

Defective Metabolic Signaling in Adenylate Kinase AK1 Gene Knock-out Hearts Compromises Post-ischemic Coronary Reflow*

Received for publication, June 27, 2007, and in revised form, August 10, 2007 Published, JBC Papers in Press, August 17, 2007, DOI 10.1074/jbc.M705268200

Petras P. Dzeja¹, Peter Bast[‡], Darko Pucar[‡], Be Wieringa[§], and Andre Terzic^{‡2}

From the [‡]Departments of Medicine, Molecular Pharmacology and Experimental Therapeutics, and Medical Genetics, Mayo Clinic, Rochester, Minnesota 55905 and the [§]Department of Cell Biology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, 6500 HB Nijmegen, The Netherlands

Matching blood flow to myocardial energy demand is vital for heart performance and recovery following ischemia. The molecular mechanisms responsible for transduction of myocardial energetic signals into reactive vasodilatation are, however, elusive. Adenylate kinase, associated with AMP signaling, is a sensitive reporter of the cellular energy state, yet the contribution of this phosphotransfer system in coupling myocardial metabolism with coronary flow has not been explored. Here, knock out of the major adenylate kinase isoform, AK1, disrupted the synchrony between inorganic phosphate P_i turnover at ATP-consuming sites and γ -ATP exchange at ATP synthesis sites, as revealed by ^{18}O -assisted ^{31}P NMR. This reduced energetic signal communication in the post-ischemic heart. AK1 gene deletion blunted vascular adenylate kinase phosphotransfer, compromised the contractility-coronary flow relationship, and precipitated inadequate coronary reflow following ischemia-reperfusion. Deficit in adenylate kinase activity abrogated AMP signal generation and reduced the vascular adenylate kinase/creatinine kinase activity ratio essential for the response of metabolic sensors. The sarcolemma-associated splice variant AK1 β facilitated adenosine production, a function lost in the absence of adenylate kinase activity. Adenosine treatment bypassed AK1 deficiency and restored post-ischemic flow to wild-type levels, achieving phenotype rescue. AK1 phosphotransfer thus transduces stress signals into adequate vascular response, providing linkage between cell bioenergetics and coronary flow.

Coronary blood flow is tightly linked to myocardial oxygen consumption securing adequate oxygenation and nutrient delivery and preventing underperfusion (1–4). Mismatch between myocardial blood supply and energy metabolism precipitates heart dysfunction, leading to aberrant force-frequency relationships in acute coronary and chronic metabolic syn-

dromes (4–7). The no-reflow phenomenon, observed after vascular reperfusion, precipitates poor outcome (8). Despite advances made in elucidating components that regulate vascular tone, the intimate mechanisms integrating metabolic signals and securing adequate vasodilatation under stress remain partially understood (4, 9–13).

Regulation of vascular tone involves several effectors, including ATP-sensitive potassium (K_{ATP}) channels, adenosine and purinergic signaling, AMP-activated protein kinase (AMPK),³ nitric oxide (NO), and neurohormonal factors (1–4, 9–13). Intra- and extracellular nucleotide-mediated signaling pathways catalyzed by phosphotransfer enzymes may provide linkage between these otherwise distinct systems (14–16). In particular, adenylate kinase phosphotransfer couples with K_{ATP} channels, facilitating decoding of metabolic signals critical in adjusting excitability-dependent functions in response to demand (17, 19–21). Adenylate kinase, with a unique property to catalyze the reaction $2ADP \leftrightarrow ATP + AMP$, is a sensitive reporter of the cellular energy state, translating small changes in the ATP/ADP balance into relative larger changes in AMP concentration, so that AMP-regulated enzymes and metabolic sensors can respond with high sensitivity and fidelity to stress signals (15–21). Adenylate kinase interacts with creatine kinase, a parallel phosphotransfer system, conveying positive and negative signals to stress response elements (7, 19–23). An imbalance between phosphotransfer enzymes compromises signal reception by metabolic sensors and disrupts maintenance of bioenergetic homeostasis (17, 20, 23–27). Although altered phosphotransfer enzyme activities are associated with abnormal vascular tone, such as hypertension (26, 27), evidence demonstrating the significance of phosphotransfer-mediated signaling in the vascular response is still lacking (11, 28).

In myocardial and vascular tissue, AK1 is the major adenylate kinase isoform. AK1 is localized in the cytosol and myofibrils where it serves as a metabolic hub connecting minor adenylate kinase isoforms, AK3 in the mitochondrial matrix, AK2 in the intermembrane space, AK6 in the nucleus, and ecto-AK1 in the extracellular space (16, 17, 25, 29–31). The AK1 splice variant, AK1 β , is targeted to the plasma membrane and linked to K_{ATP} channel gating (32). Such strategic distribution of adenylate

* This work was supported by grants from the National Institutes of Health, Marriott Heart Disease Research Program, Marriott Foundation, Ted Nash Long Life Foundation, Netherlands Organization for Scientific Research, Council for Medical Research (NOW-GMW) Program (901-01-095) and the Dutch Cancer Society (KWF) (KUN 98-1808). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ To whom correspondence should be addressed: 200 First St. SW, Mayo Clinic, Rochester, MN 55905. Tel.: 507-284-2747; Fax: 507-266-9936; E-mail: dzeja.petras@mayo.edu.

² Holds the Mayo Clinic Marriott Family Professorship in Cardiovascular Research.

³ The abbreviations used are: AMPK, AMP-activated protein kinase; NO, nitric oxide; eNOS, endothelial NO synthase; AK1, adenylate kinase 1; KO, knockout.

kinase isoforms creates a continuous phosphotransfer network, mediating energy transfer and metabolic signaling between cell compartments and along the interstitial space (14–16). Disruption in the network function, by deletion of the *AK1* gene, lowers the muscle energetic efficiency, compromising relaxation kinetics and increasing vulnerability to stress (31, 33–35). *AK1*-deficient phenotype is characterized by a reduced maintenance of adenine nucleotide pools and inefficient signal communication to the nucleus and cytosolic metabolic sensors, including K_{ATP} channels and AMPK (17, 31, 33, 36). However, linkage between adenylate kinase-mediated AMP signaling and the regulation of vascular flow has so far not been established.

Here, analysis of the *AK1*-null mutant demonstrated that deficit in vascular and myocardial adenylate kinase catalysis blunts energetic communication, AMP, and adenosine metabolic signal transduction, compromising post-ischemic coronary reflow. These data provide the first direct evidence for the role of adenylate kinase as a metabolic monitor supporting regulation of the reactive vascular response in the stressed heart.

EXPERIMENTAL PROCEDURES

AK1 Knock-out Mice—*AK1* knock-out mice were generated from embryonic stem cells carrying a replacement mutation in the *AK1* gene (31, 33, 34). 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 *AK1* (33). Male homozygous *AK1* null (*AK1*^{-/-}) mice were compared with age- and sex-matched wild-type controls. The investigation conformed to the National Institutes of Health guidelines regulating the care and use of laboratory animals and was approved by the Institutional Animal Care and Use Committee.

