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Original Paper

Repeated Non-Invasive Limb Ischemic Preconditioning Confers Cardioprotection Through PKC-Ɛ/STAT3 Signaling in Diabetic Rats

Chunyan Wang[a,b]   Haobo Li[a,c]   Sheng Wang[d]   Xiaowen Mao[a]   Dan Yan[a]
Stanley S. Wong[a]   Zhengyuan Xia[a,c]   Michael G. Irwin[a]

[a]Department of Anesthesiology, The University of Hong Kong, Hong Kong SAR, [b]Department of Anesthesiology, Shenzhen People's Hospital and Shenzhen Anesthesiology Engineering Center, the Second Clinical Medical College of Jinan University, Shenzhen, [c]State Key Laboratory of Pharmaceutical Biotechnology, The University of Hong Kong, Hong Kong, [d]Department of Anesthesiology, Guangdong Cardiovascular Institute, Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangdong, China

Abstract

Background/Aims: Protein kinase C(PKC)-ε activation is a mechanism of preconditioning cardioprotection but its role in repeated non-invasive limb ischemic preconditioning (rNLIP) mediated cardioprotection against myocardial ischemia/reperfusion (I/R) injury in diabetes is unknown. ***Methods:*** Eight-week streptozotocin-induced diabetic and non-diabetic Sprague-Dawley rats were subjected to I/R without or with rNLIP. *In vitro*, H9C2 cells were cultured with high glucose (HG) and subjected to hypoxia/re-oxygenation (H/R) without or with PKC-ε or STAT3 gene knock-down in the absence or presence of remote time hypoxia preconditioning (HPC). ***Results:*** Diabetic rats displayed larger post-ischemic myocardial infarct size and higher troponin-I release with concomitant cardiac PKC-ε overexpression and activation manifested as increased membrane translocation, while phosphorylated STAT3 (p-STAT3) and Akt (p-Akt) were lower compared to non-diabetic rats (all $P<0.05$). rNLIP reduced infarct size in both non-diabetic and diabetic rats. rNLIP reduced post-ischemic cardiac PKC-ε activation in diabetic while increased PKC-ε activation in non-diabetic rats, resulting in increased cardiac p-STAT3 and p-Akt. In H9C2 cells, HG increased PKC-ε expression and exacerbated post-H/R injury, accompanied with reduced p-STAT3 and p-Akt, which were all reverted by HPC. These HPC protective effects were abolished by either PKC-ε or STAT3 gene knock-down, except that PKC-ε gene knock-down reverted HG and H/R-induced reduction of p-STAT3. ***Conclusion:***

Michael G. Irwin and Zhengyuan Xia

Department of Anaesthesiology, University of Hong Kong,
Hong Kong SAR (China)
Tel. (852) 22553303, (852) 39179794, E-Mail mgirwin@hku.hk, zyxia@hku.hk

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;45:2107-2121

DOI: 10.1159/000488047
Published online: March 12, 2018

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www.karger.com/cpb

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rNLIP attenuates diabetic heart I/R injury by mitigating HG-induced PKC-ε overexpression and, subsequently, activating STAT3.

Introduction

Myocardial ischemia, post-ischemic myocardial injury and heart failure are leading causes of death worldwide, particularly in patients with diabetes [1]. Paradoxically, re-establishment of blood flow to ischemic myocardium may cause further damage, a phenomenon referred to as ischemia/reperfusion injury (I/RI) [2-4]. Brief episodes of ischemia intermitted with reperfusion before prolonged ischemia can reduce post-ischemic myocardial infarct size, a phenomenon termed ischemic preconditioning (IPC) [5]. IPC-mediated protection of one organ or tissue (even peripheral muscle) also protects distant organs/tissues (e.g. heart or spinal cord) from ischemic injury through a different signaling pathway [6, 7], and this phenomenon is termed remote ischemic preconditioning (RIPC) [8]. Interestingly, brief cycles of non-invasive limb ischemia and reperfusion (NLIP), a type of RIPC, has been proven to be cardioprotective in experimental settings which is easy to operate [9, 10]. However, NLIP is less efficacious in certain pathological states, such as diabetes, senescence and obesity. Interestingly, we showed that application of 3 cycles of NLIP *per* day for 3 days (repeated NLIP, rNLIP) attenuated myocardial I/RI in diabetic rats [11-13], suggesting that, in order to confer cardioprotection under pathological conditions, the threshold of NLIP should be increased. However, the mechanism whereby rNLIP may confer cardioprotection in diabetes is unclear.

Activation of the signal transducer and activator of transcription 3 (STAT3) is critical for NLIP to confer cardioprotection [14] but cardiac STAT3 is reduced in diabetes [15], which might reflect why diabetic hearts are less responsive to NLIP [16]. We previously found that rNLIP conferred cardioprotection in diabetic rats [11] whose cardiac STAT3 was initially reduced [15]. It is possible, therefore, that enhancing cardiac STAT3 activation may be a mechanism to restore rNLIP in diabetes. However, the mechanism governing STAT3 activation in rNLIP, especially in diabetic hearts, is unclear.

Besides STAT3, activation of the epsilon isoform of protein kinase C (PKC-ε) is crucial for IPC and NLIP to confer protection against myocardial I/RI given that PKC-ε knock-out cancels IPC [17] and inhibition of PKC-ε membrane translocation blocked NLIP-mediated cardioprotection [18]. The role of PKC in cardioprotecton is contentious [19], being either isoform specific or dependent on the extent of its activation. PKC-ε is essential for IPC to activate STAT3 at site Ser727 and confer its late phase cardioprotection [17, 20]. Activation of PKC-ε induces STAT3 phosphorylation, while PKC-ε knockdown reduces STAT3 phosphorylation [21]. In diabetes, PKC-ε is over-expressed and activated [22] contributing to the onset of hyperinsulinemia, hyperglycaemia [23] and cardiac remodelling in type-2 diabetic mice [24]. On the other hand, we recently showed that cardiac STAT3 is reduced in diabetic rats [25]. However, the potential causal link between PKC-ε and STAT3 in diabetes has not been explored. In particular, whether or not over-activation of cardiac PKC-ε in diabetes is responsible for the loss of sensitivity of the diabetic heart to rNLIP cardioprotection is unknown.

Therefore, we hypothesized that over-activation of PKC-ε, by down-regulating cardiac STAT3, renders the diabetic heart susceptible to myocardial I/R injury and not responsive to rNLIP cardioprotection.

