Disruption of Hexokinase II–Mitochondrial Binding Blocks Ischemic Preconditioning and Causes Rapid Cardiac Necrosis


Rationale: Isoforms I and II of the glycolytic enzyme hexokinase (HKI and HKII) are known to associate with mitochondria. It is unknown whether mitochondria-bound hexokinase is mandatory for ischemic preconditioning and normal functioning of the intact, beating heart.

Objective: We hypothesized that reducing mitochondrial hexokinase would abrogate ischemic preconditioning and disrupt myocardial function.

Methods and Results: Ex vivo perfused HKII+/− hearts exhibited increased cell death after ischemia and reperfusion injury compared with wild-type hearts; however, ischemic preconditioning was unaffected. To investigate acute reductions in mitochondrial HKII levels, wild-type hearts were treated with a TAT control peptide or a TAT-HK peptide that contained the binding motif of HKII to mitochondria, thereby disrupting the mitochondrial HKII association. Mitochondrial hexokinase was determined by HKI and HKII immunogold labeling and electron microscopy analysis. Low-dose (200 nmol/L) TAT-HK treatment significantly decreased mitochondrial HKII levels without affecting baseline cardiac function but dramatically increased ischemia-reperfusion injury and prevented the protective effects of ischemic preconditioning. Treatment for 15 minutes with high-dose (10 μmol/L) TAT-HK resulted in acute mitochondrial depolarization, mitochondrial swelling, profound contractile impairment, and severe cardiac disintegration. The detrimental effects of TAT-HK treatment were mimicked by mitochondrial membrane depolarization after mild mitochondrial uncoupling that did not cause direct mitochondrial permeability transition opening.

Conclusions: Acute low-dose dissociation of HKII from mitochondria in heart prevented ischemic preconditioning, whereas high-dose HKII dissociation caused cessation of cardiac contraction and tissue disruption, likely through an acute mitochondrial membrane depolarization mechanism. The results suggest that the association of HKII with mitochondria is essential for the protective effects of ischemic preconditioning and normal cardiac function through maintenance of mitochondrial potential. (Circ Res. 2011;108:1165-1169.)

Key Words: mitochondria ■ ischemia ■ reperfusion ■ TAT peptide ■ cardiac physiology

Hexokinase (HK), a glycolytic enzyme, is overexpressed in cancer cells. The enzyme binds to mitochondria and appears to regulate mitochondria-associated cell death induced by oxidative stress such as ischemia-reperfusion (I/R) injury.1–3 Mitochondrial HK (mitoHK) probably confers on cancer cells their resilience against cell death.2 Ischemic preconditioning (IPC), which is one of the most powerful interventions known to protect the heart against I/R injury, is

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believed to operate through complex mitochondrial signaling cascades.6,7 Indeed, translocation and activation of mitochondrial kinases have been suggested to be involved in preconditioning.8 Although various cardioprotective interventions, including IPC, cause the redistribution of hexokinase to mitochondria,9–11 it remains unclear whether hexokinase translocation and binding to mitochondria constitutes a requirement for or is simply an epiphenomenon of IPC. Furthermore, although hexokinase is known to associate with mitochondria in many tissues and organs within the body, the functional significance of this association in relation to normal cardiac physiology remains unclear.

In the present work, we used a combination of genetic and pharmacological tools to address the role of mitoHK in hearts under normal and stress conditions. We show for the first time that the physical binding of hexokinase II (HKII) to the mitochondria not only plays an essential role in IPC, but the attachment is also crucial for normal cardiac function, through maintenance of mitochondrial polarization.

