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## Pathophysiological Consequences of TAT-HKII Peptide Administration Are Independent of Impaired Vascular Function and Ensuing Ischemia

Rianne Nederlof,\* Chaoqin Xie,\* Otto Eerbeek, Anneke Koeman, Dan M.J. Milstein, Markus W. Hollmann, Egbert G. Mik, Alice Warley, Richard Southworth, Fadi G. Akar, Coert J. Zuurbier

**Rationale:** We have shown that partial dissociation of hexokinase II (HKII) from mitochondria in the intact heart using low-dose transactivating transcriptional factor (TAT)-HKII (200 nmol/L) prevents the cardioprotective effects of ischemic preconditioning, whereas high-dose TAT-HKII (10  $\mu$ mol/L) administration results in rapid myocardial dysfunction, mitochondrial depolarization, and disintegration. In this issue of *Circulation Research*, Pasdois et al argue that the deleterious effects of TAT-HKII administration on cardiac function are likely because of vasoconstriction and ensuing ischemia.

**Objective:** To investigate whether altered vascular function and ensuing ischemia recapitulate the deleterious effects of TAT-HKII in intact myocardium.

**Methods and Results:** Using a variety of complementary techniques, including mitochondrial membrane potential ( $\Delta\Psi_m$ ) imaging, high-resolution optical action potential mapping, analysis of lactate production, nicotinamide adenine dinucleotide epifluorescence, lactate dehydrogenase release, and electron microscopy, we provide direct evidence that refutes the notion that acute myocardial dysfunction by high-dose TAT-HKII peptide administration is a consequence of impaired vascular function. Moreover, we demonstrate that low-dose TAT-HKII treatment, which abrogates the protective effects of ischemic preconditioning, is not associated with ischemia or ischemic injury.

**Conclusions:** Our findings challenge the notion that the effects of TAT-HKII are attributable to impaired vascular function and ensuing ischemia, thereby lending further credence to the role of mitochondria-bound HKII as a critical regulator of cardiac function, ischemia-reperfusion injury, and cardioprotection by ischemic preconditioning. (*Circ Res.* 2013;112:e8-e13.)

**Key Words:** hexokinase ■ ischemia ■ mitochondrial membrane potential ■ TAT peptide ■ vasoconstriction

Warburg et al<sup>1</sup> were the first to report markedly elevated levels of the glycolytic enzyme hexokinase (HK) in aggressive tumors. Indeed, increased HK expression in cancer cells was mechanistically linked to their resilience against apoptosis. These pioneering studies established a central role for HK expression in cancer biology. In recent years, we extended the role of HK isoforms from the pathophysiology of cancer to that of ischemic heart disease.<sup>2,3</sup> Specifically, we showed that the expression and subcellular distribution of the HK isozyme, HKII, is a major determinant

of ischemia-reperfusion (IR) injury.<sup>4-6</sup> Furthermore, binding of HKII to mitochondria, which is increased by ischemic preconditioning (IPC),<sup>2,5</sup> protects against oxidative stress and IR injury, potentially by limiting cellular reactive oxygen species levels<sup>7</sup> and preserving the mitochondrial membrane potential ( $\Delta\Psi_m$ ).<sup>4</sup>

In our most recent work, we undertook a small molecule approach designed to acutely dislodge HKII from mitochondria.<sup>4</sup> Specifically, we used a transactivating transcriptional factor peptide (TAT-HKII) that contains the mitochondrial