Materials and Methods

Induction of diabetes

Male Sprague-Dawley rats (250 g, 6-8 weeks) obtained from the Laboratory Animal Unit (The University of Hong Kong) were used in this study. Diabetes were induced by single dose (65 mg/kg) streptozotocin

(STZ) (Sigma-Aldrich, St. Louis, MO) injection via tail vein as described [26, 27]. Rats were housed in the Laboratory Animal Service Center (University of Hong Kong) and received standard care in accordance with the principles of Animal Care of the University of Hong Kong. The experimental protocols were approved by the committee on the Use of Live Animals in Teaching and Research (CULATR). The I/R and rNLIP protocol and grouping (n= 6 rats per group) were described in the diagram below (Fig. 1).

In vivo model of myocardial ischemia reperfusion (I/R) and rNLIP

Eight weeks after diabetes induction, rNLIP was implemented by applying 3 cycles of 5- minute occlusion/5- minute reperfusion in a unilateral hindlimb daily for 3 days. The unilateral right hindlimb was selected to apply rNLIP because it is easier to tie up and closer to the heart relative to

Fig. 1. *In vivo* experimental protocols. I/R: ischemia/reperfusion; rNLIP: repeated non-invasive limb ischemia preconditioning. Normal(C): non-diabetic control rats; D8w: 8 weeks diabetic rats.

the left hindlimb. Twenty-four hours after the completion of rNLIP, the rats were subjected to myocardial I/RI. Rats in the respective subgroups were anesthetized using phenobarbital sodium; ischemia was achieved by occluding the left anterior descending (LAD) artery for 30 minutes followed by reperfusion for 2 hours as described [28]. Infarct size (IS) was assessed using TTC (1% 2, 3, 5-triphenyltetrazolium chloride) staining and expressed as a percentage of the anatomic area at risk (AAR) [29, 30]. Five percent Evans Blue was injected through the right jugular vein to mark the normal region of the left ventricle (LV) and to calculate AAR. At the completion of the experiments, the rats were euthanized with overdose pentobarbital injection, and the hearts were quickly removed and cut into 5 slices of 1mm cross-sections, followed by incubation in 1% TTC in PBS at room temperature for 20 minutes. The slices were then fixed with 10% formalin overnight. Parts of the LV tissues were immediately frozen in liquid nitrogen until processed for Western Blotting analysis.
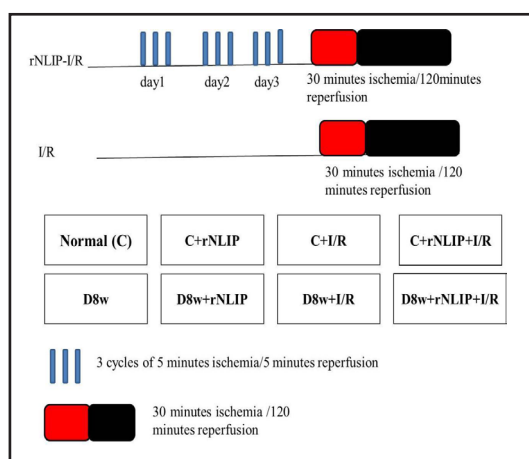
In vitro models of cardiac H9C2 cells hypoxia reoxygenation (H/R) and remote time hypoxic preconditioning (HPC)

Embryonic rat cardiac H9C2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS in a humidified atmosphere (5% CO_2) at 37°C. To mimic high glucose (HG) condition in diabetes, high D-glucose (25mM) was added to the medium to make the final glucose concentration at 30 mM, and the glucose concentration in normal medium (glucose: 5.5 mM) was used as normal glucose control group. Mannitol/glucose was added (19.5mM mannitol plus 5.5mM glucose) for osmotic control.

To partially mimic the *in vivo* situation in which rNLIP was completed 24 hours before I/R initiation, remote time repeated hypoxic preconditioning (HPC, which was achieved by 3 cycles of 5 minutes hypoxia followed by 5 minutes of re-oxygenation) was completed 24 hours before inducing hypoxic/ re-oxygenation (H/R) in cultured cardiac H9C2 cells as mentioned below. To verify the effects of HPC on cellular injury and cardiac signaling proteins in the absence of hypoxia/reoxygenation, we also performed the preliminary experiment of HPC alone under NG or HG conditions.

PKC-ε or STAT3 small interference RNA (siRNA) studies, hypoxia reoxygenation (H/R), and remote time hypoxic preconditioning (HPC) in H9C2 cells

Gene silencing was performed using PKC-ε siRNA or STAT3 siRNA, control siRNA and transfection reagent, purchased from Santa Cruz Biotechnology (Dallas, Texas, USA) following the manufacturer's instructions. In brief, $2*10^5$ H9C2 cells were seeded and maintained in DMEM containing 10% FBS in a humidified atmosphere (5% CO_2) at 37℃ and were allowed to reach 60–80% confluence. Then the cells were transfected with targeted siRNA and control siRNA. Six hours after transfection, normal growth medium was added and the cells were incubated at 37℃ in a humidified atmosphere (5% CO_2) for 24

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;45:2107-2121

DOI: 10.1159/000488047
Published online: March 12, 2018

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www.karger.com/cpb

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hours. Then the cells were subjected to 6 hours of hypoxia and 12 hours of re-oxygenation with or without high glucose (30 mM). HPC and hypoxic conditions were performed as described above.

Hypoxic conditions were obtained by equilibrating a humidified plexiglass chamber containing myocytes with 95% N_2 and 5% CO_2 and confirmed by measuring chamber O_2 concentration falling to 0.1%. Re-oxygenation was achieved by exposing cells to room air (CO_2 incubator). Remote time HPC was achieved by 3 cycles of 5 minutes hypoxia followed by 5 minutes of re-oxygenation 24 hours before hypoxic/ re-oxygenation (H/R).

Assessment of lactate dehydrogenase (LDH) and Troponin-I (Tn-I) release

After 2 hours of reperfusion (i.e. the completion of the experiment), blood samples were collected from the carotid artery and then centrifuged to separate serum. For H9C2 cells, after H/R, cellular medium was collected and then centrifuged for detection of LDH. LDH and Tn-I are specific markers of tissue damage. Therefore, plasma levels of LDH and Tn-I release as well as level of LDH in cell supernatant were analyzed using a LDH cytotoxicity assay kit (Roche USA) and Tn-I ELISA kit (Cloud-Clone Corp) following the manufacturer's instructions as described.

Determination of mitochondrial membrane potential (MMP) loss

MMP was an important indicator of cell death. MMP loss was measured using a JC-1 mitochondrial membrane potential kit (Cayman Chemical) as described [31]. JC-1 is a lipophilic, cationic dye and its advantage is that it can selectively enter into mitochondria based on the aggregation of the probe and reversibly change color from green to red as the membrane potential increases. Cells that had the strongest J-aggregation stained red (560/595nm) due to high MMP and injured cells that stained green (485/535nm) had low MMP. JC-1 stained cells were analyzed using a Live Cell Microscope (Nikon, Melville, New York, USA) (magnification, ×10) and expressed as the percentage of green cells in the total cells.