Heart Perfusion—Hearts from heparinized (50 units, intraperitoneal) and anesthetized (75 mg/kg pentobarbital, intraperitoneal) mice were rapidly excised and perfused on a Langendorff apparatus with 95% O₂/5% CO₂-saturated Krebs-Henseleit (K-H) buffer (in mmol/liter: 118 NaCl, 5.3 KCl, 2.0 CaCl₂, 19 NaHCO₃, 1.2 MgSO₄, 11.0 glucose, 0.5 EDTA; 37 °C) at a perfusion pressure of 70 mmHg (34, 37). Hearts were perfused for 45 min and subjected to 30 min of no-flow normothermic ischemia followed by a 30-min-long reperfusion. Left ventricular developed pressure (LVDP), left ventricular end diastolic pressure, rate-pressure product, and heart rate were derived from the left ventricular pressure signal monitored with a fluid-filled balloon-tipped pressure transducer (Harvard Apparatus). Through the protocol, coronary flow was recorded on-line using a small animal blood flow meter (Transonics System Inc.) interphased with a data acquisition system (MP150; Biopac Systems Inc.).

¹⁸O-assisted ³¹P NMR Spectroscopy—The ¹⁸O labeling procedure is based on incorporation of one ¹⁸O atom, provided from [¹⁸O]H₂O, into P_i with each ATP hydrolysis act and the subsequent distribution of ¹⁸O-labeled phosphoryls among other high energy phosphoryl-carrying molecules (37–39). This permits monitoring of energetic dynamics with determination of ATP consumption and synthesis rates, high energy phosphoryl transfer by creatine kinase and adenylate kinase reactions, and energetic communication between intracellular

ATP hydrolysis and ATP synthesis sites (18, 33, 37–43). To follow the kinetics of ¹⁸O incorporation into phosphoryls, ¹⁸O-assisted ³¹P NMR spectroscopy, based on the ¹⁸O-induced shift in ³¹P NMR spectra, was applied (18, 37, 41). This method permits in a single run, without prior separation of phosphoryl-containing metabolites, the determination of concentrations and metabolic turnover rates of main components in the cellular energetic network and metabolic flux rates (37, 41–44). Accordingly, hearts were labeled with ¹⁸O, introduced for 30 s with the K-H buffer supplemented with 30% of ¹⁸O-labeled H₂O (Isotec) at the end of reperfusion (18, 33, 37). Hearts were freeze-clamped, pulverized under liquid N₂, and extracted in a solution containing 0.6 M HClO₄ and 1 mM EDTA. Extracts were neutralized with 2 M KHCO₃ and used to determine ¹⁸O incorporation. ¹⁸O labeling of P_i and γ -ATP was determined with ¹⁸O-assisted ³¹P NMR spectroscopy. High resolution ³¹P[¹⁶O] and ³¹P[¹⁸O] spectra were acquired at 202.5 MHz on a Bruker 11 T spectrometer (Avance) in 5-mm tubes at ambient temperature. For ³¹P spectra, signal accumulation (36,000 scans) was run without relaxation delay (acquisition time 1.61 s) with a pulse width of 10 μ s (53° angle). Incorporation of each ¹⁸O induces isotope shifts of 0.0210 and 0.0228 ppm in the ³¹P NMR spectrum of P_i and γ -ATP, respectively. During signal acquisition, proton decoupling was applied with a 506- μ s pulse width using the WALTZ-16 sequence at a 3-kHz radio frequency (37).

¹⁸O Labeling Rates and Metabolite Levels—¹⁸O labeling rates of P_i and γ -ATP were calculated as described (37–44). Percentages of ¹⁶O, ¹⁸O₁, ¹⁸O₂, and ¹⁸O₃ phosphoryl species in γ -ATP and percentages of ¹⁶O, ¹⁸O₁, ¹⁸O₂, ¹⁸O₃, and ¹⁸O₄ phosphoryl species in P_i were proportional to the integrals of their respective peaks in the ³¹P NMR spectrum (37). Cumulative percentage of ¹⁸O labeling in P_i was calculated as [%¹⁸O₁ + 2(%¹⁸O₂) + 3(%¹⁸O₃) + 4(%¹⁸O₄)]/[4(%¹⁸O in H₂O)], whereas the ¹⁸O labeling percentage values in γ -ATP was calculated as [%¹⁸O₁ + 2(%¹⁸O₂) + 3(%¹⁸O₃)]/[3(%¹⁸O in H₂O)] (18, 37). The amount of ¹⁸O incorporated into P_i and γ -ATP expressed as nmol/min/mg protein was calculated by multiplying tissue levels of P_i and ATP by corresponding fractions of labeled phosphoryls. Tissue levels of P_i and ATP were determined by ³¹P NMR using methylene diphosphonic acid as a standard (18, 37). The P_i/ γ -ATP ¹⁸O labeling ratio, an index of intracellular energetic communication (37, 42, 43), was calculated using the amount and percentage of ¹⁸O incorporated into P_i and γ -ATP. Such comparison is necessary for the proper interpretation of ¹⁸O labeling results (37–40).

Vascular Tissue Harvest—Thoracic aorta (arch and descending segments) was isolated from heparinized and anesthetized mice, washed in oxygenated heart perfusion medium, and cleared of connective tissue and fat (33). Tissue was frozen by immersion in liquid nitrogen and stored in liquid nitrogen until processing. To simulate ischemia *in vitro*, portions of aortic tissue samples were transferred into deoxygenated heart perfusion medium saturated with 95% N₂ and 5% CO₂ at 37 °C for 10 min and snap-frozen in liquid nitrogen.

Cardiomyocyte and Sarcolemma Isolation—Adult cardiomyocytes and sarcolemma fractions from mice hearts were isolated as described (17, 23). Hearts were homogenized (in mM:

Adenylate Kinase and Coronary Reflow

10 HEPES, 1 EGTA, 1 dithiothreitol, 1 aprotinin, 0.2 phenylmethylsulfonyl fluoride, and 1 $\mu\text{g}/\text{ml}$ leupeptin, pH 7.4) and were spun at $5,000 \times g$. Supernatant was centrifuged at $100,000 \times g$ and membrane pellets suspended by sonication in (in mM) 20 HEPES, pH 7.4, 140 NaCl, 5 KCl, 2 MgCl_2 , 0.5 dithiothreitol, 1 aprotinin, 0.2 phenylmethylsulfonyl fluoride, and 2 $\mu\text{g}/\text{ml}$ leupeptin. Sarcolemmal fraction was purified by sucrose density gradient centrifugation, and the degree of enrichment was determined based on sodium, K-ATPase activity (23). Adenylate kinase-mediated generation of AMP from added 0.2 mM ADP and subsequent adenosine production by 5'-nucleotidase present in cardiac sarcolemma was measured using high performance liquid chromatography (18, 33).