Non-standard Abbreviations and Acronyms

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<tr>
<th>Abbreviation</th>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>CCCP</td>
<td>HK</td>
<td>hexokinase</td>
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<tr>
<td>HKII+/−</td>
<td>homozogate hexokinase II−/− mice</td>
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<tr>
<td>IPC</td>
<td>ischemic preconditioning</td>
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<td>I/R</td>
<td>ischemia-reperfusion</td>
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<td>mitoHK</td>
<td>mitochondria-bound hexokinase</td>
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<td>TAT</td>
<td>transactivating transcriptional factor from human immuno-deficiency virus 1</td>
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Methods

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

TAT Peptides

The soluble peptides TAT-CON (TAT control peptide), TAT-HK, TAT-HK-FITC, and TAT were produced by Pepscan Presto (Lelystad, Netherlands). Peptides were administered during the last 15 minutes before the 30-minute ischemic period and during the first 5 minutes of reperfusion.

Statistical Analysis

Data are presented as mean ± SEM. Data were analyzed by ANOVA followed by Fisher post hoc test.

Results

Low-Dose TAT-HK Prevents the Cardioprotective Effects of IPC by Decreasing MitoHKII Levels

We first examined hearts from HKII+/− mice. Although genetic reduction of HKII decreased ischemic tolerance, no effects on IPC were observed (Online Figure). To determine whether an acute pharmacological reduction in mitoHKII levels can alter IPC, isolated hearts were perfused with a cell-permeable peptide that contained the HKII mitochondrial binding motif. This peptide has been shown in cellular studies to decrease HKII association with mitochondria.12 Cellular uptake of this peptide was confirmed in cellular studies to decrease HKII association with mitochondria.12 Cellular uptake of this peptide was confirmed in the intact heart with FITC labeling. Fluorescence imaging of isolated hearts perfused with various concentrations of the peptide showed a progressive increase in fluorescence intensity with increasing peptide doses. As shown, homogeneous fluorescence could be readily observed even after prolonged periods of peptide washout (Figure 1A).

Baseline perfusion of hearts with a low concentration (200 nmol/L) of TAT-HK for 15 minutes did not cause major changes in myocardial function, as evidenced by maintained mitochondrial polarization and normal mitochondrial bioenergetics (Figure 1B, C).

Figure 1. Low-dose (200 nmol/L) TAT-HK decreases mitoHKII and prevents IPC effects. A, TAT-HK-FITC images of hearts obtained by an imaging cryomicrotome.13 Hearts were perfused with no peptide (1), 15 minutes of 10 μmol/L TAT-HK-FITC plus 10 minutes of washout (2), 15 minutes of 1 μmol/L TAT-HK-FITC plus 10 minutes of washout (3), 15 minutes of 200 nmol/L TAT-HK-FITC plus 10 minutes of washout (4), or 15 minutes of 200 nmol/L TAT-HK-FITC plus 30 minutes of washout (5). B, C, Mitochondrial (Mito) binding ratios (y-axes) for HKII and HKI as determined from HK immunogold labeling and electron microscopy analysis (n = 3 or 4 in each group). D, Lactate dehydrogenase (LDH) release during reperfusion of isolated Langendorff-perfused hearts. E, End-diastolic pressure (EDP) at end of reperfusion. F, Rate-pressure product development (RPP) at end of reperfusion relative to baseline values. G, Cytosolic Cyt C (cyt C) content in cytosolic fractions of reperfused hearts. H, 19-kDa Bax protein (P) content in mitochondrial fractions of reperfused hearts. n = 6 to 7 hearts per group. CON indicates control; A.U., arbitrary units. #P < 0.05 vs TAT-CON; *P < 0.05 IPC, TAT-HK vs CON, TAT-HK.
changes in cardiac contractility (rate-pressure product decreased slightly from 101±3% in TAT-CON–treated hearts to 92±3% in TAT-HK–treated hearts) or perfusion pressure. Using electron microscopy analysis and immunogold labeling for HKI and HKII, we observed a significant decrease (by 40%) in mitoHKII (Figure 1B), with no alterations in mitoHKI levels (Figure 1C). Representative electron microscopy images indicated an intact, well-preserved cardiac ultrastructure with clear localization of HKI and HKII at the outer mitochondrial membrane or in the cytosol in hearts treated with TAT-CON (Figures 2A–C) and TAT-HK (Figures 2D–F). Similar effects of the 200-nmol/L TAT-HK peptide compared with the TAT-CON peptide were observed at 5 minutes of reperfusion (Online Figure I).

