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 and Lawrence H. Young

Circ. Res. 2005;96;337-345; originally published online Jan 13, 2005;
DOI: 10.1161/01.RES.0000155723.53868.d2
Circulation Research is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0009-7330. Online ISSN: 1524-4571

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

Original received July 20, 2004; resubmission received December 10, 2004; accepted January 3, 2005.

From the Section of Cardiovascular Medicine (S.J.B., J.L., R.R.R., E.J.M., L.H.Y.), Yale University School of Medicine, New Haven, Conn; the Institute of Cell Biology (D.N., R.T., T.W.), Swiss Federal Institute of Technology, ETH-Hoenggerberg, Zurich, Switzerland; the Department of Medicine and Biochemistry (R.L.H., L.A.W.), Dartmouth Medical School, and the Department of Biological Sciences, Dartmouth College, Hanover, NH.

*These authors contributed equally to this work.

Correspondence to Lawrence H. Young, MD, 333 Cedar St, FMP3, New Haven, CT 06520. E-mail lawrence.young@yale.edu

Circulation Research **is available at http://www.circresaha.org** **DOI: 10.1161/01.RES.0000155723.53868.d2**

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 $\alpha_1\beta_1\gamma_1$ rAMPK containing an R70Q mutation in the γ_1 AMP-binding site, wild-type $\alpha_1\beta_1\gamma_1$, or $\alpha_2\beta_1\gamma_1$ 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 to 10 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 $\alpha_1\beta_1\gamma_1$ 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

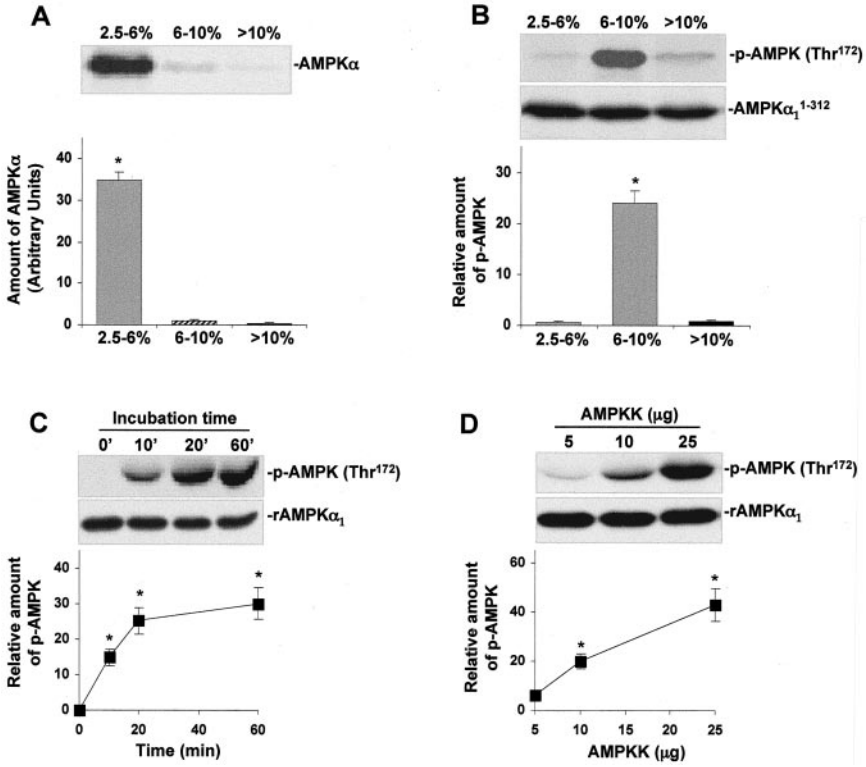

Figure 1. Enrichment of AMPK and AMPK kinase (AMPKK) activity in heart homogenate fractions. A, Ischemic heart homogenates were fractionated using PEG precipitation and immunoblotted with pan-α AMPK antibody (*$P<0.01$ vs both 6% to 10% and >10%). B, PEG fractions (25 μg protein) were incubated with 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 $\alpha_1\beta_1\gamma_1$ rAMPK (*$P<0.01$ vs 0 minutes). D, AMPKK was incubated with 5 pmol $\alpha_1\beta_1\gamma_1$ 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 $\alpha_1\beta_1\gamma_1$ 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 $\alpha_1\beta_1\gamma_1$ 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.

