Ischemic Postconditioning Reduces Infarct Size Through the α1-Adrenergic Receptor Pathway

Bruno Buchholz, MD, PhD,* Verónica D’Anunzio, MD, PhD,* Jorge F. Giani, PhD,† Nadezda Siachoque, PhD,* Fernando P. Dominici, PhD,† Daniel Turyn, PhD,† Virginia Perez, PhD,* Martín Donato, MD, PhD,* and Ricardo J. Gelpi, MD, PhD*

**Abstract:** The α1-adrenergic receptors (α1-ARs) are involved in preconditioning. Given that certain intracellular pathways seem to be shared by preconditioning and postconditioning, it is possible that postconditioning could also be mediated by α1-ARs. The objective was to evaluate, by analyzing infarct size, if α1-ARs activation could trigger postconditioning and also determine Akt and glycogen synthase kinase 3β (GSK-3β) phosphorylation. Langendorff-perfused rat hearts were subjected to 30 minutes of ischemia and 120 minutes of reperfusion (I/R; n = 8). After 30 minutes of global ischemia, we performed 6 cycles of reperfusion/ischemia of 10 seconds each, followed by 120 minutes of reperfusion [ischemic postconditioning group (postcon); n = 9]. In another postcon group, we administered prazosin during postcon protocol (postcon + prazosin; n = 7). Finally, we repeated the I/R group, but prazosin (prazosin; n = 7), phenylephrine (PE; n = 5), and clonidine (CL; n = 6) were administered during the first 2 minutes of reperfusion. Infarct size was measured using the triphenyltetrazolium chloride technique. Total and phosphorylated Akt and mitochondrial GSK-3β expression were measured by Western blot. Infarct size was 58.1 ± 5.1% in I/R, Postcon and PE reduced infarct size to 40.1 ± 2.9% and 35.3 ± 5.5%, respectively (P < 0.05 vs. I/R). Postcon + prazosin administration abolished the beneficial effect on infarct size (61.6 ± 4.5%; P < 0.05 vs. postcon). Cytosolic Akt phosphorylation and mitochondrial GSK-3β phosphorylation were higher in the postcon and PE groups compared with the I/R and postcon + prazosin groups. Prazosin or clonidine administration did not modify neither protein expression nor infarct size. Our data demonstrate that postconditioning decrease infarct size by activation of the α1-AR pathway through Akt and GSK-3β phosphorylation.

**Key Words:** ischemic postconditioning, α1-adrenergic receptor, infarct size, Akt, glycogen synthase kinase 3β

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**INTRODUCTION**

Brief intermittent periods of ischemia and reperfusion performed before long ischemia (termed ischemic preconditioning) have demonstrated protection of the myocardium against ischemia/reperfusion (I/R) injury. This cardioprotective strategy has limited clinical application in patients with acute myocardial infarction because of the inability to predict the moment of coronary artery occlusion. The concept of ischemic postconditioning may be useful, as it has been described as an entity by which brief periods of I/R, performed at the onset of reperfusion, reduce infarct size.