Cytoplasm and membrane protein extraction

Membrane and cytoplasm separation was performed as described [32] with modification [33]. Briefly, frozen heart tissue was homogenized in lysis buffer A (20 mM Tris-Hcl, PH7.5, 2mM EDTA, 2 mM EGTA, 6 mM β-mercaptoethanol, 50 μg/ml aprotinin, 48μg/ml leupetin, 5μM pepstatin, 1mM PMSF, 0.1 mM $NaVO_3$ and 50 mM NaF), with a ultrasonic homogenizers. The obtained homogenate was then centrifuged at 100, 000 g for 60 minutes, and the supernatant was collected as cytosol fraction. The pellet was suspended in buffer B (buffer A with 0.1% Triton X-100) and incubated on ice for 30 minutes, vibrated every 10 minutes on the oscillators, then centrifuged at 100, 000 g for 60 minutes, The resulting supernatant, containing the membrane fraction, was collected.

Western blot analysis

Frozen ventricular tissue samples were homogenized in lysis buffer (from Cell Signaling Technology (Beverly, MA) and centrifuged at the speed of 13200 g for 30 minutes. And the proteins from H9C2 cells were also homogenized in lysis buffer and centrifuged at speed of 13200 g for 15 minutes. The supernatant was collected as total protein and protein concentrations were determined using the Bradford protein assay. Equal amounts of protein from H9C2 cells and rat heart homogenate were resolved by 7.5-12.5% SDS-PAGE and subsequently transferred to polyvinylidene nitrocellulose membranes and processed as described [15]. Membranes were blocked in TBST containing 5% (w/v) non-fat milk and dried for 1 hour at room temperature, following by strips incubation with primary antibodies overnight at 4℃. The primary antibodies against PKC-ε, Akt, STAT3, phosphorylation-Akt (Ser-473), STAT3 at sites of Ser 727 and Tyr 705 and GAPDH were purchased from Cell Signaling Technology (Beverly, MA). Following extensive washing, membrane strips were incubated with secondary antibody 1:5000 anti-rabbit IgG (Cell Signaling Technology, Inc. MA) conjugated to horseradish peroxidase for 1 hour. Protein bands were detected by a standard ECL method and images were measured by a densitometer.

Statistical analysis

All values are expressed as mean ± standard error of the mean (S.E.M.). All data are normally distributed as confirmed by Graphpad Prism normality test. Comparisons between multiple groups and treatment for data obtained from *in vivo* studies were analyzed by two-way ANOVA, while data obtained

from *in vitro* studies were analyzed by one-way ANOVA, followed by the Tukey test for multiple comparisons (GraphPad Prism version 7, GraphPad Software, Inc., Lajolla, CA). *P* values less than 0.05 indicate statistical significance.

Results

rNLIP conferred cardioprotective effects against myocardial I/RI in diabetes

As shown in Fig. 2A-C, post-ischemic myocardial infarct size (IS) was significantly larger in diabetic than in non-diabetic rats (*P*<0.05), which was associated with significant increased release of Tn-I (Fig. 2D) and LDH (Fig. 2E) (all *P*<0.05, diabetic *vs.* non-diabetic control, diabetic-I/R *vs.* non-diabetic-I/R). rNLIP significantly reduced post-ischemic IS and decreased Tn-I and LDH in both non-diabetic and diabetic rats.
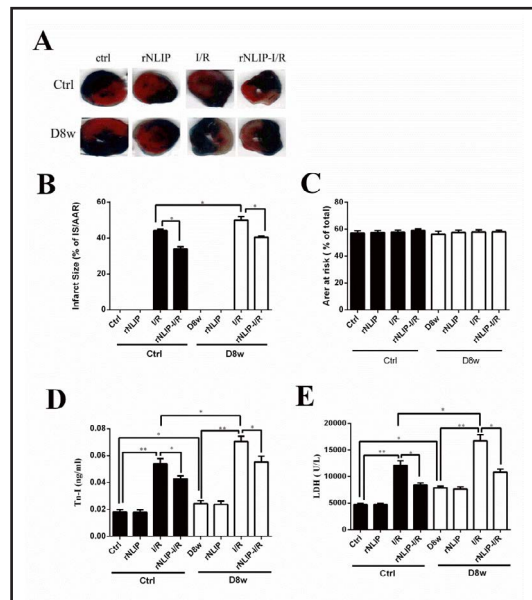
rNLIP reduced post-ischemic cardiac PKC-ε and enhanced STAT3 and Akt in diabetes

After activation, PKC-ε translocates to the membrane to exert its function [34]. As shown in Fig. 3, cardiac PKC-ε protein expression as well as its activation, are reflected as an increased ratio of membrane/cytosolic PKC-ε, and were significantly augmented in 8 weeks diabetic rats as compared to non-diabetic controls at basal conditions (Fig. 3A-C). In non-diabetic control rats, I/R induced significant increases in PKC-ε protein expression (Fig. 3B) and activation (Fig. 3C) (*P*<0.05,

Fig. 2. Effect of rNLIP on post-ischemic myocardial injury in non-diabetic control (Ctrl) and 8 weeks diabetic (D8w) rats. A: Representative images of myocardial necrosis (infarct size) determined by TTC and Evans blue staining; B and C: Post-ischemic infarct size expressed as percentage of infarct size (IS) to the area at risk (AAR); D: Serum troponin-I(Tn-I) level; E: Serum LDH level. rNLIP was achieved through 3 cycles of 5 minutes ischemia followed by 5 minutes reperfusion in right unilateral hindlimb every day for 3 days, the color of foot during ischemia became purple and was used as an indicator of successful occlusion of blood flow. Ischemia reperfusion (I/R) was achieved by 30 minutes ischemia and 120 minutes reperfusion. Data are expressed as mean ± SEM, n=6 per group. *P<0.05.

non-diabetic+ I/R *vs.* non-diabetic control), which were further significantly increased by rNLIP (*P*<0.05, non-diabetic+rNLIP+I/R *vs.* non-diabetic +I/R). In diabetic rats, I/R caused significant further increases in PKC-ε expression (Fig. 3B) and activation (Fig. 3C) (*p*<0.05, diabetic+I/R *vs.* diabetic-control) while rNLIP significantly reduced I/R induced increases in both PKC-ε protein expression and activation (*P*<0.05, diabetic+rNLIP+I/R *vs.* diabetic+I/R). rNLIP per se did not have a significant impact on cardiac PKC-ε activation in sham-operated groups both in non-diabetic and diabetic rats (Fig. 3B&C).

