrogenase (38), an enzyme involved in the anaerobic process of glycolysis. By upregulating the expression of all of these genes, HIF-1 acts to increase oxygen delivery to peripheral tissues and to keep the cell well-supplied with glucose and the enzymes necessary to produce energy in an anaerobic manner.

Hypoxia has also been shown to stimulate other transcription factors, such as nuclear factor κB (NF-κB) and heat shock factor-1 (HSF-1) (39) (40) (41) (42). Hypoxic stimulation causes NF-κB to increase the expression of cytokines, especially IL-8, (43) and intracellular adhesion molecules (ICAMs) (44). Both the cytokines and ICAMs are involved in attracting neutrophils and other inflammatory cells to a site of hypoxic injury. Hypoxia increases levels of HSF-1, which is a molecule involved in modulating the expression of heat shock proteins (HSPs). HSPs are a family of proteins that facilitate the folding of newly synthesized proteins and have been implicated in protection of the ischemic myocyte (45) (46). Thus, as the myocardial cell is exposed to the toxic stress of

hypoxia, the gene expression of immediate and long-term protective proteins are upregulated through multiple signaling pathways.

Changes in whole body substrate metabolism and circulating substrate levels can also trigger the activation of cardiac metabolic pathways through a variety of mechanisms. A well-studied set of transcription factors that is regulated by shifts in the level of free fatty acids is the peroxisome proliferator-activated receptor family (PPARs). Studies have demonstrated that PPARα is involved in upregulating genes associated with several steps of fatty acid β-oxidation (47) (48) (49) (50). Following even short-term starvation, fatty acid uptake into the myocyte increases as the cell readies itself to call upon fat stores to continue vital energy-consuming processes. Accordingly, medium and long chain fatty acids have been identified as directly-binding ligands of PPARα (51). Recent studies involving transgenic mice, deficient in PPARα, concluded that PPARα activation was absolutely necessary for the increased expression of fatty acid β-oxidation enzymes observed during short-term starvation and thus for survival (52). Such a study indicates the necessity of tight regulation of cardiac energy metabolism, to which the PPAR family contributes. Another signaling system that is exquisitely sensitive to changes in cardiac metabolic homeostasis and responds with both acute and chronic adjustment of energetic processes is centered around the AMP-activated protein kinase, which is the topic of this investigation.

The AMP-Activated Protein Kinase

The Structure of AMPK

AMPK is a heterotrimeric serine/threonine protein kinase, consisting of a catalytic α subunit, a regulatory γ subunit and the β subunit, responsible for connecting the α and γ subunits. Different isoforms of each of the subunits of AMPK have been identified and specific isoforms have been associated with specific tissue distribution and varying levels of activity. The α subunit (63 kDa) is composed of three domains. The kinase domain of the α subunit (amino acids 1-312) is located near the N terminus and is responsible for the catalytic activity associated with the α subunit (53). Researchers have determined that phosphorylation of a threonine residue (Thr^{172}) located within this kinase domain is necessary for AMPK activity. Indeed, site-specific mutagenesis of Thr^{172} to an alanine residue has been found to result in an inactive catalytic subunit (53) (54). Adjacent to the kinase domain of the α subunit lies an amino acid sequence (termed 312-392) that has been shown to serve as an autoregulatory sequence. Truncation of the α subunit to amino acid 392 (1-392) results in a complete loss of catalytic activity, while further truncation to amino acid 312 (1-312) results in a protein kinase fragment that is no longer dependent on the allosteric activation of AMP, and thus is constitutively active (53). Taken together, these findings suggest that the 312-392 sequence acts as an autoinhibitory domain. Lastly, the C-terminus of the α subunit (392-548) is responsible for binding the β and γ subunits, as demonstrated by a loss of β-γ binding when the C terminus of the α subunit is removed (53).

The α subunit is present in two different isoforms (α_1 and α_2). While the α_1 subunit seems to be more prevalent in the lung, kidney and testis (55), high levels of the

α_2 subunit have been demonstrated in the heart and skeletal muscle (55) (56). Furthermore upon activation, the α_2 subunit has been associated with greater kinase activity in skeletal muscle (57) (58) (59) and in heart (60).

The β subunit (38 kDa) provides a scaffold for the catalytic α subunit and the regulatory γ subunit to assemble. The C-terminus of the β subunit contains an 84 amino acid domain, which serves to bind the α and γ subunits (61) (62). The rest of the β subunit is comprised of a sequence that has been shown to bind glycogen, thereby facilitating an interaction between AMPK and glycogen stores (63). Two isoforms (β_1 and β_2) have been identified for the β subunit of AMPK, with the β_2 subunit being highly expressed in skeletal and cardiac tissue and the β_1 subunit showing high levels primarily in the liver (62).

The final subunit (γ) of AMPK is thought to be responsible for much of the molecular and physiologic regulation of AMPK. The γ subunit consists of four repeats of the CBS domain, a structural protein motif that is involved in the allosteric regulation in numerous other proteins, most notably in cystathione β-synthase (64). Similarly, the CBS domains of the γ subunit of AMPK also seem to be involved in allosteric regulation of the kinase, as shown by labeling studies, which have demonstrated that the AMP analogue, 8-azido-[^{32}P]AMP, binds directly to the γ subunit (55). Furthermore, mutations within the CBS domains of the γ subunit of AMPK result in a defective kinase that is no longer activated by AMP (65).

Researchers have isolated three isoforms (γ_1, γ_2, γ_3) of the γ subunit of AMPK. In most tissues (lung, liver, heart, kidney, pancreas and skeletal muscle), γ_1 is the most

prevalent isoform, accounting for 80-90% of the AMPK seen in the tissues, while the remaining 10-20% of AMPK is formed using γ_2, with γ_3 making a very minor contribution to AMPK activity (55) (66). In the testis and the brain, γ_2 and γ_3 presence were found to be significantly increased, especially in the brain where the three γ isoforms were present in almost equal amounts (55). Each of the γ isoforms also affected the degree to which AMP stimulated the heterotrimeric AMPK complex. Indeed, AMPK proteins containing the γ_2 subunit showed the greatest dependence on AMP, followed by γ_1 and then γ_3, which had a markedly lower dependence on AMP than either of the other two γ isoforms (55).

The Activation of AMPK

AMPK has been characterized as part of a highly sensitive protein kinase cascade. Within this cascade, AMPK is activated via phosphorlyation by AMP-activated protein kinase kinase (AMPKK) (67). The high-energy phosphate group is subsequently removed by protein phosphatase 2C (PP2C) (68), thereby returning AMPK to an inactivated state. This cycle of phosphorylation and dephosphorylation is commonly seen within other protein kinase families, especially the mitogen-activated protein (MAP) kinase family (69). Many researchers have speculated that the reason for this cyclical arrangement of protein kinase cascades is related to the increased sensitivity of a cascade to activating factors (70) (71). Modeling of the AMPK system demonstrated that the cascade was indeed exquisitely sensitive to activating nucleotides such that a 6-fold increase in AMP resulted in a change of AMPK activity from 10% to 90% maximal activity (72). Thus, the arrangement of AMPK within a protein kinase cascade serves to

increase the responsiveness of the protein to the factors in charge of regulating its activity.

The AMPK cascade is regulated by the energy status of the cell, as determined by levels of adenine nucleotides. Almost all energy-requiring cellular reactions are associated with the breakdown of ATP to ADP. Since cells require ATP to function, the cell has developed mechanisms such that depleted stores of ATP are recognized and replaced extremely rapidly via the reaction 2ADP ←→ ATP + AMP, catalyzed by adenylate kinase. The cell's rapid response to a depletion of ATP results in the maintenance the ratio of ADP to ATP within a very narrow limit. The minimally changing ratio of ADP to ATP suggests that anabolic processes are almost exactly balanced by catabolic processes within the cell by a system that monitors the depletion of ATP and then subsequently adjusts catabolic and anabolic reactions as needed (73).

Since ATP can be degraded to either ADP (by ATPases) or to AMP (by ligases), the question arises as to whether the cell responds to the AMP:ATP ratio of the ADP:ATP ratio. Upon examination of the reaction 2ADP ←→ ATP + AMP at equilibrium, researchers have determined that the AMP:ATP ratio varies as the square of the ADP:ATP ratio (74). For example, if the ADP:ATP ratio were to rise by a factor of 3, the AMP:ATP ratio will increase 9-fold. Such an example clearly demonstrates the increased sensitivity of AMP levels in indicating the energy status of the cell. Thus, it stands to reason that AMP levels as opposed to ADP levels are most likely to serve as the molecule that is responsible for regulating catabolic and anabolic processes (73) (74).

Multiple studies have demonstrated the activating effects of an increased AMP:ATP ratio on the AMPK cascade. Indeed, AMP has been shown to not only

increase the activity of AMPK 5-fold (75) (76), but also to increase the activity of the upstream kinase, AMPKK (77) (78). Furthermore, the binding of AMP to AMPK affects the ability of other proteins (i.e. AMPKK and PP2C) to interact with it. Indeed, inactivation of AMPK by PP2C is almost completely inhibited by the binding of AMP to AMPK (68), while the AMP-AMPK complex (78) increased AMPKK activity. Environments that serve to decrease the AMP:ATP ratio also affect the activity of the AMPK cascade. The dephosphorylating activity of PP2C on AMPK was increased and the phosphorylating activity of AMPKK was decreased in the presence of extremely high concentrations of ATP (68) (78). Clearly then, the AMPK cascade is affected at multiple steps by an increased AMP:ATP ratio in the surrounding environment.

Physiological regulation of AMPK occurs in situations when the cell is under energetic stress and the AMP:ATP ratio is thereby affected. Often, pathological conditions, such as ischemia, are responsible for energetic stress. Indeed, AMP levels are dramatically increased following global ischemia in rat heart, and correspondingly, both the phosphorylation of AMPK and AMPK kinase activity were also found to be increased (79) (80) (81). Since ischemic conditions result in both a hypoxic environment and an environment characterized by low extracellular glucose, researchers have studied the separate effects of each of these physiological conditions on AMPK. Low glucose levels in pancreatic cell lines was found to increase the AMP:ATP ratio, while consequently increasing AMPK activity (82). Furthermore, skeletal muscle cells incubated in buffers containing 95% N2-5% CO2 were also found to increase AMPK activity 7-fold (83).

Physiological stress, such as exercise, has been shown to activate AMPK as well as pathological stress. Using different methodologies, several researchers have shown

that contraction of skeletal muscle fibers result in an increase in the AMP:ATP ratio in muscle and in an increase in AMPK activity. Rats, who were exercised on a treadmill, showed increased AMPK activity in muscle removed from the leg (84) (85). Repeated electrical stimulation of the sciatic nerve also resulted in a rise in AMPK activity in the gastronemius muscle as well as in an increase in free AMP levels (86). Furthermore, in vitro contraction of isolated muscle fibers also serves to increase the activity of AMPK significantly (83) (85) (87). Recently, exercise has been shown to increase the AMPK activity and AMPK phosphorylation in the heart as well as in skeletal muscle. Rats were exercised on a treadmill at different intensities and researchers discovered that as exercise intensity increases, AMPK activity in the heart increases proportionately (60).

AMPK has been also been found to be stimulated pharmacologically. Certainly, many studies have utilized the drug 5-aminoimidazole-4-carboxmide-riboside (AICAR) in the study of AMPK. AICAR is a nucleoside that is converted into ZMP by adenosine kinase (88). ZMP has been shown to act as an AMP analog and thus allosterically activate AMPK as well as enhance the activation of AMPK by AMPKK (88) (89). More recently, two classes of drugs, metformin and the thiazolidinediones, used to treat type 2 diabetes mellitus have been found to activate AMPK. Metformin acts to lower blood glucose levels by increasing muscle glucose uptake (90) and by decreasing glucose production in the liver (91). Incubation of muscle cells with metformin resulted in a significant increase in AMPK activity (92). Furthermore, activation of AMPK has been implicated in the mechanism by which metformin acts to lower blood glucose levels (93). The thiazolidinediones are also used to treat type 2 diabetes by increasing insulin sensitivity in peripheral tissues through their actions on the transcription factor

peroxisome proliferator-activated receptor γ (94) (95) (96). AMPK phosphorylation and kinase activity was found to increase with time following incubation of rosiglitazone, a thiazolidinedione, with muscle cells (92). Thus, AMPK can be activated pharmacologically as well as during physiologic and pathologic stress.

The Actions of AMPK

Just as there are many activating environments for AMPK, AMPK has many targets of action once activated. Since the ultimate goal of AMPK is to return the ADP:ATP ratio back to physiological equilibrium, it follows that AMPK generally acts to turn on catabolic processes and switch off anabolic processes in multiple areas of metabolism. Several of the first AMPK targets to be identified were in the areas of lipid and sterol metabolism and include both hydroxymethylglutaryl-CoA (HMG-CoA) reductase, acetyl-CoA carboxylase (ACC), malonyl-CoA decarboxylase (MCD), hormone sensitive lipase (HSL) and glycerophosphate acyltransferase (GPAT). HMG-CoA reductase catalyzes the regulatory step that converts hydroxymethylglutarate to mevalonic acid in the synthesis of sterols, such as cholesterol. Researchers discovered that activated AMPK phosphorylates a serine residue (Ser 871) on the catalytic subunit of HMG-CoA reductase, thereby inhibiting the enzyme and decreasing sterol synthesis (88) (97) (98).

Regulation of fatty acid synthesis and oxidation by AMPK occurs primarily interactions with acetyl-CoA carboxylase (ACC) and malonyl-CoA decarboxylase (MCD). ACC is responsible for catalyzing the committed step in fatty acid synthesis, in which acetyl-CoA is converted into malonyl-CoA. When ACC is inhibited, not only is

fatty acid synthesis decreased, but fatty acid oxidation is also increased. The rate limiting step of fatty acid oxidation, the transport of fatty acids into the mitochondria by carnitine-palmitoyl transferase I (CPT1), is inhibited by high levels of malonyl CoA (99). By inhibiting ACC, malonyl-CoA levels decrease, thereby lifting the inhibition on CPT1 and allowing fatty acid oxidation to proceed. Multiple studies have shown that activated AMPK inhibits acetyl-CoA carboxylase in liver, heart and skeletal muscle via phosphorylation, thereby producing decreased malonyl CoA levels (84) (88) (98) (100) and resulting in the inhibition of fatty acid synthesis and the stimulation of fatty acid oxidation. Malonyl-CoA levels are also affected by MCD, which is involved in degrading malonyl-CoA. Researchers have determined that AMPK activates MCD in skeletal muscle, liver and adipose tissue, thereby decreasing the amount of malonyl-CoA in cells and further stimulating fatty acid oxidation (101) (102). Chronic AMPK activation has also been linked with a decrease in the gene expression of ACC and fatty acid synthase, an enzyme responsible for catalyzing several of the reactions involved in fatty acid synthesis (103).

AMPK is also intimately involved in the regulation of triglyceride formation and degradation. Glycerophosphate acyltransferase (GPAT) is involved in catalyzing the synthesis of triacylglycerols. AMPK was found to inhibit GPAT in muscle, liver and skeletal muscle, resulting in the decreased formation of triglycerides (102) (104). Hormone–sensitive lipase (HSL) is an enzyme involved in the breakdown of triglycerides into fatty acids and glycerol in adipocytes. Cyclic AMP-dependent protein kinase (PKA) activates HSL by phosphorylation at a serine residue (Ser-563) (105). AMPK acts to inhibit HSL by phosphorylating the protein at a serine site (Ser-565) that is nearly

adjacent to the phosphorylation site of PKA, thereby preventing PKA from phosphorylating the activating site (Ser 563) (106). In congruence with these findings, researches have found that when cells from adipose tissues are incubated with AICAR, an AMPK activator, lipolysis is decreased. This finding may seem inconsistent with the notion that AMPK is usually involved in switching on catabolic processes, as opposed to decreasing these processes as seemingly is the case with HSL and lipolysis. Nevertheless, scientists have speculated that by decreasing lipolysis in adipose tissue, AMPK is attempting to conserve ATP, an action consistent with AMPK's general goal of replacing depleted energy stores. Unused fatty acids are recycled back into triglycerides and cholesterol esters in an energy-consuming process. By decreasing lipolysis, AMPK may be working to ensure that the rate at which triglycerides are broken down does not exceed the rate at which fatty acids are consumed, thereby reducing the amount of ATP-depleting fatty acid recycling (106).

The effects of AMPK on glucose transport into the cell have also been widely studied. An increase in glucose uptake into the cell is usually associated with the translocation of GLUT4 transporters to the sarcolemma, so as to facilitate the movement of glucose across the plasma membrane. Insulin is commonly associated with stimulating the movement of GLUT4 transporters to the sarcolemma and exerts this effect via the activation of phosphatidylinositol 3-kinase (107) (108). Ischemia, hypoxia and contraction of skeletal muscle have all been associated with the translocation of GLUT4 transporters via a mechanism that is independent of insulin-associated phosphatidylinositol 3-kinase activation (108) (109) (110). Initial studies utilizing AICAR found that glucose uptake into skeletal and cardiac muscle increased 2-fold in

association with an increase in AMPK activity (111) (112) (113). Using a transgenic mouse that expressed a dominant-negative kinase-dead AMPK, researchers found that hypoxia was unable to stimulate glucose uptake or increase GLUT4 translocation in either in the heart (113) or in skeletal muscle (114). Furthermore, chronic activation of AMPK by long-term AICAR administration has been shown to result in an increase in GLUT4 gene expression (115). Taken together, these findings strongly suggest that AMPK stimulates glucose transport via increased expression and translocation of GLUT4 transporters by a mechanism that is not related to the insulin-linked phosphatidylinositol 3-kinase pathway.

Once glucose has been taken into the cell, AMPK is able to exert both acute and chronic control over the storage, breakdown and synthesis of glucose. Excess glucose is often stored as glycogen, an energy-requiring process that is mediated by the enzyme glycogen synthase. Glycogen synthase is de-activated by phosphorylation at a site (Ser-10) by casein kinase-1 (116). AMPK has been shown to phosphorylate glycogen synthase at a serine residue (Ser-7) (117), which serves to promotes the phosphorylation of Ser-10 by Casein kinase-1 and thereby results in the inactivation of glycogen synthase (116). Glucose is broken down through the process of glycolysis, during which 2 net molecules of ATP are made. A potent regulator of glycolysis is fructose 2,6-bisphosphate, a molecule that stimulates 6-phosphofructo-1-kinase, which is the enzyme that mediates the rate-limiting step of glycolysis. Fructose 2,6-bisphosphate is made using the enzyme 6-phosphofructo-2-kinase (PFK-2). AMPK has been shown to phosphorylate heart PFK-2 at Serine-466, thereby leading to the stimulation of fructose 2,6-bisphosphate production and thus, the stimulation of glycolysis (118) (119). Over the

long term, researchers have determined that chronic stimulation of AMPK results in the change in gene expression of several gluconeogenic enzymes. AMPK leads to decreased expression of glucose-6-phosphatase (120) (121), pyruvate kinase (122) (123), and phosphoenolpyruvate carboxykinase (120), all enzymes involved in the key steps of gluconeogenesis. All in all, the effects of AMPK on glucose metabolism serve to increase the energy stores available to the cell by stimulating catabolic processes and inhibiting anabolic processes.

In addition to carbohydrate and lipid metabolism, AMPK has been implicated in regulating protein metabolism through effects on p70 ribosomal protein S6 kinase (p70S6K) and eukaryotic elongation factor 2 kinase (eEF2K). Once activated, p70S6K is involved in the synthesis of proteins in the liver (124). Several researchers have found that activated AMPK is associated with the inhibition of p70S6K, although the specific mechanism of this inhibition remains elusive (125) (126) (127). Eukaryotic elongation factor 2 (eEF2) is another enzyme involved in protein synthesis in the liver. Upon hypoxic stimulation, eEF2 becomes phosphorylated, which serves to inhibit the enzyme and thus inhibit protein synthesis. Upon further study, it was determined that AMPK was responsible for activating the upstream protein, eEF2 kinase, thereby promoting the inhibitory phosphorylation of eEF2 (128). Thus AMPK serves to inhibit hepatic protein synthesis by at least two different mechanisms.

Recently, researchers have discovered that AMPK interacts directly with transcription factors in order to regulate gene expression. Hepatocyte nuclear factor (HNF) 4a is associated with regulating the expression of genes involved in glucose and triglyceride metabolism, including pyruvate kinase, Apoprotein B and Apoprotein C III

(129). HNF4a has been identified as a substrate for AMPK, which has been shown to phosphorylate HNF4a on a serine residue both in vitro and in cell lines. Researchers have speculated that the phosphorylation of HNF4a results in the degradation of the transcription factor (as evidenced by the disappearance of the protein) and the resulting reduction of gene transcription (130) (131). Carbohydrate-response-element-binding protein (ChREBP) is another hepatic transcription factor that is involved in regulating genes encoding metabolic enzymes, including pyruvate kinase, fatty acid synthase and acetyl-CoA carboxylase. AMPK has been shown to phosphorylate ChREBP, thereby inhibiting ChREBP from binding to DNA (132). Inhibition of ChREBP could serve as another way that AMPK is able to regulate anabolic processes, specifically by interfering with the expression of gluconeogenic proteins. The transcription cofactor, p300, has also been identified as a substrate for AMPK (133). Researchers have found that p300 is involved in regulating the transcriptional activity of peroxisome-proliferator-activated-receptors (PPARs), which are transcription factors involved in the differentiation of adipose tissue (134) (135). PPAR-γ activity has been found to be inhibited when activated AMPK is present, and recent studies suggest that this effect may stem from the decreased activity of p300, due to phosphorylation by AMPK (133). Although the exact mechanisms of the effect of AMPK on gene transcription still need to be further studied and elucidated, it seems clear that AMPK exerts an effect on gene expression by interacting with nuclear transcription factors.