Different authors have provided evidence that the reduction on infarct size conferred by postconditioning could be triggered by activation of multiple types of cell membrane receptors, which include adenosine A1, bradykinin B2, and opioids. However, other types of membrane receptors that could be involved in the postconditioning mechanism are the α-adrenergic receptors (α1-ARs). The beneficial effects of preischemic α-ARs activation were shown by Gao et al, who found that activation of α1-ARs maintains cytosolic and mitochondrial calcium homeostasis, improves contraction, and preserves ATP content under simulated I/R. Studies by Banerjee et al concluded that transient ischemic preconditioning is mediated by sympathetic neurotransmitter release and α1-ARs stimulation. Conversely, Vasara et al suggested that α1-ARs stimulation mimics ischemic preconditioning, but that it is not an essential component of the mechanism. However, there is no evidence of the possible role of α1-ARs as a trigger for postconditioning. Given that certain intracellular pathways, recruited at the time of myocardial reperfusion, reduce infarct size, Akt, and glycogen synthase kinase 3β shared by preconditioning and postconditioning, it is possible that postconditioning could be mediated by α1-ARs. The objective was to evaluate, by analyzing infarct size, if α1-ARs activation could trigger postconditioning and also determine Akt and glycogen synthase kinase 3β (GSK-3β) phosphorylation.
the possibility of Akt phosphorylation through an α1-AR pathway in this protective mechanism. The inactivation of glycogen synthase kinase 3β (GSK-3β), as a consequence of its phosphorylation by Akt, has been implicated in the ischemic preconditioning mechanism<sup>10</sup> and in postconditioning.<sup>11</sup> However, the effect of α1-ARs stimulation, as part of the mechanism of ischemic postconditioning on Akt and GSK-3β expression and phosphorylation, is unknown. Thus, the aim of this study is to evaluate, by analyzing infarct size, if α1-ARs may trigger postconditioning. Because it is known that Akt and GSK-3β phosphorylation participate in the protection mechanism of ischemic postconditioning, a second objective was to determine if the reduction of the infarct size by the induced activation of α1-ARs requires Akt and GSK-3β phosphorylation.

**MATERIALS AND METHODS**

Wistar male rats (200–250 g) were killed with an intraperitoneal injection of pentobarbital (150 mg/kg).<sup>12</sup> The heart was excised from the animals and placed in a perfusion system, according to the Langendorff technique. All hearts were perfused with a Krebs–Henseleit buffer containing 118.5 mM of NaCl, 4.7 mM of KCl, 24.8 mM of NaHCO₃, 1.2 mM of KH₂PO₄, 1.2 mM of MgSO₄, 1.8 mM of CaCl₂, and 10 mM of glucose, pH 7.2–7.4, and gassed with 95% of O₂ and 5% of CO₂ at 37°C. Two electrodes were sutured and connected to a pacemaker with a constant heart rate of 275 beats per minute. The experiments were approved by the Animal Care and Research Committee of the University of Buenos Aires (Protocol # 2948/2010). All the procedures were performed in compliance with the guidelines of the American Physiological Society “Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training.”

A latex balloon, connected to a pressure transducer (Deltram II; Utah Medical System) through a polyethylene cannula, was inserted into the left ventricle (LV) for measurement of left ventricular pressure. The latex balloon was filled with water to achieve a left ventricular end diastolic pressure (LVEDP) of 8–10 mm Hg. In addition, coronary perfusion pressure (CPP) was recorded using a pressure transducer connected to the perfusion line. All hearts were perfused at a constant flow. Coronary flow was adjusted to obtain a CPP of 70.5 ± 4.2 mm Hg during the initial stabilization period. This flow level was maintained constant throughout the experiment.

**Experimental Protocols**

**I/R Group (n = 8)**

Myocardial infarction was induced by 30 minutes of global no-flow ischemia followed by 120 minutes of reperfusion. Global no-flow ischemia was induced by abruptly decreasing the total coronary flow provided by the perfusion pump.

**Ischemic Postconditioning Group (n = 9)**

The same protocol as in I/R group was repeated; but at the onset of reperfusion, we performed 6 cycles of 10 seconds of reperfusion followed by 10 seconds of ischemia (2 minutes total intervention, ischemic postconditioning protocol). Reperfusion was then continued for 120 minutes.

**Ischemic Postconditioning + Prazosin Group (n = 7)**

The same protocol as ischemic postconditioning group was followed except for the administration of prazosin hydrochloride (Sigma) (α1-ARs blocker), diluted in isotonic saline solution at an 1 μM concentration during ischemic postconditioning. It is important to remark that prazosin was administered simultaneously while performing the postconditioning protocol. It was dissolved in the Krebs solution during the 6 cycles of short ischemia/reperfusion (10 seconds each). Once the postconditioning protocol was performed, hearts were perfused solely with Krebs–Henseleit solution until the protocol was complete.

