

## Myocardial Ischemia Differentially Regulates LKB1 and an Alternate 5'-AMP-activated Protein Kinase Kinase\*

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Judith Y. Altarejos, Masayuki Taniguchi, Alexander S. Clanachan, and Gary D. Lopaschuk‡

From the Cardiovascular Research Group, University of Alberta, Edmonton T6G 2S2, Canada

**During myocardial ischemia, activation of 5'-AMP-activated protein kinase (AMPK) leads to the stimulation of glycolysis and fatty acid oxidation. Together these metabolic changes contribute to cardiac dysfunction. Although AMPK signaling in the ischemic heart is well characterized, the relative contribution of phosphorylation by AMPK kinase (AMPKK), and positive allosterism by the ratios of AMP:ATP and creatine (Cr):phosphocreatine (PCr), in stimulating AMPK during ischemia are unknown. In hearts subjected to severe ischemia, the ratios of AMP:ATP and Cr:PCr were significantly elevated as compared with aerobic hearts. Severe ischemia stimulated AMPK signaling, as demonstrated by an increase in both AMPK activity and acetyl-CoA carboxylase phosphorylation. Although AMPK phosphorylation was increased by severe ischemia, the protein abundance and activity of the recently identified AMPKK, LKB1, were similar between aerobic and severely ischemic hearts. However, in contrast to LKB1, the activity of AMPKK was stimulated in severely ischemic hearts. To further delineate the relative roles of positive allosterism and AMPKK in the regulation of AMPK during ischemia, hearts were subjected to mild ischemia. Although mild ischemia did not alter the ratios of AMP:ATP and Cr:PCr, mild ischemia increased AMPK activity and increased AMPK phosphorylation. Mild ischemia also stimulated the activity of AMPKK. In summary, we demonstrate that myocardial ischemia stimulates AMPK via an AMPKK other than LKB1. Additionally, we show that changes in high energy phosphates are not essential for the activation of AMPK by ischemia. Our data emphasize the critical role AMPKK plays in mediating AMPK signaling during myocardial ischemia.**

During myocardial ischemia, the oxidative metabolism of glucose and fatty acids decreases due to the limited availability of oxygen (1). Consequently, glycolysis initially increases to compensate for the decreased supply of ATP from mitochondrial oxidative metabolism (2). Although oxidative metabolism is decreased during ischemia (1), fatty acid oxidation predominates over glucose oxidation and hence is the major consumer of residual oxygen (3, 4). Importantly, the combined preferential oxidation of fatty acids and elevated rates of glycolysis lead

to an increased production of protons and lactate in the ischemic heart, which contribute to cardiac inefficiency and contractile dysfunction (5).

Several lines of evidence implicate 5'-AMP-activated protein kinase (AMPK)<sup>1</sup> as an important mediator of the metabolic changes in glycolysis and fatty acid oxidation that arise during and following myocardial ischemia. AMPK is rapidly activated during myocardial ischemia (6–8) and remains high during early reperfusion (6). Activation of AMPK promotes glucose uptake (9, 10) by stimulating the translocation of GLUT4 transporters to the sarcolemmal membrane (9). In addition, AMPK indirectly stimulates phosphofructokinase-1 activity by phosphorylating and activating the heart isoform of phosphofructokinase-2 (11), which synthesizes the allosteric activator of phosphofructokinase-1, fructose 2,6-bisphosphate (12). Furthermore, AMPK also stimulates myocardial fatty acid oxidation (6) by phosphorylating and inhibiting acetyl-CoA carboxylase (ACC) (13). The AMPK-mediated inhibition of ACC results in a decreased synthesis of malonyl-CoA (6), a potent inhibitor of mitochondrial fatty acid uptake (14). Thus, activation of AMPK, during and following ischemia, is associated with enhanced glucose uptake (10), increased PFK-2 activity (11), and diminished ACC activity (6, 13). These events culminate in an accelerated rate of glycolysis and the preferential oxidation of fatty acids over glucose.

AMPK is a member of an evolutionarily conserved family of serine/threonine protein kinases that function as sensors of the energy state of the cell (15). The heterotrimeric kinase is composed of a catalytic subunit ( $\alpha$ ) and two regulatory subunits ( $\beta$  and  $\gamma$ ) (16). The catalytic activity of AMPK is increased following association of the  $\beta$  and  $\gamma$  subunits with the  $\alpha$  subunit (17). Additionally, AMPK activity is altered by changes in the cellular levels of high energy phosphates (18). Specifically, AMPK is allosterically activated by increases in the ratios of AMP:ATP (18) or creatine (Cr):phosphocreatine (PCr) (19). Although positive allosterism plays an important role in regulating AMPK (18, 19), it is now evident that it is not essential for AMPK activation (20, 21). Importantly, it has been found that changes in AMPK activity following ischemia, insulin, metformin, or hyperosmotic stress, do not correlate with the ratio of AMP:ATP (8, 20, 21).

The catalytic activity of AMPK is also governed by the reversible phosphorylation of the residue Thr-172 (22, 23), which is situated within the activation loop of the kinase domain of the AMPK  $\alpha$  subunit (22). AMPK kinase (AMPKK) phosphorylates and activates AMPK (22), whereas the protein phosphatases 2A and 2C dephosphorylate and thereby decrease the activity of AMPK (23). Although early studies by Hardie's laboratory demonstrated that AMP enhances AMPKK activity

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‡ An Alberta Heritage Foundation for Medical Research Medical Scientist. To whom correspondence should be addressed: 423 Heritage Medical Research Bldg., The University of Alberta, Edmonton, Alberta T6G 2S2, Canada. Tel.: 780-492-2170; Fax: 780-492-9753; E-mail: gary.lopaschuk@ualberta.ca.

<sup>1</sup> The abbreviations used are: AMPK, 5'-AMP-activated protein kinase; AMPKK, AMPK kinase; ACC, acetyl-CoA carboxylase; Cr, creatine; PCr, phosphocreatine; DTT, dithiothreitol; PEG, polyethylene glycol.

TABLE I  
Severe ischemia increases the AMP:ATP and Cr:PCr ratios

High energy phosphates were measured at the end of 60 min of aerobic perfusion ( $n = 6$ ) or at the end of a 30-min severe ischemic period ( $n = 6$ ). Values represent mean  $\pm$  S.E. Differences were determined using an unpaired Student's  $t$  test.

