Reduction in Hexokinase II Levels Results in Decreased Cardiac Function and Altered Remodeling After Ischemia/Reperfusion Injury


Rationale: Cardiomyocytes switch substrate utilization from fatty acid to glucose under ischemic conditions; however, it is unknown how perturbations in glycolytic enzymes affect cardiac response to ischemia/reperfusion (I/R). Hexokinase (HK) II is a HK isofrom that is expressed in the heart and can bind to the mitochondrial outer membrane.

Objective: We sought to define how HKII and its binding to mitochondria play a role in cardiac response and remodeling after I/R.

Methods and Results: We first showed that HKII levels and its binding to mitochondria are reduced 2 days after I/R. We then subjected the hearts of wild-type and heterozygote HKII knockout (HKII+/-) mice to I/R by coronary ligation. At baseline, HKII+/- mice have normal cardiac function; however, they display lower systolic function after I/R compared to wild-type animals. The mechanism appears to be through an increase in cardiomyocyte death and fibrosis and a reduction in angiogenesis; the latter is through a decrease in hypoxia-inducible factor–dependent pathway signaling in cardiomyocytes. HKII mitochondrial binding is also critical for cardiomyocyte survival, because its displacement in tissue culture with a synthetic peptide increases cell death. Our results also suggest that HKII may be important for the remodeling of the viable cardiac tissue because its modulation in vitro alters cellular energy levels, O2 consumption, and contractility.

Conclusions: These results suggest that reduction in HKII levels causes altered remodeling of the heart in I/R by increasing cell death and fibrosis and reducing angiogenesis and that mitochondrial binding is needed for protection of cardiomyocytes. (Circ Res. 2011;108:60-69.)

Key Words: hexokinase | ischemia/reperfusion | mitochondria | vascular endothelial growth factor (VEGF) | apoptosis

The process of glycolysis starts with glucose uptake by glucose transporters (GLUTs) and phosphorylation by hexokinases (HKs). The reaction catalyzed by HKs maintains the concentration gradient required for GLUTs to facilitate the transport of glucose into the cell.1-4 The major GLUTs in the heart are GLUT1 and GLUT4. GLUT1 transgenic mice are resistant to heart failure from pressure overload,5,6 and GLUT4 knockout mice display an increase in hypertrophy.5 However, it is not known how alterations of HKs would affect cardiac response to ischemia/reperfusion (I/R). There are 4 mammalian HK isoforms: HKI, HKII, HKIII, and HKIV, which is also known as glucokinase.7,8 Whereas HKI is ubiquitously expressed, HKII is primarily expressed in skeletal and cardiac muscle and fat tissue.9 The expression of HKII is regulated by insulin at transcriptional level, and its overexpression in tissue culture results in protection against oxidant-induced cell death.10,11
HKII contains an N-terminal, 21-aa sequence that forms a hydrophobic α helix\(^\text{12,13}\) and enables HKII to bind to the outer mitochondrial membrane.\(^\text{14–17}\) HKII mitochondrial translocation increases in response to insulin and ischemia; a phenomenon also observed with cardioprotective treatments such as ischemia preconditioning (IPC) and morphine.\(^\text{18,19}\) When the endogenous HKII is displaced from the mitochondria, cells become more susceptible to an injurious insult.\(^\text{11,20,21}\) Furthermore, overexpression of full-length HKII leads to greater protection than a mutant construct that lacks the mitochondrial binding domain.\(^\text{11}\) The binding to mitochondria may also allow preferable access of HKII to mitochondrially generated ATP\(^\text{22}\) and reduce cellular production of reactive oxygen species.\(^\text{11,23}\) This latter function may potentially contribute to the protective effects of HKII.

Although it is well demonstrated that cardiomyocytes switch substrates under ischemic conditions, it is unknown how a reduction in glycolytic enzymes alters cardiac function in response to I/R. We chose to study the effects of a reduction in HKII on cardiac response to I/R injury in intact hearts and to characterize the mechanism for this process. We first showed that the levels of HKII and its binding to mitochondria are decreased in response to I/R. HKII\(^{-/-}\) hearts have normal cardiac function at baseline; however, when they are subjected to I/R, they display increased cardiac dysfunction both in vivo and ex vivo. The mechanism for the lower systolic function appears to be mediated by an increase in cell death and cardiac fibrosis and a reduction in angiogenesis. The decrease in angiogenesis is through a reduction in hypoxia-inducible factor (HIF)-mediated vascular endothelial growth factor (VEGF) production. HKII binding to mitochondria is important for cardiomyocyte survival, because its displacement in vitro results in an increase in cell death at baseline and in the absence of any injurious agents. Furthermore, modulation of HKII expression in isolated cardiomyocytes results in changes in ATP production, O\(_2\) consumption, and contractility, suggesting that HKII may be an important player in cardiac remodeling by improving energetic and contractility of the viable cells. These results suggest that HKII and its mitochondrial binding play a role in cardiomyocyte survival and that a decrease in HKII levels in the heart worsens cardiac function after I/R by increasing cardiomyocyte death and fibrosis and reducing angiogenesis.

**Methods**

HKII\(^{-/-}\) mice were mated with wild-type (WT) (C57BL/6J) to generate HKII\(^{+/+}\) and HKII\(^{-/-}\) mice. Experiments were performed on 10- to 12 week-old female and male mice weighing 20 to 25 g. All mice were maintained and handled in accordance with the Northwestern Animal Care and Use Committee and by the animal ethics committee of the Academic Medical Center, Amsterdam, The Netherlands. Animals were initially bred with WT C57BL/6J mice (The Jackson Laboratory) and subsequently with WT offspring and were backcrossed with C57BL/6J for at least 10 generations. The genomic DNA was prepared using the PureGene DNA isolation kit according to the protocol of the manufacturer (Gentra Systems). Approximately 10 ng of the genomic DNA was used for PCR using primers against the HKII genomic DNA. The offspring carrying disrupted HKII alleles was identified by PCR amplification with forward primer HK2KO-F2 (5’-ACTCTCCTGCGCCCTG-3’) and reverse primers Neo-R1 (5’-TGCCAGTCATAGCGAATAGC-3’) and HK2KO-R1 (5’-CCC-CTC-ATC-GCC-ACC-GC-3’).

