Hexokinase II and Reperfusion Injury
TAT-HK2 Peptide Impairs Vascular Function in Langendorff-perfused Rat Hearts

Philippe Pasdois, Joanne E. Parker, Elinor J. Griffiths, Andrew P. Halestrap

Rationale: Mitochondrial-bound hexokinase II (HK2) was recently proposed to play a crucial role in the normal functioning of the beating heart and to be necessary to maintain mitochondrial membrane potential. However, our own studies confirmed that mitochondria from ischemic rat hearts were HK2-depleted, yet showed no indication of depolarization and responded normally to ADP.

Objective: To establish whether the human TAT-HK2 peptide used to dissociate mitochondrial-bound HKII in the Langendorff-perfused heart may exert its effects indirectly by impairing coronary function.

Methods and Results: Ischemic preconditioning was blocked in rat hearts perfused with 2.5 µmol/L TAT-HK2 before ischemia or at the onset of reperfusion. However, TAT-HK2 also decreased the phosphocreatine:ATP ratio that correlated with reduced rate pressure product and increased diastolic pressure. These effects were preceded by increased aortic pressure (Langendorff constant flow) or decreased coronary flow (Langendorff constant pressure), which was also observed, albeit less pronounced, at 200 nmol/L TAT-HK2 and was prevented by coperfusion with the NO-donor diethylamine NONOate. Mitochondria from TAT-HK2–perfused hearts showed no loss of bound HK2, unlike mitochondria from ischemic hearts where the expected loss was prevented by ischemic preconditioning.

Conclusions: In the perfused rat heart, TAT-HK2 should be used with caution and careful attention to dosage because some of its effects may be mediated by vasoconstriction of the coronary vasculature rather than dissociation of HK2 from myocyte mitochondria. (Circ Res. 2013;112:e3-e7.)

Key Words: hexokinase □ hypoxia □ phosphocreatine □ TAT-HK2 □ vasoconstriction

The predominant hexokinase isoform expressed in the myocardium is hexokinase (HK2), some of which is bound to mitochondria (mitoHK2) where it acts as an important regulator of mitochondria-induced cell death. It dissociates during prolonged ischemia. This is associated with greater reactive oxygen species production, which, together with calcium overload, induces mitochondrial permeability transition pore opening, a critical event in reperfusion injury. Ischemic preconditioning (IP) prevents mitoHK2 loss during ischemia, and recently Smeee et al showed that cardioprotection by IP in the perfused heart could be blocked by a peptide (TAT-HK2) containing the human HK2 mitochondrial binding motif, which others have reported to dissociate HK2 from mitochondria. They concluded that mitoHK2 is important for normal heart function by keeping mitochondria energetically charged. However, our own studies showed that HK2-deficient mitochondria from ischemic hearts responded normally to ADP while exhibiting greater reactive oxygen species emission. Thus, we decided to reevaluate the effect of TAT-HK2 perfusion of normoxic hearts on mitochondrial function. We found that human TAT-HK2 has profound effects on the coronary vasculature while not actually inducing dissociation of HK2 from myocyte mitochondria for which it was used.

Methods
Sources of reagents used are given in Online Data Supplement, which also contains details of all methods used.

Heart Perfusion
Langendorff perfusions of rat hearts (male Wistar; 225–300 g) were performed with constant flow (12 mL/min) or constant pressure (80 mm Hg) using the protocols summarized in Figure 1A and 1B, respectively. After the required perfusion protocol, hearts were either freeze-clamped to prepare heart powder (stored at −80°C for later analysis), used for the preparation of density-gradient purified mitochondria, or stained to determine infarct size.

Other Methods
MitoHK2 was determined by Western blotting and enzymatic assay of its specific activity. Phosphocreatine (PCr) and ATP were determined enzymatically after extraction from frozen heart powder.

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Statistical Analysis
Data are presented as means±SE. Statistical significance was evaluated using 1-way ANOVA (Kaleidagraph, 4.03), and differences were considered significant at $P<0.05$.