High energy phosphate metabolism	Aerobic	Severe ischemia
AMP:ATP	0.34 $\pm$ 0.15	2.32 $\pm$ 0.53 <sup>a</sup>
Cr:PCr	4.47 $\pm$ 0.65	10.51 $\pm$ 0.79 <sup>a</sup>

<sup>a</sup> Denotes  $p < 0.05$ .

*in vitro* (18, 24, 25), Hardie's group recently reported that the activity of a more purified AMPKK is not affected by AMP (26). Thus, unlike AMPK, AMPKK is not regulated by AMP.

Recently, the protein kinase LKB1 was identified as an AMPKK (26–28). However, several lines of evidence suggest that other AMPKK isoforms may also exist. First, in *Saccharomyces cerevisiae* three distinct protein kinases have been identified as activators of the AMPK orthologue, Snf1 (27, 29). Second, in LKB1-deficient cells, the activity of AMPK is mildly reduced, whereas the activities of other LKB1-regulated kinases are nearly abolished (30). Additionally, the activity of LKB1 is not affected by stimuli that activate AMPK (28, 31), whereas the activity of AMPKK has been found to increase concurrently with AMPK following exercise (32).

Because AMPK is an important mediator of the metabolic sequelae that arise during and following myocardial ischemia, we determined whether ischemia stimulates AMPK via AMPKK and/or LKB1. In addition, we investigated whether positive allosterism is required for the stimulation of AMPK by ischemia.

#### MATERIALS AND METHODS

**Heart Perfusions**—All of the animals used in this study were cared for in accordance with the guidelines provided by the Canadian Council on Animal Care.

Hearts from male Sprague-Dawley rats (300–350 g) were isolated and perfused as working preparations, as described previously (5, 33). In one series of perfusions, hearts were subjected to either 60 min of aerobic perfusion or 30 min of aerobic perfusion followed by 30 min of global severe ischemia. The left atrial inflow line and the aortic outflow line were clamped to produce a global severe ischemia (5). Isolated working hearts were perfused with a modified Krebs-Henseleit solution that contained 2.5 mM  $\text{Ca}^{2+}$ , 100 microunits/ml insulin, 11 mM glucose, and 1.2 mM palmitate bound to 3% bovine serum albumin (fraction V, Roche Molecular Biochemicals). In a second series of perfusions, hearts were subjected to either 60 min of aerobic perfusion or 30 min of aerobic perfusion followed by 30 min of mild ischemia. The one-way ball valve technique (34) was modified to produce a controlled sustained mild-ischemia. The one-way ball valve was situated in one tract of a bifurcated aortic outflow line and was designed to not impede systolic ejection (34). A backflow controller was placed in the second tract of the aortic outflow line to permit a proportion of ejected perfusate to bypass the one-way ball valve and perfuse the coronary circulation during diastole in a controlled manner. The perfusate was a modified Krebs-Henseleit solution that contained 2.5 mM  $\text{Ca}^{2+}$ , 100 microunits/ml insulin, 5.5 mM D-[5-<sup>3</sup>H]glucose, and 1.2 mM palmitate bound to 3% fatty acid free bovine serum albumin (Sigma). As described previously, rates of glycolysis and palmitate oxidation were determined by measuring the rate of production of <sup>3</sup>H<sub>2</sub>O and <sup>14</sup>CO<sub>2</sub>, respectively (35). At the end of the perfusions, all hearts were quickly frozen with Wollenberger clamps cooled to the temperature of liquid N<sub>2</sub>.

**High Energy Phosphate Measurements**—The tissue contents of AMP, ATP, Cr, and PCr were determined in neutralized perchloric acid extracts of frozen ventricular tissue by high performance liquid chromatography, as described previously (35, 36).

**Tissue Extractions**—Heart tissue was homogenized in a buffer containing 50 mM Tris-HCl (pH 8 at 4 °C), 1 mM EDTA, 10% (w/v) glycerol, 0.02% (v/v) Brij-35, 1 mM dithiothreitol (DTT), protease inhibitors (Sigma), and phosphatase inhibitors (Sigma). The homogenate was centrifuged at 13,000  $\times g$  for 20 min at 4 °C. The resulting supernatant was adjusted to contain 120 mM NaCl and 5% (w/v) PEG6000, mixed for

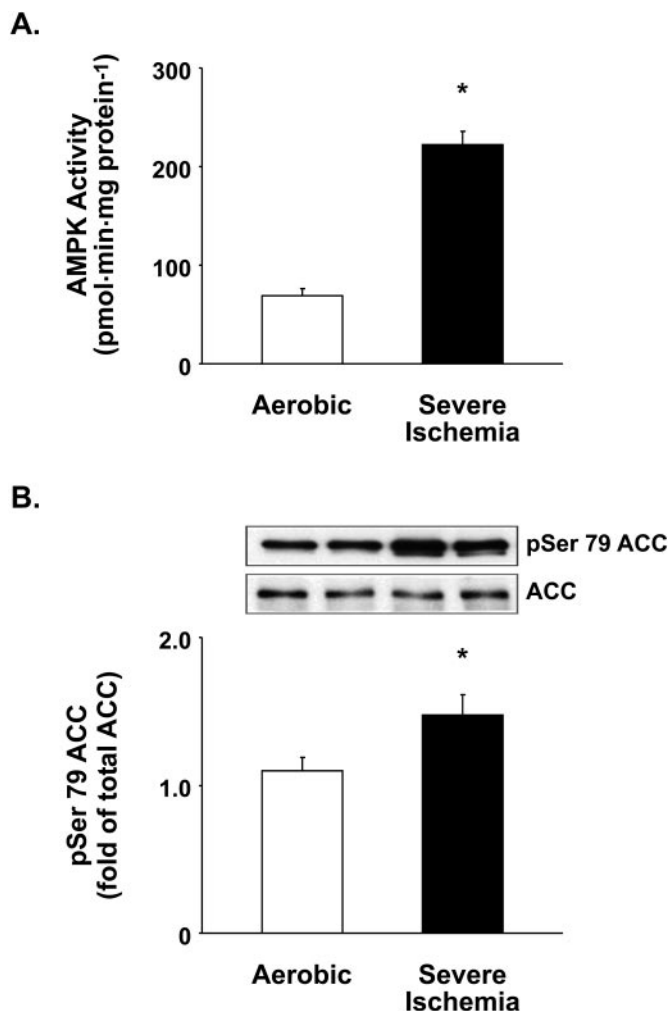


FIG. 1. Severe ischemia stimulates AMPK signaling. **A**, AMPK activity in aerobic ( $n = 8$ ) and severely ischemic ( $n = 9$ ) hearts. **B**, densitometric analysis of the phosphorylation state of Ser-79 within ACC in aerobic ( $n = 10$ ) and severely ischemic hearts ( $n = 10$ ). Representative immunoblots are shown. Values represent the mean  $\pm$  S.E. Differences were determined using an unpaired Student's  $t$  test; \*,  $p < 0.05$ .