Neonatal rat cardiomyocytes (NRCMs) were prepared from 1- to 2-day-old Sprague–Dawley rats and cultured as described previously.\(^\text{24}\) Statistical significance was assessed with ANOVA and the unpaired Student t test.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Results**

**Total and Mitochondrial-Bound HKII Levels Are Reduced in Response to I/R**

To assess the effects of ischemia and oxidant stress on the total and mitochondrial-bound HKII levels, we exposed NRCMs to H\(_2\)O\(_2\) (20 \(\mu\)mol/L, 6 hours) or doxorubicin (1 \(\mu\)mol/L, 24 hours). Total HKII levels did not change significantly, but the mitochondrial-bound HKII levels were reduced in response to both H\(_2\)O\(_2\) and doxorubicin (Figure 1A and 1B). We also measured the total and mitochondrial-bound HKII levels in the hearts of WT and HKII\(^{-/-}\) mice subjected to sham or I/R surgery. Consistent with results obtained in vitro, mitochondrial-bound HKII levels were significantly reduced in response to I/R in both WT and HKII\(^{-/-}\) mice (Figure 1C). Furthermore, total HKII levels were also reduced in WT mice 2 days after I/R but did not reach statistical significance in HKII\(^{-/-}\) mice (Figure 1D).

**HKII\(^{-/-}\) Mice Have Reduced Cardiac Function After I/R**

To determine the role of HKII in cardiac response to I/R, we studied heterozygote HKII-deficient mice. Homozygote deletion of HKII is embryonic lethal, but HKII\(^{-/-}\) mice are viable.\(^\text{25,26}\) As expected, HKII protein levels were significantly reduced in the hearts of HKII\(^{-/-}\) mice (Online Figure I, A and B). Total HK activity in the heart (which represents both HKI and HKII activity) was also significantly reduced in total cellular extracts and mitochondrial fractions of HKII\(^{-/-}\) hearts both at baseline and in response to I/R (Online Figure I, C through F). HKI levels were not changed in HKII\(^{-/-}\) mice (Online Figure II).

We first assessed cardiac function in WT and HKII\(^{-/-}\) mice at baseline using echocardiography and by measurement of hemodynamics. HKII\(^{-/-}\) mice displayed similar
cardiac size and function compared to WT animals (Online Figure III), suggesting that a reduction in the total and mitochondrial HK activity does not alter cardiac function at baseline.

We then subjected the hearts of WT and HKII/−/− mice to I/R by coronary ligation for 45 minutes followed by reperfusion. A summary of the echo results of WT and HKII/−/− hearts at baseline and 2, 14, and 28 days after I/R is shown in the Table. HKII/−/− mice had a significantly lower cardiac function after I/R, as assessed by ejection fraction, fractional shortening, and cardiac output compared to WT animals (Table and Figure 2A). We also assessed cardiac function by invasive hemodynamics. dP/dt, a marker of cardiac contractility, was significantly lower in HKII/−/− mice compared to WT animals 28 days after I/R (Figure 2B). Consistently, left ventricular end diastolic pressure was higher in HKII/−/− mice in response to I/R after 28 days (Figure 2C). However, −dP/dt (which represents cardiac relaxation) was similar between HKII/−/− and WT mice after I/R (Figure 2D). These data collectively suggest that a reduction in HKII protein results in

Table. Echocardiographic Analysis in Mice at Baseline and 2, 14, and 28 Days After I/R

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>I/R (2 Days)</th>
<th>I/R (14 Days)</th>
<th>I/R (28 Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (n=9)</td>
<td>HKII/−/− (n=9)</td>
<td>WT (n=10)</td>
<td>HKII/−/− (n=10)</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>417±18.6</td>
<td>430±14.1</td>
<td>387±16.1</td>
<td>390±21.3</td>
</tr>
<tr>
<td>EF (%)</td>
<td>65.31±2.81</td>
<td>59.39±2.18</td>
<td>44.80±4.88†</td>
<td>39.25±1.53†</td>
</tr>
<tr>
<td>FS (%)</td>
<td>35.33±2.17</td>
<td>32.53±1.36</td>
<td>25.60±2.25*</td>
<td>19.87±1.14†</td>
</tr>
<tr>
<td>CO (mL/min)</td>
<td>14.69±0.72</td>
<td>13.94±1.15</td>
<td>12.14±1.14†</td>
<td>9.18±0.05†</td>
</tr>
<tr>
<td>IVS (mm)</td>
<td>0.57±0.04</td>
<td>0.61±0.07</td>
<td>0.63±0.06</td>
<td>0.66±0.04</td>
</tr>
<tr>
<td>LVPW (mm)</td>
<td>0.55±0.04</td>
<td>0.61±0.07</td>
<td>0.60±0.05</td>
<td>0.57±0.03</td>
</tr>
<tr>
<td>LVID (mm)</td>
<td>3.33±0.28</td>
<td>3.56±0.04</td>
<td>3.98±0.11*</td>
<td>4.01±0.15*</td>
</tr>
</tbody>
</table>

Doppler measurement of aortic valve

P Vel (mm/sec) 985.8±25.3 971.0±22.8 713.6±32.6† 761.8±44.8† 811.83±33.04†† 778.72±25.8†† 791.2±35.9† 678.5±28.9††

All measurements were performed at baseline, 2, 14, and 28 days after IR. CO, cardiac output; EF, ejection fraction; FS, fractional shortening; HR, heart rate; IVS, end-systolic interventricular septum thickness; LVPW, end-diastolic posterior wall thickness; LVID, left ventricular diastolic internal diameter; LVID-s, left ventricular systolic internal diameter; P Vel, peak velocity. Data are means±SEM. *P<0.01 vs baseline; †P<0.05 vs baseline; ††P<0.05 (WT vs HKII/−/−).
a more prominent reduction in cardiac contractility compared to WT animals in response to I/R.

