The role of mitochondrial hexokinase II in ischemia-reperfusion damage

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Reduced Hexokinase II Impairs Muscle Function Two Weeks After Ischemia-Reperfusion Through Increased Cell Necrosis and Fibrosis


Submitted for publication
Abstract

We previously demonstrated that hexokinase (HK) II plays a key role in the pathophysiology of ischemia/reperfusion (I/R) injury of the heart. However, it is unknown whether HKII also plays a key role in I/R injury and healing thereafter in skeletal muscle, and if so, through which mechanisms. We used male wild-type (WT) and heterozygous HKII knockout mice (HK +/-) and performed in vivo unilateral skeletal muscle I/R, executed by 90 min hind limb occlusion using orthodontic rubber bands followed by 1 h, 1 d or 14 d reperfusion. The contralateral (CON) limb was used as internal control.

No difference was observed in muscle glycogen turnover between genotypes at 1 h reperfusion. At 1 day reperfusion, the model resulted in 36% initial cell necrosis in WT gastrocnemius medialis (GM) muscle that was doubled (76% cell necrosis) in the HK +/- mice. I/R-induced apoptosis (29%) was similar between genotypes. HKII reduction eliminated I/R induced mitochondrial Bax translocation and oxidative stress at 1 d reperfusion. At 14 d recovery, the tetanic force deficit of the reperfused GM (relative to control GM) was 35% for WT that was doubled (70%) in HK +/- mice, mirroring the initial damage observed for these muscles. I/R increased muscle fatigue resistance equally in GM of both genotypes. The number of regenerating fibers in WT muscle (17%) was also approximately doubled in HK +/- IR muscle (44%), thus again mirroring the increased cell death in HK +/- mice at day 1 and suggesting that HKII does not significantly affect muscle regeneration capacity. Reduced HKII was also associated with doubling of I/R-induced fibrosis.

In conclusion, reduced muscle HKII protein content results in impaired muscle functionality during recovery from ischemia-reperfusion. The impaired recovery seems to be mainly a result of a greater susceptibility of HK +/- mice to the initial I/R-induced necrosis (not apoptosis), and not a HKII-related deficiency in muscle regeneration.
**Introduction**

Skeletal muscle ischemia-reperfusion (I/R) frequently occurs in many acute (surgical intervention, circulatory shock, trauma) or chronic (decubitus, diabetes, obstructive vascular disease) clinical situations, resulting in muscle dysfunction and myocyte death which in turn contributes to increased morbidity and mortality even when limb circulation is restored. Elucidating and subsequent targeting of underlying cellular mechanisms may help alleviate the burden of skeletal muscle I/R. The current study examines a possible role of the glycolytic enzyme hexokinase (HK) II in skeletal muscle I/R injury.

Earlier cellular studies have demonstrated that the glycolytic enzyme HKI or HKII may have strong protective effects against stress-induced cell death (30). In addition, the increased expression of HK in malignant tumors is thought to contribute to the immortalization of cancer cells (25). Recently, we have demonstrated that HKII also plays an important role in the degree of acute injury and remodeling following I/R in the heart (33; 38). In the heart, HKI and HKII are approximate equally distributed. However, in skeletal muscle, HKII is the predominant HK isoform present (34), suggesting an even more prominent role of HKII in skeletal muscle I/R injury as compared to the heart. Indeed, we found that reduced HKII resulted in cell death in a non-injurious I/R model of intact mouse skeletal muscle (32). However, in this preliminary study, I/R injury was evaluated after a short period of ischemia (60 min) and reperfusion (90 min) only, resulting in minor I/R injury. Moreover, skeletal muscle has the capacity to regenerate and therefore recover from injury, in contrast to the very limited regenerative capacity of the heart. To our knowledge, no information exists concerning a possible role of HK in severe skeletal muscle I/R injury and regeneration. Therefore, in the present study, an extended period of ischemia (90 min) and prolonged periods of reperfusion (1 day and 14 days) were examined, to investigate the effect of HKII on severe muscle I/R injury and subsequent muscle regeneration.

The mechanisms through which HK offers protection are unresolved, but probably pertain to its binding to the mitochondria (30; 31; 40). It has been suggested that mitochondrial HK prevents apoptosis through inhibition of mitochondrial binding of the pro-apoptotic protein Bax (29), although Majewski et al. (24) demonstrated that HK protective effects
were still present in Bax-deficient cells. We were also unable to confirm a role for mitochondrial Bax in causing increased I/R injury in HKII-reduced hearts (33). Other mechanisms through which mitochondrial HK may alleviate injury relates to inhibition of necrosis and/or attenuation of oxidative stress through regulation of mitochondrial ATP/ADP exchange (9).

In the present study, we addressed whether HKII reduction affects 1) acute, severe, I/R injury of skeletal muscle, with a possible role for necrosis, apoptosis, glycogen turnover, mitochondrial Bax and oxidative stress, and 2) structural and functional healing through regeneration of postischemic, reperfused skeletal muscle tissue. A recently developed, non-invasive approach of the I/R intervention was chosen (7; 26), using orthodontic rubber bands in order to obtain complete hind limb ischemia and reperfusion.

**Material and Methods**

All procedures were in accordance with the guidelines of and approved by the Animal Ethical Committee of the University of Amsterdam and conform to NIH guidelines.