Next, hearts were subjected to I/R with and without preceding IPC. The acute decrease in mitoHKII levels in TAT-HK–treated hearts was associated with more injury, as reflected by a more pronounced rise in release of lactate dehydrogenase (Figure 1D) and cardiac contracture at the end of reperfusion (Figure 1E). Remarkably, IPC in hearts with acutely decreased mitoHKII levels was completely ineffective in mediating protection against I/R injury, except for a nominal improvement in rate-pressure product recovery (Figure 1F).

TAT-HK–treated hearts also displayed increased cytosolic cytochrome C, which indicates damage and mitochondrial leakage (Figure 1G). Analysis of mitochondrial monomeric Bax (Figure 1H) suggested that the increased I/R damage in TAT-HK–treated hearts could not be explained by increased Bax translocation to mitochondria. Similar results were obtained for mitochondrial Bax oligomers (Online Figure II).

**High-Dose TAT-HK Decreases MitoHKI and MitoHKII, Damages Mitochondria, and Results in Acute Mitochondrial Depolarization**

To determine the pathophysiological consequences of severe disruption of mitochondrial HK binding to the intact myocardium, hearts were treated with a high dose (10 μmol/L) of TAT-HK for 15 minutes. The TAT-CON peptide was without effect on cardiac structure (Figure 2G through 2I); however, TAT-HK treatment caused a significant reduction in mitoHKII (Figure 3A) but not mitoHKI (Figure 3B) levels. Electron microscopy analysis demonstrated extensive structural disruption of cardiac tissue, with 78% of mitochondria exhibiting swelling or gross damage (Figures 2J through 2L and 3C). Profound cardiac dysfunction was observed, with rate-pressure product reduced to zero within 15 minutes of peptide perfusion (rate-pressure product 1±1% versus 93±2% for TAT-HK and TAT-CON, respectively), and perfusion pressure was doubled (81±7 versus 161±13 mm Hg, for TAT-CON and TAT-HK, respectively).

Mechanisms underlying the detrimental effects of acute dissociation of HK from mitochondria on cardiac structure and function of the intact heart are not clear. We hypothesized that infusion of the TAT-HK peptide altered the mitochondrial membrane potential (ΔΨm). ΔΨm was unaffected by treatment with the TAT-CON peptide; however, administration of the TAT-HK peptide resulted in a rapid and sustained decrease in
Regions of mitochondrial depolarization developed heterogeneously across the epicardial surface of the heart, which indicates global mitochondrial de-energization at the intact heart level (Figure 3D; Online Figure V). Finally, we examined in isolated cardiomyocytes whether mitochondrial depolarization in and of itself may indeed cause cell death and whether TAT-HK is still detrimental when mitochondria are already depolarized. Similar to our findings in the intact heart, treatment of myocytes with 10 μmol/L TAT-HK resulted in cell death (Figure 3E). Remarkably, mitochondrial depolarization caused by incubation of myocytes with a submaximal dose (1 μmol/L) of the uncoupling agent carbonyl cyanide 3-chlorophenylhydrazone (CCCP) resulted in a degree of cell death comparable to that elicited by treatment with the TAT-HK peptide (Figure 3F). Interestingly, cotreatment of CCCP incubated with 10 μmol/L TAT-HK did not cause additional cell death. Similar data were obtained for tetramethylrhodamine ethyl ester fluorescence analysis in cardiomyocytes (Online Figure V). These data suggest that the development of cell death in the heart during detachment of HK from mitochondria is most likely caused by a mechanism that involves mitochondrial depolarization.

