The role of mitochondrial hexokinase II in ischemia-reperfusion damage

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Citation for published version (APA):
Smeele, K. M. A. (2012). The role of mitochondrial hexokinase II in ischemia-reperfusion damage
APPENDIX B

SUPPLEMENTAL MATERIAL & METHODS
AND FIGURES TO CHAPTER 7

PUBLISHED ONLINE:
http://circres.ahajournals.org/cgi/content/full/CIRCRESAHA.111.244962/DC1
Animals
C57BL/6J HK+/- were obtained from Vanderbilt University, Nashville (generous gift of dr. David H. Wasserman). The HK +/- mice were first described by Heikkinen et al. (3), and have a partial deletion to the HKII gene. HK+/- mice were initially bred with wild-type C57BL/6J mice (Jackson laboratories) and subsequently with wild-type offspring. The HK+/- 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+/- 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 (3). 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\(^{-1}\)). 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\(_2\) 2.25, MgSO\(_4\) 1.2, NaHCO\(_3\) 25, KH\(_2\)PO\(_4\) 1.2, EDTA 0.5 and glucose 11, glutamine 0.5, lactate 1.0 and pyruvate 0.1, gassed with 95% O\(_2\)/5% CO\(_2\). 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\(_2\)/5% CO\(_2\).

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)EEEAKNAAKLAVEILNKEKK-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 (10) 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 µmol/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 µmol/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 µm with excitation set at 480 nm (bandwidth 20 nm) and fluorescence detected at 520 nm (bandwidth 20 nm).

**Immunogold labeling 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 (9). All sample preparation and analysis was performed in a randomized 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. 1-2 mm 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 (3 x 5 minutes) with standard buffer. Each grid was then incubated on an individual 30 μL droplet of primary antibody for 90 minutes (diluted in standard buffer as follows: anti-HKI 1:100; anti-HKII 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 μL 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 min) in PBS containing 0.1% azide before post-fixation using PBS + 2% glutaraldehyde (10 min), and then washed again in distilled water (3 x 5 min) 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 visualization of gold labeling (and unintentional skewing of the results), but sufficiently high to visualize 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 labeling counting. Thus, for each heart, 20 random images of 3 grids per hexokinase isoform, plus controls, were obtained (= 60 images/antibody/heart). The labeling densities of
compartments and membranes were calculated using systematic random sampling and stereological techniques adapted from those devised by Mayhew et al. (6) as described in our preceding paper (1), and expressed as gold particles per unit compartment area.

*Generation of a “mitochondrial binding ratio”*. Since the labeling 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 (9), 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 labeling density} \times 1000}{\text{cytosolic labeling density}}
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**Mitochondrial membrane potential (ΔΨ_m) of the intact heart**

High-resolution optical ΔΨ_m imaging using the fluorescent dye tetramethylrhodamine methylester (TMRM), was performed in the intact, beating heart as recently reported (5). 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 (250 nM; 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_m with subcellular resolution (50 µm) over a 4x4mm window of the murine epicardial surface. To measure TMRM background fluorescence, hearts were excited with filtered light (525 ± 20 nm) 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 ($\Delta \Psi_m$) 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 $\Delta \Psi_m$ measurements across the imaged 4x4 mm 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 $\Delta \Psi_m$.

**TAT-HK treatment in isolated cardiomyocytes**

In neonatal rat cardiomyocytes (NRCM), incubated for 2 hours 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 (11). 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 (2). LDH release is used as index
of necrosis, as other studies have shown a good correlation between LDH release and TTC staining (4; 8).

**Biochemical analysis**

At the end of the experiments, hearts were homogenized as described previously (2). 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 (7). Mitochondrial Bax (monomers and oligomers) was determined by standard Western blot technique (2) 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**


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Supplemental figure 1. Ischemic preconditioning (IPC) is still effective in HK+/- hearts, despite increased IR injury. Although HK+/- hearts exhibit higher lactate dehydrogenase (LDH) release and cell injury during reperfusion, IPC was as effective in HK+/- hearts as in wild-type (WT) hearts (A). IPC was also effective in HK+/- 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 x developed left ventricular pressure) at end reperfusion (C). *P<0.05 IPC vs. respective CON heart; #P<0.05 vs. respective CON, WT heart. Data presented as means ± SEM (n=6-7 each group).
Supplemental figure 2. Low-dose TAT-HK decreases mitoHKII at 5 min reperfusion. Typical examples showing intact ultrastructure (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). 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.
Supplemental figure 3. 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.

Supplemental figure 4. TAT-HK and cardiac mitochondrial potential heterogeneity. Temporal ΔΨm 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).

Supplemental figure 5. Mitochondrial uncoupling prevents TAT-HK detrimental effects. Cardiomyocytes were loaded with the ΔΨm - 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.