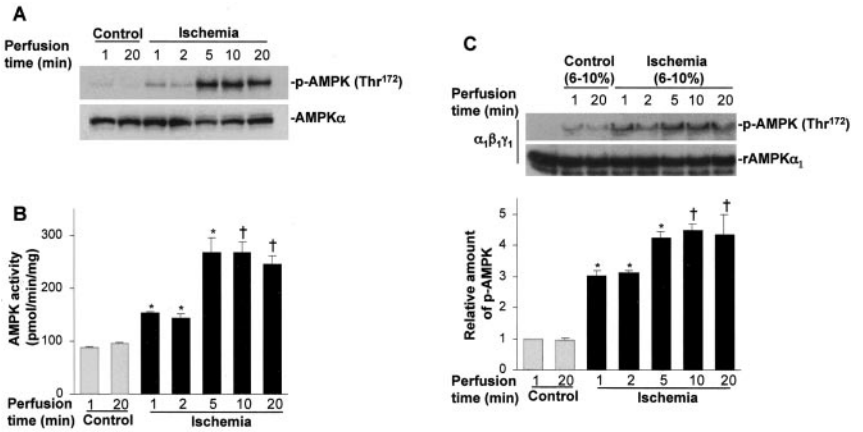

Figure 2. Kinetics of AMPK and AMPKK activation in the in vitro ischemic heart. AMPK and AMPKK activities were assessed in the working heart model after control or low-flow ischemic perfusions. A, Endogenous heart AMPK was immunoblotted with pThr^{172} and pan-α AMPK antibodies. B, Endogenous AMPK activity 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, $\alpha_1\beta_1\gamma_1$ 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.