Immunocytochemistry—Localization of the membrane-associated AK1 β isoform was detected using the anti-AK1 β antibody (CIM Antibody Core, University of Texas-Southwestern), the Alexa Fluor 568 secondary antibody (Molecular Probes), and a standard immunostaining protocol (32).

Enzyme Activities and Metabolite Levels—Blood vessel tissue was ground in liquid nitrogen and extracted with buffer containing (in mM) 150 NaCl, 60 Tris-HCl, pH 7.5, 5 EDTA, 1 phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ aprotinin, and 0.2% Triton X-100 and centrifuged ($10,000 \times g$, 4 $^\circ\text{C}$). Adenylate kinase activity was measured with a Beckman DU 7400 spectrophotometer at 340 nm in medium containing (in mM) 100 K^+ -acetate, 20 HEPES, pH 7.5, 20 glucose, 4 MgCl_2 , 2 NADP^+ , 1 EDTA, 1 dithiothreitol, 2 ADP, 4.5 units/ml hexokinase, and 2 units/ml glucose-6-phosphate dehydrogenase (17, 33). Creatine kinase activity was measured using the CK-20 reagent kit (Sigma). Adenine nucleotide and nucleoside levels were determined in perchloric acid extracts using high performance liquid chromatography (33, 44). Freeze-clamped hearts or aortic tissues were pulverized in mortar with liquid nitrogen and extracted in a solution containing 0.6 M HClO_4 and 1 mM EDTA (33, 37). Proteins were pelleted by centrifugation ($15,000 \times g$, 10 min) and protein content determined with a DC Protein Assay kit (Bio-Rad). Extracts were neutralized with 2 M KHCO_3 and analyzed using high performance liquid chromatography (33, 44).

Statistical Analysis—Data are expressed as mean \pm S.E. Student's *t* test was used for statistical analysis and $p < 0.05$ predetermined.

RESULTS

AK1 Knock Out Mitigates Energetic Signal Communication—The difference in the kinetics of P_i and $\gamma\text{-ATP}$ ^{18}O labeling, detected by ^{18}O -assisted ^{31}P NMR spectroscopy (Fig. 1), indicates the degree of synchronization between ATP consumption and ATP production sites. Control hearts, not subjected to ischemia-reperfusion, demonstrate a $\text{P}_i/\gamma\text{-ATP}$ labeling ratio of 1.2–1.5 expressed in mass units of incorporated ^{18}O and 0.90–0.95 expressed as percentage of phosphoryl oxygens replaced by ^{18}O (37, 42, 43), indicating efficient energetic communication between ATP consumption and ATP production sites. In ischemia-reperfusion-challenged wild-type hearts, perfused with medium containing $\text{H}_2[^{18}\text{O}]$, the $\text{P}_i/\gamma\text{-ATP}$ labeling ratio was 3.83 ± 0.50 , expressed in mass units of ^{18}O -incorporated in P_i , and $\gamma\text{-ATP}$ versus 0.65 ± 0.08 , expressed in percentage of

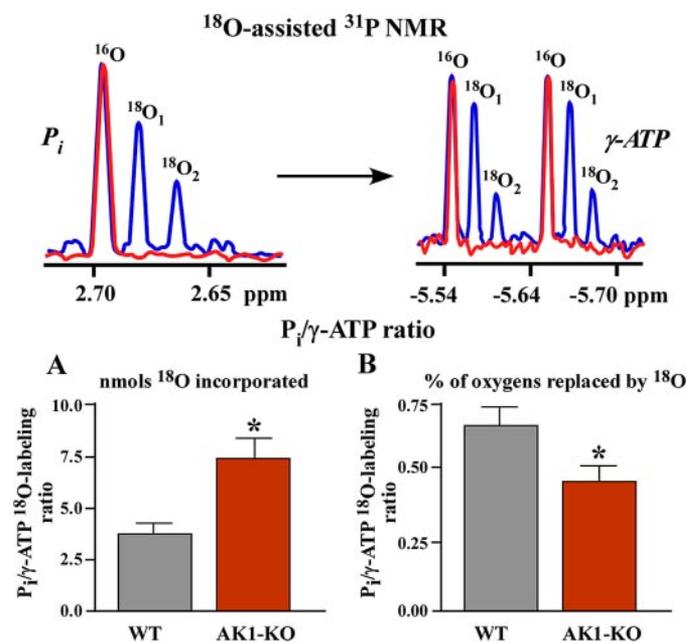


FIGURE 1. Disturbed intracellular energetic communication in AK1-KO hearts after ischemia-reperfusion assessed by ^{18}O -assisted ^{31}P NMR. Upper panel, ^{18}O -assisted ^{31}P NMR recordings of ^{18}O -induced shift (blue) in ^{31}P NMR spectra of P_i (red) and $\gamma\text{-ATP}$ (blue and red, respectively) indicating incorporation of one and two atoms of ^{18}O and metabolic activity of these energetic intermediates. Lower panels, A and B, changes in $\text{P}_i/\gamma\text{-ATP}$ labeling ratios (by ^{18}O mass (A) and by percentage of ^{18}O (B) incorporated into corresponding phosphoryls) in wild-type (WT) and adenylate kinase knock-out (AK1-KO) hearts after ischemia-reperfusion. Increased ^{18}O mass in P_i compared with $\gamma\text{-ATP}$ (A) along with diminished percentage labeling ratio (B) indicates disrupted communication between ATP consumption and ATP synthesis sites in AK1-KO hearts.

phosphoryl oxygens replaced by ^{18}O ($n = 5$; Fig. 1A). In the AK1 null mutant, with an adenylate kinase-catalyzed phosphotransfer rate blunted by 60% (34), the $\text{P}_i/\gamma\text{-ATP}$ labeling ratio expressed in mass units increased to 7.44 ± 0.80 while the percentage ratio decreased to 0.46 ± 0.06 ($n = 4$) compared with wild type ($p < 0.05$; Fig. 1B). This indicates that AK1 knock out causes accumulation of ^{18}O -labeled P_i species at ATPases and ineffective delivery to ATP synthesis or $\gamma\text{-ATP}$ labeling sites, indicating disturbed energetic signal communication.

