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RESEARCH ARTICLE | *Integrative Cardiovascular Physiology and Pathophysiology*

Adenosine A_1 receptors and mitochondria: targets of remote ischemic preconditioning

Diamela T. Paez,[1,2] Mariana Garces,[3,4] Valeria Calabró,[3,4] Eliana P. Bin,[1,2] Verónica D'Annunzio,[1,2] Julieta del Mauro,[5] Timoteo Marchini,[3,4] Christian Höcht,[5] Pablo Evelson,[3,4] Ricardo J. Gelpi,[1,2]* and Martín Donato[1,2]*

[1]*Faculty of Medicine, Department of Pathology, Institute of Cardiovascular Pathophysiology, University of Buenos Aires, Buenos Aires, Argentina;* [2]*National Council of Scientific and Technological Research (CONICET), Institute of Biochemistry and Molecular Medicine (IBIMOL), Faculty of Medicine, University of Buenos Aires, Buenos Aires, Argentina;* [3]*CONICET, IBIMOL, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina;* [4]*Faculty of Pharmacy and Biochemistry, Department of Analytical Chemistry and Physic Chemistry, General and Inorganic Chemistry, University of Buenos Aires, Buenos Aires, Argentina; and* [5]*Faculty of Pharmacy and Biochemistry, Department of Pharmacology, University of Buenos Aires, Buenos Aires, Argentina*

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Paez DT, Garces M, Calabró V, Bin EP, D'Annunzio V, del Mauro J, Marchini T, Höcht C, Evelson P, Gelpi RJ, Donato M. Adenosine A_1 receptors and mitochondria: targets of remote ischemic preconditioning. *Am J Physiol Heart Circ Physiol* 316: H743–H750, 2019. First published January 25, 2019; doi:10.1152/ajpheart.00071.2018.—Adenosine is involved in classic preconditioning in most species and acts especially through adenosine A_1 and A_3 receptors. The aim of the present study was to evaluate whether remote ischemic preconditioning (rIPC) activates adenosine A_1 receptors and improves mitochondrial function, thereby reducing myocardial infarct size. Isolated rat hearts were subjected to 30 min of global ischemia and 60 min of reperfusion [ischemia-reperfusion (I/R)]. In a second group, before isolation of the heart, a rIPC protocol (3 cycles of hindlimb I/R) was performed. Infarct size was measured with tetrazolium staining, and Akt/endothelial nitric oxide (NO) synthase (eNOS) expression/phosphorylation and mitochondrial function were evaluated after ischemia at 10 and 60 min of reperfusion. As expected, rIPC significantly decreased infarct size. This beneficial effect was abolished only when 8-cyclopentyl-1,3-dipropylxanthine (adenosine A_1 receptor blocker) and N^G-nitro-L-arginine methyl ester (NO synthesis inhibitor) were administered during the reperfusion phase. At the early reperfusion phase, rIPC induced significant Akt and eNOS phosphorylation, which was abolished by the perfusion with an adenosine A_1 receptor blocker. I/R led to impaired mitochondrial function, which was attenuated by rIPC and mediated by adenosine A_1 receptors. In conclusion, we demonstrated that rIPC limits myocardial infarct by activation of adenosine A_1 receptors at early reperfusion in the isolated rat heart. Interestingly, rIPC appears to reduce myocardial infarct size by the Akt/eNOS pathway and improves mitochondrial function during myocardial reperfusion.

NEW & NOTEWORTHY Adenosine is involved in classic preconditioning and acts especially through adenosine A_1 and A_3 receptors. However, its role in the mechanism of remote ischemic preconditioning is controversial. In this study, we demonstrated that remote ischemic preconditioning activates adenosine A_1 receptors during early reperfusion, inducing Akt/endothelial nitric oxide synthase phosphorylation and improving mitochondrial function, thereby reducing myocardial infarct size.

mitochondria; myocardial infarction; remote ischemic preconditioning

INTRODUCTION

Remote ischemic preconditioning (rIPC) is a cardioprotective phenomenon by which transient nonlethal ischemia and reperfusion of one organ or tissue confers resistance to a later episode of lethal ischemia-reperfusion (I/R) injury in a remote organ or tissue (20, 41).

The rIPC phenomenon has been extensively studied in the last 25 yr. However, some aspects of its mechanism remain unknown (27). Some authors have shown that the cardioprotective signal transference from the peripheral organ to the target organ involved humoral factors (10), neural pathways (12, 36), or a neurohumoral interaction (21). In a previous study, we showed that hindlimb rIPC triggers cardiac signaling before myocardial ischemia (13). However, we did not study the cellular and molecular changes that occur during myocardial reperfusion.

Adenosine is an endogenous nucleoside that modulates cellular homeostasis (42), and different studies have shown that it could likely trigger limb, renal, mesenteric, and carotid artery rIPC (11, 15, 40, 43). In this sense, it is unknown whether rIPC increases the local adenosine concentration in the remote tissue/organ and stimulates the neurogenic pathway (31) and/or whether adenosine participates directly at the cardiac level. Different authors have shown adenosine participation before myocardial ischemia (31, 40). However, the role of this nucleoside during myocardial reperfusion in hearts subjected to rIPC has not been studied, nor has its potential modulatory effect over mitochondrial function.