**Prazosin Group (n = 7)**

The same protocol was followed as in I/R, except for the administration of prazosin hydrochloride (Sigma) at an 1 μM concentration during the entire first 2 minutes of the reperfusion period. This was performed to rule out the effects of the drug itself on myocardial viability.

**Phenylephrine Group (n = 5)**

To assess if α1-ARs activation would trigger the postconditioning protection mechanism, we administered L-phenylephrine hydrochloride (Sigma), a selective α1-ARs agonist, diluted in isotonic saline solution at 50 μM, 5 μM, and 50 nM concentrations during 2 minutes at the beginning of reperfusion (after 30 minutes of global ischemia). Reperfusion was then continued for 120 minutes.

**Clonidine Group (n = 6)**

In this group, we administered clonidine hydrochloride (Research Biochemicals International), a selective α2-ARs agonist, to confirm our hypothesis that α1-ARs receptors, and not α2-ARs, are the ones involved in myocardial protection. Clonidine was administered diluted in isotonic saline solution at a 10⁻⁶ M concentration during the first 2 minutes of reperfusion. Again, this administration was performed after 30 minutes of global ischemia. Reperfusion was then continued for 120 minutes.

**Normoxic Group (n = 4)**

This group was only used as a control group for the Western blot determinations. Hearts were stabilized during 20 minutes, and then LV samples were taken and deep frozen at −180°C for their further processing. All the experimental groups were repeated, and LV samples were taken at 20 minutes of reperfusion for the Western blot determination (n = 4 per group). In the groups where the Western blot determinations were performed, the total perfusion time was of approximately 20 minutes, similar to the normoxic (Nx) used as the control for Western blot.

**Infarct Size Measurement**

Infarct size was evaluated using 2, 3, 5-Triphenyl/tetrathiazolium chloride. After 120 minutes of reperfusion,
the hearts were deep frozen at −20°C during 30 minutes and then cut into 2 mm transverse slices from apex to base. The sections were incubated for 20 minutes in a 1% of 2,3,5-Triphenyltetrazolium chloride (Sigma) solution diluted in Tris buffer (AppliChem) (pH 7.4, 37°C) and then immersed in 10% of formalin. This technique stained viable sections red, whereas non viable sections remained unstained. All the sections were scanned and planimetered (Image Pro Plus, version 4.5). Infarct size was expressed as a percentage of the left ventricular area.

Mitochondrial and Cytosolic Isolation and Fractioning

Frozen samples were homogenized in 5 volumes of buffer MSHE (pH 7.6) with phosphatase and protease inhibitors (Halt Protease & Phosphatase Inhibitor Cocktail EDTA-free form Pierce), using a Potter Elvehjem homogenizer. Homogenates were centrifuged at 657g for 10 minutes, filtered, and centrifuged again at 8000g for 10 minutes. Pellets were resuspended in MSHE buffer (0.21 M of mannitol/0.07 M of sucrose/10 mM of Heps, pH 7.4/1 mM of EDTA/1 mM of EGTA) with bovine serum albumin (BSA) and 30% of Percoll. Suspensions were centrifuged at 8000g for 30 minutes at 4°C. Mitochondrial fractions were separated and rinsed once with MSHE buffer with BSA at 10,509g for 10 minutes at 4°C, twice with 150 mM of KCl, and twice with MSHE buffer without BSA.