**HKII**<sup>−/−</sup> Hearts Display Altered Cardiac Function in Response to I/R Ex Vivo

To confirm our results, we also performed studies in an ex vivo system. The baseline cardiac characteristics were similar in the WT and HKII<sup>−/−</sup> mice (Online Table I). When these hearts were subjected to 40 minutes of ischemia and 60 minutes of reperfusion, the hearts from the HKII<sup>−/−</sup> mice demonstrated more injury compared to WT, as measured by lactate dehydrogenase release (Figure 3A). The recovery of the rate–pressure product following I/R was similar between the 2 groups (Figure 3B), and the development of end-diastolic pressure during ischemia showed a trend of increased contracture for HKII<sup>−/−</sup> hearts as compared to WT (data not shown). During reperfusion, end-diastolic pressure was significantly elevated at 1 hour of reperfusion for HKII<sup>−/−</sup> hearts (Figure 3C), whereas the developed left ventricular pressure (peak systolic pressure–end-diastolic pressure) was not changed (Figure 3D).

**Figure 2.** HKII<sup>−/−</sup> mice display lower cardiac function after I/R. Cardiac function was assessed via Doppler echocardiography and by hemodynamic analysis. **A,** Summary of fractional shortening (FS) measurements in WT and HKII<sup>−/−</sup> mice at baseline and 2, 14, and 28 days after I/R. **B,** dP/dt after sham operation or 28 days after I/R based on hemodynamic measurements from a pressure–volume loop in WT and HKII<sup>−/−</sup> mice. **C,** Left ventricular end-diastolic pressure (LVEDP) in WT and HKII<sup>−/−</sup> mice 28 days after I/R. **D,** –dP/dt in WT and HKII<sup>−/−</sup> mice. *P*<0.05; n=8 to 10 animals. Data are presented as means±SEM.

**Figure 3.** HKII<sup>−/−</sup> hearts have lower function and increased cell death after I/R ex vivo. HKII<sup>−/−</sup> hearts display a higher degree of lactate dehydrogenase (LDH) release and cell injury (A), no change in the rate–pressure product (RPP) (B), a higher end diastolic pressure (EDP) (C), and no change in developed left ventricular pressure (DLVP) (D). Lactate dehydrogenase release and rate–pressure product were measured during 60 minutes of reperfusion following 40 minutes of ischemia. End-diastolic pressure was assessed at baseline and after 60 minutes of reperfusion. *P*<0.05; n=6. Data are presented as means±SEM.
HKII−/− Mice Display Increased Cell Death and Fibrosis and Reduced Angiogenesis

Because HKII is protective against cell death and the levels of HKII are reduced in HKII−/− hearts, we hypothesized that the reduction in cardiac function in HKII−/− mice is partially attributable to an increase in cardiomyocyte death. To test this hypothesis, we first assessed the degree of cardiac damage with 2,3,5-triphenyltetrazolium chloride (TTC)/thiazolyl blue tetrazolium bromide (MTT) staining for the whole heart, and hematoxylin/eosin staining in the periinfarct zone of the WT and HKII−/− hearts 2 days after I/R. HKII−/− mice had a higher degree of damage to their heart compared to WT (Figure 4A and 4B). To assess whether there was an increase in cardiomyocyte apoptosis, we used terminal transferase dUTP nick end labeling (TUNEL) and measured cytochrome c release into the cytoplasm. HKII−/− mice displayed a significantly higher degree of apoptosis compared to WT based on both of these measurements (Figure 4C through 4E). Furthermore, fibrosis, as assessed by Masson trichrome stain, was significantly higher in HKII−/− mice (Figure 4F). These results all demonstrate an increase in cardiac damage and cell death in HKII−/− mice compared to WT in response to I/R.

In addition to cell death and fibrosis, angiogenesis in the ischemic border zone may affect the remodeling process.27 HKII−/− mice displayed a significantly lower capillary density in the border zone compared to WT animals 28 days after I/R, as assessed by lectin–GSL-1, a marker for endothelium (Figure 5A).28,29 The reduction in angiogenesis was associated with decreased VEGF mRNA levels in the HKII−/− hearts compared to WT animals after I/R (Figure 5B). To confirm that a reduction in HKII leads to a decrease in VEGF levels, we performed in vitro studies and used RNA interference to reduce HKII levels in NRCMs and H9c2 cells (Online Figure IV). Treatment of NRCMs with HKII small interfering (si)RNA resulted in a significant decrease in VEGF mRNA and protein levels in the media under hypoxic conditions (Online Figure V, A and B).

The mechanism for the decrease in VEGF levels in response to HKII reduction is not clear. Because HIF1α activity and levels are influenced by cellular glucose availability and metabolism,30,31 we hypothesized that the decrease in VEGF in response to a reduction in HKII is through a HIF-dependent pathway. To test this hypothesis, we measured HIF1α protein levels in WT and HKII−/− hearts 2 days after I/R injury. The levels of HIF1α were significantly lower in HKII−/− hearts compared to WT.
in HKII+/− mice (Figure 5C). Similar results were obtained in NRCMs, where treatment with HKII siRNA and hypoxia resulted in a significant decrease in HIF1α protein (Figure 5D) and mRNA levels (Online Figure V, C) compared to control siRNA. Furthermore, the mRNA levels of another target of HIF (GLUT1) were also reduced with HKII siRNA treatment in response to hypoxia (Online Figure V, D). We also transfected H9c2 cells with a construct containing 3 sequences of HIF response elements attached to a luciferase reporter sequence or a control vector, followed by treatment with HKII siRNA. HKII knockdown resulted in a reduction in HIF activity in normoxia and hypoxia (Figure 5E), further supporting that HKII can influence HIF activity. Overall, these results suggest that a reduction in HKII reduces angiogenesis and VEGF levels likely through a HIF dependent pathway.

