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Hexokinase cellular trafficking in ischemia–reperfusion and ischemic preconditioning is altered in type I diabetic heart

Ebru Gurel · Savas Ustunova · Aysegul Kapucu · Nadim Yilmazer · Otto Eerbeek · Rianne Nederlof · Markus W. Hollmann · Cihan Demirci-Tansel · Coert J. Zuurbier

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Abstract Diabetes mellitus (DM) has been reported to alter the cardiac response to ischemia–reperfusion (IR). In addition, cardioprotection induced by ischemic preconditioning (IPC) is often impaired in diabetes. We have previously shown that the subcellular localisation of the glycolytic enzyme hexokinase (HK) is causally related to IR injury and IPC protective potential. Especially the binding of HK to mitochondria and prevention of HK solubilisation (HK detachment from mitochondria) during ischemia confers cardioprotection. It is unknown whether diabetes affects HK localisation during IR and IPC as compared to non-diabetes. In this study we hypothesize that DM alters cellular trafficking of hexokinase in response to IR and IPC, possibly explaining the altered response to IR and IPC in diabetic heart. Control (CON) and type I diabetic (DM) rat hearts (65 mg/kg streptozotocin, 4 weeks) were isolated and perfused in Langendorff-mode and subjected to 35 min I and 30 min R with or without IPC (3 times 5 min I). Cytosolic and mitochondrial fractions were obtained at (1) baseline, i.e. after IPC but before I, (2) 35 min I, (3) 5 min R and (4) 30 min R. DM improved rate-pressure product recovery (RPP; 71 ± 10 % baseline (DM) versus 9 ± 1 % baseline (CON) and decreased contracture (end-diastolic pressure: 24 ± 8 mmHg (DM) vs 77 ± 4 mmHg (CON)) after IR as compared to control, and was associated with prevention of HK solubilisation at 35 min I. IPC improved cardiac function in CON but not in DM hearts. IPC in CON prevented HK solubilisation at 35 min I and at 5 min R, with a trend for increased mitochondrial HK. In contrast, the non-effective IPC in DM was associated with solubilisation of HK and decreased mitochondrial HK at early reperfusion and a reciprocal behaviour at late reperfusion. We conclude that type I DM significantly altered cellular HK translocation patterns in the heart in response to IR and IPC, possibly explaining altered response to IR and IPC in diabetes.

Keywords Diabetes · Ischemia–reperfusion · Cardioprotection

E. Gurel · S. Ustunova · A. Kapucu · C. Demirci-Tansel
Department of Biology, Faculty of Science, İstanbul University, Istanbul, Turkey

N. Yilmazer
Department of Biology, Faculty of Science, Namik Kemal University, Tekirdag, Turkey

O. Eerbeek
Department of Physiology, Academic Medical Center, Amsterdam, The Netherlands

R. Nederlof · M. W. Hollmann · C. J. Zuurbier (✉)
Laboratory of Intensive Care and Anesthesiology, Department of Anesthesiology, Academic Medical Center, Amsterdam, The Netherlands
e-mail: c.j.zuurbier@amc.uva.nl

Introduction

Ischemia–reperfusion (IR) injury of organs and/or tissues is a main contributor to several acute and chronic pathologies, with injury not only developing during ischemia, but paradoxically also during reperfusion [1, 2]. It is therefore not surprising that the discovery of a latent endogenous cellular protection program against IR injury [3], a program that can be acutely activated by several different interventions, has received much attention and has tremendously intensified the search for the cellular protective signaling molecules. For the heart transient nonlethal periods of ischemia and reperfusion, i.e. ischemic preconditioning, are one of the strongest

interventions known to activate the endogenous cardioprotective phenotype.

The reperfusion injury salvage kinase pathway (RISK) and survivor activating factor enhancement (SAFE) pathway describe the most common cellular signaling factors involved in cardioprotection [4]. Both pathways converge on the mitochondria where they prevent the mitochondrial permeability transition pore (mPTP) from opening. The opening of the mPTP is currently viewed as a key event in inducing lethal reperfusion injury [5]. We have found that the glycolytic enzyme hexokinase can be considered as a main cardioprotective-sensitive regulator of the mPTP, probably constituting one of the end-effectors of cardioprotective signaling [6, 7]. Specifically, we have shown that altered expression and subcellular trafficking of HKII are major determinants of cardiac ischemia–reperfusion (IR) injury and ischemic preconditioning [8–11]. Increasing the amount of HK binding to mitochondria during ischemia and early reperfusion seems to contribute largely to the protective effects of IPC in the healthy heart.

