Glucose Metabolism and Energy Homeostasis in Mouse Hearts Overexpressing Dominant Negative $\alpha_2$ Subunit of AMP-activated Protein Kinase*

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AMP-activated protein kinase (AMPK) is an energy-sensing enzyme that plays a pivotal role in regulating cellular metabolism for sustaining energy homeostasis under stress conditions. Activation of AMPK has been observed in the heart during acute and chronic stresses, but its functional role has not been completely understood because of the lack of effective activators and inhibitors of this kinase in the heart. We generated transgenic mice (TG) with cardiac-specific overexpression of a dominant negative mutant of the AMPK $\alpha_2$ catalytic subunit to clarify the functional role of this kinase in myocardial ischemia. In isolated perfused hearts subjected to a 10-min ischemia, AMPK $\alpha_2$ activity in wild type (WT) increased substantially (by 4.5-fold), whereas AMPK $\alpha_2$ activity in TG was similar to the level of WT at base line. Basal AMPK $\alpha_1$ activity was unchanged in TG and increased normally during ischemia. Ischemia stimulated a 2.5-fold increase in 2-deoxyglucose uptake over base line in WT, whereas the inactivation of AMPK $\alpha_2$ in TG significantly blunted this response. Using $^{31}$P NMR spectroscopy, we found that ATP depletion was accelerated in TG hearts during no-flow ischemia, and these hearts developed left ventricular dysfunction manifested by an early and more rapid increase in left ventricular end-diastolic pressure. The exacerbated ATP depletion could not be attributed to impaired glycolytic ATP synthesis because TG hearts consumed slightly more glycogen during this period of no-flow ischemia. Thus, AMPK $\alpha_2$ is necessary for maintaining myocardial energy homeostasis during ischemia. It is likely that the functional role of AMPK in myocardial energy metabolism resides both in energy supply and utilization.

The AMP-activated protein kinase (AMPK)3 functions as a fuel gauge, such that when the cell is exposed to stresses associated with energy depletion, it switches off ATP-utilizing pathways and switches on ATP-generating pathways to restore energy homeostasis (1, 2). This kinase is activated by decreases in ATP/AMP and in phosphocreatine (PCr)/creatinine through Thr$^{172}$ phosphorylation by one or more upstream kinases (AMPK kinase) and through allosteric modification by AMP (1, 2). AMPK is a heterotrimeric protein consisting of a catalytic subunit (\(\alpha\)) and two regulatory subunits (\(\beta\) and \(\gamma\)) (1, 3). Each subunit has two or more different isoforms; the \(\alpha_1\) subunit is widely expressed, whereas the \(\alpha_2\) subunit is expressed primarily in liver, heart, and skeletal muscle (2, 4, 5).

It has been suggested that AMPK regulates glucose and fatty acid metabolism in striated muscles (6, 7). Studies from our group and others showed increased AMPK activity during acute and chronic stresses, such as hypoxia and exercise in skeletal muscle and ischemia and pressure overload in the heart (8–12). Activation of AMPK in the heart is associated with enhanced glucose uptake and glycolysis (10, 11, 13). As glycolysis is a major source of ATP during ischemia, stimulation of glucose uptake and glycolysis by AMPK in the ischemic heart is consistent with the overall function of this enzyme in restoring cellular energy levels during stress. To establish a causal role of AMPK for these stress responses, however, inhibition of AMPK is required during stress. This has not been possible because of the lack of a specific inhibitor of AMPK in the heart. Furthermore, the inability to block AMPK activation during ischemia makes it difficult to test whether AMPK also functions to preserve energy by reducing ATP consumption by the heart during ischemia.

In the present study, we sought to inhibit AMPK activity by generating transgenic mice (TG) overexpressing a dominant negative mutant of the AMPK $\alpha_2$ catalytic subunit in the heart. This approach led to a selective inhibition of AMPK $\alpha_2$ activity in the heart. Here we report that AMPK $\alpha_2$ mediates critical cellular responses in maintaining energy homeostasis in the ischemic heart.