Therefore, the aim of this work was to evaluate the role of the adenosine A_1 receptor in the cardioprotective effects of rIPC, focusing on the mechanisms involving Akt/endothelial

* R. J. Gelpi and M. Donato contributed equally to this work.

Address for reprint requests and other correspondence: R. J. Gelpi, Institute of Cardiovascular Pathophysiology, Faculty of Medicine, Univ. of Buenos Aires, 950 J. E. Uriburu, 2nd floor, Buenos Aires C1114AAD, Argentina (e-mail: rgelpi@fmed.uba.ar).

nitric oxide (NO) synthase (eNOS) phosphorylation and mitochondrial function.

MATERIALS AND METHODS

Ethical Approval

Experiments were performed on male Sprague-Dawley rats (60–70 days old, 200−250 g) supplied by the animal facility of the Faculty of Pharmacy and Biochemistry, University of Buenos Aires. The procedures used in this study were approved by the Animal Care and Research Committee of the University of Buenos Aires (protocol no. 2948/10) and were in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (2011).

Surgical Procedures

In accordance with guidelines for experimental models of myocardial ischemia and infarction (3, 5, 32), rats were anesthetized with urethane (65 mg/kg ip) and intubated for mechanical ventilation. The level of anesthesia was assessed by loss of pedal reflex (toe pinch). Afterward, the left femoral or axillar artery was dissected and exposed, and animals were randomized into different experimental groups (see below).

After completion of the in vivo surgical procedure, animals were euthanized, and hearts were rapidly excised and mounted on a Langendorff apparatus by the aortic root in <1 min. Each heart was perfused with Krebs-Henseleit buffer containing 118.5 mM NaCl, 4.7 mM KCl, 24.8 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, and 10 mM glucose and bubbled with a 95% O_2-5% CO_2 gas mixture at 37°C with final pH at 7.2–7.4. Two electrodes were secured to the epicardial surface and connected to a pacemaker with a constant heart rate of 275 beats/min.

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

Experimental Groups

I/R group. Rats were anesthetized, and the left femoral artery was dissected and exposed as described above (Fig. 1). After 30 min of monitoring, 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 60 min of reperfusion. Global no-flow ischemia was induced by abruptly decreasing the total coronary flow provided by the perfusion pump.

rIPC group. After rats had been anesthetized and the left femoral artery dissected and exposed, animals were remotely preconditioned by a three-cycle hindlimb ischemia (5 min) and reperfusion (5 min) protocol by occlusion of the femoral artery with a vascular clamp. Subsequently, hearts were subjected to the same protocol used in the I/R group (30 min of global no-flow ischemia followed by 60 min of reperfusion) to induce myocardial infarction.

rIPC + 8-cyclopentyl-1,3-dipropylxanthine group. The same protocol as used in the rIPC group was performed, but 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 100 μg/kg iv), a selective adenosine A_1 receptor blocker, was administered 5 min before the rIPC protocol. Myocardial infarction was induced using the above-mentioned protocol.

rIPC + DPCPX (reperfusion) group. The same protocol as used in the rIPC group was performed, but at the onset of myocardial reperfusion, DPCPX (1 μM) was added to Krebs-Henseleit buffer. The drug was perfused during the first 10 min of reperfusion [rIPC + DPCPX (R) group].

rIPC + N^G-nitro-L-arginine methyl ester (reperfusion) group. The same protocol as used in the rIPC group was performed, but at the onset of myocardial reperfusion, N^G-nitro-L-arginine methyl ester (L-NAME; 100 mmol/l) was administered. The drug was perfused during the first 10 min of reperfusion [rIPC + L-NAME (R) group].

Two additional groups were performed in which the adenosine A_1 receptor blocker (DPCPX) and the NO synthase inhibitor (L-NAME) were administered in nonpreconditioned ischemic hearts during the first 10 min of reperfusion.

Infarct Size Measurement

After 1 h of reperfusion, hearts were frozen and cut into 2-mm-thick transverse slices from the apex to base. Sections were incubated for 20 min in 1% triphenyltetrazolium chloride (pH 7.4, 37°C) and then immersed in 10% formalin. With this technique applied, viable sections are stained red, whereas infarct areas remain unstained. Finally, all slices were then scanned on a flatbed digital scanner. Infarct size was determined by semiautomatic computer planimetry

