

# Compromised Energetics in the Adenylate Kinase *AK1* Gene Knockout Heart under Metabolic Stress\*

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Rapid exchange of high energy carrying molecules between intracellular compartments is essential in sustaining cellular energetic homeostasis. Adenylate kinase (AK)-catalyzed transfer of adenine nucleotide  $\beta$ - and  $\gamma$ -phosphoryls has been implicated in intracellular energy communication and nucleotide metabolism. To demonstrate the significance of this reaction in cardiac energetics, phosphotransfer dynamics were determined by [ $^{18}\text{O}$ ]phosphoryl oxygen analysis using  $^{31}\text{P}$  NMR and mass spectrometry. In hearts with a null mutation of the *AK1* gene, which encodes the major AK isoform, total AK activity and  $\beta$ -phosphoryl transfer was reduced by 94% and 36%, respectively. This was associated with up-regulation of phosphoryl flux through remaining minor AK isoforms and the glycolytic phosphotransfer enzyme, 3-phosphoglycerate kinase. In the absence of metabolic stress, deletion of *AK1* did not translate into gross abnormalities in nucleotide levels,  $\gamma$ -ATP turnover rate or creatine kinase-catalyzed phosphotransfer. However, under hypoxia *AK1*-deficient hearts, compared with the wild type, had a blunted AK-catalyzed phosphotransfer response, lowered intracellular ATP levels, increased  $\text{P}_i/\text{ATP}$  ratio, and suppressed generation of adenosine. Thus, although lack of *AK1* phosphotransfer can be compensated in the absence of metabolic challenge, under hypoxia *AK1*-knockout hearts display compromised energetics and impaired cardioprotective signaling. This study, therefore, provides first direct evidence that *AK1* is essential in maintaining myocardial energetic homeostasis, in particular under metabolic stress.

Adenylate kinase (AK)<sup>1</sup> catalyzes reversible phosphotransfer,  $2 \text{ADP} \leftrightarrow \text{AMP} + \text{ATP}$ , and participates in *de novo* synthesis, regeneration and salvage of adenine nucleotides (1–5). AK is particularly abundant in tissues with high energy turnover, where it facilitates transfer of energy-rich  $\beta$ - and  $\gamma$ -phosphoryls and regulates vital ATP-dependent cellular processes (6–10).

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<sup>1</sup> The abbreviations used are: AK, adenylate kinase; CK, creatine kinase; KH, Krebs-Henseleit; CrP, creatine phosphate; HPLC, high performance liquid chromatography; PGK, 3-phosphoglycerate kinase.

In fact, AK may serve as an integral component of phosphotransfer networks, along with creatine kinase (CK) and glycolysis, effectively coupling ATP-generating with ATP-consuming or ATP-sensing intracellular sites (11–15).

In the heart, CK-catalyzed phosphotransfer is the major pathway that can transfer high energy phosphoryls derived from the  $\gamma$ -phosphoryl of ATP (10, 16–18). Although less active than CK, AK catalysis provides a unique mechanism for transfer and utilization of both  $\gamma$ - and  $\beta$ -phosphoryls in the ATP molecule (10, 15). In this way, AK-catalyzed phosphotransfer doubles the energetic potential of ATP and could provide an additional energetic source under conditions of increased energy demand (10, 19). However, due to lack of membrane permeant and selective AK inhibitors, the biological importance of AK in heart muscle and its role in sustaining myocardial energetics under conditions of metabolic stress have not been established.

We have recently demonstrated that deletion of the *AK1* gene, which encodes the major AK isoform, produces a phenotype with reduced skeletal muscle energetic economy despite multiple metabolic adaptations (20). Here, the contribution of *AK1*-catalyzed phosphotransfer to cardiac energetics was determined using *AK1*-deficient hearts. Cellular energetics and phosphotransfer kinetics, under normal and hypoxic conditions, were monitored using a newly developed technique based on [ $^{18}\text{O}$ ]phosphoryl labeling in conjunction with  $^{31}\text{P}$  NMR and mass spectrometry. In *AK1*-knockout hearts, we report a significantly compromised adenine nucleotide  $\beta$ -phosphoryl transfer. Although lack of *AK1* appears to be compensated under normal conditions, under hypoxic stress *AK1*-deficient hearts have a reduced ability to sustain intracellular energetics and cardioprotective signaling. This study demonstrates that *AK1*-catalyzed phosphotransfer is essential in the maintenance of myocardial energetic homeostasis.

## MATERIALS AND METHODS

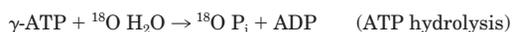
***AK1*-knockout Mice**—Gene-targeted mice were derived from mouse ES cells carrying a replacement mutation in the *AK1* gene using established procedures (12, 13). Complete inactivation of *AK1* expression was achieved by homologous DNA recombination, with a HygroB cassette vector used to replace the entire exon 3–5 region in the *AK1* gene (Fig. 1A) as described in detail elsewhere (20). In this way, homozygous *AK1*-deficient (*AK1*<sup>-/-</sup>) mice were generated and their hearts compared with those of age-matched wild-type controls (50–50% C57BL/6  $\times$  129/Ola mixed inbred background mice). The investigation conformed to the *Guidelines for the Care and Use of Laboratory Animals* of the Dutch Council and the National Institutes of Health, and was approved by the Institutional Animal Care and Use Committee at the Mayo Clinic.