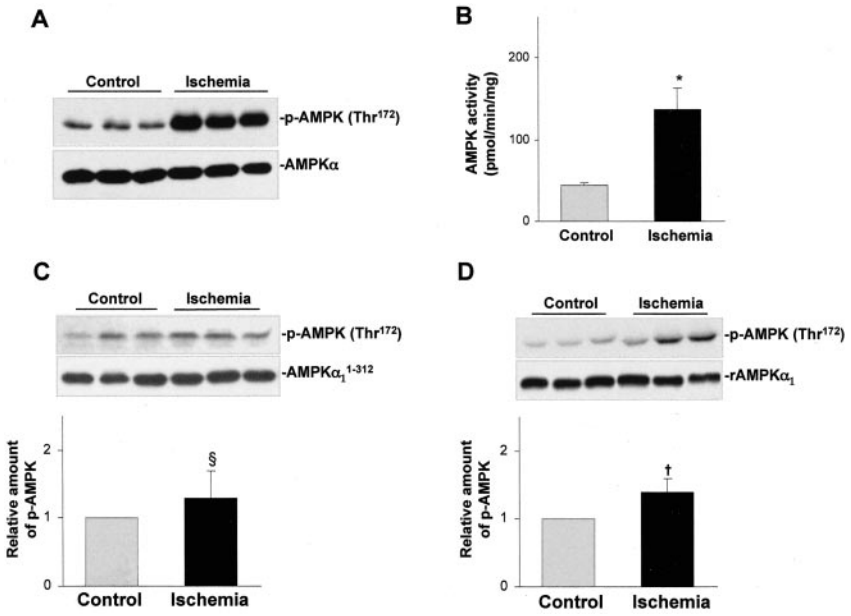

Figure 3. AMPK and AMPKK activation in the in vivo ischemic heart. Heart AMPK and AMPKK activity after sham operation (n=7) or regional ischemia (n=6) for 10 minutes in vivo. A, Endogenous heart AMPK was immunoblotted with pThr^{172} and pan-α AMPK antibodies. B, AMPK activity was measured in 2.5% to 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 $\alpha_1\beta_1\gamma_1$ 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 ($\alpha_1\beta_1\gamma_1$ or $\alpha_2\beta_1\gamma_1$). AMP clearly augmented the ability of ischemic heart AMPKK to activate the $\alpha_1\beta_1\gamma_1$ and $\alpha_2\beta_1\gamma_1$ 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 $\alpha_1\beta_1\gamma_1$ 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 $\alpha_1\beta_1\gamma_1$ 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 $\alpha_1\beta_1\gamma_1$ 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 $\alpha_1\beta_1\gamma_1$ 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