EXPERIMENTAL PROCEDURES

Generation of Transgenic Mice—A full-length cDNA of rat AMPK $\alpha_2$ subunit was tagged at the 5’ end with a HA epitope. The aspartic acid at residue 157 was changed to alanine to render the catalytic subunit inactive (14, 15). The TG mice with cardiac-specific overexpression of

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the mutant α2 subunit were generated by injecting the recombinant DNA construct, regulated by a mouse α-myosin heavy chain promoter, into fertilized Friend virus B-type mouse oocytes. Transgenic mouse founders were identified by the polymerase chain reaction-based method, and transgene expression was confirmed by Western blotting of the HA tag. Three transgenic lines were established. Mice from F1 and F2 generations were used for this study, and TG mice were compared with their wild type (WT) littermates. All the procedures were approved by the Institutional Animal Care and Use Committee of the Harvard Medical School.

Isolated Perfused Heart Experiments—Hearts were perfused in the Langendorff mode with phosphate-free Krebs-Henseleit buffer containing (in millimoles/liter) NaCl (118), NaHCO3 (25), KCl (5), CaCl2 (2.5), MgSO4 (1.2), EDTA (0.5), glucose (10), and pyruvate (0.5) as described previously (16). All hearts were perfused with a constant perfusion pressure of 80 mm Hg, and the left ventricular function was continuously monitored using a water-filled balloon (16). Fig. 1 illustrates the protocols for isolated perfused heart experiments. After stabilization, one base-line 31P NMR spectrum was collected (16), and one-half of the hearts were subjected to a 10-min no-flow ischemia and the other half to a 10-min normal perfusion. During ischemia, four consecutive 2-min 31P NMR spectra were collected to monitor the dynamic changes in high energy phosphate content. At the end of the 10-min period, a subgroup of hearts was freeze-clamped for biochemical analysis, and the rest were reperfused with a buffer in which glucose was replaced with 5 mM 2-deoxyglucose (2-DG). Five consecutive 4-min 31P NMR spectra were collected for determination of the time-dependent accumulation of 2-DG-phosphate. The rate of glucose uptake was estimated by the slope of the fitted line as described previously (11, 17). During 2-DG perfusion, 1.2 mM KH2PO4 and 5 mM pyruvate were supplied to replenish the intracellular inorganic phosphate pool and to maintain ATP synthesis. AMPK Activity Assay and Western Blotting—Freeze-clamped heart samples were homogenized as described previously, and lysates were used for AMPK activity assays and for Western blotting (18). AMPK activity was measured after immunoprecipitation 200 μg of protein using antibodies made against the amino acid sequences 339–358 of rat AMPK α1, 352–366 of α2, and 2–16 of both α1 and α2 (pan-α) (18). The kinase reaction was done using synthetic peptide with sequence HMR-SAMSGLHLVKRR as substrate, and AMPK activity is expressed as incorporated ATP (picomoles) per mg of protein per min (19). Western blotting was done with antibodies against AMPK α1, α2, pan-α, HA (Roche Diagnostics), GLUT1, GLUT4 (Chemicon, Intl., Inc., Temecula, CA), SERCA2 (Affinity BioReagents, Golden, CO), and Na+/Ca2+ exchanger (Swant, Bellinzona, Switzerland).

HPLC Measurements and Glycogen Assay—Freeze-clamped tissues were used for determination of myocardial content of adenine nucleotides, nucleosides, and purine bases by a HPLC method as reported previously (20). Myocardial glycogen content was determined by measuring the amount of glucose released from glycogen by use of an alkaline extraction to separate glycogen and exogenous glucose (21). Glucose content in the extract was measured using a Sigma assay kit.

Data Analysis and Statistics—Myocardial ATP content obtained by HPLC was converted to [ATP] assuming an intracellular water content of 0.48 ml and a protein content of 0.15 g/g of blotted wet tissue (22). The mean value of [ATP] for WT or TG hearts was used to calibrate the ATP peak area of the base-line 31P NMR spectrum. Concentrations of other metabolites were calculated using the ratio of their peak areas to the ATP peak area, and intracellular pH (pHi) was determined by the chemical shift of inorganic phosphate (Pi) relative to PCr (20). The values of [ATP] and [PCr] in the ischemic hearts were obtained from summed spectra of 3–4 hearts. Each data point represents the average of four summed results from a total of 13 hearts. Differences in results obtained from WT and TG hearts were compared by 2-tailed Student's t test or one-way factorial ANOVA. Changes during ischemia and 2-DG perfusion were compared by repeated-measures ANOVA. All the statistical analyses were performed with Statview (Brainpower Inc.), and a value of p < 0.05 was considered significant.