Results
TAT-HK2 Perfusion Impaired Hemodynamic Function and Increased Infarct Size
Hearts were perfused with 2.5 μmol/L TAT-HK2 or control peptide (TAT-CON) before ischemia. TAT-HK2 perfusion of the control group (CP) decreased rate pressure product (RPP) from 26600±970 to 13900±2600 (n=6; $P<0.01$) after 8 minutes, whereas TAT-CON had no effect (Figure 2A). In the IP group, TAT-HK2 also decreased RPP (Figure 2C). In both groups, RPP impairment during TAT-HK2 perfusion was accompanied by increased aortic pressure (Figure 2B and 2D) caused by elevated diastolic pressure (Online Table I). TAT-HK2 perfusion inhibited RPP recovery in the IP group while having little effect in the CP group (Figure 2C and 2A, respectively). At the onset of reperfusion, aortic pressures in the CP and IP groups perfused with TAT-HK2 were higher than the corresponding TAT-CON–perfused controls (Figure 2B and 2D). TAT-HK2 perfusion also increased infarct size (% of the whole heart) from 12±4 to 30±4 in the IP group but had no significant effect in the CP group (Figure 2E). TAT-HK2 was equally effective at inhibiting RPP recovery when present only at the onset of reperfusion (Figure 2C) and strongly increased aortic pressure from 67±14 to 120±3 mm Hg throughout its perfusion (Figure 2D; n=5). This was accompanied by a greater infarct size (Figure 3E), whereas TAT-CON had no effect (data not shown).

TAT-HK2 Perfusion Decreased PCr Content in Normoxic Hearts
The effects of TAT-HK2 perfusion on the bioenergetic status of hearts were determined by measuring their PCr and ATP content. After stabilization, hearts were perfused with the TAT peptides before freeze-clamping and metabolite assay. RPP impairment accompanying TAT-HK2 perfusion correlated with a decrease in the PCr:ATP ratio (Figure 2F), reflecting a decreased PCr without significant change in ATP (Online Figure IA). We also determined the l-lactate content in these hearts and observed that its accumulation increased in the TAT-HK2–treated hearts compared with the controls (Online Figure IIA), and this increase correlated with a decrease in the PCr:ATP ratio (Online Figure IIB). TAT-CON perfusion was without effect on these parameters. These data suggest that the myocardium was becoming hypoxic during TAT-HK2 perfusion.

TAT-HK2 Perfusion Induces Hypoxia in the Perfused Heart
We hypothesized that TAT-HK2 might cause vasoconstriction of the coronary vessels, resulting in reduced coronary flow in hearts perfused at constant pressure. In the presence of TAT-HK2, RPP depression was accompanied by decreased coronary flow from 9.2±0.2 to 3.8±0.9 mL/min per g; both parameters returned to baseline after washout of TAT-HK2 (Figure 3A and 3B). It should be noted that as the coronary flow decreased during perfusion with TAT-HK2, the peptide concentration increased by ≈80% from the initial concentration of 2.5 μmol/L (Online Table II). This was not the case in
the constant flow mode. When TAT-HK2 was coperfused with the NO-donor diethylamine NONOate (0.5 µmol/L), neither RPP nor coronary flow was decreased (Figure 3C and 3D). Thus, we concluded that TAT-HK2 caused vasoconstriction of the coronary vessels, decreasing perfusion quality and causing hypoxia.

**TAT-HK2 Peptide Perfusion Failed to Dissociate HK2 Bound to Mitochondria**

Mitochondria were isolated from 6 different groups of hearts, and the specific activity of bound HK isoforms (Figure 4A) and HK2 protein content (Western blotting) were determined (Figure 4B and 4C). TAT-HK2 perfusion had no effect on either parameter, whereas ischemia significantly reduced HK2 binding measured by both techniques, and this decrease was largely prevented by IP as reported previously.6,7