**Animals.**
C57BL/6J mice containing a partial deletion of the HKII gene (HK+/-) (17), were in-house bred under standard housing conditions (12 h dark /12 h light cycle; water and food given ad libitum) and fed Purina Laboratory Rodent Diet 5001 standard chow (PURINA; PMI Nutrition International, USA). These animals were shown to have reduced HK activity and HKII isoform levels in both heart and gastrocnemius medialis (GM) skeletal muscle (32; 34), without effect on HKI protein levels. Genotypes were determined by standard PCR technique after extraction of DNA from toe tissue after weaning, and verified from ear tissue analysis after experiment termination. Male mice of 3-4 months were used.

**Experimental procedures.**
Prior to performing the actual experiments, we performed pilot experiments in wild-type (WT) animals to determine the amount of injury created with increasing ischemic periods of 1, 1.5 or 2 hours followed by 24 hours reperfusion (n=3-5 per group). Injury was
determined by the amount of released lactate dehydrogenase (LDH) enzyme from I/R muscle and calculated as the ratio of LDH in the I/R and contralateral, control, muscle. Subsequently, WT and HK<sup>+/−</sup> mice were studied in four different experiments: group A) 90 min ischemia followed by 24 hours reperfusion (n=6-8 per group); group B) 90 min ischemia followed by 14 days reperfusion (n=5-7 per group); Additionally, in order to examine a possible role of glycogen among the genotypes, glycogen content of GM was determined in two separate groups: C) in I/R GM of WT animals immediately after ischemia (n=6), and in D) in control and I/R GM of WT (n=6) and HK<sup>+/−</sup> animals (n=9) at 1 h reperfusion following 90 min of unilateral ischemia. For group D muscles, caspase-3/7 activity was also determined. At the end of each experiment, all animals were sacrificed using cervical dislocation during a surgical plane of anesthesia.

**Validation of ischemia and reperfusion.** A laser speckle imaging (LSI) technique was used to assess limb perfusion (6; 11) during ischemia and reperfusion in separate experiments using WT animals (n=3). For LSI measurements, a commercially available system was used (Moor Instruments Ltd, UK). A 785 nm class 1 laser diode was employed for illumination of the tissue and light directly reflected by the tissue surface was blocked by a tunable polarizer in front of the lens system. Laser speckle images were captured using a 576 by 768 pixels grayscale CCD camera at a frame rate of 25 Hz and converted to pseudo-color images, where the level of perfusion was scaled from blue (low perfusion) to red (high perfusion). I/R was performed in the exact manner and duration as described below. The LSI was positioned about 50 cm above the animal and focused such that both hind limbs could be monitored simultaneously. Laser speckle images of both GMs were obtained at baseline, ischemia and reperfusion. The perfusion of the I/R muscle was calculated relative to the perfusion of the contralateral muscle, and normalized to baseline values.

**Ischemia and reperfusion procedure.** *In vivo* ischemia-reperfusion (I/R) was performed slightly modified from the method described by Crawford *et al.* (7). Briefly, induction of anesthesia was performed with an intraperitoneal injection of 80 mg/kg sodium pentobarbital, and was followed by a subcutaneous injection of 1 ml saline for fluid support. Body temperature of each mouse was monitored continuously with a rectal thermometer and maintained at 37°C throughout the complete procedure using heating.
pads. Ischemia for 90 min was applied to one hind limb using two orthodontic, rubber bands (#110, 3.2 mm, Latex O-Rings; Miltex Instruments, USA) placed together at the most proximal side of the thigh as close to the pelvic as possible using a McGivney Haemorrhoidal Ligator (Miltex Instruments, USA). During ischemia anesthesia was maintained by intraperitoneal injection of 20 mg/kg pentobarbital at 30 min and 75 min after induction. Additionally, 10 mg/kg pentobarbital was injected 100 min after induction (i.e. just before reperfusion), in order to prevent the mice to move at the first, crucial minutes of reperfusion. Reperfusion was created by cutting the rubber bands and visually confirmed by the color change of the paw from blue to red. Throughout the I/R procedure the contralateral (CON) hind limb was left untreated to serve as internal control. Animals returned to the (non-heated) cage after 1 hour reperfusion. Animals of experimental group A were anaesthetized after 1 day reperfusion with ketamine (125 mg/kg), medetomidine (0.2 mg/kg) and atropine (0.5 mg/kg) (41) and GM and gastrocnemius lateralis (GL) of both hind limbs excised for further analysis. This procedure was also performed for animals of group B after 14 days reperfusion with the exception that force measurements (described below) were performed prior to tissue excision.

**Table 1.** Body weight and gastrocnemius medialis muscle mass of wild-type (WT) and HK\(^{+/−}\) animals at 1 d reperfusion (n=8 each group) and 14 d reperfusion (n=7 each group).

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<tr>
<th></th>
<th>WT</th>
<th>HK(^{+/−})</th>
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<td></td>
<td>CON</td>
<td>1/R</td>
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<td>1 day reperfusion</td>
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<tr>
<td>GM wet weight (mg)</td>
<td>49 ± 3</td>
<td>56 ± 2(^*)</td>
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<tr>
<td>Body weight (g)</td>
<td>27.8 ± 0.8</td>
<td>27.4 ± 0.6</td>
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<tr>
<td>14 days reperfusion</td>
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<tr>
<td>GM wet weight (mg)</td>
<td>47 ± 2</td>
<td>27 ± 2(^*)</td>
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<tr>
<td>Body weight (g)</td>
<td>24.9 ± 0.6</td>
<td>25.1 ± 0.7</td>
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\(^*\) p<0.05 WT, CON vs. HK\(^{+/−}\), CON; \(^*\) p<0.05 1/R vs. CON, same genotype.

