Cardiovascular pharmacology

Cardioprotective efficacy against reperfusion injury of EMD-87580: Comparison to ischemic postconditioning

Juliana Fantinelli a,1, Luisa F González Arbeláez b,1, Susana M Mosca a,*

a Fellow of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Centro de Investigaciones Cardiovasculares, Universidad Nacional de La Plata, La Plata, Buenos Aires, Argentina
b These authors collaborated equally to the work.

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ABSTRACT

Previous results show that prolonged treatment with EMD-87580 (EMD) NHE-1 blocker attenuates and reverses postinfarction remodelling. Our aim was to evaluate the effects of the treatment of EMD compared to ischemic postconditioning (IPO) in a model of regional ischemia. Isolated hearts were subjected to 40-min coronary occlusion followed by 60-min reperfusion (IC). Other hearts were treated with EMD 5 μM during the first 10 min of reperfusion or submitted to one cycle of 2 min of reperfusion and 2 min of ischemia as IPO protocol.

Infarct sizes (IS), postischemic myocardial and vascular functions were assessed. The concentration of thiobarbituric reactive substances (TBARS), reduced glutathione (GSH) and expression of phosphorylated forms of ERK1/2, Akt, GSK-3β, eNOS were analyzed. MnSOD cytosolic activity – as an index of mitochondrial permeability – was also measured. EMD treatment and IPO decreased IS~−50% and significantly improved the postischemic recovery of contractility and coronary perfusion. TBARS decreased and GSH increased after interventions compared to the values observed in IC hearts. MnSOD cytosolic activity increased in IC group and was significantly attenuated by EMD and abolished in IPO hearts. The content of P-ERK1/2 increased whereas P-Akt, P-GSK-3β and P-eNOS decreased in IC hearts. EMD treatment and IPO reversed these changes.

The present data show that EMD treatment at the beginning of reperfusion-similarly to IPO- limited infarct size and attenuated the postischemic impairment of myocardial function through reactive oxygen species-mediated ERK1/2/Akt/GSK-3β/eNOS pathways.

1. Introduction

Activation of the cardiac isoform of Na+ / H+ exchanger (NHE-1) is an important regulator of intracellular pH myocardium during ischemia (Piper et al., 1996) extruding H+ and causing intracellular Na+ overload (Lazdunski et al., 1985), which leads to intracellular Ca2+ overload by activation of reverse mode Na+/Ca2+ exchanger (NCX) (Tani and Neely, 1989). Reactive oxygen species increase at the beginning of reperfusion and together with Ca2+ overload are two mechanisms proposed to explain the postischemic cellular damage (Webster, 2012; Murphy and Steenbergen, 2008). It is difficult to separate cause and effect between these two variables since reactive oxygen species mediate the activation of upstream kinases that promote NHE-1 activation (Sabri et al., 1998), and consequently increase of intracellular Ca2+ concentration. Moreover, increases of Ca2+ and reactive oxygen species are regulators of the formation and/or opening of mitochondrial permeability transition pore (mPTP), a key step in the process of reperfusion injury (Halestrap et al., 2004; Javadov and Karmazyn, 2007). Studies using different experimental models show that NHE-1 inhibition performed before or after ischemia improves the postischemic recovery of myocardial function and limits the infarct size (Mosca and Cingolani, 2008; Scholz et al., 1993; Karmazyn et al., 2001; Avkiran and Marber, 2002; An et al., 2001; Hurtado and Pierce, 2000). It has also been reported that the cardioprotection afforded by the NHE-1 blockers cariporide and KR-32560 (Fantinelli et al., 2006; Jung et al., 2010) is associated to an attenuation of oxidative stress. This beneficial effect appears to be mediated by mitochondrial actions (Jung et al., 2010; Pérez Nuñez et al., 2011; Javadov et al., 2008). However, the effects of NHE-1 specific inhibitor EMD-87580 (2-methyl-4,5-di-(methylsulfonyl-benzoyl)-guanidine), during ischemia and reperfusion are still unclear.