The AMP-activated Protein Kinase Kinase

As mentioned above, the primary molecular activator of AMPK is the upstream kinase, AMP-activated protein kinase kinase (AMPKK). Indeed, AMPKK has been shown to produce a 50-fold increase in AMPK activity (88) through phosphorylation. Early studies identified the primary site phosphorylated by AMPKK as Thr^{172} on the catalytic subunit (67), a site that has been deemed necessary for AMPK activity as demonstrated by the lack of AMPK activity in Thr^{172} deficient proteins (53) (54). Clearly then, AMPKK is an important player in the AMPK cascade.

In order to further understand the regulation and activity of AMPKK, researchers have made attempts to elucidate the structure of the kinase. AMPKK purification from rat liver demonstrated a molecule quite similar in structure to AMPK (67). Indeed, AMPKK possesses a catalytic subunit of weight 58 kDa, quite similar to the α subunit of AMPK, which weighs 63 kDa. Overall molecular mass of the kinases were also similar with AMPKK measuring in at 195 kDa as compared to the 190 kDa weight of AMPK.

Seeing as the structure of the two kinases is quite similar, studies have investigated the likely possibility that similar molecular and physiologic environments might activate AMPK and AMPKK. Since AMPK was found to be significantly activated by AMP and inhibited by high concentrations of ATP (75) (76), researchers have examined the effects of AMP and ATP on AMPKK activity. Indeed, an elegant study was performed in which it was found that the addition of AMP resulted in a 1.5 fold increase in the phosphorylation of the catalytic subunit of AMPK after incubation with AMPKK purified from rat liver (78). Similarly, other researchers found that AMP addition resulted in increased AMPK activity after AMPK was incubated with purified

rat liver AMPKK (77). Furthermore, studies found that high concentrations of ATP could completely inhibit the activation of AMPK by AMPKK (67). Such findings lend credence to the notion that the kinases are similarly regulated.

Despite similar stimulation by adenosine molecules, recent work has found that AMPKK may not be subject to the same physiologic regulation as AMPK. Since AMPK is activated by hypoglycemia, hypoxia and AICAR, it was expected that AMPKK would show increased activity under these conditions as well. Experiments using a bacterially expressed recombinant AMPK α_1 subunit demonstrated no change in AMPKK activity in insulinoma cells after AICAR stimulation or exposure to a hypoglycemic environment (136). This finding has led scientists to conclude that AMPKK may be constituitively active. Such results are puzzling in light of the apparent similarities between AMPKK and AMPK both in structure and in response to AMP.

In order to fully understand the AMPK cascade, it is necessary to elucidate the role that AMPKK plays in the regulation of AMPK activity. Clearly, some studies have suggested that AMPKK may not be an important player in modulating the actions of AMPK, and that instead, AMPK activation may be modified by proteins other than AMPKK (136). Further research is needed to understand the regulation of AMPKK activity, so as to better understand the effects of AMPKK on AMPK activity and the entire AMPK cascade.

Specific Aims of Study

This research seeks to further clarify the physiologic regulation of AMPKK and the role of AMPKK in the AMPK cascade. The first aim was to develop methods that could enrich AMPKK and could be used to measure AMPKK activity. The second aim was to assess the suitability of different substrates as tools for investigating AMPKK as well as to assess the kinetics of the AMPKK reaction in order to optimize the assay. The third aim was to utilize these newly developed methods to address the specific physiological effects of *in vitro* global or *in vivo* regional ischemia on AMPKK activity in myocardial tissue.

Methods

Male Sprague-Dawley rats (250-350g) were housed in an animal facility and given standard chow and water before experiments. All procedures were approved by the Yale University Animal Care and Use Committee.

In Vitro Ischemia Protocol

An *in vitro* model of global ischemia was used to assess the effects of ischemia on AMPKK activity. The *in vitro* ischemic model is a simple model in which the degree of ischemia is able to be controlled and other potentially confounding variables, such as hormones and neurosympathetic activation, which may have some effect on AMPK and AMPKK activation, are able to be excluded.

Rats were anesthetized using an intraperitoneal injection of pentobarbital sodium (60 mg/kg) and were heparinized with 300 units intraperitoneally. Hearts were excised and placed in Krebs-Henseleit bicarbonate buffer at 4° C. The aorta was then cannulated and the heart perfused anterogradely with Krebs-Henseleit buffer (2.5 mM Ca^{2+}) containing glucose (5 mM) in order to wash out any remaining blood. Subsequently, a cannula was inserted into the pulmonary vein and the hearts were perfused in the working heart mode (138) at 37° C with Krebs-Henseleit bicarbonate buffer containing 1% BSA (fraction V, fatty acid free), oleate (0.4 mM), glucose (5 mM), calcium (2.5mM) and bubbled with 95% O_2 /5% CO_2. Control hearts were subjected to 40 minutes of perfusion with a preload of 15 cm H_2O and an afterload of 100 cm H_2O. Ischemia heart perfusion protocol went as follows : during the first 20 minutes, hearts were perfused with a preload of 15 cm H_2O and an afterload of 100 cm H_2O; during minutes 20-40, hearts were

perfused with a preload of 15 cm H_2O and an afterload of 30 cm H_2O to produce a flow that was 15% of control flow. After 40 minutes, the hearts were freeze-clamped and stored at –80° C. The heart perfusion procedures were performed by Monica Palmieri under the supervision of Raymond R. Russell III M.D. to provide myocardial tissue for these experiments.

In Vivo Ischemia Protocol

An *in vivo* model of regional ischemia was also utilized during these experiments in order to further apply these findings to syndromes seen in clinical practice. Since coronary artery disease is a phenomenon of regional ischemia caused by coronary artery occlusion, it was appropriate to investigate the effects of regional ischemia produced *in vivo*. Furthermore, the *in vivo* model includes other factors which may have an effect on AMPK and AMPKK activity, such as varying intracardiac pressures and circulating neurohormonal influences, thereby making the *in vivo* ischemic model an appropriate and useful tool for investigation.

Rats were anesthestized using an intravenous injection of pentobarbital sodium (60 mg/kg IP). The rats were subsequently intubated and ventilated at 80 breaths/min and a tidal volume of 2.5 cc with 100% oxygen. A left lateral thoracotomy was performed and the proximal left anterior descending artery was ligated with a 6.0 silk suture (137). After 10 minutes, the heart was excised and freeze-clamped with aluminum tongs, which were cooled in liquid nitrogen. Control rats underwent the same procedures, excepting that the left anterior descending artery was not occluded. The

hearts were stored at –80° C. The surgical procedure was performed by Xiaoyue Hu M.D under the supervision of Dr. Lawrence H. Young to provide cardiac tissue for this study.

Tissue Homogenization

All procedures occurred at 4° C. Heart tissue was homogenized for 60 seconds in homogenization buffer (125mM Tris, 1mM EDTA, 1mM EGTA, 250mM Mannitol, 50mM NaF, 5mM NaPPi, 1mM DTT, 1mM Benzamedine, 0.004% Trypsin Inhibitor, 3mM NaN_3, pH 7.5). The homogenate was then centrifuged at 10,900 RPM (14,400g) on a SS34 Rotor for 30 minutes. The pellet was discarded and the supernatant was saved. 25% Polyethylene Glycol (PEG) was added to each supernatant volume to a final concentration of 2.5% PEG. The samples were then agitated for 10 minutes and subsequently centrifuged at 9,200 RPM (10,000g) for 10 minutes. Again, the pellet was discarded and the supernatant was placed into a separate set of test tubes. 25% PEG was added to each sample to a final concentration of 6% PEG. The samples were agitated for 10 minutes and then centrifuged at 9,200 RPM (10,000g) for 10 minutes. The pellet (2.5-6% PEG precipitate) was resuspended in homogenization buffer. The supernatant was collected and placed into another set of test tubes. Again, 25% Polyethylene Glycol (PEG) was added to each sample to a final concentration of 10% PEG. The samples were shaken for 10 minutes and centrifuged at 9,200 RPM (10,000g) for 20 minutes. The pellet (6-10% PEG precipitate) was resuspended in homogenization buffer and the supernatant was collected (>10% supernatant).

Protein Concentration Assay

Preparation of reagents: BioRad reagent was diluted 1:4 in distilled water. Bovine serum albumin (BSA) was prepared at a concentration of 1mg/1ml and then diluted 1:10 in distilled water. Sample protein was diluted 1:20 in distilled water.

Determination of protein concentration: 800λ of diluted BioRad reagent was added to each test tube. For the BSA standards: 0λ of diluted BSA was added for the zero standard, 25λ of diluted BSA was added for the 2.5μg standard, 50λ of diluted BSA was added for the 5.0 μg standard and 75λ of diluted BSA was added for the 7.5μg standard. 20λ of diluted protein extract was added to each sample test tube. Distilled water was then added so as to bring the total volume in each test tube to 1ml. All conditions were run in duplicate. Standards and samples were then transferred to cuvettes and placed in a spectrophotometer with wavelength set at 595λ in the absorbance mode. The zero standard was set as reference and the samples and standards were subsequently read. The BSA standard curve was determined using Microsoft Excel, and sample protein concentrations were ascertained based on the standard curve using linear regression.

Recombinant AMPK Phosphorylation by cellular AMPKK

10 μg of PEG precipitated protein (6-10% fraction) was incubated with 10 pmol of a recombinant AMPK α_1 subunit, which consisted of α_1^{1-312} with an N-terminal maltose binding protein (gift from Dr. Lee Witters) (136) or 5 pmol of recombinant heterotrimeric (α_1-β_1-γ_1) AMPK (gift from Dr. Diebert Neumann) (139) (140) in 25 μl of incubation buffer (20mM Tris, 5mM $MgCl_2$, 0.2mM ATP, 0.5mM DTT, 0.1% Tween,

1mg/ml BSA, pH 7.5) for 10 minutes at 30° C. The reaction was stopped by a 2-fold dilution with Laemelli Sample Buffer (20% glycerol, 2% SDS, 10mM Tris, 1.2% Mercaptoethanol, 5% Bromethylene Blue, pH 6.8). Samples were boiled for 10 minutes prior to gel electrophoresis on an 8% gel.

Gel Electrophoresis and Immunoblotting

The running gels were made by combining 8% bis-Acrylamide, 0.375M Tris, 0.1% SDS, 0.045% APS and 0.004% TEMED, adjusted to a pH of 8.8. After the running gel had set, the stacking gel (4% bis-Acrylamide, 0.125M Tris, 0.001% SDS, 0.001% APS, 0.001% Temed, pH 6.8) was added. 40 μg of PEG-precipitated protein (2.5-6% fraction) was combined with Laemelli Sample buffer and boiled for 10 minutes. 20λ of sample was loaded into each lane and then subjected to electrophoresis (200V) for 75 minutes in electrode buffer (19.3mM Tris, 18.6mM Glycine, 5mM SDS). The proteins were subsequently transferred to methanol-soaked PDVF membranes in transfer buffer (19.2mM Tris, 192.2mM glycine, 0.2% methanol) at 200 mAmps for 90 minutes. Membranes were then blocked overnight with specific buffers to prevent non-specific binding. Membranes to be blotted for $pThr^{172}$ AMPK were blocked with TBS-T milk buffer (15mM Tris, 137mM NaCl, 5% nonfat dry milk, 0.1% Tween-20, pH 7.6). Membranes to be blotted for total AMPK were blocked with 5% Tris milk buffer (10mM Tris, 500mM NaCl, 5% nonfat dry milk, 1% Tween-20, pH 7.4).

$pThr^{172}$ Immunoblotting: The membrane was incubated with the primary antibody, anti-$pThr^{172}$ AMPK (Cell Signaling, Beverly, MA), at 1:5,000 dilution in 10ml of primary antibody dilution buffer (15mM Tris, 137mM NaCl, 5% BSA, 0.1% Tween-20,

pH 7.6) for 1 hour. Membranes were subsequently washed 3 times for 10 minutes each with wash buffer (15mM Tris, 0.137M NaCl, 0.1% Tween-20, ph 7.6). A 60-minute incubation with the secondary antibody, HRP-conjugated goat-antirabbit IgG (Zymed, San Francisco, CA) followed. Membranes were then washed 6 times for 15 minutes each with wash buffer. 10ml of ECL (company name) was incubated with the membranes for 1 minute. After membranes were blotted dry, they were placed in a plastic sheet protector and exposed to autoradiographic film.

Total AMPK Immunoblotting: The membrane was incubated with the primary antibody, anti-pan-α AMPK (gift from Dr. M. Birnbaum), at 1:20,000 dilution in 10ml of 5% tris milk buffer for 1 hour. Membranes were subsequently washed 3 times for 10 minutes each with 5% tris milk buffer. A 60-minute incubation with the secondary antibody, HRP-conjugated goat-antirabbit IgG (Zymed, San Francisco, CA) followed. Membranes were then washed a total of 6 times – 2 times for 15 minutes each with 5% tris milk buffer, 2 times for 15 minutes each with PBS and 2 times for 15 minutes each with distilled water. Membranes were visualized as described above.

AMPK Assay

10μg of heart homogenates (2.5-6% PEG fractions) or 0.16 pmol of recombinant heterotrimeric AMPK were added to 25λ AMPK kinase assay buffer (0.8mM DTT, 0.2mM AMP. 0.048M Hepes-NaOH ph 7.0, 0.096M NaCl, 9.5% Glycerol, 0.96mM EDTA) with or without 0.2mM of the AMPK substrate, SAMS peptide (HMRSAMSGLHLVKRR; see (79) (113)). All samples, both with and without SAMS, were run in duplicates. A set of blanks, in which no protein was added, was also included

in the assay. 3λ of an ATP solution (5mM $MgCl_2$, 0.2mM ATP, [^{32}P]ATP (New England Nuclear, Boston, MA)) was added to the kinase assay mixture. The assay continued for 10 minutes at 37° C. Aliquots (15λ) of the reaction mixture were then spotted on Whatman filter paper (P81) and the filter papers were dropped into cold 150mM phosphoric acid to stop the reaction. The filter papers were washed 4 times for 10 minutes each with cold 150mM phosphoric acid and then once for 20 minutes with acetone. After the filter papers had dried, they were placed into scintillation vials with 5ml scintillation fluid and subjected to scintillation counting.

Statistics

Results were determined using the student's t test and are presented as means ± SEM.

Results

Enrichment of AMPK in PEG precipitated fractions: In order to partially enrich AMPK in heart homogenates, protein from each PEG precipitated fraction (2.5-6%, 6-10% and the 10% supernatant) was immunoblotted for total AMPK (see Figure 1A). A 28-fold increase in AMPK levels was seen in the 2.5-6% PEG fraction as compared to the 6-10% PEG fraction and the 10% supernatant PEG fraction ($p < 0.0005$ vs. 6-10% PEG fraction and $p < 0.0005$ vs. 10% supernatant PEG fraction). These findings suggest that the majority of AMPK is present in the 2.5-6% PEG-precipitated fraction with only minor amounts appearing in the remaining PEG fractions.

Enrichment of AMPKK activity in PEG precipitated fractions: In order to enrich AMPKK in heart homogenates, protein from each PEG precipitated fraction (2.5-6%, 6-10% and the 10% supernatant) was incubated with recombinant α_1 AMPK subunit. Incubation samples were then immunoblotted for $pThr^{172}$ and total AMPK (Figure 1B). A 22-fold increase in Thr^{172} phosphorylation of the recombinant α-1 AMPK subunit was seen when the 6-10% PEG fraction is included in the incubation as compared to the 2.5-6% PEG fraction and the 10% supernatant PEG fraction ($p < 0.0005$ vs. 2.5-6% PEG fraction and $p < 0.0005$ vs. 10% supernatant PEG fraction). These results clearly suggest that the majority of AMPKK is present in the 6-10% PEG-precipitated fraction.

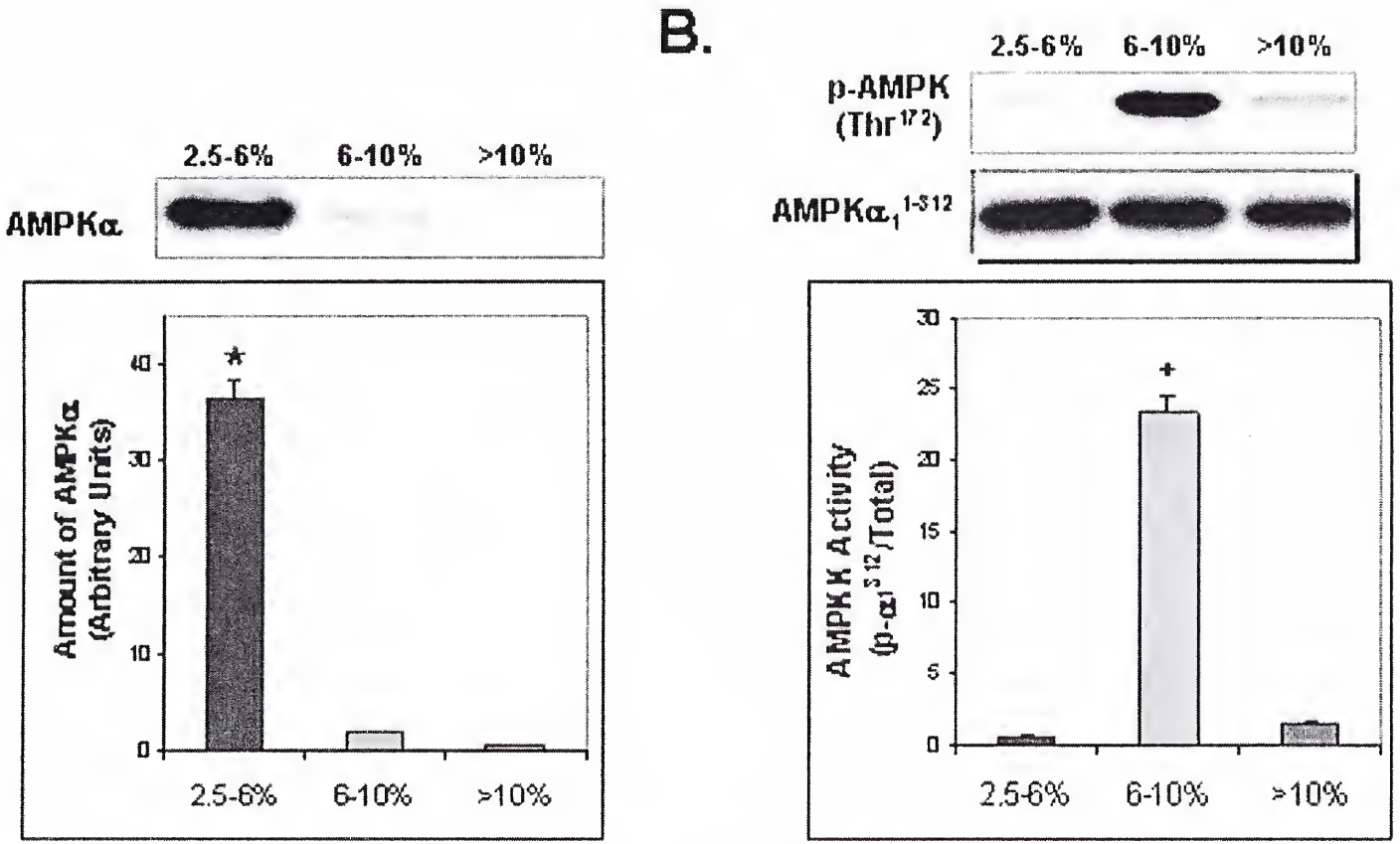

Figure 1: AMPK and AMPKK enrichment in heart polyethylene glycol (PEG) extracts. *Panel A – Enrichment of AMPK in Heart Extracts.* 40 μg of protein extract from the 2.5-6% PEG fraction, 6-10% PEG fraction and the supernatant from the 10% fraction (n=3) was immunoblotted using pan-α-AMPK antibodies (* $p < 0.0005$ vs. both 6-10% and >10%). *Panel B – Enrichment of AMPKK activity in Heart Extracts.* 10 pmol of recombinant α1 AMPK subunit was incubated for 10 minutes with protein extract (25 μg) from the 2.5-6% PEG fraction, 6-10% PEG fraction and the supernatant from the 10% fraction (n=3) in 25 μl of kinase buffer. Incubations were then immunoblotted using pThr172 and pan-α-AMPK antibodies to show the amount of phosphorylated recombinant α1 AMPK as a measure of AMPKK activity (+ $p < 0.0005$ vs. both 2.5-6% and >10%).