Myocardial Energetics Versus Coronary Flow—In wild-type hearts, correlation coefficients of energetic parameters reflecting ATPase and adenylate kinase velocities, P_i , and $\beta\text{-ADP}$ and $\beta\text{-ATP}$ turnover rates (34) versus basal coronary flow were 0.58, 0.58 and 0.53. A significant negative correlation, $r = -0.97$ ($p < 0.05$, $n = 5$), was observed between myocardial GTP levels (34) and basal coronary flow, indicating distribution of control among energetic signaling systems. AK1 knock-out hearts had a higher correlation coefficient for ATPase velocity or P_i ^{18}O labeling and basal coronary flow, $r = 0.80$, and a negative correlation between creatine kinase velocity or creatine phosphate ^{18}O labeling, creatine phosphate levels (34), and basal coronary flow, $r = -0.85$ and $r = -0.95$ ($p < 0.05$, $n = 4$), indicating imbalanced phosphotransfer signaling. Post-ischemic coronary flow recovery of AK1 knock-out hearts correlated with $\beta\text{-ADP}$ and $\beta\text{-ATP}$ turnover rates (34), reflecting the remaining adenylate kinase isoform velocity, $r = 0.98$ and $r = 0.99$ ($p < 0.05$, $n = 4$), respectively. This tight relationship suggests that adenylate kinase phosphotransfer becomes rate-limiting in the myocar-

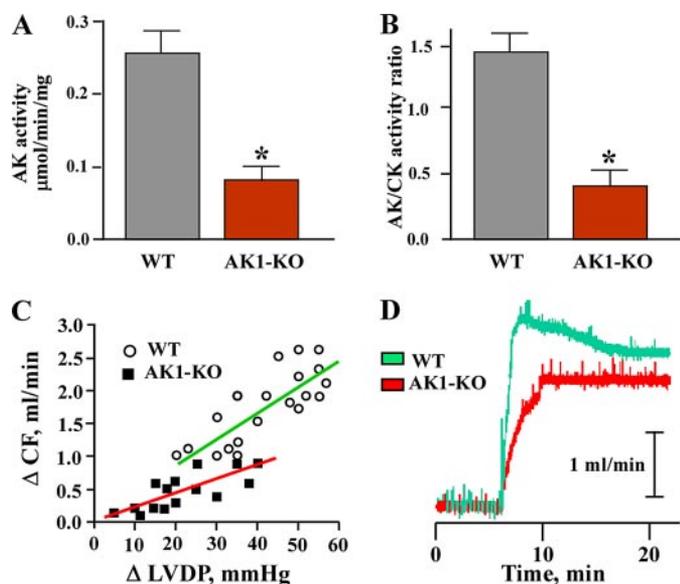


FIGURE 2. Adenylate kinase AK1 gene knock out reduces vascular AK activity, shallows the relationship between coronary flow and heart contractility, and compromises coronary reflow after ischemia-reperfusion. A and B, compared with wild-type (WT), adenylate kinase knock-out (AK1-KO) hearts display markedly diminished AK activity in vascular (aorta) tissue along with a decreased AK/creatine kinase (CK) activity ratio, indicating cellular phosphotransfer imbalance. C, the relationship between natural fluctuations in heart contractility (left ventricle developed pressure, Δ LVDP) and increments in coronary flow (Δ CF) is dampened in AK1-KO hearts. D, coronary reflow after ischemia-reperfusion is blunted in AK1-KO hearts (red) compared with WT counterparts (green).

dial-vasculature energetic communication in ischemia-injured hearts.

AK1 Deficiency Alters the Heart Contractility-Flow Relationship and Abrogates Coronary Reflow after Ischemia-Reperfusion—The AK1 null mutant displayed reduced vascular adenylate kinase activity, at $0.08 \pm 0.02 \mu\text{mol}/\text{min}/\text{mg}$ ($n = 5$) compared with $0.25 \pm 0.04 \mu\text{mol}/\text{min}/\text{mg}$ ($n = 4$) in wild type ($p < 0.001$; Fig. 2A). Vascular creatine kinase activity, the parallel phosphotransfer pathway, was essentially unchanged at $0.17 \pm 0.04 \mu\text{mol}/\text{min}/\text{mg}$ in wild type ($n = 4$) and $0.19 \pm 0.05 \mu\text{mol}/\text{min}/\text{mg}$ protein in AK1 knockouts ($n = 5$). However, the adenylate kinase/creatine kinase activity ratio was significantly diminished, from 1.45 ± 0.12 in wild type to 0.42 ± 0.13 in AK1 knockouts ($p < 0.01$; Fig. 2B). AK1 knock-out hearts demonstrated a dampened oscillation of contractility and coronary flow, which occurs in the wild type and has been related to alternations of myocardial metabolic cycles (11, 45). While the relationship between changes in heart contractility above base line and corresponding increments in coronary flow was steep in wild-type hearts (Fig. 2C), the slope was significantly shallower in AK1-knock-out hearts, indicating altered signal communication from the myocardium to the vasculature. The slope of the left ventricular developed pressure-coronary flow relation was halved, from 0.040 ± 0.007 in wild-type to 0.021 ± 0.004 in AK1 knock-out hearts (Fig. 2C). Despite this difference in the heart contractility-flow relationship, basal coronary flow under well oxygenated conditions was on average similar among groups. However, recovery of flow after ischemia-reperfusion was markedly compromised in AK1 knock-out hearts (Fig. 2D).

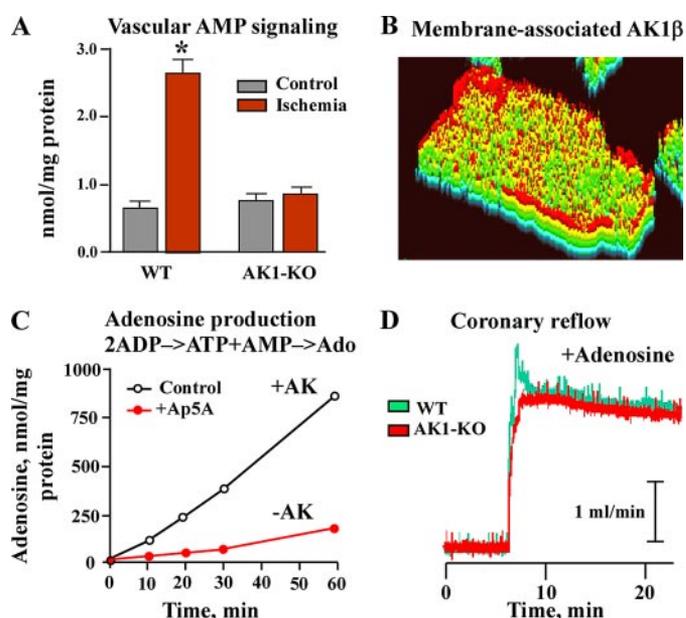


FIGURE 3. Adenylate kinase deficiency reduces vascular AMP signaling and cardiac membrane adenosine generation, while supplementation of adenosine rescues disrupted coronary reflow in AK1-KO hearts. A, generation of vascular (aorta) AMP signal is markedly diminished in AK1-KO mice in response to ischemia. B, isolated myocytes stained with anti-AK1 β antibody. Color-transformed image indicates predominantly sarcolemmal localization of the AK1 β splice isoform (red). C, isolated sarcolemma demonstrates bound AK activity (AK1 β splice isoform) that facilitates adenosine production from added 0.2 mM ADP. Inhibition of AK activity with a specific inhibitor, diadenosine pentaphosphate (Ap₅A, 25 μM), abrogated adenosine production. D, compromised reflow in AK1-KO hearts after ischemia-reperfusion can be rescued by perfusion with adenosine (50 μM).

