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Deficiency of LKB1 in heart prevents ischemia-mediated activation of AMPKα2 but not AMPKα1

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Abstract

Recent studies indicate that the LKB1 is a key regulator of the AMP-activated protein kinase (AMPK), which plays a crucial role in protecting cardiac muscle from damage during ischemia. We have employed mice that lack LKB1 in cardiac and skeletal muscle and studied how this affected the activity of cardiac AMPKα1/α2 under normoxic, ischemic, and anoxic conditions. In the heart lacking cardiac muscle LKB1, the basal activity of AMPKα2 was vastly reduced and not increased by ischemia or anoxia. Phosphorylation of AMPKα2 at the site of LKB1 phosphorylation (Thr^{172}) or phosphorylation of acetyl-CoA carboxylase-2, a downstream substrate of AMPK, was ablated in ischemic heart lacking cardiac LKB1. Ischemia was found to increase the ADP-to-ATP (ADP/ATP) and AMP-to-ATP ratios (AMP/ATP) to a greater extent in LKB1-deficient cardiac muscle than in LKB1-expressing muscle. In contrast to AMPKα2, significant basal activity of AMPKα1 was observed in the lysates from the hearts lacking cardiac muscle LKB1, as well as in cardiomyocytes that had been isolated from these hearts. In the heart lacking cardiac LKB1, ischemia or anoxia induced a marked activation and phosphorylation of AMPKα1, to a level that was only moderately lower than observed in LKB1-expressing heart. Echocardiographic and morphological analysis of the cardiac LKB1-deficient hearts indicated that these hearts were not overtly dysfunctional, despite possessing a reduced weight and enlarged atria. These findings indicate that LKB1 plays a crucial role in regulating AMPKα2 activation and acetyl-CoA carboxylase-2 phosphorylation and also regulating cellular energy levels in response to ischemia. They also provide genetic evidence that an alternative upstream kinase can activate AMPKα1 in cardiac muscle.

Keywords

cellular energy metabolism; hypoxia; cardiovascular physiology; AMP-activated protein kinase

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The AMP-activated protein kinase (AMPK) is switched on by increases in levels of AMP, resulting from reduced availability of ATP. AMPK functions to restore ATP concentrations by stimulating energy-producing processes, such as nutrient uptake and oxidation of fatty acids, and inhibiting unnecessary energy-consuming processes, such as protein synthesis and cell proliferation (reviewed in Refs. 8,11). AMPK is a heterotrimeric complex comprising a catalytic α-subunit and regulatory β- and γ-subunits. AMP activates the AMPK complex by binding to the Bateman domains made up of pairs of CBS sequences located on the γ-subunit and by stimulating the phosphorylation of Thr172 in the T-loop of both mammalian AMPKα catalytic subunits, termed AMPKα1 and AMPKα2.

There has been much interest in the mechanism by which AMPK is regulated and the identities of the upstream protein kinase(s) that phosphorylate Thr^{172}. Elegant studies performed in *Saccharomyces cerevisiae* (14,15,21,29) indicated that enzymes homologous to the mammalian LKB1 tumor suppressor kinase and calmodulin-dependent protein kinase kinase (CAMKK) would mediate the activation of the yeast homolog of AMPK. This prompted studies in the mammalian system that resulted in the finding that LKB1 phosphorylated AMPK at Thr^{172} in vitro and that, in LKB1-deficient cell lines, AMPK could not be activated by a variety of agonists and stresses (12,27,33). More recently, muscle contraction and other agonists were unable to activate AMPKα2 in mouse skeletal muscle lacking the expression of LKB1 (26), and AMPK phosphorylation at Thr^{172} was markedly diminished in mouse liver deficient in LKB1 (28). Although these studies support the notion that LKB1 is a regulator of AMPK, the finding that AMPK possessed significant basal activity and phosphorylation at Thr^{172} in LKB1-deficient cells (12,27) suggested that there were alternative regulators. Recent studies revealed that CAMKK isoforms are likely to also phosphorylate AMPK at Thr^{172}, based on the finding that the CAMKK inhibitor, STO-609, as well as short interfering (si)RNA-mediated knockdown of CAMKK isoforms, inhibited the basal AMPK activity in LKB1-deficient cell lines, as well as the activation of AMPK that is observed in response to agents that elevate cellular Ca^{2+} levels (13,16,32). CAMKK isoforms are highly expressed in neuronal tissue, and K^+-induced depolarization of rat cerebrocortical slices, which increases Ca^{2+} without affecting ATP levels, was observed to activate AMPK in a manner that was inhibited by STO-609. This study suggested that CAMKK rather than LKB1 controls AMPK in Ca^{2+}-regulated pathways, at least in neuronal tissues. Although expression of CAMKK isoforms was detected in tissues, including testis, spleen, and heart at low levels (3), whether CAMKKs function to activate AMPK in these tissues is unknown.

AMPK plays a key role in regulating lipid and glucose metabolism in cardiac muscle, where it is activated when oxygen and/or blood supply is compromised during hypoxic and/or ischemic conditions. Activation of AMPK in cardiac muscle stimulates fatty acid oxidation (18), glucose uptake (23), and glycolysis (20), to generate ATP and thereby protect cardiac tissues during and following ischemic or hypoxic stress. Mice that have reduced AMPK activity in cardiac muscle caused by the overexpression of a dominant-negative form of AMPK are more susceptible to cardiac damage during ischemia and reperfusion experiments (24). Although LKB1 appears to be a major regulator of AMPKα2 in skeletal muscle (26), the identity of the upstream kinase(s) that regulates AMPK in the cardiac muscle is less certain. Previous studies have suggested that at least two separate activities that phosphorylate and activate AMPK could be resolved from heart extracts, and the activity of one of these was reportedly stimulated by ischemia (2,4). Although the identity of this ischemia-stimulated enzyme is unknown, immunoprecipitation studies indicated that it was not LKB1 (2). In this study, we employed mice that were deficient in cardiac and skeletal muscle LKB1, to define the role that cardiac muscle LKB1 plays in regulating the activity of AMPK isoforms, as well as cellular energy levels, in the heart under normoxic, no-flow ischemic, and anoxic conditions.

EXPERIMENTAL PROCEDURES

Materials

Protease inhibitor cocktail tablets were obtained from Roche (no. 1697498, Lewes, Sussex, UK), protein G-Sepharose, and [γ-^{32}P]ATP were purchased from Amersham Biosciences (Little Chalfont, UK), precast SDS-polyacrylamide Bis-Tris gels were from Invitrogen, phosphocellulose P81 paper was from Whatman. Medium 199, pronase E, proteinase K, bovine albumin, and collagenase were from Sigma. All peptides were synthesized by Dr. Graham Bloomberg at the University of Bristol, UK.