Since diabetic patients are at high risk for IR injury, the development of effective cardioprotective interventions for this group will be tremendously worthwhile, especially when one considers the increased prevalence of diabetes. However, the translation of cardioprotective interventions from the pre-clinical laboratory setting to the clinical condition is hampered by recent observations that diabetes impairs cardioprotective signaling [12–14]. Knowing that cardiac hexokinase is one of the first proteins affected during the development of diabetes [15], we considered it likely that cellular HK trafficking within the heart with IR and IPC is altered in the diabetic heart. The relation between diabetes and HK cellular trafficking in response to IR and IPC remains, however, to be elucidated. Alterations in HK cellular behavior may offer a possible explanation for the well described altered response of the diabetic heart to IR and IPC. Therefore, the aim of the current study was to (1) characterize and compare HK cellular trafficking in the diabetic heart at different time points throughout ischemia–reperfusion with that of the healthy heart, and (2) evaluate whether DM alters IPC-effects on HK cellular trafficking throughout ischemia–reperfusion of the heart.

Methods

Animals

Animal experiments were reviewed and approved by the Animal Care and Use Committee of Istanbul University. Wistar albino male rats (120 total) were housed in groups of two to four per cage in a controlled temperature room (22 ± 3 °C). They were fed with standard pellet diet and tap water ad libitum.

Induction of diabetes

Experimental diabetes was induced by a single intraperitoneal injection of freshly prepared streptozotocin (STZ; Sigma S0130, single dose of 65 mg/kg bw) in 0.1 M citrate buffer (pH 4.5). After 10 days and 4 weeks of STZ administration, plasma glucose levels from tail bleeding of each rat were determined using a glucometer (Glucotech α, YD Diagnostics, Korea). In the successful experiments, a total of 104 rats divided into sixteen groups of 6–8 each were used. The rats were used after 4 weeks of STZ administration.

Heart perfusion

Animals were anesthetized with pentobarbital sodium (65 mg/kg). Tracheotomy was performed and mechanical ventilation initiated. Following the opening of the thorax and administration of intravenous heparin (150 IU), the aorta was cannulated in situ, and perfusion was started before excision of the heart. Hearts were Langendorff-perfused *(PowerLab ML870B2)* at 37 °C with Krebs-Henseleit buffer containing (mM) 118 NaCl, 0.5 EDTA, 4.7 KCl, 2.25 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11 glucose, 1 lactate, 0.5 glutamine, and 0.1 pyruvate, gassed with 95 % O_2–5 % CO_2. A water-filled polyethylene balloon was inserted into the left ventricle through the left atrium and connected to a pressure transducer for assessment of contractile performance. End-diastolic pressure was set at 3-6 mmHg by adjusting balloon volume. Hearts were perfused at a constant flow with initial perfusion pressure set at 80 mmHg. After stabilization of pressure development during the first 20 min of Langendorff-perfusion, 16 groups of hearts (*n*: 6–8 each group) were studied (Fig. 1a). The ischemia–reperfusion protocol existed of 35 min perfusion before ischemia, 35 min global no-flow ischemia, and 30 min of reperfusion. The ischemic preconditioned (IPC) groups received three 5-min periods of ischemia each followed by 5 min reperfusion, except that the last reperfusion lasted for 10 min, before the start of 35 min ischemia. To determine the temporal and subcellular localization of HK activity, hearts from non-IPC groups (CON and DM) and IPC groups (CON and DM) were examined at four different time points: (1) after IPC, just before ischemia, (2) 35 min of ischemia, (3) 5 min of reperfusion, and (4) 30 min of reperfusion. During the 35 min global ischemia the volume of the balloon was kept intact and hearts were submerged in the Krebs-Henseleit solution (37 °C) gassed with 95 % N_2–5 % CO_2.