**Heart Isolation**—Wild-type and *AK1*-knockout mice (40–50 g) were heparinized (250 units, administered intraperitoneally), and anesthetized with pentobarbital (100 mg/kg, administered intraperitoneally). Hearts were rapidly excised, washed from blood, and used immediately

for [ $^{18}\text{O}$ ]phosphoryl labeling or freeze-clamped in liquid  $\text{N}_2$  for enzymatic analysis.

**Western Blot and Zymogram Analysis**—Homogenates from freshly excised hearts (10% w/v) were prepared in SETH buffer (250 mM sucrose, 2 mM EDTA, 10 mM Tris-HCl (pH 7.4)) at 4 °C. Heart extracts were diluted 1:1 in 30 mM  $\text{Na}_3\text{PO}_4$  buffer (pH 7.4), containing 0.05% v/v Triton X-100, 0.3 mM dithiothreitol and a complete protease inhibitor mixture (Roche Molecular Biochemicals). For Western blot analysis, extracted proteins were separated on 10% SDS-polyacrylamide gels and proteins electrophoretically transferred onto nitrocellulose membranes. AK1 and CK-M proteins were detected using anti-mouse AK1 and CK-M antibodies raised in rabbit against purified recombinant proteins produced in *Escherichia coli* (13). Immunocomplexes were visualized by chemiluminescence using goat anti-rabbit immunoglobulin G coupled to horseradish peroxidase. For zymogram analysis, extracts were centrifuged for 20 min at  $11,000 \times g$ , and aliquots (1–5  $\mu\text{l}$ ) applied to agarose gels. AK1 and CK isoenzymes were separated electrophoretically and stained for enzyme activity (13).

**Phosphotransfer Rates**—AK- and CK-catalyzed phosphotransfers were measured in intact cardiac muscle using the [ $^{18}\text{O}$ ]phosphoryl-labeling technique (10). This procedure is based on the incorporation of one  $^{18}\text{O}$  atom, provided from [ $^{18}\text{O}$ ]water, into inorganic phosphate ( $\text{P}_i$ ) with each act of ATP hydrolysis and the distribution of  $^{18}\text{O}$ -labeled phosphoryls among high energy phosphoryl-carrying molecules depending on the following reactions, shown in Scheme 1.



SCHEME 1

Up to three  $^{18}\text{O}$  atoms can be incorporated in phosphoryls of  $\gamma$ -ATP,  $\beta$ -ATP, and creatine phosphate (CrP), while a maximum of four  $^{18}\text{O}$  atoms can be incorporated into  $\text{P}_i$ .

**[ $^{18}\text{O}$ ]Phosphoryl Labeling**—Isolated hearts were superfused with Krebs-Henseleit (KH) buffer (in mM: NaCl 137, KCl 5,  $\text{CaCl}_2$  2,  $\text{MgCl}_2$  1, EDTA 0.05,  $\text{NaH}_2\text{PO}_4$  1, HEPES 20, glucose 5,  $\text{NaHCO}_3$  24 (pH 7.45)) saturated with 95%  $\text{O}_2$ , 5%  $\text{CO}_2$ , and paced at a constant rate of 2 Hz using platinum electrodes. Under this condition, due to a small size and thin walls, hearts continuously beat and preserve intracellular adenine nucleotide ratios throughout the duration of experiments (Ref. 21; see also Fig. 2A). In control protocols, hearts were incubated for 3 min in KH buffer then transferred for an additional 3 min to a KH buffer supplemented with 20% of  $^{18}\text{O}$ -containing water. In hypoxia-simulated protocols, the mitochondrial cytochrome *c* oxidase inhibitor KCN (2 mM) was added to buffers. At the 6-min point, hearts were freeze-clamped, pulverized in mortar with liquid  $\text{N}_2$ , and extracted in a solution containing 600 mM  $\text{HClO}_4$  and 1 mM EDTA. Proteins were pelleted by centrifugation ( $15,000 \times g$ , 10 min) and protein content determined with a  $\text{D}_c$  Protein Assay kit (Bio-Rad). Extracts were neutralized with 2 M  $\text{KHCO}_3$  and used for determination of  $^{18}\text{O}$  labeling by  $^{31}\text{P}$  NMR spectroscopy and mass spectrometry.

