Remote Ischemic Preconditioning Prevents Sarcolemmal Associated Protein Proteolysis by Mmp-2 Inhibition

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Abstract

The death of myocytes occurs through different pathways, but a key point in the transition from reversible to irreversible injury is the rupture of the plasma membrane. Three major groups of structural proteins that link the extracellular and intracellular milieu and confer structural stability to the cell membrane are present in cardiac myocytes: the dystrophin-associated protein complex, the vinculin–integrin link, and the spectrin-based submembranous cytoskeleton.

The objective was to determine if rIPC preserves membrane-associated cytoskeletal proteins (dystrophin and β-dystroglycan) through the inhibition of metalloproteinase type 2 (MMP-2) activity. A second objective was to describe some of the intracellular signals of the rIPC, that modify mitochondrial function and are activated during early reperfusion.

Methods: Isolated rat hearts were subjected to 30 min of global ischemia and 60 min of reperfusion (I/R). rIPC was performed by 3 cycles of ischemia/reperfusion in the lower limb (rIPC). Results: As we expected, rIPC significantly decreased the infarct size. rIPC induced an Akt/GSK-3β phosphorylation and the inhibition of the MPTP opening, improving mitochondrial function, increasing membrane potential, ATP production and respiratory control. I/R induced ONOO⁻ production, which activates MMP-2. This enzyme degrades β-dystroglycan and dystrophin and collaborates to sarcolemmal disruption.

Conclusion: rIPC attenuates the breakdown of β-dystroglycan and dystrophin through the inhibition of MMP-2 activity. Furthermore, rIPC activates different intracellular pathway that involves the an Akt/Gsk3β and MPTP pore with preservation of mitochondrial function.

Introduction

Remote ischemic preconditioning (rIPC) is a cardioprotective phenomenon by which transient non-lethal ischemia and reperfusion of one organ or tissue confers resistance to a later episode of lethal ischemia reperfusion injury in a remote organ (1). In the last years, rIPC has been translated from experimental animal studies to human studies demonstrating the protection of the heart, brain, and kidney by using surrogate endpoints (2–4). However, the intracellular pathway of cardioprotection is not yet fully known (5). In a previous paper we demonstrated that rIPC reduces infarct size activating parasympathetic nervous system and muscarinic receptor (6).

During the evolution of a myocardial infarction, the death of myocytes occurs through different pathways, but a key point in the transition from reversible to irreversible injury is the rupture of the plasma membrane. In this sense, three major groups of structural proteins that link the extracellular and intracellular milieu and confer structural stability to the cell membrane are present in cardiac myocytes: the dystrophin-associated protein complex, the vinculin–integrin link, and the spectrin-based submembranous cytoskeleton (7). Dystrophin and spectrin breakdown are the molecular basis for membrane fragility during the transition from reversible to irreversible ischemic myocardial injury. Particularly, dystrophin is most sensitive to ischemia and its breakdown occurred during this period, by
MMP-2 enzyme (8). Additionally, we showed that classic ischemic preconditioning inhibits MMP-2 activity, preventing the proteolysis of dystrophin, and revealing a new protective mechanism of ischemic preconditioning (8). However, the effect of rIPC on the dystrophin-associated protein complex and MMP-2 activity has not been studied yet.

The objective was to determine if rIPC modifies the metalloproteinase type 2 (MMP-2) activity and thus preserves membrane-associated cytoskeletal proteins (dystrophin and β-dystroglycan). A second objective was to describe some of the intracellular signals activated during early reperfusion which are involved in the rIPC cardioprotection.

**Material And Methods**

**Ethical approval**

The experiments were performed on male Sprague Dawley rats, 60-70 days old (200 to 250 g). The procedures were approved by the Animal Care and Research Committee of the University of Buenos Aires (Protocol number 2948/10) and were in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health in 2011.

**Surgical procedure (in vivo)**

Rats were anesthetized with urethane (65 mg/kg, i.p.) and intubated for mechanical ventilation. Level of anesthesia was assessed by loss of pedal reflex (toe pinch). Afterwards, the left femoral artery were dissected and exposed and the animals were randomized into different experimental groups (see below).

