Dual Mechanisms Regulating AMP-Activated Protein Kinase Action in the Ischemic Heart

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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 Thr172 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 α₁βγ1 or α₂βγ1 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 α₁, α₁βγ, or α₂βγ rAMPK containing an R70Q mutation in the γ1 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 AMPKK 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;96:000-000.)

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, promotes glucose transport,2,3 accelerates glycolysis,4 and inhibits triglyceride5 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 expression7 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 Thr172 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,1,2 exercise,13 hypoglycemia,14 and hypoxia15 is associated with ATP breakdown and increases in intracellular AMP. However, AMPK is also phosphorylated through AMP-independent pathways during osmotic stress16 and metformin17 or leptin18 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 Thr172-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 AMPKK23 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
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) 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 mM/mL oleate, and 5 mM/0.01 L glucose, and equilibrated with 95% O2/5% CO2 at 37°C. Control hearts were perfused at a preload of 15 cm H2O and an afterload of 100 cm H2O 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 H2O) 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-aminooimidazolecarboxamide (AICAR) (Sigma, St. Louis, Mo), which is converted to the monophosphorylated metabolite ZMP that is an AMP mimetic, was administered intravenously (80 mg/kg bolus and 7.5 mg/kg per minute infusion for 10 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 H2O) for 1 to 20 minutes. Hearts were freeze-clamped in liquid nitrogen and stored at −80°C.

Tissue Fractionation
Heart tissue was homogenized in buffer containing 125 mM/L Tris, 1 mM/L EDTA, 1 mM/L EGTA, 250 mM/L mannitol, 50 mM/L NaF, 5 mM/L NaPi, 1 mM/L DTT, 1 mM/L benzamidine, 0.004% trypsin inhibitor, and 3 mM/L Na3 (pH 7.5). 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% PEG fraction for 70 minutes to chronically caliheterized rats. Control rats received saline infusions. Plasma glucose was maintained constant with a variable infusion of 20% dextrose to prevent hypoglycemia, as previously described. At the end of the infusion, the rat was anesthetized with intravenous pentobarbital (60 mg/kg), and the hearts were rapidly excised and freeze-clamped in liquid nitrogen.

Immunoblotting
Proteins were diluted in Laemmli sample buffer before SDS-PAGE. After transfer to PVDF membranes, proteins were immunoblotted with pan-α (α/α) AMPK antibody at 1:10 000 dilution (kind gift from Dr M. Birnbaum) and anti-pThr172 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 Thr172 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 truncated α1/312 fusion protein (N-terminal maltose binding protein), or 5 pmol α1/γ1 rAMPK containing an R70Q mutation in the γ1 AMP-binding site, wild-type α1/γ1, or α1/γ1 rAMPK. Incubations were performed in 25 μL of AMPKK assay buffer (20 mM/L Tris, 5 mM/L MgCl2, 0.2 mM/L ATP, 0.5 mM/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 μM/L), ATP (400 μM/L to 10 mM/L), and ZMP (0 to 1000 μM/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-pThr172 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-32P]ATP into the SAMS peptide. Endogenous AMPK activity was measured using 10 μg 2.5% to 6% PEG fraction protein prepared from heart homogenates. The activity of α1/312 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 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 Thr172 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 α1/γ1 AMPK 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...
maximal after 5 to 20 minutes. Because AMPK was not present in the AMPKK fraction, there was no detectable endogenous phosphorylated Thr172 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 (<0.01) (Figure 3B). Regional ischemia also stimulated AMPKK activity: phosphorylation of α1γ1 rAMPK increased significantly (<0.05) (Figure 3D), whereas the phosphorylation of the α1γ1 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 α1γ1γ1 fusion protein or α1γ1γ1 rAMPK containing an R70Q mutation in the γ1 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.
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 (αβγ1 or α2γ1). AMP clearly augmented the ability of ischemic heart AMPKK to activate the αβγ1 and α2γ1 rAMPKs (Figure 5). Although AMP had little discernible effect to increase rAMPK Thr172 phosphorylation (Figure 5A and 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 Thr172 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. AICAR is converted to the AMP mimetic compound ZMP in the heart and is known to activate heart AMPK activity in vivo. To further examine the physiological importance of nucleotide interaction with the heterotrimeric AMPK complex, we assessed the mechanisms by which AICAR activates AMPK in the heart. AICAR is converted to the AMP mimetic compound ZMP and is known to activate heart AMPK activity in vivo. AICAR infusion increased heart AMPK Thr172 phosphorylation (Figure 6A) but had no effect on heart AMPKK activity, as assessed in vitro with either the truncated AMPKK (Figure 6B) but it clearly increased the ability of heterotrimeric αβγ1 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; 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 α1-312 fusion protein or heterotrimeric αβγ1 rAMPK (Figure 7). ATP (5 to 10 mmol/L) had no effect on AMPKK-mediated phosphorylation of the αβγ1 rAMPK but did significantly inhibit the ability of AMPKK to phosphorylate αβγ1 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 heart12,20,27 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 Thr172 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 activation in the absence of increased AMPKK activity. Hypoglycemia increased Thr172 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 α1βγ1 rAMPK fusion protein24 and heterotrimeric rAMPKs26 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 AMPKK substrates without normally functional AMP-binding sites (α1βγ1 rAMPK fusion protein and αβγ1γ1 rAMPK R70Q mutation) in the AMPKK assays also enabled us to demonstrate that AMPKK 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 αβγ1 rAMPK, we observed an increase in Thr172 phosphorylation and AMPK activity. These results, taken together with the αβγ1 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 pThr172 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 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 conditions12,27 clearly inhibited AMPKK phosphorylation of rAMPK substrate that contained an intact subunit AMP binding site. However, these same concentrations of ATP had no discernible effect to inhibit AMPKK activity directly, as assessed using the α1 subunit rAMPK (C) or α2 subunit rAMPK (D) were isolated and their activities measured using the SAMS peptide as a substrate. Values are means±SE for 3 independent experiments (*P<0.01 vs control group, †P<0.05 vs 0 μmol/L AMP).