In 8 weeks diabetic rats, both baseline and post-ischemic myocardial phosphorylation of STAT3 (Fig. 3D&E) and p-Akt(Ser473) (Fig. 3F) were significantly lower than those in the non-diabetic groups and were further significantly reduced by I/R. rNLIP corrected I/R induced reductions of p-STAT3 at both sites of Ser727 (Fig. 3D) and Tyr705 (Fig. 3E) and p-Akt (Ser473) (Fig. 3F) in both non-diabetic and diabetic rats.

Remote time HPC attenuated H/R induced cell injury under high glucose conditions

As shown in Fig. 4A, in cardiac H9C2 cells, H/R induced significant cell injury under both normal (NG) and high glucose (HG) conditions with more severe injury seen in HG (*P*<0.05,

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;45:2107-2121

DOI: 10.1159/000488047
Published online: March 12, 2018

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Fig. 3. Effects of rNLIP on post-ischemic myocardial injury in non-diabetic control (Ctrl) and 8 weeks diabetic (D8w) rats. A : Representative bands of protein PKC-ε-cytosol, PKC-ε-membrane, PKC-ε, p-STAT3(Tyr705), p-STAT3(Ser727), T-STAT3, p-Akt(Ser473) and T-Akt; B: protein expression of PKC-ε; C: Ratio of membrane/cytosol of PKC-ε to; D: protein expression of p-STAT3(Ser727); E: protein expression of p-STAT3(Tyr705); F: protein expression of p-Akt(Ser473). rNLIP was achieved through 3 cycles of 5 minutes ischemia followed by 5 minutes reperfusion in unilateral hindlimb every day for 3 days, the color of foot during ischemia became purple and was used as an indicator of successful occlusion of blood flow. I/R was achieved by 30 minutes ischemia and 120 minutes reperfusion. Phosphorylated proteins were assessed by phosphorylation/total protein expression. Data are expressed as mean ± SEM, n=6 per group.*P<0.05.
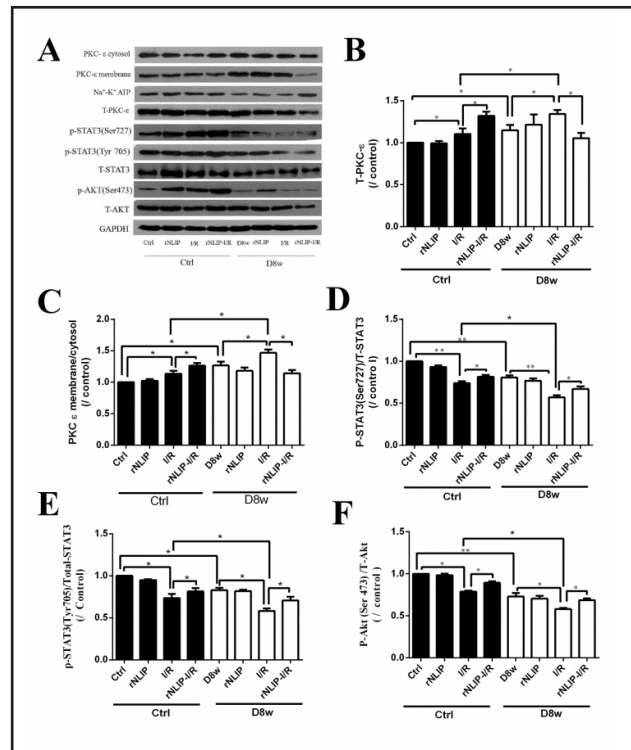
Fig. 4. Effects of remote time HPC on cellular injury in H9C2 cells exposed to normal glucose (NG) and high glucose (HG) in the presence or absence of hypoxia-reoxygenation (H/R). A: LDH release to culture medium; B: ratio of pro-apoptosis and anti-apoptosis proteins Bax/Bcl2; C: MMP assessed by JC-1 staining. In the HG group, H9C2 cells were subjected 30 mM high glucose for 48 hours before being subjected to 6 hours hypoxia and 12 hours of reperfusion. Remote time HPC was achieved by 3 cycles of 5 minutes hypoxia followed by 5 minutes re-oxygenation 24 hours before H/R. *P<0.05. Data are expressed as mean ± SEM of two independent experiments each performed in triplicate. n=6 per group.
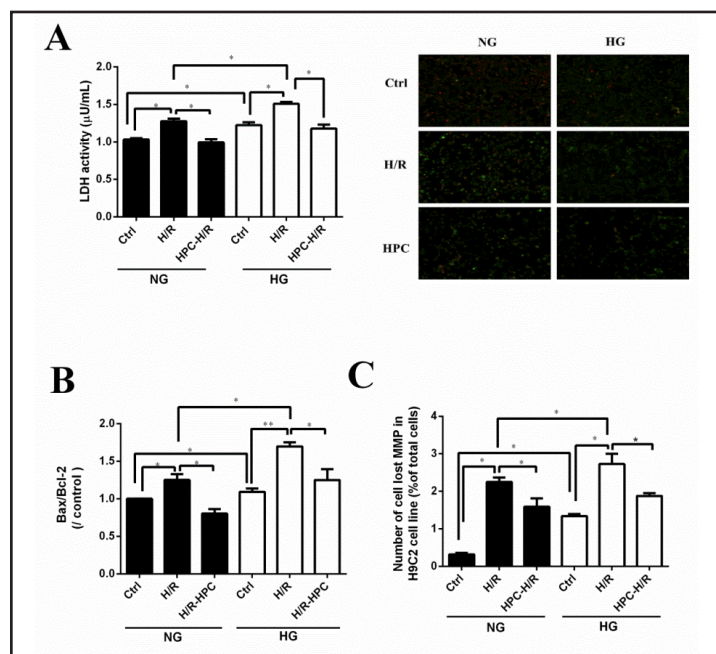
NG-H/R *vs.* NG-control, *P*<0.05, HG+H/R *vs.* NG + H/R). This was accompanied by increased ratio of Bax/Bcl-2 protein expression (Fig. 4B) and an increased number of cells that lost mitochondrial membrane potential (MMP, Fig. 4C) manifest as significantly more cells with positive JC-1 staining, which is indicative of increased cell death, in HG conditions relative to that in NG (all *P*<0.05). Application of HPC 24 hours prior to inducing H/R (i.e., remote time HPC) significantly attenuated H/R-induced increases in LDH release and Bax/Bcl-2

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;45:2107-2121

DOI: 10.1159/000488047
Published online: March 12, 2018

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www.karger.com/cpb

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expression and reduced the number of cells that lost MMP under both NG and HG conditions (Fig. 4A-C). The osmotic control, mannitol, had no significant effects on protein expression such as Bax/Bcl-2 and did not affect LDH release (data not shown).