The protein load was confirmed with an anti–VDAC-1 antibody (1:700) (Santa Cruz Biotechnology Inc.) and an anti–β-actin antibody (1:1000) (Millipore) used as markers for outer mitochondrial membrane and cytosolic fraction, respectively. Removing the possibility that the mitochondrial fraction was contaminated, Western blot was performed where mitochondrial and cytosolic fractions were seeded and incubated with anti–β-actin. As shown in Figure 1A, we used anti-calreticulin antibody (1:1500) (Pierce) as an endoplasmic reticulum marker. Calreticulin markings were observed in the cytosolic fraction, but were not observed in the mitochondrial fraction, discarding the possibility of contamination with endoplasmic reticulum. In Figure 1B, in the lanes where mitochondrial fractions were seeded, there is no evidence of the presence of β-actin, ruling out the possible contamination of the cytosolic fraction. To determine mitochondrial purity, 2 mitochondrial specific antibodies were used: anti-TOM40 antibody (1:1000) (Santa Cruz Inc.) and anti-VDAC. TOM40 was included in Figure 1C, and it shows positive marking only in the mitochondrial fraction lanes. These findings, and the fact that VDAC was used as a loading control for the mitochondrial fractions, support the fact that the separation of the mitochondrial fraction was adequate.

Western Blot

Protein samples from mitochondrial fractions (30 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The samples were then incubated for 1 hour at room temperature in a blocking buffer containing 5% of non fat milk and 5% of BSA. Blots were then incubated overnight at 4°C with rabbit polyclonal anti-GSK-3β (1:1000) (Santa Cruz Biotechnology Inc.), mouse monoclonal anti–Ser9 phospho GSK-3β (1:300) (Millipore), rabbit monoclonal anti-Akt (1:1000) (Cell Signaling Technology), and rabbit monoclonal anti-Ser473 phospho Akt antibodies (1:1000) (Cell Signaling Technology). The blots were then incubated with goat anti-rabbit (1:5000) and goat anti-mouse antibodies (1:3000) (Millipore) at room temperature for 1 hour. Afterwards, they were rinsed with Tris buffered saline and tween 20 (TBST). Blots were developed using the enhanced chemiluminescence method (Pierce) according to the manufacturer’s instructions. Relative levels of GSK-3β, phospho GSK-3β, Akt, and phospho Akt were quantified by densitometric analysis using Image Gauge 4.0 software (Fujiﬁlm).

Statistical Analysis

Data are expressed as mean ± SEM. Intergroup comparisons were carried out using analysis of variance (one-way analysis of variance) 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

Table 1 shows the behavior of the CPP and the infarct size after the administration of different concentrations of phenylephrine. Concentrations of 5 and 50 μM produced a significant increase in CPP at 30 minutes of reperfusion, and an increase in the infarct size in both groups compared with group I/R. This is the reason why we administered a lower concentration of phenylephrine (50 nM) where we could evidence a protective effect on the infarct size (P < 0.05 vs. I/R) without significant changes in CPP at 30 minutes of reperfusion.

Table 2 shows the left ventricular developed pressure (LVDP), the LVEDP, and the CPP values at baseline and during different reperfusion times. In all groups, LVDP, a contractility index, was significantly lower compared with the respective preischemic values, but showed no significant differences among groups throughout the procedure. Regarding myocardial stiffness, represented by LVEDP, a significant increase at 30 minutes of reperfusion without differences among the groups was observed. Phenylephrine is a selective α1-ARs agonist and potent vasoconstrictor. We would like to point out that the CPP values, where phenylephrine was administered, were similar to the other groups.

Figure 2 shows infarct size after 30 minutes of global no-flow ischemia and 120 minutes of reperfusion, expressed as a percentage of the total left ventricular area. In Figure 2A, infarct size was 58.1 ± 3.9% in the I/R group. Ischemic postconditioning significantly decreased infarct size to 40.1 ± 2.9% (P < 0.05 vs. I/R group). The administration of prazosin during the postconditioning protocol abolished the protective effect of postconditioning (63.5 ± 3.4%, P < 0.05 vs. postcon group). The infarct size in the group treated with prazosin alone during the first 2 minutes of reperfusion was similar to that observed in the I/R group (58.8 ± 3.6%),
ruling out any possible effect of the drug per se. However, the activation of the α1-ARs with phenylephrine during reperfusion significantly reduced the infarct size compared with the I/R group (35.3 ± 5.5%, P < 0.05 vs. I/R). On the contrary, the activation of α2-ARs by the administration of clonidine during the first 2 minutes of reperfusion did not modify the infarct size compared with the I/R group (55.4 ± 2.7%), demonstrating that α2-ARs do not mediate protection. Figure 2B shows representative slices of the different experimental groups.