1 h, and centrifuged at 10,000  $\times g$  for 10 min. The activities of AMPKK and AMPK were determined from the 5% PEG6000 supernatant and cleared homogenate, respectively. Protein concentrations of the cleared homogenate and 5% PEG6000 supernatant were determined by the method of Bradford (37).

**Immunoblotting**—Equal amounts of cleared homogenate were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were probed first with an anti-AMPK antibody (Cell Signaling Technologies), an anti-phosphoThr-172 AMPK antibody (Cell Signaling Technologies), an anti-phosphoSer-79 ACC antibody (Cell Signaling Technologies), or an anti-LKB1 antibody (Cell Signaling Technologies), and then probed with a horseradish peroxidase-coupled anti-rabbit secondary antibody (Jackson ImmunoResearch). Immunoblots for ACC were conducted using horseradish peroxidase-labeled streptavidin (Kirkegaard and Perry Laboratories). Blots were developed using enhanced chemiluminescence reagent (Amersham Biosciences). Detected immunocomplexes were scanned using a calibrated densitometer (Bio-Rad) and quantified using Quantity One software (Bio-Rad).

**Recombinant Protein Expression and Purification**—The C-terminal truncation mutant of the AMPK  $\alpha 1$  subunit encoding amino acids 1–312 ( $\alpha_{312}$ ) was amplified from the full-length rat  $\alpha_1$  cDNA (a gift from Dr. D. Carling, Imperial College of London) by the polymerase chain reaction. The sense and antisense primers used were: 5'-GTCGTACCATGGC-CGAGAAGCAGAGCAGCA-3' and 5'-CACTAGCTCGAGGTACAG-CGAGCTGAGGA-3', respectively. The amplicon was cloned into pCR2.1-TOPO (Invitrogen) and then subcloned into the bacterial expression vector pET30a using the restriction sites NcoI and XhoI. The

resulting construct ( $\alpha_{312}$ -pET30a) encodes  $\alpha_{312}$  with an S tag and His<sub>6</sub> tag at the N terminus and a His<sub>6</sub> tag at the C terminus.

The *Escherichia coli* strain BL21(DE3) was transformed with the  $\alpha_{312}$ -pET30a construct. Cells were grown to the mid-log phase and were then induced to express  $\alpha_{312}$  by incubating the cells for 3 h at 37 °C in the presence of 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside. Cells were pelleted by centrifugation at 10,000  $\times$  *g* for 10 min at 4 °C. Cells were lysed by freeze thawing and sonication in 50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 8 at 4 °C), 500 mM NaCl, 10% (w/v) glycerol, 0.02% Brij-35, 5 mM  $\beta$ -mercaptoethanol, 100  $\mu$ g/ml lysozyme (Sigma), and protease inhibitors (Sigma). The lysate was cleared by centrifugation at 13,000  $\times$  *g* for 30 min, adjusted to pH 8 at 4 °C, and then filtered through a 0.45- $\mu$ m filter.

Recombinant  $\alpha_{312}$  was purified using a Biologic HR chromatography system (Bio-Rad). All of the purification procedures were conducted at 4 °C. The cleared lysate was loaded onto a Ni<sup>2+</sup> chelating Sepharose column (Amersham Biosciences) that was equilibrated in buffer A (50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 8 at 4 °C), 10% w/v glycerol, 0.02% Brij-35, and 500 mM NaCl). The column was washed with 5 column volumes of buffer A, and  $\alpha_{312}$  was eluted using a step gradient of imidazole. Fractions that contained purified  $\alpha_{312}$  were pooled and exchanged into buffer B (50 mM Tris (pH 8 at 4 °C), 100 mM NaCl, 10% (w/v) glycerol, 0.02% (w/v) Brij-35, and 5 mM  $\beta$ -mercaptoethanol) by dialysis. Purified  $\alpha_{312}$  was concentrated by centrifugation using Amicon Ultra (Millipore) centrifugal filters with a molecular mass cutoff of 10 kDa. Protease inhibitors (Sigma) and 1 mM DTT were added to the purified  $\alpha_{312}$ , which was then stored in aliquots at -80 °C. In this study,  $\alpha_{312}$  was thawed immediately prior to use in the AMPKK assay.

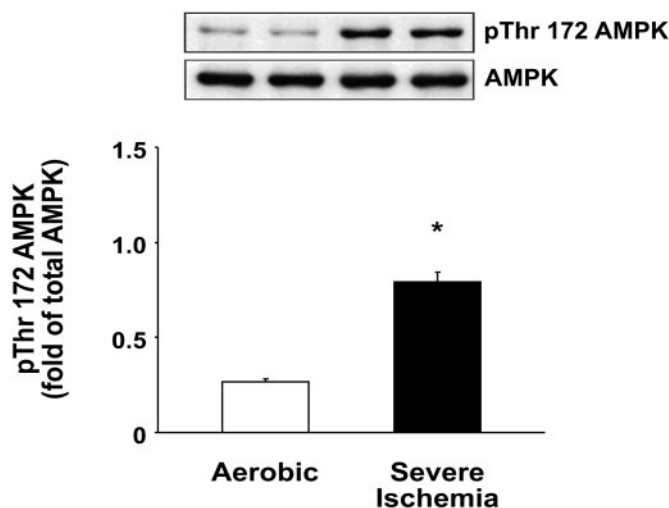
**AMPK Assay**—AMPK activity was assayed in homogenates as described previously (6), except the synthetic AMARA peptide (AMARAASAAALARRR) (38) was used as the substrate.