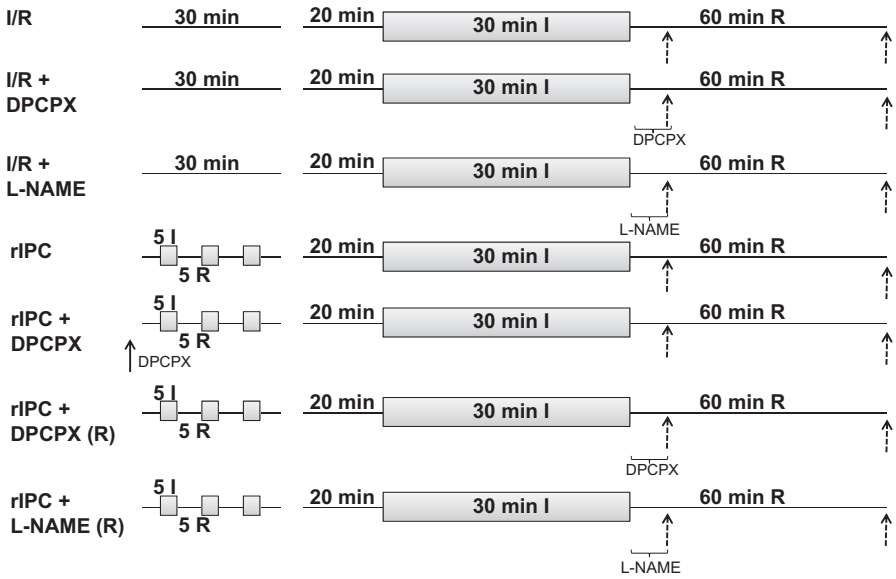

Fig. 1. Representative scheme of the experimental protocols. Dashed arrows indicate the time when tissue samples were collected to measure protein expression and mitochondrial function involved in the remote ischemic preconditioning (rIPC) mechanism. DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; I/R, ischemia (I)-reperfusion (R); L-NAME, N^G-nitro-L-arginine methyl ester.

(Image Pro Plus, version 4.5, Media Cybernetics). Infarct size was expressed as a percentage of the LV area.

Western Blot

Samples from LV of animals subjected to I/R, rIPC, and rIPC + DPCPX (R) protocols were taken at 10 min of reperfusion. Samples were homogenized in 3 volumes (wt/vol) of ice-cold homogenization buffer [150 mM NaCl, 50 mM Trizma·HCl, 1% (vol/vol) sodium deoxycholate, 1 mM EGTA, 1 mM NaF, 1 mM PMSF, and 1 mM sodium pervanadate]. All reactants (Sigma-Aldrich, St. Louis, MO) and a protease inhibitor cocktail (4%, Roche, Hertfordshire, UK) at pH 8.0 were centrifuged at 10,507 *g* for 10 min at 4°C. These proteins in the supernatant were quantified by the Lowry method. Equal amounts of proteins (80 μg) were subjected to 8% SDS-PAGE and transferred to nitrocellulose membranes. After being blocked for 1 h in 3% (wt/vol) BSA in PBS, membranes were incubated overnight at 4°C with the corresponding primary antibodies: rabbit anti-phospho-Akt antibody (catalog no. 5012, RRID:AB_2224726, 1:1,000 dilution, Cell Signaling Technology, Danvers, MA) (12) and rabbit anti-phospho-eNOS (catalog no. 9570, 1:750 dilution, Cell Signaling Technology) (12). Blots were hybridized with secondary antibody coupled to horseradish peroxidase (dilution 1:5,000, Santa Cruz Biotechnology, Dallas, TX). Complexes were visualized by chemiluminescence detection (Pierce ECL Western blotting substrate). Membranes were stripped and reprobed with anti-Akt (Ser^{473}) (catalog no. 4691, 1:1,000 dilution, Cell Signaling Technology) (12) and anti-eNOS antibody (Ser^{1177}) (catalog no. 1133, 1:750 dilution, Cell Signaling Technology) (12). Densitometric analysis of the bands was performed using ImageJ (National Institutes of Health, Bethesda, MD). Protein band densities were normalized to Akt and eNOS content.

Mitochondrial Respiration

Samples from the LV ($n = 6$ LVs/group) were taken at 10 and 60 min of reperfusion, and mitochondrial purified fractions were obtained from tissue homogenates by differential centrifugation (34). Mitochondrial O_2 consumption was followed polarographically with a Clark-type O_2 electrode (Hansatech Oxygraph, Hansatech Instruments, Norfolk, UK) for high-resolution respirometry at 30°C. Freshly isolated heart mitochondria (0.15 mg protein/ml) were incubated in respiration buffer (120 mM KCl, 5 mM KH_2PO_4, 1 mM EGTA, 3 mM HEPES, and 1 mg/ml fatty acid-free BSA, pH 7.4) supplemented with 2 mM malate and 5 mM glutamate. An initial rest state respiration (state 4) was established under these conditions, which was then switched to active state respiration (state 3) by the addition of 125 μM ADP. The respiratory control ratio (RCR) was calculated as state 3/state 4 respiration rates. Results are expressed as nanograms of atmospheric O per minute per milligram of protein (34).

Mitochondrial ATP Production Rate

A chemiluminescent assay based on the luciferin-luciferase system was used (16). Freshly isolated heart mitochondria (0.25 mg protein/ml) were incubated in respiration buffer supplemented with 40 μM D-luciferin and 0.05 μg/ml luciferase. ATP production was triggered by the addition of 3 mM malate, 1.25 mM glutamate, and 1 mM ADP to the reaction well. Chemiluminescence emission was followed as a function of time in a Varioskan LUX multimode microplate reader (ThermoFisher Scientific, Waltham, MA) at 30°C. Oligomycin (2 μM) was used as a negative control to establish basal signal levels. A calibration curve was performed using ATP as the standard, and the ATP production rate was expressed as nanomoles of ATP per minute per milligram of protein.