Effects of time and concentration on AMPKK activity: We next examined the effects of time and concentration on AMPKK activity in order to obtain the optimal conditions under which to assess the effects of ischemia on AMPKK activity. In the investigation of the time dependence of AMPKK activity, 6-10% PEG precipitated heart protein was incubated with recombinant heterotrimeric AMPK for 10, 20 and 60 minutes. Incubation samples were then immunoblotted for $pThr^{172}$ and total AMPK (Figure 2A). As incubation time increased, $pThr^{172}$ levels on the recombinant heterotrimeric protein also increased, with the steepest part of the curve appearing between 10 and 20 min, thereby suggesting that experiments involving AMPKK would be best performed at times within this range.

Subsequently, the concentration dependence of AMPKK activity was examined by incubating recombinant heterotrimeric AMPK with 5 μg, 10 μg, and 25 μg of 6-10% PEG precipitated heart protein and then immunoblotting the incubation samples for $pThr^{172}$ and total AMPK (Figure 2B). As the concentration of 6-10% PEG precipitated protein increased, the level of $pThr^{172}$ increased as well in a nearly linear fashion. It was determined that 10 μg was an optimal concentration at which to perform further AMPKK studies since it was in the linear part of the curve, it produced adequate phosphorylation at the Thr^{172} site, and it did not require excessive amounts of protein.

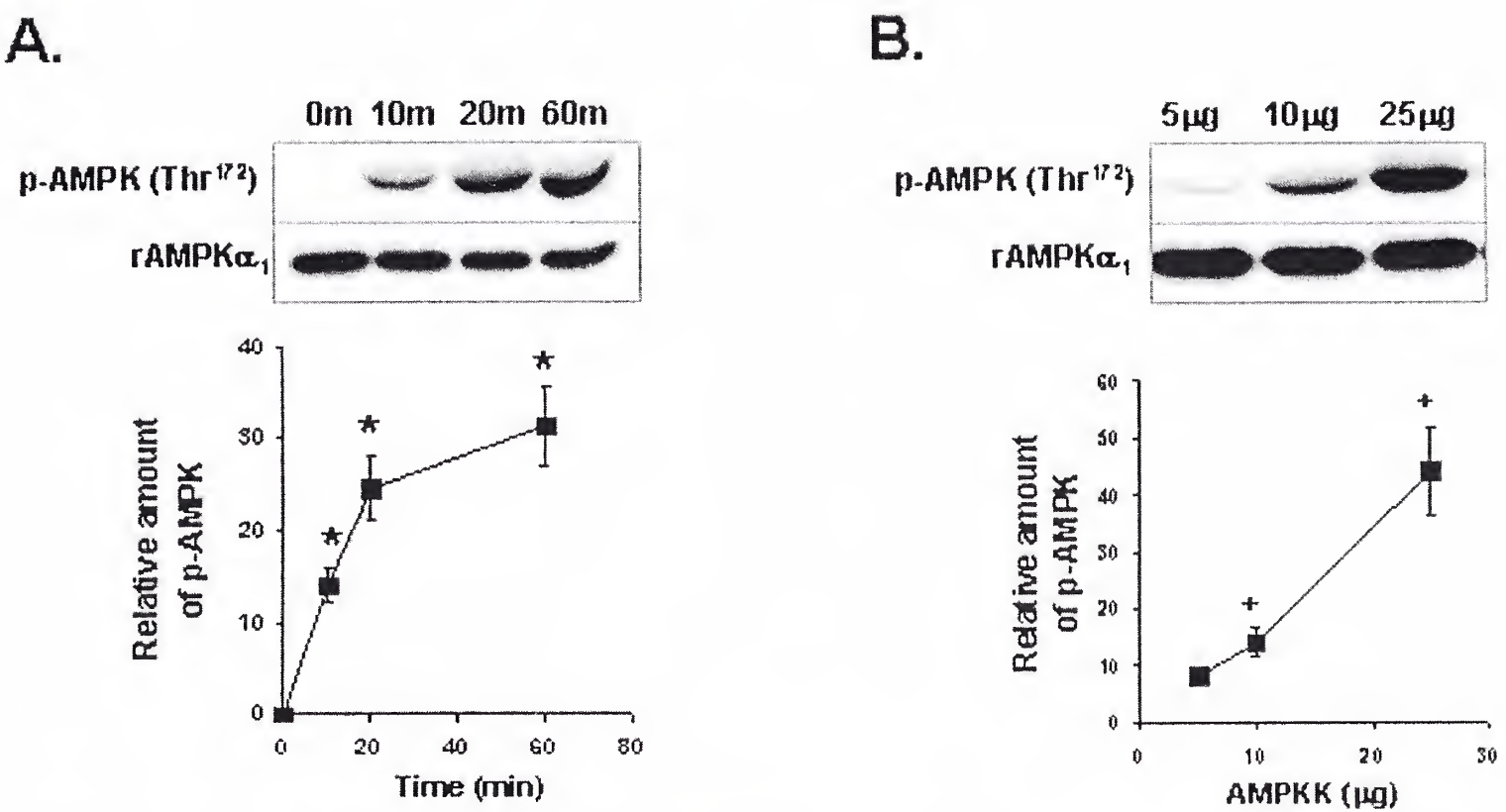

Figure 2: AMPKK dependence on time and concentration. *Panel A -- Incubation Time.* Recombinant heterotrimeric AMPK (5 pmol) was incubated for 0, 10, 20 and 60 minutes with AMPKK in the 6-10% PEG fraction (10 μg) in 25 μl of kinase buffer (n=3). Incubations were then immunoblotted using pThr172 and pan-α-AMPK antibodies (* $p < 0.01$ vs. 0 min). *Panel B -- Protein Extract Concentration.* Recombinant heterotrimeric AMPK (5 pmol) was incubated with 5 μg, 10 μg, and 25 μg of AMPKK in the 6-10% PEG fraction for 10 min in 25 μl of kinase buffer (n=3). Incubations were immunoblotted using pThr172 and α-AMPK antibodies (+ $p < 0.01$ vs. 5μg AMPKK). Immunoblots were quantified using densitometry.

Effects of *in vitro* ischemia on AMPK phosphorylation and activity in the heart: AMPK activity has been recently shown to be cardioprotective during ischemia (150), although the mechanism for this effect and the role that AMPKK plays remains unclear. Having obtained a basic understanding of some of the characteristics of AMPKK activity, we were now able to investigate the effects of global low-flow ischemia on the level of AMPKK activity as measured by phosphorylation of Thr^{172}. In order to provide an index of AMPK activity in the intact heart during *in vitro* ischemia, we compared the degree of endogenous AMPK phosphorylation at Thr^{172} as well as endogenous AMPK activity during both control and ischemic conditions. Our results demonstrated a 2.6-fold increase ($P < 0.002$) in $pThr^{172}$ levels in the ischemic heart (Figure 3A) and a 3.4 fold increase ($p < 0.01$) in AMPK activity as measured by the SAMS kinase assay (Figure 3B). Increased Thr^{172} phosphorylation of AMPK may reflect either increased AMPKK activity, or decreased phosphatase activity during ischemia.

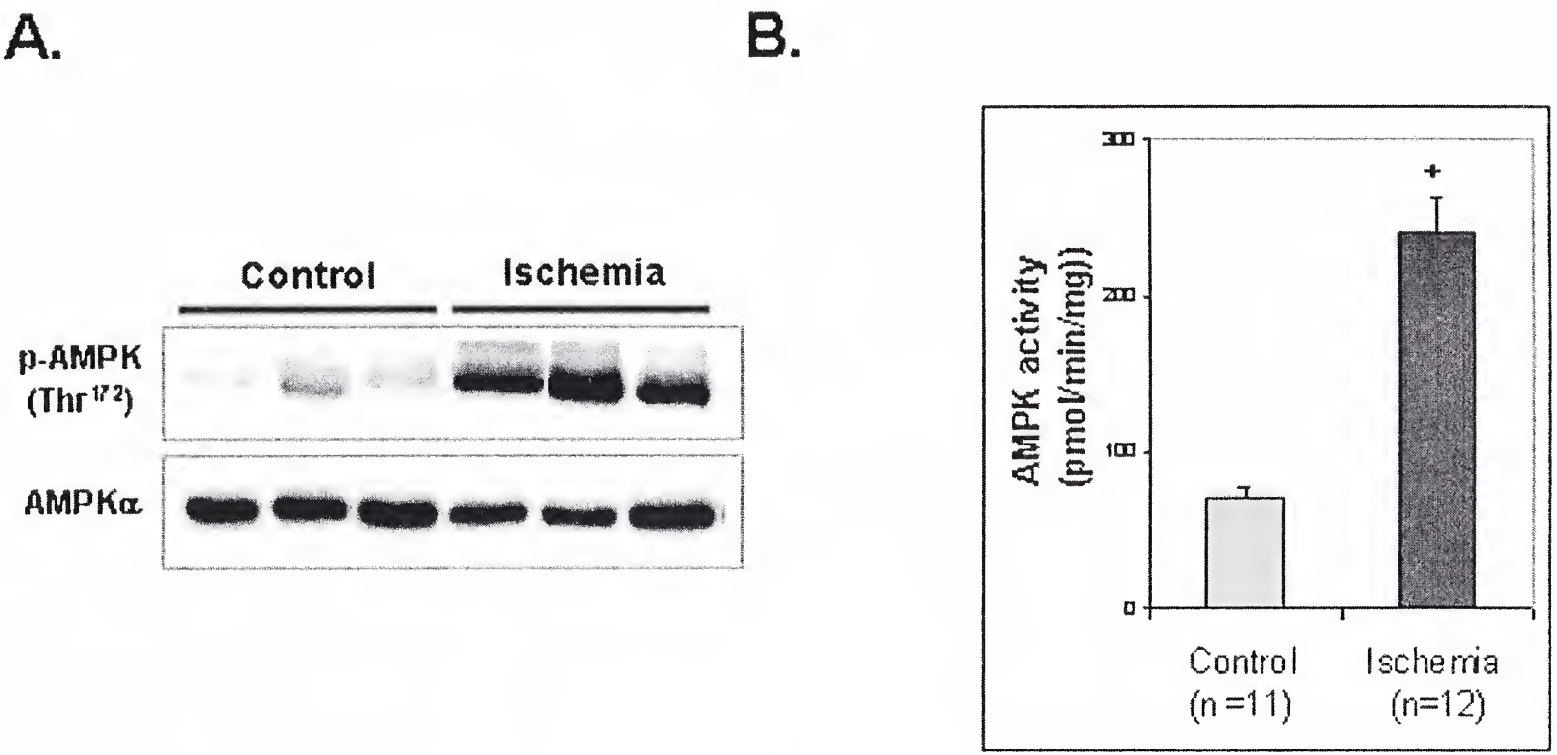

Figure 3: AMPK activation in the *in vitro* ischemic heart. Rat hearts were perfused using the working heart model under control (n=11) and low-flow (15%) ischemic conditions (n=12). *Panel A – Thr172 Phosphorylation*: Phosphorylated and total AMPK in the 2.5-6& PEG fraction was immunoblotted with pThr172 and pan-α-AMPK antibodies. Immunoblots were quantified using densitometry ($p < 0.002$ vs control). *Panel B – AMPK kinase activity*: Endogenous AMPK activity was measured using the SAMS kinase assay with 10μg of control and ischemic AMPK from the 2.5-6% PEG precipitate (+$p < 0.01$ vs. control).

Kinetics of AMPK activation during *in vitro* ischemia: Having determined that ischemia does indeed activate AMPK, we next examined the effects of varying time courses of *in vitro* ischemia on the degree of AMPK Thr^{172} phosphorylation and kinase activity. Hearts were subjected to 1 or 20 minutes of control perfusion or to 1, 2, 5, 10 or 20 minutes of global low-flow ischemia, before being homogenized and precipitated into PEG fractions. The 2.5-6% PEG fraction, containing endogenous AMPK, was then immunoblotted for $pThr^{172}$ and total AMPK levels (Figure 4A) and the SAMS kinase assay was performed to assess for AMPK activity (Figure 4B) (SAMS kinase assay was performed by Ji Li PhD.). Global ischemia resulted in a significant increase in Thr^{172} phosphorylation ($p < 0.05$) with maximal Thr^{172} phosphorylation observed after 5 minutes (Figure 4A). Endogenous AMPK activity was also found to be increased 2- to 3-fold ($p < 0.01$) during global ischemia, with maximal activity observed after 5 minutes, thereby mirroring the observed increase in Thr^{172} phosphorylation (Figure 4B). Based on these experiments, it was determined that AMPKK activation was very rapid in this ischemic model.

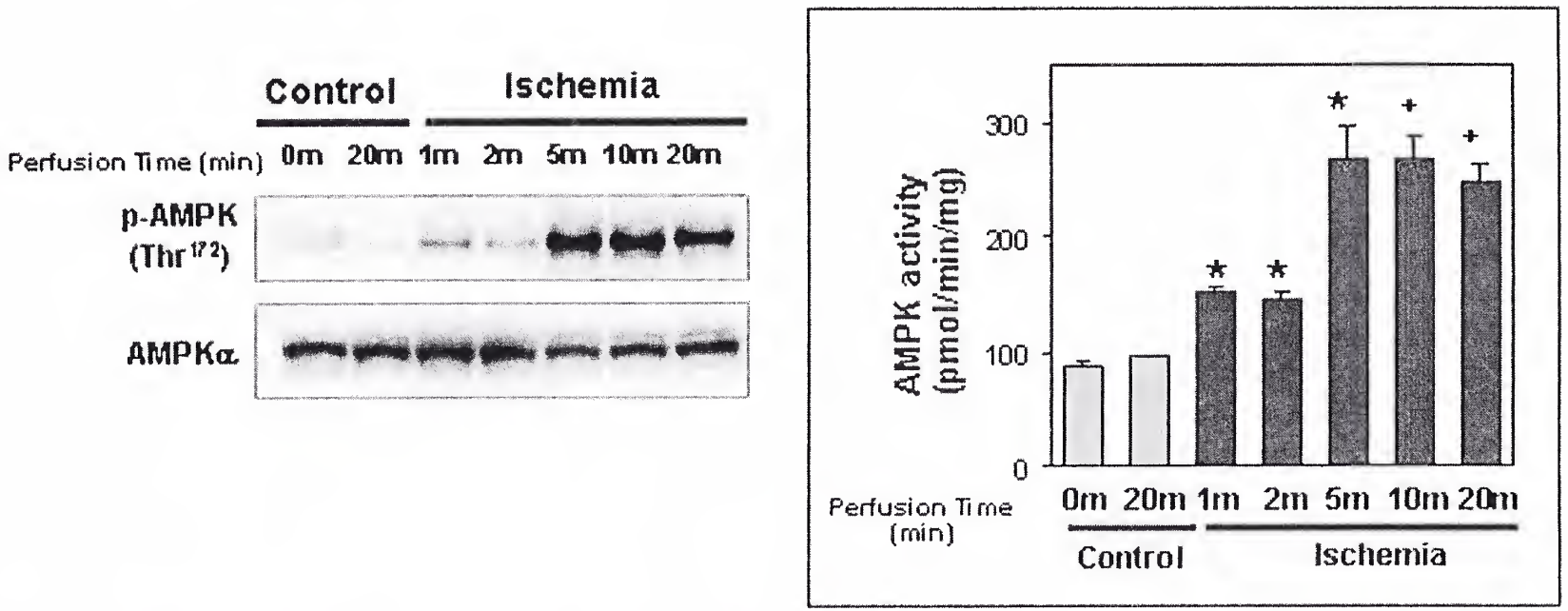

Figure 4: Kinetics of AMPK activation in the *in vitro* ischemic heart. Rat hearts were perfused using the working heart model for varying time courses under control (1 min, 20 min) (n=3) and low-flow (15%) ischemic conditions (1 min, 2 min, 5 min, 10 min, 20 min) (n=3). *Panel A – Thr172 Phosphorylation*: Phosphorylated and total AMPK in the 2.5-6& PEG fraction was immunoblotted with pThr172 and pan-α-AMPK antibodies. Immunoblots were quantified using densitometry ($p < 0.05$ vs control). *Panel B – AMPK kinase activity*: Endogenous AMPK activity was measured using the SAMS kinase assay with 10μg of control and ischemic AMPK from the 2.5-6% PEG precipitate (* $p < 0.01$ vs. 1 min control; + $p < 0.01$ vs 20 min control).

Effects of *in vitro* ischemia on AMPKK activity: In order to directly examine the possibility that AMPKK activity is increased by *in vitro* ischemia, 6-10% PEG-precipitated protein from both control and ischemic tissue was incubated with either the recombinant α_1 AMPK subunit or recombinant heterotrimeric AMPK (Figure 5A, 5B). The incubations were then immunoblotted for $pThr^{172}$ and total AMPK. Under ischemic conditions, Thr^{172} phosphorylation was increased 1.5-fold ($P < 0.04$) on the recombinant α-1 AMPK subunit (Figure 5A) and 2-fold ($P < 0.00002$) on the recombinant heterotrimeric AMPK (Figure 5B) during ischemia as compared to control conditions. Together, these results indicate that *in vitro* global ischemia acts as a physiological activator of AMPKK.

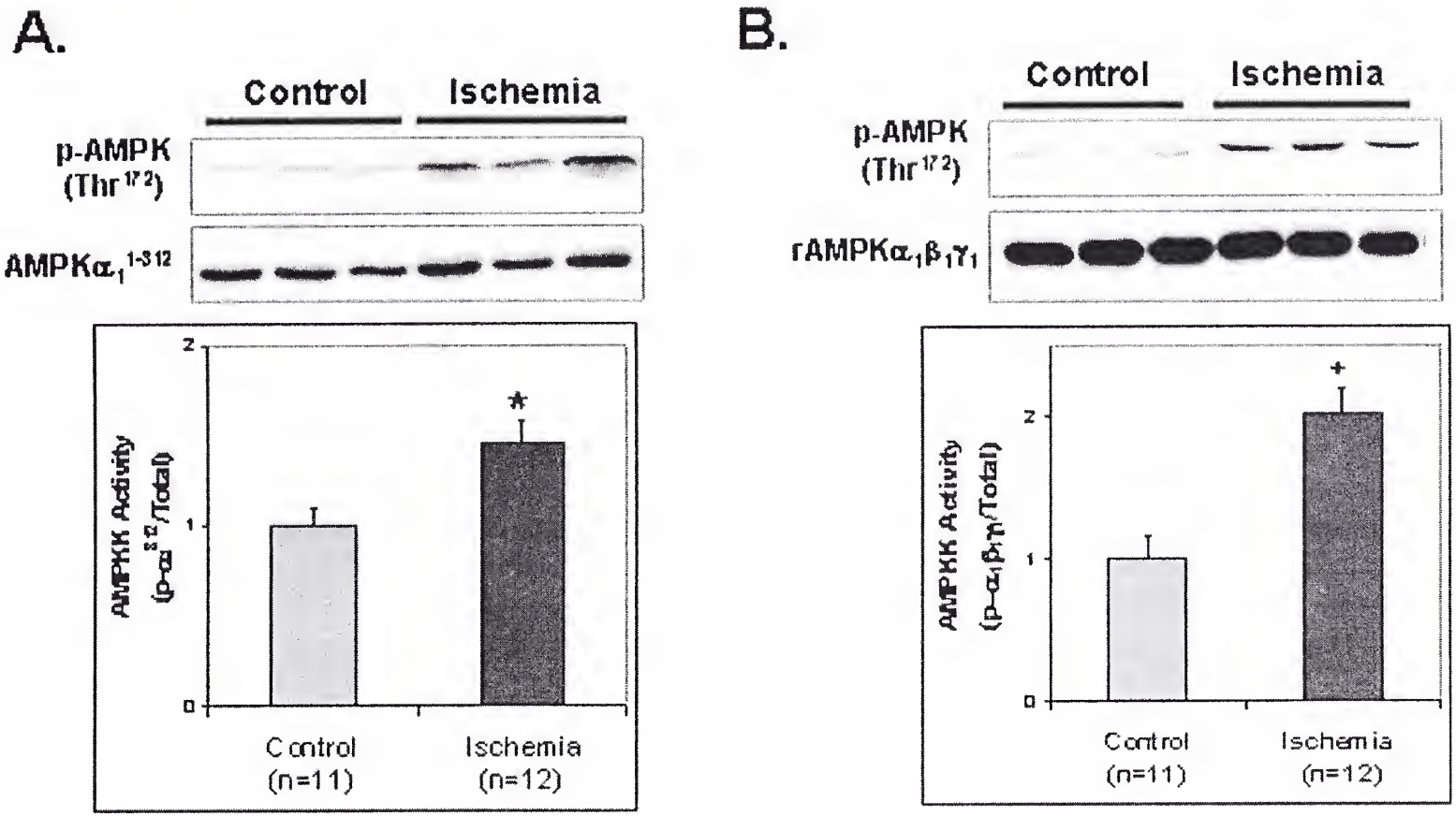

Figure 5: AMPKK activation in the in vitro ischemic heart. Rat hearts were perfused using the working heart model under control (n=11) and low-flow (15%) ischemic conditions (n=12). Recombinant AMPK α1 subunit (*Panel A*) and recombinant heterotrimeric AMPK (*Panel B*) were incubated with control and ischemic heart AMPKK from the 6-10% PEG precipitate for 10 minutes in 25μl of kinase buffer. Incubations were then immunoblotted with pThr172 and pan-α-AMPK antibodies (* $p < 0.04$ vs control. + $p < 0.00002$ vs control).