Cardiac Akt protein expression was shown in Figures 3, A and C and phosphorylation was shown in Figures 3, B and D in the cytosolic fraction. Cardiac Akt protein expression was displayed as a percentage of the Nx. There were no significant changes in cytosolic Akt protein expression; however, ischemic postconditioning induced a significant increase in Akt phosphorylation compared with the I/R and postconditioning + prazosin groups (Fig. 3B). The activation of α1-ARs, when phenylephrine was administered during reperfusion, shows a significant increase in Akt phosphorylation compared with I/R group, demonstrating that α1-ARs would be involved in the protection. When we administered prazosin without ischemic postconditioning, we did not detect any changes compared with I/R group in the cytosolic fraction (Fig. 3D).

Figure 4 shows cardiac GSK-3β protein expression in the mitochondrial fraction. Cardiac GSK-3β mitochondrial protein expression was shown as a percentage of the Nx. There were no differences among groups in total cardiac GSK-3β protein expression (Figs. 4, A and C). In Figure 4B, we show the Nx, I/R, postconditioning, and postconditioning + prazosin groups. Ischemic postconditioning induced significant GSK-3β phosphorylation compared with the I/R group. Administration of prazosin during the ischemic postconditioning protocol decreased protein phosphorylation. In Figure 4D, we observed that prazosin without ischemic postconditioning did not modify GSK-3β phosphorylation compared with the I/R group. However, α1-AR activation with phenylephrine administration increased GSK-3β phosphorylation compared with the I/R group, reaching similar values to the Nx. This finding suggests a link between mitochondrial GSK-3β and α1-AR activation.

DISCUSSION

The beneficial effect on infarct size was abolished by prazosin administration during the postconditioning protocol. The phenylephrine administration, an α1-ARs agonist, mimics the postconditioning effect and protected the heart against I/R injury. Also, the activation of α1-ARs involves Akt and GSK-3β phosphorylation. Therefore, the results of this study demonstrate that α1-ARs triggers the protective effect of postconditioning in the model of isolated rat hearts used herein.

As we mentioned, ischemic postconditioning and phenylephrine increased Akt phosphorylation in the cytosol. Given that GSK-3β is a downstream substrate of Akt and is able to phosphorylate it, this study sought to analyze the expression/phosphorylation state of this enzyme. The obtained results showed that GSK-3β mitochondrial phosphorylation is increased in isolated rat hearts subjected to ischemic postconditioning and also in the presence of phenylephrine. In addition, it is noteworthy that administration of prazosin alone, without the postconditioning protocol, had no effect on infarct size, ruling out any possible effect of the drug per se.

Previous works used phenylephrine as a pharmacological tool with the aim of blocking α1-ARs in higher concentrations than the ones we used in this research. An

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**TABLE 1. Response of Different Concentrations of Phenylephrine Administration on Infarct Size and CPP**

| Groups       | Baseline CPP (mm Hg) | 30 Minutes Reperfusion CPP (mm Hg) | Infarct Size (%) |
|--------------|----------------------|-----------------------------------|------------------|
| I/R          | 66.1 ± 1.1           | 102.3 ± 3.1*                      | 58.1 ± 3.9       |
| PE, 50 μM    | 67.9 ± 0.9           | 152.8 ± 13.5†                    | 82.5 ± 5.3†      |
| PE, 12.5 μM  | 67.8 ± 3.3           | 145.5 ± 9.6†                     | 74.4 ± 6.2†      |
| PE, 50 nM    | 63.1 ± 3.2           | 103.4 ± 7.3*                     | 35.3 ± 5.5†      |