Mitochondrial H_2O_2 Production Rate

The mitochondrial H_2O_2 production rate was evaluated by the Amplex red-horseradish peroxidase (HRP) method (8). The reaction mixture consisted of 125 mM sucrose, 65 mM KCl, 10 mM HEPES, 2 mM KH_2PO_4, 2 mM $MgCl_2$, and 0.01% (wt/vol) BSA (pH 7.2) supplemented with 2 mM malate and 5 mM glutamate as mitochondrial respiratory substrates. After an initial stabilization period, freshly isolated heart mitochondria (0.25 mg protein/ml) were added to the reaction mixture together with 25 μM Amplex red and 0.5 U/ml HRP. Resorfurin formation resulting from Amplex red oxidation by HRP bound to H_2O_2 was measured in a Varioskan LUX multimode microplate reader (ThermoFisher Scientific) at 563 nm (excitation) and 587 nm (emission) at 30°C. Controls in the absence of isolated mitochondria or HRP or in the presence of catalase indicated that nonspecific probe oxidation was minimal (<1%). A calibration curve was performed using H_2O_2 solutions as standards, and the mitochondrial H_2O_2 production rate was expressed as nanomoles per minute per milligram of protein.

Mitochondrial Membrane Potential

Freshly isolated heart mitochondria (25 μg protein/ml) were incubated in respiration buffer with the potentiometric cationic probe 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$; 30 nM) in the dark at 37°C for 20 min. After the incubation period, mitochondria were acquired in a FACScalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ). Samples were gated based on light-scattering properties, and 20,000 events/sample were collected. 10-*N*-nonyl acridine orange (100 nM) was used to selectively stain mitochondria and to evaluate their purity due to its ability to selectively bind to cardiolipin at the inner mitochondrial membrane (20). To quantify the resulting changes in membrane potential after the addition of 2 mM malate and 5 mM glutamate to the reaction mixture, the $DiOC_6$ signal was analyzed in the FL-1 channel with FlowJo software (FlowJo, Ashland, OR), and the arithmetic mean values of the median fluorescence intensities were obtained. Total depolarization induced by 2 μM m-CCCP was used as a positive control. Mitochondrial preparations that showed no changes in membrane potential under this condition were discarded (35).

Statistical Analysis

Data are expressed as means ± SE. Intergroup comparisons were carried out using one-way ANOVA followed by *t*-tests, with the *P* value adjusted for multiple comparisons using the Bonferroni test. Data comparisons were not significant unless the corresponding *P* value was $<0.05/k$, where *k* represents the number of comparisons. Intragroup comparisons were analyzed by two-factor repeated-measures ANOVA.

RESULTS

Figure 2 shows the infarct size induced by 30 min of global ischemia followed by 120 min of reperfusion expressed as a percentage of the LV area. In the I/R group, infarct size was 46.9 ± 0.8%, whereas rIPC decreased infarct size to 34.7 ± 1.3% ($P < 0.05$ vs. the I/R group), thus displaying significant cardioprotection by rIPC. The administration of DPCPX (adenosine A_1 receptor blocker) before the rIPC protocol did not influence the infarct-limiting effects of rIPC. However, blockade of the adenosine receptor during 10 min of reperfusion completely abolished the beneficial effect of rIPC, resulting in an infarct size of 56.4 ± 4.1% ($P < 0.05$ vs. the rIPC group). The possible participation of NO synthase in the rIPC cardioprotection was investigated by using L-NAME (NO synthase inhibitor) during 10 min of reperfusion, which abolished the effect of rIPC on infarct size (45.8 ± 2.6%, $P < 0.05$ vs. the rIPC group). It is important to mention that the administration of the adenosine A_1 receptor blocker and NO synthase inhibitor in nonpreconditioned ischemic hearts did not show any significant differences in infarct size.

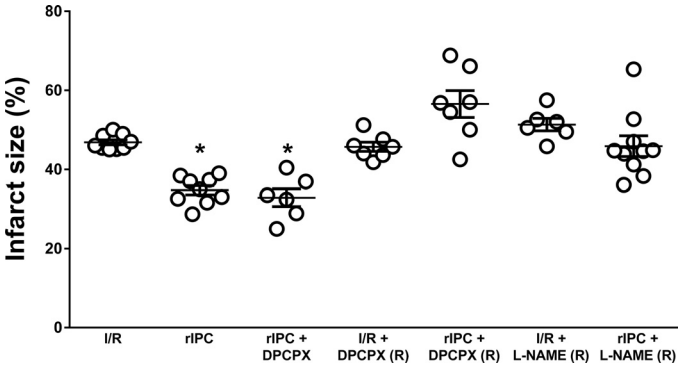

Fig. 2. Infarct size expressed as a percentage of the left ventricular area. Remote ischemic preconditioning (rIPC) significantly reduced the infarct size, and this effect was abolished by the administration of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and N^G-nitro-L-arginine methyl ester (L-NAME) during 10 min of reperfusion (R). ○, Individual infarct sizes. I/R, ischemia-reperfusion. I/R group: $n = 9$, rIPC group: $n = 9$, rIPC + DPCPX group: $n = 6$, rIPC + DPCPX (R) group: $n = 7$, rIPC + L-NAME group: $n = 10$, I/R + DPCPX (R) group: $n = 7$, and I/R + L-NAME (R) group: $n = 6$. *$P < 0.05$ vs. the I/R group.