Antibodies

The specific AMPKα1 antibody was raised against the peptide CTSPPDSFLDDHHLTR, residues 344–358 of rat AMPKα1; the specific AMPKα2 antibody was raised against the peptide CMDDSAMHIPPGLKPH, residues 352–366 of rat AMPKα2; the phosphospecific antibodies recognizing AMPK phosphorylated on the T-loop were generated against the peptide KFLRT(P)SCGSPNYA, residues 168–180 of rat AMPKα1. The LKB1 antibody used for immunoblotting and immunoprecipitation was raised in sheep against the NH_2-terminal peptide TFIHRIDSTEVIYQPR, residues 24–39 of human LKB1, and the phosphospecific antibody recognizing mouse acetyl-CoA carboxylase-2 (ACC2; GenBank no. NP_598665) phosphorylated on Ser^{212} was generated against the peptide TMRPSMS(P)GLHLVK, corresponding to residues 215–227 of human ACC2. ExtrAvidin peroxidase conjugate, used to detect total ACC2 that has a naturally conjugated biotin, was from Sigma. Anti-total ERK1/ERK2 antibody (no. 9102) and anti-total AMPKα1/α2 (no. 2532) were from Cell Signaling Technology. Secondary antibodies coupled to horseradish peroxidase were from Pierce.

Muscle-specific LKB1 knockout and LKB1 hypomorphic mice

All animal studies and breeding were approved by the University of Dundee Ethics Committee and performed under a UK Home Office project license, and also the studies were approved by the Animal Research Committee at the Université catholique de Louvain. LKB1$^{fl/fl}$ mice were generated, bred, and genotyped as previously described (26). These mice were crossed to transgenic mice expressing Cre recombinase from the muscle creatine kinase promoter [expressed in skeletal as well as cardiac muscle (7)], which had been backcrossed for seven generations to the C57BL/6J strain. The LKB1$^{fl/fl}$ mice have 5- to 10-fold lower expression and activity of LKB1 in various tissues, including skeletal muscle, heart, testis, lung, liver, and kidney (26).

Echocardiographic analysis

Mice were anesthetized by intraperitoneal injection of 0.3 mg/g body wt of Avertin (tribromoethanol). Left ventricular function was assessed by a two-dimensional echocardiography, using a 15-MHz probe (Philips Medical System). Left ventricular function was evaluated by measuring the percentage of anterior wall thickening (AWT) and the ejection fraction (EF) (fractional area shortening) from short-axis view. The percentage of AWT is [(AWT in end-systole − AWT in end-diastole)/AWT in end-systole] × 100. Left ventricular areas during end-diastole ($LVED_{area}$) and end-systole ($LVES_{area}$) were measured to calculate the percentage of EF {%EF = [($LVED_{area}$ − $LVES_{area}$)/$LVED_{area}$] × 100}.

Isolated heart perfusion

Hearts from mice [2–3 mo old, anesthetized by intraperitoneal injection of ketamine-xylazine (0.34/0.03 mg/g body wt)] were perfused retrogradely at 37°C and at a constant pressure of 75 mmHg with a Krebs-Henseleit buffer containing 1.5 mM $CaCl_2$ and 11 mM glucose and in equilibrium with a 95% O_2-5% CO_2 gas phase. After an equilibrium period of 15 min, the

hearts were subjected to ischemia study (10 min of normoxia or no-flow ischemia) or anoxia study (15 min of normoxia or anoxia). No-flow ischemia was obtained by interrupting the flow, and anoxia was achieved by replacing O_2 with N_2 in the gas phase (5). The ischemic hearts were maintained at 37°C by a thermostated air reservoir. At the end of the procedure, the hearts were freeze-clamped, and samples were stored at −80°C.

Preparation of tissue lysates

Freeze-clamped heart tissues were pulverized to a powder in liquid nitrogen. A 15-fold mass excess of ice-cold lysis buffer containing 50 mM Tris·HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (by mass) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% (by volume) 2-mercaptoethanol, and "complete" proteinase inhibitor cocktail (1 tablet per 50 ml) was added to the powder tissue and homogenized on ice using Kinematica Polytron (Brinkmann, CT). Homogenates were centrifuged at 13,000 *g* for 10 min at 4°C to remove insoluble material. The supernatant was collected, and protein concentration was measured by the Bradford method using bovine serum albumin as the standard. Lysates were snap frozen in aliquots in liquid nitrogen and stored at −80°C.

Immunoblotting

Heart tissue extracts (20–40 μg) were heated at 95°C for 5 min in SDS sample buffer and subjected to SDS-polyacrylamide gel electrophoresis and electrotransfer to nitrocellulose membranes. Membranes were then blocked for 1 h at room temperature in 50 mM Tris·HCl, pH 7.5, 0.15 M NaCl, 0.1% (by vol) Tween (TBST), containing 10% (by mass) skimmed milk for the sheep antibodies and 5% (by mass) bovine serum albumin for ExtrAvidin-peroxidase antibody. The membranes were then incubated for 16 h at 4°C with 0.5–1 μg/ml for the sheep antibodies or 1,000-fold dilution for commercial antibodies in TBST, 5% (by mass) skimmed milk for sheep antibodies, or 5% (by mass) and bovine serum albumin for commercial antibodies. Detection of proteins was performed using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence reagent.

Quantitative immunoblot by Li-Cor analysis

The immunoblots were incubated with antibodies in TBST containing 5% (by mass) skimmed milk overnight at 4°C. The blots were washed and incubated for 1 h with fluorescently labeled anti-sheep secondary antibody at room temperature. The blots were analyzed using a Li-Cor Odyssey infrared detection system following the manufacturer's guidelines. The band intensity was quantified using Li-Cor software. Using this approach, a more quantitative analysis of immunoblots can be achieved than using the standard chemiluminescence techniques (see http://www.licor.com).

Immunoprecipitation and assay of LKB1 and AMPK

Five hundred-microgram heart tissue lysates were used to immunoprecipitate LKB1, and 50-μg lysates were used to immunoprecipitate AMPKα1 and AMPKα2. The lysates were incubated at 4°C for 1 h on a shaking platform with 5 μl of protein G-Sepharose coupled to 3 μg of LKB1 and 2 μg of AMPKα1 or AMPKα2 antibodies. The immunoprecipitates were washed twice with 1 ml of lysis buffer containing 0.5 M NaCl, and twice with 1 ml of buffer A [50 mM Tris·HCl, pH 7.5, 0.1 mM EGTA, and 0.1% (by volume) 2-mercaptoethanol]. Phosphotransferase activity toward the LKBtide peptide [SNLYHQGKFLQTFCGSPLYRRR residues 241–260 of human NUAK2 with 3 additional Arg residues added to the COOH-terminal to enable binding to P81 paper (19)] for LKB1 or *AMARA* peptide for AMPKα1 and AMPKα2 were then measured in a total assay volume of 50 μl, consisting of 50 mM Tris·HCl, pH 7.5, 0.1 mM EGTA, 0.1% (by volume) 2-mercaptoethanol, 10 mM magnesium acetate, 0.1

mM [γ-^{32}P]ATP (~200 counts·min^{-1}·pmol^{-1}), and 200 μM LKBtide or 200 μM *AMARA* peptide. The assays were carried out at 30°C with continuous shaking, to keep the immunoprecipitates in suspension, and were terminated after 20 min by applying 40 μl of the reaction mixture onto P81 papers. These were washed in phosphoric acid, and the incorporated radioactivity was measured by scintillation counting. One milliunit (mU) of activity was defined as that which catalyzed the incorporation of 1 pmol of ^{32}P into the substrate per minute.