**$^{31}\text{P}$  NMR Spectroscopy**— $^{18}\text{O}$  incorporation in  $\gamma$ -phosphoryl of ATP or phosphoryls of CrP was measured by  $^{31}\text{P}$  NMR spectroscopy.  $^{18}\text{O}$  incorporation induces an isotope shift in the  $^{31}\text{P}$  NMR spectrum and is used to study enzymatic reactions *in vitro* (22). Based on this principle, we here developed a novel approach to monitor phosphotransfer kinetics in intact heart muscle. Perchloric acid extracts were pre-cleaned with Chelex 100 resin (Sigma), supplemented with 2 mM EDTA and methylene diphosphonate. Samples, concentrated by vacuum-centrifugation to obtain higher NMR signals, were supplemented with deuterium water (10%).  $^{31}\text{P}$  NMR spectra were recorded at 202.5 MHz on a Bruker 11 T spectrometer (Avance) in 5-mm tubes at 5 °C. In hearts superfused with regular,  $^{16}\text{O}$ -containing medium, CrP appears as a single peak in the NMR spectrum (Fig. 1B, left panel). In  $^{18}\text{O}$ -containing medium, CK-catalyzed incorporation of  $^{18}\text{O}$  into CrP results in the appearance of  $^{18}\text{O}_1$  and  $^{18}\text{O}_2$  phosphoryl species (Fig. 1B, right panel). As a consequence, the CrP  $^{31}\text{P}$  NMR signal is split into three peaks corresponding to  $^{16}\text{O}$ ,  $^{18}\text{O}_1$ , and  $^{18}\text{O}_2$  phosphoryl species (Fig. 1B, right panel). At 20% of  $^{18}\text{O}$ -containing water, a fourth peak, corresponding to  $^{18}\text{O}_3$  phosphoryl species, was usually at the limit of detection. Isotope shifts were also observed following incorporation of  $^{18}\text{O}$  into phosphoryls of  $\gamma$ -ATP.

Percentages of  $^{16}\text{O}$ ,  $^{18}\text{O}_1$ ,  $^{18}\text{O}_2$ , and  $^{18}\text{O}_3$  phosphoryl species in  $\gamma$ -ATP and CrP were proportional to the integrals of respective lines in the NMR spectrum. The cumulative percentage of phosphoryl oxygens replaced by  $^{18}\text{O}$  in  $\gamma$ -ATP and CrP was calculated as  $[\%^{18}\text{O}_1 + 2(\%^{18}\text{O}_2) + 3(\%^{18}\text{O}_3)]/[3(\%^{18}\text{O} \times \text{H}_2\text{O})]$  (8). In addition to  $^{18}\text{O}$  incorporation into CrP,  $\beta$ -ATP, or  $\gamma$ -ATP,  $^{31}\text{P}$  NMR [ $^{18}\text{O}$ ]phosphoryl-labeling permits simultaneous measurements of tissue levels of  $\text{P}_i$ , CrP, and ATP. [ $^{18}\text{O}$ ]Phosphoryl-labeling values for  $\gamma$ -ATP obtained by  $^{31}\text{P}$  NMR correlated well ( $r = 0.94$ ) with those obtained by mass spectrometry. Although the  $^{31}\text{P}$  NMR [ $^{18}\text{O}$ ]phosphoryl-labeling technique is faster than mass spectrometry, its lower sensitivity precludes reliable monitoring of relatively slower  $^{18}\text{O}$  labeling of  $\beta$ -ATP under our experimental conditions. Therefore, [ $^{18}\text{O}$ ]phosphoryl labeling of  $\beta$ -ATP was determined by mass spectrometry.

**Mass Spectrometry**—Incorporation of  $^{18}\text{O}$  into  $\beta$ -phosphoryls of ATP was determined by mass spectrometry using established procedures (10, 19). ATP from heart extracts was purified and quantified by high performance liquid chromatography (HPLC System Gold, Beckman) using a Mono Q HR 5/5 ion-exchange column (Amersham Pharmacia Biotech). Elution was accomplished using a linear gradient of triethylammonium bicarbonate buffer (pH 8.8) from 1 to 950 mM. The  $\beta$ -phosphoryl of ATP was transferred to glycerol by a combined catalytic action of AK and glycerokinase. Samples that contained phosphoryls of  $\beta$ -ATP, as glycerol 3-phosphate, were converted to a respective trimethylsilyl derivative using Tri-Sil/BSA (Pierce) as the derivatization agent.  $^{18}\text{O}$ -Enrichment of phosphoryls in glycerol 3-phosphates was determined with a Hewlett-Packard 5973 gas chromatograph-mass spectrometer operated in the select ion-monitoring mode. Specifically, mass ions ( $m/z$ ) of 357, 359, 361, and 363 that correspond to phosphoryl species of  $^{18}\text{O}_0$ ,  $^{18}\text{O}_1$ ,  $^{18}\text{O}_2$ , and  $^{18}\text{O}_3$  were determined. The cumulative percentages of phosphoryl oxygens replaced by  $^{18}\text{O}$  in  $\beta$ -ATP were calculated as described above.

**Metabolite Levels**—Adenosine, AMP, ADP, and ATP were purified and quantified with HPLC (10). The levels of ATP, CrP, and  $\text{P}_i$  were determined using  $^{31}\text{P}$  NMR spectroscopy comparing respective peak areas with peak area of 250 nmol of methylene diphosphonate used as an internal standard (23).