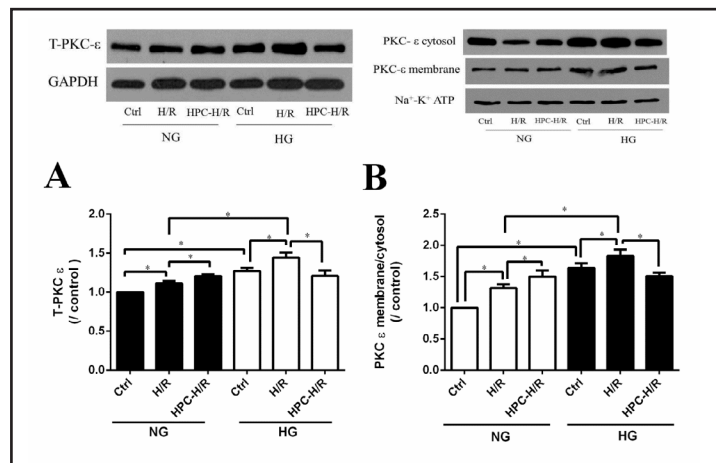*Effects of remote time HPC on PKC-ε activation in H9C2 cells subjected to H/R*

As shown in Fig. 5, expression of both total (Fig. 5A) and the ratio of membrane/cytosol (Fig. 5B) PKC-ε were increased in H9C2 cells exposed to HG as compared to NG ($P<0.05$). H/R significantly increased the expression of total (Fig. 5A) and the ratio of membrane/cytosol (Fig.

Fig. 5. Effects of remote time HPC on activity of PKC-ε in H9C2 cells subjected to H/R. A: changes of protein PKC-ε expression; B：Ratio of membrane/cytosol of PKC-ε in H9C2 cells exposed to normal glucose (NG, 5.5 mM) or high glucose (HG, 30 mM) and subjected to 6 hours hypoxia and 12 hours re-oxygenation with or without remote time HPC. Remote time HPC was achieved by 3 cycles of 5 minutes hypoxia followed by 5 minutes re-oxygenation 24 hours before H/R. *P<0.05. Data are expressed as mean ± SEM of two independent experiments each performed in triplicate. n=6 per group.

5B) PKC-ε under both NG and HG conditions ($p<0.05$, NG-H/R *vs*.NG-control, HG-H/R *vs*. HG-control). Remote time HPC significantly further increased post-hypoxic total and membrane/cytosol PKC-ε under NG condition but reduced both post-hypoxic total and membrane/cytosol PKC-ε under HG conditions. Given that translocating from cytosol to membrane represents activation of PKC-ε, these results indicate that remote time HPC reduces post-hypoxic PKC-ε activation to confer cardiac protection under HG conditions. The osmotic control, mannitol, exerted no effects on PKC-ε expression. We also did the experiments to verify the effects of HPC alone on PKC translocation and found that HPC alone had no significant effect on PKC-ε translocation (data not shown).

Effects of remote time HPC on the protein expression of STAT3 and Akt in H9C2 cells subjected to H/R

Akt and STAT3 are the major pro-survival proteins in the RISK and SAFE signaling pathways related to IPC. As shown in Fig. 6A-D, the expression levels of proteins p-STAT3-Ser727 (Fig. 6B) and p-STAT3-Tyr 705 (Fig. 6C) were significantly lower in the HG than the NG group under both baseline and H/R conditions (all $p<0.05$). HPC significantly attenuated H/R-induced reductions of p-STAT3 both under NG and HG conditions (all $p<0.05$). H/R significantly reduced the expression of p-Akt(Ser473) under HG conditions ($p<0.05$, HG+H/R *vs.* HG-control, Fig. 6D) but not during NG ($P>0.05$, Fig. 6D). HPC significantly increased the expression of p-Akt(Ser473) during NG and reverted H/R induced reduction of p-Akt(Ser473) under HG conditions ($p<0.05$, NG+H/R+HPC *vs.* NG+H/R, HG+H/R+HPC *vs.* HG+H/R, Fig. 6D).

STAT3 gene knock-down cancelled the protective effects of remote time HPC in cardiac H9C2 cells

In an animal study we found that rNLIP conferred cardioprotection by activating PKC-ε, while in diabetes rNLIP moderately but significantly reduced the expression and activation of PKC-ε and increased the activation of STAT3 to confer cardioprotection. While the roles