**AMPKK Assay**—AMPKK activity was determined by measuring the activation of  $\alpha_{312}$ . Kinase buffer A contained 50 mM Tris (pH 8), 80 mM NaCl, 1 mM EDTA, 0.02% (w/v) Brij-35, 1 mM DTT, 100  $\mu$ M ATP, 10 mM MgCl<sub>2</sub>, a mixture of serine/threonine phosphatase inhibitors (Sigma), a tyrosine phosphatase inhibitor mixture (Sigma), a protease inhibitor mixture (Sigma), and 5  $\mu$ g  $\alpha_{312}$ , unless otherwise indicated. Reactions were initiated by the addition of sample (3  $\mu$ g) that was incubated in kinase buffer A with or without  $\alpha_{312}$ , for 30 min or for the times indicated. The reactions were then adjusted to contain 50 mM Tris (pH 8), 80 mM NaCl, 1 mM EDTA, 0.02% (w/v) Brij-35, 1 mM DTT, 200  $\mu$ M ATP, 7.5 mM MgCl<sub>2</sub>, 1  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP, a serine/threonine phosphatase inhibitor mixture (Sigma), a tyrosine phosphatase inhibitor mixture (Sigma), a protease inhibitor mixture (Sigma), and 200  $\mu$ M AMARA peptide. The reactions were further incubated for 10 min at 30 °C and subsequently terminated by the addition of H<sub>3</sub>PO<sub>4</sub> (1% v/v, final concentration). An aliquot of each reaction was spotted into a well of a Unifilter P81 (Whatman) 96-well filterplate. Each well was washed 10 times with 300  $\mu$ l of 1% (v/v) H<sub>3</sub>PO<sub>4</sub> and twice with methanol. The filterplate was air dried, and 50  $\mu$ l of MicroScint PS scintillant (PerkinElmer Life Sciences) was added to each well. Each well of the filterplate was counted in a MicroBeta Trilux (Wallac) scintillation counter.

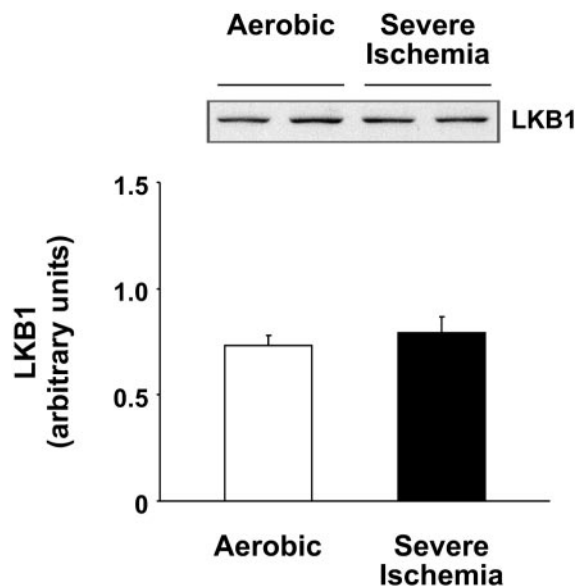
AMPKK activity was also directly determined by measuring the phosphorylation of Thr-172 within  $\alpha_{312}$ . Samples were incubated in kinase buffer A for 60 min, and the reactions were terminated by the addition of SDS-PAGE loading buffer. Reactions were subsequently boiled, separated by SDS-PAGE, and then transferred to polyvinylidene difluoride membranes. Membranes were then immunoblotted for phospho-Thr-172, as described above.

**Immunoprecipitations**—The ability of the goat anti-LKB1 antibody (M-18; Santa Cruz Biotechnology) to immunoprecipitate LKB1 was tested by incubating 0, 150, or 300  $\mu$ g of cleared homogenate with 1  $\mu$ g of antibody, which was prebound to the wells of a protein A/G-coated eight-well strip (Pierce). The strips were incubated with gentle shaking for 4 h at 4 °C. Wells were then washed four times with a buffer containing 50 mM Tris-HCl (pH 8 at 4 °C), 1 mM EDTA, 10% (w/v) glycerol, 0.02% (v/v) Brij-35, 150 mM NaCl, protease inhibitors (Sigma), and phosphatase inhibitors (Sigma). After the last wash, the immunoprecipitate and supernatant were resuspended in protein sample buffer lacking  $\beta$ -mercaptoethanol. Samples were boiled, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Membranes were probed first with a rabbit anti-LKB1 antibody (Cell Signaling Technologies) and then probed with a horseradish peroxidase-coupled donkey anti-rabbit IgG antibody that was preabsorbed to goat IgG (Jackson ImmunoResearch). Immunocomplexes were detected as described above.

All subsequent immunoprecipitations were conducted, as described above, except LKB1 was immunoprecipitated from 0 or 150  $\mu$ g of



**FIG. 2. Severe ischemia stimulates phosphorylation of AMPK.** Densitometric analysis of the phosphorylation state of Thr-172 within AMPK in aerobic ( $n = 10$ ) and severely ischemic hearts ( $n = 12$ ). Representative immunoblots are shown.



**FIG. 3. LKB1 protein levels are not altered by severe ischemia.** Representative immunoblot and densitometric analyses of LKB1 protein levels in aerobic ( $n = 8$ ) and severely ischemic hearts ( $n = 10$ ). Values represent the mean  $\pm$  S.E.

cleared homogenate and 1 mM DTT was included in the wash buffer. In addition, after the last wash the immunoprecipitate was immediately assayed for AMPKK activity by the aforementioned methods.

## RESULTS

**Cardiac Function and High Energy Phosphates in Severely Ischemic Hearts**—Hearts were perfused either for 60 min aerobically or for 30 min aerobically followed by 30 min of severe ischemia. As expected, cardiac work ceased in hearts subjected to severe ischemia ( $62 \pm 4$  and  $0$  mm Hg $\cdot$ ml $\cdot$ min<sup>-1</sup> $\cdot$ 10<sup>-2</sup>, in aerobic and ischemic hearts, respectively). In addition to a depression in cardiac function, 30 min of severe ischemia caused a perturbation in the cellular levels of high energy phosphates that reflected an increase in the ratios of AMP:ATP and Cr:PCr (Table I).