Basal coronary flow was $2.83 \pm 0.31 \text{ ml}/\text{min}$ ($n = 5$) and $2.68 \pm 0.24 \text{ ml}/\text{min}$ ($n = 4$) in weight-matched wild-type and AK1 knock-out hearts, respectively. Following ischemia-reperfusion, recovery of flow essentially reached pre-ischemic levels, *i.e.* $98 \pm 4.0\%$, in the wild type but remained at $67 \pm 5.0\%$ of pre-ischemic levels in AK1-deficient hearts ($p < 0.05$). Thus, a reduced adenylate kinase activity and an altered adenylate kinase/creatine kinase activity ratio in the setting of AK1 deficiency contribute to inadequate coronary reflow after ischemia-reperfusion.

Defective Vascular AMP and Adenosine Signaling in AK1 Knock-out Mice—Deletion of the AK1 gene reduced the generation of vascular AMP signaling in response to ischemia (Fig. 3A). In isolated wild-type aorta, AMP levels increased from $0.65 \pm 0.08 \text{ nmol}/\text{mg}$ protein in well oxygenated conditions to $2.56 \pm 0.24 \text{ nmol}/\text{mg}$ protein ($n = 5$, $p < 0.01$) after 10 min of simulated ischemia. However, AK1 knockouts lacked this response as AMP levels remained essentially unchanged, $0.78 \pm 0.09 \text{ nmol}/\text{mg}$ protein in normoxic conditions and $0.93 \pm 0.11 \text{ nmol}/\text{mg}$ protein ($n = 4$) following 10 min of ischemia. The AK1 gene produces two transcripts, the cytosolic AK1 isoform and the membrane-associated AK1 β splice variant (32). Immunocytochemistry, using a specific AK1 β antibody, demonstrated a predominant sarcolemmal distribution of the AK1 β isoform (Fig. 3B). The membrane-associated adenylate kinase-generated AMP is efficiently transformed by 5'-nucleotidase into adenosine, a mediator implicated in the vascular response to metabolic stress (Fig. 3C). Inhibition of sarcolem-

Adenylate Kinase and Coronary Reflow

mal adenylate kinase activity with the specific inhibitor diadenosine pentaphosphate (Ap_5A) abrogated adenosine production. The rate of adenosine production was 18.3 ± 1.2 nmol/min/mg protein in the presence of adenylate kinase activity and 4.1 ± 0.3 nmol/min/mg protein in its absence ($n = 3, p < 0.01$). Thus, adenylate kinase deficiency compromises vascular AMP signal generation and adenosine efflux in the stressed myocardium.

Rescue of Defective Phenotype in AK1 Knock-out Hearts—To test whether addition of adenosine could rescue disrupted coronary reflow in AK1 knock-out hearts, perfusion medium was supplemented with adenosine. Addition of adenosine ($50 \mu M$) nullified the difference in recovery of coronary flow between wild-type and AK1-deficient hearts following ischemia-reperfusion (Fig. 3C). Coronary flow, in the presence of adenosine, was 3.65 ± 0.31 ml/min in wild-type ($n = 4$) and 3.60 ± 0.28 ml/min in AK1 knock-out ($n = 4$) hearts. Adenosine supplementation thus rescues defective coronary flow in AK1 knock-out hearts, bypassing the deficit in adenylate kinase-mediated generation of AMP/adenosine and the malfunction in linkage between cell bioenergetics and coronary flow.

DISCUSSION

The molecular components supporting metabolic linkage between the ischemic myocardium, experiencing oxygen and nutrient deficit, and the vascular response, adjusting flow to meet energy demand, are only partially understood. Here, we identify adenylate kinase as a necessary element in facilitating energy communication and transduction of metabolic signals from the myocardium to the vasculature, regulating the contractility-flow relationship and coronary reflow in the post-ischemic heart.

Efficient intracellular and intercellular communication of energetic and metabolic signals is critical for maintenance of cell homeostasis and induction of adequate functional response (5–7, 14–25). Using the ^{18}O -assisted ^{31}P NMR technique, we demonstrate that energetic communication between ATPases and ATP generation sites is compromised in AK1-deficient hearts, as indicated from altered P_i/γ -ATP ^{18}O labeling ratios (37). These findings are in accord with the function of adenylate kinase-catalyzed phosphorelay to support communication between mitochondria and myofibrils, nucleus or membrane metabolic sensors (17, 19, 22, 43, 44, 46). Thus, deletion of AK1 mitigates intracellular metabolic signaling disrupting integration and synchronization of ATP-consuming and ATP-producing processes in response to stress.

This study also demonstrates that along with defective myocardial intracellular energetic communication, AK1 deficiency blunts vascular AMP signal generation. Moreover, deficiency in AK1 and the membrane-associated AK1 β splice variant reduced adenosine generation, critical in the vascular response. Deletion of AK1 also diminishes myocardial AMP and adenosine generation (31, 34). Thus, metabolic signaling is compromised in both tissues, preventing cross-talk in response to stress. Poor coronary reflow in AK1 knock-out hearts after ischemia-reperfusion was rescued with adenosine treatment, which bypassed AK1 deficiency and restored post-ischemic flow to wild-type levels. Adenosine protects against vascular

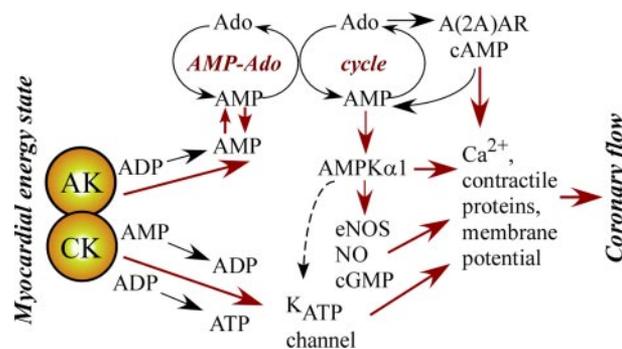


FIGURE 4. Summary of the myocardial-vascular metabolic signal transduction cascades initiated by phosphotransfer redistribution between adenylate kinase (AK) and creatine kinase (CK) governing the AMP/adenosine (Ado) cycle and the response of metabolic sensors (ATP-sensitive potassium channel, K_{ATP} , and AMP-activated protein kinase, AMPK). Hypoxia or metabolic stress diminishes CK and increases AK flux, inducing AMP generation and subsequent AMP/adenosine signaling events. Adenosine/AMP signals delivered to vascular tissue through intercellular and paracellular pathways induce signaling through adenosine receptors A(2A)AR, AMPK α 1, and K_{ATP} channels. AMPK α 1 activates eNOS, inducing NO/cGMP signaling, and could regulate K_{ATP} channels. Collectively, A(2A)AR, AMPK α 1, eNOS, and K_{ATP} signaling converge on contractile protein, Ca $^{2+}$, and membrane potential regulation, critical determinants of vascular tone.