Interesting results from our (Fantinelli and Mosca, 2007) and other laboratories (Zhao et al., 2003; Yang et al., 2004; Iliodromitis et al., 2006) show that the application of brief episodes of
ischemia-reperfusion at the onset of reperfusion intervention called “ischemic postconditioning” (IPO) decreases postischemic alterations. Several kinases such as phosphatidylinositol 3-kinase PI3K/Akt and GSK-3β, ERK1/2 MAPKs have been involved in the mechanism of IPO-mediated cardioprotection (Zhu et al., 2006; Miura and Miki, 2009). However, the participation of those kinases in the actions of NHE-1 blockers during ischemia and reperfusion has not been clearly defined.

The purpose of the present study was to assess the effects of EMD-87580 NHE-1-selective inhibitor applied at the onset of reperfusion compared to IPO on myocardial infarct size and tissue oxidative stress in a model of regional ischemia in rats analyzing the pathways involved.

2. Material and methods

2.1. Isolated heart preparation

All procedures followed during the present investigation conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and to the guidelines laid down by the Animal Welfare Committee of La Plata School of Medicine.

Hearts from male Wistar rats were quickly isolated and perfused with Ringer’s solution containing (in mmol/L) 118 NaCl, 5.9 KCl, 1.2 MgSO4, 1.35 CaCl2, 20 NaHCO3 and 11.0 glucose (gassed with 95% O2–5% CO2, pH 7.4, 37°C) through the Langendorff technique and using the perfusion pump Masterflex Model 7016–21 (Cole-Parmer). The conductive tissue in the atrial septum was damaged with a fine needle to achieve atrioventricular block, and the right ventricle was paced at 280 ± 10 beat/min. A latex balloon tied to the end of a polyethylene tube was passed into the left ventricle through the mitral valve; the opposite end of the tube was then connected to a Statham P23XL pressure transducer. The balloon was filled with water to deliver a left ventricular end-diastolic pressure (LVEDP) of 8–12 mmHg, and this volume remained unchanged for the rest of the experiment. Coronary perfusion pressure (CPP) was monitored at the point of cannulation of the aorta and was adjusted to approximately 60–70 mmHg. Coronary flow (CF), which was controlled with a peristaltic pump, was 11 ± 2 mL/min. Left ventricular pressure (LVP) and its first derivative (dP/dt) were recorded with a direct writing recorder.

2.2. Experimental protocols

After 20-min stabilization, the following experimental protocols were performed (Fig. 1):

Non-ischemic control hearts (NIC; n = 8): Hearts were perfused for 120 min without any treatment.

Ischemic control hearts (IC; n = 9): Heats were subjected to 40 min of occlusion of the left anterior descending (LAD) artery followed by 60 min of reperfusion.

EMD-87580 (EMD, n = 8): EMD was a gift from Merck KGaA, Darmstadt, Germany. Hearts were treated during 120 min after the beginning of reperfusion with a dose of 5 μM of EMD NHE-1 blocker. This dose was found to completely inhibit NHE-1 activity in myocytes in which intracellular acidosis was induced by ammonium chloride pulsing technique (Gan et al., 2010).

Ischemic postconditioning (IPO; n = 8): One cycle of ischemia-reperfusion 2 min each was applied at the onset of reperfusion.

The effects of EMD-87580 in non-ischemic hearts (NIC+EMD; n = 4) administered at the same time as the EMD group were assessed.

Fig. 1. Scheme of the different experimental groups. NIC: Non-ischemic control; NIC+EMD: treatment with the NHE-1 blocker EMD-87580; IC: Ischemic control; EMD: treatment with EMD-87580 and IPO: ischemic postconditioning.