**Force measurements.** Preparation and force measurements were identical as previously described (32). In brief, animals were ventilated mechanically after tracheotomy. In order to perform *in situ* stimulation, the medial head of the gastrocnemius was prepared free from surrounding tissue, leaving the origin on the femur and blood supply intact. Distally, the Achilles tendon was cut and attached through a metal hook to a force transducer (42). The muscle was stimulated via the severed sciatic nerve, with temperature kept at 37ºC throughout the experiment. Muscle optimal length using twitch contractions was determined (10) and maximum twitch and tetanic force was measured. Twitch and tetanic
forces were measured as mN and normalized to GM wet muscle mass (Table 1). Both were used as index of functional recovery of I/R relative to CON GM performance. Finally, muscle fatigue was assayed by application of 30 repetitive tetani (stimulation frequency 150 Hz, stimulation duration 50 ms, pulse width 40 μs, one contraction every 250 ms) as described previously (19).

**GM cryopreservation and homogenization.** CON and I/R GMs from group A and group B were excised from the anesthetized animals and weighed. Each GM was cut in half over the length-axis. One part was immediately cryopreserved for immunohistological determination by embedding in Tissue-Tek (Sakura Finetek, The Netherlands) and quickly frozen in liquid-nitrogen pre-chilled isopentane (Sigma-Aldrich, Germany) and directly stored at -80°C until further analysis. The second GM part was directly homogenized in 0.8 ml ice-cold isolation buffer containing (mM): 250 sucrose, 20 HEPES buffer (pH 7.4), 10 KCl, 1.5 MgCl₂, 1 EDTA, 0.1 PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin and 1 μg/ml pepstatin A. Half of the homogenate was centrifuged at 10,000 g for 10 min. The pellet contained the crude mitochondrial fraction and the supernatant the cytosolic fraction. The other half of homogenate was determined as whole-cell fraction. Samples were directly stored at -80°C until further analysis. Muscles from group C and D were excised, immediately frozen in liquid nitrogen and stored at -80°C until further analysis for glycogen content and caspase-3/7 activity. Mice were subsequently sacrificed by cervical dislocation.

**Edema measurement.** After excision of GMs, the gastrocnemius lateralis (GL) was excised for group B, weighted (to obtain the GL wet weight) and dried in a stove at 60°C for 48 hours and weighted again to obtain GL dry weight. Edema was calculated as ratio of wet to dry GL weight.

**Postexperimental analysis.**

**Immunohistochemistry.** For the immunofluorescence and the TUNEL-reaction we used 5-μm thick serial tissue specimen. The specimen from the I/R and CON GM were placed on the same glass slide per experiment. Necrotic cells were detected by intracellular caveolin-staining, based on the premise that loss of plasma membrane integrity is a primary characteristic of necrosis. Apoptotic cells were detected by the TUNEL assay (In situ Cell
Death Detection Kit, POD, Roche), based on the premise that DNA fragmentation is a primary characteristic of apoptosis (22). For counting the amount of necrotic cells (group A, 1 day reperfusion) and analysis of regeneration by measuring cell surface and counting number of cells with centralized nuclei (group B, 14 day reperfusion), GM slices were incubated with anti-caveolin (red; CAV; 1:100; BD Biosciences), anti-laminin (green; LAM; 1:50; Sigma) and anti-DAPI (blue; 300nM; Invitrogen), staining for necrotic cells, cell membrane and nuclei, respectively. Cells were counted as necrotic when the intracellular surface stained for caveolin. Number of necrotic cells and number of cells with centralized nuclei were counted by hand and analyzed as ratio to the total cell number counted in each picture. The number of apoptotic cells equalled the number of myocyte related TUNEL-positive nuclei divided by total number of myocyte related nuclei (= nuclei (DAPI-staining) close to cell membrane (LAM staining)). Total cell surface was determined by drawing the borders of the laminin stained cells by hand, hereby eliminating (non-stained) intracellular space, followed by software-determined calculation of the selected area. Subsequently, mean cell surface was determined by dividing total cell surface by total number of cells counted in each picture. Other GM tissue slices were incubated with anti-CD31 (red; 1:100; BD Biosciences) together with anti-laminin (blue; 1:50; Sigma) to stain for capillaries and cell membrane, respectively. Angiogenesis in the GM after 14 days reperfusion was determined by counting the number of CD31-stained capillaries within the LAM-stained myocytes in each image. The number of capillaries was normalized to total cell area, which borders were drawn by hand and area was measured automated and shown as mm². Fibrosis, the amount of intercellular collagen formed, was analyzed by measuring the total collagen surface (red) and total cell surface (pink) in each image after performing a Sirius Red staining.

All abovementioned analyses were performed in a blinded fashion, both to genotype as to condition. Each parameter was determined in 3-5 images per GM, with each image analyzed 2-3 times. All images were 20x magnifications and analysis was performed using Lucia G/F imaging analysis software (Laboratory Imagin, CZ).