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Non-standard Abbreviations and Acronyms	
AP	action potential
APD	action potential duration
$\Delta\Psi_m$	mitochondrial membrane potential
EM	electron microscopy
HK	hexokinase
IR	ischemia-reperfusion
IPC	ischemic preconditioning
LDH	lactate dehydrogenase
NADH	nicotinamide adenine dinucleotide
TAT	transactivating transcriptional factor from human immunodeficiency virus 1
TAT-HKII	cell permeable peptide containing hexokinase II mitochondrial binding motif

binding motif of HKII<sup>8,9</sup> to investigate the functional impact of reduced mitochondrial-HKII association in the intact heart. Using electron microscopy (EM) and HKII immunogold labeling, we demonstrated that treatment of hearts with TAT-HKII reduced mitochondrial-HKII localization.<sup>4</sup> Using this pharmacological approach, we showed that pretreatment of hearts with low-dose TAT-HKII (200 nmol/L) abrogated the protective effects of IPC on IR injury. Moreover, treatment of hearts with high-dose TAT-HKII (10  $\mu$ mol/L) caused abrupt contractile and electric dysfunction during basal perfusion of the heart. Interestingly, severe myocardial dysfunction elicited by TAT-HKII in the absence of additional stress (such as IR injury) was associated with rapid and sustained  $\Delta\Psi_m$  depolarization and the disintegration of mitochondrial structure. Because these pathological changes developed during the same time-course as the reduction in mitochondrial-HKII levels, we concluded that the interaction between mitochondria and HKII is not only required for the protective effects of IPC but also for normal cardiac function.

In this issue of *Circulation Research*, Pasdois et al<sup>10</sup> offer an alternative interpretation of our recent findings. Specifically, they argue that the deleterious effects of TAT-HKII peptide administration on cardiac function, including the abrogation of IPC by low-dose TAT-HKII treatment and myocardial dysfunction with high-dose perfusion, are likely attributed to impaired vascular properties and ensuing ischemic damage. They base their conclusion, in large part, on the fact that TAT-HKII treatment increases perfusion pressure under constant flow conditions and decreases perfusion flow under constant pressure conditions. Because Pasdois et al attributed the deleterious effects of TAT-HKII on myocardial function to impaired vascular properties and ensuing ischemia/hypoxia, we conducted a series of additional experiments to directly address the discrepancy between our conclusions and theirs. Specifically, we used a variety of complementary techniques to evaluate the presence of ischemia in our preparations and to compare the functional effects of ischemia and vascular dysfunction on metabolic and electrophysiological properties with those elicited by TAT-HKII treatment.

We provide direct evidence that acute myocardial dysfunction elicited by high-dose TAT-HKII administration cannot be ascribed to impaired vascular function or ensuing ischemia.

Moreover, we demonstrate that low-dose TAT-HKII treatment, which abrogates the protective effects of IPC, is not associated with functionally significant ischemia. Taken together, our data refute the notion that the deleterious effects of TAT-HKII are attributable to impaired vascular function and ischemia, thereby lending further credence to our previous conclusion that mitochondria-bound HKII is indeed a critical regulator of cardiac function, IR injury, and cardioprotection by IPC in the intact heart.

## Methods

A detailed Materials and Methods section is available in the Online Data Supplement.

All experiments were approved by the animal ethics committee of the Academic Medical Center, Amsterdam, The Netherlands or by the Animal Care and Use Committee of the Mount Sinai School of Medicine and adhered with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Hearts were Langendorff-perfused.

### TAT-Peptides

The soluble peptides MIASHMIACLFTELN( $\beta$ -Ala)GYGRKKR-RQRRRG-amide (TAT-HK) and GYGRKKRRQRRRG-amide (TAT-CON) were produced by Pepsan 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.

The effect of endothelin-induced vasoconstriction on tissue structure of the mouse heart was evaluated using transmission EM of cryosections. High-resolution optical  $\Delta\Psi_m$  imaging using the fluorescent dye tetramethylrhodamine methylester was performed in the intact, beating rat heart. Action potentials (APs) were measured using a charge-coupled device-based high-resolution optical mapping system. In a separate series of mice experiments, effluent lactate and lactate dehydrogenase (LDH) were measured spectrophotometrically, and nicotinamide adenine dinucleotide (NADH) fluorescence of the surface of the heart was evaluated using a videofluorometer.