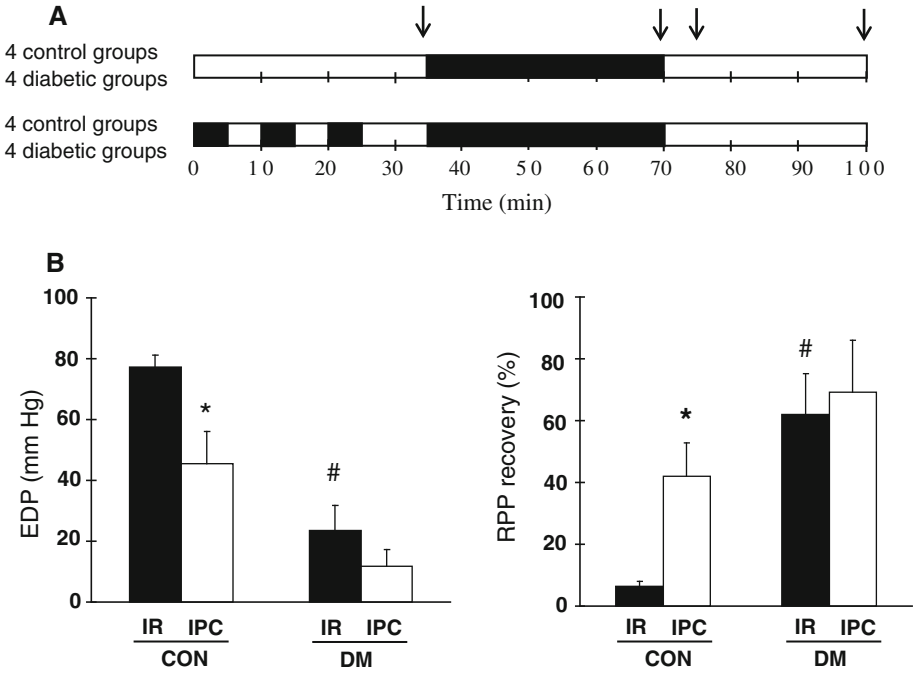

Fig. 1 **a** Experimental protocol for the isolated perfused rat heart studies. ↓, time at which hearts were homogenized for both control (CON) and IPC groups. Filled bar indicate period of global ischemia of the heart. **b** End-diastolic pressure (EDP) and Rate-Pressure-Product recovery (RPP %) at 30 min reperfusion for CON and diabetic (DM) hearts following ischemia–reperfusion (IR) without or with ischemic preconditioning (IPC). *$P < 0.05$ versus IR of similar group; #$P < 0.05$ versus IR of CON group

Heart homogenization and fractionation

At the designated time points, the heart was immediately minced in 8 ml ice-cold homogenization medium (pH 7,4) containing (mM) 250 sucrose, 20 HEPES (pH 7.4), 10 KCl, 1.5 $MgCl_2$, EDTA, 1 dithiothreitol, 0.1 PMSF, 5 μg/ml leupeptin and aprotinin, and 1 μg/ml pepstatin, and homogenized on ice. A small part of the homogenate was not centrifuged and represented the whole cell fraction. Subsequently, most of the homogenate was immediately centrifuged at 10,000 *g* for 10 min at 4 °C. The supernatants and pellets were recovered and represented the soluble (cytosolic) and mitochondrial fractions, respectively.

Determination of enzyme activities

Whole cell homogenates, mitochondrial fractions and cytosolic fractions were treated with 0.5 % Triton X-100 and 0.9 M KCl to maximally solubilize HK [15], followed by centrifugation in an Eppendorf microcentrifuge (12,000 *g*; 10 s). Enzyme activities were determined in the supernatant of these fractions. HK activity was measured spectrophotometrically at 25 °C with glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*), glucose, ATP, and NAD^+, in the presence of rotenone (1 μM) to inhibit the mitochondrial respiration chain. Protein content of the different fractions was determined by the Bradford method in the supernatant of the 12,000 g, 10 s centrifugation step.

Statistics

The results are expressed as mean ±SE. Student's t test was used to compare group means (CON versus DM) of baseline characteristics of the animal and heart model. ANOVA with Dunnett's post hoc tests was used to compare HK activity within one treatment group with baseline values. ANOVA followed by Fisher's post hoc tests was used to compare IPC effects at one time point within each group (CON or DM). A value of $P < 0.05$ was considered statistically significant.