Displacement of HKII From Mitochondria Increases Cardiomyocyte Death

We next studied the mechanism for the increase in cell death in HKII+/− mice in response to I/R. HKII mitochondrial binding is important for cell survival, and the displacement of the protein from the mitochondria makes cancer cells susceptible to cell injury.20,21 We thus assessed the role of HKII binding to the mitochondria in cardiomyocyte death. HKII was displaced from the mitochondria in NRCMs using a cell permeable peptide (n-HKII) against its N-terminal hydrophobic domain. Treatment with the peptide at a concentration of 5 μmol/L resulted in a small but nonsignificant reduction in the mitochondrial-bound HKII, whereas the 20 μmol/L concentration of the peptide resulted in almost complete displacement of HKII from the mitochondria (Figure 6A). Consistent with the levels of displacement, the 20 μmol/L dose resulted in a significant reduction in the mitochondrial membrane potential, as assessed by TMRE fluorescence (Figure 6B). Treatment with the HKII peptide also resulted in a dose-dependent increase in cell death, as assessed by trypan blue and propidium exclusion (Figure 6C and Online Figure VI, A, respectively). Treatment with HKII peptide did not result in changes in the levels of mitochondrial HKI (Online Figure VI, B). These results are in contrast to those obtained in cancer cells, where treatment with HKII peptide does not increase cell death but makes the cells more susceptible to injury.20,32 Thus, we conclude that HKII binding to mitochondria is important for cardiomyocyte viability, and
decreased mitochondrially bound HKII in HKII+/− mice could explain the higher degree of cell death in those mice in response to I/R.

Modulation of HKII Levels Alter Cardiomyocyte Energy Levels and Contractility
To better characterize the mechanism for the reduction in cardiac function in HKII+/− mice, we assessed the role of HKII in cardiomyocyte energy production and contractility. We hypothesized that because HKII is a rate-limiting enzyme in glucose metabolism, it plays a major role in cellular ATP production, O2 consumption, and cardiac contractility, and a reduction in its levels results in decreased contractility of the viable cells and altered remodeling after I/R. To test this hypothesis, we overexpressed or downregulated HKII in NRCMs using adenoviral and RNA interference techniques and assessed ATP levels and O2 consumption. Overexpression of HKII resulted in an increase in both ATP levels and O2 consumption in NRCMs (Figure 7A and 7C), whereas its downregulation reduced both of these parameters (Figure 7B and 7D). We then assessed cellular contractility by measuring Ca2+ transients. HKII overexpression was associated with greater transient amplitude, duration at 50% recovery, integral, fall time, and half width (Online Figure VII). These data suggest that HKII plays an important role in cardiomyocyte cellular energy production and contractility and that the reduction in cardiac function in HKII+/− mice after I/R may be partly attributable to lower energy production and cellular contractility of the viable cells.

Discussion
In this study, we examined the hearts of HKII+/− mice in response to I/R. We hypothesized that because glucose becomes the major substrate for energy production in hypoxic conditions, a perturbation in its phosphorylation would lead to a more exacerbated damage from I/R. HKII is of particular interest because: (1) it becomes the rate limiting enzyme in glucose metabolism in the heart under hyperinsulinemic conditions; (2) its overexpression is protective against cell death in tissue culture; (3) it can bind to the mitochondria and this binding contributes to the protective effects of the enzyme; (4) it has been shown to reduce the cellular levels of reactive oxygen species; and (5) cardioprotection by ischemic preconditioning increases HKII binding to mitochondria.19,33

Our results demonstrated that hearts from HKII+/− mice display lower systolic function after I/R. We then showed that HKII+/− animals display: (1) an increase in cell death, suggesting that a reduction in HKII leads to more cardiomyocyte injury in response to ischemia; (2) an increase in fibrosis; and (3) a reduction in angiogenesis. For the latter process, we demonstrated that the mechanism appears to be through a reduction in a HIF-dependent production of VEGF in cardiomyocytes. We further show that the HKII binding to mitochondria is required for survival of cardiomyocytes and that a reduction in the mitochondrially bound HKII in HKII+/− mice is possibly a major contributor to cell death after I/R. Finally, our results demonstrate that modulation of HKII in NRCMs leads to a change in cardiomyocyte ATP production, O2 consumption, and contractility, suggesting that HKII may...
play a role in cardiac remodeling likely by providing more energy and altering the contractility of viable cardiomyocytes. These results suggest that stimulation of HKII may lead to an increase in glucose metabolism and energy production, a decrease in cardiomyocyte cell death and fibrosis, and an increase in angiogenesis in response to I/R. Collectively, these changes would all lead to an improvement in cardiac function and remodeling of the heart after I/R. Although this study focuses on HKII in the heart, it is possible that defects in HKI could have similar effects as HKII.

The product of glucose phosphorylation, ie, glucose-6-phosphate, can undergo glycolysis or glycogen synthesis or, alternatively, enter the pentose phosphate or hexosamine pathways. Thus, one may argue that a reduction in HKII activity in HKII+/− mice results in reduced substrate availability for at least 4 different pathways, and our data do not differentiate which of these pathways plays a role in the phenotypic abnormality of HKII+/− mice in response to I/R. Cardiac glucose uptake, glycogen content, and glucose metabolism in HKII+/− mice have been studied in the past and have been shown to be similar to WT animals at baseline.25,34 However, HKII+/− mice display lower glucose metabolism in response to stress conditions such as exercise, whereas glycogen content may actually be increased under these conditions.25,34 Thus, we propose that the attenuated cardiac function in response to I/R in HKII+/− mice is attributable to decreased glucose metabolism that may occur in these mice in response to low oxygen, in addition to decreased mitochondrial-bound HKII. Our results, along with those reported previously, argue against reduced glycogen content to contribute to the worsened cardiac function in HKII+/− mice in response to I/R.