Effects of *in vivo* ischemia on AMPK and AMPKK activity: The same experiments were performed using tissue from hearts subjected to regional ischemia by left coronary occlusion. We first examined endogenous AMPK Thr^{172} phosphorylation as well as endogenous AMPK activity in control and ischemic homogenates. We found that there was a 2-fold increase ($p < 0.03$) in $pThr^{172}$ levels in the ischemic heart (Figure 6A) and a 3-fold increase ($p < 0.01$) in AMPK activity (Figure 6B). Subsequently, we incubated AMPKK protein from the 6-10% PEG fraction with either the recombinant α_1 AMPK subunit or the recombinant heterotrimeric AMPK (Figure 7A, 7B). Our results demonstrated an insignificant increase ($p < 0.15$) in Thr^{172} phosphorylation of the recombinant α-1 AMPK subunit (Figure 7A); however there was a 1.3-fold increase ($p < 0.04$) in $pThr^{172}$ on the recombinant heterotrimeric AMPK (Figure 7B) during ischemia in comparison to control hearts. These results suggest that regional ischemia produced via ligation of the left coronary artery serves to increase AMPKK activity, although to a lesser extent when compared to the increase in AMPKK activity observed during *in vitro* ischemia.

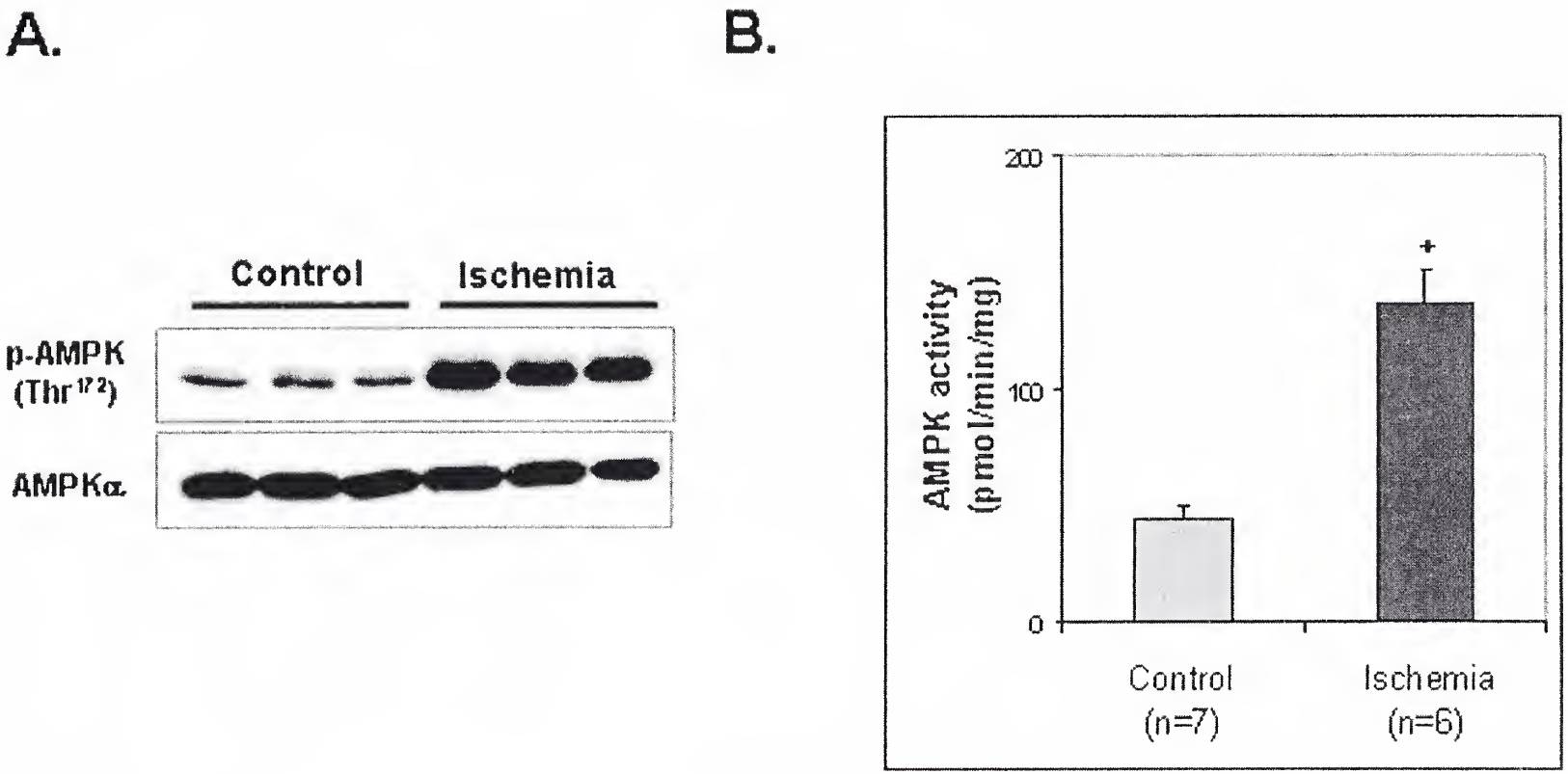

Figure 6: AMPK activation in the *in vivo* ischemic heart. Rat hearts were subjected to either regional ischemia via ligation of the LCA for 10 minutes (n=6) or to control conditions (sham operation) (n=7). *Panel A – Thr172 Phosphorylation*: Phosphorylated and total AMPK in the 2.5-6& PEG fraction was immunoblotted with pThr172 and pan-α-AMPK antibodies. Western blots were quantified using densitometry ($p < 0.04$ vs control). *Panel B – AMPK kinase activity*: Endogenous AMPK activity was measured using the SAMS kinase assay with 10μg of control and ischemic AMPK from the 2.5-6% PEG precipitate (+ $p < 0.01$ vs. control).

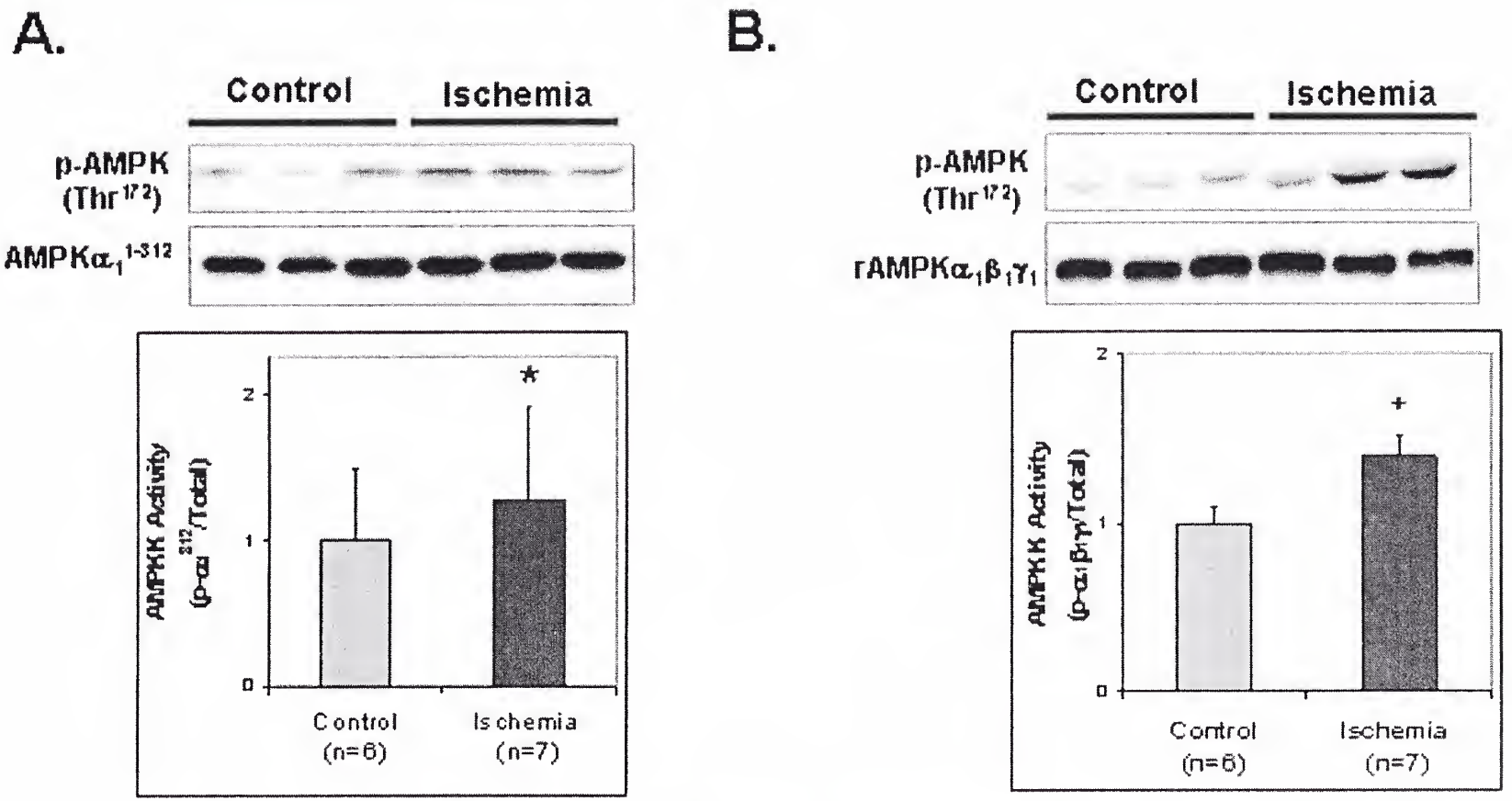

Figure 7: AMPKK activity in the *in vivo* ischemic heart. Rat hearts were subjected to either regional ischemia via ligation of the LCA for 10 minutes (n=6) or to control conditions (sham operation) (n=7). Recombinant AMPK α1 subunit (*Panel A*) and recombinant heterotrimeric AMPK (*Panel B*) were incubated with control and ischemic heart AMPKK from the 6-10% PEG precipitate for 10 minutes in 25μl of kinase buffer. Incubations were then immunoblotted with pThr172 and pan-α-AMPK antibodies (* $p < 0.15$ vs control. + $p < 0.04$ vs control).

Discussion

The overall aim of this study was to examine the effect of ischemia on the AMPK-AMPKK cascade in the heart. Our initial results demonstrated that ischemic conditions resulted in increased Thr^{172} phosphorylation along with increased kinase activity of AMPK, thereby reaffirming previous research that ischemia acts as an activator of AMPK (79) (80) (81). This finding is consistent with the notion that AMPK is activated by physiological environments associated with metabolic stress, including exercise (60), hypoglycemia (82), hypoxia (83), and heat shock (98).

While it has been presumed that the activation of AMPKK, the upstream kinase of AMPK, is responsible for the phosphorylation of AMPK during metabolic stress, the scientific literature on this subject has been limited and has not supported this theory. Indeed, initial studies have indicated that AMPKK was constitutively active in insulinoma cell lines (136), liver (146) and skeletal muscle (147). In order to study the question of the regulation of AMPKK, we developed a novel enzymatic assay with which we were able to show a significant increase in AMPKK activity during *in vitro* and *in vivo* ischemia in myocardial tissue. Our results represent the first demonstration, in any tissue, of the activation of AMPKK by a physiological stimulus, thereby suggesting that increased AMPK phosphorylation during metabolic stress is due to the increased activity of AMPKK as opposed to the inhibition of phosphatase activity, or to changes in the structure of AMPK that may have led to an increased ability of the protein to become phosphorylated, or to autophosphorylation by AMPK itself.

Development of an AMPKK Assay

There were several experimental parameters that needed to be considered when devising an AMPKK assay, such as enriching the kinase from myocardial homogenates, establishing the concentration and time dependence of the enzyme, understanding the kinetics of the AMPK-AMPKK reaction in the heart, and determining appropriate substrates to use in the assay. Early research that had examined liver AMPKK found that AMPKK activity could be enriched using protein fractionation methods with polyethylene glycol precipitation (67), thereby providing a starting point for the development of the AMPKK assay detailed herein. Our results clearly demonstrated that heart AMPKK was enriched in the 6-10% PEG fraction. Furthermore, the virtual absence of AMPKK activity observed in the 2.5-6% PEG fraction and the 10% PEG supernatant suggests that we were able to enrich AMPKK without losing AMPKK activity in other PEG fractions during the enrichment process. It should also be noted that AMPK was found to be present exclusively in the 2.5-6% PEG fraction. The fact that AMPK and AMPKK activity were enriched in different PEG fractions was important, since it guaranteed that endogenous AMPK would not be present and thereby would not introduce background signal during experiments that used the recombinant heterotrimeric AMPK as a substrate. There was no possibility of overlap with endogenous AMPK signal using the other substrate, the recombinant α_1 AMPK subunit, since it migrated more slowly during SDS-PAGE because of a higher molecular weight.

After achieving the enrichment of AMPKK, it was next necessary to detail the optimal experimental conditions for the assay. Specifically, we studied the effects of the duration of the assay as well as the effects of the amount of AMPKK protein on AMPKK

activity. Our results allowed us to choose parameters that ensured a linear relationship between these variables (amount of AMPKK or the time course of the assay) and the observed AMPKK activity, as well as confirming that the substrate would not be a limiting factor for the assay.

Further experiments were performed in order to examine the time course of AMPK activation during low-flow ischemia in the heart. Our results clearly demonstrated that AMPK is activated early on during ischemia and remains significantly activated after 20 minutes, thereby indicating that a relatively short duration of ischemia is sufficient to produce AMPK activation. Such results would suggest that AMPKK activity is rapidly activated in a similar fashion; however, the question arises as to whether AMPKK follows a prolonged course of activation similar to that of AMPK, or whether AMPKK may instead experience a transient peak of activation at an early time point during ischemia and then return to baseline levels of activity at later time points. Recent research from our laboratory has reported significant AMPK activation after 30 minutes of ischemia that persists during 30 minutes of reperfusion in perfused mouse hearts (150). These findings would suggest that AMPKK activity is consistently as opposed to briefly elevated during ischemia. Additional experiments performed in our laboratory by Dr. Ji Li have confirmed this theory by demonstrating early and persistent activation of AMPKK after 20 minutes of ischemia (Appendix A – Figure 1) (141).

An important component of an assay examining enzymatic activity is the utilization of an appropriate substrate. We were fortunate to be able to obtain two different recombinant AMPK substrates through scientific collaboration with researchers at Dartmouth University and from Zurich. This study initially utilized a recombinant α_1

AMPK subunit, which is a truncated synthetic version of the α_1 subunit containing amino acids 1-312 with a N-terminal maltose binding protein, that had been used in other studies in order to assess AMPKK activity in insulinoma cells (136). Since this truncated recombinant α_1 subunit contained the Thr^{172} phosphorylation site, we believed that it might be a suitable substrate with which to assess AMPKK activity as a measure of Thr^{172} phosphorylation. Initial experiments demonstrated that this substrate was readily phosphorylated by AMPKK. We then obtained a more physiologic substrate, a recombinant heterotrimeric $\alpha_1\beta_1\gamma_1$ AMPK protein. Since AMPK is a heterotrimeric protein, it was highly possible that AMPK phosphorylation by AMPKK is affected by the tertiary structure of the AMPK heterotrimeric complex. Subsequent experiments were performed using the recombinant heterotrimeric $\alpha_1\beta_1\gamma_1$ AMPK protein. While the findings were largely similar in experiments using the recombinant heterotrimeric $\alpha_1\beta_1\gamma_1$ AMPK protein and the recombinant α_1 AMPK subunit, the experiments involving the recombinant heterotrimeric $\alpha_1\beta_1\gamma_1$ AMPK protein produced more consistent results.

One additional possible explanation for the observed difference in results is that the presence of the regulatory γ subunit in the recombinant heterotrimeric AMPK protein may have resulted in the steric hindrance of phosphatases, thereby leading to a decreased susceptibility of the catalytic α subunit to dephosphorylation. Without such steric hindrance, the recombinant α_1 AMPK subunit may have been more vulnerable to phosphatase activity, thus leading to less consistent results. If this hypothesis were accurate, we would have expected to see less Thr^{172} phosphorylation across both control and ischemic samples during experiments using the recombinant α_1 AMPK subunit as compared to experiments using the recombinant heterotrimeric $\alpha_1\beta_1\gamma_1$ AMPK protein.

Nevertheless, overall level of Thr^{172} phosphorylation was comparable between experiments using either the recombinant α_1 AMPK subunit or the recombinant heterotrimeric $\alpha_1\beta_1\gamma_1$ AMPK protein, thereby suggesting that variable results obtained using the recombinant α_1 AMPK subunit were not likely due to phosphatase action.

Certainly, the presence of the β and γ subunits may have been responsible for the more consistent results seen during experiments utilizing the recombinant heterotrimeric $\alpha_1\beta_1\gamma_1$ AMPK protein. In particular, the γ subunit has been implicated in binding AMP, which results in a conformational change of the kinase that leads to greater Thr^{172} phosphorylation (55), probably due to the fact that an AMP-AMPK complex provides a better substrate for AMPKK (78). With the heterotrimeric AMPK protein, AMP may have been able to complex with AMPK to produce a better site of action for AMPKK, thereby magnifying the effects of the activated AMPKK. While this is a theoretical explanation for the observed effects, the experiments were performed in the absence of AMP in that no AMP was added to the incubation buffer and carry over of AMP from heart homogenate in the 6-10% PEG fraction was likely minimal, thereby making this hypothesis less likely.

With regards to the recombinant heterotrimeric $\alpha_1\beta_1\gamma_1$ AMPK protein as a substrate for the AMPKK assay, it should be noted that multiple isoforms of AMPK subunits exist. In fact, the α_2 isoform is predominant in myocardial tissue. Thus, although the recombinant heterotrimeric $\alpha_1\beta_1\gamma_1$ AMPK protein was an excellent substrate for the AMPKK assay, it should be noted that findings related to this recombinant heterotrimeric $\alpha_1\beta_1\gamma_1$ AMPK protein are slightly limited as it is not the dominant isoform in heart (55). A recombinant heterotrimeric $\alpha_2\beta_1\gamma_1$ AMPK protein has since become

available, and recent studies in our laboratory, performed by Dr. Ji Li, have demonstrated results using the recombinant heterotrimeric $\alpha_2\beta_1\gamma_1$ AMPK protein that are similar to the results described herein using the recombinant heterotrimeric $\alpha_1\beta_1\gamma_1$ AMPK protein (Appendix A – Figure 2) (141).

Thus, this study was able to develop an effective assay with which to examine AMPKK activity. This assay should serve as a useful research tool in the further study of AMPKK regulation in the heart as well as in other tissues.

Ischemia is an activator of AMPKK

The predominant mechanism responsible for the increased level of Thr^{172} phosphorylation on heart AMPK observed during ischemia has been previously unknown. The results presented herein suggest, for the first time, that ischemic conditions do increase AMPKK activity as measured by increased levels of Thr^{172} phosphorylation and increased kinase activity of the recombinant heterotrimeric protein, thereby challenging the prior notion that AMPKK is constitutively active (136).

Two different experimental ischemia preparations were used in this study. We utilized *in vivo* regional ischemia in order to examine AMPKK activity under integrated physiologic conditions; however, while using the *in vivo* protocol, we found that control tissues sometimes displayed increased baseline AMPKK activity. Such findings led to the speculation that AMPKK is extremely labile, such that even a short course of ischemia, as produced during the time when the control heart was excised and frozen, would lead to increased AMPKK activity. Therefore, it was determined that it would be advantageous to utilize an *in vitro* tissue preparation protocol in which the tissue could be

immediately frozen when the control and ischemic protocol ended. Furthermore, our laboratory has recently demonstrated that AMPK plays a critical role in preventing myocyte apoptosis after *in vitro* low-flow ischemia (151), thus increasing our interest in using this experimental ischemic model to assess AMPKK activity.

There are several limitations that need to be recognized when making generalizations using results from *in vitro* experiments about AMPKK activity. First, global as opposed to regional ischemia is produced during the *in vitro* protocol, which does not mimic the typical clinical situation of coronary artery disease. Also, the neurohumoral activation and influence of other substances, such as catecholamines or angiotensin II that may affect cardiac energy metabolism *in vivo*, are absent in an *in vitro* tissue preparation. Lastly, a heart perfused under *in vitro* conditions experiences a lower workload than a heart *in vivo*, thereby reducing the need for increased energy metabolism and subsequently potentially decreasing the degree of AMPK activation. In order to circumvent the issue of a decreased workload, the working heart model was utilized as it produces a greater workload than the Langendorf model of *in vitro* retrograde perfusion.