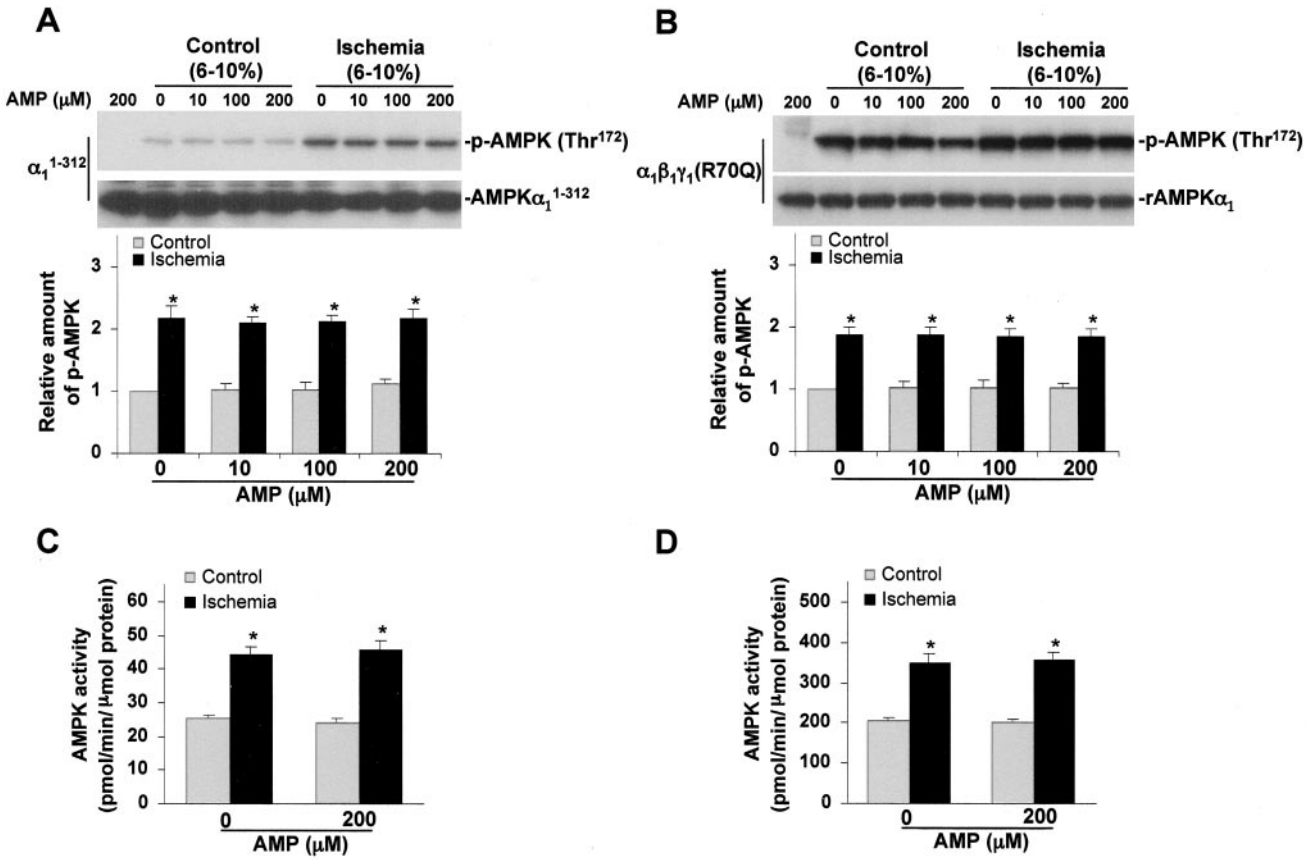

Figure 4. Effect of AMP on heart AMPKK phosphorylation of rAMPK substrates lacking AMP-binding capacity. Heart AMPKK activity after control or low-flow ischemic perfusions was measured as the phosphorylation of $\alpha_1^{1\text{-}312}$ fusion protein (A) or $\alpha_1\beta_1\gamma_1$ rAMPK containing 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