dysfunction via A1 receptors and determines coronary flow via A2A and A2B receptors (1, 47). Also, adenosine present in the extracellular space can be transported back into myocytes where it is phosphorylated to AMP and ADP by adenylate kinase and creatine kinase, respectively (48). Such changes in nucleotide distribution would alter intracellular metabolic dynamics that govern ion channels and contractile behavior as well as the activity of energy-sensing protein kinases (7, 11, 25, 48–52) and, thus, coronary vasoreactivity. Although we cannot exclude altered nitric oxide signaling and/or other mechanisms, that adenosine supplementation rescued deficient reflow in AK1 knock-out hearts suggests that defective AMP/adenosine signaling directly contributes to the abnormal phenotype.

In the heart, under normoxia creatine kinase suppresses adenylate kinase phosphotransfer by scavenging cellular ADP and maintaining K_{ATP} channels closed with low signaling through the AK \rightarrow AMP \rightarrow AMPK and AK \rightarrow AMP \rightarrow adenosine systems (Fig. 4) (7, 19–21, 25). Hypoxia or metabolic stress diminishes creatine kinase and increases adenylate kinase flux, inducing AMP generation and downstream AMP/adenosine signaling (18, 23). Deletion of AK1 shifts this balance toward creatine kinase, compromising metabolic signaling and coronary reflow after ischemia-reperfusion. This is supported by the observed shift in the negative correlation of coronary flow toward the creatine kinase system in AK1 knock-out hearts. AK1 deficiency compromises metabolic signal reception by metabolic sensors such as K_{ATP} channels and AMPK (17–21, 36), and altered adenylate and creatine kinase phosphotransfer activities are associated with abnormal vascular tone (26, 27). The significance of adenylate kinase-mediated myocardial to vascular metabolic signaling is underscored by the tight association between ATP and ADP β -phosphoryl turnover and recovery of coronary flow following ischemia, as well as by a compromised contractility-flow relationship in AK1-deficient hearts.

Muscles from AK1-deficient mice display increased glycolytic enzyme activity and mitochondrial volume along with specific variations in transcripts involved in glycolysis and mitochondrial metabolism and in gene products defining structural and myogenic events (31, 33, 34, 53). Such wide scale remodeling in the AK1 knock-out muscles suggests a compensatory response capable of reducing, and not exuberating, the energetic burden caused by AK1 gene deficiency (17, 43, 53). Electron microscopy and gene array analysis, as well as energetic and functional ischemia-reperfusion studies, do not indicate increase in tissue damage, changes in apoptotic signaling, or deficit in contractile recovery in the AK1 knockout compared with wild type (33, 53). The fact that adenosine addition rescued coronary flow defects caused by AK1 deletion suggests that vascular viability is not compromised (54, 55). Thus, changes in AMP/adenosine signaling in the AK1-deficient environment are critical in defining the outcome of metabolic regulation on vascular tone.

Intrinsic regulation of vascular tone depends on the arrangement of the cellular energetic system and on the distinct contribution of ATP-producing and phosphotransfer pathways (1, 4–7, 26–28, 48, 50, 56). These pathways regulate energy-dependent steps responsible for coupling vascular metabolism with contraction, including myosin ATPase, myosin light chain kinase, and ATP-dependent Ca^{2+} pump (4, 11, 28, 50, 56, 57). Our data demonstrate that vascular tissue displays a comparable activity of adenylate and creatine kinase phosphotransfer systems that maintain a balance of positive and negative signals to metabolic sensors and glycolytic enzymes (17, 20, 23). Glycolytic metabolism, which supports membrane ATPases and ion channel activity, has a privileged role in vascular contractile reactivity and vasorelaxation (56, 57). In this regard, the vascular metabolic signal transduction cascade would be initiated by phosphotransfer redistribution between adenylate kinase and creatine kinase, governing AMP/adenosine signaling and the response of metabolic sensors, such as K_{ATP} channels and AMPK (Fig. 4) (7, 17–26, 51, 52). Subsequently, regulation of vascular tone would proceed through modulation of the resting membrane potential and Ca^{2+} influx, which is partially governed by K_{ATP} channels (2, 3, 7, 9, 58, 59). Recent studies indicate that knock out of vascular K_{ATP} channels disrupts coupling of coronary vasoreactivity with myocardial energy demand (58, 59). K_{ATP} channel or adenosine receptor blockade does not, however, abolish coronary vasodilatation (3, 56), indicating the existence of parallel signaling pathways (12, 48).

Signals originating in the myocardium must be transmitted to the vasculature to adjust coronary flow (1–4). Coupled intracellular and extracellular (ecto-AK) adenylate kinases regulate nucleotide equilibrium and propagate nucleotide-based signals at cellular and intercellular distances (14, 16, 19, 46). The heart also possesses a potent AMP-adenosine cycle/relay operating through the myocardium and vascular tissue (Fig. 4) (60, 61). Adenosine signals are tightly regulated in cardiomyocytes and vascular endothelial cells. As such, adenosine is constantly released and taken up through adenosine transporters (61). Inside the cell, adenosine is converted to AMP by the adenosine kinase while intracellular AMP levels are regulated by adenylate kinase (19, 61). Normally this cycle operates without, or with

little, output of adenosine to vascular tissue. Metabolic imbalances trigger AK-mediated AMP generation and release of adenosine (61). This signal is modulated by transporters and by the adenylate kinase reaction to prevent excess of adenosine signaling, regimenting the coronary response. Disruption in tuning of adenosine signals by adenosine transporter inhibitors results in excessive adenosine signaling unleashing maximal vascular response, *i.e.* the coronary flow reserve (4, 62). Delivered to the vasculature, adenosine can act through endothelial and smooth muscle adenosine receptors to generate cyclic AMP, which signals to the Ca^{2+} transport machinery (Fig. 4). Consequently cyclic AMP could be converted to AMP by phosphodiesterase, thus contributing to metabolic signaling through AMPK and endothelial NO synthase (eNOS) pathways (63, 64). In this regard, coronary flow in AK1-deficient hearts remains responsive to exogenous adenosine signals, indicating an intact intrinsic vascular endothelium and smooth muscle signal reception system.