*Mitochondrial Bax amount.* The pellet of the crude mitochondrial fraction from experimental group A (1 day reperfusion) was resuspended in isolation buffer and incubated for 1 hour with 2% CHAPS in order to contain Bax oligomers formed due to I/R (1). For tissue
HKII reduction affects necrosis and fibrosis after skeletal muscle I/R injury

Lysation the samples were sonicated and debris was removed by centrifugation at 100,000 g for 30 min. The supernatant, consisting of the solubilized mitochondrial fraction, was incubated with 1 mM disuccinimidyl suberate (DSS, Thermo Scientific, USA) dissolved in DMSO for cross-linking of Bax complexes. The chemical reaction was stopped by addition of loading buffer containing mercaptoethanol and analyzed for total (monomer and oligomer) mitochondrial Bax amount by Western Blotting. 15 µg protein per slot was loaded on a 10% SDS-polyacrylamide denaturing gel. PVDF membranes were incubated with anti-Bax (1:1,000; BD Biosciences, USA) and anti-VDAC (1:10,000; Calbiochem, USA) as loading control, followed by horseradish peroxidase-conjugated secondary goat anti-mouse antibodies (1:10,000; Jackson ImmunoResearch, Germany) and anti-biotin (1:2,000; Cell Signaling, USA). Immunoreactive bands were visualized by chemiluminescence on X-ray film (Hyperfilm ECL, Amersham, USA) using enhanced chemiluminescence solution (Santa Cruz, USA). For quantification a Kodak Image Station 440CF (Eastman Kodak Company, USA) was used. Each sample was analyzed in duplo.

Oxidative stress. Homogenized I/R and CON GM tissue samples of experimental group A (1 day reperfusion) were analyzed. Oxidative stress was determined by analyzing the degree of protein carbonylation in the supernatant of whole-cell homogenate, obtained after 0.5% Triton X-100 treatment and centrifugation at 12,000 g for 10 min. The method uses derivatization of protein carbonyl groups with 2,4-dinitrophenyl hydrazine (DNPH) producing hydrazone, which is detected spectrophotometrically (Protein Carbonyl Assay kit; Cayman Chemical Company, USA). Values were normalized to sample protein concentration (Bradford assay).

Enzyme activity measurements. GM whole-cell homogenate of experimental group B was treated with 0.5% Triton X-100 to solubilize hexokinase (21), followed by centrifugation (12,000 g; 1 min) to pellet crude undissolved remnants. All enzyme activities were spectrophotometrically determined in the resulting supernatant at 25°C. Hexokinase (HK) and citrate synthase (CS) activity was measured as reported previously (16). HK activity was normalized to citrate synthase (CS) activity. Lactate dehydrogenase (LDH), as index of necrosis used in the pilot experiments, was measured using KH₂PO₄ buffer, NADH and pyruvate and normalized to sample protein (Bradford assay) concentration.
Glycogen amount. Frozen muscles were grinded into powder in liquid nitrogen, and a small part of the powder was used for glycogen determination, the other part for caspase 3/7 activity analysis. Glycogen was enzymatically converted to glucose using amylglucosidase (Roche Diagnostics). Glucose was determined by a two step enzymatic method using hexokinase and glucose-6-phosphate dehydrogenase (both Roche Diagnostics) as described by (3). Liquid handling was performed by a Freedom EVO 100 robot (Tecan) and formation of NADPH was measured fluorometrically using an Infinite plate reader (Tecan). Values were normalized to sample protein concentration (Bradford assay).

Caspase-3/7 activity assay. Part of the grinded GM was dissolved in 0.6 ml ice-cold isolation buffer containing (mM): 250 sucrose, 20 HEPES buffer (pH 7.4), 10 KCl, 1.5 MgCl2, 1 EDTA, 0.1 PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A and 0.5% CHAPS (Sigma-Aldrich). After 5 min incubation at room temperature each sample was sonicated for 5 sec and subsequently centrifuged at 12,000 g for 10 min at 4°C. The supernatant was transferred into a new vial and stored at -80°C until further analysis. Caspase-3/7 activity was assessed in the supernatant with the Caspase-Glo 3/7 assay kit (G8090, Promega, Madison, Wis.). 75 μl supernatant was diluted in 75 μl assay buffer and luminescence detected according to the manufacturer protocol. Values were normalized to sample protein concentration (Bradford).

Statistics.
Values are presented as mean ± SEM. Differences between WT and HK+/- groups were analyzed by a two-way ANOVA (main effects: genotype and I/R-treatment; interaction effect: genotype x I/R-treatment) for repeated measurements followed by contrast comparison (SPSS version 18). Differences were considered statistically significant at $P<0.05$. 
Results

Model characteristics
Optimization of ischemic model. The degree of I/R damage is critically dependent on the duration of ischemia. Therefore, we performed pilot experiments in order to determine the optimal duration of ischemia at which damage would be severe enough, but still be amenable to decreases or increases. Ischemia was applied for 1, 1.5 or 2 hours and followed by 24 hours reperfusion. LDH measurements, as parameter of necrosis and therefore muscle damage, showed that with each 30 min prolongation of ischemia the degree of damage was increased proportionally (Figure 1A). The optimal duration of ischemia to test our hypotheses appeared to be 90 min resulting in approximately 60% muscle damage as measured by LDH leakage from the I/R GM muscle.

Muscle perfusion. In order to validate successful ischemia and reperfusion in this non-invasive I/R model, we monitored relative muscle perfusion simultaneously in both hind limbs using laser speckle imaging. Figure 1B (middle picture) clearly shows immediate, near-complete occlusion of limb perfusion upon application of two rubber bands in the tight. Removal of the occlusion immediately restored blood flow in the whole limb (Figure 1B, right panel). Applying I/R to one hind limb appeared to be without influence on muscle perfusion in the contralateral, control hind limb (Figure 1B). Hyperemia was clearly detectable during the first minutes of reperfusion, after which perfusion returned close to normal from 5 minutes reperfusion on (Figure 1C).

Thus, the used method of I/R application appeared to be non-damaging to the limb vasculature and resulted in severe ischemic injury when applied for 90 minutes, and successful reperfusion of the hind limb in vivo.