2.3. Infarct size determination

At the end of reperfusion, the LAD was occluded again and the myocardium was perfused for 1 min with a 0.1% solution of Evans blue. This procedure delineated the non-ischemic myocardium as dark blue. After staining, the hearts were frozen and cut into six transverse slices, which were incubated for 15 min at 37°C in 1% solution of triphenyltetrazoliumchloride (TTC). All atrial and right ventricular tissues were excised. To measure myocardial infarction the slices were weighed and scanned. The infarcted (pale), viable ischemic/reperfused (red), and non-ischemic (blue) areas were measured by computed planimetry (Scion Image 1.62; Scion Corp., Frederick, Maryland, USA). By TTC technique non-infarcted viable myocardium containing dehydrogenase stained brick red; whereas the infarcted tissue remained unstained because of the lack of the enzyme. The area at risk (AAR) was the portion of the left ventricle supplied by the previously occluded coronary artery identified by the absence of blue dye. Infarct weights were calculated as (A1 × t1)/(A1 × t1 + (A2 × t2) + (A3 × t3) + (A4 × t4) + (A5 × t5) + (A6 × t6)), where A is the area of infarct in the slice and W is the weight of the respective section. The weight of the AAR was calculated in a similar fashion. IS was expressed as a percentage of AAR (Fantinelli et al., 2013).

2.4. Systolic and diastolic function

The systolic function was assessed by the left ventricular developed pressure (LVEDP) – calculated by subtracting left ventricular end-diastolic pressure (LVEDP) from the left ventricular peak pressure values – and the maximal velocity of rise of LVP (+dP/dtmax). The diastolic function was evaluated through the maximal velocity of relaxation (−dP/dtmax) and LVEDP values.

2.5. Coronary resistance (CR) determination

CR was calculated as a quotient between CPP and CF and expressed as difference between the values obtained at the end of reperfusion period and that observed in the preischemic period.

2.6. Preparation of tissue homogenate

At the end of reperfusion a portion of left ventricle (LV) was homogenized in 5 volumes of PO4KH2 25 mM and CIK 140 mM at pH = 7.4 with a polytron homogenizer. Aliquots of homogenate were used for assessing reduced glutathione content (GSH) and
lipid peroxidation. The remaining homogenate was centrifuged at 12,000g for 5 min at 4 °C and the supernatant stored at −70 °C until superoxide dismutase (SOD) activity was assayed.

2.6.2. Assessment of reduced glutathione (GSH) proteins

Lipid peroxidation was estimated by the measure of the concentration of thiobarbituric acid reactive substances (TBARS) according to the technique described by Buege and Aust (1978). Briefly, hearts were homogenized in physiological saline solution and centrifuged at 2500 rpm, and the reaction was performed in the supernatant. The absorbance was measured at 535 nm and TBARS was expressed in nmol/mg protein using an extinction coefficient of 1.56 × 10^5 (M cm)^{-1}.

2.6.2. Assessment of reduced glutathione (GSH)

GSH was determined by Ellman’s method (Sedlak and Lindsay, 1968). This method is based on the reaction of GSH with 5,5′-dithiobis (2-nitrobenzoic acid) to give a compound that absorbs at 412 nm. GSH levels were expressed as μg/mg of protein.

2.6.3. Measurement of MnSOD cytosolic activity

SOD activity was measured by means of the nitroblue tetrazolium (NBT) method (Beauchamp and Fridovich, 1971). Briefly, the supernatant was added to the reaction mixture of NBT with xanthine–xanthine oxidase, and the SOD activity was measured colorimetrically as inhibitory activity toward blue formazan formation. For measuring MnSOD activity, KCN 5 mmol/L was added to inhibit Cu-ZnSOD.

2.6.4. Immunoblotting

Other portion of LV was homogenized and cytosolic fraction was isolated by differential centrifugation. Briefly, LVwas homogenized in ice-cold RIPA buffer (300 mmol/L sacarosa, 1 mmol/L DTT, 4 mmol/L EGA, 20 mmol/L TRIS pH 7.4, 1% Triton X, 10% protease cocktail, 25 μmol/L FNa, 1 mmol/L Ortovanadate) and centrifuged at 12,000g for 15 min at 4 °C. Supernatant proteins (60 μg) were resolved on SDS-PAGE and transferred to PVDF membrane (2 h). Equal loading of samples was confirmed by Ponceau S staining. Membranes were blocked with 5% non-fat milk in Tris-buffered saline (pH 7.5) containing 0.1% Tween (TBS-T), and probed overnight at 4 °C with antibodies anti-P-GSK-3β (1:1000, Santa Cruz Biotechnology), anti-Akt (1:1000, Santa Cruz Biotechnology), anti-P-ERK1/2 (1:1000, Millipore), anti-P-ENOS, and anti-P-14-3-3 protein (1:1000, Millipore). The phospho-Ser 14-3-3 binding motif antibody represents a useful tool for estimating NHE-1 Ser703 phosphorylation as previously reported (Lehoux et al., 2001) and recently applied in our laboratory (Garciaarena et al., 2008). Membranes were washed four times for 10 min in TBS-T prior to the addition of anti-rabbit secondary antibody (1:5000, Santa Cruz Biotechnology) and protein bands were analyzed by a chemiluminescent system (ECL Plus; Amersham Biosciences). Total content of enzyme or GAPDH signal was used as a loading control.