Recent data indicate that extracellular adenosine can activate AMPK through a mechanism that requires uptake of adenosine and conversion to AMP (65). Vasorelaxation induced by activation of AMPK is abolished in AMPK $\alpha 1^{-/-}$ (major vascular isoform), but not in AMPK $\alpha 2^{-/-}$ -deficient, mice (66). It is also known that AMPK can phosphorylate and activate eNOS (10), which contributes to elevated eNOS activity and subsequent NO production (64). However, inhibition of eNOS does not prevent AMPK activation-induced aortic relaxation (66), suggesting direct effects of AMP signaling on smooth muscle cells. In this regard, inhibition of NO synthesis attenuates coronary dilation during adenosine infusion or pacing-induced increase in metabolic demand (67). Thus, AK1 deficiency and changes in adenine nucleotide metabolism could impair eNOS activation and NO generation. Collectively with the present data, emerging evidence suggests the existence of an integrated metabolic signaling system securing transmission and tuning of energetic signals from the myocardium to vasculature (Fig. 4).

In summary, myocardial and vascular adenylate kinase phosphotransfer is necessary in facilitating intracellular and intercellular energetic communication and AMP metabolic signal transduction, regulating myocardial-vasculature cross-talk and coronary reflow after ischemia-reperfusion. In this way, adenylate kinase is a pivotal monitor of cellular energetic imbalances, generating and transmitting a feedback reply to metabolic sensors to adjust vascular blood flow.

Acknowledgment—We thank Dr. Slobodan Macura, Mayo Clinic Analytical Nuclear Magnetic Resonance Facility, for the use of the spectrometer.

REFERENCES

- Berne, R. M. (1980) *Circ. Res.* **47**, 807–813
- Daut, J., Klieber, H. G., Cyrus, S., and Noack, T. (1994) *Cardiovasc. Res.* **28**, 811–817
- Ishibashi, Y., Duncker, D. J., Zhang, J., and Bache, R. J. (1998) *Circ. Res.* **82**, 346–359
- Tune, J. D., Gorman, M. W., and Feigl, E. O. (2004) *J. Appl. Physiol.* **97**, 404–415
- Dzeja, P. P., Redfield, M. M., Burnet, J. C., and Terzic, A. (2000) *Curr.*