**Enzyme Activity**—Frozen samples were powdered in liquid  $\text{N}_2$  with mortar and pestle and extracted with 150 mM NaCl, 60 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu\text{g}/\text{ml}$  leupeptin, and 1  $\mu\text{g}/\text{ml}$  aprotinin. Extracts were centrifuged (10 min,  $8,000 \times g$ , 4 °C), and enzyme activities measured using coupled enzyme assays with a Beckman DU 7400 spectrophotometer at 340 nm. The activity of CK was measured with a commercial kit (47-20, Sigma). The activity of AK was measured in 100 mM potassium acetate, 10 mM HEPES (pH 7.5), 20 mM glucose, 4 mM  $\text{MgCl}_2$ , 2 mM  $\text{NADP}^+$ , 2 mM ADP, 2 mM EDTA, 2 mM dithiothreitol, 4.5 units/ml hexokinase, and 2 units/ml glucose-6-phosphate dehydrogenase (10, 15). The activity of 3-phosphoglycerate kinase (PGK) was measured using a reaction mixture containing 50 mM imidazole buffer (pH 7.6), 2 mM  $\text{MgCl}_2$ , 0.1 mM EDTA, 1 mM ATP, 5 mM 3-phosphoglycerate, 0.2 mM NADH, and 5 units/ml glyceraldehyde-3-phosphate dehydrogenase. The reaction was started by addition of 40  $\mu\text{l}$  of heart extract. Changes in absorbance were recorded at 340 nm.

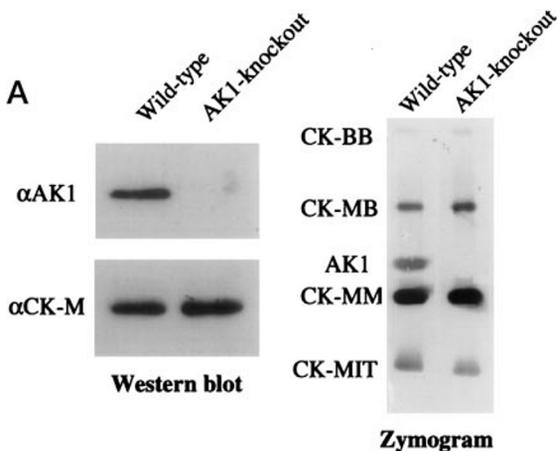
**Statistical Analysis**—Data are expressed as mean  $\pm$  S.E. The Student's *t* test for unpaired samples was used for statistical analysis, and a difference at  $p < 0.05$  was considered significant.

## RESULTS

**AK1-knockout Heart Energetics under Control Conditions**—A targeted replacement mutation in the *AK1* gene was engineered<sup>2</sup> by positioning the HygroB selection cassette in lieu of the exon 3–5 segment of the *AK1* gene, which normally encodes the ATP-binding domain of the protein (Fig. 1A). That the mutant *AK1* allele was rendered dysfunctional was confirmed in heart muscle extracts, which demonstrate lack of *AK1* mRNA,<sup>2</sup> absence of AK1 protein (Fig. 2A, left panel), and loss of AK1-related enzymatic activity (Fig. 2A, right panel). No apparent up-regulation of CK isoforms (Fig. 2A, left panel) and related enzymatic activities (Fig. 2A, right panel) was detected following deletion of AK1.

In the wild-type mouse heart, total AK activity was  $1180 \pm 220$  nmol of ATP $\cdot\text{min}^{-1}\cdot\text{mg}$  of protein $^{-1}$  (Fig. 2B). In the *AK1*-knockout, total cardiac AK activity was dramatically reduced to  $65 \pm 2$  nmol of ATP $\cdot\text{min}^{-1}\cdot\text{mg}$  of protein $^{-1}$  (Fig. 2B).

FIG. 1. *A*, structural organization of the mutant *AK1* gene. Gene-targeting with a HygroB cassette-bearing vector was used to replace the ATP binding site-coding exons 3, 4 and 5 and mutate the *AK1* gene. A schematic drawing of the exon-intron organization and positioning of the HygroB cassette in the mutant locus is provided. Coding exon segments are shown as black boxes, while white boxes represent 5'- and 3'-untranslated regions. In addition, restriction sites for the endonuclease *Bam*HI is provided. *B*, incorporation of  $^{18}\text{O}$  into high energy phosphoryls induces an isotopic shift in  $^{31}\text{P}$  NMR spectrum, a novel approach in assessing phosphotransfer rates in intact heart muscle.  $^{18}\text{O}$  induces an isotopic shift in cellular phosphoryl-containing metabolites, including those of  $\text{P}_i$ , CrP, and  $\gamma$ - and  $\beta$ -ATP. As an example, the  $^{31}\text{P}$  NMR spectra at the resonance position of CrP are presented from extracts of hearts superperfused with buffer containing either regular  $\text{H}_2^{16}\text{O}$  (*B*, left panel) or 20%  $\text{H}_2^{18}\text{O}$ -enriched water (*C*, right panel). Creatine kinase phosphotransfer rate can be monitored through the appearance of phosphoryl species of CrP containing  $^{18}\text{O}_1$  and  $^{18}\text{O}_2$ .



Thus, total AK activity in *AK1*-knockout hearts was diminished by more than 94% when compared with the wild-type. The remaining 6% of AK activity could be attributed to minor AK isoforms, such as AK2, still present in *AK1*-knockout hearts.<sup>2</sup> In fact, we observed a reduction in  $\beta$ -phosphoryl transfer by only 36% (see Fig. 4B), suggesting a marked compensatory up-regulation of phosphoryl flux through remaining minor AK isoforms.