2.7. Statistical analysis

Data were expressed as means ± S.D. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Newman–Keul’s post test used for multiple comparisons among groups. Values of P < 0.05 were considered to indicate statistical significance.

3. Results

In NIC group the IS was small and it was not modified after EMD addition (Table 1). Forty min of coronary occlusion followed by 1-h reperfusion in isolated rat hearts without any treatment caused an IS of ~40% of the AAR. The AAR for all interventions was similar and represented ~32% of the left ventricle. A significant reduction in IS was obtained when 5 μmol of EMD-85780 (EMD) NHE-1 specific blocker was added to the perfusate during the first 10 min of reperfusion or when ischemic postconditioning was applied (Fig. 2).

A the end of stabilization period, the absolute values of LVDP, LVEDP, +dP/dt_max and −dP/dt_max were similar for all experimental groups (Table 1). At the end of 2-h non-ischemic control hearts exhibited a decrease in contractility of approximately 10%. This behaviour was not altered when NIC hearts were treated with EMD. In ischemic control hearts a decrease in LVDP to values of 34 ± 5% from baseline was detected at the end of the reperfusion period. At this time the addition of EMD improved LVDP reaching values close to 70%. IPO, similarly to EMD, significantly increased the postischemic recovery of systolic function. A similar pattern was observed when +dP/dt_max was analyzed (Fig. 3A). By examining −dP/dt_max an improvement of relaxation velocity after treatment with EMD or IPO application was also evident. The LVEDP (an index of diastolic stiffness) was approximately 13 mmHg at the end of the stabilization period in all experimental groups. This parameter significantly increased reaching a value of approximately 50 mmHg at the end of reperfusion after 40-min coronary occlusion. EMD treatment and IPO significantly reduced the LVEDP increase. In other words, myocardial diastolic stiffness of EMD treated and postconditioned hearts was lesser in comparison to ischemic control hearts (Fig. 3B).

No-reflow phenomenon, a disorder that interrupts the microcirculation during reperfusion, is also involved in reperfusion injury (Schwartz and Kloner, 2012). Although we did not assess the effects of our intervention on that phenomenon, changes of coronary perfusion pressure (CPP) at constant coronary flow could be indicating changes in the area of no-reflow. Thus, our data show

<table>
<thead>
<tr>
<th>Group</th>
<th>IS (%)</th>
<th>LVDP (mmHg)</th>
<th>LVEDP (mmHg)</th>
<th>+dP/dt_max (mmHg/s)</th>
<th>−dP/dt_max (mmHg/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
<td>120</td>
<td>20</td>
<td>120</td>
<td>20</td>
</tr>
<tr>
<td>NIC</td>
<td>1.6 ± 0.4</td>
<td>78 ± 3</td>
<td>68 ± 3</td>
<td>13.3 ± 1.4</td>
<td>16 ± 4</td>
</tr>
<tr>
<td>NIC + EMD</td>
<td>2.0 ± 0.5</td>
<td>79 ± 3</td>
<td>65 ± 5</td>
<td>12.8 ± 0.9</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>IC</td>
<td>37 ± 2a</td>
<td>81 ± 4</td>
<td>28 ± 4b</td>
<td>12.6 ± 0.5</td>
<td>54 ± 9b</td>
</tr>
<tr>
<td>IC + EMD</td>
<td>12 ± 2b</td>
<td>80 ± 4</td>
<td>59 ± 5b</td>
<td>13.1 ± 0.6</td>
<td>23 ± 3b</td>
</tr>
<tr>
<td>POS</td>
<td>12 ± 2b</td>
<td>81 ± 3</td>
<td>55 ± 3b</td>
<td>13.1 ± 0.6</td>
<td>22 ± 3b</td>
</tr>
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</table>