**AMPK Activity and ACC Phosphorylation Following Severe Ischemia**—Accordant with previous findings (6, 11), AMPK activity was markedly stimulated in severely ischemic hearts compared with aerobic hearts (Fig. 1A). During severe ischemia

## A.

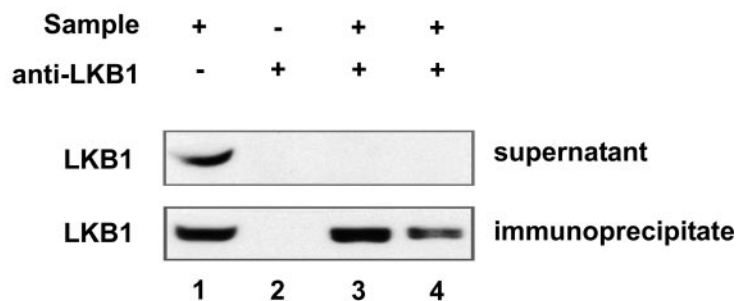
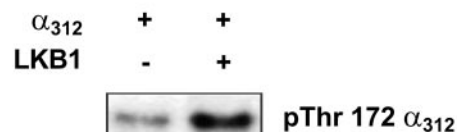
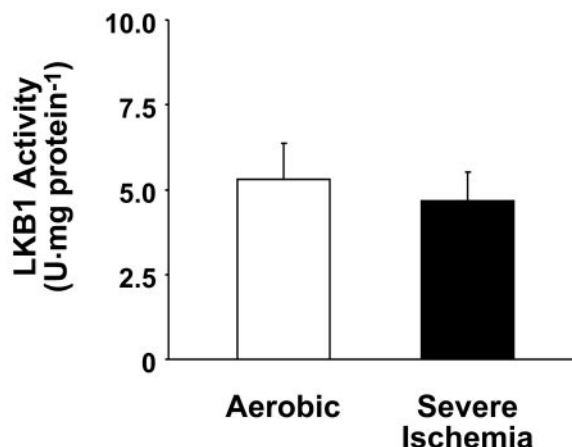


FIG. 4. LKB1 is not the myocardial AMPKK activated by ischemia. A, LKB1 immunoblot of supernatants and precipitates following immunoprecipitation of LKB1. Lane 1 is a non-immunoprecipitated sample. Lanes 2–4 are immunoprecipitates and supernatants obtained from 0, 300, and 150  $\mu$ g of homogenate, respectively. B, immunoblot for phospho-Thr-172 AMPK following a kinase assay of immunoprecipitated LKB1. Immunoprecipitated LKB1 was incubated with recombinant  $\alpha_{312}$  for 60 min. C, kinase assay of immunoprecipitated LKB1 from aerobic ( $n = 5$ ) and severely ischemic ( $n = 5$ ) hearts. Immunoprecipitated LKB1 was incubated with  $\alpha_{312}$  for 60 min prior to determining the activity of  $\alpha_{312}$  as described under “Materials and Methods.” Values represent the mean  $\pm$  S.E. Differences were determined using an unpaired Student’s *t* test; \*,  $p < 0.05$ .

## B.



## C.



it is not possible to measure the rate of fatty acid oxidation as a downstream marker of AMPK activity; thus, we determined whether the increased activity of AMPK was associated with an increase in ACC phosphorylation. As expected, a significant increase in the phosphorylation of ACC at Ser-79 was observed in severely ischemic hearts as compared with aerobic hearts (Fig. 1B). The total abundance of ACC protein was similar between aerobic and severely ischemic hearts ( $0.80 \pm 0.03$  and  $0.89 \pm 0.05$  arbitrary units, respectively).

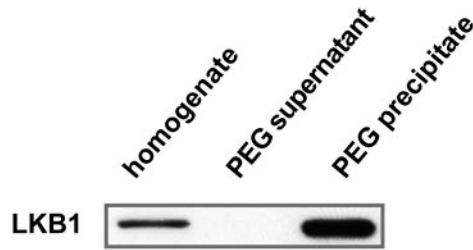
**Phosphorylation State of AMPK Thr-172 following Severe Ischemia**—An important mechanism that governs the catalytic activity of AMPK is the phosphorylation of the key residue, Thr-172 (23). Accordingly, the stimulation of AMPK activity following no-flow ischemia was paralleled by an increase in the phosphorylation state of Thr-172 (Fig. 2). The total amount of AMPK did not differ between aerobic and severely ischemic hearts ( $1.20 \pm 0.05$  and  $1.14 \pm 0.04$  arbitrary units, respectively).

**Effect of Ischemia on LKB1 Protein Abundance and Activity**—Because previous studies have implicated LKB1 as an important AMPKK (26–28), and it has been demonstrated that endurance training, which stimulates AMPK activity (39, 40), results in increased LKB1 protein abundance in skeletal muscle (41), we examined whether LKB1 protein abundance and/or activity is enhanced by severe ischemia. As depicted in Fig. 3,

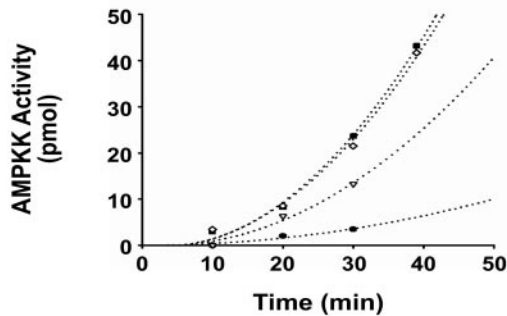
LKB1 protein abundance was similar between aerobic and severely ischemic hearts. LKB1 was efficiently immunoprecipitated from 0, 300, or 150  $\mu$ g of homogenate and was not detected in the supernatant (Fig. 4A). Consistent with the results of others (26–28), immunoprecipitated LKB1 phosphorylated  $\alpha_{312}$  at Thr-172 and also activated  $\alpha_{312}$  (Fig. 4, B and C, respectively). Interestingly, the activity of LKB1 was not different between aerobic and severely ischemic hearts (Fig. 4C).