Table 1. *Mitochondrial respiration and RCR of isolated mitochondria from the left ventricle of animals subjected to 30 min of ischemia followed by 60 min of reperfusion (late reperfusion)*

Experimental Group	State 4 Respiration, ng atm O·min^{-1}·mg protein^{-1}	State 3 Respiration, ng atm O·min^{-1}·mg protein^{-1}	RCR
Nx	29 ± 3	162 ± 21	5.6 ± 0.5
I/R	25 ± 1	43 ± 6†	1.7 ± 0.3§
I/R + DPCPX (R)	17 ± 5	24 ± 3†	2.0 ± 0.3§
I/R + L-NAME (R)	24 ± 3	40 ± 1†	1.9 ± 0.2§
rIPC	18 ± 3	69 ± 12†	3.8 ± 0.7
rIPC + DPCPX (R)	26 ± 3	72 ± 7*	2.7 ± 0.8‡
rIPC + L-NAME (R)	16 ± 2	33 ± 5†	1.9 ± 0.3§

Values are means ± SE; $n = 6$/group. RCR, respiratory control ratio; Nx, normoxic; I/R, ischemia-reperfusion; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; L-NAME, N^G-nitro-L-arginine methyl ester; rIPC, remote ischemic preconditioning; (R), when the drug was administered at reperfusion. RCR values were calculated as state 3/state 4 respiration rates. *$P < 0.01$ and †$P < 0.001$ vs. state 3 respiration in the Nx group; ‡$P < 0.05$ and §$P < 0.001$ vs. RCR in the Nx group.

The role of the Akt/eNOS pathway in the rIPC cardioprotection effect was confirmed by evaluating phosphorylated Akt (Ser^{477}) and eNOS (Ser^{1177}) (Fig. 3). rIPC induced a significant increase in the phosphorylation of both enzymes, which were abolished by the administration DPCPX (adenosine A_1 receptor blocker) during the first 10 min of reperfusion.

Table 1 shows the effect of rIPC on mitochondria at 60 min of reperfusion (late reperfusion). Whereas no changes were observed in state 4 respiration rates, state 3 respiration was significantly decreased in the I/R, rIPC, and rIPC + DPCPX (R) groups compared with the normoxic (control) group ($P < 0.001$). As indicated by RCR values, I/R induced a significant impairment of mitochondrial function ($P < 0.001$) and the rIPC protocol attenuated the mitochondrial damage, whereas this effect was abolished by the administration of DPCPX and L-NAME.

In the following experiments, we focused on the study of the events that occur at early reperfusion (10 min of reperfusion). As shown in Table 2, compared with the normoxic group, a slight, nonsignificant decrease in the mitochondrial rest state respiration (state 4) rate was observed for the I/R and rIPC groups, whereas the active state respiration (state 3) rate was significantly decreased ($P < 0.001$). Therefore, mitochondrial RCR was significantly decreased in the I/R group compared with the normoxic group ($P < 0.05$), which was partially recovered in the rIPC group. These results suggest that the I/R protocol impairs mitochondrial function and that rIPC attenuates this effect. Moreover, a significant decrease in mitochondrial RCR was observed in the rIPC + DPCPX (R) and rIPC + L-NAME (R) groups ($P < 0.05$), suggesting that blockade of adenosine A_1 receptor and NO synthase inhibition at the beginning of reperfusion reversed the protective effect of rIPC on mitochondrial function. Of note is that DPCPX seemed to induce a transient effect over mitochondrial O_2 consumption in the I/R and rIPC groups, particularly over rest state respiration at early (10 min) reperfusion. However, this effect was not observed at later time points (Table 1) or in the normoxic group (data not shown) and did not interfere with the blocking effect over rIPC cardioprotection (Fig. 2).

To deeply characterize the involvement of mitochondria in the protective mechanisms triggered by rIPC, mitochondrial inner membrane potential, H_2O_2, and ATP production rates were assessed at 10 min of reperfusion. As shown in Fig. 4*A*, the I/R protocol induced a significant decrease, by 47%, in the $DiOC_6$ signal ($P < 0.05$) compared with the normoxic group, which was reverted in the rIPC group. This result indicates that

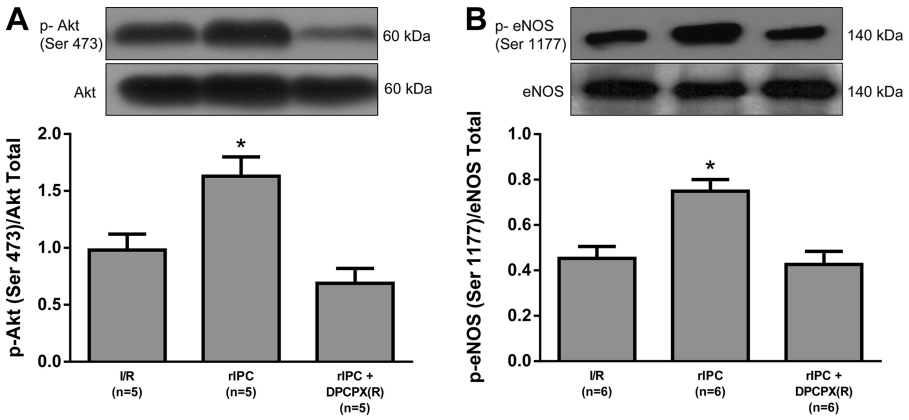

Fig. 3. Cardiac expression of phosphorylated Akt (Ser^{473}; *A*) and endothelial nitric oxide synthase [eNOS (Ser^{1177}); *B*] can be observed in the ischemia-reperfusion (I/R), remote ischemic preconditioned (rIPC), and rIPC + 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) groups ($n = 5$–6/group) at 10 min of reperfusion (R). rIPC induced a significant increase of Akt and eNOS phosphorylation, which was abolished by the administration of DPCPX at early reperfusion. *$P < 0.05$ vs. the I/R and DPCPX (R) groups.