Results

A total of 120 rats were initially included in this experiment (55 healthy rats and 65 STZ-treated rats). 16 rats were excluded: 5 rats died after STZ-treatment, 7 heart preparations failed (5 STZ and 2 healthy hearts) due to heart rate <100 beats/min (3 STZ hearts, 2 control (CON) hearts) or low perfusion pressure (2 STZ hearts) during baseline perfusion, and 4 rats died during anesthesia (2 CON and 2 DM). A total of 104 successful isolated heart experiments were performed. Blood glucose was elevated 10 days after STZ administration in DM animals. 4 weeks after inclusion, the STZ group showed persistent significantly elevated blood glucose levels and decreased body and heart weight. Heart weight relative to body weight was, however, similar between groups (Table 1). There was no significant difference in cardiac coronary flow at baseline (all hearts

perfused at an initial perfusion pressure of 80 mmHg). DM hearts demonstrated significant impaired cardiac performance at baseline as reflected by a lower heart rate and a lower left ventricular peak systolic pressure (Table 1).

Thirty-five min ischemia and 30 min reperfusion resulted in severe myocardial dysfunction in CON hearts, as reflected by high diastolic contracture and low recovery of RPP (Fig. 1b). DM was associated with significant improved recovery of RPP and less diastolic contracture after IR as compared to CON hearts. Ischemic preconditioning decreased diastolic contracture and increased the recovery of left ventricular pressure development in CON hearts. In contrast, no additional beneficial effects of IPC on myocardial function after IR were observed in DM hearts.

Whole heart HK activity throughout IR and IPC for CON and DM hearts is depicted by Fig. 2. Short-term DM resulted in a 24 % decrease in total cardiac HK activity (29.6 ± 1.9 vs 38.8 ± 2.3 nmol min^{-1} mg $protein^{-1}$ for DM vs CON, respectively). HK activity throughout IR was constant in CON hearts, whereas in DM hearts there was a small, but significant, decrease upon reperfusion. IPC had only minor effects on total heart HK activity for both CON and DM hearts, except that HK activity was decreased at early reperfusion for CON hearts.

Despite the decrease in total HK activity in DM hearts, cytosolic HK activity at baseline was not different between DM and CON hearts (Fig. 3). Compared to baseline, ischemia–reperfusion significantly increased cytosolic HK activity at end ischemia in CON but not in DM hearts, indicating that DM prevented solubilisation of HK during ischemia. It was only with IPC that solubilisation of HK at end ischemia and early reperfusion in CON hearts was prevented. An almost opposite behavior was observed for DM hearts: IPC decreased cytosolic HK activity immediately after IPC at baseline, but significantly increased cytosolic HK activity during early and late reperfusion.

The decrease in whole heart HK activity observed with DM was reflected by a significant 28 % decrease in

Table 1 Characteristics of experimental animal model and baseline cardiac function of Langendorf-perfused heart model

Groups	Control ($n = 50$)	Diabetic ($n = 54$)
Intact animal		
10 days-Glucose (mg/dl)	N.D.	530 ± 10
4 weeks-Glucose (mg/dl)	116 ± 2	$554 \pm 9^{***}$
Body weight (g)	282 ± 4	$208 \pm 3^{***}$
Heart weight (g)	1.06 ± 0.03	$0.82 \pm 0.02^{***}$
HW/BW (mg/g)	3.8 ± 0.1	4.0 ± 0.01
Langendorf-perfused hearts		
Flow (ml min^{-1} g^{-1})	14 ± 0.5	13 ± 0.5
EDP (mmHg)	3 ± 1	4 ± 1
Psys (mm Hg)	150 ± 4	$120 \pm 3^{***}$
Heart Rate (beats min^{-1})	228 ± 4	$180 \pm 5^{***}$
RPP $\times 10^3$ (mm Hg min^{-1})	33.9 ± 1.1	$20.5 \pm 0.7^{**}$

N.D. not determined, *EDP* end-diastolic pressure, *Psys* peak systolic pressure, *RPP* rate-pressure-product (Psys times HR)