The findings presented herein, that AMPKK activity is increased by ischemia, are directly contradictory to the previous belief that AMPKK is constitutively active (136). This discrepancy may be explained by the differences in experimental methodology employed by Hamilton et al (136) and this current study. Firstly, Hamilton et al (136) employed an insulinoma cell line as opposed to the whole heart tissue preparation used in this study. Certainly, in a whole tissue preparation, multiple factors, including cell-to-cell interaction and synchronous contractility, can influence energy metabolism, and these additional factors may be involved in regulating AMPKK activity. Also, Hamilton et al

(136) incubated the synthetic AMPK substrate with crude cell lysate as opposed to enriching AMPKK through PEG fractionation. It is unknown what other proteins, specifically phosphatases, may have been present in the cell lysate and how those proteins might have affected the levels of Thr^{172} phosphorylation observed. Furthermore, Hamilton et al (136) used only the recombinant α_1 AMPK subunit as a substrate, which is likely not the optimal substrate to study the subtle differences in AMPKK activity under control and experimental conditions as has been previously discussed.

This study raises the question of how ischemia activates AMPKK on a molecular basis. Initial experiments had suggested that increased levels of AMP, such as those produced during ischemia (79), might serve to activate AMPKK (78). Using the methodology developed in this study, subsequent experiments, performed in our laboratory by Dr. Ji Li, were developed to address the effects of AMP on AMPKK activation during myocardial ischemia. The results of these experiments involving AMP have been subsequently published in conjunction with the experiments described herein (See Appendix B) (141). AMPKK was incubated with a series of substrates (the recombinant α_1 AMPK subunit, a recombinant heterotrimeric AMPK protein containing a γ subunit with the R70Q mutation, which decreases the γ subunit's ability to bind AMP, and a recombinant wild-type heterotrimeric $\alpha_1\beta_1\gamma_1$ AMPK protein) in the presence or absence of AMP. AMP had no effect on AMPKK activity as assessed by unchanging levels of Thr^{172} phosphorylation from both control and ischemic samples when the recombinant isolated α_1 AMPK subunit or the R70Q mutated AMPK was used as a substrate (Appendix A – Figures 3 and 4) (141). However, similar experiments using the recombinant heterotrimeric $\alpha_1\beta_1\gamma_1$ AMPK protein as a substrate, demonstrated increased

levels of Thr^{172} phosphorylation by AMPKK in the presence of AMP (Appendix A – Figure 5) (141). These results suggest that AMP acts to make AMPK a better substrate by interacting with the γ subunit, but has no direct effect on AMPKK activity. Interestingly, when AMPKK activity was studied in the presence of ATP, there was no change in AMPKK activity when the recombinant α_1 AMPK subunit was used as a substrate; however, when the recombinant heterotrimeric $\alpha_1\beta_1\gamma_1$ AMPK protein was used as a substrate, AMPKK activity was found to be decreased by increasing levels of ATP, thereby suggesting that ATP acts to indirectly inhibit AMPKK activity by transforming AMPK into a less suitable substrate for phosphorylation (See Appendix A – Figure 6) (141). These results, when taken together, suggest a model of the AMPK-AMPKK cascade, in which ischemia activates AMPKK through an AMP-independent mechanism and AMP and ATP function to either promote or inhibit respectively the ability of AMPK to be phosphorylated by AMPKK in the heart (Figure 8).

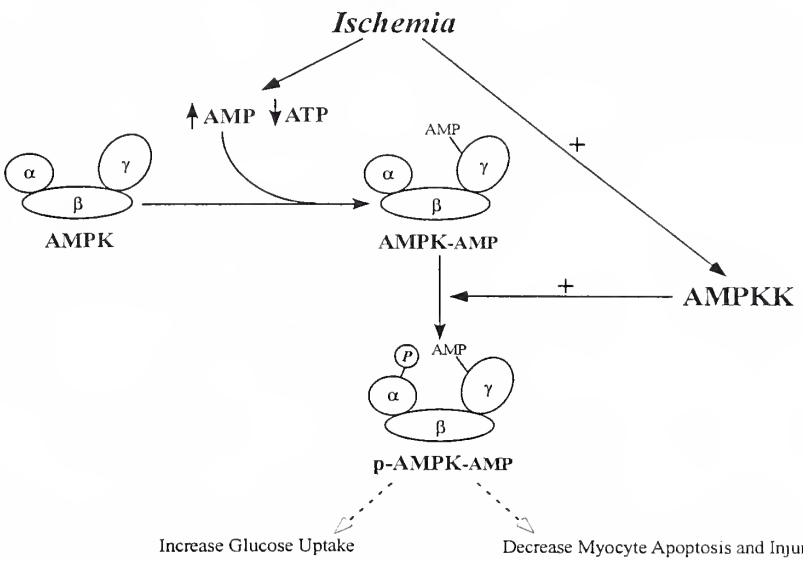

Figure 8: Model of AMPKK and AMPK activation by Ischemia: Ischemia increases AMP levels and decreases ATP levels. AMP then binds the γ subunit of AMPK, leading to an AMPK-AMP complex. The AMPK-AMP complex is a more suitable substrate for AMPKK to phosphorylate at the Thr^{172} site, thereby leading to the activation of AMPK. Ischemia also acts to activate AMPKK by a mechanism that is independent of AMP.

Recent research, published in January 2005, has also demonstrated that AMPKK activity is increased during myocardial ischemia (142), and serves to further confirm the findings of this thesis. Methodology similar to the methods described here was employed, although a 5% PEG supernatant (that may have contained endogenous AMPK activity) as opposed to a 6-10% PEG precipitant was utilized for AMPKK enrichment and only the recombinant α_1 subunit was used as a substrate (142). These authors also suggested that heart AMPKK was activated by ischemia in the absence of changes in AMP concentration (142). This conclusion was based on cardiac tissue analysis of the cellular content of AMP using high performance liquid chromatography (HPLC). Since most AMP is bound to cellular proteins, the free cytosolic AMP is present at much lower concentrations than the total AMP (i.e. μM versus near mM), and is also likely responsible for regulating AMPK activity. Furthermore, studies have shown that AMPK is activated in the intact heart by minor changes (i.e. 1μM) in the concentration of free AMP (151). Since HPLC measures total levels of AMP, it seems likely that the methods used by these authors were not sensitive to support the conclusion that AMP concentrations do not change in the ischemic heart. Thus, while the study by Altarejos et al (142) confirm our results that AMPKK is activated by myocardial ischemia, they do not provide convincing proof that the mechanism by which AMPKK is activated is independent of AMP.

Other physiologic activators of AMPK that do not appear to rely on AMP as a molecular mediator do exist. Researchers have found that hyperosmotic stress and metformin both result in an increase in Thr^{172} phosphorylation and increased AMPK activity in the absence of any detectable change in AMP levels (92). Further studies

revealed that neither phosphatidylinositol 3-phosphate kinase, protein kinase C, mitogen-activated protein (MAP) kinase kinase, nor p38 MAP kinase were involved in the activation of AMPK by hyperosmotic stress (92). The here-to-uncharacterized AMP-independent mechanism by which hyperosmotic stress and metformin activate AMPK may or may not be the same mechanism by which AMPKK is activated. Further research is needed to identify and characterize the mechanisms responsible for AMPKK activation in the heart.

Other Phosphorylation Sites on AMPK

This study concentrated on the phosphorylation of Thr^{172}, which is the critical activating site on the α catalytic subunit of AMPK (53) (54). Recent research has found that there are two other sites (Thr^{258}, Ser^{485}) besides Thr^{172} present on the α subunit (143), as well as multiple sites on the β subunit ($Ser^{24/25}$, Ser^{108}, Ser^{182}) that are phosphorylated by upstream kinases (152). Site specific mutagenesis of Thr^{258} and Ser^{485} revealed no difference in AMPK activity, thereby suggesting that these sites are not important for activation of AMPK. Furthermore, examination of the amino acid sequence surrounding Thr^{258} and Ser^{485} reveals sequences significantly different from that surrounding Thr^{172}, leading researchers to speculate that two separate upstream kinases are responsible for phosphorylating Thr^{172} and Thr^{258}/Ser^{485} (143). Thus, it seems likely that ischemia has a primary effect on the AMPKK that is primarily responsible for regulating Thr^{172} phosphorylation, while other upstream kinases, which are not involved in Thr^{172} phosphorylation, may not be similarly affected by ischemia or other environments involved in modulating AMPK activity.

Other Upstream Kinases of AMPK

This study focused on the physiologic activation of upstream kinase activity of AMPK, which has been generically termed AMPKK; however, the molecular identity responsible for the kinase activity observed in this study is still unknown. Early studies in liver have shown that Ca^{2+}/calmodulin (CaM)-dependent protein kinase I kinase (CAMKIK) is able to activate AMPK, although this study clearly demonstrated that other proteins besides CAMKIK existed with greater specificity and activity for AMPK activation (78).

More recent research in yeast has identified three upstream kinases, Pak1p, Tos3p and Elm1p, responsible for phosphorylating and activating Snf1 kinase, the yeast analogue of AMPK (144). Such findings led to the identification of a mammalian relative of Pak1p, Tos3p and Elm1p, known as LKB1, a kinase that shares a similar catalytic domain as the three upstream yeast kinases (144). LKB1 is a tumor suppressor protein involved in the inhibition of cellular propagation. Mutations in the LKB1 gene have been found to cause Peutz-Jeghers syndrome, an autosomal dominant condition associated with hamartomatous polyps of the colon and small intestine, melanotic nodules of lips and hands as well as an increased risk for malignancies in the stomach, breast and ovary (145). When isolated, LKB1 was found to phosphorylate AMPK at Thr^{172} and activate the protein kinase (144) (146). Furthermore, when LKB1 was removed from purified extracts of rat liver containing AMPKK activity, the extracts were no longer able to phosphorylate and activate AMPK (146).

The role of LKB1 in the heart is unclear at this time. Recent preliminary studies in our laboratory have shown that, although LKB1 is present in the heart, it is found in PEG fractions that do not demonstrate AMPKK activity (unpublished data). Furthermore, additional research has demonstrated that, while AMPKK activity is increased during ischemia, there is no difference in immunoprecipitated LKB1 activity in ischemic myocardium as compared to control tissue (142). Taken together, these observations seem to suggest that another protein besides LKB1 is responsible for the AMPKK activity observed during cardiac ischemia. Nevertheless, it has been difficult to assess the activity of LKB1 *in vitro* due to the dissociation of LKB1 from STRAD and MO25, which are two modifier proteins that form a functional complex with LKB1 (146) and may be necessary for LKB1 activity.

While it seems likely that LKB1 may not be responsible for the AMPKK activity described herein, LKB1 does seem to share the characteristic with heart AMPKK of being unresponsive to AMP. Studies have demonstrated that skeletal muscle LKB1 was unaffected by AICAR or exercise (147) and that liver LKB1 activity was unaltered by AMP (146). These results regarding LKB1 activity are consistent with findings that AMP does not increase AMPKK phosphorylation of either the recombinant α_1 subunit or of a recombinant AMPK with the R70Q mutation in the γ subunit (141). Further investigation is needed to understand the physiological environments responsible for activating LKB1 as well as to define the role of LKB1 in the heart.

Conclusion

In summary, this study is the first to demonstrate that AMPKK is not constitutively active, but is indeed activated by ischemia, thereby suggesting that the regulation of Thr^{172} phosphorylation of AMPK is mediated by upstream kinases. Further experiments are needed to define the molecular identity of heart AMPKK and to study the molecular mechanism by which AMPKK is activated. By elucidating the regulation of AMPKK, a greater understanding of AMPK activation can be obtained

As research continues into the upstream regulation of AMPK, scientists have been investigating the large-scale physiological role of AMPK through the utilization of mice genetically engineered to be deficient in AMPK. AMPK-deficient mice demonstrate multiple defects in metabolism, including glucose intolerance, decreased sensitivity to insulin in muscle, decreased glycogen in skeletal muscle (148) and increased catecholamine production (149). The variety of metabolic irregularities produced in an AMPK-deficient organism illustrate the complex role that AMPK plays in the regulation of metabolic processes. Future research, employing genetically altered mice and other technological advances, will serve to further clarify AMPK's function as a cornerstone in the regulation of metabolism and perhaps point to possible treatments for diseases of metabolic dysfunction and the ischemic heart.

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Appendix A: Additional Figures

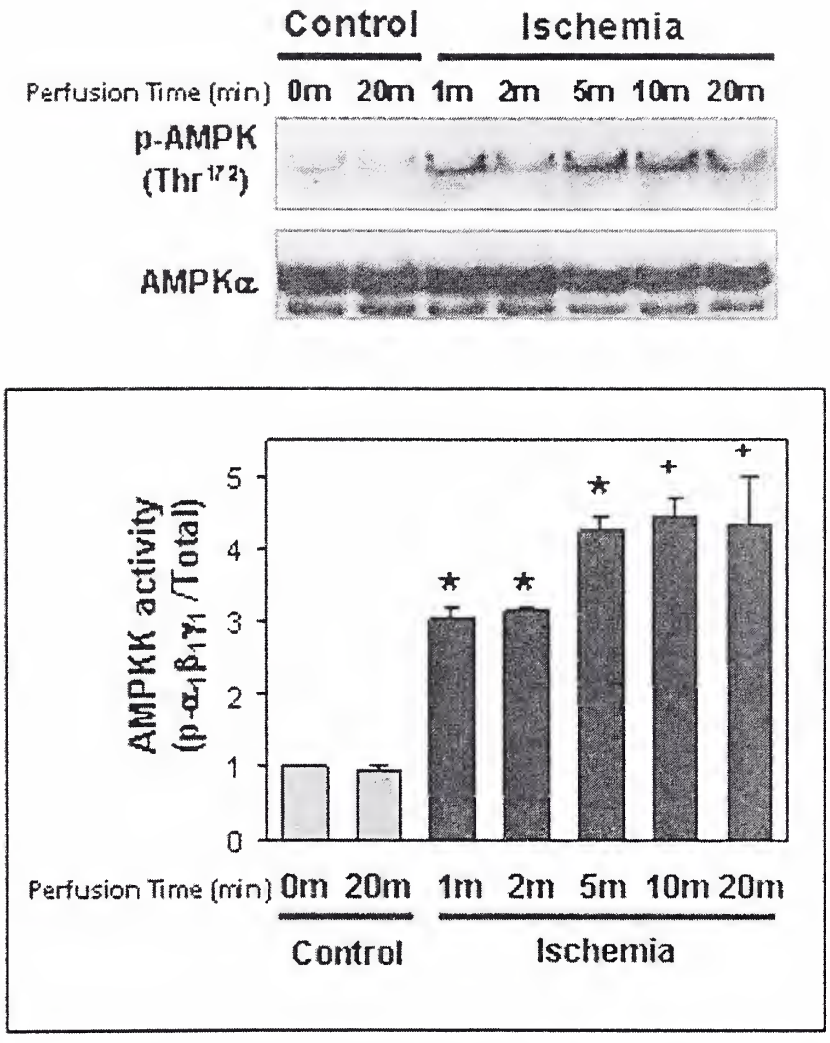

Figure A1: Kinetics of AMPKK activation in the *in vitro* ischemic heart: AMPKK activities were assessed in the working heart model after control or low-flow ischemic perfusions. $\alpha_1\beta_1\gamma_1$ rAMPK (5pmol) was incubated with heart AMPKK (10 μg) for 10 minutes and then immunoblotted with pThr^{172} and pan-α AMPK antibodies to measure AMPKK activity. Immunoblots were quantified using densitometry. (* $p < 0.05$ vs 1 minute control; + $p < 0.01$ vs 20-minute control). Values are means ± SE for 3 independent experiments.

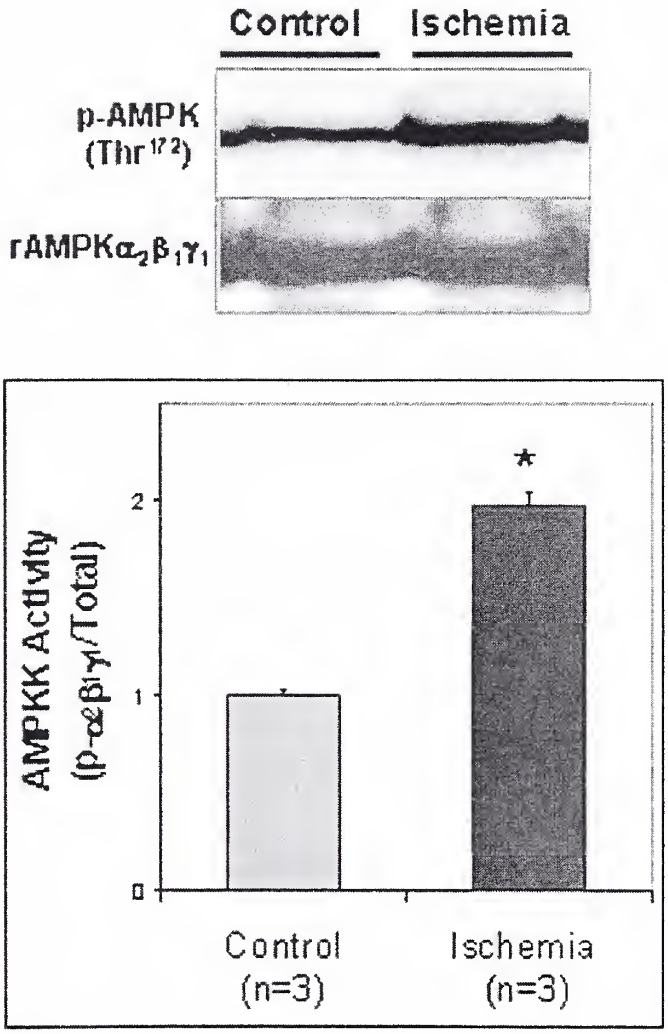

Figure A2: AMPKK activation in the in vitro ischemic heart using recombinant heterotrimeric $\alpha_2\beta_1\gamma_1$ AMPK protein as substrate. Rat hearts were perfused using the working heart model under control (n=3) and low-flow (15%) ischemic conditions (n=3). Recombinant heterotrimeric $\alpha_2\beta_1\gamma_1$ AMPK were incubated with control and ischemic heart AMPKK from the 6-10% PEG precipitate for 10 minutes in 25µl of kinase buffer. Incubations were then immunoblotted with pThr^{172} and pan-α-AMPK antibodies and quantified using densitometry. Values are means ± SE for 3 independent experiments (* $p < 0.01$ vs control).

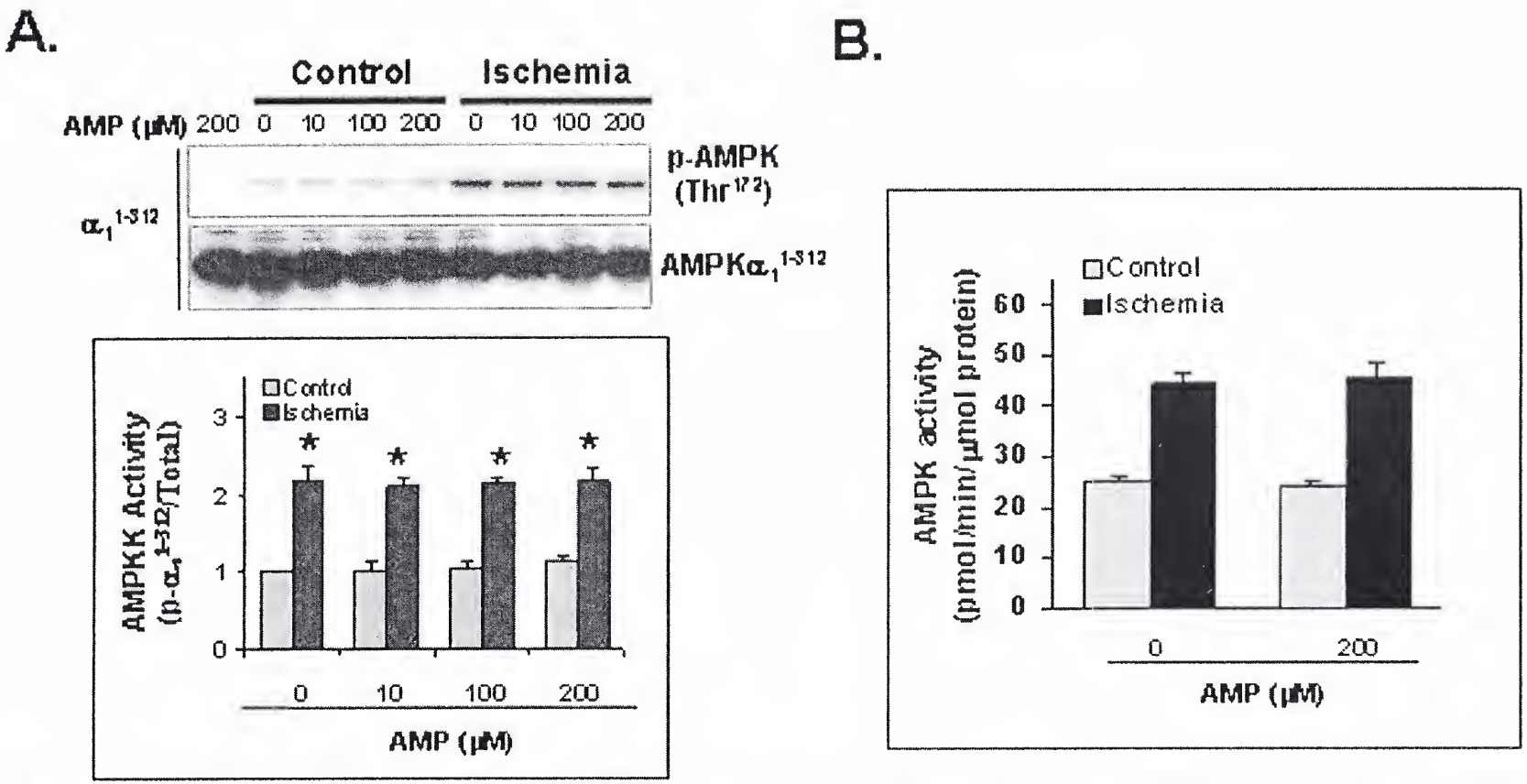

Figure A3: Effect of AMP on heart AMPKK phosphorylation of the recombinant α_1 AMPK subunit. Heart AMPKK activity after control or low-flow ischemic perfusions was measured as the phosphorylation of $\alpha_1^{1\text{-}312}$ in the absence or presence of AMP (10 to 200 µmol/L). *Panel A:* After incubations with AMPKK, substrates were immunoblotted with pThr^{172} and pan-α AMPK antibodies and their relative phosphorylation was quantified using densitometry. *Panel B:* The activity of the isolated recombinant $\alpha_1^{1\text{-}312}$ AMPK subunit were then measured using the SAMS peptide as a substrate. The AMPK concentration in *Panel B* refers to that present during incubations with AMPKK. Values are means ± SE for 3 independent experiments (* $p < 0.01$ vs control group).