Table 2. *Mitochondrial respiration and RCR of isolated mitochondria from the left ventricle of animals subjected to 30 min ischemia, followed by 10 min of reperfusion (early reperfusion)*

Experimental Group	State 4 Respiration, ng atm O·min^{-1}·mg protein^{-1}	State 3 Respiration, ng atm O·min^{-1}·mg protein^{-1}	RCR
Nx	35 ± 7	210 ± 13	5.8 ± 0.8
I/R	21 ± 4	79 ± 16†	3.9 ± 0.5‡
I/R + DPCPX (R)	39 ± 6	93 ± 17†	3.2 ± 0.5‡
I/R + L-NAME (R)	29 ± 4	91 ± 20†	3.6 ± 0.4‡
rIPC	16 ± 3	87 ± 14†	5.4 ± 1.0
rIPC + DPCPX (R)	61 ± 9*	146 ± 16§	2.5 ± 0.8‡
rIPC + L-NAME (R)	28 ± 2	90 ± 3†§	2.9 ± 0.2‡

Values are means ± SE; $n = 6$/group. RCR, respiratory control ratio; Nx, normoxic; I/R, ischemia-reperfusion; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; L-NAME, N^G-nitro-L-arginine methyl ester; rIPC, remote ischemic preconditioning; (R), when the drug was administered at reperfusion. RCR values were calculated as state 3/state 4 respiration rates. *$P < 0.01$ vs. state 4 respiration in the Nx group; †$P < 0.001$ vs. state 3 respiration in the Nx group; ‡$P < 0.05$ vs. RCR in the Nx group; §$P < 0.05$ vs. state 3 respiration in the I/R and rIPC groups.

I/R induces strong mitochondrial depolarization and that rIPC prevents this effect. Moreover, a slight, nonsignificant decrease in the $DiOC_6$ signal was observed in the rIPC + DPCPX (R) group, whereas the rIPC + L-NAME (R) group showed a significant decrease, by 37% ($P < 0.05$), compared with the normoxic group. These data suggest that adenosine exerts a minor contribution in the protective effect of rIPC over mitochondrial membrane potential, yet NO plays a more relevant role in this scenario.

Regarding mitochondrial H_2O_2 production (Fig. 4*B*), the I/R protocol induced a significant increase, by 33%, compared with the normoxic group ($P < 0.001$), whereas no differences were observed in the rIPC group. Although no changes were also observed in the rIPC + DPCPX (R) group, the rIPC + L-NAME (R) group showed a significant increase in mitochondrial H_2O_2 production rate, by 79% ($P < 0.001$ vs. the normoxic group and $P < 0.01$ vs. the rIPC group).

The mitochondrial ATP production rate (Fig. 4*C*) was significantly decreased, by 67%, in the I/R group compared with the normoxic group ($P < 0.01$). This effect was attenuated by rIPC, as the ATP production rate decreased by only 28% ($P < 0.05$) compared with the normoxic group. This effect was abolished by blockage of adenosine A_1 receptors and NO synthase inhibition at the beginning of reperfusion, as the mitochondrial ATP production rate in the rIPC + DPCPX (R) and rIPC + L-NAME (R) groups were significantly decreased, by 85% and 87%, respectively, compared with the normoxic group. These findings indicate that rIPC prevents the induced decrease in mitochondrial ATP production by the I/R protocol. This effect seems to be dependent on adenosine signaling through adenosine A_1 receptor activation, in which NO plays a central role.

It is important to mention that the administration of the adenosine A_1 receptor blocker and NO synthase inhibitor in nonpreconditioned ischemic hearts did not show any significant differences in the different variables of mitochondrial function.

DISCUSSION

In the present study, we demonstrated the participation of adenosine A_1 receptors in the rIPC mechanism, considering its activation before myocardial ischemia and at the time of myocardial reperfusion. Moreover, the activation of adenosine A_1 receptors at early reperfusion is associated with higher phosphorylation of Akt/eNOS and an improvement of mitochondrial function, supporting the notion that the mitochondrion is a myocardial target organelle in the mechanisms of the rIPC-protective effect.

Adenosine was the first signaling molecule involved as a trigger of "classic" ischemic preconditioning (33). During normoxia, most synthesized adenosine is derived from *S*-adenosylhomocysteine. During ischemia or hypoxia, however, the major pathway of adenosine production is shifted to the 5′-AMP pathway (25). This adenosine could activate adenosine receptors. In this sense, at least by classic ischemic preconditioning, Kuno et al. (30) demonstrated that this cardioprotective strategy increases the heart's sensitivity to adenosine so that endogenous adenosine can activate survival kinases during reperfusion.