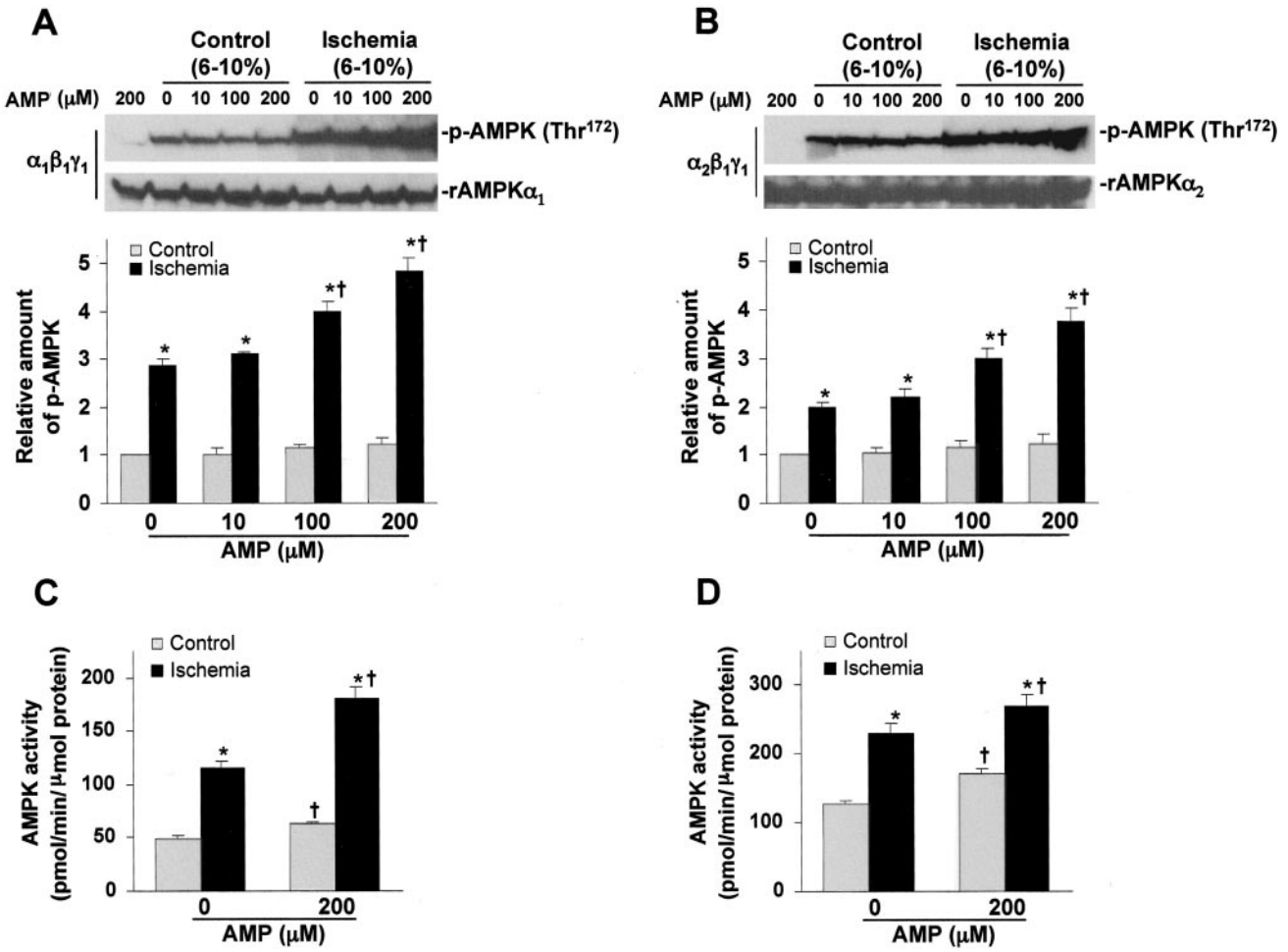

Figure 5. Effects of AMP on the action of heart AMPKK to phosphorylate intact heterotrimeric rAMPK. Heart AMPKK activity after control or low-flow ischemic perfusions was measured as the phosphorylation of $\alpha_1\beta_1\gamma_1$ (A) or $\alpha_2\beta_1\gamma_1$ (B) rAMPK substrates in the absence 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). $\alpha_1\beta_1\gamma_1$ rAMPK (C) or $\alpha_2\beta_1\gamma_1$ 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]

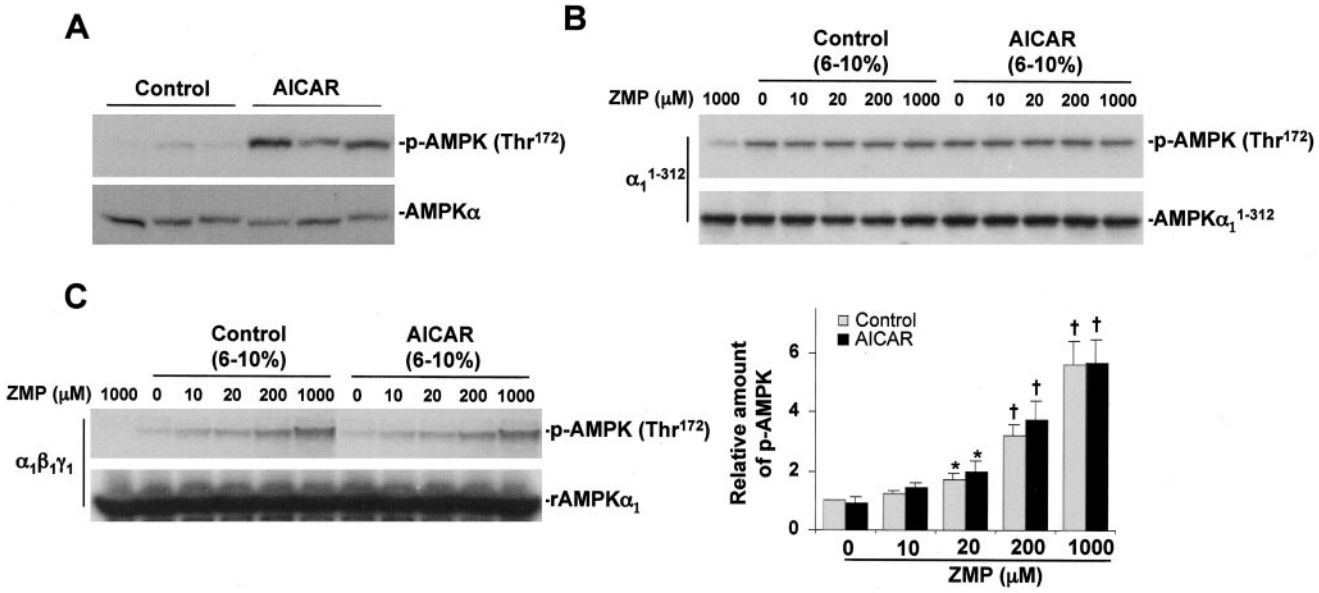

Figure 6. Effects of AICAR infusion in vivo and ZMP in vitro on heart AMPKK action. After AICAR or saline infusions in vivo, heart AMPK phosphorylation and AMPKK activity were assessed. A, Phosphorylated and total endogenous heart AMPK were immunoblotted with pThr^{172} and pan-α AMPK antibodies, respectively. 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). 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.