**Ischemia/reperfusion (in vitro)**

After completion of the in vivo surgical procedure, animals were euthanized and each heart was rapidly excised and mounted on a Langendorff apparatus. Each heart was perfused with Krebs-Henseleit buffer and bubbled with 95% O₂ – 5% CO₂ gas mixture at 37°C, with a final pH 7.2-7.4. The heart rate was maintained constant (300 beats per min) using a pacemaker.

A saline-filled latex balloon connected by a catheter to a pressure transducer (Deltram II, Utah Medical System) was inserted into the left ventricle. The volume of the balloon was adjusted to achieve a left ventricular end diastolic pressure of 8-10 mmHg. Coronary perfusion pressure (CPP) was also recorded and the coronary flow was adjusted to obtain a CPP around 70 mmHg during the initial stabilization period. This flow was kept constant throughout the experiment.

**Experimental groups**

I/R (n=9): Rats were anesthetized, and the left femoral artery was dissected and exposed as described above. After 30 min of monitoring, the hearts were excised and perfused according to the Langendorff technique. After 15 min of stabilization myocardial infarction was induced by 30 min of global no-flow
ischemia followed by 120 min of reperfusion. Global no-flow ischemia was induced by abruptly decreasing the total coronary flow provided by the perfusion pump.

rIPC (n=9): After the rats were anesthetized and the left femoral artery dissected and exposed, the animals were remotely preconditioned by a 3-cycle hindlimb ischemia (5 min) and reperfusion (5 min) protocol by occlusion of the femoral artery with a vascular clamp. Subsequently, the hearts were subjected to the same protocol used in I/R group (30 min of global no-flow ischemia followed by 120 min of reperfusion) to induce myocardial infarction.

In order to study the opening of mitochondrial permeability transition pore (MPTP) 5 hearts perfused in normoxic conditions (Nx) were used as control.

**Infarct size measurement**

After 1 h of reperfusion in the Langendorff apparatus the hearts were frozen and cut into 2 mm thick transverse slices from apex to base. Sections were incubated for 20 min in 1% triphenyltetrazolium chloride (pH 7.4, 37°C) and then immersed in 10% formalin. The sections were scanned and viable and infarcted areas were measured (Image Pro Plus, version 4.5, Media Cybernetics, Rockville, USA). The infarct size was expressed as a percentage of the left ventricular area.

**Western blot**

Samples from left ventricles of animals subjected to I/R and rIPC (n=5 per group) were taken at 5 min of reperfusion; homogenized in 3 vol (w.v⁻¹) of ice-cold homogenization buffer and a 4% protease inhibitor cocktail (Roche Hertfordshire, UK), pH 8.0, and centrifuged at 10507 g for 10 min at 4 °C. The protein content in the supernatant was quantified by the Lowry method using bovine serum albumin as standard, resuspended in 2X solution of SDS-sample buffer (62.5 mM Tris-HCl buffer, pH 6.8 containing 2% w/v SDS, 25% w/v glycerol, 5% v/v β-mercaptoethanol and 0.01% w/v bromophenol blue) and heated at 95 °C for 5 min. Equal amounts of proteins (80 μg) were loaded onto 8% SDS-PAGE and transferred to nitrocellulose membranes. After blocking for 1 h in 3% w/v BSA in PBS buffer, membranes were incubated overnight at 4 °C with the corresponding primary antibodies: rabbit anti-phospho-Akt antibody (dilution 1:1000, Cell Signalling Technology, MA, USA), mouse monoclonal anti-phospho GSK-3b (dilution: 1:300, Millipore, MA, USA). The blots were hybridized with a secondary antibody coupled to horseradish peroxidase (dilution 1: 5000, Santa Cruz Biotech Inc., Dallas, TX, USA). Complexes were visualized by chemiluminescence detection (Pierce ECL Western blotting Substrate). The membranes were stripped and reprobed with anti-Akt (dilution 1:1000, Cell Signalling Technology, MA, USA) and anti-GSK-3b (dilution: 1:300, Millipore, Massachusetts, USA). Protein band densities were normalized to the Akt, and GSK-3b content.