Adenosine A_1 receptors are localized on cardiomyocytes (38), and all adenosine receptors are coupled to G proteins (G_s or G_i) (37, 38). Downstream of the different G proteins, the eNOS/PKG and reperfusion injury salvage kinase (RISK) signal transduction pathways are activated by adenosine (24). We and others have evaluated the effect of exogenous adenosine administration on infarct size and ventricular function (2, 14). Other authors studying the role of the adenosine receptor as a mediator of rIPC have explored its contribution either in the remote organ or tissue or in the heart but before myocardial ischemia (25, 40). Pell et al. (40) demonstrated that a remote renal preconditioning reduces myocardial infarction. This cardioprotective effect was abolished by a nonselective adenosine receptor blocker, suggesting the involvement of adenosine receptors in mediating acute preconditioning of the myocardium. Similarly, Takaoka et al. (46) also described that remote renal preconditioning significantly reduces infarct size and improves myocardial energy metabolism in rabbit hearts subjected to I/R through the activation of adenosine receptors. Dong et al. (15) showed that remote limb ischemic preconditioning that induces protective effects against sustained I/R injury was abolished after femoral nerve transection and DPCPX administration, suggesting the involvement of local adenosine release and activation of the neural pathway in mediating remote limb ischemic preconditioning. Other authors have demonstrated the participation of adenosine A_1 receptors in rIPC cardioprotective effects, but with the use of an adenosine A_1 receptor knockout mice model (43). However, none of these studies investigated the participation of adenosine receptors at the beginning of reperfusion, considering the effect of its activation on mitochondrial function. In the present study, we showed the relevance of adenosine A_1 receptor activation only during reperfusion to achieve cardioprotection and improved of mitochondrial function.

As mentioned above, adenosine is able to directly induce NO production in cardiomyocytes by activation of eNOS (48). Adenosine can activate phosphatidylinositol 3-kinase (29), leading to increased eNOS activity. In the present study, we showed that rIPC increases Akt phosphorylation during reper-

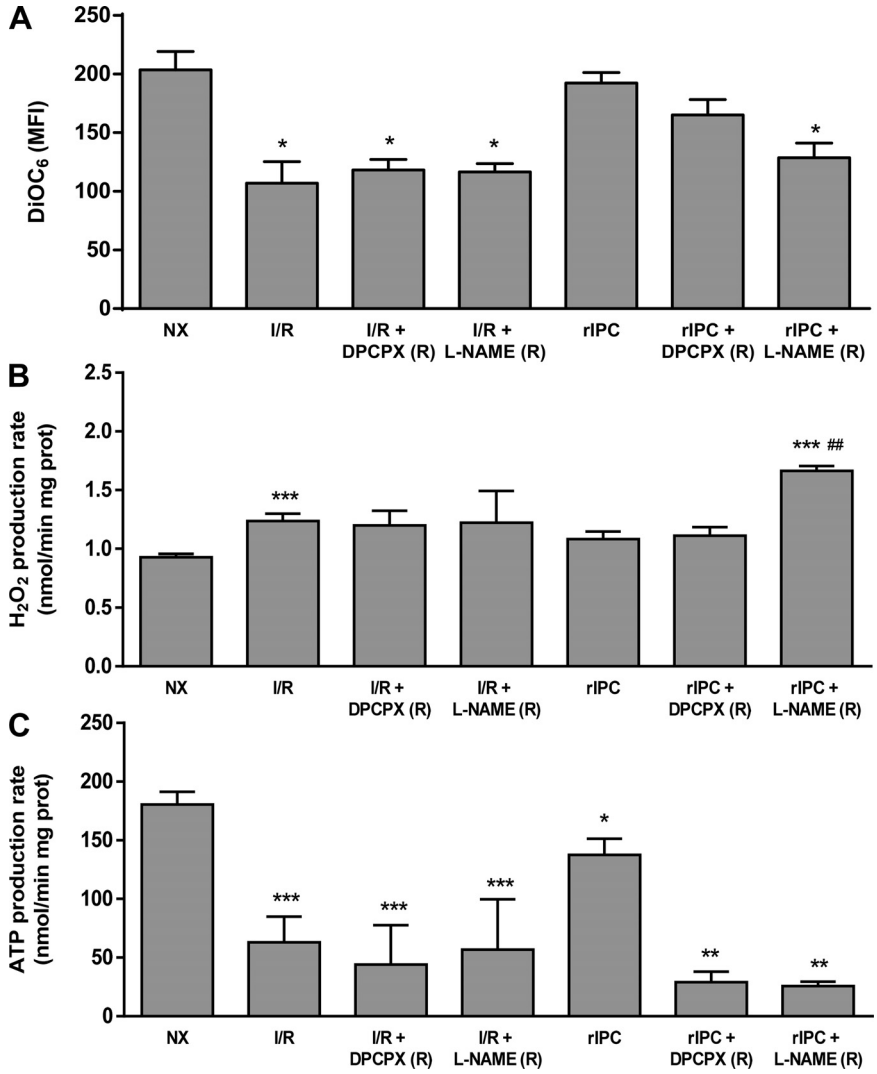

Fig. 4. Evaluation of mitochondrial function in freshly isolated heart mitochondrial samples ($n = 6$/group). *A*: inner membrane potential was studied by flow cytometry and the potentiometric cationic probe 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$). *B*: fluorometric assessment of the H_2O_2 production rate by the Amplex red/horseradish peroxidase method. *C*: ATP production rate evaluation by the luciferin-luciferase method. $*P < 0.05$ vs. the normoxic (Nx) group; $**P < 0.01$ vs. the Nx group; $***P < 0.001$ vs. the Nx group; $\#\#P < 0.01$ vs. the ischemia-reperfusion (I/R) group.

fusion and that this effect is mediated by adenosine A_1 receptor activation. We also noted that rIPC induced eNOS phosphorylation, which was inhibited by L-NAME administration.