Discussion

MitoHKII and Ischemic Tolerance

TAT-HK peptide–mediated mitochondrial HKII dissociation is associated with an extensive decrease in the tolerance of the heart to an I/R insult. Because it is unlikely that total cellular HK activity is altered with this peptide, the increased cell death can only be ascribed to decreases in mitoHKII and not to decreases in cardiac HK activity. One mechanism through which detachment of HK from mitochondria could cause cell death is through the translocation of proapoptotic Bax to mitochondria3,4; however, this was not observed in the present experiments. Although the large increase in release of lactate dehydrogenase suggests that the detrimental effects of HKII detachment from mitochondria may be mediated through necrosis, further experiments are needed to establish this more firmly.

MitoHKII and IPC

The present study indicates that a basal level of HKII binding to mitochondria is required for IPC-mediated cardioprotection against I/R injury; however, the observation that IPC was still effective in the HKII+/− hearts but not in TAT-HK–treated hearts, with both models having similar reductions in mitoHKII (30% to 40%), suggests that it is not just a reduction of overall HKII binding to mitochondria that prevents IPC. Although not investigated, it is possible that the TAT-HK peptide used in the present study occupied mitochondrial binding sites that were specifically targeted for IPC-induced translocation of HKII to mitochondria. This is commensurate with the observation that cardioprotective

ΔΨm (Figure 3D).
signaling was associated with a protein kinase C-ε mitochondrial protein complex that contained HK in the amount of only 1% of total HK present. Further experiments are required to clarify this issue.

**MitoHKII and Normal Cardiac Function**

The present data demonstrate that HKII detachment from mitochondria results in acute mitochondrial depolarization, which is the likely mechanism for cardiac dysfunction. Together with our observation that CCCP mediated cell death through mitochondrial depolarization, without directly affecting mitochondrial permeability transition pore opening, suggests that mitochondrial membrane depolarization per se may be the execution trigger for HKII-related cardiac dysfunction. Previous literature has indicated that mitochondrial membrane depolarization may induce opening of the mitochondrial permeability transition pore, especially when pH is above 7.0. Overall, the present data suggest that mitochondria-bound HKII is required for the polarization and stability of mitochondrial energetics within the intact heart.

**Sources of Funding**

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**Disclosures**

None.

**References**


**Novelty and Significance**

**What Is Known?**

- The glycolytic enzyme hexokinase (HK) translocates to the mitochondria during insulin stimulation, ischemia, and ischemic preconditioning.
- The association of hexokinase with the mitochondria is thought to both promote glycolysis and prevent mitochondrial permeability transition pore opening and may be an important end effector for cardioprotection by ischemic preconditioning.

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

- Acute targeted disruption of HK-mitochondria binding with low doses of a cell-permeable peptide homologous to the binding region of HK had no effect on basal cardiac function but compromised ischemic tolerance and blocked ischemic preconditioning.
- Genetic reduction of HKII (by 30% at the mitochondria) also compromised ischemic tolerance but had no effect on ischemic preconditioning.
- Administration of the same peptide at a higher dose caused rapid and dramatic mitochondrial damage, necrosis, and cardiac dysfunction.
- Detachment of HK from mitochondria appears to cause acute necrosis through mitochondrial depolarization, swelling, and rupture rather than Bax-mediated apoptosis.

The data suggest that the glycolytic enzyme HK is an important guardian of the mitochondrion in the beating, intact heart. It could be argued that its key integrative role between glycolysis and mitochondria makes it an ideal candidate as a metabolic sensor or cell death switch; its upregulation in cancer cells as part of the Warburg effect provides some evidence for this. Our findings establish the essential importance of HK in maintaining mitochondrial (and hence myocyte) viability and provide further evidence that upregulation of HK-mitochondria binding may underlie the protective effect of ischemic preconditioning.
Disruption of Hexokinase II–Mitochondrial Binding Blocks Ischemic Preconditioning and Causes Rapid Cardiac Necrosis