Measurement of nucleotides

AMP, ADP, and ATP levels were measured in neutralized perchloric acid extracts of the frozen hearts after their separation by high-performance liquid chromatography (31).

Isolation of adult ventricular cardiomyocytes

Ventricular cardiomyocytes were isolated from adult mice using an established enzymatic digestion procedure (22). Mice were killed by dislocation of the neck, and the heart was excised and immediately transferred to a 35-mm Petri dish containing low-Ca^{2+} *solution 1* (100 mM NaCl, 10 mM KCl, 1.2 mM KH_2PO_4, 5 mM $MgSO_4$, 20 mM glucose, 50 mM taurine, 10 mM HEPES, and 100 μM $CaCl_2$) at room temperature. The aorta was cannulated and tied using 4-0 silk suture, and hearts were retrogradely perfused at 37°C using a Langendorff perfusion system. All solutions used in the isolated heart perfusion procedure were continuously gassed with 95% O_2-5% CO_2. Hearts were initially perfused with medium 199, followed by EGTA-buffered low-Ca^{2+} *solution 2* (low-Ca^{2+} *solution 1* containing 2 mM EGTA), then with low-Ca^{2+} *solution 3* [low-Ca^{2+} *solution 1* containing pronase E (8 mg/100 ml), proteinase K (1.7 mg/100 ml), bovine albumin (0.1 g/100 ml, fraction V), and 200 μM $CaCl_2$]. Following digestion, the ventricles were cut into fragments (2–5 mm^3) in low-Ca^{2+} *solution 1* at room temperature. Tissue fragments were then transferred to a 50-ml beaker containing low-Ca^{2+} digestion *solution 3* supplemented with collagenase (5 mg/10 ml), and cells were isolated by stirring the tissue for 10 min at 37°C. The cell suspension was then filtered through a nylon sieve and centrifuged for 1 min (at 300–400 *g*). Cell pellets were washed three times in low-Ca^{2+} *solution 1*. To confirm the purity of the cardiomyocyte preparation, we employed laser confocal microscopy LSM-510 (Zeiss, Gottingen, Germany), and images were taken (see Fig. 4A). The pellets were then lysed in 10–15 volume of lysis buffer and centrifuged at 13,000 *g* for 10 min at 4°C, the supernatant was collected, and protein concentration was measured. Lysates were snap frozen in aliquots in liquid nitrogen and stored at −80°C.

Calculation and statistical analysis

Data are expressed as means ± SE. Statistical analysis was undertaken by one-way analysis of variance followed by Fisher's least significant difference post hoc test. Differences between groups were considered statistically significant when $P < 0.05$.

RESULTS

Cardiac phenotype of muscle LKB1-deficient mice

Our laboratory (26) has previously described the generation of LKB1$^{fl/fl}$ mice in which the LKB1 gene is flanked by the *loxP* Cre excision sequence. Even in the absence of Cre recombinase expression, the LKB1$^{fl/fl}$ mice had a hypomorphic phenotype, expressing 5- to 10-fold lower levels of LKB1 in all tissues, including the heart. To ablate LKB1 expression in skeletal and cardiac muscle, the LKB1$^{fl/fl}$ mice were crossed with transgenic mice expressing the Cre recombinase under the muscle creatine kinase promoter, which induces expression of the Cre recombinase specifically in skeletal and cardiac muscle, just before birth (7). The resulting LKB1$^{fl/fl}$ Cre$^{+/-}$ mice were found to completely lack LKB1 expression in skeletal and cardiac muscle, but displayed no marked phenotype having normal body weights, as well

as fasted and fed blood glucose levels (26). We have now maintained the LKB1$^{fl/fl}$ Cre$^{+/-}$ mice up to ~26 wk of age and observed that these animals survive normally and have thus far not developed any obvious phenotype.

Although the LKB1-deficient and LKB1 hypomorphic mice survive normally and display no obvious adverse phenotypes, the heart weight-to-body weight ratio of LKB1 hypomorphic LKB1$^{fl/fl}$ Cre$^{-/-}$ and LKB1-lacking LKB1$^{fl/fl}$ Cre$^{+/-}$ mice were found to be 18 and 31%, respectively, lower than those of LKB1$^{+/+}$ wild-type hearts (Fig. 1A). Moreover, as shown in Fig. 1B, the heart lacking LKB1 in cardiac muscle possessed enlarged atria (15.8 ± 1.35 mg) compared with LKB1$^{fl/fl}$ hypomorphic (6.9 ± 0.30 mg) or wild type (7.6 ± 0.50 mg). Thus the reduced heart weight in mice lacking cardiac LKB1 results from a smaller ventricle size (LKB1$^{fl/fl}$ Cre$^{+/-}$ 69.3 mg compared with LKB1$^{+/+}$ 117.5 mg). There was no marked difference in the cardiomyocyte cell arrangement (Fig. 1B, bottom). To determine whether LKB1 regulates muscular size in general, we measured skeletal muscle mass from tibialis and gastrocnemius muscles and found no difference between wild-type and LKB1-deficient skeletal muscles in both weight and muscle weight-to-body weight ratio (K. Sakamoto, data not shown). Echocardiographic analysis of LKB1 wild-type, LKB1 hypomorphic, and cardiac muscle LKB1 knockout hearts revealed that the AWT in end-diastole and in end-systole was similar in both cardiac muscle LKB1 knockout and wild-type hearts. The left ventricular area in end-diastole and in end-systole was slightly reduced in the hearts lacking cardiac muscle LKB1, consistent with the smaller heart phenotype. Importantly, however, the resulting EF in the hearts deficient in cardiac muscle LKB1 was similar to that observed in LKB1$^{+/+}$ wild-type hearts. These results suggested that, despite reduced heart size and enlarged atria, there is no apparent sign of heart failure or ventricular dysfunction in the hearts lacking cardiac muscle LKB1.