*P < 0.05 versus respective baseline value. †P < 0.05 versus I/R group and PE 50 nM group. ‡P < 0.05 versus I/R group. PE, Phenylephrine.
important difference is that, in those studies, phenylephrine was administered before the ischemia period, and when trying to use the 50 nM, according to previous works, we evidenced a powerful vasoconstrictor effect. This, added to the fact that we administered it 30 minutes after ischemia, produced a significant increase on the infarct size and the CPP values (Table 1). Therefore, we performed experiments with different decreasing concentrations of phenylephrine, until we obtained a concentration that did not produce significant changes in the CPP behavior compared with the I/R protocol. We were able to evidence in this way a protective effect on the infarct size.

There is evidence supporting the hypothesis that α1-AR stimulation is an important mechanism in the protection conferred by ischemic preconditioning. A number of authors found that ischemic preconditioning and postconditioning share intracellular mechanisms, particularly during early reperfusion. In this regard, stimulation of any Gi protein-coupled receptor has been shown to trigger preconditioning, and a similar involvement of G protein-coupled receptors is being revealed for postconditioning. This study contributes to the understanding of the mechanisms involved in postconditioning because of the fact that the blockade of α1-ARs during ischemic postconditioning modulates infarct size. This

**TABLE 2. Left Ventricular Function and CPP**

| Groups                        | Baseline | 2 Minutes Reperfusion | 15 Minutes Reperfusion | 30 Minutes Reperfusion |
|-------------------------------|----------|-----------------------|------------------------|------------------------|
| LVDP (mm Hg)                  |          |                       |                        |                        |
| I/R                           | 101.6 ± 3.6 | 15.8 ± 3.1*          | 11.3 ± 3.9*           | 12.6 ± 2.6*           |
| Postcon                       | 105.4 ± 4.7 | 9.1 ± 1.4*           | 7.4 ± 1.4*           | 11.4 ± 1.8*           |
| Postcon + Prazosin 1 μM       | 103.9 ± 4.3 | 8.1 ± 1.2*           | 7.6 ± 0.8*           | 10.3 ± 2.3*           |
| Prazosin 1 μM                 | 109.5 ± 3.2 | 15.4 ± 6.1*          | 6.7 ± 1.7*           | 9.9 ± 2.5*            |
| Phenylephrine 50 nM           | 102.1 ± 2.8 | 13.1 ± 1.7*          | 17.2 ± 4.9*          | 19.6 ± 3.8*           |
| Clonidine 10⁻⁶ M              | 99.7 ± 5.2 | 14.2 ± 8.5*          | 18.9 ± 5.2*          | 21.3 ± 6.5*           |
| LVEDP (mm Hg)                 |          |                       |                        |                        |
| I/R                           | 9.4 ± 0.3 | 110.0 ± 4.8*         | 110.9 ± 4.1*         | 104.7 ± 4.5*          |
| Postcon                       | 7.8 ± 0.4 | 117.9 ± 4.2*         | 121.2 ± 4.2*         | 107.1 ± 4.5*          |
| Postcon + Prazosin 1 μM       | 7.3 ± 0.4 | 115.8 ± 9.9*         | 118.9 ± 7.7*         | 108.1 ± 9.8*          |
| Prazosin 1 μM                 | 7.6 ± 0.8 | 92.8 ± 12.7*         | 126.4 ± 12.2*        | 111.3 ± 3.7*          |
| Phenylephrine 50 nM           | 7.9 ± 0.8 | 94.1 ± 4.8*          | 104.1 ± 5.9*         | 100.3 ± 9.7*          |
| Clonidine 10⁻⁶ M              | 8.1 ± 0.7 | 99.8 ± 5.9*          | 112.9 ± 7.2*         | 107.8 ± 8.6*          |
| CPP (mm Hg)                   |          |                       |                        |                        |
| I/R                           | 66.1 ± 1.1* | 87.1 ± 2.3*          | 90.4 ± 2.7*          | 102.3 ± 3.1*          |
| Postcon                       | 67.9 ± 0.9* | 90.1 ± 2.5*          | 89.9 ± 2.7*          | 99.7 ± 3.5*           |
| Postcon + Prazosin 1 μM       | 67.8 ± 3.3 | 87.5 ± 2.7*          | 84.7 ± 3.6*          | 91.8 ± 9.3*           |
| Prazosin 1 μM                 | 63.1 ± 3.2 | 85.8 ± 4.1*          | 101.1 ± 4.9*         | 106.1 ± 11.8*         |
| Phenylephrine 50 nM           | 64.1 ± 2.7 | 83.3 ± 4.5*          | 89.3 ± 3.2*          | 103.4 ± 7.3*          |
| Clonidine 10⁻⁶ M              | 71.1 ± 3.9 | 89.5 ± 6.2*          | 93.2 ± 4.9*          | 101.8 ± 8.9*          |