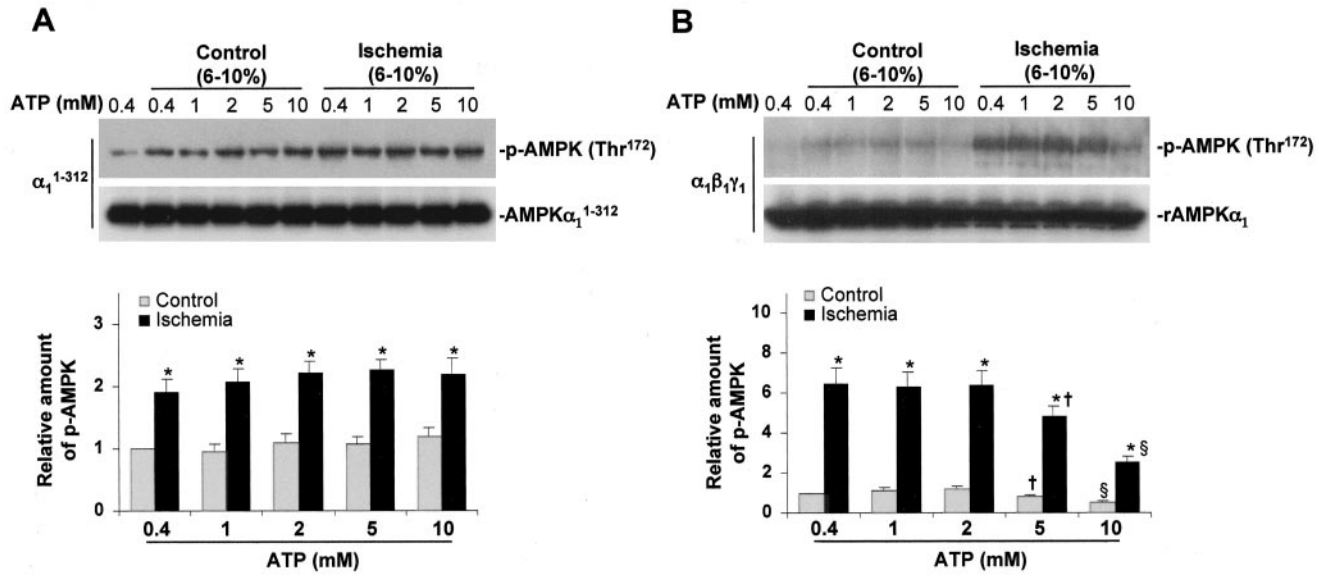

Figure 7. Effect of ATP on the action of heart AMPKK to phosphorylate rAMPK. Heart AMPKK activity after control or low-flow ischemic perfusions was measured as the phosphorylation of $\alpha_1^{1\text{-}312}$ fusion protein (A) or $\alpha_1\beta_1\gamma_1$ rAMPK (B) in the presence of varying concentrations 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.

Acknowledgments

This work was supported by grants from the United States Public Health Service: RO1 HL63811 (L.H.Y.), K08 HL04438 (R.R.R.), T32 HL07950 (E.J.M.); and by the Swiss National Science Foundation: 3100AO-102075/1 (T.W.). This work was presented in part at the 57th Scientific Sessions of the American Heart Association. We thank Monica Palmeri and Richard M. Reznick for expert assistance.