LKB1 is not activated by ischemia

We next assayed LKB1 activity following its immunoprecipitation from heart extracts and found that ischemia did not stimulate LKB1 activity in wild-type hearts (Fig. 2). Consistent with our previous study, the hypomorphic LKB1$^{fl/fl}$ Cre$^{-/-}$ mice possessed markedly lower LKB1 activity and protein levels than observed in wild-type hearts. LKB1 was not detectable in cardiac LKB1-deficient heart (Fig. 2).

LKB1 is required for ischemia- or anoxia-induced AMPKα2 activation

To investigate the role of LKB1 in regulating AMPK in cardiac muscle, we performed perfusion studies under basal normoxic or no-flow ischemic conditions, using 9- to 13-wk-old muscle LKB1-deficient LKB1$^{fl/fl}$ Cre$^{+/-}$, LKB1 hypomorphic LKB1$^{fl/fl}$ Cre$^{-/-}$, and wild-type LKB1$^{+/+}$ Cre$^{-/-}$ control mice, generated using a previously described breeding strategy (26). We first measured the activity of AMPKα2 and phosphorylation of Thr^{172}, the site of LKB1 phosphorylation. In wild-type LKB1$^{+/+}$ hearts, no-flow ischemia stimulated AMPKα2 activity approximately fourfold, to a specific activity of ~200 mU/mg (Fig. 3A) and robustly increased phosphorylation at Thr^{172} (lower migrating band in Fig. 3B, top). In hypomorphic LKB1$^{fl/fl}$ Cre$^{-/-}$ hearts, the basal AMPKα2 activity under normoxic conditions was decreased approximately twofold (25 mU/mg), but still substantially stimulated by ischemia to an activity of 130 mU/mg. Ischemia also enhanced the phosphorylation of AMPKα2 at Thr^{172} in the LKB1 hypomorphic mice, albeit to a lower level than was observed in LKB1$^{+/+}$ wild-type hearts. Strikingly, however, in cardiac muscle LKB1-lacking hearts, although AMPKα2 was expressed normally, the basal AMPKα2 activity of 1.2 mU/mg was vastly lower than the ~50 mU/mg observed in wild-type LKB1$^{+/+}$, or the ~25 mU/mg hypomorphic LKB1$^{fl/fl}$ Cre$^{-/-}$ heart (Fig. 3A). Moreover, in the heart lacking cardiac LKB1, ischemia did not stimulate AMPKα2 activity (Fig. 3A) or Thr^{172} phosphorylation (Fig. 3B).

We next examined AMPKα1 activity in wild-type LKB1$^{+/+}$ heart under normoxic conditions and found that its activity was only slightly lower than that of AMPKα2 (compare Fig. 3, A and C). In wild-type heart, ischemia induced marked activation of AMPKα1 to an activity ~125 mU/mg, which was accompanied by phosphorylation of AMPKα1 at Thr^{172} (upper migrating band in Fig. 3B, top). In the hypomorphic LKB1$^{fl/fl}$ Cre$^{-/-}$ heart, AMPKα1 activity was ~50% lower than that observed for wild-type LKB1$^{+/+}$ hearts (~20 mU/mg), but ischemia still induced robust activation of AMPKα1 to an activity of ~100 mU/mg (Fig. 3C), as well as marked phosphorylation of Thr^{172} (Fig. 3B). Interestingly, however, in cardiac muscle LKB1-deficient LKB1$^{fl/fl}$ Cre$^{+/-}$ heart, the basal AMPKα1 activity under normoxic condition was not any lower than observed in the hypomorphic LKB1$^{fl/fl}$ Cre$^{-/-}$ hearts. Moreover, AMPKα1 was still significantly activated by ischemia to an activity ~60 mU/mg, only moderately lower than observed in the LKB1 wild-type or hypomorphic hearts. Ischemia also induced phosphorylation of AMPKα1 (but not AMPKα2) at Thr^{172}, in cardiac LKB1-lacking hearts, but to a lower extent than was observed in LKB1 hypomorphic or wild-type heart (Fig. 3B, top). In LKB1-deficient LKB1$^{fl/fl}$ Cre$^{+/-}$ skeletal muscle, we previously found that AMPKα1 protein levels were increased approximately twofold (26). By contrast, in cardiac muscle LKB1-deficient hearts, AMPKα1 as well as AMPKα2 levels were judged normal (Fig. 3B, middle).

To rule out the possibility that acidosis induced by the lack of metabolic waste removal (e.g., lactate accumulation) due to no-flow affected AMPK activity in cardiac muscle LKB1-deficient heart, we also performed the anoxia/hypoxia experiment by perfusing isolated hearts with N_2 instead of O_2, as described in EXPERIMENTAL PROCEDURES. Anoxia robustly stimulated AMPKα2 and AMPKα1 activity as well as AMPKα1/α2 phosphorylation at Thr^{172} in LKB1$^{+/+}$ hearts (Fig. 3, D, E, and F). In cardiac muscle LKB1-deficient heart, anoxia-induced AMPKα2 activation and phosphorylation were completely abolished (Fig. 3, D and F), whereas AMPKα1 activity and phosphorylation were only moderately inhibited, as observed with ischemia (Fig. 3, E and F).

We next analyzed the phosphorylation of a downstream target of AMPK, namely the muscle isoform of ACC2, at the primary site phosphorylated by AMPK [Ser^{212}, equivalent to Ser^{79} phosphorylated on rat ACC1 (10)]. In wild-type LKB1$^{+/+}$ and hypomorphic LKB1$^{fl/fl}$ Cre$^{-/-}$ hearts, ischemia profoundly enhanced phosphorylation of ACC2 at this site (Fig. 3B, bottom). In contrast, phosphorylation of ACC2 was undetectable in both normoxic and ischemic hearts from the cardiac muscle LKB1-deficient mice.

AMPKα1 but not AMPKα2 is activated in LKB1-deficient cardiomyocytes

Myocytes constitute ~75% of the total volume of the myocardium (6), and the rest of the volume contains nonmyocyte cells that do not express the Cre recombinase. It might be argued that the significant AMPKα1 activity measured in cardiac muscle LKB1-deficient LKB1$^{fl/fl}$ Cre$^{+/-}$ heart extracts in Fig. 3C was derived from nonmuscle cell types, such as fibroblasts and endothelial cells, present in the heart. To investigate this possibility, we isolated cardiomyocytes from wild-type LKB1$^{+/+}$ and cardiac muscle LKB1-lacking hearts using a protocol in which >99% of the isolated cells are cardiomyocytes (22). We observed that only cardiomyocytes were recovered from the pellet (Fig. 4A; depicted rod-shaped cells are intact cardiomyocytes, while few rounded cells are cardiomyocytes in hypercontractured state). The cardiomyocytes were lysed, and AMPKα1 and AMPKα2 activity was assayed. Although no detectable AMPKα2 activity was observed in the LKB1-deficient cardiomyocytes, the activity of AMPKα1 was only moderately reduced in these cells compared with LKB1$^{+/+}$ wild-type cells, thereby indicating active AMPKα1 is indeed expressed in LKB1-deficient cardiomyocytes (Fig. 4, B and C).