### Discussion

There is evidence that mitoHK2 stabilizes mitochondria against mitochondrial permeability transition pore opening12 and that ischemia dissociates mitoHK2.6,7 Thus, it would seem likely that mitoHK2 dissociation plays a role in mitochondrial permeability transition pore opening during reperfusion, and we have suggested that this may be mediated by greater oxidative stress.7 Indeed, IP prevents mitoHK2 dissociation during ischemia,4 and this is accompanied by less oxidative stress and less mitochondrial permeability transition pore opening.13 Dissociation of mitoHK2 should prevent IP, which is what Smeele et al9 observed using immunogold electron microscopy. However, when we performed such studies using Western blotting of isolated mitochondria (as used by the authors in previous studies),6 we observed the expected decrease in bound HK2 after ischemia but not after treatment with TAT-HK2 at either 2.5 µmol/L (Figure 4) or 200 nmol/L (Online Figure IV).

**TAT-HK2 Exerts Effects on Heart Function Independently of mitoHK2 Dissociation**

Our data imply that TAT-HK2 may have effects on heart function that are independent of HK2 binding to myocyte mitochondria. We performed the majority of our studies with 2.5 µmol/L TAT-HK2, a concentration intermediate between 200 nmol/L and 10 µmol/L used by Smeele et al.9 In HeLa cells, dissociation of mitoHK2 required incubation with 20
µmol/L TAT-HK2 for 1 hour.10 For Rat1a cells 30 minutes was used to induce dissociation, but several hours were required to induce mitochondrial depolarization and apoptosis.14 This is much longer than the 2 to 4 minutes of exposure to 2.5 µmol/L TAT-HK2 that induced the changes in hemodynamic function we observed and which were not accompanied by mitoHK2 dissociation. We have repeated our experiments using the peptide at 200 nmol/L which Smeele et al9 reported inhibited IP, and these data are reported in Online Figures II–IV. Interestingly, Smeele et al9 themselves noted that RPP decreased to 92±3% in hearts treated with 200 nmol/L TAT-HK2, whereas at 10 µmol/L the peptide abolished heart function after 15 minutes of perfusion, which was associated with mitochondrial depolarization and ultrastructural damage. Here, we demonstrate that 2.5 µmol/L TAT-HK2 impaired vascular function as revealed by an increased aortic pressure in the constant flow model (Figure 2) and a reduced coronary flow in the constant pressure model (Figure 3). Smaller effects were observed at 200 nmol/L peptide (Online Figure II). Even in the constant flow model it would seem that oxygen supply to the heart was restricted by the presence of 2.5 µmol/L TAT-HK2 (but not 200 nmol/L) because RPP impairment correlated with a decrease in the PCr:ATP ratio (Figure 2F). This drop in RPP was prevented when diethylamine NONOate was added to reverse the vasoconstriction (Figure 3).

Although we confirmed that perfusion with TAT-HK2 reduced the ability of IP to protect hearts from ischemia–reperfusion injury (Figure 2A, 2C, and 2E), this might be explained if TAT-HK2–induced vasoconstriction caused hypoxia and mitochondrial dysfunction on reperfusion. Consistent with this, 2.5 µmol/L TAT-HK2 exerted similar effects on infarct size, whether it was added before the IP protocol or during reperfusion (Figure 2C and 2E). At 200 nmol/L the damaging effects of TAT-HK2 were less but still observed. Hypercontracture was significantly increased (Online Figure IIIE), but the slight increase in infarct size of the IP group was not significant (Online Figure IIIF), unlike the increase in LDH used by Smeele et al9 as an indicator of necrotic damage. Even at 2.5 µmol/L TAT-HK2, the infarct size of the IP group peptide was significantly less than the non-IP hearts (Figure 2E), despite being associated with complete inhibition of hemodynamic function recovery (Figure 2C). These data show that in the presence of TAT-HK2 peptide there was a disparity between the reduction in infarct development and recovery of the hemodynamic function. We suggest that this is likely to be secondary to its effect on vasculature because the effects of 200 nmol/L TAT-HK2 on both parameters were abolished in the presence of the NO-donor diethylamine NONOate (Online Figure IIIB and IIIF). We do not know the mechanism by which the TAT-HK2 peptide causes vasoconstriction.