1 h reperfusion
Glycogen content. No differences were observed between glycogen content in control muscles between genotypes (Figure 2A). At 1 h reperfusion, there was a clear trend of diminished glycogen content in I/R muscles relative to control muscles, indicating glycogen breakdown during the ischemic period. However, no differences in I/R-induced glycogen breakdown between genotypes were detected. In a second series of experiments we measured glycogen content in I/R wild-type muscle immediately after ischemia, thus
Figure 1. Model characterization measurements. (A) Cytosolic LDH activity measurements as parameter for cellular injury, shown as % I/R to CON values as measured in pilot experiments with 1, 1.5 or 2 hours ischemia followed by 1 day reperfusion. Blood flow velocity was measured using laser speckle imaging. (B) Images of the same mouse of both hindlimbs at the moment of baseline (BL), 1 min ischemia (I-01) and 1 min reperfusion (R-01). Left limb is I/R and right limb is CON limb. Degree of perfusion is visualized by colour: blue=none to low, yellow=medium and red=high. (C) Muscle perfusion during I/R (n=3), normalized to CON limb and relative to BL values. Values are given as mean ± SEM.

Figure 3. Tissue damage after ischemia followed by 1 day reperfusion. Cellular necrosis and apoptosis were histologically evaluated using caveolin (red; CAV), laminin (green; LAM), and DAPI (blue) for necrosis (A) and using LAM (red), DAPI (blue) and TUNEL (green) for apoptosis (C) staining, respectively. The number of necrotic cells (B) and apoptotic cells (D) are shown relative to total cell count in each image. Increased edema (E) was found in I/R muscle tissue compared to CON as measured by gastrocnemius lateralis (GL) wet-to-dry weight ratio. Values are given as mean ± SEM. * p < 0.05 versus WT group. # p < 0.05 versus CON group of same genotype.
without reperfusion. The measured amount of glycogen (2.51 ± 0.36 μM/mg protein) in ischemia-only muscle is almost similar to the glycogen content of 1 h reperfused muscles (2.54 ± 0.52 μM/mg protein), indicating that 1 h reperfusion does not substantially recover muscle glycogen content.

Caspase-3/7 activity. Caspase activity was measured in control and I/R muscle of both genotypes at 1 h reperfusion (Figure 2B). No differences in caspase activity in control muscles between WT and HK+/− mice were detected. Surprisingly, caspase activity in I/R muscle of WT was significantly decreased as compared to control muscle. However, no differences in I/R-induced alterations in muscle caspase activity between WT and HK+/− mice were observed.

24 h reperfusion
Cellular damage. I/R-induced muscle necrosis and apoptosis were clearly present after 24 hours reperfusion, as assessed using histological analysis. As the representative images in Figure 3A show, CON myocytes of both genotypes were unaffected by the I/R procedure performed with the opposite limb as they had retained their normal morphological features – polygonal shape and tightly aligned - and did not stain intracellular for caveolin.

In I/R GM of WT animals 36% of myocytes were necrotic (Figure 3B) and were morphologically mostly rounded and separated from each other (Figure 3A). The number of necrotic myocytes was significantly increased to 76% in HK+/− animals (Figure 3B). Apoptotic cells were barely detectable in control muscle of both genotypes (Figure 3C and 3D). I/R induced an increase in apoptotic cells to 26 ± 3% (WT) and 33 ± 6% (HK+/−) muscle. However, the I/R-induced increase in apoptosis was not significantly different between WT and HK+/− muscle. I/R-induced edema was approximately 50% for both WT and HK+/− animals (Figure 3E).

I/R-induced mitochondrial Bax and oxidative stress. Since HKII may exert its cellular protective role by directly influencing the amount of mitochondrial bound pro-apoptotic protein Bax - due to competition for the same mitochondrial binding site (29) - we investigated the total amount of mitochondrial Bax (Figure 4A). Mitochondrial Bax more
Figure 2. Glycogen content and caspase-3/7 activity after ischemia followed by 1 h reperfusion. Glycogen was measured in control and I/R muscle tissue of WT and HK⁻/⁻ mice (A). Caspase-3/7 activity was determined in control and I/R muscle tissue of WT and HK⁻/⁻ mice (B). Values are given as mean ± SEM. # p < 0.05 versus CON group of same genotype.

Figure 4. Pro-apoptotic Bax and oxidative stress were investigated at 1 day reperfusion. (A) Mitochondrial Bax normalized to VDAC content of I/R and CON GM muscles of WT and HK⁺/⁻. (B) Protein carbonylation content of WT and HK⁺/⁻ GM and shown as carbonyl content normalized to protein content. Values are given as mean ± SEM. # p < 0.05 versus corresponding CON group. $ p < 0.05 for interaction effect (I/R-treatment effect in WT vs. that in HK⁺/⁻).

Figure 5. Mitochondrial capacity and hexokinase (HK) activity measured in WT and HK⁺/⁻ whole-cell homogenate of ischemia-reperfusion treated (I/R) and contralateral (CON) GM after 14 days reperfusion. (A) Citrate synthase (CS) activity values normalized to protein content as parameter of mitochondrial capacity. (B) HK activity shown as ratio to CS enzyme activity. Values are given as mean ± SEM. * p < 0.05 vs. corresponding WT group. # p < 0.05 vs. CON group of same genotype.
than doubled in WT animals upon I/R, indicating that skeletal muscle I/R is indeed associated with a translocation of Bax to mitochondria. Remarkably, this effect of I/R on mitochondrial Bax was absent in HK+/− animals. No differences were observed in amount of mitochondrial Bax in CON GM between WT and HK+/− animals.