RESULTS

Expressing Dominant Negative α2 Subunit Resulted in Isoform-Specific Inhibition of AMPK Activity—The mutant α2 subunit of AMPK was expressed in TG hearts as evidenced by the presence of HA-tagged protein (Fig. 2A). The expression of mutant α2 subunit caused an upward shift of the AMPK α2 band (due to the HA tagging). The presence of a single shifted AMPK α2 band in the TG hearts suggests that the native AMPK α2 protein is replaced by the mutant protein (Fig. 2A). Because monomeric α subunit protein is unstable and has a short life span (23), those endogenous α subunits that were displaced by the mutant protein and failed to bind with β and γ subunits are likely to be degraded. In contrast to the changes in AMPK α2, the amount of AMPK α1 was unchanged in the TG hearts (Fig. 2B). AMPK α2 activity was reduced in the TG hearts at base line by 78% (Fig. 3A). Ischemia increased AMPK α2 activity by
at base line, and it increased normally in response to ischemia (Fig. 3B). These findings suggest that the dominant negative transgenic approach used here resulted in specific inhibition of AMPK α2 activity in the heart. Blocking the activation of AMPK α2 during ischemia led to a 65% reduction in total AMPK activity in the heart (Fig. 3C), suggesting that AMPK α2 is a major contributor to ischemia-stimulated AMPK activity in the heart.

**General Characteristics and Cardiac Function of the TG Mice**—Table I summarizes the base-line characteristics of the mice used for this study. There was no difference in the body weight and heart weight between TG and WT mice. The TG mice were grossly normal, and no premature death was observed up to 1 year (data not shown). Left ventricular contractile function at base line was similar for TG and WT hearts, suggesting that inhibition of AMPK α2 activity does not alter cardiac function during normal perfusion. When subjected to ischemia, however, the TG hearts showed a more rapid increase in left ventricular end-diastolic pressure (Fig. 4, **LVEDP**). This is unlikely because of a decrease in the amount of calcium-handling proteins in TG hearts. The protein levels for SERCA2 and Na⁺/Ca²⁺ exchanger were unchanged in TG hearts (SERCA2: 31 ± 1 versus 32 ± 2 absorbance units; and Na⁺/Ca²⁺ exchanger: 79 ± 2 versus 76 ± 2 absorbance units for WT and TG, respectively, n = 4 in each group). The rapid increase in left ventricular end-diastolic pressure is consistent with the observation that ATP depletion was accelerated during ischemia in TG hearts (see below), implying a faster development of myofibril rigor force in these hearts.

**High Energy Phosphate Content**—Dynamic changes in high energy phosphate content during ischemia were monitored by $^{31}$P NMR spectroscopy and shown in Fig. 5. Pre-ischemic values of [PCr], [ATP], and [P] are not different in WT and TG hearts. [PCr] decreased rapidly in both groups during ischemia; the PCr peak was no longer detectable in TG hearts after 4 min of ischemia, whereas a very low but detectable PCr peak remained for 2 more minutes in WT hearts. The rate and extent of ATP depletion was also accelerated in TG hearts; [ATP] decreased by 80 and 55% from the pre-ischemic value in TG and WT hearts, respectively (p < 0.05). The [P] rose markedly (by 10-fold) during ischemia. The pH decreased significantly in both groups, but pH$_i$ was lower in WT hearts than in TG hearts at the end of ischemia (6.40 ± 0.03 and 6.58 ± 0.05, respectively, p < 0.05).

Table II shows ATP degradation and accumulation of adenine nucleosides and purine bases during ischemia measured by HPLC. Myocardial content of adenine nucleotides was similar in WT and TG hearts during base-line perfusion. At the end of a 10-min ischemia, ATP content in TG and WT hearts decreased by 80 and 60% from the base-line value, respectively (p < 0.05). Thus, the measurement by HPLC was consistent with the NMR measurement. Increased ATP degradation in the TG hearts during ischemia resulted in a tendency for