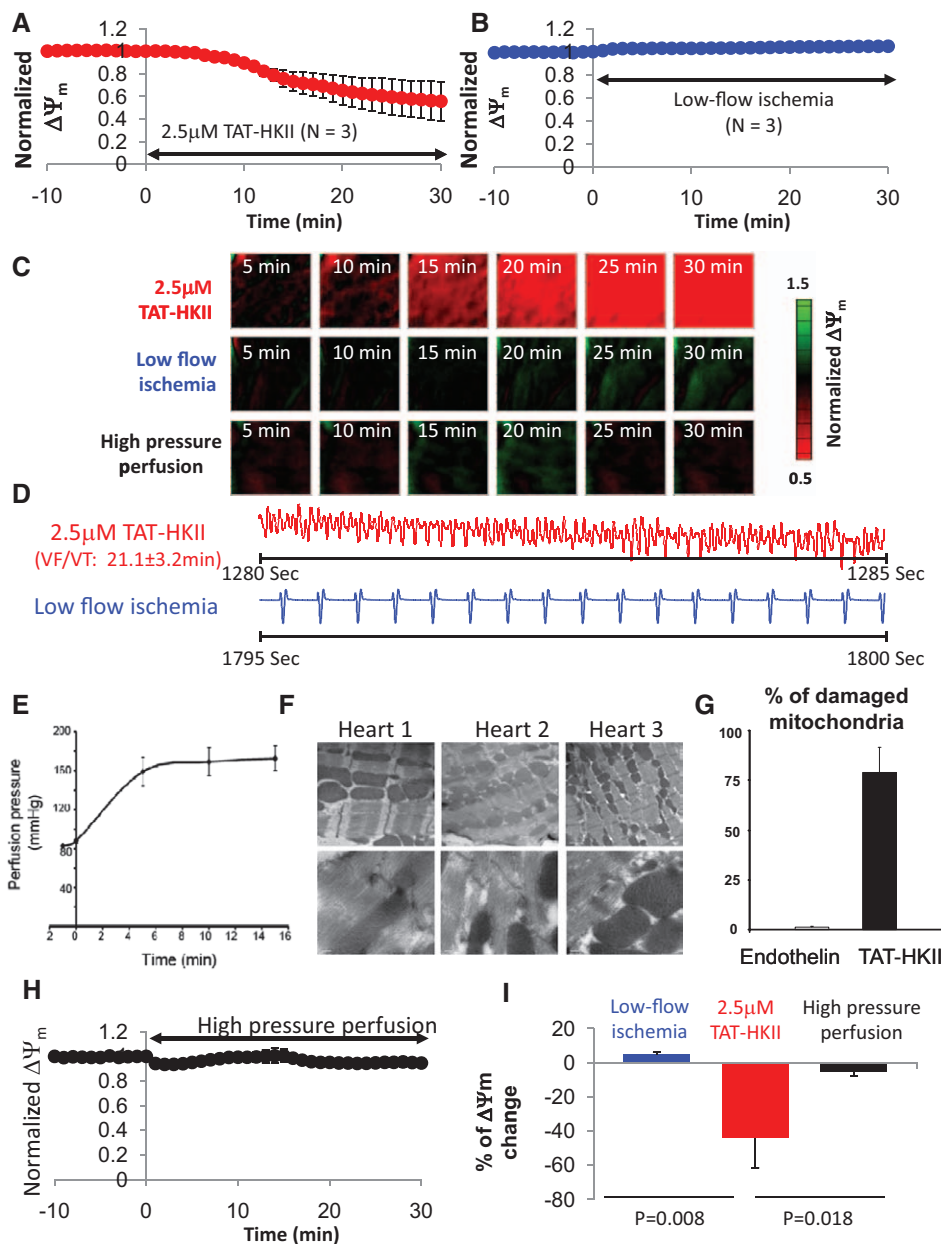
## Results

### Myocardial Dysfunction Caused by High-Dose TAT-HKII Administration Cannot Be Attributed to Impaired Vascular Function

The study by Pasdois et al revealed a 58% reduction in perfusion flow to the rat heart after administration of 2.5  $\mu$ mol/L TAT-HKII but not TAT-con. They concluded that increased vascular resistance by TAT-HKII caused functionally significant ischemia, which in turn, likely altered myocardial function. Reproducing their experimental conditions (60% flow reduction) and using the same species and peptide concentrations, we set out to formally investigate whether changes in perfusion flow and pressure could indeed account for the functional changes that we recently reported.