- Cardiol. Rep.* **2**, 212–217
6. Ingwall, J. S. (2004) *J. Mol. Cell. Cardiol.* **37**, 613–623
 7. Saks, V., Dzeja, P., Schlattner, U., Vendelin, M., Terzic, A., and Wallimann, T. (2006) *J. Physiol.* **571**, 253–273
 8. Ito, H. (2001) *J. Cardiol.* **37**, 39–42
 9. Melchert, P. J., Duncker, D. J., Traverse, J. H., and Bache, R. J. (1999) *Am. J. Physiol.* **277**, H617–H625
 10. Morrow, V. A., Fougelle, F., Connell, J. M., Petrie, J. R., Gould, G. W., and Salt, I. P. (2003) *J. Biol. Chem.* **278**, 31629–31639
 11. Rubin, L. J., Magliola, L., Feng, X., Jones, A. W., and Hale, C. C. (2005) *J. Appl. Physiol.* **98**, 296–306
 12. Kakkar, R., Ye, B., Stoller, D. A., Smelley, M., Shi, N. Q., Galles, K., Hadhazy, M., Makielski, J. C., and McNally, E. M. (2006) *Circ. Res.* **98**, 682–689
 13. Guns, P. J., Korda, A., Crauwels, H. M., Van Assche, T., Robaye, B., Boeynaems, J. M., and Bult, H. (2005) *Br. J. Pharmacol.* **146**, 288–295
 14. Yegutkin, G. G., Henttinen, T., and Jalkanen, S. (2001) *FASEB J.* **15**, 251–260
 15. Dzeja, P. P., and Terzic, A. (2003) *J. Exp. Biol.* **206**, 2039–2047
 16. Picher, M., and Boucher, R. C. (2003) *J. Biol. Chem.* **278**, 11256–11264
 17. Carrasco, A. J., Dzeja, P. P., Alekseev, A. E., Pucar, D., Zingman, L. V., Abraham, M. R., Hodgson, D., Bienenraeber, M., Puceat, M., Janssen, E., Wieringa, B., and Terzic, A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 7623–7628
 18. Pucar, D., Dzeja, P. P., Bast, P., Gumina, R. J., Drahl, C., Lim, L., Juranic, N., Macura, S., and Terzic, A. (2004) *Mol. Cell. Biochem.* **256–257**, 281–289
 19. Dzeja, P. P., Zeleznikar, R. J., and Goldberg, N. D. (1998) *Mol. Cell. Biochem.* **184**, 169–182
 20. Dzeja, P. P., and Terzic, A. (1998) *FASEB J.* **12**, 523–529
 21. Selivanov, V. A., Alekseev, A. E., Hodgson, D. M., Dzeja, P. P., and Terzic, A. (2004) *Mol. Cell. Biochem.* **256–257**, 243–256
 22. Dzeja, P. P., Bortolon, R., Perez-Terzic, C., Holmuhamedov, E. L., and Terzic, A. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 10156–10161
 23. Abraham, M. R., Selivanov, V. A., Hodgson, D. M., Pucar, D., Zingman, L. V., Wieringa, B., Dzeja, P. P., Alekseev, A. E., and Terzic, A. (2002) *J. Biol. Chem.* **277**, 24427–24434
 24. Hodgson, D. M., Zingman, L. V., Kane, G. C., Perez-Terzic, C., Bienenraeber, M., Ozcan, C., Gumina, R. J., Pucar, D., O’Cochlain, F., Mann, D. L., Alekseev, A. E., and Terzic, A. (2003) *EMBO J.* **22**, 1732–1742
 25. Dzeja, P. P., and Terzic, A. (2006) in *Creatine Kinase* (Vial, C., ed) pp. 195–221, Nova Science, New York
 26. Brewster, L. M., Mairuhu, G., Bindraban, N. R., Koopmans, R. P., Clark, J. F., and van Montfrans, G. A. (2006) *Circulation* **114**, 2034–2039
 27. Seccia, T. M., Atlante, A., Vulpis, V., Marra, E., Passarella, S., and Pirrelli, A. (1998) *Clin. Exp. Hypertens.* **20**, 345–358
 28. Ishida, Y., Riesinger, I., Wallimann, T., and Paul, R. J. (1994) *Mol. Cell. Biochem.* **133–134**, 39–50
 29. Kurokawa, Y., Takenaka, H., Sumida, M., Oka, K., Hamada, M., and Kuby, S. A. (1990) *Enzyme* **43**, 57–71
 30. Tanabe, T., Yamada, M., Noma, T., Kajii, T., and Nakazawa, A. (1993) *J. Biochem.* **113**, 200–207
 31. Pucar, D., Janssen, E., Dzeja, P. P., Juranic, N., Macura, S., Wieringa, B., and Terzic, A. (2000) *J. Biol. Chem.* **275**, 41424–41429
 32. Janssen, E., Kuiper, J., Hodgson, D., Zingman, L. V., Alekseev, A. E., Terzic, A., and Wieringa, B. (2004) *Mol. Cell. Biochem.* **256–257**, 59–72
 33. Janssen, E., Dzeja, P. P., Oerlemans, F., Simonetti, A. W., Heerschap, A., de Haan, A., Rush, P. S., Terjung, R. R., Wieringa, B., and Terzic, A. (2000) *EMBO J.* **19**, 6371–6381
 34. Pucar, D., Bast, P., Gumina, R. J., Lim, L., Drahl, C., Juranic, N., Macura, S., Janssen, E., Wieringa, B., Terzic, A., and Dzeja, P. P. (2002) *Am. J. Physiol.* **283**, H776–H782
 35. Hancock, C. R., Janssen, E., and Terjung, R. L. (2005) *Am. J. Physiol.* **288**, C1287–C1297
 36. Hancock, C. R., Janssen, E., and Terjung, R. L. (2006) *J. Appl. Physiol.* **100**, 406–413
 37. Pucar, D., Dzeja, P. P., Bast, P., Juranic, N., Macura, S., and Terzic, A. (2001) *J. Biol. Chem.* **276**, 44812–44819
 38. Dawis, S. M., Walseth, T. F., Deeg, M. A., Heyman, R. A., Graeff, R. M., and Goldberg, N. D. (1989) *Biophys. J.* **55**, 79–99
 39. Zeleznikar, R. J., and Goldberg, N. D. (1991) *J. Biol. Chem.* **266**, 15110–15119
 40. Dzeja, P. P., Zeleznikar, R. J., and Goldberg, N. D. (2002) *J. Biol. Chem.* **271**, 12847–12851
 41. Dzeja, P. P., Pucar, D., Bast, P., Juranic, N., Macura, S., and Terzic, A. (2002) *MAGMA* **14**, 180–181
 42. Gumina, R. J., Pucar, D., Bast, P., Hodgson, D. M., Kurtz, C. E., Dzeja, P. P., Miki, T., Seino, S., and Terzic, A. (2003) *Am. J. Physiol.* **284**, H2106–H2113
 43. Janssen, E., Terzic, A., Wieringa, B., and Dzeja, P. P. (2003) *J. Biol. Chem.* **278**, 30441–30449
 44. Dzeja, P. P., Terzic, A., and Wieringa, B. (2004) *Mol. Cell. Biochem.* **256–257**, 13–27
 45. Sutherland, F. J., Baker, K. E., Shattock, M. J., and Hearse, D. J. (2003) *Clin. Exp. Pharmacol. Physiol.* **30**, 879–884
 46. Dzeja, P. P., and Terzic, A. (2006) in *Handbook of Neurochemistry and Molecular Neurobiology: Brain Energetics. Integration of Molecular and Cellular Processes* (Gibson, G., and Diemel, G., eds) pp. 641–666, Springer, New York
 47. Flood, A. J., Willems, L., and Headrick, J. P. (2002) *Cardiovasc. Res.* **55**, 161–170
 48. Evans, A. M., Mustard, K. J., Wyatt, C. N., Peers, C., Dipp, M., Kumar, P., Kinnear, N. P., and Hardie, D. G. (2005) *J. Biol. Chem.* **280**, 41504–41511
 49. Dzeja, P. P., Vitkevicius, K. T., Redfield, M. M., Burnett, J. C., and Terzic, A. (1999) *Circ. Res.* **84**, 1137–1143
 50. Savabi, F., Geiger, P. J., and Bessman, S. P. (1986) *Biochem. Med. Metab. Biol.* **35**, 227–238
 51. Frederich, M., and Balschi, J. A. (2002) *J. Biol. Chem.* **277**, 1928–1932
 52. Hardie, D. G. (2004) *J. Cell Sci.* **117**, 5479–5487
 53. Janssen, E., de Groof, A., Wijers, M., Fransen, J., Dzeja, P. P., Terzic, A., and Wieringa, B. (2003) *J. Biol. Chem.* **278**, 12937–12945
 54. Hayashi, M., Kim, S. W., Imanaka-Yoshida, K., Yoshida, T., Abel, E. D., Eliceiri, B., Yang, Y., Ulevitch, R. J., and Lee, J. D. (2004) *J. Clin. Investig.* **113**, 1138–1148
 55. Ikeno, F., Inagaki, K., Rezaee, M., and Mochly-Rosen, D. (2007) *Cardiovasc. Res.* **73**, 699–709
 56. Paul, R. J. (1989) *Annu. Rev. Physiol.* **51**, 331–349
 57. Pastorino, J. G., and Hoek, J. B. (2003) *Curr. Med. Chem.* **10**, 1535–1551
 58. Miki, T., Suzuki, M., Shibasaki, T., Uemura, H., Sato, T., Yamaguchi, K., Koseki, H., Iwanaga, T., Nakaya, H., and Seino, S. (2002) *Nat. Med.* **8**, 466–472
 59. Kane, G. C., Lam, C. F., O’Cochlain, F., Hodgson, D. M., Reyes, S., Liu, X. K., Miki, T., Seino, S., Katusic, Z. S., and Terzic, A. (2006) *FASEB J.* **20**, 2271–2280
 60. Kroll, K., Decking, U. K., Dreikorn, K., and Schrader, J. (1993) *Circ. Res.* **73**, 846–856
 61. Decking, U. K., Schlieper, G., Kroll, K., and Schrader, J. (1997) *Circ. Res.* **81**, 154–164
 62. Nitenberg, A., and Antony, I. (1995) *Eur. Heart J.* **16**, 7–21
 63. Morrison, R. R., Talukder, M. A., Ledent, C., and Mustafa, S. J. (2002) *Am. J. Physiol.* **282**, H437–H444
 64. Zhang, Y., Lee, T. S., Kolb, E. M., Sun, K., Lu, X., Sladek, F. M., Kassab, G. S., Garland, T., Jr., and Shyy, J. Y. (2006) *Arterioscler. Thromb. Vasc. Biol.* **26**, 1281–1287
 65. da Silva, C. G., Jarzyna, R., Specht, A., and Kaczmarek, E. (2006) *Circ. Res.* **98**, e39–47
 66. Goirand, F., Solar, M., Athea, Y., Viollet, B., Mateo, P., Fortin, D., Leclerc, J., Hoerter, J., Ventura-Clapier, R., and Garnier, A. (2007) *J. Physiol.* **581**, 1163–1171
 67. Jones, C. J., Kuo, L., Davis, M. J., DeFily, D. V., and Chilian, W. M. (1995) *Circulation* **91**, 1807–1813