*P < 0.05 versus baseline.
Postcon, ischemic postconditioning group.

There is evidence supporting the hypothesis that α1-AR stimulation is an important mechanism in the protection conferred by ischemic preconditioning. A number of authors found that ischemic preconditioning and postconditioning share intracellular mechanisms, particularly during early reperfusion. In this regard, stimulation of any Gi protein-coupled receptor has been shown to trigger preconditioning, and a similar involvement of G protein-coupled receptors is being revealed for postconditioning. This study contributes to the understanding of the mechanisms involved in postconditioning because of the fact that the blockade of α1-ARs during ischemic postconditioning modulates infarct size. This

**FIGURE 2. Infarct size.** A, Infarct size expressed as a percentage of the total left ventricular area. Infarct size decreased significantly with ischemic postconditioning, and ischemic postconditioning + prazosin treatment abolished this beneficial effect. Administration of prazosin or clonidine alone, without postconditioning, did not modify infarct size, but phenylephrine administration reduced infarct size. B, Representative slices of the different experimental groups. *P < 0.05 versus I/R, postcon, postcon + prazosin, and prazosin. Postcon, ischemic postconditioning group; PE, phenylephrine group; CL, clonidine group.
suggests that these receptors may trigger the cardioprotection exerted by ischemic postconditioning. Consistent with our hypothesis, the lack of protection on the infarct size after the activation of α2-ARs with clonidine shows that protection would not be conferred by the activation of α2-ARs. Therefore, as it was mentioned, although we are not showing in a direct way the activation of α1-ARs, we strongly suggest that these receptors take part in the postconditioning protective mechanism, at least on isolated rat hearts.

The PI3K–Akt pathway is activated in response to the activation of a wide range of receptors, including G protein-coupled receptors. PI3K–Akt participates in different cellular processes by phosphorylating diverse substrates, including GSK-3β (glycogen and protein metabolism). Tsang et al. demonstrated that ischemic postconditioning protects the isolated rat heart by activating the PI3K–Akt pathway at the time of myocardial reperfusion. Different studies have suggested that Akt may protect the myocardium against I/R injury by acting at the level of the mitochondria. In this regard, Juhaszova et al. demonstrated that ischemic preconditioning protects myocytes by phosphorylating GSK-3β, a downstream target of Akt. In this study, we showed that ischemic postconditioning phosphorylates Akt in the cytosol and GSK-3β in the mitochondria. Regarding α1-ARs in myocardial ischemic protection, Zhao et al. demonstrated the protective effect of preconditioning in rats overexpressing α1-ARs compared with nontransgenic rats as it can be evidenced by a reduction on infarct size. According to the authors, this protective effect involves an increase in extracellular signal-regulated kinases, the mitogen-activated protein kinase phosphorylation, and also the inducible nitric oxide synthase activity and expression. Their findings are in agreement with this study’s results showing that blockade of α1-AR receptors abolishes the protective effect of ischemic postconditioning. The latter involves Akt and GSK-3β, which together with extracellular signal-regulated kinases, mitogen-activated protein kinase, and inducible nitric oxide synthase constitute the RISK-pathway, a group of proteins that activate during reperfusion and that are involved in the protection elicited by ischemic postconditioning. In concordance, our results showed that α1-ARs activation with phenylephrine increased Akt and GSK-3β phosphorylation.