### Differential Effects of Global Low-Flow Ischemia Versus High-Dose TAT-HKII Administration

Consistent with our previous findings, treatment of rat hearts with 2.5  $\mu$ mol/L TAT-HKII (Figure 1A and 1C) caused a rapid (within 20 minutes) and sustained depolarization of  $\Delta\Psi_m$  across the intact heart. Interestingly,  $\Delta\Psi_m$  depolarization was associated with the initiation of sustained ventricular fibrillation within 21.1 $\pm$ 3.2 minutes of TAT-HKII administration in all treated hearts (Figure 1D, red trace). In sharp contrast,



**Figure 1. Change in the normalized mitochondrial membrane potential ( $\Delta\Psi_m$ ) within the intact heart elicited by 2.5  $\mu\text{mol/L}$  TAT-HKII administration (A) and a 60% reduction in perfusion flow (ie, global low-flow ischemia; B). C, Representative  $\Delta\Psi_m$  contour maps measured during 30 minutes in each group (TAT-HKII, low-flow ischemia, high-pressure perfusion). Red indicates depolarized  $\Delta\Psi_m$ . D, Representative volume-conducted electrocardiograms in hearts perfused with 2.5  $\mu\text{mol/L}$  TAT-HKII (red) compared with low-flow ischemia (blue). E, Endothelin-1-mediated rise in perfusion pressure. F, Typical electron microscopic images obtained at low-magnification (top row) and high magnification (bottom row) of cardiac ultrastructure in endothelin-1 treated hearts. G, Percentage of visibly damaged mitochondria as determined by electron microscopy analysis. The TAT-HKII data are from reference 4. H,  $\Delta\Psi_m$  in hearts subjected to an acute elevation of perfusion pressure by increased flow. I, Average normalized  $\Delta\Psi_m$  in all 3 groups. TAT-HKII indicates cell-permeable peptide containing hexokinase II mitochondrial binding motif.**

global low-flow ischemia mimicking the flow reduction reported by Pasdois et al (ie, by 60%) completely failed to alter  $\Delta\Psi_m$  within the same time-course (Figure 1B and 1C). In fact,  $\Delta\Psi_m$  was unchanged ( $P=NS$ ) even after 1 hour of low-flow perfusion compared with baseline levels. Interestingly, unlike TAT-HKII administration, global low-flow ischemia to the rat heart was not associated with the incidence of sustained ventricular arrhythmias because all hearts exhibited relatively stable electric rhythm (3/3 hearts, Figure 1D, blue trace). In further support of these findings, we performed an additional series of studies using high-resolution optical AP mapping to assess whether 60% flow reduction produces adverse electrophysiological changes that are consistent with ischemic damage. Indeed, although global no-flow ischemia to the heart elicited significant AP duration (APD) shortening and conduction velocity slowing (known hallmarks of ischemia), a 60% reduction in flow failed to alter either parameter (Online

Figure I). These findings confirm the notion that the effects of TAT-HKII administration cannot be reproduced by low-flow conditions, which contrary to the suggestion by Pasdois et al do not produce adverse mitochondrial (Figure 1) or electrophysiological (Online Figure I) alterations.