**AMPKK Is Activated in Severely Ischemic Hearts**—Neither the abundance of LKB1 nor the activity of LKB1 was stimulated by severe ischemia, we further investigated the mechanism responsible for the increased phosphorylation state of Thr-172 within AMPK. We hypothesized that other AMPKK(s) may regulate AMPK phosphorylation. To determine whether an additional AMPKK is present in the heart, LKB1 was precipitated with 5% PEG6000, and the 5% PEG supernatant was assayed for AMPKK activity. As shown in Fig. 5A, LKB1 was precipitated by 5% PEG and was not present in the 5% PEG supernatant. Although the 5% PEG supernatant was depleted of LKB1, a significant amount of AMPKK activity remained in the LKB1-depleted supernatant (Fig. 5B). Moreover, the *in vitro* activity of AMPKK increased in a time-dependant manner when assayed with various concentrations of the substrate,  $\alpha_{312}$  (Fig. 5B).

A.



B.



C.

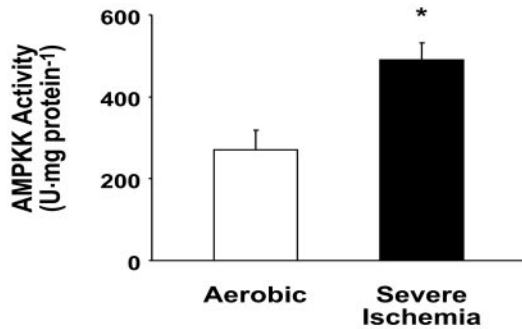


FIG. 5. AMPKK activity is stimulated by severe ischemia. A, immunoblot for LKB1 in homogenate, 5% PEG supernatant, and 5% PEG precipitate. B, AMPKK assay progress curve. The activity of AMPKK was determined during a 10, 20, 30, or 39 min incubation of 3  $\mu$ g of 5% PEG6000 supernatant with the following amounts of  $\alpha_{312}$ : 1  $\mu$ g (filled circles), 2  $\mu$ g (open triangles), 5  $\mu$ g (filled squares), or 10  $\mu$ g (open diamonds). C, AMPKK activity in aerobic ( $n = 9$ ) and severely ischemic hearts ( $n = 9$ ). Values represent the mean  $\pm$  S.E. Differences were determined using an unpaired Student's  $t$  test; \*,  $p < 0.05$ .

Having established that LKB1 is not activated by severe ischemia and that there is an AMPKK in LKB1-depleted samples, the activity of this AMPKK was determined in aerobic and severely ischemic hearts. Notably, it was found that the activity of AMPKK was 1.8-fold greater in severely ischemic hearts than in aerobic hearts (Fig. 5C). Importantly, these data provide the first evidence that ischemia stimulates an LKB1-distinct myocardial AMPKK and demonstrates that this AMPKK is coordinately regulated with AMPK in the heart.

**Cardiac Function and Levels of High Energy Phosphates following Mild Ischemia**—Thus far, AMPK activation has primarily been studied in models of severe myocardial ischemia where dramatic changes in the AMP:ATP and Cr:PCr ratios occur (8). We attempted to distinguish between the regulatory roles of allosterism and phosphorylation by studying the regulation of AMPK in a model of mild ischemia. A series of hearts

TABLE II

Mild ischemia decreases cardiac function but not the AMP:ATP or the Cr:PCr ratios

High energy phosphates were measured at the end of 30 min of aerobic perfusion ( $n = 8$ ) or at the end of the a 30-min mild ischemic period ( $n = 10$ ). Values represent mean  $\pm$  S.E. Differences were determined using an unpaired Student's  $t$  test.

High energy phosphate metabolism	Aerobic	Mild ischemia
AMP:ATP	0.15 $\pm$ 0.05	0.13 $\pm$ 0.01
Cr:PCr	2.23 $\pm$ 0.42	1.98 $\pm$ 0.25

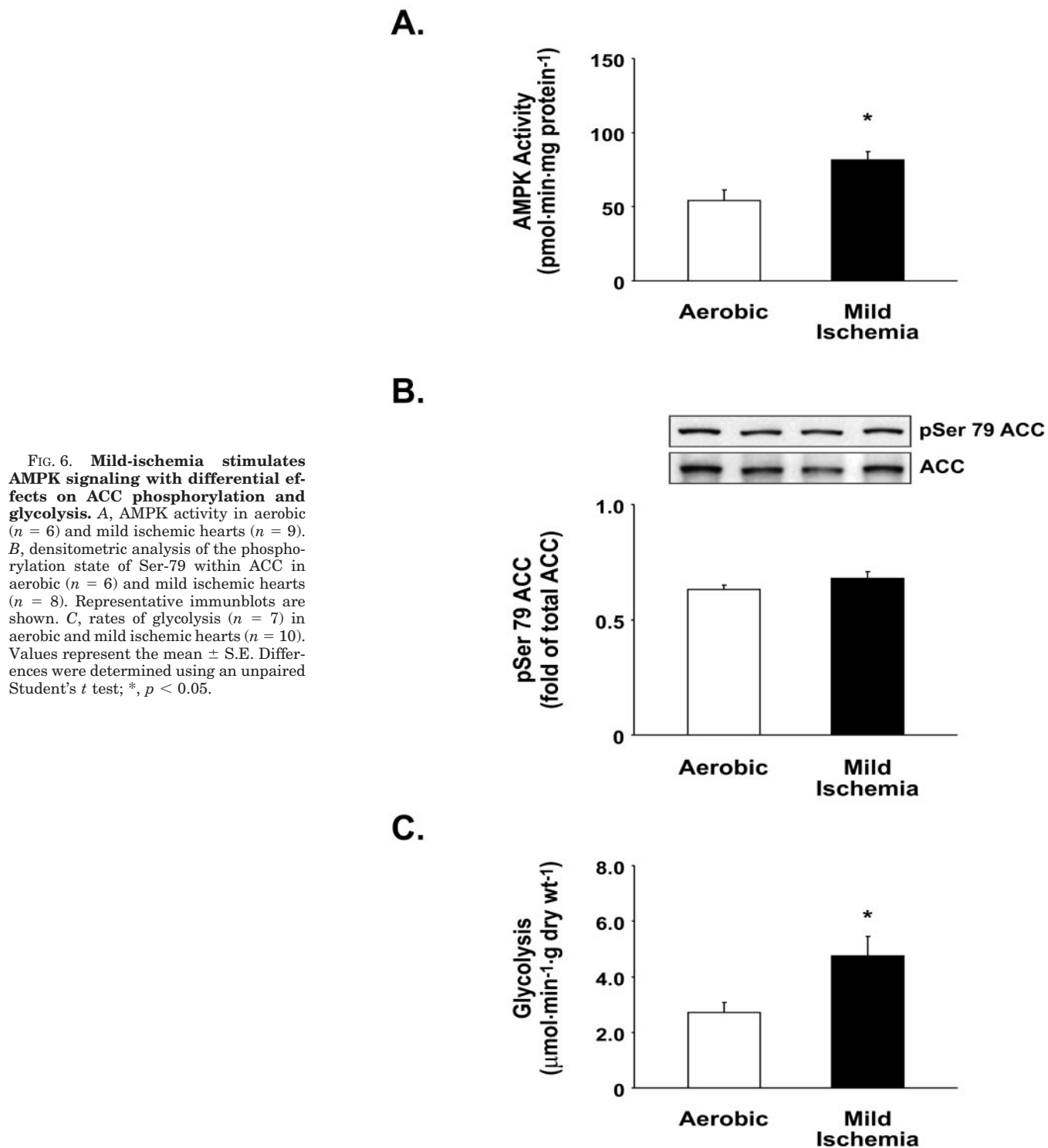
was subjected to either 60 min of aerobic perfusion or 30 min of aerobic perfusion followed by 30 min of mild ischemia. In this model of ischemia, coronary flow was reduced by 39% compared with aerobic hearts (23  $\pm$  2 and 14  $\pm$  0.4 ml $\cdot$ min<sup>-1</sup> in aerobic and mild ischemic hearts, respectively). The reduction in coronary flow resulted in a 53% decrease in cardiac work compared with aerobic hearts (73  $\pm$  8 and 34  $\pm$  4 mm Hg $\cdot$ ml $\cdot$ min<sup>-1</sup> $\cdot$ 10<sup>-2</sup> in aerobic and mild ischemic hearts, respectively). The decreases in cardiac work and coronary flow remained constant throughout the entire 30 min of ischemia (data not shown). Despite the impairments in coronary flow and cardiac work, the ratios of AMP:ATP and Cr:PCr were similar between aerobic and mildly ischemic hearts (Table II).