Nucleotide levels in wild-type ( $n = 5$ ) and *AK1*-knockout ( $n = 5$ ) hearts were not significantly different (Fig. 3A). AMP was  $4.9 \pm 0.8$  and  $5.7 \pm 0.7$  nmol·mg of protein<sup>-1</sup>, ADP was  $11.1 \pm 0.8$  and  $10.9 \pm 0.5$  nmol·mg of protein<sup>-1</sup>, while ATP was  $29.3 \pm 1.6$  and  $26.2 \pm 2.8$  nmol·mg of protein<sup>-1</sup>, in the wild-type and *AK1*-knockout hearts, respectively. Moreover, the percentage of phosphoryl oxygens replaced by  $^{18}\text{O}$  in  $\gamma$ -ATP, an indicator of the ATP synthetic activity in the myocardium, was similar between wild-type ( $n = 4$ ) and *AK1*-knockout ( $n = 4$ ) hearts ( $48 \pm 5\%$  versus  $50 \pm 3\%$ ,  $p > 0.05$ ; Fig. 3B). In addition, total CK activity in wild-type and *AK1*-knockout hearts was not significantly different,  $4770 \pm 490$  ( $n = 4$ ) and  $5050 \pm 410$  ( $n = 3$ ) nmol of ATP·min<sup>-1</sup>·mg of protein<sup>-1</sup>, respectively ( $p > 0.05$ ; Fig. 3C). In fact, CK-catalyzed phosphotransfer, expressed as the percentage of phosphoryl oxygens replaced by  $^{18}\text{O}$  in CrP, was essentially the same in wild-type ( $77 \pm 3\%$ ;  $n = 4$ ) and