In order to study a possible effect of HKII on oxidative stress due to I/R, the degree of protein carbonylation in the whole-cell homogenate of the GM was studied. As Figure 4B shows, increases in protein carbonylation in the I/R muscle were observed for WT animals, indicating that skeletal muscle I/R is indeed associated with elevated oxidative stress. Surprisingly, I/R was not associated with increased carbonylation in the GM of the HK+/−, whereas basal levels of carbonylation in the CON muscle were similar between genotypes. The data therefore indicate that the increased injury following I/R in the HK+/− muscle cannot be explained through increases in mitochondrial Bax or oxidative stress.

14 days reperfusion
As a result of the finding that HKII reduction does severely increase the amount of cells injured due to the I/R procedure, we investigated whether HKII reduction would also influence the subsequent muscle repair processes. GM tissue was studied after 90 min ischemia and a subsequent period of 14 days reperfusion, a moment at which all injured cells have been eliminated and different processes of skeletal muscle regeneration are in progress (20; 27).

Mitochondrial capacity and HK activity. In order to examine whether I/R and regeneration affected mitochondrial capacity and total hexokinase activity relative to mitochondrial content, and to what extent this was affected by the deletion of one HKII allele, citrate synthase (CS) and HK activity were determined. CS was not different between genotypes and was unaffected by I/R and regeneration (Figure 5A). Contrastingly, I/R and regeneration resulted in large increases in HK activity relative to CS activity. This increase was unaffected by the ablation of one HKII allele (Figure 5B). The data indicate that the response of skeletal muscle to a severe ischemic period followed by 14 days of healing contains a large of the hexokinase enzyme without alterations in mitochondrial capacity. Ablation of one HKII allele resulted in approximately 50% reduction in total HK activity as compared to WT, indicating that HK activity in GM is predominantly HKII activity.
Functional recovery and fatigue resistance. Both twitch (8.2 ± 0.6 mN/mg vs 10.4 ± 0.6 mN/mg) and tetanic force production (31.5 ± 2.1 mN/mg vs 34.0 ± 2.0 mN/mg) were similar for control GM between WT and HK+/− mice, respectively. However, HKII reduction diminished both twitch (Figure 6A) and tetanus (Figure 6B) force recovery following ischemia and 14 d healing; functional recovery in HK+/− GM was approximately 50% of the recovery observed for WT GM. Resistance to fatigue was investigated by measuring the developed force upon application of 30 repetitive tetani and relate the value measured for each tetanus to the value of the first tetanus during reperfusion (Figure 6C). The high-intensity fatigue protocol showed a strong decrease in force in the control GM, which was not significant different between WT and HK+/−. I/R and 14 d healing resulted in increased fatigue resistance of the GM of both genotypes. The I/R-induced increase in fatigue resistance was not affected by ablation of one HKII allele.

Muscle regeneration. Muscle regeneration is characterized by the presence of immature fibers recognized as centronucleated fibers of small size. Representative images in Figure 7A clearly demonstrate the smaller (-52 to -55%) cell size and increased incidence of

Figure 6. Functional recovery of GM after ischemia and 14 days reperfusion. Twitch (A) and tetanus (B) recovery shown as ratio I/R to CON GM values. (C) Muscle fatigue as tested by applying 30 repetitive tetani. Developed force at each tetanus for both CON and I/R GM was measured and shown as ratio to first tetanus of same group. Values are given as mean ± SEM. * p<0.05 vs. WT group. # p<0.01 vs. CON group of same genotype.
HKII reduction affects necrosis and fibrosis after skeletal muscle I/R injury

Figure 7. Muscle regeneration after ischemia followed by 14 days reperfusion. Cell plasma membranes were stained by laminin (green) and nuclei were stained by DAPI (blue). Representative pictures (A) of CON and I/R GM tissue of WT and HK +/- animals with both stainings shown in each image. Mature myocytes are recognized by nuclei at the border of each cell and polygonal shape, while immature myocytes are recognized by nuclei in the center of the cell, the rounded shape and smaller size. (B) Average cell size was measured in both CON and I/R GM and (C) the number of cells with centralized nuclei relative to the total number of cells were counted in GM of WT and HK +/- animals. Data of centralized nuclei are shown as % I/R to CON GM. Values are given as mean ± SEM. * p<0.05 vs. corresponding WT group. # p<0.01 vs. CON group of same genotype.

Figure 8. Angiogenesis in skeletal muscle after ischemia followed by 14 days reperfusion. Capillaries were visualized by CD31 staining (pink) and plasma membranes were stained by laminin (blue). Representative pictures (A) of CON and I/R GM tissue of WT and HK +/- animals with both stainings shown in each image. (B) Number of capillaries was measured and normalized to the total area of cells in each image in CON and I/R GM of WT and HK +/- animals. Values are given as mean ± SEM. # p<0.01 vs. CON group of same genotype.