**Effect of Mild Ischemia on AMPK Activity, ACC Phosphorylation, and Metabolism**—Although the ratios of AMP:ATP and Cr:PCr were similar between aerobic and mildly ischemic hearts, AMPK activity was significantly elevated in hearts subjected to mild ischemia, as compared with aerobically-perfused hearts (Fig. 6A). Although the activity of AMPK was elevated in mildly ischemic hearts, this did not translate to an increase in the phosphorylation of ACC at Ser-79 (Fig. 6B). However, a significant increase in glycolytic rates was evident in hearts subjected to mild ischemia (Fig. 6C).

**Alterations in AMPK Phosphorylation and AMPKK Activity following Mild Ischemia**—Because the activation of AMPK following mild ischemia occurred in the absence of detectable changes in the ratios of AMP:ATP or Cr:PCr, we addressed whether the activation of AMPK was due to an increase in the phosphorylation state of Thr-172 and to an increase in AMPKK activity. Immunoblot analysis revealed that, although the total abundance of AMPK was similar between aerobic and mildly ischemic hearts (0.95  $\pm$  0.05 and 0.92  $\pm$  0.02 arbitrary units, respectively), the phosphorylation state of Thr-172 was significantly increased in hearts exposed to mild ischemia (Fig. 7A). In parallel, the activity of AMPKK was also elevated in hearts subjected to mild ischemia when compared with hearts perfused aerobically (Fig. 7B). Thus, mild ischemia stimulates AMPKK independently of changes in the AMP:ATP or Cr:PCr ratios. Increased AMPKK activity is the primary mechanism by which mild ischemia stimulates AMPK.

## DISCUSSION

Although it is evident that myocardial AMPK activity, Thr-172 phosphorylation, and the ratios of AMP:ATP and Cr:PCr are increased during severe ischemia (6–8,10), the role of LKB1 and/or AMPKK in modifying AMPK activity during ischemia has not been previously addressed. Importantly, the present study demonstrates that neither LKB1 protein levels nor LKB1 activity are stimulated in the ischemic heart. We show for the first time that the activity of an AMPKK, distinct from LKB1, is stimulated in conjunction with an increase in AMPK activity, Thr-172 phosphorylation, and changes in cellular high energy phosphate content. Additionally, utilizing a model of mild ischemia, we demonstrate an important regulatory role of AMPKK on AMPK activity.



**FIG. 6. Mild-ischemia stimulates AMPK signaling with differential effects on ACC phosphorylation and glycolysis.** A, AMPK activity in aerobic ( $n = 6$ ) and mild ischemic hearts ( $n = 9$ ). B, densitometric analysis of the phosphorylation state of Ser-79 within ACC in aerobic ( $n = 6$ ) and mild ischemic hearts ( $n = 8$ ). Representative immunoblots are shown. C, rates of glycolysis ( $n = 7$ ) in aerobic and mild ischemic hearts ( $n = 10$ ). Values represent the mean  $\pm$  S.E. Differences were determined using an unpaired Student's  $t$  test; \*,  $p < 0.05$ .

Recently, the Ser/Thr protein kinase LKB1 was identified as an AMPKK that is not coordinately stimulated with AMPK (28). LKB1 was found to co-purify with liver AMPKK and phosphorylate recombinant AMPK complexes (26, 28). Strikingly, the activity of LKB1 was not increased by stimuli that increased AMPK activity (28, 30, 31). In the present study, we also found that LKB1 was not activated with AMPK in severely ischemic hearts. Although immunoprecipitated LKB1 phosphorylated and activated recombinant  $\alpha_{312}$ , the activity of myocardial LKB1 was not increased by severe ischemia. Moreover, LKB1 protein abundance was not altered by ischemia. We further show that in LKB1-depleted samples, the activity of

AMPKK is stimulated by severe ischemia. Taken together, these data demonstrate that the activities of myocardial LKB1 and AMPKK are differentially regulated by ischemia. This suggests that an additional AMPKK, distinct from LKB1, is present in the heart. Unlike LKB1, this AMPKK is coordinately stimulated with AMPK by ischemia. The existence of multiple mammalian AMPKKs is not without precedence, because three AMPKK orthologues have been identified in *S. cerevisiae* (27, 29). Because the AMPKK characterized in this study plays an important role in the regulation of AMPK and hence metabolism, both during and following ischemia, we are currently determining the identity of this kinase.