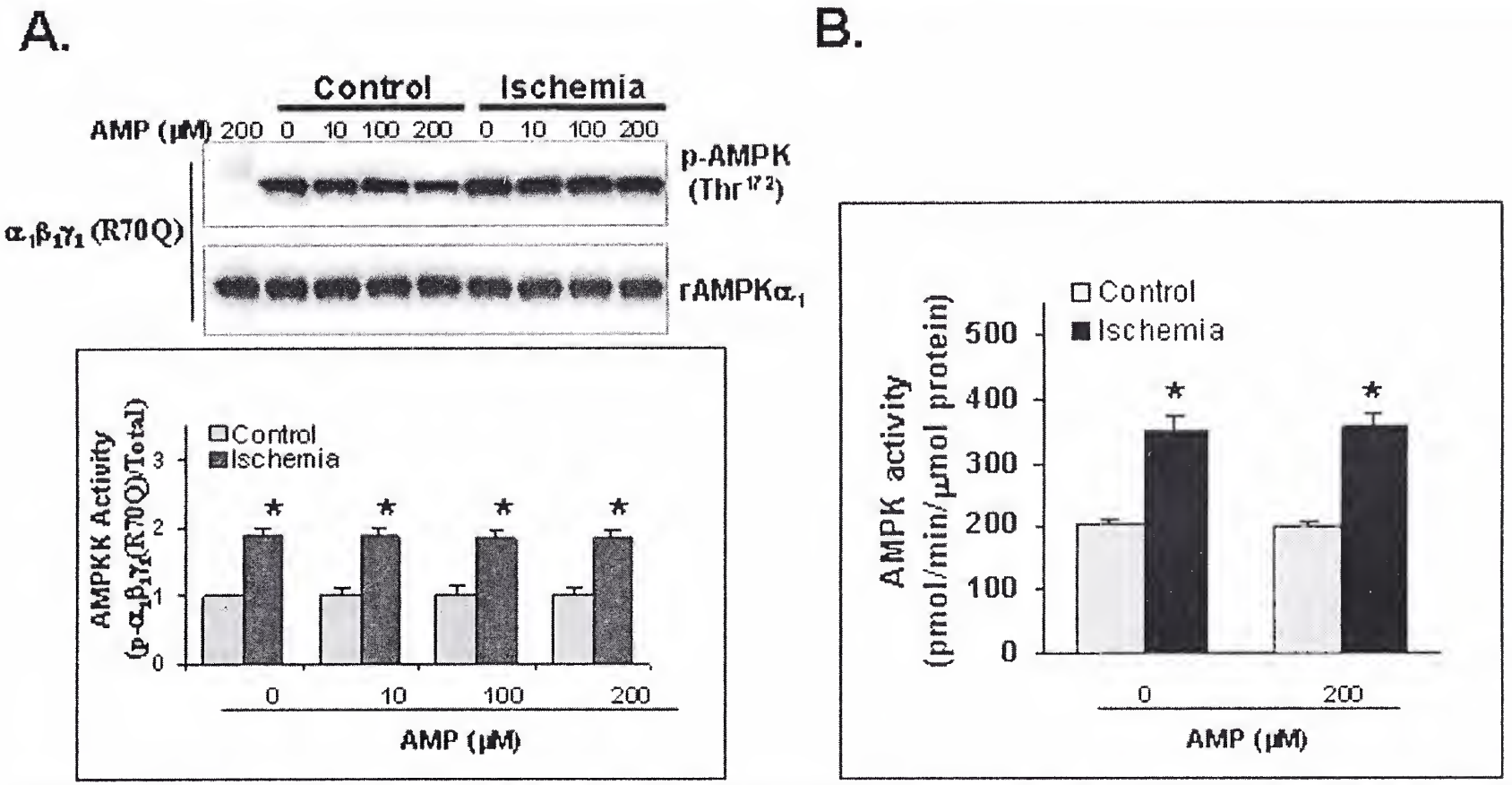

Figure A4: Effect of AMP on heart AMPKK phosphorylation of the recombinant $\alpha_1\beta_1\gamma_1$ AMPK with the R70Q mutation. Heart AMPKK activity after control or low-flow ischemic perfusions was measured as the phosphorylation of $\alpha_1\beta_1\gamma_1$ AMPK with the R70Q mutation in the γ_1 AMP-binding site in the absence or presence of AMP (10 to 200 µmol/L). *Panel A:* After incubations with AMPKK, substrates were immunoblotted with pThr^{172} and pan-α AMPK antibodies and their relative phosphorylation was quantified using densitometry. *Panel B:* The activity of the R70Q mutated recombinant $\alpha_1\beta_1\gamma_1$ AMPK were then measured using the SAMS peptide as a substrate. The AMP concentration in *Panel B* refers to that present during incubations with AMPKK. Values are means ± SE for 3 independent experiments (* $p < 0.01$ vs control group).

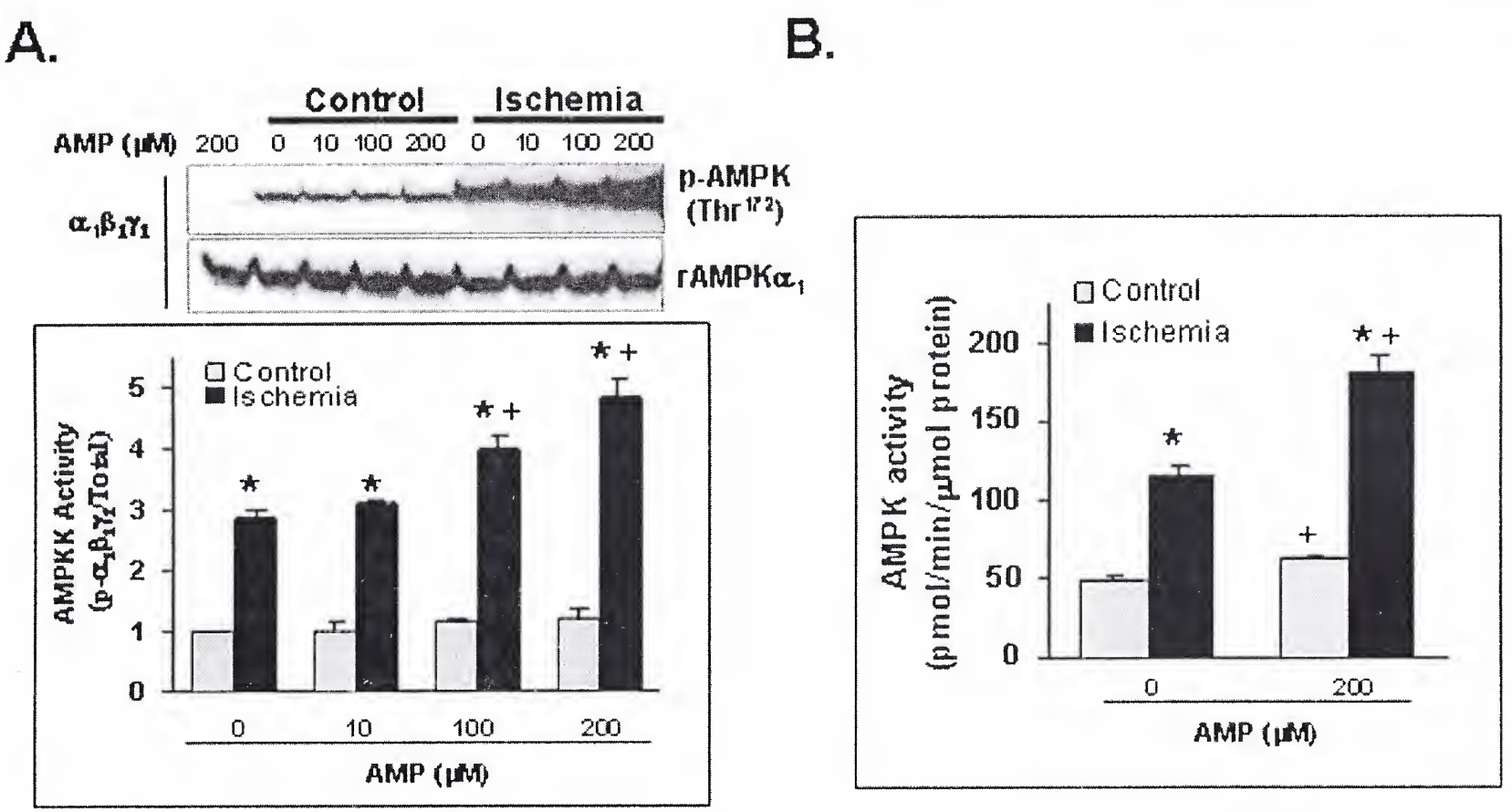

Figure A5: Effect of AMP on heart AMPKK phosphorylation of the recombinant $\alpha_1\beta_1\gamma_1$ AMPK. Heart AMPKK activity after control or low-flow ischemic perfusions was measured as the phosphorylation of $\alpha_1\beta_1\gamma_1$ AMPK in the absence or presence of AMP (10 to 200 µmol/L). *Panel A:* After incubations with AMPKK, substrates were immunoblotted with pThr^{172} and pan-α AMPK antibodies and their relative phosphorylation was quantified using densitometry. *Panel B:* The activity of recombinant $\alpha_1\beta_1\gamma_1$ AMPK was then measured using the SAMS peptide as a substrate. The AMP concentration in *Panel B* refers to that present during incubations with AMPKK. Values are means ± SE for 3 independent experiments (* $p < 0.01$ vs control group; + $p < 0.05$ vs 0 µmol/L AMP).

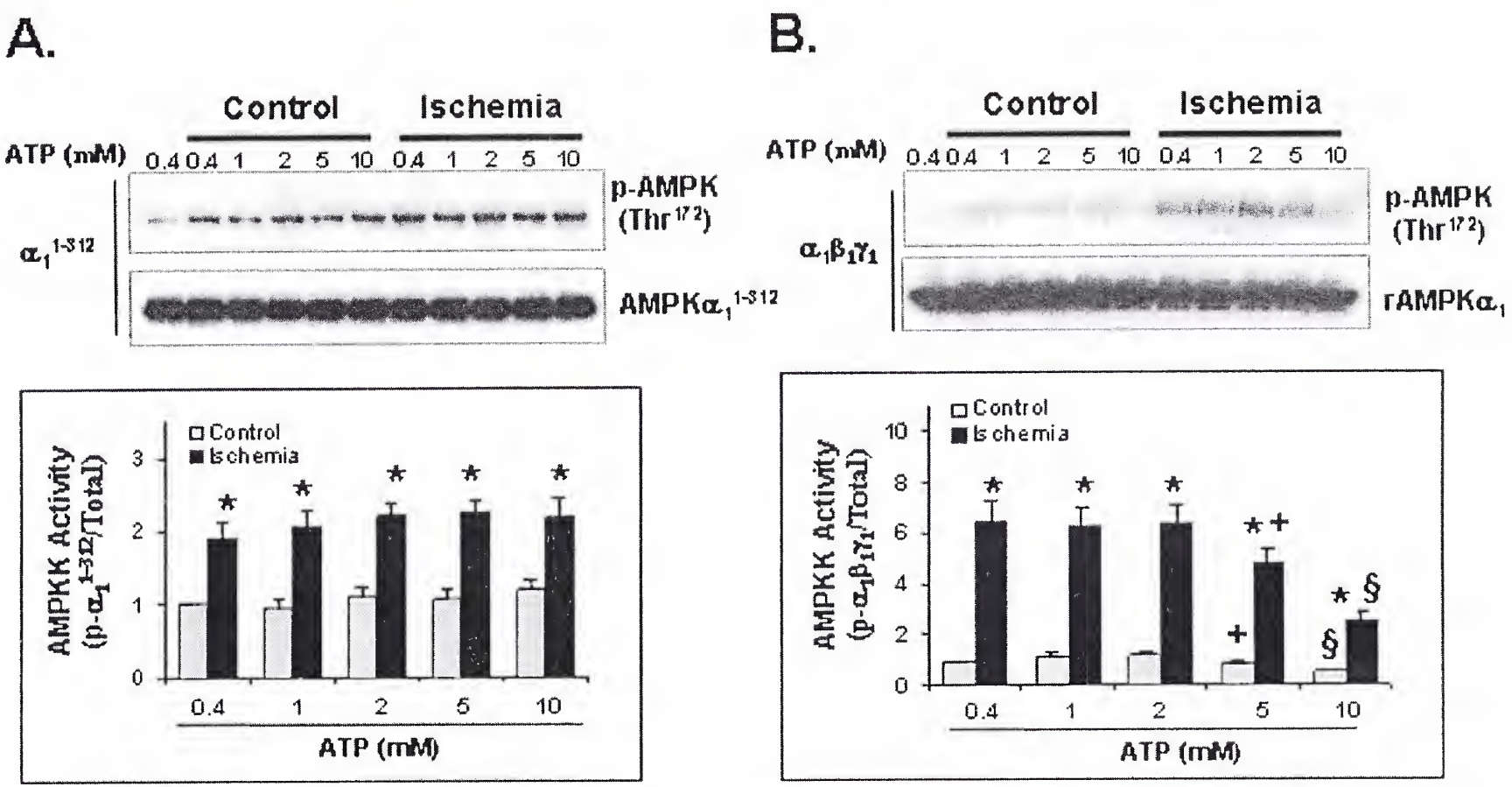

Figure A6: **Effect of ATP on heart AMPKK phosphorylation of recombinant AMPK.** Heart AMPKK activity after control or low-flow ischemic perfusions was measured as the phosphorylation of the recombinant α_1^{1-312} AMPK subunit *(Panel A)* or the recombinant heterotrimeric $\alpha_1\beta_1\gamma_1$ AMPK *(Panel B)* in the absence or presence of ATP (0.4 to 10 mmol/L). After incubations with AMPKK, substrates were immunoblotted with pThr^{172} and pan-α AMPK antibodies *(upper panels)* and their relative phosphorylation was quantified using densitometry *(lower* panels). Values are means ± SE for 3 independent experiments (* $p < 0.01$ vs control group; + $p < 0.05$ vs 0.4 mmol/L ATP; § $p < 0.01$ vs 0.04 mmol/L ATP).

Appendix B: Reprint of Journal Article

Dual Mechanisms Regulating AMPK Kinase Action in the Ischemic Heart

Suzanne J. Baron,* Ji Li,* Raymond R. Russell III, Dietbert Neumann, Edward J. Miller, Roland Tuerk, Theo Wallimann, Rebecca L. Hurley, Lee A. Witters, Lawrence H. Young

Abstract—AMP-activated protein kinase (AMPK) is emerging as an important signaling protein during myocardial ischemia. AMPK is a heterotrimeric complex containing an α catalytic subunit and β and γ regulatory subunits. Phosphorylation of Thr^{172} in the activation loop of the α subunit by upstream AMPK kinase(s) (AMPKK) is a critical determinant of AMPK activity. However, the mechanisms regulating AMPK phosphorylation in the ischemic heart remain uncertain and were therefore investigated. In the isolated working rat heart, low-flow ischemia rapidly activated AMPKK activity when measured using recombinant AMPK (rAMPK) as substrate. The addition of AMP (10 to 200 μmol/L) augmented the ability of heterotrimeric $\alpha_1\beta_1\gamma 1$ or $\alpha_2\beta_1\gamma 1$ rAMPK to be phosphorylated by heart AMPKK in vitro, whereas physiologic concentrations of ATP inhibited rAMPK phosphorylation. However, neither AMP nor ATP directly influenced AMPKK activity: they had no effect on AMPKK-mediated phosphorylation of rAMPK substrates lacking normal AMP-binding γ subunits (isolated truncated $\alpha_1^{1\text{-}312}$ or $\alpha_1\beta_1\gamma 1$ rAMPK containing an R70Q mutation in the γ_1 AMP-binding site). Regional ischemia in vivo also increased AMPKK activity and AMPK phosphorylation in the rat heart. AMPK phosphorylation could also be induced in vivo without activating AMPKK: AICAR infusion increased AMPK phosphorylation without activating AMPKK; however, the AMP-mimetic AICAR metabolite ZMP enhanced the ability of heterotrimeric rAMPK to be phosphorylated by AMPKK. Thus, heart AMPKK activity is increased by ischemia and its ability to phosphorylate AMPK is highly modulated by the interaction of AMP and ATP with the heterotrimeric AMPK complex, indicating that dual mechanisms regulate AMPKK action in the ischemic heart. **(*Circ Res*. 2005;96:337-345.)**

Key Words: AMP-activated protein kinase ■ AMPK kinase ■ ischemia

AMP-activated protein kinase (AMPK) regulates energy generating metabolic and biosynthetic pathways during physiologic and pathologic cellular stress. AMPK activation stimulates fatty acid oxidation,[1] promotes glucose transport,[2,3] accelerates glycolysis,[4] and inhibits triglyceride[5] and protein synthesis.[6] By increasing ATP synthesis and decreasing ATP utilization, AMPK functions to maintain normal cellular energy stores during ischemia. Chronic activation of AMPK also phosphorylates transcription factors altering gene expression[7] and modulates muscle mitochondrial biogenesis.[8]

AMPK is a heterotrimer consisting of an α catalytic subunit and β and γ regulatory subunits. The primary mechanism responsible for AMPK activation involves phosphorylation of the Thr^{172} residue located within the activation loop of the α catalytic subunit.[9] Additional phosphorylation sites have been identified on the α and β subunits, but their functional roles remain uncertain.[10,11] Activation of AMPK during myocardial ischemia,[1,12] exercise,[13] hypoglycemia,[14] and hypoxia[15] is associated with ATP breakdown and increases in intracellular AMP. However, AMPK is also phosphorylated through AMP-independent pathways during osmotic stress[16] and metformin[17] or leptin[18] stimulation.

Activation of AMPK is very sensitive to an increase in the intracellular concentration of AMP, which promotes its allosteric activation and phosphorylation.[19,20] Phosphorylation of the α subunit Thr^{172}-activating site is mediated by one or more upstream kinases, termed AMPK-activating protein kinases or AMPKK(s).[21] AMP increases liver AMPKK(s) activity through binding to the AMPK γ subunit, which renders AMPK a better substrate for AMPKK, and by direct activation of AMPKK by AMP.[22] However, recent findings challenge the notion that AMP has a direct effect on AMPKK[23] and have also raised the possibility that AMPKK is constitutively active.[24]

The physiological mechanisms responsible for the regulation of AMPKK in the heart remain uncertain. The aims of this study were to assess whether AMPKK is activated by ischemic stress and the extent to which AMP and ATP

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modulate heart AMPKK action. The results indicate that heart AMPKK is activated by ischemia, but that it is not directly affected by either increases in AMP or decreases in ATP concentration. Instead, AMP augments and ATP inhibits the action of AMPKK to phosphorylate and activate the AMPK α subunit by interacting with the heterotrimeric AMPK complex.

Materials and Methods

Male Sprague–Dawley rats (250 to 350 grams; Charles River Laboratories, Inc, Wilmington, Mass) were given standard chow and water before experiments. All procedures were approved by the Yale University Animal Care and Use Committee.

In Vitro Low-Flow Ischemia

Rats were anesthetized with pentobarbital sodium (60 mg/kg intraperitoneal) and heparinized (300 U intraperitoneal). Hearts were excised and anterogradely perfused in the working mode with Krebs–Henseleit buffer containing 1% bovine serum albumin, 0.4 mmol/L oleate, and 5 mmol/L glucose, and equilibrated with 95% O_2/5% CO_2 at 37°C.[25] Control hearts were perfused at a preload of 15 cm H_2O and an afterload of 100 cm H_2O for 40 minutes. Ischemic hearts were perfused normally for 20 minutes and then flow was reduced to 15% of control (by decreasing afterload pressure to 30 cm H_2O) for 1 to 20 minutes. Hearts were freeze-clamped in liquid nitrogen and stored at −80°C.