Figure 9. Fibrosis in skeletal muscle after ischemia followed by 14 days reperfusion. Representative pictures (A) showing Sirius Red staining for collagen (red) and muscle tissue (pink) of CON and I/R GM tissue of WT and HK +/- animals. (B) Total area of collagen was normalized to the total area of cells in each image for I/R and CON GM of WT and HK +/- animals. Values are given as mean ± SEM. # p<0.01 vs. CON group of same genotype. $ p<0.05 HK +/- 1/R to CON ratio vs. WT I/R to CON ratio.
centronucleated cells following I/R for both WT and HK⁺/⁻ mice. No centronucleated myocytes were found in CON muscles of both genotypes. Surprisingly, cell size in the HK⁺/⁻ CON muscle was significantly smaller (-14%) than that in the WT CON muscle (Figure 7B), indicating that HKII may be a determinant of myocyte cell size. I/R reduced cell size to a large extent in our model, which reduction was not different between WT and HK⁺/⁻ mice. In contrast, the number of centronucleated fibers in the I/R muscle was significantly larger for HK⁺/⁻ GM (44%) compared to that in the WT GM (17%; Figure 7C).

**Angiogenesis.** Capillaries were detected using the endothelial marker CD31 (Figure 8A). No significant differences were observed in capillary density of CON muscle between WT and HK⁺/⁻ mice. Ischemia-reperfusion and 14 d healing resulted in increased capillary density of the GM, which increase, however, was not affected by the partial deletion of the HKII allele (Figure 8B). The data indicate therefore that HKII does not play a critical role in skeletal muscle angiogenesis following I/R.

**Fibrosis.** The degree of fibrosis that developed in reaction to the I/R intervention was investigated by measuring the amount of interstitial collagen. In both Figure 9A (representative images) and Figure 9B it is shown that fibrosis amounted to 1.3% and 0.9 % of muscle area in control GM of WT and HK⁺/⁻ mice, respectively. I/R increased fibrosis to 4.5% in WT mice and to 6.8% in HK⁺/⁻ mice, where the increase in the HK⁺/⁻ mice was significantly larger compared to that in the WT mice. The almost doubling of I/R-induced fibrosis at 14 d healing in the HK⁺/⁻ mice mirrors the doubling of acute I/R-induced cell necrosis in these muscles (Figure 3B). This indicates that HKII probably does not play a major role in the development of fibrosis during muscle healing.

**Discussion**

The major findings of this study are that reductions in HKII in a skeletal muscle model of ischemia-reperfusion, result in 1) increased myocyte cell necrosis at day 1 of reperfusion, without alterations in I/R-induced glycogen turnover or apoptosis, or increases in mitochondrial Bax and oxidative stress; 2) reduced functional recovery, and 3) increased fibrosis without affecting I/R-induced angiogenesis at day 14 of reperfusion. The
observation that the doubling of acute I/R injury at day 1 translates directly into a
doubling of the number of regenerating fibers and a doubling of fibrosis at day 14,
indicates that HKII mainly affects the acute I/R intervention and not the subsequent
regeneration capacity of skeletal muscle.

**HKII and acute I/R cell damage.**

We observed about 40% injured GM fibers following 90 min ischemia and 24 h
reperfusion, which value falls within the reported range of 30-60% GM injury following
90-120 min ischemia and 24 h reperfusion (5; 26; 39). In a previous study in skeletal
muscle, we showed that non-injurious I/R for WT muscle became mildly injurious with
reductions in HKII (32). The present work underscores the importance of HKII in injurious
I/R of skeletal muscle. In heart, we recently showed that upon 25% reduction of cardiac
HKII, I/R injury was increased by 60% (38). In the present study, skeletal muscle HKII
content was decreased by 40% (34), and I/R injury was increased by 109%. Even though
obtained in different tissues, these results imply that the amount of HKII is inversely
related to the degree of I/R injury and therefore emphasize the importance of HKII
protein levels in relation to acute skeletal muscle cell survival.

In the past, mitochondrially bound HK was found to be the key factor in reducing
apoptosis through inhibition of the mitochondrial permeability transition pore (mPTP)
opening. The proposed mechanism was that HK prevented pro-apoptotic Bax binding to
the mitochondrion by direct competition for the same mitochondrial binding site (29).
However, the importance of Bax in the process of I/R damage and thereby HKII cellular
protection is under debate since Majewski et al. (24) showed that HKII protection was
found with and without Bax present. Very recently, we did not find Bax-mediated
apoptotic pathway to be involved in mitochondrial HKII dissociation-mediated I/R injury
in the intact heart (33). The present results support our previous work and that of
Majewski et al. (24) in that mitochondrial Bax is probably not the mediator through which
reductions in total cellular HKII result in increased I/R cell death in muscle. Moreover,
our results clearly indicate that HKII content mainly affects the necrotic pathway of cell
death in a model of *in vivo* skeletal muscle I/R. No changes in apoptotic cells or caspase
activity were observed with reduced skeletal muscle HKII content. It should be realized
that necrotic and apoptotic pathways of cell death are not completely separate entities,
they are often interconnected (22). Although not directly examined in the present study, it is possible that the decreased caspase activity in the I/R muscle of WT mice at 1 h reperfusion is a consequence of disruption of the plasma membrane (= necrosis) and therefore leakage of the caspase protein out of the myocyte. This could be one example as to how necrosis may affect the apoptotic machinery. In addition, increases in oxidative stress have also been proposed to mediate the increased I/R-induced cell death with decreases in mitochondrial HK (9, 28). In our experimental model, I/R was indeed associated with increases in oxidative stress in WT animals. Surprisingly, this increase in oxidative stress was significantly attenuated in the HK+/− mice, making it unlikely that increases in oxidative stress may explain the increased cell death with chronic decreases in HKII. We previously demonstrated that mitochondrial HKII is needed to keep mitochondria polarized (33). It is possible that in the HK+/− mice, the reduction in mitochondrial HKII results in an increased number of depolarized mitochondria, normally occurring during ischemia-reperfusion (23), which is directly translated into increased I/R damage.