Limitations and Possible Explanations of Disparities Between Studies
A key difference between our studies and those of Smeele et al9 is that they used immunogold electron microscopy to demonstrate colocalization of HK2 with mitochondria rather than determining HK2 remaining bound to isolated mitochondria as we do here and had been done in previous studies.4 Another difference is that our studies were performed with rat hearts, whereas those of Smeele et al9 used mouse hearts. It is possible that rat and mouse hearts respond differently to the peptide, although the N-terminal sequences of mouse and rat HK2 are identical (MIASHMIAZCFLTELN), which makes this less likely. It should also be noted that the rodent sequence has 4 different residues (italic) to the human sequence.
sequence (MIASHLLAYFFTELN) on which the TAT-HK2 peptide used in both studies is based. We cannot rule out that this difference might make the human TAT-HK2 peptide inefficient at displacing rat and mouse mitoHK2. Nor can we be certain that under the conditions used the peptide reaches the myocyte mitochondria at sufficient concentration and for sufficient time to dissociate mitoHK2. However, Smeee et al11 performed experiments with fluorescein isothiocyanate–labeled TAT-HK2 peptide to show its uniform distribution in the myocardium at concentrations from 200 nmol/L to 10 μmol/L, although it remains possible that the presence of the hydrophobic fluorescein moiety might enhance the permeability of peptide across the plasma membrane, enabling it to distribute more uniformly than the unlabeled peptide. Importantly, both studies suffer the same limitations in this regard.

Conclusion
In the perfused rodent heart, human TAT-HK2 should be used with considerable caution and careful attention to dosage because some of its effects may be mediated by vasoconstriction of the coronary vasculature rather than dissociation of HK2 from myocyte mitochondria.

Sources of Funding
This work was supported by a Program Grant from the British Heart Foundation (RG/08/001/24717).

Disclosures
None.

References
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Supplemental Material

Antibodies and chemicals

The following antibodies were used in this study: hexokinase 2 (HK, rabbit monoclonal, Cell Signaling), ADP-ATP translocase (ANT, homemade antibody). All chemicals used in this study were purchased from Sigma unless otherwise stated.

Heart Perfusion

All procedures conformed to the UK Animals (scientific Procedures) Act 1986 and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23. revised 1996). Male Wistar rats (225-300 g) were killed by stunning and cervical dislocation and hearts (~0.75 g) were rapidly removed into ice-cold Krebs-Henseleit buffer containing (mmol / L) NaCl 118, NaHCO₃ 25, KCl 4.8, KH₂PO₄ 1.2, MgSO₄ 1.2, glucose 11 and CaCl₂ 1.2 gassed with 95% O₂ / 5% CO₂ at 37°C (pH 7.4). Langendorff heart perfusions were performed as described previously.¹ Hearts were perfused in a constant flow mode (12 ml / min) or in constant pressure (80 mmHg) according to the protocol schematically described in Figure 1A and 1B (main manuscript), respectively. Global normothermic ischemia (index ischemia) was induced by halting perfusion for 30 min and immersing the heart in perfusion buffer at 37°C. At the end of the pre-ischemic or ischemic period the hearts were either removed from the perfusion cannula for the preparation of mitochondria or, freeze-clamped using liquid-nitrogen cooled tongues. In the latter case, hearts were grounded under liquid nitrogen, and stored at -80°C for later analysis.

TAT-peptides synthesis and perfusion:
The soluble peptides TAT-HK2 (MIASHLLAYFFTELNbA-GYGRKKRRQRRRQ) and TAT-CON (GRKKRRQRRPRPKRPPTTLNLFPQVPRSQDT) were synthesized by Dr Will Mawby in the Proteomics Facility, University of Bristol using FMOC amino acids on a Millipore Pioneer peptide synthesizer and were purified by Reverse Phase HPLC on a Vydac C18 column using a Waters 510 gradient system. Peptides were dissolved in Krebs-Henseleit buffer and perfused at a final concentration of 2.5 µM using a syringe-pump (0.5 ml / min) connected to the top of the cannula. In order to assess the effects of a low dose of the soluble peptides separate groups of hearts were used. The soluble peptides (TAT-HK2 and TAT-CON) were perfused at a final concentration of 200 nM for 15 to 16 min prior to the index ischemia and during the first 5 min of reperfusion as described by Smeele et al² in the presence or absence of 1 µM of the NO-donor diethylamine NONOate (DEA).