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METHODS

Animals
C57BL/6J HK<sup>+/−</sup> were obtained from Vanderbilt University, Nashville (generous gift of Dr David H Wasserman). The HK<sup>+/−</sup> mice were first described by Heikkinen et al., and have a partial deletion to the HKII gene. HK<sup>+/−</sup> mice were initially bred with wild-type C57BL/6J mice (Jackson laboratories) and subsequently with wild-type offspring. The HK<sup>+/−</sup> mice were backcrossed with C57BL/6J background for at least 8 generations. The wild-type animals in the present study were either littermates of the HK<sup>+/−</sup> breeding colony or wild-type C57Bl/6J (Charles River). Genotyping was performed with the polymerase chain reaction on genomic DNA obtained and isolated from toe biopsies, as described before. Mice were fed a Purina Laboratory Rodent Diet 5001 standard chow ad libitum and studied at 3-4 months of age. All experiments were approved by the animal ethics committee of the Academic Medical Center, Amsterdam, The Netherlands.

Heart Perfusion
Experiments were performed with male mice only. Mice were heparinized (15 IU) and anesthetized with pentobarbital (80 mg kg<sup>−1</sup>). 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 mm Hg) at 37°C with Krebs-Henseleit solution containing (mmol/L) NaCl 118, KCl 4.7, CaCl<sub>2</sub> 2.25, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, KH<sub>2</sub>PO<sub>4</sub> 1.2, EDTA 0.5 and glucose 11, glutamine 0.5, lactate 1.0 and pyruvate 0.1, gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>. 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 minus the end-diastolic pressure. During ischemia, the hearts were submerged in Krebs-Henseleit perfusate gassed with 95% N<sub>2</sub>/5% CO<sub>2</sub>.

Model of IR injury and IPC
Isolated. Langendorff-perfused, hearts were subjected to 30 min global ischemia and 45 min reperfusion. IPC consisted of 3 x 5 min global ischemia interspersed with 5 min reperfusion, with the last reperfusion period lasting 15 min.

TAT-peptides
The soluble peptides MIASHLLAYFFTELN(β-Ala)GYGRKKRRQRRRG-amide (TAT-HK), GYGRKKRRQRRRG(β-Ala)EEEAKNAAKLANKEEK-amide (TAT-CON), GYGRKKRRQRRRG-amide (TAT only) and FITC-MIASHLLAYFFTELN(β-Ala)GYGRKKRRQRRRG-amide (TAT-HK-FITC) were produced by Pepscan presto (Lelystad, The Netherlands). Peptides were dissolved in perfusate Krebs-Henseleit solution and administered through a side-arm connected to a mixing chamber above the heart, at 1% of total perfusion flow, during the last 15 min prior to the 30 min ischemic period and during the first 5 min reperfusion.

Imaging cryomicrotome
Serial FITC fluorescence images at 27 μm separation from base to apex of each heart were obtained with an imaging cryomicrotome<sup>2</sup> for five hearts in total: 1) no peptide treatment, 2) 15 min treatment with 10 μmol/L TAT-HK-FITC min followed by
10 min washout peptide, 3) 15 min treatment with 1 \( \mu \text{mol/L} \) TAT-HK-FITC min followed by 10 min washout peptide, 4) 15 min treatment with 200 nmol/L TAT-HK-FITC min followed by 10 min washout peptide, and 5) 15 min treatment with 10 \( \mu \text{mol/L} \) TAT-HK-FITC min followed by 30 min washout peptide. Following the Langendorff-perfusion, all hearts were simultaneously submerged in a solution of carboxymethylcellulose sodium solvent (Brunschwig Chemie, Amsterdam) and Indian Ink (Royal Talens, Apeldoorn) and frozen at -20°C in an imaging cryomicrotome. The frozen hearts were simultaneously sectioned, with images taken after each cut of 27 \( \mu \text{m} \) with excitation set at 480 nm (bandwidth 20 nm) and fluorescence detected at 520 nm (bandwidth 20 nm).