References

1. Kudo N, Barr AJ, Barr RL, Desai S, Lopaschuk GD. High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels due to an increase in 5′-AMP-activated protein kinase inhibition of acetyl-CoA carboxylase. *J Biol Chem*. 1995; 270:17513–17520.
2. Hayashi T, Hirshman MF, Kurth EJ, Winder WW, Goodyear LJ. Evidence for 5′ AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. *Diabetes*. 1998;47:1369–1373.
3. Russell RR, Bergeron R, Shulman GI, Young LH. Translocation of myocardial GLUT4 and increased glucose uptake through activation of AMPK by AICAR. *Am J Physiol*. 1999;277:H643–H649.
4. Marsin AS, Bertrand L, Rider MH, Deprez J, Beauloye C, Vincent MF, Van den Berghe G, Carling D, Hue L. Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. *Curr Biol*. 2000;10:1247–1255.
5. Winder WW, Hardie DG. AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am J Physiol*. 1999;277: E1–E10.
6. Kimura N, Tokunaga C, Dalal S, Richardson C, Yoshino K, Hara K, Kemp BE, Witters LA, Mimura O, Yonezawa K. A possible linkage between AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signalling pathway. *Genes Cells*. 2003;8:65–79.
7. Woods A, Azzout-Marniche D, Foretz M, Stein SC, Lemarchand P, Ferre P, Foufelle F, Carling D. Characterization of the role of AMP-activated protein kinase in the regulation of glucose-activated gene expression using constitutively active and dominant negative forms of the kinase. *Mol Cell Biol*. 2000;20:6704–6711.
8. Zong H, Ren JM, Young LH, Pypaert M, Mu J, Birnbaum MJ, Shulman GI. AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. *PNAS*. 2002;99: 15983–15987.
9. Stein SC, Woods A, Jones NA, Davison MD, Carling D. The regulation of AMP-activated protein kinase by phosphorylation. *Biochem J*. 2000; 345:437–443.
10. Warden SM, Richardson C, O'Donnell J, Jr., Stapleton D, Kemp BE, Witters LA. Post-translational modifications of the beta-1 subunit of AMP-activated protein kinase affect enzyme activity and cellular localization. *Biochem J*. 2001;354:275–283.
11. Carling D, Fryer LG, Woods A, Daniel T, Jarvie SL, Whitrow H. Bypassing the glucose/fatty acid cycle: AMP-activated protein kinase. *Biochem Soc Trans*. 2003;31:1157–1160.
12. Russell RR, 3rd, Li J, Coven DL, Pypaert M, Zechner C, Palmeri M, Giordano FJ, Mu J, Birnbaum MJ, Young LH. AMP-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis, and injury. *J Clin Invest*. 2004;114: 495–503.
13. Coven DL, Hu X, Cong L, Bergeron R, Shulman GI, Hardie DG, Young LH. Physiological role of AMP-activated protein kinase in the heart: graded activation during exercise. *Am J Physiol Endocrinol Metab*. 2003; 285:E629–E636.
14. Salt I, Celler JW, Hawley SA, Prescott A, Woods A, Carling D, Hardie DG. AMP-activated protein kinase: greater AMP dependence, and preferential nuclear localization, of complexes containing the alpha2 isoform. *Biochem J*. 1998;334:177–187.
15. Hayashi T, Hirshman MF, Fujii N, Habinowski SA, Witters LA, Goodyear LJ. Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism. *Diabetes*. 2000;49:527–531.
16. Fryer LGD, Parbu-Patel A, Carling D. The anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct pathways. *J Biol Chem.* 2002;M202489200.
17. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest*. 2001;108:1167–1174.
18. Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Muller C, Carling D, Kahn BB. Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature*. 2002;415:339–343.
19. Hardie DG, Salt IP, Hawley SA, Davies SP. AMP-activated protein kinase: an ultrasensitive system for monitoring cellular energy charge. *Biochem J*. 1999;338:717–722.
20. Frederich M, Balschi JA. The relationship between AMP-activated protein kinase activity and AMP concentration in the isolated perfused rat heart. *J Biol Chem*. 2002;277:1928–1932.
21. Hawley SA, Davison M, Woods A, Davies SP, Beri RK, Carling D, Hardie DG. Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. *J Biol Chem*. 1996;271:27879–27887.
22. Hawley SA, Selbert MA, Goldstein EG, Edelman AM, Carling D, Hardie DG. 5′-AMP activates the AMP-activated protein kinase cascade, and Ca2+/calmodulin activates the calmodulin-dependent protein kinase I cascade, via three independent mechanisms. *J Biol Chem*. 1995;270: 27186–27191.
23. Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Makela TP, Alessi DR, Hardie DG. Complexes between the LKB1 tumor suppressor, STRADalpha/beta and MO25alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *J Biol*. 2003;2:28.
24. Hamilton SR, O'Donnell JB, Jr., Hammet A, Stapleton D, Habinowski SA, Means AR, Kemp BE, Witters LA. AMP-activated protein kinase kinase: detection with recombinant AMPK alpha1 subunit. *Biochem Biophys Res Commun*. 2002;293:892–898.
25. Russell RR, Cline GW, Guthrie PH, Goodwin GW, Shulman GI, Taegtmeyer H. Regulation of exogenous and endogenous glucose metabolism by insulin and acetoacetate in the isolated working rat heart: A three tracer study of glycolysis, glycogen metabolism and glucose oxidation. *J Clin Invest*. 1997;100:2892–2899.
26. Neumann D, Woods A, Carling D, Wallimann T, Schlattner U. Mammalian AMP-activated protein kinase: functional, heterotrimeric complexes by co-expression of subunits in Escherichia coli. *Protein Expr Purif*. 2003;30:230–237.