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Fig. 6. Remote time HPC activated protein STAT3 and Akt expressions in H9C2 cells subjected to hypoxic reoxygenation (H/R). A: Representative bands of protein p-STAT3(Tyr705), p-STAT3(Ser727), T-STAT3, p-Akt(Ser473), T-Akt; B: protein expression of p-STAT3(Ser727); C: protein expression of p-STAT3(Tyr705); D: protein expression of p-Akt(Ser473) in cells exposed to normal glucose and high glucose in the presence or absence of HPC. In HG group, H9C2 cells were subjected 30 mM high glucose for 48 hours, and then all cells subjected to 6 hours hypoxia and 12 hours reperfusion. Remote time HPC was achieved by 3 cycles of 5 minutes hypoxia followed by 5 minutes re-oxygenation 24 hours before H/R. Phosphorylated proteins were assessed by phosphorylation/total protein expression. *P<0.05. Data are expressed as mean ± SEM of two independent experiments each performed in triplicate. n=6 per group.
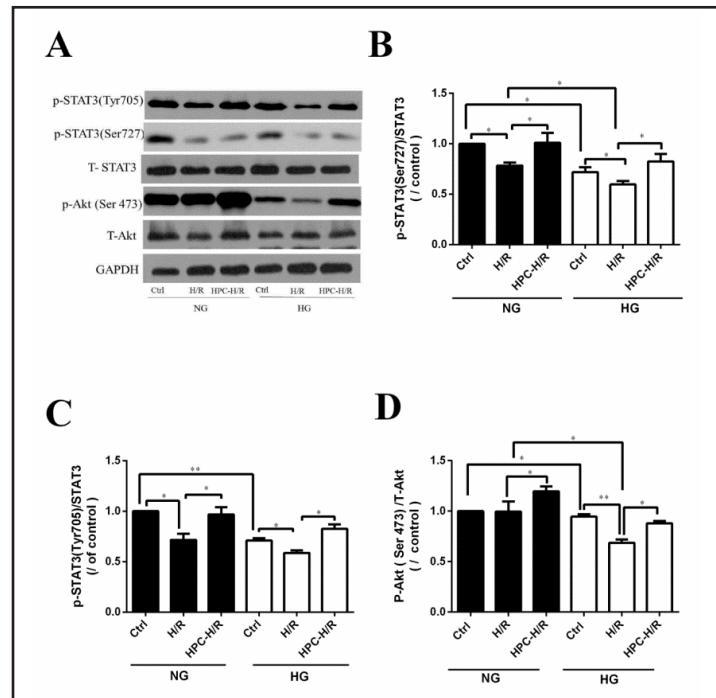
of STAT3 and PKC-ε in the relationship between STAT3 and PKC-ε in rNLIP in diabetic or hyperglycemic conditions have yet to be explored. To further confirm the role of STAT3 activation in remote time HPC cellular protection during HG, siRNA was used to knock down STAT3 in cells treated with or without remote time HPC. As shown in Fig. 7A and 7B, STAT3 gene knock-down decreased the expression of STAT3 significantly compared to control (*P*<0.01). Control siRNA did not significantly affect the protein expression of STAT3 (data not shown). Further, STAT3 gene knock-down canceled remote time HPC mediated cellular protection manifest as elevated LDH release (*P*<0.05, HG+H/R+HPC+siRNA STAT3 *vs*. HG+H/R+HPC; *P*>0.05, HG+H/R+HPC+siRNA STAT3 *vs*. HG+H/R, Fig. 7D). As shown in Fig. 7E, STAT3 gene knock-down had no effect on PKC-ε protein expression under H/R, and remote time HPC significantly attenuated the H/R-induced increase of post-hypoxic PKC-ε expression under HG conditions (*p*<0.05, HG+HPC+H/R group *vs.* HG+H/R group) both in the absence or presence of siRNA STAT3. This suggests that PKC-ε works upstream of STAT3 in this experimental setting. HG exposure significantly reduced STAT3 activation manifested as reduced phosphorylation at both sites Tyr705 (*p*<0.05, HG-control *vs*. NG-control, Fig. 7F) and Ser727(*p*<0.05, HG-control *vs*. NG-control, Fig. 7G), which were further significantly reduced by H/R (*p*<0.05, HG+H/R *vs*. HG-control, Fig. 7 F&G). These changes were reverted by HPC (*p*<0.05, HG+H/R+HPC *vs*. HG+H/R, Fig. 7 F&G). Further, STAT3 gene knock-down prevented remote time HPC to revert H/R induced reductions in p-STAT3 at both Tyr705 and Ser727(*P*>0.05, HG+H/R+HPC+siRNA STAT3 *vs*. HG+H/R+HPC, Fig. 7 F&G). As shown in Fig. 7H, p-Akt(Ser473) was significantly decreased after H/R, and HPC increased the expression of p-Akt(Ser473) (all *p*<0.05, HG+H/R *vs*. HG-control, HG+H/R+HPC *vs*. HG+H/R, Fig. 7H), while STAT3 gene knock-down canceled remote time HPC-induced increase of p-Akt(Ser473) (Fig. 7H).

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;45:2107-2121

DOI: 10.1159/000488047
Published online: March 12, 2018

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www.karger.com/cpb

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Fig. 7. Effects of STAT3 gene knockdown on remote time HPC cellular protection in H9C2 cells. A: Representative band of protein T-STAT3 expression; B: changes of expression of STAT3 after gene knock down STAT3; C: Representative bands of protein of PKC-ε, p-STAT3(Tyr705), p-STAT3(Ser727), T-STAT3, p-Akt(Ser473), T-Akt ; D: LDH release to culture medium; E: protein expression of PKC-ε; F: protein expression of p-STAT3(Tyr705); G: protein expression of p-STAT3(Ser727); H: protein expression of p-Akt(Ser473) in cells with STAT3 gene knockdown (siRNA STAT3) in the presence or absence of remote time HPC. In HG group, H9C2 cells were subjected to 30 mM high glucose for 48 hours, and then all cells subjected to 6 hours hypoxia and 12 hours reperfusion. Remote time HPC was achieved by 3 cycles of 5 minutes hypoxia followed by 5 minutes re-oxygenation 24 hours before H/R. Phosphorylated proteins were assessed by phosphorylation/total protein expression.*P<0.05. Data are expressed as mean ± SEM of two independent experiments each performed in triplicate. n=6 per group.
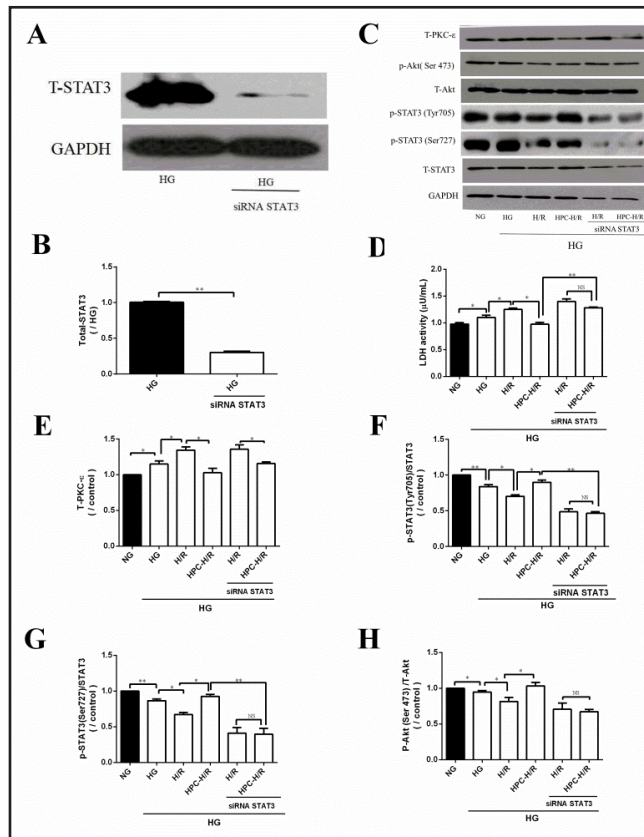

Effects of PKC-ε gene knock-down on post-hypoxic p-STAT3 and p-Akt during remote time HPC