Assessment of infarct size:
At the end of the reperfusion period hearts were stained with triphenyltetrazolium chloride (TTC) as described previously³ with slight modification. Briefly, hearts were perfused for 2 min at 10 ml / min with a 1% (w / v) TTC solution. Hearts were then detached from the cannula and incubated for an additional 5 min at 37°C before being sliced perpendicular to the longitudinal axe in 6 slices. The slices were then fixed in 4% (w / v) formalin solution overnight at 4°C and weighed. Both sides of each slice were photographed. Surface of the necrotic and area at risk of each side for each slice were determined by planimetry (AlphaEase v5.5), and since global ischemia was employed, infarct size was expressed as a percentage of the total cross-sectional area of the heart.

Isolation of mitochondria
All steps were performed essentially as described previously⁴ with slight modification. Each heart was homogenized at 4°C in 6 ml ISA using a Polytron tissue disruptor (Kinematica) at 10,000 rpm for 2 bursts of 5 s and 1 of 10 s. The homogenate was diluted with 3 volumes of
ISAPP (ISA: 300mM sucrose, 2mM EGTA, 10mM Tris-HCl, pH 7.1 at 4°C supplemented with inhibitors of proteases (Roche complete) and phosphatases (Sigma cocktail 1)) and further homogenized for 2 min in a 50 ml glass Potter homogenizer using a motorized Teflon pestle. The homogenate was centrifuged at 7,500 g for 7 min and the resulting pellet rinsed twice with 5 ml isolation buffer, resuspended in 20 ml isolation buffer and subject to further homogenization for 3 min as described above. The homogenate was then centrifuged at 700 g for 10 min and the resultant supernatant centrifuged at 7,000 g for 10 min to yield a crude mitochondrial pellet that was resuspended in ISAPP containing 25% (w/v) Percoll (pH 7.1-7.2 at 4°C) and centrifuged at 17,000 g for 10 min. The resulting pellet was resuspended again at 7,000 g for 10 min. The final purified mitochondrial pellet was resuspended in ISAPP and the protein concentration determined by Biuret method using bovine serum albumin (BSA) as a standard. Mitochondria were kept on ice at a final concentration of 50 mg / ml and stored at -80°C for later analysis.

Hexokinase and Citrate Synthase specific activity

Aliquots (0.75 mg protein) of frozen mitochondria prepared by the polytron method (see above) were solubilized by brief sonication at 4°C and protein concentration was adjusted at 2 mg / ml in a buffer containing 33 mM KH$_2$PO$_4$ and 50 µM dithiothreitol (pH 7.2). For assay of hexokinase, samples (20, 30 or 40 µl) were added to 1 ml assay buffer (pH 7.4 at room temperature) containing 100 mM Tris-HCl, 0.4 mM NAD P$^+$, 10 mM MgCl$_2$, 5 mM ATP, 0.3% (v/v) Triton X-100 and 0.5 units / ml of glucose-6-phosphate dehydrogenase and incubated for 2 min at 37°C before addition of glucose (1 mM final) to start the reaction. Hexokinase activity was calculated from the rate of NADPH production corrected for glucose-independent rates of NADPH formation determined in parallel assays lacking glucose. For assay of citrate synthase (E.C. 2.3.3.1 formerly E.C. 4.1.3.7) mitochondrial samples (20, 30 or 40 µl of a solution at 0.2 mg / ml) were added to 1 ml assay buffer (pH 7.4 at room temperature) containing 50 mM Tris-HCl, 0.3% (v/v) Triton X-100 and 150 µM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and incubated for 2 min in the presence of 0.3 mM acetyl-CoA, before addition of 500 µM final oxaloacetic acid to start the reaction. One unit of citrate synthase was defined as the reduction of 1 µmole DTNB per min.