*** $P < 0.001$ or ** $P < 0.01$, respectively

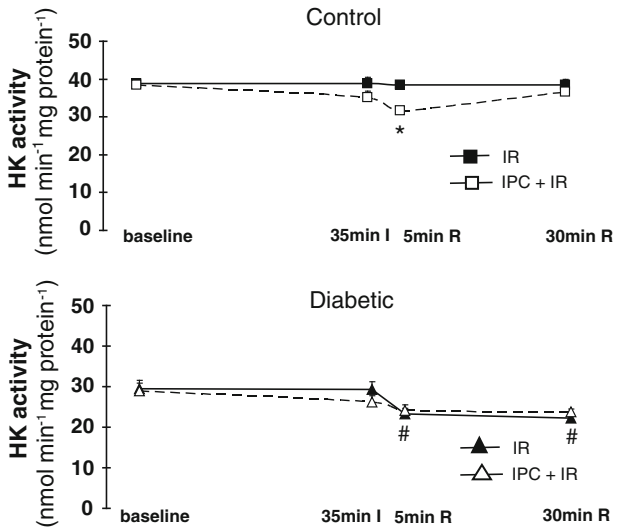

Fig. 2 Time course of hexokinase (HK) activity in the whole heart fraction for the IR and IPC groups of control and diabetic hearts throughout the experimental protocol. HK acitivity is normalized to protein content of fraction. *$P < 0.05$ versus IR of similar group and at similar time point; #$P < 0.05$ versus baseline of similar group

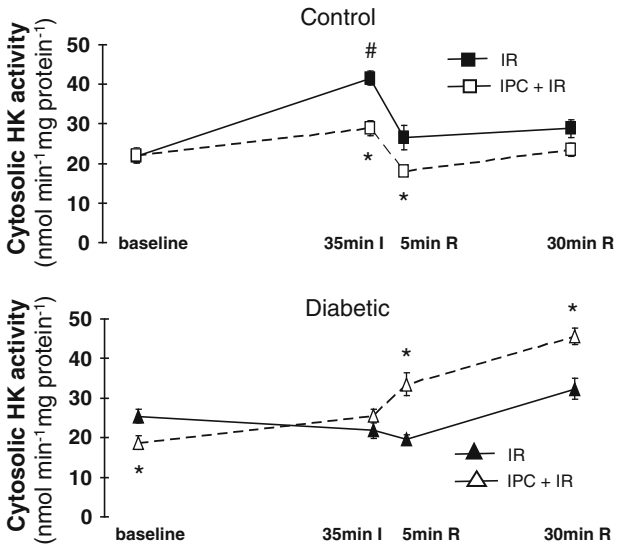

Fig. 3 Time course of hexokinase (HK) activity in the cytosolic fraction for the IR and IPC groups of control and diabetic hearts throughout the experimental protocol. HK acitivity is normalized to protein content of fraction. *$P < 0.05$ versus IR of similar group and at similar time point; #$P < 0.05$ versus baseline of similar group

mitochondrial HK activity (46.4 ± 3.0 vs 64.6 ± 4.4 nmol min^{-1} mg $protein^{-1}$ for DM vs CON, respectively; Fig. 4). Ischemia–reperfusion *per se* was without effect on mitochondrial HK activity for CON hearts. However, IR induced a significant increase in mitochondrial HK activity at early reperfusion for DM hearts. IPC increased mitochondrial HK immediately after IPC at baseline for CON hearts ($P = 0.053$), and was associated with a non-significant trend of increased mitochondrial HK throughout IR for CON hearts. IPC significantly increased mitochondrial HK activity at end ischemia and end reperfusion for DM hearts. Surprisingly, DM decreased mitochondrial HK activity at early reperfusion (Fig. 4).

Discussion

This study shows that hearts 4 weeks after STZ treatment have improved functional recovery after ischemia–reperfusion as compared to control hearts. The functional improvement was associated with the prevention of HK solubilisation during ischemia. Ischemic preconditioning strongly improved cardiac function after IR in healthy hearts, and this was associated with reduced HK solubilization during ischemia and early reperfusion. Ischemic preconditioning did not further improve functional recovery in diabetic hearts, and this was associated with solubilisation of HK at early reperfusion with increases in mitochondrial HK activity during ischemia and late reperfusion. In overall, HK cellular trafficking induced by IR and IPC was significantly altered with DM.