HKII+/− mice displayed lower angiogenesis in response to I/R than WT mice. Because the animals have HKII knockout in every tissue, it is difficult to determine whether the reduction in HKII levels in cardiomyocytes versus endothelial cells is responsible for the decrease in angiogenesis. To better define the role of cardiomyocytes in the reduction of angiogenesis in HKII+/− mice, we studied the effects of HKII knockdown in isolated cardiomyocytes on VEGF production. Our results showed that HKII knockdown results in a decrease in VEGF levels under hypoxic conditions. We then studied the mechanism for a reduction in VEGF production in response to HKII knockdown. Previous studies had shown that a reduction in glucose results in a decrease in the expression of HIF-dependent genes and the HIF1α response to hypoxia31 and that glucose metabolites like pyruvate inactivate HIF1α decay.30 We thus hypothesized that a reduction in VEGF and angiogenesis in response to HKII knockdown is through a HIF-dependent pathway. Our results demonstrate that treatment of cells with HKII siRNA results in reduced HIF levels and activity. Thus, we conclude that decreased angiogenesis in HKII+/− mice may be through a reduction in HIF activity and VEGF production.

Cardiomyocyte contractility is highly dependent on the ATP levels within the cell. Thus, a reduction in HKII levels may hinder the ability of cardiomyocytes in the remote area to increase their contractility and overall remodeling of the heart. We assessed this possibility by overexpressing and downregulating HKII in isolated cardiomyocytes. The down-regulation of HKII in NRCMs resulted in lower ATP levels and O2 consumption, whereas HKII overexpression had the opposite effect and increased cardiomyocyte contractility.
Thus, the decrease in cardiac function in HKII−/− mice after I/R could be partially attributable to a reduction in cardiomyocyte contractility from a decrease in energy production. It is important to mention that although our data suggest an association between cardiac contractility and bioenergetic compromise through reductions in HKII, it does not establish a direct cause-and-effect relationship.

In summary, our results indicate that the HKII−/− mice are more susceptible to ischemic injury to their heart. This is attributable to an increase in cell death and fibrosis, a decrease in angiogenesis, and possibly a reduction in the contractility of the viable cardiomyocytes because of reduced energy production. Furthermore, the binding of HKII to mitochondria plays an important role in cardiomyocyte survival and protection against cell death. Our results provide the first description of the role of HKII and its mitochondrial binding in acute cardiac I/R injury and subsequent remodeling of the intact heart. Thus, in addition to their role in cancer, HKII and its mitochondrial binding play a prominent role in the pathogenesis of ischemic heart disease. These data suggest that targeting HKII and its cellular distribution may provide a novel therapeutic option in ischemic heart disease.

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Disclosures
None.

References


**Novelty and Significance**

**What Is Known?**

- Cardiomyocytes switch substrate utilization from fatty acid to glucose under stress conditions, such as ischemia and hypertrophy.
- Hexokinase (HKII) carries out the first step in glycolysis by phosphorylating glucose in muscle and fat tissue and can bind to the outer mitochondrial membrane.
- Overexpression of HKII or increasing its mitochondrial binding protects against cell death.
- Homozygote deletion of HKII is embryonic lethal, but HKII+/− mice are viable and have minimal abnormalities.

**What New Information Does This Article Contribute?**

- Mice with heterozygote deletion of HKII have normal cardiac function at baseline, but display lower systolic function after ischemia/reperfusion (I/R) compared with wild-type animals.
- Following I/R, HKII+/− mice display a reduction in angiogenesis through a decrease in HIF-dependent pathway signaling and an increase in cell death and fibrosis.
- Displacing HKII from mitochondria in tissue culture increases cell death, suggesting that the binding of HKII to the mitochondria is critical for cardiomyocyte survival.

It is well accepted that cardiomyocytes switch substrate preference from fatty acid to glucose in ischemia. However, the effects of alterations in glycolytic enzymes on cardiac response to I/R are not known. HKII is the major isoform expressed in the heart and can bind to the mitochondrial outer membrane. This study shows that HKII+/− mice display lower systolic function after I/R compared to wild-type animals. They show a reduction in angiogenesis via reduced HIF signaling, as well as an increase in cardiomyocyte death and fibrosis. Additionally, binding of HKII to the mitochondria appears to be critical for cardiomyocyte survival, because dissociation of the protein from the mitochondria in tissue culture increased cell death. HKII may also be important for the remodeling of the viable cardiac tissue because its modulation in vitro altered cellular energy levels and contractility. Our results suggest that targeting HKII or its cellular distribution may provide a novel therapeutic option for treating ischemic heart disease.
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SUPPLEMENTAL MATERIAL

METHODS

In vivo ischemia-reperfusion

The surgical protocol were performed as previously described with some modifications.\(^1\) Briefly, mice were anesthetized with isoflurane with induction at 3% and maintenance at 1.5 to 2%. The animals were placed in a supine position and ECG leads were attached. The body temperature was monitored using a rectal probe and was maintained at 37°C with heating pads throughout the experiment. A catheter was inserted into the trachea and was then attached to the mouse ventilator via a Y-shaped connector. The mice were ventilated at a tidal volume of 200 \(\mu\)l and a rate of 105 breaths/min using a rodent ventilator.

The chest was then opened by an incision of the left fourth intercostal space. A 1 mm section of PE-10 tubing was placed on top of left anterior descending artery (LAD), and a knot was tied on the top of the tubing to occlude the coronary artery with an 8-0 silk suture. Ischemia was verified by pallor of the anterior wall of the left ventricle and by ST segment elevation and QRS widening on the ECG. After occlusion for 45 minutes, reperfusion occurred by cutting the knot on top of the PE-10 tubing. The chest was then closed in layers. The mice were kept warm with heating pats and on 100% oxygen via nasal cannula. Animals were given buprenorphine for post operative pain.

Echocardiography

Echocardiography was performed using Vevo 770 High-resolution imaging system at 2, 14 and 28 days after I/R as previously described.\(^2\) Animals were anesthetized with isoflurane and were placed in a supine position. The chest was shaved and the parasternal short- and long-axis views were used to obtain two-dimensional and M-mode images. At least 10 independent cardiac cycles per each experiment were obtained. The cardiac output values were corrected to the animals' weights to obtain cardiac index.