To verify the role of PKC-ε in remote time HPC mediated cardiac protection and its impact on pro-survival signaling under HG conditions, PKC-ε siRNA was used to knock down PKC-ε. As shown in Figs. 8A and 8B, PKC-ε gene knock-down decreased the expression of PKC-ε significantly compared to control (*P*<0.05). Control siRNA did not significantly affect the protein expression of PKC-ε (data not shown). Under HG, post-hypoxic LDH release (Fig. 8 D) was significantly increased in cells subjected to H/R which was significantly reduced by HPC and also by PKC-ε gene knock-down separately (all *p*<0.05, HG+H/R *vs*. HG-control, HG+H/R+HPC *vs*. HG+H/R, HG+H/R+siRNA PKC-ε *vs*. HG+H/R), while HPC plus PKC-ε gene knock-down did not have significantly added effects in reducing post-hypoxic LDH release, indicating that HPC conferred cellular protection primarily via reducing H/R induced increases in PKC-ε under HG conditions. The expression of PKC-ε was significantly increased during HG exposure and further increased after H/R, with HPC significantly reducing the HG+H/R-induced increase in PKC-ε expression (*P*<0.05, HG+H/R+HPC *vs.* HG+H/R, Fig. 8E). PKC-ε gene knock-down resulted in a profound decrease of post-hypoxic PKC-ε expression in both the H/R and HPC+H/R groups (*P*<0.05, HG+H/R+siRNA PKC-ε *vs.* HG+H/R, HG+H/R+HPC+siRNA PKC-ε *vs.* HG+H/R+HPC, Fig. 8E), while HPC did not have significant impact on post-hypoxic PKC-ε expression in the presence of PKC-ε gene knock-down.

Compared to control, phosphorylation/activation of STAT3 at sites Tyr705 (Fig. 8F) and Ser727 (Fig. 8G) decreased significantly under HG conditions and further significantly reduced after H/R, while remote time HPC reverted H/R induced reduction of STAT3 activation at both sites Tyr 705 (Fig. 8F) and Ser727 (Fig. 8G). PKC-ε gene knock-down did not have a significant impact on either HG+H/R induced reduction nor HG+H/R+HPC induced elevation in p-STAT3(Tyr 705) (Fig. 8F), but resulted in a significant increase in p-STAT3(Ser727)

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;45:2107-2121

DOI: 10.1159/000488047
Published online: March 12, 2018

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Fig. 8. Protein expressions changes and LDH release in H9C2 cells after gene knockdown PKC-ε subjected to H/R with or without remote time HPC. A: Representative band of protein PKC-ε expression; B: effect of siRNA on PKC-ε protein expression; C: protein expression of PKC-ε, p-STAT3(Tyr705), p-STAT3(Ser727), T-STAT3, p-Akt(Ser473), T-Akt; D: LDH release to culture medium; E: protein expression of PKC-ε: protein expression of p-STAT3(Tyr 705); G: protein expression of p-STAT3(Ser727); H: protein expression of p-Akt(Ser473) in H9C2 cells exposed to HG for 48 hours and subjected to 6 hours hypoxia followed by 12 hours re-oxygenation in the presence or absence of remote time HPC and/or PKC-ε gene knockdown. Remote time HPC was achieved by 3 cycles of 5 minutes hypoxia followed by 5 minutes re-oxygenation 24 hours before H/R. Phosphorylated proteins were assessed by phosphorylation/total protein expression.*P<0.05. Data are expressed as mean ± SEM of two independent experiments each performed in triplicate. n=6 per group.
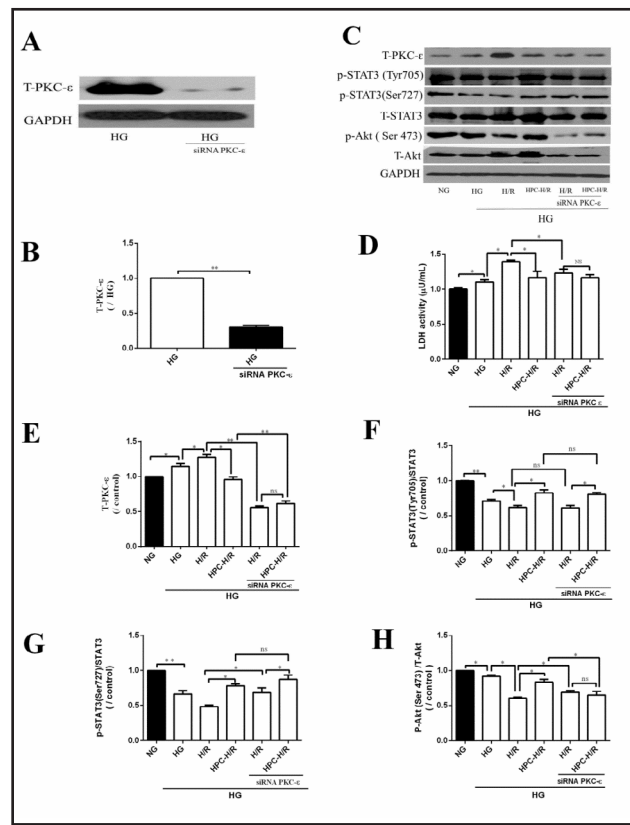
expression in cells subjected to HG+H/R (*P*<0.05, HG+H/R +siRNA PKC-ε *vs.* HG+H/R, Fig. 8G), while PKC-ε gene knock-down did not further significantly enhance HG+H/R+HPC induced increases in p-STAT3(Ser727) expression (*p*>0.05,HG+H/R+HPC+siRNA PKC-ε *vs.* HG+H/R+HPC, Fig. 8G). As shown in Fig. 8H, HG resulted in a moderate but statistically significant reduction in p-Akt (Ser473). p-Akt(Ser473) was further significantly reduced after H/R, while HPC reverted H/R induced reduction of p-Akt(Ser473) (*p*<0.05, HG+HPC+H/R *vs.* HG+H/R). PKC-ε gene knock-down increased post-hypoxic p-Akt (*p*<0.05, HG+H/R+ siRNA PKC-ε *vs.* HG+H/R, Fig. 8H). However, PKC-ε gene knock-down significantly compromised and prevented the HPC-mediated increase in post-hypoxic p-Akt(Ser473) (*p*<0.05, HG+H/R+HPC+siRNA PKC-ε *vs.* HG+H/R+HPC).

Discussion

We found that rNLIP reduced myocardial I/RI in both non-diabetic and diabetic rats. In non-diabetic hearts rNLIP conferred cardioprotection by enhancing PKC-ε activation, while in diabetes, cardiac PKC-ε was over activated which concurs with a previous study [35]. rNLIP conferred cardioprotection by reducing PKC-ε activation which is the opposite of what is seen in non-diabetic conditions. These results were confirmed by inducing H/R and applying remote time HPC in cardiomyocyte H9C2 cells without or with PKC-ε gene knock-down and high glucose (HG) exposure. This showed that HG exposure significantly increased PKC-ε activation and exacerbated H/R-induced cellular injury, and all these changes were reverted by remote time HPC.