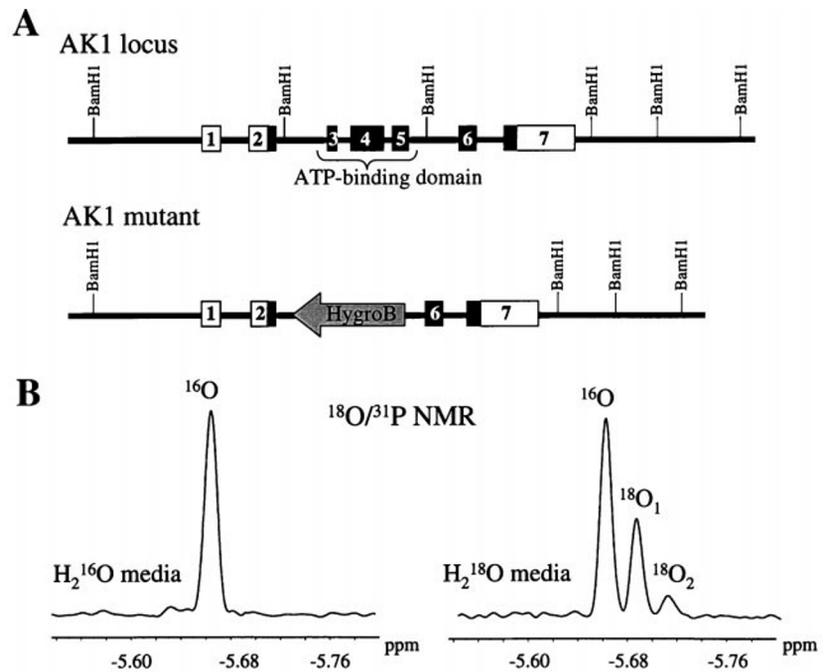


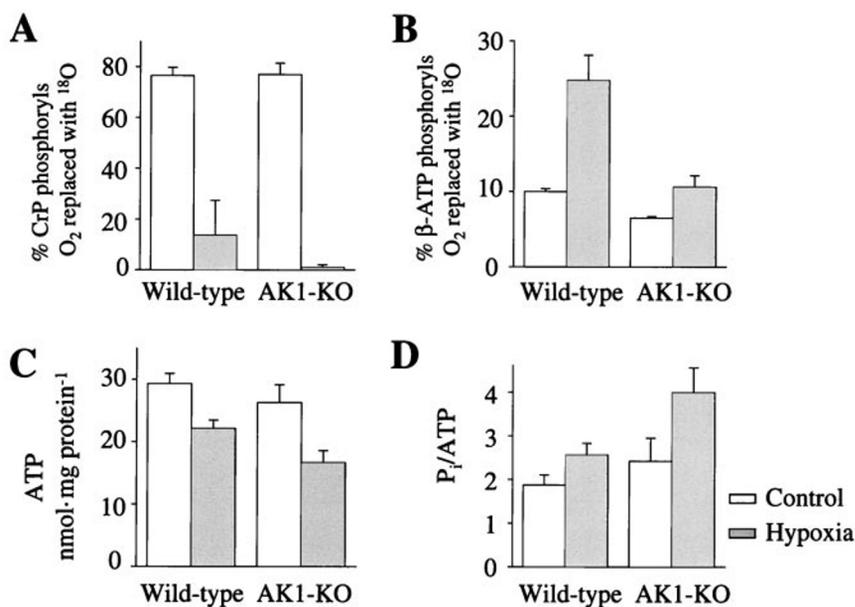
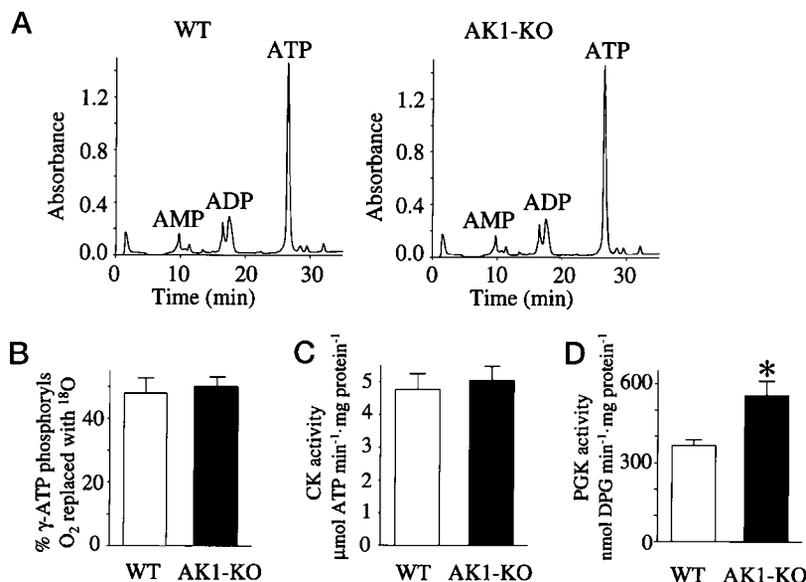
FIG. 2. *AK1*-knockout hearts lack AK1 and have reduced total AK activity. *A*, Western blot (left panel) indicates lack of AK1 protein in heart muscle of *AK1*-knockout mice. As a control, Western blot of the CK-M isoform is also shown. Zymogram analysis (right panel) of heart homogenates shows abundant AK1, along with mitochondrial (*CK-MIT*), muscle type (*CK-MM*), and muscle/brain type (*CK-MB*) creatine kinase isoenzyme activity. The brain type (*CK-BB*) is marginally expressed. Heart tissue from *AK1*-knockout mice lacks AK1 activity, but retains creatine kinase isoenzyme activities. *B*, total AK activity in extracts from wild-type ( $n = 4$ ) and *AK1*-knockout ( $n = 3$ ) hearts. AK activity was measured spectrophotometrically in the direction of ATP formation. Asterisk indicates significant difference between the two groups.

*AK1*-knockout ( $77 \pm 4\%$ ;  $n = 4$ ) hearts (Fig. 4A). However, the activity of the glycolytic phosphotransfer enzyme, PGK, was significantly increased from  $370 \pm 20$  to  $560 \pm 60$  nmol of 1,3-diphosphoglycerate·min<sup>-1</sup>·mg of protein<sup>-1</sup>, in wild-type ( $n = 4$ ) and *AK1*-knockout ( $n = 3$ ) hearts, respectively ( $p < 0.05$ ; Fig. 3D). Thus, under control conditions, deletion of the *AK1* gene, which produces marked reduction in total myocardial AK activity, did not translate into abnormal nucleotide levels or ATP production. Moreover, in *AK1*-deficient hearts, there was no change in CK activity and CK-catalyzed phosphotransfer, but rather a compensatory increase in the activity of PGK, a key phosphotransfer enzyme in the glycolytic pathway (14).

*Deficient Energetics in the AK1-knockout Heart under Hypoxic Stress*—Available evidence indicates that AK-catalyzed phosphotransfer increases under metabolic stress (14, 19). Therefore, the absence of AK1, the major AK isoform in heart muscle (4, 24), may impose an energetic disadvantage under stress conditions.

Down-regulation of the CK/CrP system is a sensitive marker of the myocardial response to hypoxic stress (25). In hypoxia, induced with the mitochondrial poison KCN (2 mM), CK-catalyzed phosphotransfer was markedly suppressed in both wild-type and *AK1*-knockout hearts (Fig. 4A). In contrast, in hypoxia AK-catalyzed phosphotransfer was increased in both wild-type

**FIG. 3. *AK1*-knockout hearts have preserved nucleotide levels, ATP turnover, and CK activity, but increased PGK activity.** *A*, HPLC chromatograms of nucleotide profiles in wild-type (*WT*; *left panel*) and *AK1*-knockout (*AK1-KO*; *right panel*) heart extracts under control conditions. *B*, percentage of  $\gamma$ -ATP phosphoryl oxygens replaced with  $^{18}\text{O}$  in wild-type ( $n = 4$ ) and *AK1*-KO hearts ( $n = 4$ ) as an indicator of total ATP production. *C*, CK activity in *WT* ( $n = 4$ ) and *AK1*-KO ( $n = 3$ ) hearts. CK activity was measured spectrophotometrically in the direction of ATP formation. *D*, PGK activity in *WT* ( $n = 4$ ) and *AK1*-KO ( $n = 3$ ) hearts. PGK activity was measured spectrophotometrically in the direction of 1,3-diphosphoglycerate formation. *Asterisk* indicates significant difference between groups.