**Immunogold labelling of hexokinase biodistribution**

*Antibodies* Monoclonal anti-rabbit HK I & affinity purified anti-rabbit HK II were supplied by Chemicon Europe Ltd, Hampshire, UK, and goat anti-rabbit 10nm colloidal gold antibodies were supplied by BB International, Cardiff, UK.

*Sample preparation and sectioning* Sample preparation was slightly modified from that previously described. All sample preparation and analysis was performed in a randomised blinded manner. At the end of each perfusion experiment, longitudinal left ventricular sections were cut, and lightly fixed for 24 hours in 2% formaldehyde + 0.2% glutaraldehyde in KHB (pH 7.4), during which time they were shipped from the Netherlands to the UK for analysis. Upon arrival, sections were placed in 2.3mol/L sucrose overnight at 4°C. 1-2mm square mid-myocardial longitudinal sections were then cut from these samples, mounted on specimen pins, cryofixed by plunging into liquid nitrogen and stored in liquid nitrogen prior to cryosectioning. 70nm thick sections were cut using glass knives at -80°C using a Leica ultramicrotome, and mounted on 3mm nickel grids coated on one side with 0.3% Pioloform film. The grids were then floated on standard buffer consisting of PBS + 0.1% BSA-c (Aurion) + 0.1% sodium azide (pH 8.2) until all required sections had been cut. 12 grids per sample were used (3 each for, HK I, HK II analysis, and 2 in each control group). The grids were then transferred from the standard buffer onto a droplet of PBS + 0.05 mol/L glycine for 20 minutes, and then washed (3x5 minutes) with standard buffer. Each grid was then incubated on an individual 30\( \mu \text{L} \) droplet of primary antibody for 90 minutes (diluted in standard buffer as follows: anti-HK I 1:100; anti-HK II 1:800, previously titrated to optimal concentrations, data not shown). Controls were incubated on standard buffer only. Grids were then washed (6 x 5 minutes) in the standard buffer before incubation on 30\( \mu \text{L} \) droplets of the gold-conjugated secondary antibody for 60 minutes with goat anti-rabbit 10nm colloidal gold antibody diluted 1:100 in standard buffer. Again, optimal concentrations of secondary antibodies had been previously determined, data not shown. Grids were washed (3 x 5 mins) in PBS containing 0.1% azide before post-fixation using PBS + 2% glutaraldehyde (10 mins), and then washed again in distilled water (3 x 5 mins) prior to embedding in 9 parts 2% methyl cellulose, 1 part 3% uranyl acetate on ice, and blotted dry prior to analysis.

**Transmission Electron Microscopy of Cryosections**

Micrographs of the sections were obtained using an FEI T20 transmission electron microscope at an accelerating voltage of 80kV. At a magnification sufficiently low to prevent visualisation of gold labelling (and unintentional skewing of the results), but sufficiently high to visualise the distribution of the tissue sections present in each grid (typically 1500x), 20 representative co-ordinates were randomly assigned. Magnification was then increased to 11,500x, and the stage advanced through each randomly assigned co-ordinate, and an image captured for gold labelling counting. Thus, for each heart, 20 random images of 3 grids per hexokinase isoform, plus controls, were obtained (=60 images/antibody/heart). The labelling densities of
compartments and membranes were calculated using systematic random sampling and stereological techniques adapted from those devised by Mayhew et al as described in our preceding paper and expressed as gold particles per unit compartment area.

**Generation of a “mitochondrial binding ratio”**

Since the labelling efficiencies of the anti-HK I and anti-HK II antibodies for their respective targets could not be assumed to be the same, direct comparison of absolute amounts of these proteins is not possible. By generating an artificial “mitochondrial binding ratio”, defined below, and described previously, we obtained an index of the percentage of each HK isoform bound to the mitochondria in each of our experimental groups.