### Increased Vascular Resistance Does Not Cause Mitochondrial Damage, Whereas 10 $\mu\text{mol/L}$ TAT-HKII Does

High-dose TAT-HKII administration to the intact heart is also associated with increased vascular resistance in the constant flow mode, as initially reported by us<sup>4</sup> and later confirmed by Pasdois et al.<sup>10</sup> It was therefore possible that the ultrastructural damage to mitochondria was a consequence of increased perfusion pressure. To address this issue, a series of mouse hearts were first treated with the vasoconstrictor endothelin-1 (7.5 nmol/L). As expected, perfusion pressure rose significantly

(173±13 mm Hg, Figure 1E) after 15 minutes of endothelin-1 administration, comparable ( $P=0.66$ ) with that elicited by 10  $\mu\text{mol/L}$  TAT-HKII (161±13 mm Hg).<sup>4</sup> Analysis of mitochondrial morphology using EM on tissue sections from these hearts revealed normal ultrastructure (Figure 1F). On average, the percentage of mitochondria exhibiting abnormal morphology in endothelin-1-treated hearts was only 1.01±1.25%, well below the reported value<sup>4</sup> (78±21%) for high-dose TAT-HKII administration (Figure 1G).

These qualitative ultrastructural findings were corroborated by our functional measurements of  $\Delta\Psi_m$  within the intact heart. Specifically, in a series of 3 additional rat hearts, we increased perfusion flow to achieve an artificially high perfusion pressure that mimics values reported by Pasdois et al. Despite a major rise in perfusion pressure ( $\approx 150$  mm Hg), these hearts exhibited a minor reduction (<8%) in  $\Delta\Psi_m$  compared with >40% by TAT-HKII (Figure 1C, 1H, and 1I). On average,  $\Delta\Psi_m$  depolarization after 30 minutes of perfusion was markedly greater ( $P<0.018$ ) in TAT-HKII compared with high-pressure hearts. Taken together, our findings argue against high perfusion pressure as a major cause of mitochondrial dysfunction.

### Low-dose TAT-HKII Administration Does Not Result in Ischemia

A major finding of our previous report was the abrogation of the protective effects of IPC on IR injury by low-dose TAT-HKII (200 nmol/L) administration. Pasdois et al speculated that this is likely attributed to ischemic damage by the peptide, although their data on perfusion flow and pressure did not support that view. Nonetheless, we set out to determine whether low-dose TAT-HKII peptide administration results in functionally significant ischemic damage that can potentially confound the interpretation of our earlier findings. Using optical AP mapping, we asked whether low-dose administration of TAT-HKII altered ischemia-sensitive functional parameters (particularly APD and conduction velocity). Shown in Figure 2 are representative AP traces recorded during steady-state pacing from TAT-con (blue) and TAT-HKII (red) hearts (Figure 2A), as well as average APD75 and APD90 measurements performed over a wide range of pacing cycle lengths (Figure 2B and 2C). Also shown are representative AP upstrokes indicating the delay in AP propagation (Figure 2D), a series of isopotential contour maps that depict the spread of the AP wavefront in TAT-con and TAT-HKII hearts (Figure 2E), and average conduction velocity measurements from each group (Figure 2F). Clearly, low-dose TAT-HKII (200 nmol/L) administration did not alter key electrophysiological properties (APD75, APD90, or conduction velocity). These findings argue against the development of functionally significant ischemia with low-dose administration of the peptide that could potentially explain the abrogation of IPC, as speculated by Pasdois et al.

### Ischemia Does Not Explain the Adverse Effects of High-Dose TAT-HKII Administration and Is Not Present With Low-Dose Administration

Finally, we evaluated the presence of ischemia by measuring lactate production (Figure 2G) and NADH epifluorescence (Figure 2H and Online Figure II). LDH release was