LKB1 controls cellular energy levels in cardiac muscle

As AMPK regulates cellular energy balance, we measured ATP, ADP, and AMP levels in normoxic and ischemic heart by HPLC (Fig. 5). We observed that, in wild-type $LKB1^{+/+}$ and hypomorphic $LKB1^{fl/fl}$ $Cre^{-/-}$ heart in normoxic conditions, ADP/ATP and AMP/ATP were similar and that ischemia increased ADP/ATP by ~50% (Fig. 5A) and AMP/ATP two-to threefold (Fig. 5B). In the cardiac muscle LKB1-deficient hearts, ADP/ATP and AMP/ATP were moderately higher in normoxic hearts than those measured in $LKB1^{+/+}$ and $LKB1^{fl/fl}$ $Cre^{-/-}$ hearts. Ischemia increased ADP/ATP and AMP/ATP in LKB1-lacking heart to significantly higher levels than those observed in wild-type heart. The higher ADP/ATP and AMP/ATP in cardiac muscle LKB1-lacking heart in both normoxic and hypoxic conditions are largely due to reduced ATP level (normoxia: 2.1 μmol/g wet wt, ischemia: 2.0 μmol/g) compared with $LKB1^{+/+}$ wild-type heart (normoxia: 3.5 μmol/g wet wt, ischemia: 3.6 μmol/g).

DISCUSSION

Our results establish that LKB1 is a major in vivo upstream regulator of AMPKα2 in cardiac muscle and that the lack of LKB1 is not compensated by other kinases. However, an unexpected finding was that, in the heart lacking cardiac muscle LKB1 or LKB1-deficient isolated cardiomyocytes, AMPKα1 was still significantly active, and its activity as well as phosphorylation at Thr^{172} were markedly stimulated by ischemia or anoxia/hypoxia. Therefore, at least in the absence of LKB1, an alternative upstream activator can phosphorylate AMPKα1 at Thr^{172} in cardiac muscle. In skeletal muscle (mixed tibialis anterior and extensor digitorum longus), although the basal AMPKα1- and AMPKα2-specific activities are similar, in situ muscle contraction induced by sciatic nerve stimulation only activated AMPKα2 but not AMPKα1 (25,26). One interpretation for this observation is that AMPKα1 detected in total skeletal muscle extract might be largely derived from nonmuscle cells (e.g., fibroblasts, endothelial cells). This would also account for our previous observation that AMPKα1, unlike AMPKα2, still possessed substantial activity in LKB1-lacking $LKB1^{fl/fl}$ $Cre^{+/-}$ skeletal muscle extracts (26), as the Cre recombinase would not be expressed in nonmuscle cells. However, the finding in this study that cardiac AMPKα1 can be stimulated independently of LKB1 would suggest that AMPKα1 expressed in skeletal muscle might also be activated by an LKB1-independent mechanism. Previous studies have reported that AMPKα1 activity can be stimulated by high-intensity exercise protocol in human skeletal muscle (9). Furthermore, a recent study demonstrated that low-frequency electrical stimulation of isolated rat epitrochlearis muscle only activated AMPKα1 under conditions in which the intracellular AMP level was not elevated (30). Taken together, these findings suggest that the AMPKα1 isoform is expressed within skeletal and cardiac muscle cells and is regulated by a Thr^{172} upstream kinase that is not LKB1.

As mentioned in the introductory section, recent studies have suggested that, in addition to LKB1, another AMPK activity phosphorylated AMPK at Thr^{172} in the heart (2). The activity of this LKB1-independent enzyme was stimulated by ischemia, under conditions in which LKB1 activity was not enhanced (2). Consistent with the previous study, we have also found that LKB1 was not activated by ischemia (Fig. 2). This observation supports the notion that LKB1 is a constitutively active enzyme, and that binding of AMP to the AMPKγ subunit regulates the activation of AMPK by LKB1 (12,19,25). Interestingly, Altarejos et al. (2) deployed recombinant AMPKα1 kinase domain (1–312) rather than AMPKα2 to identify the ischemia-stimulated AMPK-activating activity present in heart extracts. It would be necessary to establish the identity of this ischemia-activated enzyme and investigate whether it possesses an intrinsic preference for AMPKα1 over AMPKα2. It would also be necessary to verify whether the ischemia-stimulated activity was CAMKKα and/or CAMKKβ, which can act as

upstream regulators of AMPK (13,16,32). It has not been reported whether CAMKK isoforms have a preference for activating AMPKα1 or AMPKα2. The active LKB1:STRAD:MO25 complex appears to have no marked preference for AMPK isoforms, as it activated complexes of AMPKα1:AMPKβ1:AMPKγ1 with similar efficiency as AMPKα2:AMPKβ1:AMPKγ1 in cell-free studies (12). It is possible that differences in subcellular localization of AMPKα1, AMPKα2, LKB1, and other AMPK activators in cardiomyocytes contribute to the observed differences in their regulation. To our knowledge, the cellular localization of AMPKα isoforms or LKB1 in cardiomyocytes has not previously been investigated.

In the hearts lacking cardiac muscle LKB1 in response to ischemia, despite activation of AMPKα1, we observed that ACC2 was not phosphorylated. This suggested that AMPKα2 rather than AMPKα1 is the dominant enzyme controlling ACC2 phosphorylation. As both AMPKα1 and AMPKα2 would be expected to phosphorylate ACC2 with similar catalytic efficiency, at least in vitro, the preferential phosphorylation of ACC2 by AMPKα2 in cardiomyocytes might also account for the differences in subcellular localizations of AMPK isoforms and/or ACC2. ACC2 is reported to be associated with mitochondria in neonatal rat cardiomyocytes (1), whereas this is not the case for AMPKα1/α2, at least in HeLa cells (D. G. Hardie, unpublished). AMPKα2 knockout mice have recently been generated and, although ACC2 phosphorylation has not been investigated in the heart, in skeletal muscle, ACC2 was normally phosphorylated following contraction in these animals (17). However, it should be noted that AMPKα1 protein expression was significantly increased in the AMPKα2 knockout muscle, which may compensate for the loss of AMPKα2 (17). We also found that, in the skeletal muscle of LKB1-deficient $LKB1^{fl/fl}$ $Cre^{+/-}$ animals, AMPKα1 protein levels were increased approximately twofold (26). By contrast, this compensatory mechanism may not operate in cardiac muscle, as neither AMPKα1 nor AMPKα2 expression was elevated in the heart of cardiac muscle LKB1-deficient $LKB1^{fl/fl}$ $Cre^{+/-}$ mice (Fig. 3B). The finding that cardiac muscle LKB1-deficient hearts possess abnormal elevation of AMP-to-ATP ratio suggests that AMPKα1 activity is also unable to fully compensate for lack of AMPKα2 activity in the maintenance of cellular energy levels.