Because of this, mitochondria are viewed as end effectors of ischemic preconditioning (49), and the preservation of mitochondrial function after I/R is a key event that might determine the survival of cardiomyocytes (19, 22, 23, 28). In rats, the myocardial infarct size reduction by rIPC was associated with preserved mitochondrial morphology (6, 18) and retained mitochondrial membrane potential and increased mitochondrial manganese superoxide dismutase activity (5, 26). Furthermore, reactive oxygen species (ROS) at high levels do play an adverse role in myocardial I/R injury but contribute to endogenous cardioprotection at lower concentrations (7).

In this sense, Gedik et al. (19) showed that plasma obtained from rIPC pigs improves mitochondrial function in early reperfusion of isolated rat hearts. However, plasma of preconditioned pigs contains different substances that are released in the ischemic tissue (adenosine, bradykinin, etc.), which can protect from I/R injury. Furthermore, studies performed in patients undergoing cardiovascular surgery (28, 44) showed that rIPC preserved mitochondrial and contractile function of the human atrial trabeculae of patients.

On the other hand, NO can modulate the mitochondrial permeability transition pore (mPTP) in isolated mitochondria (1). The mPTP is a nonspecific channel that induces depolarization of the mitochondrial inner membrane, leading to ATP depletion and enhancing colloidal osmotic pressure in the mitochondrial matrix, producing matrix swelling and rupture of the mitochondrial outer membrane (21). The fact that NO can alter the sensitivity of mitochondria to mPTP inducers might be important for some physiological and pathophysiological processes where the mitochondrial permeability transition has been implicated. A physiological NO concentration may inhibit the mPTP by *S*-nitrosylation-related mechanisms (39). The mPTP induces mitochondrial swelling and membrane depolarization and provides a mechanism for cytochrome *c* release, a hallmark signal protein of the mitochondrial apoptosis pathway (9). Thus, several studies (45, 47) have shown that NO production can protect the heart against I/R injury.

In the present report, we showed that rIPC increases eNOS phosphorylation, resulting in an improvement in mitochondrial respiration and a reduction in myocardial infarct size. This beneficial effect was mediated by adenosine A_1 receptor activation.

The evaluation of mitochondrial RCR by high-resolution respirometry is the most useful general measure of mitochondrial function, as it encompasses every single aspect of the oxidative phosphorylation process, including membrane potential across the inner mitochondrial membrane (4). Improved mitochondrial respiration after rIPC was also associated with restored mitochondrial RCR at early reperfusion, suggesting a possible inhibition of mPTP opening, as indicated by mitochondrial membrane potential and H_2O_2 production measurements. It is well known that the first minutes of reperfusion after sustained ischemia have been emphasized as critical for signal transduction of cardioprotection and mitochondrial function. In the present study, we measured mitochondrial function at 10 min (early) and 60 min (late) of myocardial reperfusion. Interestingly, the I/R group showed a progressive impairment of mitochondrial function (indicated by RCR) along the reperfusion period; however, the beneficial effect of rIPC was maintained during this entire phase and was found to be dependent on adenosine A_1 receptor activation and NO release. Consequently, as indicated by ATP production measurements, this pathway triggered by rIPC preserved the mitochondrial capacity of producing energy for the cardiomyocyte, which might be associated with the infarct size reduction in I/R injury. During the evaluation of the role of adenosine A_1 receptor in rIPC cardioprotection, a transient effect of DPCPX over mitochondrial respiration was observed by a yet-unknown mechanism that did not interfere with the inhibition of rIPC protective effect over infarct size, as we (Fig. 2) and others (17) have reported. This observation would need to be clarified in further studies.

In summary, in the isolated rat heart, remote ischemic limb preconditioning limits myocardial infarction by activation of adenosine A_1 receptors and the Akt/eNOS pathway during early reperfusion. Interestingly, remote ischemic limb preconditioning appears to reduce myocardial infarct size by improving key aspects of mitochondrial function such as ATP production during myocardial reperfusion. Further studies are required to investigate the myocardial signal transduction pathways underlying rIPC to explain the association between adenosine A_1 receptor activation and mitochondrial function.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

D.T.P., M.G., V.C., E.P.B., V.D., J.D.M., T.M., C.H., and M.D. performed experiments; D.T.P., M.G., E.P.B., V.D., J.D.M., T.M., C.H., P.E., R.J.G., and M.D. analyzed data; D.T.P., M.G., V.C., E.P.B., J.D.M., T.M., C.H., P.E., R.J.G., and M.D. interpreted results of experiments; D.T.P., M.G., J.D.M., T.M., C.H., P.E., and M.D. prepared figures; D.T.P., M.G., V.C., E.P.B., V.D., T.M., P.E., R.J.G., and M.D. edited and revised manuscript; D.T.P., M.G., V.C., E.P.B., V.D., J.D.M., T.M., C.H., P.E., R.J.G., and M.D. approved final version of manuscript; J.D.M., T.M., C.H., P.E., R.J.G., and M.D. drafted manuscript; P.E., R.J.G., and M.D. conceived and designed research.

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