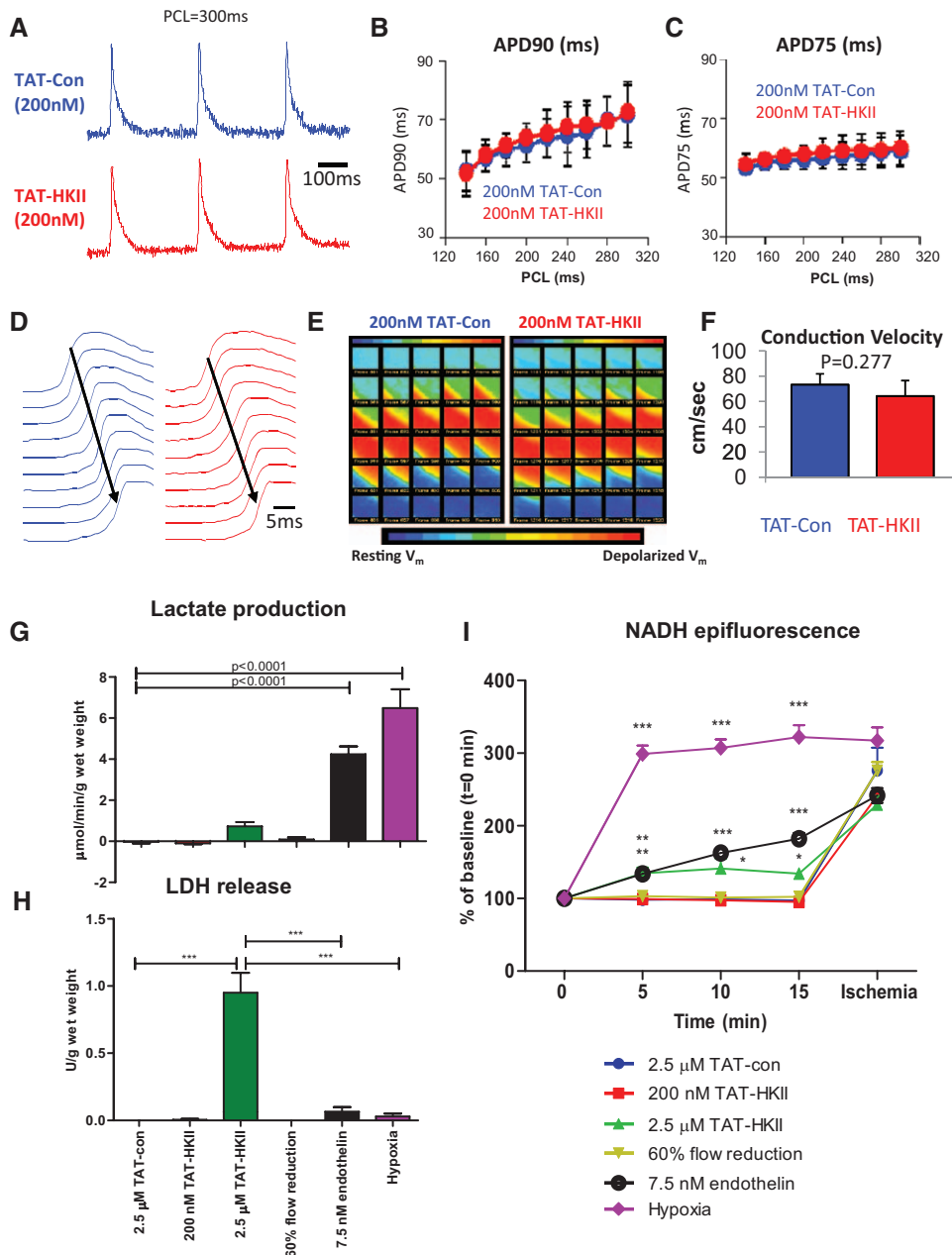
simultaneously measured as an independent index of cardiac damage (Figure 2I). Consistent with the electrophysiological measurements reported above, ischemia was undetectable in low-dose TAT-HKII hearts because NADH and lactate production were identical in low-dose TAT-HKII and TAT-Con groups, further arguing against a role of ischemia in our reported<sup>4</sup> IR and IPC-effects. Moreover, in support of the electrophysiological measurements performed during low-flow perfusion (60% flow reduction), there were also no differences in any of the ischemia-sensitive parameters. High-dose TAT-HKII peptide administration, on the contrary, resulted in a minor increase in NADH levels (by 18%,  $P<0.05$ ), a nonsignificant trend toward elevated lactate production (by 11%,  $P=\text{NS}$ ), and a large significant increase in LDH release. In contrast, the high-pressure (endothelin) and hypoxia groups exhibited significantly greater increases in ischemia-sensitive parameters (NADH and lactate production) compared with high-dose TAT-HKII administration. These rises in NADH and lactate in these groups, however, were not associated with significant cardiac damage (assayed by LDH release). This clear dichotomy between ischemia markers and cardiac damage within 15 minutes of isolated heart perfusion argues against a causative role of ischemia in the cardiac destructive effects observed with high-dose administration of the peptide.