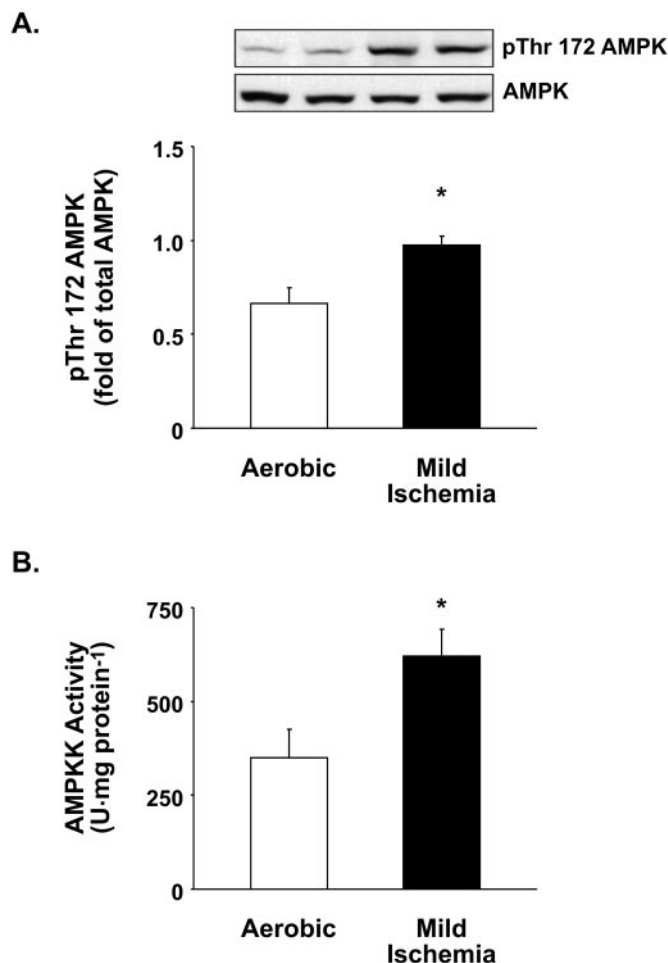


FIG. 7. Mild ischemia stimulates phosphorylation of AMPK via AMPKK. A, densitometric analysis of the phosphorylation state of Thr-172 within AMPK in aerobic ( $n = 6$ ) and mild ischemic hearts ( $n = 8$ ). Representative immunoblots are shown. B, AMPKK activity in aerobic ( $n = 7$ ) and mild ischemic hearts ( $n = 8$ ). Values represent the mean  $\pm$  S.E. Differences were determined using an unpaired Student's  $t$  test; \*,  $p < 0.05$ .

Perfusion of hearts under mild ischemic conditions, where coronary flow was reduced by 39%, resulted in decreased cardiac work and increased rates of glycolysis. Although such changes typically occur during myocardial ischemia, levels of high energy phosphates may or may not be altered, depending on the severity of ischemia (42). In the model employed in this study, the ratios of AMP:ATP and Cr:PCr were unchanged following mild ischemia, yet a marked enhancement of AMPK activity and Thr-172 phosphorylation was evident. Furthermore, the activation of AMPK by mild ischemia was accompanied by an increase in AMPKK activity. Our data suggest that mild ischemia stimulates AMPKK, and subsequently AMPK, through a high energy phosphate-independent mechanism.

Although the classic model for activation of the AMPK "cascade" is centered on the role of the positive allosteric effects of the ratios of AMP:ATP (43) and Cr:PCr (19), a study by Beauloye *et al.* (8) established precedence for an AMP-independent regulation of AMPK activity in the heart. In other cell types, it has also been shown that AMPK is regulated by various stimuli, which do not alter the AMP:ATP ratio (20, 21, 44). Our data provide additional evidence that increases in the ratios of AMP:ATP and Cr:PCr are not essential for the activation of myocardial AMPK and AMPKK during ischemia. Rather, we find that myocardial AMPK is primarily regulated via phosphorylation of Thr-172 by AMPKK.

Interestingly, AMPK was activated to a greater degree following severe ischemia than mild ischemia. Despite the differential activation of AMPK, AMPKK was stimulated to a similar extent (1.8-fold). Although it is possible that AMPKK is further stimulated by positive allosterism during severe ischemia, this effect may not have been detected by the *in vitro* assay for AMPKK. Alternatively, positive allosterism may further enhance the activity of phosphorylated AMPK during severe ischemia.

Elevated ratios of AMP:ATP and Cr:PCr may also be necessary to facilitate AMPK-mediated phosphorylation of ACC. Even though AMPK was activated with mild and severe ischemia, ACC Ser-79 phosphorylation was increased only with severe ischemia. Alternatively, it is possible that a threshold stimulation of AMPK must be achieved to confer signaling to ACC or that other protein kinases participate in ACC phosphorylation.

Altered subcellular localization of AMPK may also positively affect the activity of AMPK and/or confer signaling specificity. Specifically, although the  $\beta$  subunit was initially shown to function as a scaffold that binds the  $\alpha$  and  $\gamma$  subunits (45), recent studies suggest the  $\beta$  subunit additionally regulates the localization of AMPK (46–48). The two isoforms of the  $\beta$ -subunit contain a glycogen-binding domain that, as its name implies, facilitates its binding to glycogen (47, 48). Intriguingly, the catalytic activity of AMPK is not affected by glycogen binding (48). Thus, differences in the degree of AMPK activation and ACC phosphorylation found between severe ischemia and mild ischemia may be explained by an increase in the proportion of AMPK that is not sequestered by glycogen. Although glycogen turnover was not assessed in this study, rates of glycogenolysis have been shown to differ with ischemic severity (49).

In summary, the present study demonstrates that increased AMPKK activity is the primary mechanism responsible for the stimulation of AMPK during ischemia. In addition, we reveal that global changes in high energy phosphate homeostasis are not necessary for the activation of AMPKK and thus AMPK, but these changes may additionally activate phosphorylated AMPK. Although the results of this study confirm that LKB1 phosphorylates and activates AMPK, we demonstrate that ischemia stimulates an alternate AMPKK, which activates AMPK and thereby mediates changes in metabolism that contribute to cardiac dysfunction.

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