In Vivo Regional Ischemia

Anesthetized rats were endotracheally intubated and ventilated with a small animal respirator, and they underwent thoracotomy to ligate the proximal left coronary artery for 10 minutes. Control rats underwent sham thoracotomy. Hearts were then rapidly excised and freeze-clamped in liquid nitrogen.

In Vivo AICAR Infusion

The AMPK-activator 5-amino-4-imidazolecarboxamide (AICAR) (Sigma, St. Louis, Mo), which is converted to the monophosphorylated metabolite ZMP that is an AMP mimetic, was administered intravenously (100 mg/kg bolus and 10 mg/kg per minute infusion for 60 minutes) to chronically catheterized rats.[3] Control rats received saline infusions. Plasma glucose was maintained constant with a variable infusion of 20% dextrose to prevent hypoglycemia, as previously described.[3] At the end of the infusion, rats were anesthetized with intravenous pentobarbital (50 mg/kg), and the hearts were rapidly excised and freeze-clamped in liquid nitrogen.

Tissue Fractionation

Heart tissue was homogenized in buffer containing 125 mmol/L Tris, 1 mmol/L EDTA, 1 mmol/L EGTA, 250 mmol/L mannitol, 50 mmol/L NaF, 5 mmol/L NaPPi, 1 mmol/L DTT, 1 mmol/L benzamedine, 0.004% trypsin inhibitor, and 3 mmol/L NaN_3 (pH 7.5).[13] After centrifugation at 14 000*g* for 20 minutes, the supernatant was fractionated by the sequential addition of polyethyleneglycol (PEG) into 2.5% to 6% and 6% to 10% precipitants and >10% supernatant. Fractions were resuspended in homogenizaiton buffer without mannitol. Protein concentrations were determined using the Bradford assay (BioRad reagent).

Immunoblotting

Proteins were diluted in Laemmli sample buffer before SDS-PAGE.[13] After transfer to polyvinylidine difluoride membranes, proteins were immunoblotted with pan-α (α_1/α_2) AMPK antibody at 1:10 000 dilution (kind gift from Dr M. Birnbaum) and anti-pThr^{172} AMPK antibody at 1:5000 dilution (Cell Signaling, Beverly, Mass). Proteins were detected with enhanced chemiluminescence and autoradiographs were quantified using densitometry.

AMPKK Assay

Heart AMPKK activity was assessed by measuring the AMPKK-induced Thr^{172} phosphorylation of rAMPK substrates in vitro. Initial experiments demonstrated that AMPKK activity was present almost exclusively in the 6% to 10% PEG fraction (see Results). To assess AMPKK activity, protein (10 μg) from the 6% to 10% PEG fraction was incubated with 10 pmol of truncated $\alpha_1^{1\text{-}312}$ fusion protein (N-terminal maltose binding protein),[24] or 5 pmol of $\alpha_1\beta_1\gamma1$ rAMPK containing an R70Q mutation in the γ_1 AMP-binding site, wild-type $\alpha_1\beta_1\gamma1$, or $\alpha_2\beta_1\gamma1$ rAMPK.[26] Incubations were performed in 25 μL of AMPKK assay buffer (20 mmol/L Tris, 5 mmol/L $MgCl_2$, 0.2 mmol/L ATP, 0.5 mmol/L DTT, 0.1% Tween, 1 mg/mL bovine serum albumin; pH 7.5). In experiments designed to assess the effects of nucleotides on AMPKK activity, AMP (0 to 200 μmol/L), ATP (400 μmol/L to10 mmol/L), and ZMP (0 to 1000 μmol/L; Sigma, St. Louis, Mo) were added to the incubation mixture. Samples were diluted with Laemmli buffer, subjected to SDS-PAGE, and immunoblotted with anti-pThr^{172} AMPK and pan-α AMPK antibodies.

AMPK Activity Assay

Endogenous heart AMPK activity, as well as the catalytic activity of rAMPK incubated with AMPKK, were assessed with a kinase assay measuring the incorporation of [γ-^{32}P]-ATP into the SAMS peptide.[13] Endogenous AMPK activity was measured using 10 μg of 2.5% to 6% PEG fraction protein prepared from heart homogenates. The activity of $\alpha_1^{1\text{-}312}$ fusion protein or heterotrimeric rAMPK used as AMPKK substrates was measured after isolation with a Ni-NTA kit (Qiagen, Valencia, Calif), which bound the epitope-tagged recombinant proteins via their polyhistidine sequences.

Statistics

Results were analyzed using Student *t* test and are presented as means±SEM. Results were significant at $P<0.05$.

Results

AMPK and AMPKK Fractions

We initially evaluated whether AMPK and AMPKK might be separately enriched using PEG precipitation of heart homogenates. Immunoblots demonstrated that endogenous AMPK was present predominantly in the 2.5% to 6% PEG fraction (Figure 1A), whereas AMPKK activity was almost exclusively in the 6% to 10% fraction (Figure 1B). Conditions for optimizing the AMPKK assay were then established. AMPKK activity was found to be linear for 20 minutes (Figure 1C), using up to 25 μg of 6% to 10% PEG-precipitated protein from ischemic hearts (Figure 1D), so that AMPKK assays were subsequently performed with 10 μg protein for 10 minutes.

AMPK and AMPKK Activity During In Vitro Ischemia

We next assessed whether ischemia activated AMPK and AMPKK activity in perfused working rat hearts. Endogenous AMPK Thr^{172} phosphorylation (Figure 2A) and activity (Figure 2B) increased 2- to 3-fold ($P<0.01$) after low-flow ischemia. Incubation of the AMPKK fraction with heterotrimeric $\alpha_1\beta_1\gamma1$ rAMPK as substrate demonstrated a 4- to 5-fold ($P<0.01$) increase in AMPKK activity in ischemic hearts (Figure 2C). The increase in ischemic heart AMPKK activity was very rapid, increasing 3-fold after 1 minute and reaching maximal activity by 5 to 20 minutes. The accumulation of phosphorylated AMPK was less rapid ($P<0.05$), but also significant, during the first 2 minutes of ischemia, and was

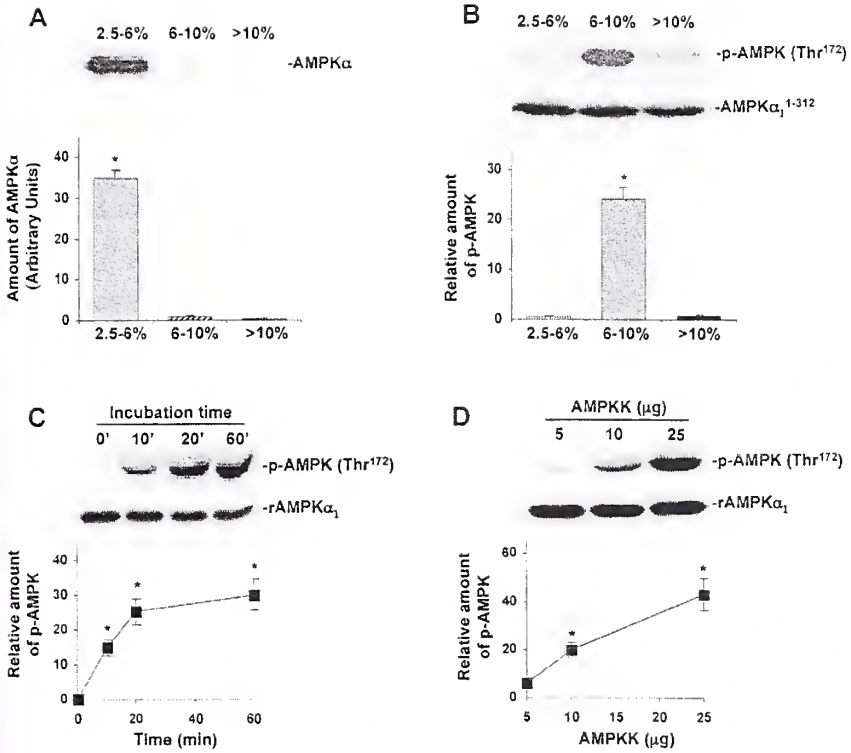

Figure 1. Enrichment of AMPK and AMPK kinase (AMPKK) activity in heart homogenate fractions. A, Ischemic heart homogenates were fractionated using PEG precipitation and immunoblotted with pan-α AMPK antibody (*P<0.01 vs both 6% to 10% and >10%). B, PEG fractions (25 μg protein) were incubated with 10 pmol $\alpha_1^{1\text{-}312}$ fusion protein for 10 minutes. $\alpha_1^{1\text{-}312}$ was then immunoblotted with pThr^{172} and pan-α AMPK antibodies to measure AMPKK activity (*P<0.01 vs both 2.5% to 6% and >10% fractions). C, AMPKK (10 μg protein from 6% to 10% fraction) from ischemic hearts was incubated with 5 pmol $\alpha_1\beta_1\gamma1$ rAMPK (*P<0.01 vs 0 minutes). D, AMPKK was incubated with 5 pmol $\alpha_1\beta_1\gamma1$ rAMPK for 10 minutes. rAMPK was then immunoblotted with pThr^{172} and pan-α AMPK antibodies to measure AMPKK activity (*P<0.01 vs 5 μg AMPKK). Values are means±SE for 3 independent experiments.

maximal after 5 to 20 minutes. Because AMPK was not present in the AMPKK fraction, there was no detectable endogenous phosphorylated Thr^{172} AMPK in the incubations.

Effects of In Vivo Ischemia on AMPK and AMPKK Activity

To determine whether AMPKK was also activated by regional ischemia in the intact rat in vivo, we measured AMPK and AMPKK activity after coronary occlusion. Regional ischemia stimulated endogenous AMPK phosphorylation (Figure 3A) and increased AMPK activity 3-fold (P<0.01) (Figure 3B). Regional ischemia also stimulated AMPKK activity: phosphorylation of $\alpha_1\beta_1\gamma1$ rAMPK increased significantly (P<0.05) (Figure 3D), and the phosphorylation of the $\alpha_1^{1\text{-}312}$ also tended to be greater after in vivo ischemia (Figure 3C).

Effects of AMP on Heart AMPKK Activity In Vitro

To determine whether heart AMPKK is activated directly by AMP, perfused heart AMPKK was incubated with varying concentrations of AMP and either the $\alpha_1^{1\text{-}312}$ fusion protein or $\alpha_1\beta_1\gamma1$ rAMPK containing an R70Q mutation in the γ_1 AMP binding site. These substrates enabled assessment of the direct effects of AMP on AMPKK, without the potentially confounding effect of AMP interacting with the heterotrimeric complex to render the substrates more effective targets for AMPKK. With the addition of physiologic concentrations of AMP (10 to 200 μmol/L) found in the ischemic heart,[20,27] there was no augmentation of AMPKK-stimulated Thr^{172} phosphorylation (Figure 4A and 4B) or the catalytic activities (Figure 4C and 4D) of these rAMPK substrates.

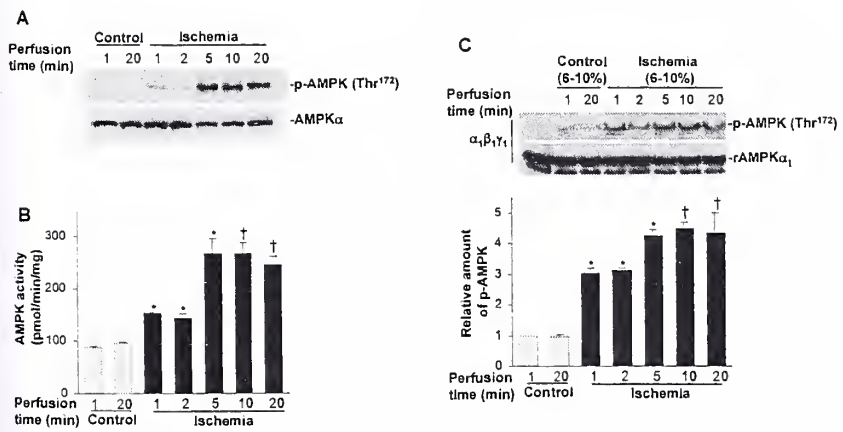

Figure 2. Kinetics of AMPK and AMPKK activation in the in vitro ischemic heart. AMPK and AMPKK activities were assessed in the working heart model after control or low-flow ischemic perfusions. A, Endogenous heart AMPK was immunoblotted with pThr^{172} and pan-α AMPK antibodies. B, Endogenous AMPK activity was measured in the 2.5% to 6% PEG fraction of heart homogenates using the SAMS peptide as a substrate (*P<0.01 vs 1-minute control; †P<0.01 vs 20-minute control). C, $\alpha_1\beta_1\gamma1$ rAMPK (5 pmol) was incubated with heart AMPKK (10 μg) for 10 minutes and then immunoblotted with pThr^{172} and pan-α AMPK antibodies to measure AMPKK activity (*P<0.05 vs 1-minute control; †P<0.01 vs 20-minute control). Values are means±SE for 3 independent experiments.

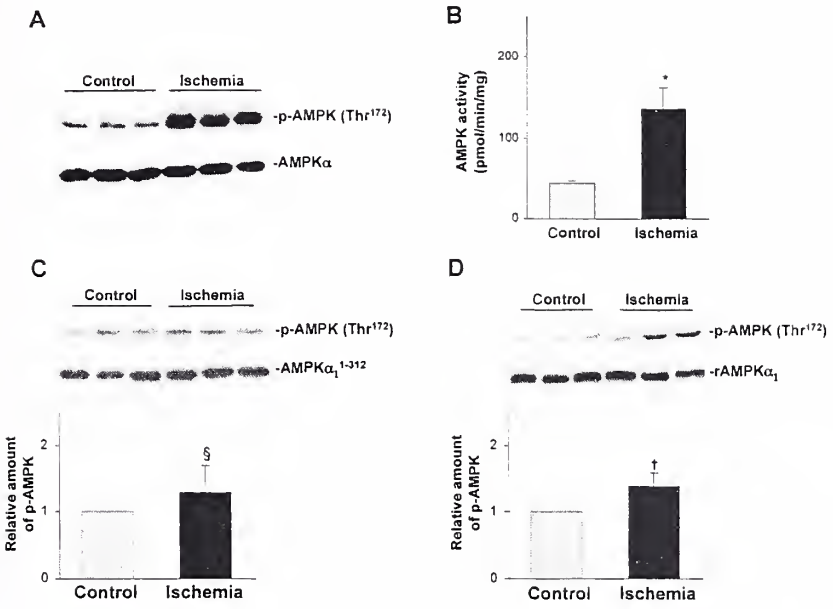

Figure 3. AMPK and AMPKK activation in the in vivo ischemic heart. Heart AMPK and AMPKK activity after sham operation (n=7) or regional ischemia (n=6) for 10 minutes in vivo. A, Endogenous heart AMPK was immunoblotted with pThr^{172} and pan-α AMPK antibodies. B, AMPK activity was measured in 2.5% to 6% PEG fractions using the SAMS peptide assay (*P<0.01 vs control). C and D, AMPKK (10 μg) activity was assessed with $\alpha_1^{1\text{-}312}$ fusion protein (10 pmol) or $\alpha_1\beta_1\gamma1$ rAMPK (5 pmol) substrates that were immunoblotted with pThr^{172} and pan-α AMPK antibodies. The relative amount of phosphorylated substrate is quantified in the bar graphs (§P<0.15, †P<0.05 vs control). Values are means±SE.

In contrast, the addition of AMP did enhance the action of heart AMPKK to phosphorylate (Figure 5A and 5B) and increase the catalytic activity (Figure 5C and 5D) of rAMPKs containing intact AMP-binding domains ($\alpha_1\beta_1\gamma1$ or $\alpha_2\beta_1\gamma1$). AMP clearly augmented the ability of ischemic heart AMPKK to activate the $\alpha_1\beta_1\gamma1$ and $\alpha_2\beta_1\gamma1$ rAMPKs (Figure 5). Although AMP had little discernible effect to increase rAMPK Thr^{172} phosphorylation (Figure 5Aand 5B), it did slightly and significantly increase the ability of control heart AMPKK to stimulate rAMPK activity (Figure 5C and 5D). Taken together, these observations suggest that AMP interaction with rAMPKs containing functional γ subunits renders the α subunits better substrates for Thr^{172} phosphorylation, particularly by ischemic heart AMPKK.

Effects of AICAR Infusion and ZMP on Heart AMPKK Activity

To further examine the physiological importance of nucleotide interaction with the γ subunit in mediating AMPK phosphorylation by heart AMPKK, we assessed the mechanisms by which AICAR activates AMPK in the heart.[3] AICAR is converted to the AMP mimetic compound ZMP[28] and is known to activate heart AMPK activity in vivo.[3] AICAR infusion increased heart AMPK Thr^{172} phosphorylation (Figure 6A) but had no effect on heart AMPKK activity, as assessed in vitro with either the $\alpha_1^{1\text{-}312}$ fusion protein or the $\alpha_1\beta_1\gamma1$ rAMPK (Figure 6B and 6C). Interestingly, ZMP had no effect to stimulate AMPKK phosphorylation of the truncated $\alpha_1^{1\text{-}312}$ fusion protein (Figure 6B), but it clearly increased the ability of heterotrimeric $\alpha_1\beta_1\gamma1$ rAMPK to be phosphorylated by AMPKK (Figure 6C). These results suggest that the AMP mimetic ZMP potentiates AMPKK action through interaction with the γ subunit, rendering AMPK a better substrate for the upstream kinase. In the absence of AMPKK activation, this physiological mechanism appears to account for AICAR-stimulated AMPK phosphorylation in the heart in vivo.

Effects of ATP on Heart AMPKK Action

ATP concentrations also decrease during ischemia;[12,27] therefore, we examined the hypothesis that normal physiologic concentrations of ATP might inhibit AMPKK directly or inhibit the ability of AMPK to be phosphorylated by heart AMPKK. Heart AMPKK was incubated with varying ATP concentrations and either the truncated $\alpha_1^{1\text{-}312}$ fusion protein or heterotrimeric $\alpha_1\beta_1\gamma1$ rAMPK (Figure 7). ATP (5 to 10 mmol/L) had no effect on AMPKK-mediated phosphorylation of the $\alpha_1^{1\text{-}312}$ fusion protein (Figure 7A) but did significantly inhibit the ability of AMPKK to phosphorylate $\alpha_1\beta_1\gamma1$ rAMPK (Figure 7B). These results indicate that physiologic intracellular concentrations of ATP indirectly inhibit the action of heart AMPKK through interaction with the heterotrimeric AMPK complex.

Discussion

These results elucidate the dual mechanisms regulating the phosphorylation and activation of AMPK by upstream AMPKK(s) in the ischemic heart. First, AMPKK activity per se is increased by both low-flow ischemia in vitro and regional ischemia in vivo. Second, AMP and ATP interactions with the heterotrimeric AMPK complex reciprocally modulate its suitability as a substrate to be phosphorylated by heart AMPKK. The findings suggest that the increases in AMP and decreases in ATP concentrations that occur in the ischemic heart[12,20,27] have an indirect influence on AMPKK action, rather than a direct effect on AMPKK activity. In addition, the results of the AICAR/ZMP experiments further demonstrate that the interaction of nucleotides with heterotrimeric AMPK are important and sufficient to increase AMPK Thr^{172} phosphorylation in vivo, even in the absence of direct heart AMPKK activation.