Mice lacking LKB1 in skeletal and cardiac muscle had normal body and skeletal muscle weight, but their hearts were 30% smaller than wild-type $LKB1^{+/+}$ hearts and possessed enlarged atria. Mice that overexpressed dominant-negative AMPK in cardiac and skeletal muscle possessed 10% smaller hearts (24). Despite the smaller size of heart left ventricle, there was no obvious modification of left ventricular systolic function, as assessed by echocardiographic analysis. It is possible that AMPKα1 activity that is present in the hearts deficient in cardiac muscle LKB1 enables them to retain normal heart function. It would be of interest if cardiac muscle LKB1-deficient hearts display defects under more stressful conditions, such as strenuous exercise or ischemia.

In conclusion, we have provided genetic evidence that LKB1 plays an essential role in activating AMPKα2 in cardiac muscle in normoxic and ischemic as well as anoxic conditions. By contrast, significant AMPKα1 activity was still detected in cardiac muscle lacking LKB1, and the AMPKα1 activity was robustly stimulated in response to ischemia or anoxia. This observation indicates that there is an AMPKα1 kinase(s) present in cardiac muscle. It would be of interest to identify this “α1 kinase” and to also investigate the role that AMPKα1 plays in regulating cardiac muscle metabolism and function during ischemia and anoxia.

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A

Morphologic	LKB1+/+ (n=8)	LKB1fl/flCre-/- (n=9)	LKB1fl/flCre+/- (n=7)
BW (g)	26.5 ± 1.21	26.7 ± 0.43	25.9 ± 1.44
HW (mg)	125.1 ± 7.13	102.5 ± 2.47 [a]	85.1 ± 7.27 [a,b]
HW/BW (mg/g)	4.72 ± 0.16	3.85 ± 0.10 [a]	3.25 ± 0.15 [a,b]
Echocardiographic	(n=7)	(n=8)	(n=8)
AWD (mm)	0.67 ± 0.04	0.65 ± 0.04	0.64 ± 0.04
AWS (mm)	1.37 ± 0.08	1.35 ± 0.07	1.37 ± 0.05
AWT (%)	51.52 ± 1.64	51.43 ± 2.02	52.77 ± 3.03
LVEDarea (mm^2)	10.11 ± 0.59	10.47 ± 0.50	8.29 ± 0.60 [a,b]
LVESarea (mm^2)	2.86 ± 0.21	3.41 ± 0.54	2.59 ± 0.09
EF (%)	71.83 ± 1.98	67.73 ± 1.94	68.74 ± 3.18
HR (bpm)	297 ± 16	298 ± 15	301 ± 17

B

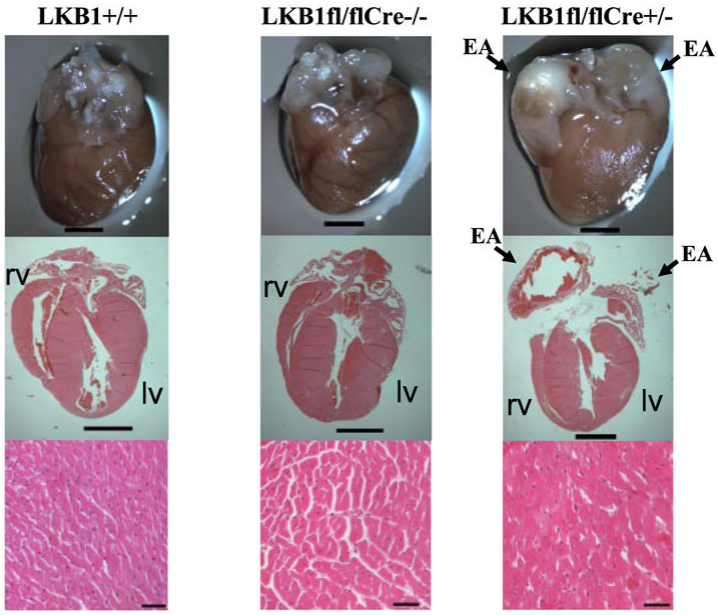

Fig. 1.

Cardiac phenotype of muscle LKB1-decient mice. *A*: mice (2–3 mo old) were anesthetized, and left ventricular function was assessed by a 2-dimensional echocardiographic analysis. Left ventricular function was evaluated by measuring the percentage of anterior wall thickening (AWT) and the ejection fraction (EF) (fractional area shortening) from short-axis view. The percentage of AWT is [(AWT in end-systole − AWT in end-diastole)/AWT in end-systole] × 100. Left ventricular areas during end-diastole ($LVED_{area}$) and end-systole ($LVES_{area}$) were measured to calculate the percentage of EF {%EF = [($LVED_{area}$ − $LVES_{area}$)/$LVED_{area}$] × 100}. Results are presented as the average ± SE; *n*, no. of animals. BW, body weight; HW, heart weight; HW/BW, ratio of HW to BW; AWD, diastolic AWT; AWS, systolic AWT; HR, heart rate; bpm, beats/min. [a]$P < 0.05$ vs. $LKB1^{+/+}$; [b]$P < 0.05$ vs. $LKB1^{fl/fl}$. *B*: morphological and histological analysis of hearts from $LKB1^{+/+}$, $LKB1^{fl/fl}$, and $LKB1^{fl/fl}$ $Cre^{+/-}$ mice (~3 mo old). The hearts were fixed in 10% formalin, embedded in wax and stained with

hematoxylin and eosin. *Top*: image of entire heart. *Middle*: representative longitudinal sections with the horizontal bar representing 2 mm. *Bottom*: higher magnification of the image of cardiomyocytes present in the longitudinal section, with the horizontal bar representing 0.2 mm. EA, enlarged atrium; rv, right ventricle; lv, left ventricle.

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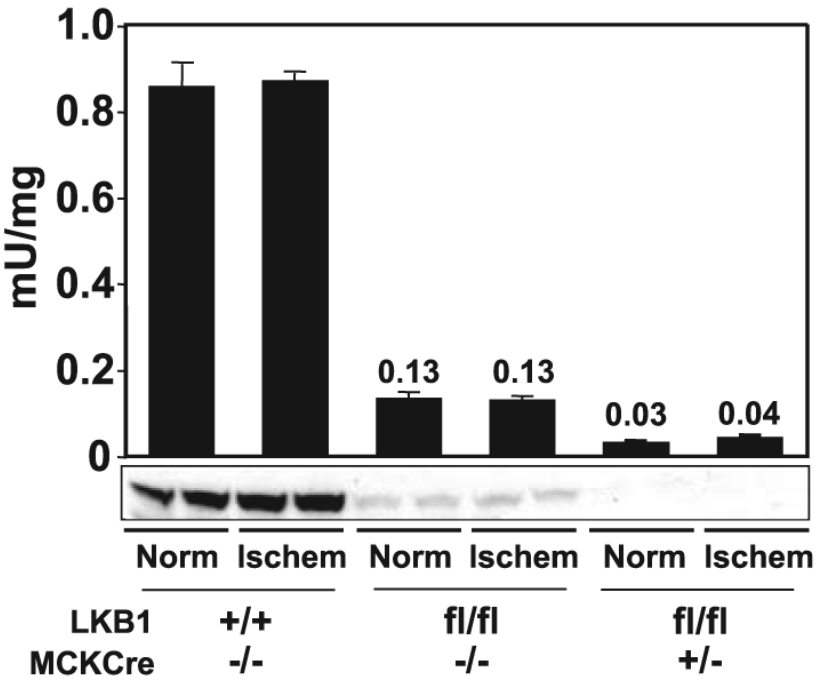

Fig. 2.