For dystrophin and β-dystroglican equal amounts of protein (100 mg) were separated on 5–10% gradient SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Thermo Scientific, USA). Blots were incubated in blocking buffer containing 5% nonfat milk for 1 h at room temperature and then
incubated with mouse monoclonal anti-dystrophin (1:1,000, MANDYS-8, D8168, Sigma-Aldrich, USA) and mouse monoclonal anti-b-dystroglican (1:50, Leica) overnight at 4°C. Later, blots were incubated with goat anti-mouse antibody (1:10,000) (Millipore, USA) at room temperature for 1 h followed by rinses with Tris-buffed saline-Tween 20. Blots were developed using the enhanced chemiluminescence method (Thermo Scientific, USA) according to the manufacturer’s instructions. Densitometric analysis of the bands was performed using Image J (National Institute of Health, MD, USA).

For protein nitration evaluation, 50 µg of protein were electrophoresed on 7.5% SDS-polyacrylamide gel, was electro transferred to polyvinylidene difluoride membranes, incubated with anti-nitrotyrosine, clone 1A6-monoclonal antibody (Millipore, Billerica, MA, USA) and detected with theECL (TM) system (Advance Western Blotting Detection Kit). Cardiac protein nitration was expressed as a percentage of maximal nitration induced by 100 µM of peroxynitrite, in non-ischemic hearts.

MMP-2 activity

For the ventricular tissue MMP-2 activity, the hearts were frozen at the end of the ischemic period (n=5 per group). The samples were concentrated at 5000 g at 4°C and, protein content was measured by the Lowry method. Metalloproteinase activity was detected by zymography. Sodium dodecyl sulphate (SDS)-polyacrylamide gels (7.5%) were copolymerized with 0.1% gelatin (G-8150, Sigma, St Louis, MO, USA). A constant amount of protein (2 µg) was loaded in each well in non-reducing conditions, and gels were run for 3 h in 25 mM Tris, 192 mM glycine and 0.1% SDS at 4°C, pH 8.3, in a Mini Protean-3 (Bio-Rad Laboratories, Hercules, CA, USA). After running, gels were rinsed with 2.5% Triton X-100 for 30 min and then incubated for 18 h in 0.15 M NaCl, 10m MCaCl$_2$ and 50 mM Tris–HCl, pH 7.4, at 37°C. After staining with Coomassie Blue R-250 (B-0149, Sigma) and destaining with acetic acid–methanol–water (1:3:6), enzyme activity was detected as colorless bands against the blue-stained background. The gelatinolytic bands disappeared in parallel zymograms in which the development buffer contained EDTA, confirming that the gelatinolytic activity was caused by metalloproteinases. Individual enzymes [MMP-2, 72 kDa (pro-form) and 64 kDa (active form)] were identified by molecular weight. Conditioned media from the promyelocyte U-937 cell line was used as activity standard for pro-MMP-2. Gels were scanned and band intensities quantified using Sion ImageJ software (Scion Corp., Frederick, MA, USA).

Immunostaining

Frozen sections (5 mm thick) were prepared using a cryostat, transferred to gelatin-covered glass slides, and fixed in 4% formaldehyde. Immunolabeling was performed using primary antibodies to dystrophin (mouse monoclonal anti-dystrophin, clone MANDYS-8, D8168, Sigma-Aldrich, USA) and b-dystroglycan (43DAG/8D5, Leica, USA). Secondary antibody was biotinylated antirabbit IgG (Biotrend, USA). Sections were analyzed using a Leica DM4000 B LED microscope and Leica DFC310 FX camera (Leica Microsystems) with Leica Application Suite LAS (version 4.2.0) software (Leica Microsystems).
Opening of mitochondrial permeability transition pore (MPTP) assay