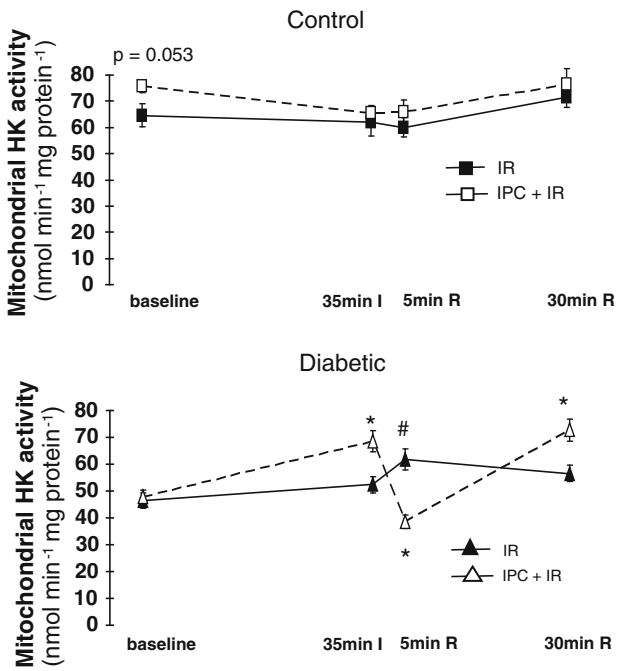

Fig. 4 Time course of hexokinase (HK) activity in the mitochondrial fraction for the IR and IPC groups of control and diabetic hearts throughout the experimental protocol. HK acitivity is normalized to protein content of fraction. $*P < 0.05$ versus IR of similar group and at similar time point; $\#P < 0.05$ versus baseline of similar group

DM and cardiac HK activity and contractility

Cardiac HK activity was decreased by 24 % with DM in the current study, which is in agreement with the DM-induced decreases (20–50 %) reported in HK activity by numerous previous studies [15–18]. Although not examined in the current study, these previous studies demonstrated that streptozotocin-treatment mainly affected HKII and not HKI. Knowing that for the heart both HK isoforms contribute approximately equal to total cardiac HK activity [19], the 24 % decrease in total HK activity observed in the present study with DM thus probably reflects a 50 % decrease in HKII protein content. The results demonstrated that the overall down-regulation of total whole heart HK activity with DM was mainly reflected by a decrease in mitochondrial HK activity with no change in cytosolic HK activity. Similar findings were recently reported by Da Silva et al. [18], showing that 3 days following STZ-treatment HK activity was mainly lowered in the mitochondrial-enriched fraction. Interestingly, these authors demonstrated that metformin administration can completely reverse the DM-induced HK cellular distribution. Knowing that metformin offers IR protection in diabetic hearts [20, 21], it is therefore possible that these protective effects of metformin are partly mediated through HK cellular distribution.

At baseline, DM was associated with significant reduced performance of the Langendorff-perfused heart as compared to the healthy heart. Although this is not a consistent finding in literature, other studies have reported decreased mechanical performance of DM heart [22–24]. One limitation of this study is that we cannot completely exclude the possibility that the decreased cardiac performance at baseline of DM hearts played a role in the cardioprotective phenotype of these hearts. However, in other studies with decreased cardiac performance of DM hearts, IR injury was actually increased [24] or unaffected [22, 23] as compared to healthy hearts, making it unlikely that the decrease in heart rate and left ventricular pressure observed in the present study for DM hearts caused the protection against IR injury.