## Discussion

We recently investigated the functional consequences of reduced mitochondrial-HKII interactions by using a small molecule approach. Specifically, we used a peptide (TAT-HKII) containing the mitochondrial binding motif of HKII,<sup>8</sup> which has been shown to dislodge HKII from mitochondria in intact cells.<sup>9</sup> In that study, we abrogated the protective effects of IPC on IR injury by low-dose TAT-HKII administration and caused rapid myocardial dysfunction and mitochondrial disintegration by high-dose TAT-HKII treatment. We attributed these functional and ultrastructural changes to reduced HKII interaction with mitochondria, as evidenced by EM and immunogold labeling of HKII. In this issue of *Circulation Research*, Pasdois et al<sup>10</sup> report similar functional changes in rat hearts but arrive at a very different conclusion with regard to underlying mechanisms.

### Deleterious Effects of High-Dose TAT-HKII In Intact Myocardium: Muscle or Vessel?

A key finding by Pasdois et al is the impairment of vascular function by high-dose TAT-HKII administration in the intact heart. This translates to a major increase in perfusion pressure (during constant flow conditions) or a decrease in flow (during constant pressure perfusion). Indeed, it is conceivable that ischemic injury caused by impaired vascular function may underlie mitochondrial dysfunction and associated electric abnormalities. We addressed this issue directly by investigating the impact of low-flow perfusion, endothelin-mediated vasoconstriction, and high perfusion pressure on electrophysiological properties, mitochondrial function, and ultrastructure. Finally, we assessed the presence of ischemia and associated damage in several groups, including low- and high-dose TAT-HKII perfused hearts. We found that neither vascular changes nor ischemic damage can explain the acute



**Figure 2. A, Representative action potential traces recorded during steady-state pacing in TAT-con and TAT-HKII hearts.** Average rate-dependence of APD90 (B) and APD75 (C) in TAT-con and TAT-HKII groups. D, Representative action potential upstrokes recorded from equidistant sites relative to the pacing electrode indicating action potential (AP) propagation delays in TAT-con and TAT-HKII hearts. E, Representative series of isopotential contour maps recorded every 1 ms from TAT-con and TAT-HKII hearts indicating sequential spread of the AP. F, Average conduction velocity in TAT-con and TAT-HKII hearts. Average Lactate production (G), LDH release (H) and NADH fluorescence levels (I) in 2.5  $\mu\text{mol}/\text{L}$  TAT-HKII (high-dose peptide group), 200 nmol/L TAT-HKII (low-dose peptide group), 2.5  $\mu\text{mol}/\text{L}$  TAT-con (control group), 60% flow reduction group, 7.5 nmol/L endothelin-1 group, and hypoxia group. One-way analysis with Dunnett's (G, H) or Bonferroni (I) post hoc tests. \*, \*\*, \*\*\*,  $P < 0.05, 0.01, \text{ or } 0.001$ , respectively. LDH indicates lactate dehydrogenase; NADH, nicotinamide adenine dinucleotide; PCL, pacing cycle length; TAT-con, transactivating transcriptional factor for control group; and TAT-HKII, cell permeable peptide containing hexokinase II mitochondrial binding motif.

and detrimental effects of TAT-HKII administration in the intact heart. However, our present findings do not exclude the theoretical possibility that some of the adverse effects of high-dose TAT-HKII administration may somehow be amplified by minor ischemic damage. Nevertheless, we believe that our present findings reaffirm the vital importance of maintaining a basal level of HKII on cardiac mitochondria to preserve mitochondrial energetics, electro-mechanical function, and cardiac structure, as we originally concluded.