The opening of MPTP was assessed in fresh isolated left ventricle mitochondria by the occurrence of cyclosporin A sensitive mitochondrial membrane potential disruption followed by swelling as determined by the changes in optical density of the mitochondrial suspension. MPTP opening causes mitochondrial swelling that is conveniently assayed as a decrease in the optical density of a mitochondrial suspension. Absorbance at 540 nm was measured using a microplate spectrophotometer at 30°C (ThermoFisher Scientific, USA). Mitochondria (1 mg of protein/ml) were added to buffer containing HEPES 40 mM, mannitol 195 mM, sucrose 25 mM, succinate 5 mM, pH 7.2. After a single pulse of 100 µM CaCl$_2$, absorbance was recorded and MPTP opening was estimated as the fraction of the initial optical density of the mitochondrial preparation, that remained after 20 min. The measurements were performed at 5 minutes of reperfusion period.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Inter-group comparisons were carried out using analysis of variance (one way-ANOVA) followed by t-tests with the p value adjusted for multiple comparisons using the Bonferroni test. The data comparisons were not significant unless the corresponding p value was less than 0.05/k, where k represents the number of comparisons.

Results

Figure 1 (Panel A and B) shows the LVDP and LVEDP behavior basal an after 30 min of global ischemia. In the I/R group LVDP decreased from 128,2±7,3 to 20.7±5,2 %, at 30 min of reperfusion. The rIPC attenuates the contractile decreased reaching a value of 48.2±8.7% (p<0.05 vs. I/R group). In the same way, LVEDP, an index of myocardial stiffness, increased during reperfusion in the I/R group to a value of 83,3±4,6 mmHg. rIPC has a beneficial effect on LVEDP, increasing to a value of 41.5±2.7 (p<0.05 vs. I/R group).

Figure 2 shows the expression of β-dystroglican (Panel A) and dystrophin (Panel B) after 30 min of ischemia and 5 min of reperfusion. The western blot analysis showed that rIPC significantly attenuates the proteolysis of both membrane proteins. These findings were confirmed by immunostaining (Panels C and D).

Since β-dystroglycan and dystrophin are cleaved by MMP-2, we measured MMP-2 activity (Figure 3, Panel A) in ventricular tissue samples and found that rIPC impaired the MMP-2 activity, respect to I/R group. It is known that MMP-2 is activated by ONOO$^-$; for this reason, we measured peroxynitrite induced protein nitration, which was significantly lower in rIPC group as compared to I/R (Figure 3, Panel B).

In a previous paper we showed that rIPC activates muscarinic receptors, which could induce a phosphorylation of Akt/Gsk3b pathway (9). To address this issue, phosphorylation of both enzymes was determined at 5 min of reperfusion in ventricular tissue samples (Figure 4, Panels A and B). A significant
increase both in Akt and Gsk3b phosphorylation was observed in the rIPC group as compared with I/R group. These results clearly indicate the involvement of Akt/Gsk3b pathway in the rIPC mechanism.

Figure 5 (Panel A) shows a representative trace of the evaluation of the opening of MPTP, assessed in fresh isolated left ventricle mitochondria of the two groups. Panel B shows an increased rate of MPTP opening (18.79%) in the I/R group. A protection against MPTP opening was evidenced in mitochondria from rIPC rats with values only 10.51% than in mitochondria from control animals for rIPC.

To deeply characterize the involvement of mitochondria in the protective mechanisms triggered by rIPC, mitochondrial inner membrane potential and ATP production rates were assessed after 10 min of reperfusion. As shown in Figure 5 (Panel C), the I/R protocol induced a significant decrease by 47% in DiOC6 signal (p<0.05) when compared to the Nx group, and this was reverted in the rIPC group. This result indicates that I/R induces strong mitochondrial depolarization, and that rIPC prevents this effect. Mitochondrial ATP production rate (Figure 5D) was significantly decreased by 67% in the I/R group as compared to Nx group (p<0.01). This effect was attenuated by rIPC, as ATP production rate only decrease by 28% (p<0.05) when compared to the Nx group. These findings indicate that rIPC prevents the decrease in mitochondrial ATP production induced by the I/R protocol.