It is known that GSK-3β contributes to different cellular processes including I/R injury. In contrast to many protein kinases, GSK-3β is active in resting cells and is inactivated by phosphorylation. Inhibition of GSK-3β has been shown to reduce apoptosis and enhance cell survival, providing a plausible mechanism by which phosphorylation and inhibition of GSK-3β might mediate cardioprotection. In this regard, Tong et al. showed that ischemic preconditioning reduces infarct size by increasing GSK-3β phosphorylation through a PI3K–Akt–dependent pathway. Also Gómez et al. demonstrated that serine 9 phosphorylation of GSK-3β is required for cardioprotection by postconditioning, and it is likely to act by inhibiting the opening of the mitochondrial permeability transition pore (mPTP) at the time of reperfusion in a transgenic mouse model of reperfused myocardial infarction. In concordance with the fact that GSK-3β has a cardioprotective effect, Terashima et al. demonstrated that the GSK-3β phosphorylation, at the time of reperfusion by a Protein Kinase C-ε, PI3K–Akt, and adenosine A2b receptor-dependent mechanism, contributes to the prevention of myocardial necrosis.
by preischemic activation of the K⁺ mitochondrial channel. Also, it is important to consider the role of matrix metalloproteinases, particularly type 2 (MMP-2), since it is known that the increase of oxidative stress that occurs during reperfusion is one of the main triggers for the activation of this enzyme, and that both preconditioning and postconditioning decrease its activity. This is a fact that could partially explain the mechanisms of cardioprotection. In relation to this concept, Kandasamy and Schulz demonstrated that MMP-2 activates GSK-3β by proteolytic removal of the N-terminal domain where the inhibitory phosphorylation of Serine 9 (Ser9) occurs. Hence, this MMP-2-mediated increase of GSK-3β kinase activity may contribute to myocardial injury observed as a result of oxidative stress. Therefore, we cannot discard that inhibition of MMP-2 by postconditioning could contribute by exerting its cardioprotective effect indirectly, given that the GSK-3β activity in a cell is dynamically regulated in response to stress.

The results of this study demonstrate that activation of α1-ARs induced phosphorylation of Ser9-GSK-3β, because administration of prazosin during the ischemic postconditioning protocol decreased phospho-Ser9-GSK-3β expression and phenylephrine increased phospho-Ser9-GSK-3β expression. Ballou et al demonstrated a differential role of GSK-3β during myocardial I/R. The authors showed that activation of GSK-3β is detrimental during reperfusion but protective during ischemia. Activation of GSK-3β during ischemia and inactivation of GSK-3β during reperfusion are both compensatory for the heart. Ischemic postconditioning acting in the reperfusion phase induces GSK-3β phosphorylation, decreasing its activity and conferring cardioprotection.

In this study, ischemic postconditioning and phenylephrine administration reduced infarct size but were unable to improve ventricular function (contractile state and myocardial stiffness). The absence of improvement of left ventricular function in our study may be due to the presence of myocardial stunning areas peripheral to the infarct zone. In this sense, it has been well shown that the presence of a certain degree of posts ischemic dysfunction (stunned myocardium) reverts approximately after 48/72 hours of reperfusion. Therefore, the change on infarct size in acute experiments does not significantly influence the ventricular function.

In conclusion, the data obtained herein suggest that ischemic postconditioning decreases infarct size by activation of the α1-AR pathway, which could involve Akt and GSK-3β phosphorylation. These current results could indicate that after being phosphorylated, Akt may phosphorylate and inhibit GSK-3β. Further research must be conducted to better understand the mechanism of ischemic postconditioning cardioprotection and to make it fully applicable in the clinical setting.
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