Histological analysis

Hearts were fixed in 10% formalin (PBS buffered), dehydrated, and embedded in paraffin. Heart architecture was determined from transverse 5-\(\mu\)m deparaffinized sections stained with H&E. Fibrosis was detected with Masson's Trichrome staining and was reported as the ratio of the length of fibrosis to
the LV circumference. To determine angiogenesis, mice were injected with 50 μl of fluorescein-BSI-Lectin I (Vector Laboratories, Burlingame, Calif) 15 minutes before euthanasia as described before. TUNEL positivity 48 hours after I/R was determined using an In Situ Detection Kit according to the manufacturer’s instructions (Roche Diagnostics). Nuclei were counterstained with DAPI and actin was stained with phalloidin-Alexa 546 dye (Molecular Probes, Eugene, OR). For 2,3,5-triphenyltetrazolium chloride (TTC) / thiazolyl blue tetrazolium bromide (MTT) staining, 2 days after ischemia reperfusion the artery was reoccluded, and 1.5% MTT (Sigma-Aldrich, St Louis, Mo) was injected into the aortic root to delineate the area at risk from not-at-risk myocardium. Hearts were then sectioned parallel to the AV groove in approximately 1-mm sections. Viable and necrotic sections of the area at risk were identified by incubating the hearts in 1% TTC (Sigma-Aldrich, St Louis, Mo) for 10 minutes at 37°C, followed by 10% neutral-buffered formaldehyde for 24 hours. Photographed using a Leica microscope, and analyzed using NIH Image software.

**Hemodynamic studies**

Hemodynamic studies were performed 28 days after I/R as previously described. A high-fidelity transducer-tipped pressure-volume catheter (Scisense Inc., London, Ontario, Canada) was calibrated in 37°C saline and was introduced into the LV of the anaesthetized mouse to determine hemodynamics. The right carotid artery was isolated, and two ties were gently pulled back using hemostats to block blood flow from the vessel. When pulsatile flow was no longer visible, a small incision was made just below the distal tie, and the catheter was placed and secured inside the carotid artery. The transducer was then advanced into the left ventricle and the mice were allowed to stabilize for 15 minutes. After stabilization, 30 seconds of data was collected. At the end of data collection, the catheter was retracted from the heart and arterial pressure was measured to demonstrate competency of the aortic valve. Signals were digitized by use of a data translation series analog-digital converter and then stored and analyzed. Values derived from pressure tracings were averaged over a minimum of 20 beats.

**Western blotting**

For Western blotting, hearts were homogenized in a modified RIPA buffer containing protease inhibitors. Antibodies against HKII, HKI, cytochrome c and GAPDH were purchased from Santa Cruz Biotechnology, and HIF1α antibody was purchased from Abcam (Cambridge, MA). The protein bands were developed with an enhanced chemiluminescence substrate kit. Quantification of blots was performed with Image J.
Measurement of HK activity in isolated hearts

The amount of HK activity was measured in separate hearts (n=5-8 per group). Briefly, hearts were excised and homogenized in 1 ml ice-cold homogenization medium (in mM: 250 sucrose, 20 Hepes (pH 7.4), 10 KCl, 1.5 MgCl₂, EDTA, 0.1 PMSF, 5 μg/ml leupeptin and aprotinin and 1 μg/ml pepstatin). Differential centrifugation was used to obtain whole-heart and mitochondrial fractions. Fractions were quickly frozen at -80 °C until further analysis. The fractions were treated with 0.5% Triton X-100 and 0.9 M KCl to solubilize hexokinase. Hexokinase activity was measured. For whole-heart, HK activity was normalized to protein content (as determined by the Bradford method); for mitochondrial fraction, HK activity was normalized to citrate synthase (CS) activity.

ELISA

Quantitative analysis of VEGF protein secretion by the cultured NRCM was performed using an ELISA kit (R&D Systems, Inc). Briefly, isolated NRCM were seeded in 6 well tissue culture plates in 2 ml fresh growth medium. Cells were transfected with either siRNA-HKII and or Luciferase as a control for 24 hours and then were exposed to 48 hours of 1.5% O₂. The conditioned media were removed and spun at 600 xg to remove cellular components. For VEGF quantification, 100 μl aliquots of the conditioned media were added to each well of 96-well assay plate. To construct a standard curve, known quantities of standard VEGF was run in parallel. The results were reported as the amount of total secreted VEGF in pg/ml.

Luciferase assays

H9c2 cells were co-transfected using Lipofectamine Plus Reagent (Invitrogen) in a serum-free medium with both the recombinant firefly luciferase plasmid containing 3 copies HRE (0.25 μg)/ control vector and the Renilla luciferase plasmid (0.025 μg). Twenty four hours after transfection, luciferase activities were measured with a dual luciferase reporter assay kit (Promega) on a Berthold Lumat LB 9570 luminometer. Firefly luciferase expression was corrected by Renilla luciferase expression in the same well to normalize for variations in transfection efficiency.

RNA isolation and quantitative real-time PCR

Total RNA samples from either cultured cardiomyocytes or mouse hearts were isolated using the RNA stat-60 reagent (Tel-Test, Inc), according to manufacturer's instructions. Samples of total RNA (500ng)
were reverse-transcribed using the Taqman reverse transcription PCR Kit (Applied Biosystems), and the resulting cDNA was used as a PCR template. The mRNA levels were determined by Real-Time PCR with the 7500 Fast Real-Time PCR system (Applied Biosystems), according to the manufacturer’s instructions. The mRNA expression levels of VEGF, GLUT1, HIF1, and HKII were determined by quantitative real-time PCR with SYBR green detection. Beta actin RNA was amplified as an internal control. The relative gene expression level was calculated using the comparative Ct method formula: $2^{-\Delta\Delta Ct}$. Primers used in our studies are listed in the Table below:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>Mouse-GLUT1</td>
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<td>GTAGCGGTGGTTCCATGTTT</td>
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<tr>
<td>Rat-GLUT1</td>
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<td>CATAGCGGTGGTTCCATGTTT</td>
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</table>