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\text{Mitochondrial binding ratio} = \frac{\text{mitochondrial labelling density}}{\text{cytosolic labelling density}} \times 1000
\]

**Mitochondrial membrane potential (ΔΨ<sub>m</sub>) of the intact heart**

High-resolution optical ΔΨ<sub>m</sub> imaging using the fluorescent dye tetramethylrhodamine methylester (TMRM), was performed in the inact, beating heart as recently reported. This method allows the assessment of mitochondrial function at a subcellular resolution within the intact organ. Briefly, following cannulation, hearts were allowed to stabilize for ten minute at physiological temperature (36 ± 1°C). Hearts were then stained with TMRM (250nM; Molecular Probes Inc.) mixed in a bolus of Tyrodes solution (dye loading phase) for 20 minutes. This was followed by a 20-30 minute dye washout phase during which perfusion was switched back to dye free Tyrodes solution. TMRM background fluorescence intensity was measured periodically throughout the dye staining and washout phases using a 6400 pixel CCD based optical imaging approach that allowed the measurement of normalized ΔΨ<sub>m</sub> with subcellular resolution (50μm) over a 4x4mm window of the murine epicardial surface. To measure TMRM background fluorescence, hearts were excited with filtered light (525±20nm) emitted from a quartz tungsten halogen lamp (Newport Corporation, CT, USA). Emitted fluorescence was filtered (585±20nm for TMRM) and focused onto the high resolution CCD camera. Background fluorescence intensity was measured as the amplitude difference before and after excitation. Peak emitted TMRM fluorescence signal from each of 6400 pixels was measured before and after excitation achieved by a computer automated filter shutter switch. Background corrected TMRM fluorescence (ΔΨ<sub>m</sub>) caused by TAT-HK or TAT-CON were normalized to the value of steady-state TMRM fluorescence achieved during the dye washout phase for each of the 6400 individual pixels. Normalized ΔΨ<sub>m</sub> measurements across the imaged 4x4mm region of the heart were plotted as contour maps using Delta Graph 5.6 (Red Rock Software). These maps served to illustrate the spatial distribution of ΔΨ<sub>m</sub>.

**TAT-HK treatment in Isolated cardiomyocytes**

In neonatal rat cardiomyocytes (NRCM), incubated for 2 h at different TAT-HK peptide concentrations (200 nmol/L, 1 μmol/L and 10 μmol/L) with or without 1 μmol/L CCCP. Cell viability and preservation of mitochondrial membrane potential was assessed as reported previously. Cell survival was determined by Trypan blue exclusion, whereas membrane potential was determined by tetramethylrhodamine
ethyl ester (TMRE; 100 nmol/L) using flow analysis with the FacsCanto (BD Biosciences, San Jose, CA).

**TAT only effects in isolated hearts**

Treatment of hearts with the TAT-CON peptide conferred a strong cardioprotective effect preventing IPC to exercise additional protection. To examine whether this effect results from the TAT moiety or the scrambled amino acids, we constructed a TAT only peptide. In a separate series, isolated mouse hearts were treated with 200 nM TAT peptide in a similar fashion as the TAT-CON peptide. The hearts were either subjected to ischemia-reperfusion only (n=3) or ischemia-reperfusion preceded by IPC (n=3) as reported for the other hearts. LDH release during reperfusion was measured as index of IPC protective effects. This peptide did not induce cardioprotection (LDH release 19.3 ± 2.4 U/g), and IPC was now able to reduce LDH release (11.7 ± 2.6 U/g), indicating that the TAT moiety does not affect IPC.