* P < 0.05 respect to NIC and NIC + EMD.

b P < 0.05 respect to IC.
that the reperfusion produced an increase in CPP and consequently an increment of coronary resistance (CR) which was significantly attenuated by EMD treatment and did not increase in postconditioned hearts (Fig. 4). The addition of EMD did not modify the CR detected in NIC hearts at 70 min of perfusion. The values were $7.00 \pm 0.28$ and $7.22 \pm 0.46 \text{mmHg/mL \times min}^{-1}$ in EMD+NIC and NIC groups, respectively.

Fig. 2. Infarct size (IS), expressed as a percentage of risk area (AAR), in ischemic control (IC, n=9), hearts treated with EMD-87580 (EMD, n=8) and postconditioned (IPO, n=8). Observe that EMD treatment and IPO decreased the IS obtained in IC hearts. *$P<0.05$ vs. IC.

Fig. 3. (A) Values of left ventricular developed pressure (LVDP) and maximal rise velocity of left ventricular pressure ($+\text{dP/dt}_{\text{max}}$) at the end of reperfusion, expressed as percentage of its respective preischemic values. (B) Data of maximal decrease velocity of left ventricular pressure ($-\text{dP/dt}_{\text{max}}$, expressed as % of preischemic value) and left ventricular end diastolic pressure (LVEDP, expressed in mmHg) at the end of reperfusion, in IC (n=9), EMD (n=8) and IPO (n=8) groups. Note that NHE-1 blockade by EMD and ischemic postconditioning (IPO) significantly improved the postischemic recovery of systolic function and relaxation velocity and attenuated the increase of diastolic stiffness detected in IC hearts. *

Fig. 4. Changes of coronary resistance (CR) expressed as mmHg/mL \times min}^{-1}, in ischemic control (IC, n=9) and EMD-87580 (EMD, n=8) treated and postconditioned hearts (IPO, n=8). The NHE-1 blockade attenuated the increase of CR detected in IC hearts whereas in preconditioned hearts CR did not increase. *$P<0.05$ vs. IC; NS: IPO at the end of reperfusion vs IPO at the baseline.

Fig. 5. Effects of NHE-1 blocker, EMD-87580 (EMD, n=6) and ischemic postconditioning (IPO, n=5) on thiobarbituric acid reactive substances concentration (TBARS, upper panel) and reduced glutathione content (GSH, lower panel). Both treatments decreased the lipid peroxidation and partially preserved the GSH levels compared with IC (n=6) hearts. GSH content was significantly lesser than that detected in NIC (n=6) hearts. *$P<0.05$ vs. NIC; *$P<0.05$ vs. IC.
Given that reactive oxygen species generation and the consequent tissue damage may be responsible for myocardial reperfusion injury (Yellon and Hausenloy, 2007; Loor et al., 2011), we next determined the impact of EMD treatment and IPO on myocardial TBARS concentration—an index of lipid peroxidation—and level of GSH. In ischemic control hearts TBARS increased and GSH decreased. These changes were significantly attenuated by EMD and IPO (Fig. 5).

Since the increase in reactive oxygen species production may stimulate the ERK1/2 pathway leading to NHE-1 phosphorylation and activation (Sabri et al., 1998; Rothstein et al., 2002), we then analyzed the phosphorylation state of the NHE-1 and its upstream kinase at the end of the reperfusion period. NHE-1 phosphorylation at Ser703 was estimated by quantifying levels of P-14-3-3 binding motif, as explained in methods. In ischemic control hearts the content of P-ERK1/2 and P-14-3-3 increased. Both interventions (EMD and IPO) attenuated these changes but the values remained elevated in comparison to that observed in non-ischemic hearts (Fig. 6).