Our finding that rNLIP conferred cardioprotection by activating PKC-ε in non-diabetic but by reverting the over-activation of PKC-ε in diabetic rats, indicates that the role of PKC-ε in rNLIP may vary under different conditions. Indeed, previous research has shown that IPC needs to activate PKC-ε to confer cardioprotection and that application of the PKC-ε inhibitor

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;45:2107-2121

DOI: 10.1159/000488047
Published online: March 12, 2018

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εv1-2 abolishes the cardioprotection of IPC in non-diabetic conditions. However, given the diversity of models and protocols and the relative but not absolute selectivity of currently available PKC inhibitors, it is difficult to set a standard for PKC- ε inhibition to determine the role of PKC- ε. Further studies are needed to incorporate the use of cardiac specific PKC-ε knockout or overexpression in control and diabetic rodents to define the role of PKC- ε in myocardial I/RI [34, 36]. One possible explanation for the difference of PKC-ε activation during rNLIP between non-diabetic and diabetic conditions could be that PKC-ε, when activated at a moderate level, stimulates a small amount of reactive oxygen species (ROS) [37], which serves as a trigger of IPC/NLIP [38] which then stimulates downstream targets to confer cardioprotection. Over-activation of PKC-ε under diabetic conditions in our current study, may have resulted in overproduction of ROS [37] which exacerbated myocardial I/R injury and diminished IPC or NLIP cardioprotection in diabetic hearts. Further research would be useful to test this intriguing hypothesis.

Studies have shown that STAT3 is not only important for cardiomyocyte function, but also modulates the cardiac microenvironment and communicates with cardiac fibroblasts. STAT3 plays major roles in I/RI [39-42], preconditioning and postconditioning cardioprotection and PKC-ε is essential for conditioning stimuli to activate STAT3 in non-diabetic conditions. However, the role of STAT3 in relation to PKC-ε in rNLIP mediated cardioprotection in diabetes has not been explored. STAT3 activation in the mitochondria is critical for cardioprotection in reducing post-ischemic infarct size [41-43], and specific inhibition of STAT3 with Stattic enhanced ROS formation and subsequently resulted in problems with mitochondrial function [42], despite the fact that ROS may have dual effects during myocardial I/RI in maintaining STAT3 activity [42]. Our finding that remote time HPC mediated up-regulation of PKC-ε and the subsequent activation of STAT3 at site Ser727 and cardiac protection against H/R injury under NG conditions were abolished by STAT3 gene knock-down, suggests that STAT3 worked as the main downstream target of PKC-ε during rNLIP. Our findings and those of others which show that PKC-ε is essential for IPC to activate STAT3 at site Ser727 to confer its late phase cardioprotection [17, 20] and that PKC-ε activation induced STAT3 phosphorylation, while PKC-ε knockdown reduced STAT3 [21], indicate the importance of PKC-ε in STAT3 activation. Our results show that the relationship between PKC-ε and STAT3 activation in myocardial I/R and IPC or rNLIP may vary depending on the environment e.g. non-diabetic or diabetic. In the study of Kim et al. [44], no direct interaction was found between PKC and STAT3 during IPC in astrocytes, suggesting that PKC-ε and STAT3 interaction may also be organ or tissue specific [45].

It is interesting to note that STAT3 is not only important for the cardioprotection mediated by ischemic and remote ischemic preconditioning [14, 46] but is also critical in postconditioning cardioprotection [47, 48]. Reduced levels of STAT3 protein in the aged hearts of rodents may be responsible for the loss of cardioprotection by preconditioning and postconditioning [47]. However, during remote ischemic preconditioning, STAT3 was not the only protein activated by RIPC and its activation by RIPC could be species and/or protocol specific. For example, in humans, RIPC has been shown to increase the phosphorylation of STAT5, and in rats and pigs, RIPC has been shown to increase the phosphorylation of STAT3 [14]. However, a recent study of ours in patients showed that RIPC, when applied about one hour before open heart surgery can significantly increase the cardiac phosphorylation/activation of both STAT3 and STAT5 and reduce the myocardial I/RI in children with tetralogy of Fallot [49]. It has also been shown that STAT3 played a central role in the development of postpartum cardiomyopathy [50]. A recent study examining the dynamic changes of cardiac STAT3 after myocardial I/RI and the effects of ischemic postconditioning, showed that cardiac p-STAT3 increased as early as 5 minutes after reperfusion following ischemic postconditioning and p-STAT3 decreased thereafter, while the ischemic postconditioning-initiated cardioprotection remained [51], suggesting a long term cardioprotective effect of STAT3 activation.

A limitation is that we only performed studies in type 1 diabetes which may differ from type 2 in terms of the mechanism of onset and prevalence. However, at the end of long term diabetes, both type1 and type 2 share common characteristics such as impaired insulin

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;45:2107-2121

DOI: 10.1159/000488047
Published online: March 12, 2018

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signaling and hyperglycemia. Therefore, insight gained from our current study may elucidate the potential interventions that could attenuate diabetic myocardial ischemia-reperfusion injury in general, although future studies in type 2 diabetic rodents and in humans are still warranted. Another limitation is that in the cell studies the "remote time" HPC protocol cannot replicate all aspects of remote IPC. The effects of what the culture media can do to naïve cells after they have undergone the HPC protocol could not be determined and this deserves further study. Also, the role of PKC-ε in high glucose induced cell injury in the absence of hypoxia/reoxygenation should be explored.

Conclusion

Overexpression and activation of cardiac PKC-ε may represent a major mechanism that renders diabetics unresponsive to conventional IPC or NLIP cardioprotection, and that rNLIP attenuates diabetic heart I/R injury by mitigating HG-induced PKC-ε overexpression and by subsequently activating cardiac STAT3 at site Tyr 727.

Patients with diabetes are more vulnerable to myocardial I/R injury. Repeated non-invasive limb ischemic preconditioning (rNLIP) protects the heart against myocardial I/RI in rats with diabetes but the underlying mechanism is unclear. The present study was designed to investigate the role of PKC-ε in diabetic myocardial I/R injury and in rNLIP cardioprotection. Our results show that, in non-diabetic hearts, rNLIP, by activating PKC-ε, activates STAT3 and Akt to confer its cardioprotection. In diabetic hearts, in which PKC-ε is over-activated, rNLIP, by down-regulating PKC-ε, restores STAT3 to confer cardioprotection. It is strongly suggestive that targeting inhibition of PKC-ε may be a potential clinical therapeutic intervention in the treatment of diabetes-associated cardiac disease

Acknowledgements

Disclosure Statement

No conflicts of interest are declared by the authors.

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DOI: 10.1159/000488047
Published online: March 12, 2018

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Cell Physiol Biochem 2018;45:2107-2121

DOI: 10.1159/000488047
Published online: March 12, 2018

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Cell Physiol Biochem 2018;45:2107-2121

DOI: 10.1159/000488047
Published online: March 12, 2018

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