**Table I**

| General characteristics and base-line cardiac function |
|--------------------------------------------------------|
| **WT** (n = 50)                                       |
| **TG** (n = 41)                                       |
| Age (weeks)                                           | 15.8 ± 1.3 | 17.3 ± 2.3 |
| Body weight (g)                                       | 26.6 ± 0.5 | 26.4 ± 0.7 |
| Heart weight (mg)                                     | 82 ± 6.4   | 82 ± 1.5   |
| LVSP (mm Hg)                                          | 108 ± 19   | 111 ± 20   |
| LVEDP (mm Hg)                                         | 7.3 ± 1.3  | 7.4 ± 1.4  |
| HR (bpm)                                              | 324 ± 56   | 316 ± 58   |
| CF (ml/min)                                           | 3.1 ± 1    | 2.8 ± 1    |

Fig. 3. **AMPK activity in the hearts.** Hearts were freeze-clamped after 10 min of no-flow ischemia or normal perfusion for measurement of AMPK α2 (A), α1 (B), and total (C) activity as described under “Experimental Procedures.” Data are means ± S.E., n = 4–6 per group; *, p < 0.05.
higher content of AMP, adenosine, and inosine compared with WT (Table II).

Glucose Metabolism—During base-line perfusion, TG hearts showed normal rates of 2-DG uptake (Fig. 6) and normal glycogen content (Table III). The protein content of GLUT1 and GLUT4, two primary glucose transporters in the heart, was also unchanged in TG hearts (Fig. 7). These results suggest that AMPK α2 has a minimum effect on glucose metabolism in hearts under unstressed conditions.

In WT hearts, ischemia caused a 2.5-fold increase in the rate of 2-DG uptake. In contrast, 2-DG uptake increased by only 1.7-fold in TG hearts after ischemia, representing a 62% reduction in the response to ischemia (Fig. 6). Thus, blocking the activation of AMPK α2 in the ischemic heart significantly blunted the increase in myocardial glucose uptake.

During no-flow ischemia, glycogen is the only substrate for anaerobic glycolysis that generates ATP. To assess glycogen consumption during ischemia, we determined the differences in average myocardial glycogen content before and after ischemia (Table III). ATP produced from glycogen consumption was estimated assuming that each glucose molecule derived from glycogen gave three molecules of ATP. Interestingly, glycogen content in TG hearts at the end of ischemia was lower than WT hearts, and ATP generated during ischemia was slightly greater in TG hearts. These results indicate that accelerated ATP depletion in TG hearts during ischemia is not because of impaired ATP generation via glycolysis.

DISCUSSION

There are three major findings in this study. First, overexpressing the dominant negative α2 subunit of AMPK in mouse hearts results in isoform-specific inhibition of AMPK α2 activity. Second, blocking the activation of AMPK α2 dramatically reduces ischemia-stimulated glucose uptake in the heart. Third, inhibition of AMPK α2 activity during ischemia leads to an accelerated depletion of ATP and exacerbated diastolic dysfunction, possibly because of increased energy consumption. These results demonstrate that AMPK α2 regulates energy metabolism in the ischemic hearts by modulating both energy supply and expenditure.

Selective Inhibition of AMPK α2 Activity—Little is known about the isoform-specific characteristics of the AMPK heterotrimers. In this study, we sought to inhibit AMPK activity by generating transgenic mice with cardiac-specific overexpression of a kinase-inactive mutant of the α2 subunit (D157A) (14, 15). Interestingly, overexpression of this mutant α2 subunit results in substantial and selective replacement of the native α2 subunit while leaving the α1 subunit unaffected. Accordingly, AMPK α1 activity in TG hearts remains unchanged and responds normally during ischemia. We found that total AMPK activity, measured by immunoprecipitation with an antibody against pan-α-AMPK was reduced by two thirds, supporting the notion that AMPK α2 contributes the majority of AMPK activity under these conditions. In a transgenic mouse model described previously, in which a different mutation of α2 subunit (R45K) was overexpressed in skeletal muscle (24), the authors reported that both native α subunits were replaced by the mutant. This apparent discrepancy between the two models raises the possibility that skeletal muscle differs from cardiac muscle in subunit composition and/or subcellular localization of AMPK heterotrimers. Selective inhibition of AMPK α2 activity in the heart, as observed in our model, offers a unique opportunity to examine isoform-specific function of AMPK in the heart.