**FIG. 4. Reduced ability of *AK1*-knockout hearts to maintain cellular energetics in hypoxia.** Hypoxia was induced by 2 mM KCN. *A*, percentage of creatine phosphate phosphoryl oxygens replaced with  $^{18}\text{O}$ , as an indicator of CK-catalyzed phosphotransfer, in wild-type ( $n = 4$ ) and *AK1*-knockout (*AK1-KO*;  $n = 4$ ) hearts under control (*open*) and hypoxic (*filled*) conditions. *B*, percentage of  $\beta$ -ATP phosphoryl oxygens replaced with  $^{18}\text{O}$ , as an indicator of AK-catalyzed phosphotransfer, in wild-type ( $n = 4$ ) and *AK1*-knockout ( $n = 4$ ) hearts under control (*open*) and hypoxic (*filled*) conditions. CK- and AK-catalyzed phosphotransfers were assessed by the [ $^{18}\text{O}$ ]phosphoryl labeling technique. *C*, ATP levels in wild-type ( $n = 5$ ) and *AK1*-knockout ( $n = 5$ ) hearts measured by  $^{31}\text{P}$  NMR under control (*open*) and hypoxic (*filled*) conditions. *D*,  $\text{P}_i/\text{ATP}$  ratio, an index of the energetic status, in wild-type ( $n = 5$ ) and *AK1*-knockout ( $n = 5$ ) hearts measured by  $^{31}\text{P}$  NMR under control (*open*) and hypoxic (*filled*) conditions. In hypoxia, ATP was significantly reduced while  $\text{P}_i/\text{ATP}$  ratio increased in *AK1*-KO compared with wild-type.

and *AK1*-knockout hearts (Fig. 4B), from  $10 \pm 0.4\%$  to  $25 \pm 3.3\%$  ( $p < 0.01$ ;  $n = 4$ ), and from  $6.5 \pm 0.2\%$  to  $11 \pm 1.5\%$  ( $p < 0.05$ ;  $n = 4$ ), respectively. Although AK-catalyzed phosphotransfer was activated by hypoxia, the increase was markedly lower in the *AK1*-knockout than wild-type, 67% and 147%, respectively (Fig. 4B). In addition, under hypoxia, ATP levels dropped to  $22.1 \pm 1.3$  in the wild-type ( $n = 5$ ), and even further, to  $16.6 \pm 1.9$  nmol·mg of protein $^{-1}$ , in *AK1*-knockout hearts ( $n = 5$ ; Fig. 4C). Thus, under hypoxic stress, ATP levels are significantly lower in *AK1*-deficient compared with wild-type hearts ( $p < 0.05$ ; Fig. 4C). Moreover, the  $\text{P}_i/\text{ATP}$  ratio, an index of cardiac energetic deficit, was significantly higher ( $4.0 \pm 0.6$  versus  $2.6 \pm 0.3$ ;  $p < 0.05$ ) in *AK1*-knockout ( $n = 5$ ) compared with wild-type ( $n = 5$ ) hearts. Thus, under hypoxic stress, a null mutation in the *AK1* gene translates into a blunted increase in AK-catalyzed phosphotransfer and is associated with lower ATP levels and higher  $\text{P}_i/\text{ATP}$  ratio.

Adenosine is a potent trigger of cardioprotective processes in the heart under metabolic stress (26–29). Although adenosine significantly increased in wild-type hearts under hypoxia (from  $3.2 \pm 1.4$  to  $7.7 \pm 1.5$  nmol·mg protein $^{-1}$ ;  $p < 0.05$ ), it remained

essentially at base-line levels in *AK1*-knockout hearts exposed to the same hypoxic stress (from  $4.2 \pm 0.8$  to  $3.3 \pm 0.6$  nmol·mg of protein $^{-1}$ ;  $p > 0.05$ ) (Fig. 5). Thus, deletion of the *AK1* gene compromises the ability of cardiac muscle to generate a cardioprotective mediator under hypoxia.

#### DISCUSSION

Although AK was discovered half a century ago and implicated in the regulation of energy metabolism (2, 14, 30), the significance of this phosphotransfer enzyme in myocardial energetic homeostasis has not been established (10). Here, using the knockout approach to delete the *AK1* gene, along with the [ $^{18}\text{O}$ ]phosphoryl oxygen exchange analysis to monitor cellular phosphotransfer dynamics, we provide direct evidence for a critical role of AK in sustaining cardiac energetics and promoting a cardioprotective response under hypoxic conditions.

Hearts from gene-targeted mice with a null mutation of the *AK1* gene lacked AK1 protein expression, which was associated with a dramatic reduction in total cardiac AK activity. This corroborates the observed absence of *AK1* gene products and AK1 activity in other tissues of these *AK1*-knockout mice.<sup>2</sup> In

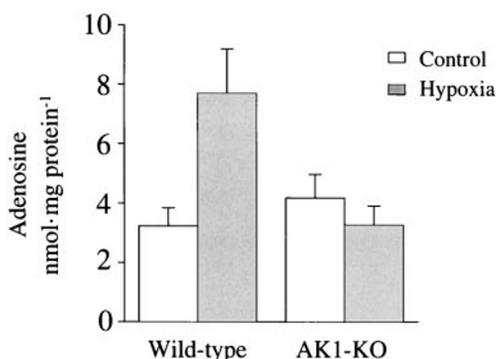


FIG. 5. **Blunted adenosine production in AK1-knockout hearts in hypoxia.** Adenosine levels in wild-type ( $n = 5$ ) and AK1-knockout ( $n = 5$ ) heart extracts measured by HPLC are shown.

the heart, under control conditions, AK1 deficiency did not translate into gross abnormalities in nucleotide levels or  $\gamma$ -ATP turnover rate. Thus, energy metabolism in AK1-deficient hearts, under normal conditions, is apparently well compensated. In this regard, the mammalian myocardium appears to adapt better than lower organisms, such as yeast, where the AK gene is essential for mitochondrial energetics and cell survival (31).