**Ex-vivo ischemia-reperfusion**

Experiments were performed with both male and female mice. Mice were heparinized (15 IU) and anesthetized with S-ketamine (125 mg/kg) and medetomidine (0.2 mg/kg). Following tracheotomy, the mice were mechanically ventilated and a thoracotomy performed. The hearts were cannulated in situ with perfusion started before excision of the heart. Hearts were Langendorff-perfused at a constant flow (initial perfusion pressure 80 mmHg) at 37°C with Krebs-Henseleit solution containing (mmol/l) NaCl 118, KCl 4.7, CaCl₂ 2.25, MgSO₄ 1.2, NaHCO₃ 25, KH₂PO₄ 1.2, EDTA 0.5 and glucose 5, glutamine 0.5, lactate 1.0, pyruvate 0.1, and insulin 100 mU/l, gassed with 95% O₂/5% CO₂. The perfusate was in-line filtered by a 0.45-μm filter. End-diastolic pressure (EDP) was set at ~4-8 mmHg using a water-filled polyethylene balloon inserted into the left ventricular (LV) cavity via the mitral valve. The hearts were continuously submerged in 37°C perfusate. LV developed pressure was calculated as the systolic pressure.
pressure minus the end-diastolic pressure. The rate-pressure product (RPP) was the product of the developed pressure and the heart rate. Following stabilization of left ventricular pressure, all hearts were subjected to 40 min no-flow ischemia and 60 min reperfusion. During ischemia, the hearts were submerged in Krebs-Henseleit perfusate gassed with 95% N\textsubscript{2}/5% CO\textsubscript{2}. Two different groups of hearts (n=12 each; 6 males and 6 females) were studied: group 1, control (WT); group 2, HK-knockout (HKII\textsuperscript{−/−}) hearts.

**Construction of adenoviruses**

For each construct, polymerase chain reaction (PCR) was used to amplify the regions of interest. These constructs were subcloned into the TOPO vector (Invitrogen, Carlsbad, CA), sequenced, and were subcloned into the pAdTrack plasmid vector downstream of the CMV promoter and upstream of the poly-A site (Stratagene, LaJolla, CA). Linearized pAdTrack plasmids were transformed into *E. coli* BJ5183 cells along with the pAdEasy adenoviral backbone (Stratagene). After antibiotic selection, positive recombinants were confirmed using a restriction digest and partial sequencing. The recombinant plasmids were linearized with *PacI*, and transfected into adenovirus packaging cell line. Viral purification of recombinants was performed on a cesium chloride gradient, as described previously.\textsuperscript{5} Virus titers were determined by plaque assays, and particle numbers were determined by absorbance at 260 nm on a DU640 spectrophotometer.

**Adenoviral treatment of the cells**

The adenoviruses were added to NRCM in the presence of 2% serum. GFP only adenovirus was used as a control in our experiments. Western blot analysis, ATP measurement, O\textsubscript{2} consumption and Calcium transients were performed 48 hours after the initial transfection.

**Lactate dehydrogenase enzyme activity in effluent**

During the reperfusion period the effluent was collected at 5, 10, 30, 60 and 60 min of reperfusion and immediately frozen at -80°C. Lactate dehydrogenase (LDH) activity was determined using spectrophotometric analysis at 340 nm. The total amount of LDH released during 60 min reperfusion was calculated and normalized to heart weight (g).

**HKII peptide treatment**
A peptide analogous to the N-terminal sequence of HKII (n-HKII Peptide: MIASHLLAYFFTELNHDQVQKVD), along with a scrambled peptide (Control Peptide: VLIQKEVTDNLAYFYMADHQLF); were synthesized by Genemed Synthesis, Inc. (San Antonio, TX). To facilitate cell permeability, each peptide included a polyarginine sequence at the C-terminal. Peptides were added to NRCM cultured in serum free media, as indicated. Changes in mitochondrial membrane potential (ΔΨm) were assessed using tetramethylrhodamine ethyl ester (TMRE) fluorescence (Invitrogen) and flow cytometry as described previously. Briefly, cells were loaded with TMRE (100nM) 10 min prior to peptide treatment. After 2h, loss of membrane potential, an early predictor of cell death, was measured by flow analysis using the the FacsCanto (BD Biosciences, San Jose, CA). Cell viability data was corroborated using both trypan blue and propidium iodide exclusion. HKII dissociation from mitochondria was confirmed via Western blot of fractionated NRCM lysates undergoing the same peptide treatment regimen.

Isolation of mitochondria

For tissue samples, we used the Pierce Mitochondria Isolation Kit for Tissue according to the manufacturer’s reagent-based protocol for hard tissue (Pierce, USA). For mitochondria isolation from NRCM, we used the Pierce isolation kit for cultured cells, and the protocol described previously. Briefly, cells were resuspended in lysis buffer (68 mM sucrose, 200 mM mannitol, 50 mM KCl, 1 mM EGTA, 1 mM EDTA, 5 mM Hepes, pH 7.4), supplemented with protease inhibitors. The cells were removed with a scraper, sonicated gently for 30 seconds, incubated on ice for 30 minutes, and then centrifuged at 500 xg for 5 minutes at 4°C to remove cellular debris. Following differential centrifugation at 4°C, the supernatant (cytosolic fraction) is removed, and the remaining pellet containing the mitochondrial enriched fraction is dissolved in the above buffer with the addition of Triton X-100 (Invitrogen). The purity of the fractionated lysates was assessed via Western blot using appropriate markers for each compartment; Actin: cytosolic; SDH 70kDa subunit: mitochondria (Santa Cruz, Molecular Probes respectively).