This study focused on AMPKK phosphorylation of the critical α subunit Thr172-activating site. The α subunits contain additional phosphorylation sites, Thr258 and Ser485 (α1)/Ser491 (α2), but they do not appear to be important determinants of AMPK catalytic activity.32 The amino acid sequences surrounding the Thr258 and Ser485 residues are significantly different from those surrounding Thr172 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 Ser108, which may be autophosphorylated by the α subunit.32 Whereas this study provides insight into the ischemic regulation of Thr172 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 heart1 and increases glucose transport by stimulating GLUT4 translocation to the sarcolemma3 and by activating phosphofructokinase-2, which accelerates glycolysis.4 Recent results indicate that transgenic mice, expressing a dominant-negative AMPK catalytic sub-

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 α1β1γ1 (A) or α2β1γ1 (B) rAMPK substrates in the absence or presence of AMP (10 to 200 μmol/L). After incubations with AMPKK, substrates were immunoblotted with pThr172 AMPK and pan α-AMPK antibodies (upper panels) and their relative phosphorylation was quantified in the bar graphs (lower panels). α1β1γ1 rAMPK (C) or α2β1γ1 rAMPK (D) were isolated and their activities measured using the SAMS peptide as a substrate. Values are means±SE for 3 independent experiments (*P<0.01 vs control group, †P<0.05 vs 0 μmol/L AMP).
unit, have impaired ischemic and postischemic glucose uptake. AMPK-deficient hearts demonstrate poor recovery of left ventricular function, increased necrosis, and myocyte apoptosis after low-flow ischemia and reperfusion, 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. 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 Thr172 phosphorylation activity. Further investigation is needed to delineate the role of LKB1, STRAD αβ, and MO25 αβ in modulating AMPKK activity in the heart. However, liver LKB1 does not appear to be AMP-responsive, consistent

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 pThr172 and pan-α AMPK antibodies, respectively. Heart AMPKK activity was measured as the phosphorylation of α11-312 fusion protein (B) or heterotrimeric α1βγ1 rAMPK (C) in the absence or presence of ZMP (0 to 1000 μmol/L). After incubations, AMPKK substrates underwent immunoblotting with pThr172 and panα AMPK antibodies. Values are means±SE for 3 independent experiments (*P<0.05 vs 0 μmol/L ZMP; †P<0.01 vs 0 μmol/L ZMP).

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 α11-312 fusion protein (A) or α11β1 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 pThr172 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).
with our findings that AMP did not directly increase heart AMPKK activity.

Although we found detectable baseline AMPKK activity and endogenous AMPK Thr172 phosphorylation in vivo and in vitro in the heart, AMPKK is clearly not fully activated in the nonischemic heart. The effects of anestesia 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, Altjelos et al have presented evidence in press that AMPKK is activated in the ischemic heart without a measurable increase in AMP concentration or change in LKB1 activity. 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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