Previous studies with CK-knockout mice have uncovered a high plasticity of muscle energetic systems in adapting to genetic disruptions in energy-supply pathways (12, 13, 16). Although AK1 is the main AK isoform, heart muscle also expresses minor isoforms, in particular AK2 and AK3 (24). In fact, AK-catalyzed phosphoryl flux in AK1-knockout hearts was reduced by 36% compared with the wild type, a decrease less pronounced than that in total AK enzymatic activity, which was reduced by 94%. This may indicate that, in AK1<sup>-/-</sup> hearts, there is a compensatory increase in AK2 and AK3-catalyzed phosphotransfer, suggesting significant functional reserve in remaining AK isoforms. AK2/AK3-processed phosphoryls in mitochondria could be handed to cytosolic CK and glycolytic systems, securing delivery of high energy phosphoryls to cellular ATP-consuming sites. This is supported by data indicating a close functional interaction of AK phosphotransfer with both the CK and/or glycolytic systems (32, 33). In the present study, AK1-deficient hearts displayed no significant compensatory increase in CK-catalyzed phosphotransfer, but the activity of 3-phosphoglycerate kinase, a critical enzyme in the glycolytic phosphotransfer pathway, was significantly augmented by 51% following deletion of AK1. Thus, under control conditions, the AK1-knockout heart could maintain an apparent energetic homeostasis by adaptive up-regulation of metabolic flux through remaining AK isoforms and glycolysis. In addition, cytoarchitectural and possibly other metabolic adaptations in the AK1-knockout heart could contribute to an overall compensation for the loss of the AK1 gene.<sup>2</sup>

The extent of adaptations was, however, insufficient to sustain cardiac energetics in the AK1<sup>-/-</sup> myocardium exposed to hypoxic stress. Under hypoxic conditions, ATP levels were significantly more depressed and the P<sub>i</sub>/ATP ratio significantly increased in AK1-deficient hearts when compared with the wild-type. Observed lower ATP levels and higher P<sub>i</sub>/ATP ratio indicate a more pronounced energetic deficit (34) in the hypoxic myocardium of AK1-deficient when compared with wild-type hearts. This may suggest a unique feature of AK catalysis, essential in sustaining optimal myocardial energetics under stress.

Indeed, AK-catalyzed phosphotransfer has the exclusive ability to transfer and make available the energy of both  $\beta$ - and  $\gamma$ -phosphoryls in the ATP molecule (10, 14). This property of

AK catalysis, not shared by other phosphotransfer enzymes, could sustain cellular energetics under hypoxia, when the major energy delivery pathway catalyzed by CK is compromised (10). Moreover, in the wild type, AK flux increases in response to hypoxia, thereby preventing a rapid decline in myocardial ATP levels. In AK1-deficient hearts, however, the AK response to hypoxia is blunted, creating a significant deficit in energy transfer and reducing the ATP-regeneration potential of the hypoxic myocardium. In this regard, hearts with genetically disrupted AK1 catalysis bear similarities with failing hearts in which the disease-compromised phosphotransfer capacity, including down-regulation in AK as well as CK and glycolytic enzyme activities, precipitates ventricular dysfunction (15, 35–37).

In addition to an energetic function, AK has a distinct signaling role through generation of AMP and activation of AMP-dependent processes (9, 38, 39), including opening of ATP-sensitive potassium (K<sub>ATP</sub>) channels (7) and adenosine production (40, 41). This is of significance in view of the role that these AK-catalysis dependent events play in protecting the myocardium under hypoxic insult (37). In fact, AK gene expression is induced by hypoxia (47), and agents that increase AK activity are beneficial in preserving tissue functions under hypoxic conditions (48). Therefore, the absence of hypoxia-induced adenosine production observed here in AK1-knockout hearts may reduce the ability of AK1-deficient heart muscle to withstand hypoxic injury. Moreover, reduced AK phosphotransfer in AK1-deficient hearts may further alter the behavior of K<sub>ATP</sub> channels, which sense changes in cellular metabolism, and contribute to cellular protection (28, 42–46). In this way, the energetic disbalance of AK1-deficient hearts would be further aggravated by compromised cardioprotective signaling under metabolic stress.

In summary, this study demonstrates that AK1 is an integral component of cardiac energetic homeostasis facilitating transduction of adenine nucleotide-associated signals into cellular response to metabolic stress. Although lack of AK1 is apparently compensated under normal conditions, absence of AK1 under hypoxic stress translates into pronounced energetic deficit associated with lowered ATP levels and depressed generation of adenosine, a major endogenous cardioprotective mediator. Thus, AK-catalyzed phosphotransfer could provide a previously unrecognized target in promoting cardiac tolerance to metabolic challenge.

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