27. Tian R, Abel ED. Responses of GLUT4-Deficient Hearts to Ischemia Underscore the Importance of Glycolysis. *Circulation*. 2001;103:2961–2966.
28. Henin N, Vincent M, Van den Berghe G. Stimulation of rat liver AMP-activated protein kinase by AMP analogues. *Biochim Biophys Acta*. 1996;1290:197–203.
29. Sakamoto K, Goransson O, Hardie DG, Alessi DR. Activity of LKB1 and AMPK-related kinases in skeletal muscle; effects of contraction, phenformin and AICAR. *Am J Physiol Endocrinol Metab*. 2004;287:310–317.
30. Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, Schlattner U, Wallimann T, Carlson M, Carling D. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr Biol*. 2003;13:2004–2008.
31. Crute BE, Seefeld K, Gamble J, Kemp BE, Witters LA. Functional domains of the alpha1 catalytic subunit of the AMP-activated protein kinase. *J Biol Chem*. 1998;273:35347–35354.
32. Woods A, Vertommen D, Neumann D, Turk R, Bayliss J, Schlattner U, Wallimann T, Carling D, Rider MH. Identification of phosphorylation sites in AMP-activated protein kinase (AMPK) for upstream AMPK kinases and study of their roles by site-directed mutagenesis. *J Biol Chem*. 2003;278:28434–28442.
33. Hardie DG. AMP-activated protein kinase: the guardian of cardiac energy status. *J Clin Invest*. 2004;114:465–468.
34. Polekhina G, Gupta A, Michell BJ, van Denderen B, Murthy S, Feil SC, Jennings IG, Campbell DJ, Witters LA, Parker MW, Kemp BE, Stapleton D. AMPK beta subunit targets metabolic stress sensing to glycogen. *Curr Biol*. 2003;13:867–871.
35. Xing Y, Musi N, Fujii N, Zou L, Luptak I, Hirshman MF, Goodyear LJ, Tian R. Glucose metabolism and energy homeostasis in mouse hearts overexpressing dominant negative alpha2 subunit of AMP-activated protein kinase. *J Biol Chem*. 2003;278:28372–28377.
36. Altarejos JY, Taniguchi M, Clanachan AS, Lopaschuk GD. Myocardial ischemia differentially regulates LKB1 and an alternate 5′AMP-activated protein kinase kinase. *J Biol Chem*. 2005;280:183–190.