### Abrogation of IPC: Partial Loss of Mitochondrial-HKII Levels or Silent Ischemia?

Pasdois et al<sup>10</sup> argued that the abrogation of IPC with low-dose peptide administration may be attributed to ischemia. Using 3 independent techniques, we were unable to detect ischemia or ischemia-mediated dysfunction in the low-dose peptide group,

arguing strongly against a role for ischemia in the abrogation of the protective effects of IPC by low-dose peptide administration. However, it is still possible that vascular changes may be unmasked by low-dose administration of the peptide during the reperfusion phase that follows an ischemic insult, an issue that will require direct examination in future studies.

Finally, using cell fractionation and Western blotting techniques on isolated mitochondria, Pasdois et al found that TAT-HKII administration did not alter HKII binding to mitochondria. One potential explanation for this discrepancy could be their reliance on a short period of peptide administration (<9 minutes). The protocol they chose is indeed problematic considering that the half-time of HK dissociation from mitochondria was recently reported to be 8.5 minutes.<sup>11</sup> In addition, the negative findings by Pasdois et al can also be explained by previous studies that highlight the importance

of maintaining the tissue intact when studying the subcellular localization of proteins whose association with mitochondria is highly regulated.<sup>12</sup> To achieve this stringent requirement, we resorted to EM and immunohistological analysis of HKII in perfusion-fixed hearts. Of note, in pilot experiments we also were unable to detect TAT-HKII-mediated changes in mitoHKII levels using standard techniques (Western blotting and HK activity measurements) that necessitate membrane fragmentation (data not shown). Because the association of HKII with the outer membrane of mitochondria is a dynamic and reversible process, which is likely dependent on the *in vivo* posttranslational status of its mitochondrial binding partner and the outer membrane potential (through binding to voltage-dependent anion channel), it is not surprising that the tissue fractionation process used by us in pilot experiments and Pasdois et al markedly disrupts HKII binding to the mitochondrial membrane.

The importance of studying mitoHKII *in situ* is also reflected by the higher concentration threshold required to elicit the detrimental effects of TAT-HKII in isolated mitochondria (30  $\mu\text{mol/L}$  range) as compared with intact cardiomyocytes ( $\approx 1 \mu\text{mol/L}$  range).<sup>9</sup> Such finding is consistent with the notion that the most dynamic and regulated subpopulation of HKII that binds mitochondria *in situ* is likely to be severely disrupted by the artificial process of membrane fragmentation used to isolate mitochondria.<sup>12</sup> Interestingly, John et al<sup>11</sup> recently reported HK dissociation on permeabilization of Chinese hamster ovary cells. A similar mechanism underlies the observation that although the outer membrane of mitochondria in their *in situ* native environment is highly impermeable, it is readily permeabilized by the mitochondrial isolation process probably through loss of tubulin binding to voltage-dependent anion channel.<sup>13</sup> This highlights major differences in mitochondrial membrane properties that likely affect the association between HKII and mitochondria using various techniques.

Finally, one must note that the technique of EM with immunogold labeling has clear limitations. For one, it only allows for very limited sampling and semiquantitative analyses, issues which we tried to minimize by choosing an identical predefined sampling procedure in all groups. We also complemented our histological examinations with integrative functional measurements, including electrophysiological and mitochondrial imaging tools, lactate production, NADH, and LDH release assays. Further refinement of biochemical tools for studying mitoHKII localization and displacement will be important in future studies because they will further complement the semiquantitative EM technique.

In conclusion, Pasdois et al report important changes in vascular function that are elicited by TAT-HKII administration. Here, we demonstrate that impaired vascular function does not account for mitochondrial dysfunction in the intact

heart. However, in light of the intriguing findings by Pasdois et al, future work directed toward understanding potential mechanisms by which HKII subpopulations may regulate vascular smooth muscle and endothelial cell function seems highly warranted.

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## Disclosures

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