Both in vitro and in vivo myocardial ischemia caused significant increases in AMPKK activity in these experiments. In contrast, previous studies in noncardiac tissues and cells have observed greater AMPK phosphorylation and

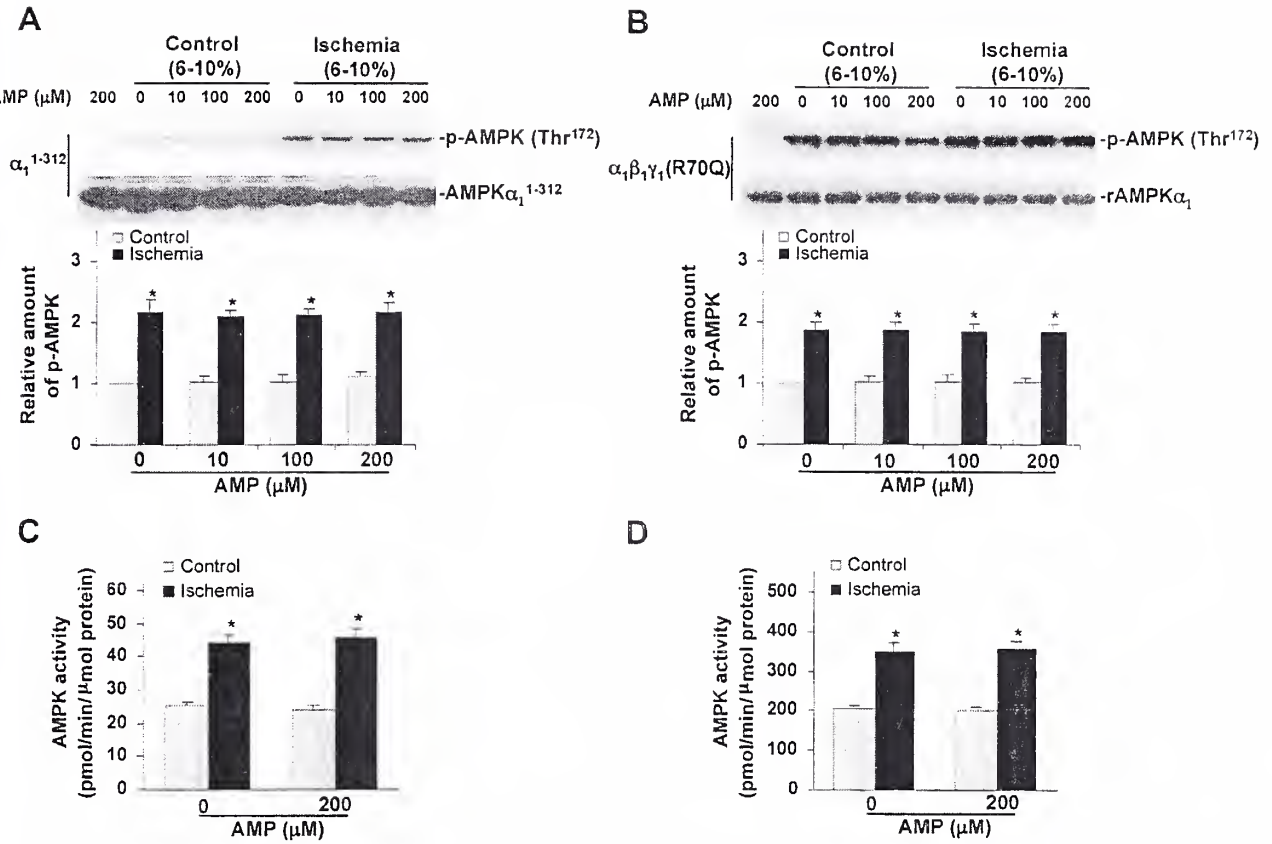

Figure 4. Effect of AMP on heart AMPKK phosphorylation of rAMPK substrates lacking AMP-binding capacity. Heart AMPKK activity after control or low-flow ischemic perfusions was measured as the phosphorylation of $\alpha_1^{1\text{-}312}$ fusion protein (A) or $\alpha_1\beta_1\gamma 1$ rAMPK containing an R70Q mutation in the γ_1 AMP-binding site (B) in the absence or presence of AMP (10 to 200 μmol/L). After incubations with AMPKK, substrates were immunoblotted with pThr^{172} and pan α-AMPK antibodies and their relative phosphorylation was quantified in the bar graphs. The activities of the isolated $\alpha_1^{1\text{-}312}$ fusion protein (C) and R70Q mutated $\alpha_1\beta_1\gamma 1$ rAMPK (D) were then measured using the SAMS peptide as a substrate. The AMP concentration in panels C and D refers to that present during incubations with AMPKK. Values are means±SE for 3 independent experiments (*P<0.01 vs control group).

activation in the absence of increased AMPKK activity. Hypoglycemia increased Thr^{172} phosphorylation and AMPK activity without altering AMPKK activity in INS-1 cells.[24] Similarly, in situ contraction increased AMPK phosphorylation in skeletal muscle without increasing the activity of LKB1,[29] a recently identified AMPKK.[23,30] Although these findings raised the possibility that AMPKK might be constitutively active, this does not appear to be the case in the heart during ischemic stress.

The mechanisms by which AMPKK action is increased in the ischemic heart were elucidated through the use of different substrates to measure AMPKK activity. Both the $\alpha_1^{1\text{-}312}$ fusion protein[24] and heterotrimeric rAMPKs[26] were effective substrates for the heart AMPKK assay in vitro. Measurement of AMPKK activity in the absence of AMP demonstrated intrinsic AMPKK activation in the ischemic heart. The use of rAMPK substrates without normally functional AMP-binding sites ($\alpha_1^{1\text{-}312}$ fusion protein and $\alpha_1\beta_1\gamma 1$ rAMPK R70Q mutation) in the AMPKK assays also enabled us to demonstrate that AMP has no direct effects to increase AMPKK activity. AMPK activation in the absence of measurable changes in the AMP concentration has been implicated in the response of noncardiac tissues to leptin,[18] osmotic stress,[16] and metformin,[16,17] but AMPKK activity has not been assessed in these experiments and the specific mediators of presumed AMPKK activation in these settings remain unknown.

In contrast, when AMP was added to ischemic heart AMPKK incubated with intact heterotrimeric $\alpha_1\beta_1\gamma 1$ or $\alpha_2\beta_1\gamma 1$ rAMPK, we observed an increase in α subunit Thr^{172} phosphorylation and AMPK activity. These results, taken together with the α_1 fusion protein and R70Q $\alpha_1\beta_1\gamma 1$ rAMPK findings, are consistent with the hypothesis that AMP-binding to the γ subunit induces a conformational change in the heterotrimeric AMPK complex, which renders the α subunit more susceptible to phosphorylation by AMPKK.[22,31,32] Interestingly, we found less striking effects of AMP to render AMPK a better substrate for nonischemic heart AMPKK, raising the possibility that activated AMPKK from the ischemic heart may better-recognize the change in AMPK conformation induced by AMP-binding to the γ subunit. Although these studies were not designed to assess protein phosphatases in the ischemic heart, it is possible that AMP binding to the γ subunit may also decrease the susceptibility of α subunit pThr^{172} to dephosphorylation by heart protein phosphatases, as previously shown in liver.[19]

In the ischemic heart, inhibition of oxidative metabolism causes ATP breakdown and leads to the formation of AMP

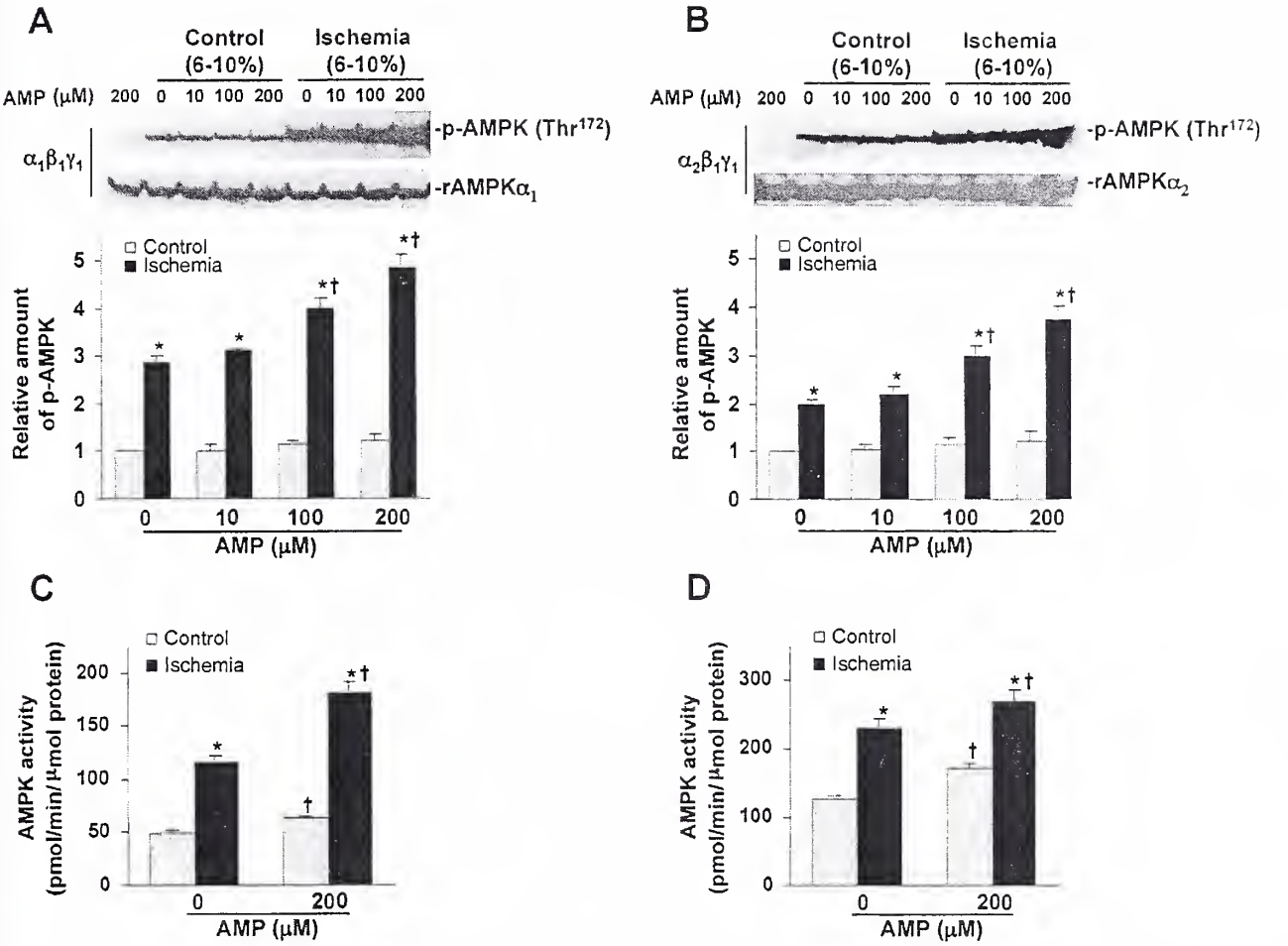

Figure 5. Effects of AMP on the action of heart AMPKK to phosphorylate intact heterotrimeric rAMPK. Heart AMPKK activity after control or low-flow ischemic perfusions was measured as the phosphorylation of $\alpha_1\beta_1\gamma1$ (A) or $\alpha_2\beta_1\gamma1$ (B) rAMPK substrates in the absence or presence of AMP (10 to 200 μmol/L). After incubations with AMPKK, substrates were immunoblotted with pThr^{172} AMPK and pan α-AMPK antibodies (upper panels) and their relative phosphorylation was quantified in the bar graphs (lower panels). $\alpha_1\beta_1\gamma1$ rAMPK (C) or $\alpha_2\beta_1\gamma1$ rAMPK (D) were isolated and their activities measured using the SAMS peptide as a substrate. The AMP concentration in panels C and D refers to that present during incubations with AMPKK. Values are means±SE for 3 independent experiments (*P<0.01 vs control group, †P<0.05 vs 0 μmol/L AMP).

through the action of adenylate kinase.[33] Our results indicate that the decline in ATP concentration, which occurs in the ischemic heart,[12,27] may also contribute to the phosphorylation and activation of AMPK. The concentrations of ATP (5 to 10 mmol/L) present in heart under nonischemic conditions[12,27] clearly inhibited AMPKK phosphorylation of rAMPK substrate that contained an intact γ subunit AMP binding site. However, these same concentrations of ATP had no discernible effect to inhibit AMPKK activity directly, as assessed using the $\alpha_1^{1\text{-}312}$ fusion protein as substrate. Thus, these findings suggest that AMP and ATP interact with the AMPK complex in a reciprocal fashion to modulate its suitability as an AMPKK substrate, rather than acting directly on AMPKK.

This study focused on AMPKK phosphorylation of the critical α subunit Thr^{172}-activating site. The α subunits contain additional phosphorylation sites, Thr^{258} and Ser^{485} (α_1)/Ser^{491} (α_2), but they do not appear to be important determinants of AMPK catalytic activity.[32] The amino acid sequences surrounding the Thr^{258} and Ser^{485} residues are significantly different from those surrounding Thr,^{172} suggesting that distinct upstream kinases are responsible for their phosphorylation.[32] In addition, glycogen may modulate AMPK activity through interaction with the β subunit glycogen binding domain.[34] The β subunit also contains several phosphorylation sites,[10,32] including Ser^{108}, which may be autophosphorylated by the α subunit.[32] Whereas this study provides insight into the ischemic regulation of Thr^{172} phosphorylation by AMPKK, the physiologic regulation and role of these additional AMPK phosphorylation sites in the heart remain to be determined.

AMPK is activated in the ischemic heart[1] and increases glucose transport by stimulating GLUT4 translocation to the sarcolemma[3] and activates phosphofructokinase-2, which accelerates glycolysis.[4] Recent results indicate that transgenic mice, expressing a dominant-negative AMPK catalytic subunit, have impaired ischemic[12] and postischemic glucose uptake.[12,35] AMPK-deficient hearts demonstrate poor recovery of left ventricular function, increased necrosis, and myocyte apoptosis after low-flow ischemia and reperfusion,[12]

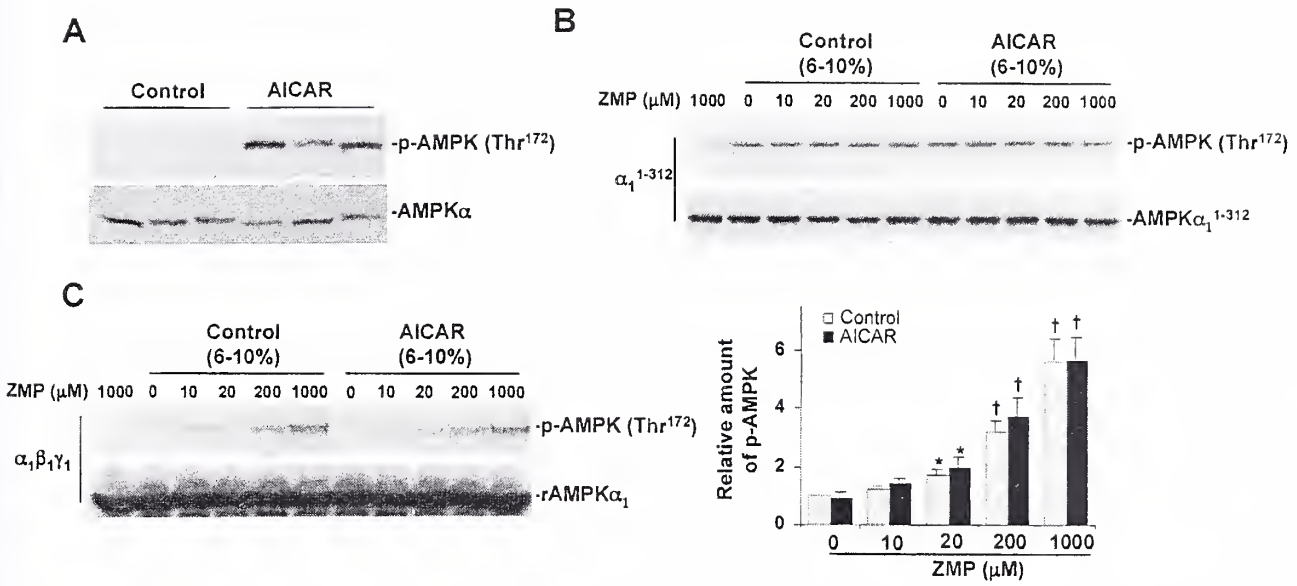

Figure 6. Effects of AICAR infusion in vivo and ZMP in vitro on heart AMPKK action. After AICAR or saline infusions in vivo, heart AMPK phosphorylation and AMPKK activity were assessed. A, Phosphorylated and total endogenous heart AMPK were immunoblotted with pThr^{172} and pan-α AMPK antibodies, respectively. Heart AMPKK activity was measured as the phosphorylation of $\alpha_1^{1\text{-}312}$ fusion protein (B) or heterotrimeric $\alpha_1\beta_1\gamma_1$ rAMPK (C) in the absence or presence of ZMP (0 to 1000 μmol/L). After incubations, AMPKK substrates underwent immunoblotting with pThr^{172} and pan-α AMPK antibodies. Results of $\alpha_1\beta_1\gamma_1$ rAMPK phosphorylation are quantified in the bar graph. Values are means±SE for 3 independent experiments (*P<0.05 vs 0 μmol/L ZMP; †P<0.01 vs 0 μmol/L ZMP).

suggesting that AMPK may have a cardioprotective role in the heart during ischemia-reperfusion. These results highlight the importance of further understanding the upstream pathways involved in AMPK activation in the ischemic heart.

Recent studies have identified the tumor suppressor LKB1 to be an upstream AMPKK in the liver.[23,30] Although we have observed that the heart AMPKK fraction contains LKB1, LKB1 is also present in PEG fractions that have no detectable AMPKK activity (unpublished data, 2004). The latter observation may be attributable to dissociation of LKB1 from STRAD and/or MO25, two modifier proteins that form a functional complex with LKB1 and potentiate its Thr^{172} phosphorylation activity.[23] Further investigation is needed to delineate the role of LKB1, STRAD α/β, and MO25 α/β in modulating AMPKK activity in the heart. However, liver LKB1 does not appear to be AMP-responsive,[23] consistent with our findings that AMP did not directly increase heart AMPKK activity.

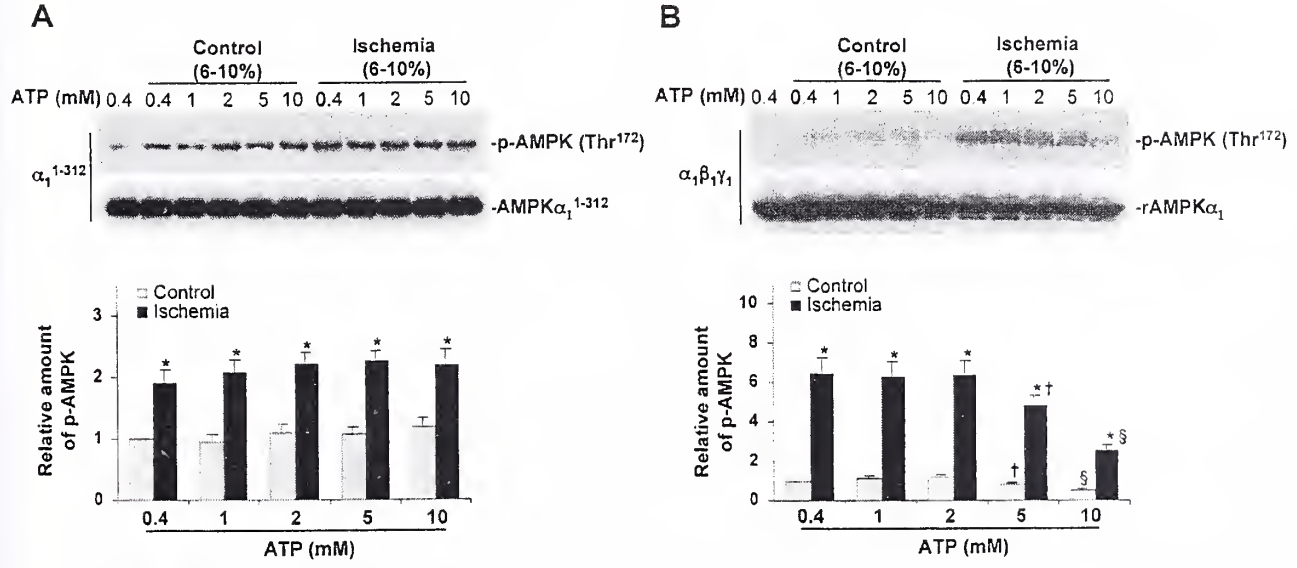

Figure 7. Effect of ATP on the action of heart AMPKK to phosphorylate rAMPK. Heart AMPKK activity after control or low-flow ischemic perfusions was measured as the phosphorylation of $\alpha_1^{1\text{-}312}$ fusion protein (A) or $\alpha_1\beta_1\gamma_1$ rAMPK (B) in the presence of varying concentrations of ATP (0.4 to 10 mmol/L). After incubations with AMPKK, rAMPK substrates underwent immunoblotting with pThr^{172} and pan α-AMPK antibodies (upper panels) and their relative phosphorylation was quantified in the bar graphs (lower panels). Values are means±SE for 3 independent experiments (*P<0.01 vs control group; †P<0.05 vs 0.4 mmol/L ATP; §P<0.01 vs 0.4 mmol/L ATP).

Although we found detectable baseline AMPKK activity and endogenous AMPK Thr[172] phosphorylation in vivo and in vitro in the heart, AMPKK is clearly not fully activated in the nonischemic heart. The effects of anesthesia or the few seconds required to excise and freeze-clamp the hearts might have contributed to the baseline AMPKK activity observed in vivo in sham-operated rats and to some extent led to underestimation of the degree of activation of AMPKK during regional ischemia. These effects together with the inherent variability of sampling in the regional model of ischemia may explain in part why the degree of activation of AMPKK in the ischemic isolated perfused hearts was greater than in the in vivo hearts.

Since the initial submission of this manuscript, Altarejos et al have presented evidence that AMPKK is activated in the ischemic heart without a measurable increase in AMP concentration or change in LKB1 activity.[36] These observations are consistent with and complement our results, further supporting the conclusion that AMPKK activation is AMP-independent in the ischemic heart and highlighting the need to identify additional AMPKK(s) in the heart and the mechanisms activating these upstream kinase(s).

In conclusion, this study demonstrates that there are dual mechanisms operative in the ischemic heart that regulate AMPKK-mediated phosphorylation and activation of AMPK. Further understanding the molecular identity of AMPKK(s) in the heart will be important as AMPK emerges as a critical signaling pathway in the ischemic heart.

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