**Lactate dehydrogenase enzyme activity in effluent**

During the reperfusion period the effluent was collected at 5, 10, 30, 60 and 120 min of reperfusion and immediately frozen at -80°C. Lactate dehydrogenase (LDH) activity was determined using spectrophotometric analysis at 340 nm [10, 11]. LDH release is used as index of necrosis, as other studies have shown a good correlation between LDH release and TTC staining.8,9

**Biochemical analysis**

At the end of the experiments, hearts were homogenized as described previously.10 Briefly, following homogenization, the homogenate was centrifuged at 800 g for 3 min, and the resultant supernatant centrifuged at 10,000 g for 10 min at 4 °C. The pellet contained the crude mitochondrial fraction, and the remaining supernatant contained the cytosolic fraction. The mitochondrial fraction was treated with 0.1% Triton and 1 mmol/L of the cross-linker disuccinimidyl substrate (DSS, Thermo Scientific) to enable examination of Bax oligomerization.11 Mitochondrial Bax (monomers and oligomers) was determined by standard Western blot technique10 using 50 μg protein per lane. Bax was detected using a mouse anti-Bax monoclonal antibody (1:1000; BD Biosciences). The cytosolic fraction was used for cytochrome C determination (ELISA, Quantikine R&D Systems). Protein content of the different fractions was determined by the Bradford method.

**References**

5. Davey KA, Garlick PB, Warley AW, Southworth R. Immunogold labelling study of the distribution of GLUT-1 and GLUT-4 in cardiac tissue following


Ischemic preconditioning (IPC) is still effective in HKII\textsuperscript{+/-} hearts, despite increased IR injury. Although HKII\textsuperscript{+/-} hearts exhibit higher lactate dehydrogenase (LDH) release and cell injury during reperfusion, IPC was as effective in HKII\textsuperscript{+/-} hearts as in wild-type (WT) hearts (A). IPC was also effective in HKII\textsuperscript{+/-} hearts for the functional parameters of end diastolic pressure (EDP) at end reperfusion (B) and the recovery of the rate-pressure product (RPP = heart rate \times developed left ventricular pressure) at end reperfusion (C). *P<0.05 IPC vs. respective control (CON) heart; #P<0.05 vs. respective CON, WT heart. Data presented as means±SEM (n=6/7 each group).

Low-dose TAT-HK decreases mitoHKII at 5 min reperfusion. Typical examples showing intact ultrastruture (A, D) and binding of HKI (B, E) and HKII (C, F) for 200 nM TAT-control peptide (A-C) and TAT-HK peptide (D-F). G,H, Mitochondrial binding ratio (y-axes) for HKII (G) and HKI (H) as determined from HK immunogold labelling and EM analysis (n=3 each group). # P< 0.05 vs TAT-CON

Western blot analysis for Bax oligomers in the mitochondrial fraction
Bax oligomers of 62 kDa (A), 78 kDa (B) and 125 kDa (C) in mitochondrial fractions of low-dose TAT-HK treated hearts. (n=6-7 each group). Data presented as means ± SEM. # P < 0.05 vs TAT-CON, CON.

TAT-HK and cardiac mitochondrial potential heterogeneity.
Temporal ΔΨ\textsubscript{m} heterogeneities during infusion of 10 μM TAT-HK or TAT-CON measured at equidistant distances on the surface of isolated hearts. (n=4 each group).

Mitochondrial uncoupling prevents TAT-HK detrimental effects.
Cardiomyocytes were loaded with the ΔΨ\textsubscript{m} - sensitive dye TMRE, treated with different doses with TAT-CON or TAT-HK peptide without (A) or with (B) a submaximal dose (1 μM) of the mitochondrial uncoupler CCCP in neonatal rat cardiomyocytes (NRCM). (n=3 each group) * P< 0.05 vs 10 μM TAT-CON.
Online Figure I

A. LDH release

B. EDP

C. RPP recovery

WT

HKII+/−
ΔΨ
m Heterogeneity

Time (min)

TAT-HK
(10 μM, n=4)

TAT-Control
(10 μM, n=4)

P<0.05  P<0.01