Preparation of freeze-clamped heart powder deproteinized sample

Frozen heart powder (150 to 200 mg) was added to 2 ml of ice cold perchloric acid (0.2M) and sonicated on ice for 3 bursts of 5 sec interspersed by 10 sec. The resulting homogenate was further homogenized in a 2ml glass Potter until complete dissolution using a motorized Teflon pestle. The homogenate was then centrifuged for 10 min at 4,000g. The resulting supernatant was neutralized with KHCO$_3$ and centrifuged for 10 min at 4,000g. The supernatant was stored at -80°C for later analysis.

Phosphocreatine (PCr) and ATP assay

To determine PCr content in deproteinized samples (see above), 20 and 40 µl were incubated at 37°C in the assay buffer (Tris-HCl 100 mM, MgCl$_2$ 10 mM, 2 mM glucose, 1 mM ADP, hexokinase 1.2 units /ml, glucose-6-phosphate dehydrogenase 2 units / ml, NADP$^+$ 1 mM, pH 7.4 at room temperature), until the A$_{340}$ stabilized. The reaction was then started by addition of 40µg of creatine kinase (from rabbit muscle, Roche products) and stopped when no further increase in A$_{340}$ was observed. ATP content was determined in the same buffer lacking hexokinase. After addition of samples (20 and 40 µl) and stabilization of the A$_{340}$, the reaction was started by addition of 1.2 units of hexokinase and stopped when no further increase in A$_{340}$ was observed. Internal calibration was performed for each sample by successive addition of two known concentrations of PCr or ATP. PCr and ATP content was expressed as nmoles / mg wet weight.
L-lactate assay in deproteinized heart powder samples

To determine L-lactate content in deproteinized samples, 200 µl were incubated for 2 to 3 hours at 37°C in 1 ml final of the following buffer (Gly- L-glycine 0.27 M, hydrazine hydrate 0.7 M, NAD⁺ 0.4 mM, L-lactate dehydrogenase 1 U / ml, pH 8 at room temperature). The A₃₄₀ was read every 30 min and the reaction was stopped when it reached a stable value. Parallel experiments were performed in the absence of lactate dehydrogenase in order to correct for the increase in baseline A₃₄₀. A standard curve was performed under identical conditions in presence of known concentrations of L-lactate. L-lactate content in the deproteinized samples was expressed as nmoles / mg wet weight.

Western-blotting

Mitochondria, prepared by the polytron method (see above), were separated by SDS-PAGE (12% for the adenine nucleotide translocase (ANT) and 5% for hexokinase) using 20 µg protein for each lane. Gels were then subjected to Western-blotting with the required primary antibody (see above) and blots developed using the required horseradish peroxidase secondary antibody, with ECL/ECL⁺ detection (Amersham Biosciences UK Limited). Appropriate exposures of the film were used to ensure that band intensities were within the linear range. Quantification of blots was performed using an Alphalnotech ChemiImager 4400 to image the blot, and analysis of band intensity with AlphaEase v5.5 software. In order to normalize band intensities, parallel blots were performed on the same samples using ANT antibodies.
Online Figure I. TAT-HK2 peptide perfusion does not alter ATP content but increases L-Lactate content in the beating heart.