In order to characterize the mitochondrial function, mitochondrial respiration and respiratory control ratio (RCR) of isolated mitochondria from the left ventricle of animals subjected to 30 min ischemia followed by 10 min of reperfusion (early reperfusion). As shown in Figure 6 (Panel A), when compared to the Nx group, a significant decrease in mitochondrial resting state, i.e. state 4, respiration rate was observed for the I/R and rIPC groups (p<0.001). In addition, active state 3 respiration rate was impaired by 80% in the I/R group and by 62% in the rIPC groups. As a result, the mitochondrial RCR was significantly decreased in the I/R group in comparison with the Nx group (p<0.05), and this effect was recovered in the rIPC group. These results suggest that the I/R protocol impairs mitochondrial function, and that rIPC attenuates this effect.

Discussion

In this study, we demonstrated that rIPC improves systolic and diastolic postischemic ventricular function and preserves sarcolemma membrane integrity through the inhibition of MMP-2. Furthermore, during early reperfusion rIPC activates Akt/Gsk3-b pathway, inhibiting MPTP opening and preserving mitochondrial function. Thus, supporting the notion that mitochondria is a target organelle in the mechanisms of rIPC protective effect.

The evaluation of dystrophin and β-dystroglycan expression showed that, at least part of the cardioprotection provided by rIPC is performed on structural components of myocyte sarcolemma, since both proteins are sparsely present in other myocardial cells. Reduction or absence of dystrophin implies rupture of the physical linkage that anchors the actin-based subsarcolemmal cytoskeleton and the sarcomeres to the sarcolemma, which may be related to impairment of contractile force transmission [7]. Concomitantly, this breakdown of the link with the cytoskeleton may impair the sarcolemmal structural
support, destabilizing and rendering it more vulnerable to subsequent damage. Although it is tempting to link the loss of dystrophin to sarcolemmal disruption, this might not necessarily be the case since it has been demonstrated that loss of dystrophin correlates with subsarcolemmal bleb formation during ischemia but poorly with cell fragility [7]. Loss of dystrophin produced by ischemia by proteolysis, as observed in this study, may also represent a greater susceptibility to further damage, which could occur during reperfusion.

Kyoi et al. (10) showed that classic ischemic preconditioning preserves dystrophin and reverses sarcolemma fragility during reperfusion by increasing mitochondrial ATP production (9). Therefore, mitochondrial protection and enhancement of ATP generation performed through ischemic preconditioning culminates in preservation of sarcolemma dystrophin and membrane stabilization during reperfusion, thus preventing reperfusion injury.

Previous studies have indicated that MMP-2 is involved in several processes responsible for ischemia/reperfusion damage (11). In addition, in a previous work we showed that MMP-2 participates in the protection mechanism of classic preconditioning (12). Thus, we hypothesized that it would be possible, that classic preconditioning and rIPC share mechanisms and thus inhibits MMP-2 activity.

Since that, MMP-2 can be activated by PKC induced phosphorylation (13) or by reaction with peroxynitrite (13). We measured protein nitration which was increased in I/R group and this finding was accompanied by a significant increase in MMP-2 activity. Interestingly, rIPC attenuated both increased MMP-2 activity and protein nitration. As we mentioned, the MMP-2 activity increases during ischemia (14) and is responsible for the dystrophin breakdown (12). In this study we showed that rIPC attenuated protein nitration and the increase of MMP-2 activity preserves dystrophin and b-dystroglycan.

Dystrophin binds to the end and along the side of F-actin filaments, either of the subsarcolemmal cytoskeleton or the sarcomeres. This complex is involved in contractile force transmission and stabilization of the plasma membrane, which has been evidenced by dystrophin involvement in cardiomyocyte remodeling in failing hearts. In addition, participation in cell signaling and calcium handling are emerging functions of this complex. In this sense, Yasuda et al. (15) showed that in the dystrophin-deficient heart, the membrane surrounding cardiac myocytes becomes susceptible to the formation of microtears during passive tension. A small amount of calcium enters through these holes and partially engages the myofilaments, generating tension that is observed as a reduction in passive compliance. Furthermore, the persistent elevation of Ca\(^{2+}\) levels/concentration during diastole results in significant reductions of resting sarcomere length in the mdx myocyte (15, 16). This membrane instability represents a major pathophysiological mechanism of the cardiomyopathy resulting from the loss of dystrophin in the heart. In our study, rIPC preserves dystrophin and as a consequence, we hypothesized that rIPC attenuates the increase of myocardial stiffness in the isolated heart.