LKB1 activity in response to ischemia in cardiac muscle. Isolated heart derived from the indicated mice (2–3 mo of age) were perfused under normoxic (Norm) or no-flow ischemic (Ischem) conditions for 10 min, as described in EXPERIMENTAL PROCEDURES. Equal amounts of protein (30 μg) from heart extracts were immunoblotted with LKB1 antibody. LKB1 was immunoprecipitated and assayed, employing the LKBtide peptide.

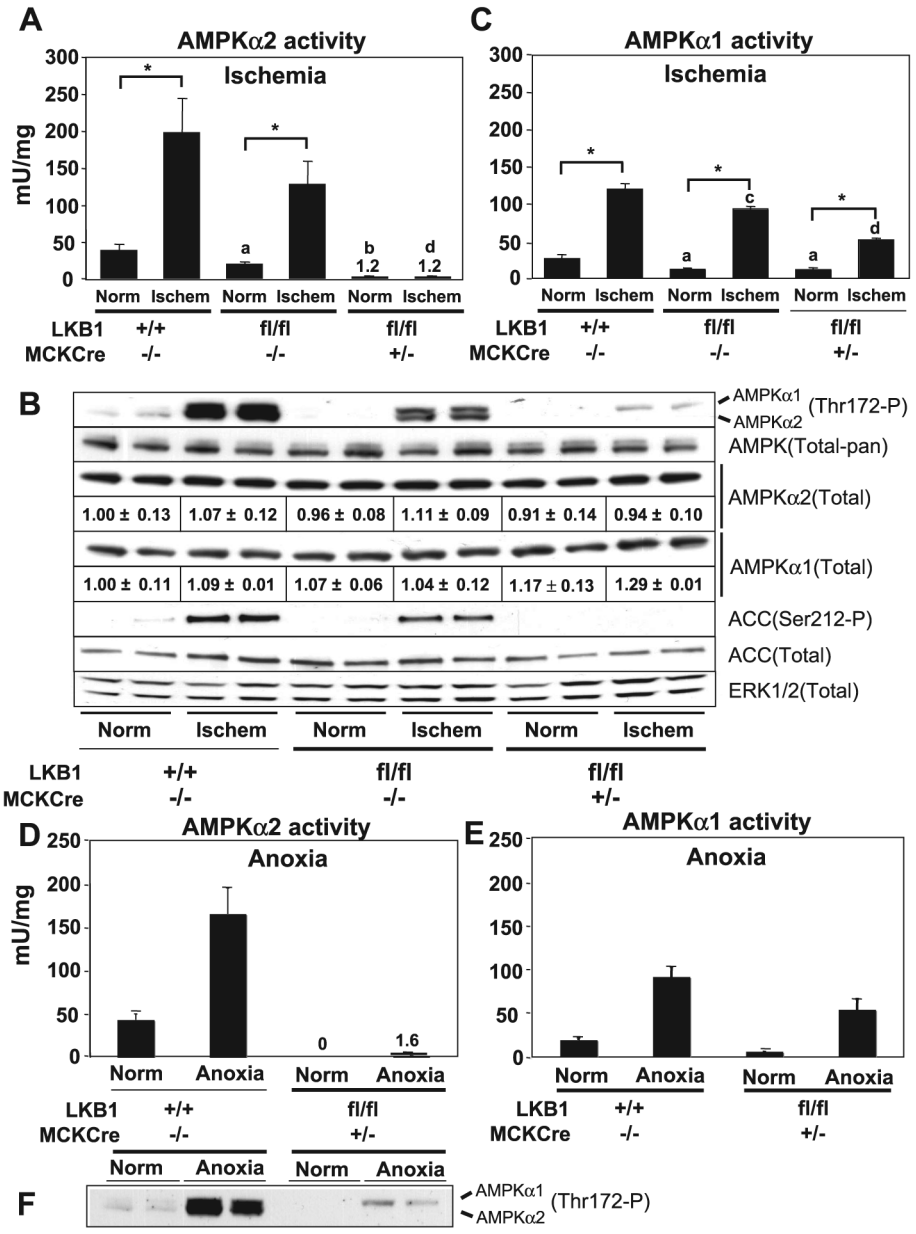

Fig. 3.

Role of LKB1 in regulating ischemia- or anoxia-induced AMP-activated protein kinase (AMPK) activation in the heart. Isolated heart derived from the indicated mice (2–3 mo of age) were perfused under normoxic, no-flow ischemic (*A*, *B*, and *C*) or anoxic (*D*, *E*, and *F*) conditions for 10 or 15 min, as described in the EXPERIMENTAL PROCEDURES. *A* and *D*: AMPKα2 was immunoprecipitated and assayed with the *AMARA* peptide. Assays were performed in duplicate from the heart tissue derived from 3–6 mice, and results are presented as the average specific activity ± SE. *B* and *F*: equal amounts of protein (20–40 μg) from heart extracts were immunoblotted with the indicated antibodies. The immunoblotting results are representative of independent experiments performed with heart from at least 3 mice. Immunoblot analysis of total AMPKα1 and AMPKα2 levels was performed by quantitative Li-Cor method. The data presented are the mean ± SE expression relative to expression in LKB1$^{+/+}$ muscle derived from

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3–5 mice. *C* and *E*: as in *A*, except that AMPKα1 was assayed. ACC, acetyl-CoA carboxylase. **P* < 0.05 basal vs. ischemia within each genotype; [a]*P* < 0.05 vs. $LKB1^{+/+}$ (normoxia); [b]*P* < 0.05 vs. $LKB1^{fl/fl}$ $Cre^{-/-}$ (normoxia); [c]*P* < 0.05 vs. $LKB1^{+/+}$ (ischemia); [d]*P* < 0.05 vs. $LKB1^{fl/fl}$ $Cre^{-/-}$ (ischemia).