AMPK α2 and Glucose Metabolism—AMPK has emerged as a potential mediator of increased glucose uptake in response to energy depletion. Pharmacological activation of AMPK by 5-aminoimidazole-4-carboxamide ribonucleoside results in increased glucose uptake in both skeletal and cardiac muscle in an insulin-independent fashion (13, 25, 26). Furthermore, a close relationship between increased AMPK activity and enhanced glucose uptake has been observed under a variety of stress conditions (8). Because ischemia also stimulates glucose uptake through an insulin-independent mechanism (27–29), the role of AMPK in this event is strongly implied. Yet, the effort of defining a causal relationship between AMPK activation and increased glucose uptake during ischemia has been hindered by the lack of an effective AMPK inhibitor for the heart. Using the transgenic approach to prevent AMPK activation, here we provide the first direct evidence that AMPK α2 plays a critical role in ischemia-stimulated glucose uptake. Our results also show that AMPK α2 is not the sole mediator for ischemia-stimulated glucose uptake. The finding that AMPK α2-specific TG hearts have a partial reduction in ischemia-stimulated glucose uptake raises the possibility that AMPK α1 is also important in the regulation of glucose uptake in the heart. Our study, however, does not rule out the possibility that other mechanisms in addition to AMPK are also responsible for ischemia-stimulated glucose uptake.

It has been suggested that AMPK contributes to enhanced glycolysis during ischemia by activating 6-phosphofructo-2-ki-
nase, the enzyme responsible for the synthesis of fructose 2,6-bisphosphate, a potent stimulator of glycolysis (10). In this study, we found a greater breakdown of glycogen in TG hearts during no-flow ischemia, suggesting that AMPK α2 activity is not required for stimulating glycolysis under our experimental conditions. Because the total AMPK activity increased partially in the ischemic TG hearts due to the activation of AMPK α1, it is possible that this increase is sufficient to activate 6-phosphofructo-2-kinase and hence stimulate glycolysis. In addition, 6-phosphofructo-2-kinase can be phosphorylated and activated by other kinases such as protein kinase A (30). Furthermore, glycolysis is also regulated by the concentrations of adenine nucleotides and intracellular pH (31, 32). Both accelerated depletion of ATP and reduced acidosis in TG hearts would favor increased glycolytic flux. Considering the essential role of glycolysis for myocardial survival during ischemia, it is conceivable that redundant signaling mechanisms exist for stimulation of glycolysis.

**Role of AMPK α2 in Energy Homeostasis during Ischemia—**

We found normal content of high energy phosphate and adenine nucleotides in the TG hearts under base-line conditions. This observation, together with the apparent normal glucose metabolism in unstressed TG hearts, support the notion that the primary function of AMPK is to regulate cellular response to stress. By subjecting hearts to ischemia, we observed a more rapid decline of ATP in TG hearts. This finding suggests that AMPK α2 plays a critical role in sustaining energy homeostasis in stressed hearts. Importantly, the exacerbated ATP degradation cannot be explained by decreased ATP synthesis via glycogenolysis. Because the no-flow ischemia protocol applied in this study does not allow utilization of energy substrates other than glycogen, our results likely indicate increased ATP consumption by TG hearts during ischemia.

It has been shown that in the liver AMPK reduces energy consumption by switching off synthetic reactions in the cell (1, 2, 15). It has not been determined if AMPK mediates energy conservation mechanisms in the heart. The majority of energy consumed by the heart supports contractile function, i.e. the
myosin ATPase reaction (33, 34). During our ischemic protocol, the heart stops contracting in less than 1 min. Increased ATP depletion in TG hearts under this condition likely reflects increased ATP consumption for noncontractile function, predominantly for maintaining basal metabolism and ion homeostasis (33, 34). On this note, we found a slower decline in pHi indicating a reduced accumulation of intracellular H+ in TG hearts. This is in contrast to the observation that degradation of ATP and glycogen is accelerated in TG hearts, which would lead to increased H+ production during ischemia. Thus, it is likely that the TG heart is more active in exporting H+. Taken together, our findings suggest that AMPK α2 plays a role in energy conservation in the ischemic heart, possibly by modifying the ion transport process.

In summary, inactivation of AMPK α2 causes accelerated ATP depletion and early development of myocardial contracture and leads to decreased glucose uptake in response to ischemia. These findings suggest that AMPK plays a critical role in sustaining energy homeostasis and myocardial protection during ischemia, possibly by modulating cellular functions for both energy supply and utilization.

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