Alternatively, alterations in glycogen turnover during I/R may also partly explain the increased injury, although the amount of glycogen in relation to I/R damage is often a double-edged sword as shown by Cross et al (8). High glycogen may protect against damage as long as it is not depleted. With lasting ischemia and consequently full depletion of initial high glycogen, a high glycogen actually may result in increased I/R damage. Our results clearly indicate that the observed increase in necrosis with reductions in HKII cannot be explained by alterations in glycogen turnover.

Although further experiments are needed to elucidate the mechanism through which chronic reductions in HKII exacerbate skeletal muscle I/R-induced myocyte necrosis, the present work discards a possible role for glycogen turnover or apoptosis in explaining increased injury with reductions in HKII in a model of in vivo skeletal muscle I/R.

**HKII and skeletal muscle recovery after I/R**

The present study clearly demonstrates reduced functional recovery and increased fibrosis in skeletal muscle of HK+/− mice at 14 d recovery following I/R injury. This observation can be explained by 1) a HKII-related deficiency in muscle regeneration following I/R injury, and/or 2) a greater susceptibility of HK+/− mice to the initial damage of I/R. In
general, HKII is increased in highly proliferating, growing tissues (25) to support the increase in biosynthetic activity in such tissues. Our observations that HKII is increased in regenerating I/R muscles and that skeletal muscle cell size in control muscle is smaller in the HK\(^{-/-}\) mice, are commensurate with this role of HKII in growth and proliferation. However, the finding of an increased number of regenerating muscle fibers in the GM of HK\(^{-/-}\) mice as compared to WT mice, suggests that reductions in HKII do not result in reduced regeneration. The similar weight of the reperfused GM muscle for both WT and HK\(^{-/-}\) mice at 14 d recovery also indicates that muscle regeneration is not reduced by reductions in HKII. Actually, the observation that the doubling in the number of regenerating fibers in HK\(^{-/-}\) muscle at 14 d recovery mirrors the doubling in number of death of fibers in HK\(^{-/-}\) muscle at 1 day reperfusion, indicates that the regeneration process is not directly affected by HKII. Our data therefore suggest that the reduced recovery of GM in HK\(^{-/-}\) mice is mainly a result of a greater susceptibility of HK\(^{-/-}\) mice to the initial damage of the I/R insult, and not to a deficiency in muscle regeneration. The increased fibrosis with reductions in HKII also probably reflects the increased initial I/R damage in these mice and is in agreement with the increased cardiac fibrosis observed using the same mouse model following an *in vivo* cardiac I/R insult (38).

The increase in capillary density at 14 d recovery is similar to previous findings of increased capillarisation following I/R in skeletal muscle (18; 35). That the I/R-induced increase in capillary density was similar for HK\(^{-/-}\) and WT muscle indicates that HKII does not affect I/R-induced angiogenesis. In a previous study using the same mouse model, we observed decreased capillary density in the border zone of the I/R-subjected heart, which decrease was exacerbated in the HK\(^{-/-}\) mice (38). However, this discrepancy is very likely caused by the fact that the heart has very limited regenerating capacity, such that the decreased capillary density observed for the reperfused heart is mainly a consequence of cell death and fibrosis in the border zone, which were both increased for HK\(^{-/-}\) mice as compared to WT mice.

*Functional recovery.* The tetanic force deficit following 14 d recovery is approximately 35% for WT mice and 70% for HK\(^{-/-}\) mice, again mirroring the initial damage observed in each genotype (cell death of 36% and 76% for WT and HK\(^{-/-}\) mice, respectively). This corroborates our morphological analyses in that the decreased functional recovery in the
HK+/− mice is probably mostly the result of the increased initial damage and not of a slower rate of recovery during the 14 days of healing. Because the force measurements are normalized to muscle mass, the diminished force production in the reperfused muscles indicates that the regenerating, centronucleated, fibers are still in the immature state after 14 days of recovery and not yet fully integrated as functional muscle units.

The increase in fatigue resistance measured after I/R correlates with other reports (2; 37) and possibly reflects a transition of muscle fiber type from fast-twitch to slow oxidative fibers (2; 36). GM muscle predominantly consists of fast-twitch fibers (4), which are relatively prone to fatigue (36). The proposed change to a more fatigue resistant fiber type due to I/R would therefore reduce fatigue vulnerability. Besides a switch in fiber type, increased fatigue resistance may also be explained by the I/R-induced increase in HK activity, as observed in the present study. Fueger and colleagues have shown in different studies that the HKII protein content is a determinant of endurance capacity (15). Additionally, during exercise muscle glucose uptake is reduced in HKII reduced animals (13), while the glucose transporter GLUT4 is unaffected by HKII reduction (14), making glucose phosphorylation a limiting factor for muscle glucose uptake (12; 13). Therefore, glucose phosphorylation by HK is a limiting factor for muscle endurance and fatigue properties, and the increase in HK activity observed in the reperfused muscles of both genotypes may partly explain the increased fatigue resistance.

In summary, our results demonstrate that reduced HKII protein content results in impaired muscle functionality at 14 d recovery following an ischemia-reperfusion episode. The impaired recovery seems to be mainly a result of a greater susceptibility of HK+/− mice to I/R-induced acute necrosis (not apoptosis) and is not related to a HKII-related deficiency in muscle regeneration. The data suggest that manipulation of HKII protein content before I/R interventions may be a promising avenue for preventing muscle dysfunction following I/R.
HKII reduction affects necrosis and fibrosis after skeletal muscle I/R injury

References