DM and ischemia–reperfusion injury

In our model, 4 weeks after STZ treatment resulted in a cardioprotective phenotype with abrogation of the IPC

potential. Previous studies have demonstrated that the duration of diabetes is one of the main determinants of IR injury. Short–term duration is hereby often associated with increased tolerance and long-term duration with decreased tolerance to IR injury [13, 14, 25]. Our findings are in support of Xu et al. [25], who also reported that 4 weeks STZ protected the heart against IR injury, whereas 20 weeks ameliorated cardiac IR injury. In the present study we hypothesized that DM alters HK cellular trafficking and that these alterations may possibly partly explain the changes in response of DM hearts to IR. The major differences in HK cellular trafficking between healthy and DM hearts during the IR intervention are (1) the prevention of HK solubilisation (i.e., increases in cytosolic HK activity) during ischemia and (2) the detectable increase in mitochondrial HK activity at early reperfusion in DM hearts. Both of these differences can indeed contribute to the cardioprotective phenotype of short-term DM hearts. Keeping HK at the mitochondria has been shown to prevent mitochondria-induced ROS production [26, 27] and activation of cell death pathways [28]. In addition, dislodging HK from mitochondria or inhibiting HK binding to mitochondria was associated with increased cardiac IR injury [10]. Thus, it is possible that these changes in HK cellular trafficking with short-term DM explain the cardioprotective phenotype. However, the present study also demonstrated an overall decrease of 28 % in mitochondrial HK activity. We previously demonstrated that, in a genetic model of HKII manipulation, a 25 % decrease in HK activity caused by a 50 % decrease in HKII protein content resulted in increased cardiac or skeletal muscle IR injury [9–11]. This was commensurate with observations whereby overexpression of yeast HK protects the diabetic heart against hypoxic dysfunction and ATP breakdown [29]. In contrast, we now observed *decreased* IR injury with *decreased* whole heart HK activity in short-term DM hearts. Obviously, many other factors may affect IR injury, and activation or upregulation of Akt, eNOS, PKC, ERK and/or heat-shock proteins with short-term DM have all been suggested to explain the protective nature of short-term DM [30]. It is likely that the activation state of some of these factors will affect HK cellular trafficking. For example, Akt activation in DM hearts may partly explain the inhibition of HK solubilisation during ischemia, because it was shown that Akt phosphorylation induced HK translocation to mitochondria in cardiomyocytes [31]. The present study suggests that altered HK cellular trafficking, and not just diminished amounts of HK, is associated with increased tolerance against IR.

DM and ischemic preconditioning

IPC-induced protection against IR injury is initiated through acute activation of complex cellular signaling networks, among which the RISK and SAFE pathway figure prominently [4, 32]. It has been shown that these cardioprotective pathways are disrupted at numerous places in long-term DM, such as diminished phosphorylation of PI3 K/Akt, GSK-3β, ERK, STAT3 and AMPK [22, 33–36]. Many of these protective signaling pathways condensate through phosphorylation and thereby inactivation of GSK-3β, that directly affects the mPTP [37]. HK is in this scheme a likely end-effector, because it was shown that inhibition of GSK-3β results in detachment of HK from mitochondria [38]. Thus, an attractive hypothesis is that the abolishment of the protective potential of IPC in long-term DM models is caused by inhibited RISK and SAFE pathway activation and consequently prevention of HK translocation to mitochondria. However, in the current study, we observed that short-term DM was also associated with abrogation of IPC protective action. In this condition the cardiac survival pathways are in a chronic activated state, not allowing additional activation by IPC. The major difference in HK cellular trafficking between healthy and DM hearts following the IPC stimulus occurred at early reperfusion: the IPC protocol in DM hearts resulted in decreased mitochondrial HK activity, which was mirrored by a significant increase in cytosolic HK activity at this period. We are unaware of any previous observation showing that an IPC protocol can actually result in decreased mitochondrial HK within the heart. It is possible that this decrease in mitochondrial HK during the critical early period of reperfusing actually mitigates any possible protective effects of IPC in short-term DM hearts. The underlying mechanisms to this different regulation of HK in DM hearts following IPC are unknown, but may relate to the fact that many of the signaling pathways are already activated in these hearts. Further research will be necessary to elucidate the precise origin of this different cellular HK trafficking in the preconditioned DM hearts.

Conclusion

The present study clearly demonstrates that diabetes not only results in a decrease in the total amount of the glycolytic enzyme hexokinase, but also, and probably more importantly, significantly distorts the cellular trafficking of HK in response to ischemia–reperfusion and ischemic preconditioning. Knowing that the spatial and temporal behavior of HK is an important determinant of IR injury and IPC protective potential, these results support the hypothesis that the altered response of the diabetic heart to IR and IPC is related to difference in cellular trafficking of HK.

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