O₂ consumption studies

The oxygen consumption of NRCM was measured at 37°C by using a Hansatech instruments as described before. The polarographic system is equipped with an oxygen electrode disc, a borosilicate reaction chamber and a magnetic stirring bar and connected to a circulating water bath. Cells were washed with PBS, trypsinized and measured in a final volume of 1ml NRCM cell culture medium.
(containing 20% 199 medium and 80% DMEM medium) without serum in an O2 consumption chamber. After O2 consumption reached steady state, the mitochondrial respiratory chain decoupler, carbonyl cyanide m-chlorophenylhydrazone (CCCP, 1 μM), was added to the chamber to establish the maximal rate of O2 consumption for each set of cells. Once a maximal steady state was reached, the mitochondrial respiratory chain terminator, myxothiazole (1mg/mL) was added to the chamber to halt mitochondrial oxygen consumption. The remaining cells were used for viable cell counting using trypan blue. The rate of myxothiazole, which represents the rate of O2 consumption within the system not attributable to mitochondrial respiration, was subtracted from the basal and maximal O2 consumption rates to establish absolute rates of mitochondrial O2 consumption. O2 consumption rates were normalized to viable cell counts. The data were analyzed with Oxyg32 (Hansatech) software.

Measurement of ATP levels

Cells were washed with PBS, trypsinized, and collected in a final volume of 400μL. Cells were divided into three aliquots of 100 μL, placed in a 96 well plate and allowed to equilibrate to room temperature for 30 minutes. One hundred μL of Cell Titer Glo reagent (Promega) was added to cells followed by agitation to facilitate lysis and incubation for 10 minutes at room temperature. Luminescence was then measured using a luminometer (Spectramas Gemini XS). The remaining cells were used for viable cell counting using trypan blue. Luminescence was averaged for each set of three samples and normalized to cell count. Normalization to cell count allowed us to determine ATP levels instead of cell viability. For the knockdown studies, the cells were incubated for 6h in HBSS starvation media prior to the assessment of ATP levels.

Calcium-transient measurements

NRCM were attached to 18-mm coverslips and loaded with 15 μM rhod-2AM for 20 minutes at 37°C, then washed in serum-free DMEM, placed in a Cell MicroControls (Norfolk, Virginia) chamber, with constant perfusion with DMEM at 35°C. After 10 minutes, the spontaneous beating rates of the NRCM were determined by measurement of Ca2+ transients using rhod-2AM fluorescence (excitation wavelength: 543 nm, emission wavelength: >560 nm) with a Zeiss LSM510 laser scanning confocal microscope. The cells were then paced at 2 Hz via field stimulation so that characteristics of Ca2+ transients could be measured at a constant rate. The ratio of maximal fluorescence (F) and ambient fluorescence during diastole (F0) was calculated by placing the scan line across the myocyte cluster,
allowing calculation of transient magnitude (F/F0), duration at 50% recovery, integral, decay time, and half width for that cluster.

REFERENCES


Supplemental Figure I. HKII level and activity are reduced in HKII knockout mice. (A) Western blot of protein extract from the hearts of wild type and HKII+/− mice. (B) Western blot of HKII levels in different tissues from WT and HKII+/− mice. HKII levels in skeletal muscle of these mice has also been reported before (Ref 24 and 26 in the manuscript). Summary of the results is shown on the bottom. (C,D) Total HK enzyme activity in the hearts of WT and HKII+/− mice at baseline (C) and after (40 min) ischemia and (60 min) reperfusion (D). * P<0.05 to WT, BL: n=5-8, I/R: n=4 hearts. (E,F) HK activity in the mitochondrial fraction of WT and HKII+/− hearts relative to citrate synthase (CS) activity at baseline (E) and after (40 min) ischemia and (60 min) reperfusion (F). * P<0.05 to WT, BL: n=5-8, I/R: n=4 hearts.
Supplemental Figure II. HKI levels are not different in WT and HKII\(^{+/--}\) mice and are not changed in response to I/R. Western Blot gels are shown on top and summary of results is shown on the bottom. \(n = 6\), Data are presented as mean ± SEM.
C. Hemodynamic analysis of WT and HKII+/− mice at baseline (n=8).

<table>
<thead>
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<td></td>
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Supplemental Figure III. HKII+/− mice display normal cardiac function at baseline. (A) Gross representation of hearts from HKII+/− and WT mice. These mice display similar heart size. (B) Ejection fraction obtained from echo on WT and HKII+/− mice. (C) Table representing hemodynamics of WT and HKII+/− mice. There is no difference in dp/dt and −dp/dt between WT and HKII+/− mice. Data are presented as mean ± SEM.
Supplemental Figure IV. HKII siRNA treatment of NRCM (A) and H9c2 cells (B) results in a decrease in HKII levels. Western blot gels are shown on top and summary of results on the bottom. *P < 0.05 vs control siRNA. n= 3. Data are presented as mean ± SEM.
Supplemental Figure V. Regulation of HIF1 and HIF1 target gene in response to HKII knockdown in NRCM. Cells were treated with either control (ctr) siRNA or HKII siRNA for 24 hours and put in normoxia or hypoxia for additional 24 hours. (A) VEGF mRNA expression as assessed by RT-PCR. (B) Quantitative analysis of VEGF protein in the cultured media was performed by an ELISA kit. (C) HIF mRNA expression assessed by RT-PCR. (D) GLUT1 mRNA expression normalized to beta actin. *P < 0.05, n=6. Data are presented as mean ± SEM.
Supplemental Figure VI. HKII peptide does not increase cell death at low concentrations and does not alter HKI binding to mitochondria. (A) NRCM were treated with different concentrations of scrambled control or HKII dissociation peptide (n-HKII) for 2h and cell death was assessed via propidium iodide exclusion. HKII peptide at 5 μM did not increase cell death, while higher concentrations all led to an increase in cell death, * P < 0.05. Data are presented as mean ± SEM. (B) HKII peptide at 10 μM (left) and 20 μM (right) does not alter mitochondrial HKI levels.
Supplemental Figure VII. HKII overexpression increases calcium transients in NRCM. Calcium transients were also monitored with the fluorescent Ca^{2+} marker rhod-2AM. HKII overexpression significantly increased the transient amplitude (P = 0.002) (A), 50% duration (P = 0.002) (B), integral (P = 0.001) (C), fall time (P = 0.022) (D), and half-width (P = 0.001) (E). n = 17 myocytes for control and 16 for HKII. Data are presented as mean ± SEM.