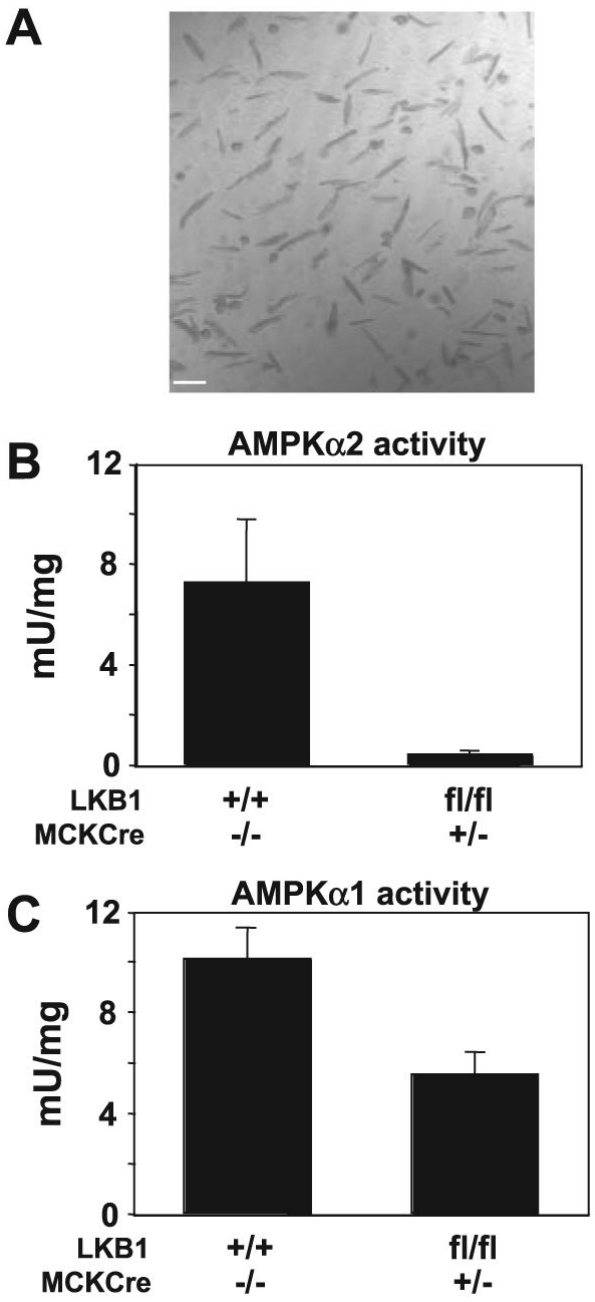

Fig. 4.

Activity of AMPKα1 and AMPKα2 in isolated cardiomyocytes from LKB1$^{+/+}$ or LKB1$^{fl/fl}$ Cre$^{+/-}$ mice. Cardiomyocytes were isolated from the 2- to 3-mo-old LKB1$^{+/+}$ or LKB1$^{fl/fl}$ Cre$^{+/-}$ mice, as described in EXPERIMENTAL PROCEDURES, and total cell extracts were immediately generated. *A*: original image taken by laser confocal microscopy LSM-510 of cardiomyocytes isolated from the mouse heart, as described in EXPERIMENTAL PROCEDURES. Magnification was ×10. Note a lack of noncardiac cells in this preparation. AMPKα1 (*B*) or AMPKα2 (*C*) was immunoprecipitated from the lysates and assayed with the *AMARA* peptide. Assays were performed from 3 mice per genotype, and results are presented as the average specific activity ± SE.

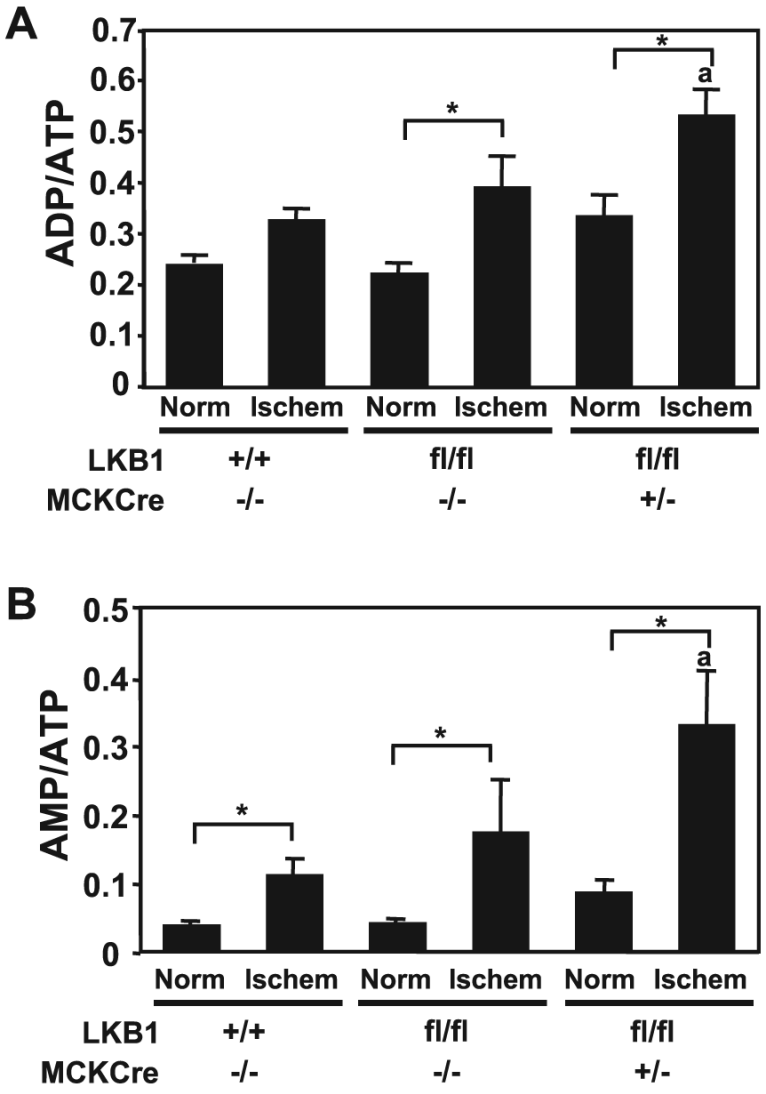

Fig. 5.

Role of LKB1 in regulating ADP-to-ATP and AMP-to-ATP ratios during ischemia in cardiac muscle. Isolated heart derived from wild-type or LKB1 mutant mice (2–3 mo of age) was perfused under normoxic or no-flow ischemic conditions for 10 min. The ratios of ADP to ATP (*A*) and AMP to ATP (*B*) derived from 4–5 heart samples for each condition were measured. The results are shown as the average ± SE. **P* < 0.05 normoxia vs. ischemia within each genotype; [a]*P* < 0.05 vs. $LKB1^{+/+}$ (ischemia).