During reperfusion, activation of the RISK pathway would lead to phosphorylation of Gsk-3b and the consequent inhibition of MPTP opening. The mitochondrial inner membrane is usually impermeable to
almost all metabolites and ions, and the MPTP is in a closed conformation. Under stress conditions such as reperfusion, the MPTP opens and allows the equilibration of small molecules. Prolonged opening of the MPTP, which occurs upon reperfusion, leads to mitochondrial swelling, which in turn induces rupture of the outer mitochondrial membrane, a decline in the activity of the electron transport chain protein complexes, and a release of pro apoptotic factors (17). Thus, inhibition of mPTP activation appears to be a key event for cardioprotection through a reduction in infarct size and an improvement in the recovery of contractile function after reperfusion (18). In the present study, we demonstrate that rIPC decreased the rate of MPTP opening probably by activation of the Akt/Gsk3-b pathway. We measured the mitochondrial function at early of reperfusion. Interestingly, the I/R group showed a progressive impairment of mitochondrial function, as indicated by RCR decay; however, the rIPC preserved mitochondrial energy production capacity in the cardiomyocytes, which might be associated with infarct size reduction.

In conclusion, these results indicate that rIPC improves systolic and diastolic function through the preservation of dystrophin-associated protein complex, inhibiting MMP-2 activity. MMP-2 could be activated by a high concentration of peroxynitrite. rIPC attenuates the increase of peroxynitrite and activates the RISK pathway preserving mitochondrial integrity, as evidenced through the inhibition of MPTP opening, the preservation of ATP production rate and membrane potential and the improvement of respiratory control.

References


Figure 1

Panel A and B shows the behavior of systolic (LVDP) and diastolic (LVEDP) ventricular function, respectively during reperfusion period. After 30 minutes of reperfusion, rIPC attenuated the contractile state alteration and the increase of myocardial stiffness. (*p<0.05 vs control)
Figure 2

Cardiac b-dystroglycan (Panel A) and dystrophin expression (Panel B) can be observed in the Nx (Normoxic), I/R and rIPC groups at 10 min of reperfusion (Panel A). r-IPC protocol attenuated the breakdown of b-dystroglycan and dystrophin. Panel C and B showed the immunostaining for both proteins. *: p<0.05 vs. Nx group. #: p<0.05 vs. I/R group. I/R: ischemia/reperfusion, rIPC: remote ischemic preconditioning.
Figure 3

rIPC attenuated the increase of MMP-2 activity in ventricular tissue (Panel A) and the peroxinitrite induced protein nitration (Panel B). *: p<0.05 vs. I/R group. I/R: ischemia/reperfusion, rIPC: remote ischemic preconditioning.
Figure 4

rIPC induced a significant increase of the cardiac Akt (Panel A) and GSK-3β phosphorylation (Panel B) at 10 min of reperfusion. *: p<0.05 vs. I/R group. I/R: ischemia/reperfusion, rIPC: remote ischemic preconditioning.
Figure 5

Panel A shows a representative trace of the evaluation of the opening of MPTP, assessed in fresh isolated left ventricle mitochondria. Panel B shows an increased rate of MPTP opening (18.79%) in the I/R group. Evaluation of mitochondrial function in freshly isolated heart mitochondrial samples. The inner membrane potential was studied by flow cytometry and the potentiometric cationic probe DiOC6 (Panel C). Panel D shows the ATP production rate evaluation by the luciferin luciferase method. *: p<0.05 vs. Nx
Figure 6

Panel A shows the effect of rIPC on mitochondria at 10 min of reperfusion. State 4 and state 3 respiration rates were significantly decreased in the I/R and groups in comparison with the Nx group (control) (*: p<0.05). As indicated by RCR values, I/R induced a significant impairment of mitochondrial function (*: p<0.05) and the rIPC protocol attenuated the mitochondrial damage.