Hearts were perfused in the constant flow mode at 12 ml / min. TAT-CON and TAT-HK2 peptides were perfused at 2.5µM final using a syringe-pump (rate 0.5 ml / min) connected to the top of the cannula. Following perfusion of the peptides hearts were freeze-clamped and grounded in liquid nitrogen before assessing metabolites content. Panel A: the left Y axis shows ATP content and the right Y axis shows L-Lactate content (black bar TAT-CON, grey bar TAT-HK2) were assayed in deproteinized heart powder using an enzymatic assay (see above). Panel B: L-Lactate content as a function of the PCr to ATP ratio (filled circle TAT-CON, grey circle TAT-HK2).
Online Figure II. Effects of 200 nM TAT-HK2 peptide on the hemodynamic function of the normoxic heart. Hearts were perfused in the constant pressure mode (A, B) or in the constant flow mode (C, D). TAT-CON (black line) and TAT-HK2 (grey line) peptides were perfused at 200 nM final using a syringe-pump (rate 0.5 ml / min) connected to the top of the cannula. Panels A and C present data on the effects of the peptides on RPP in constant pressure and constant flow modes respectively while panel B and D present the corresponding data for coronary flow and aortic pressure respectively. Data are presented as means ± SEM (n=5) for each group with the SEM represented as the error bars which in the case of Panels A, C and D provide the apparent thickness of the line.
Online Figure III. The effects of 200 nM TAT-HK2 peptide in the ischemic heart. Ischemic preconditioned hearts were perfused in the constant flow mode. TAT-CON (black line) and TAT-HK2 (light grey line) peptides were perfused at 200 nM final for 16 min prior to ischemia and 5 min during reperfusion. In a separate group, TAT-HK2 was co-perfused with the NO-donor DEA at 1 µM final (dark grey line). Panels A and B – RPP before and after global ischemia, respectively. Panels C and D - Aortic Pressure before and after global ischemia. Panel E – End Diastolic Pressure during reperfusion. Panel F – Infarct size. Data are means ± SEM (n=5). In Panels A to E the SEM is reflected by the “thickness” of the plotted lines.
Online Figure IV. The effects of TAT-peptides on mitochondrial hexokinase activity and bound HK2. Panel A shows hexokinase specific activity (normalized to citrate synthase content) in mitochondria from pre-ischemic hearts perfused for 15 min with 200 nM TAT-CON or TAT-HK2. Data are presented as Means ± SEM (n=4). Panel B shows typical Western blots for HK2 and ANT content of mitochondria from each group of hearts. Panel C shows the ratio of HK2 to ANT derived from scanning the blots. Data are presented as means ± SEM (n=4).
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<th>HR (bpm)</th>
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<th>SP (mmHg)</th>
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<tr>
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<td>Peptide</td>
<td>256 ± 20</td>
<td>59 ± 14 †</td>
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**Online Table I. Effect of TAT-peptides on pre-ischemic hemodynamic function.**
Hearts were perfused in the constant flow mode according to the protocols described in Figure 1A of the main manuscript. Values presented were obtained before (baseline) and after 8 min of perfusion with TAT-peptide at 2.5 µM (Peptide). HR, heart rate (beat per min, bpm). DP, diastolic pressure. SP, systolic pressure. Data are means ± SEM of 6 hearts for each group.

* p<0.05 CP+TATHK2 vs. CP+TATCON (n=6 each group); † p<0.05 IP+TATHK2 vs. IP+TATCON (n=6 each group).
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<th>TAT-peptides + DEA</th>
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<tr>
<td>TAT-CON</td>
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<td>TAT-HK2</td>
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</table>

Online Table II. Average concentration of the TAT-peptides and DEA during treatment.
Hearts were perfused in the constant pressure mode according to the protocol described in Figure 1B of the main manuscript. TAT-CON and TAT-HK2 were perfused at a constant rate of 0.5 ml /min using a syringe-pump connected to the top of the cannula. The concentration of the TAT-CON and TAT-HK2 in the syringe was defined based on the value of the coronary flow obtained at the end of the baseline for each individual heart. In presence of DEA the vasodilatory effect was also taken into account for the calculation of TAT-peptides stock solution. Values were averaged over the total length of TAT-peptides perfusion (3 min). Data are means ± SEM of 5 hearts for each group.
REFERENCES


4. Halestrap AP. The regulation of the oxidation of fatty acids and other substrates in rat heart mitochondria by changes in the matrix volume induced by osmotic strength, valinomycin and Ca²⁺. *Biochem J*. 1987;244:159-164