Previous studies revealed that IPO exerts its protective effects through the recruitment of prosurvival kinases such as phosphatidylinositol 3-kinase (PI3K)-Akt, GSK-3β as well as endothelial nitric oxide synthase (eNOS) (Zhu et al., 2006; Miura and Miki, 2009; Penna et al., 2006). In our experimental preparation the expression of P-Akt, P-GSK-3β and P-eNOS showed the same pattern diminishing after ischemia and reperfusion and increasing in hearts treated with EMD and postconditioned hearts (Fig. 7).

Taking into account that MnSOD is localized in mitochondria its presence in cytosol is an indirect evidence of increased mitochondrial permeability (Fantinelli et al., 2013). Thus, an increase of the MnSOD cytosolic activity was detected after ischemia and reperfusion. EMD treatment attenuated and IPO did not change the cytosolic activity of that enzyme (Fig. 8).

4. Discussion

In the current study we demonstrate that the acute treatment with EMD-87580 NHE-1 specific blocker at the beginning of reperfusion, similarly to ischemic postconditioning, reduces the infarct size, improves the postischemic recovery of contractility, and attenuates oxidative stress.

Although results obtained by us (Mosca and Cingolani, 2008; Fantinelli et al., 2006; Pérez Nuñez et al., 2011) and other laboratories (Karmazyn et al., 2001; Avkiran and Marber, 2002; An et al., 2001; Hurtado and Pierce, 2000) show that the NHE-1 blockade at the beginning of reperfusion is effective against reperfusion injury, the effects of EMD are not conclusive yet. Previously, it was demonstrated that the chronic treatment of EMD is able to attenuate postinfarction remodeling and heart failure without any modification of infarct size (Chen et al., 2004). Our data clearly show that the acute administration of EMD at the onset of reperfusion was effective to reduce the infarct size and improve the systolic and diastolic postischemic function. On the other hand, although the IPO has been extensively examined (Fantinelli and Mosca, 2007; Zhao et al., 2003; Yang et al., 2004; Iliodromitis et al., 2006), the fact that its beneficial effects were...
The no-reflow phenomenon is related to the inability to reperfuse regions of the myocardium after ischemia, despite removal of coronary artery occlusion. The no-reflow zone expands during the first few hours of reperfusion suggesting an element of reperfusion injury. In animal models, extensive no-reflow was associated with worse infarct expansion (Kloner, 2011). It was previously suggested that IPO reduces the no-reflow phenomenon (Loukogeorgakis et al., 2006). Furthermore, a recent paper shows that NHE-1 inhibition improves tissue perfusion (Wu et al., 2013). (Loukogeorgakis et al., 2006). Furthermore, a recent paper shows that reactive oxygen species can activate NHE-1, and by Rothstein et al. (2002) that approximately 50% of myocardial Ca^2+ overload after reperfusion was due to reactive oxygen species-mediated NHE-1 activation through an ERK1/2 MAP kinase signalling pathway. Considering that calcium overload is a determinant of mitochondrial permeability, a diminution of intracellular calcium consequent to NHE-1 blockade could be contributing to a reduction of mPTP opening as previously reported (Toda et al., 2007). This fact could lead to a minor reactive oxygen species release which in small amount acts as signalling molecules triggering cardioprotective pathways (Tsutsumi et al., 2007). It was also demonstrated that the persistent acidosis during the first min of reperfusion could mimic the protection of IPO (Cohen et al., 2007) and that a delayed normalization of intracellular pH by IPO could occur via inhibition of NHE-1 (Inserte et al., 2001). An increase of ERK1/2 and NHE-1 phosphorylation was detected in ischemic control hearts while lower values were found in EMD treated or postconditioned hearts allowing us to speculate that both interventions are preserving mitochondrial integrity. These results are in agreement with previous reports (Javdov et al., 2006, 2008; Garcia et al., 2008; Arcand et al., 2005). On the other hand, it has been reported by Sabri et al. (1998) and Snabaitis et al. (2008) that reactive oxygen species can activate NHE-1, and by Rothstein et al. (2002) that approximately 50% of myocardial Ca^2+ overload after reperfusion was due to reactive oxygen species-mediated NHE-1 activation through an ERK1/2 MAP kinase signalling pathway. Considering that calcium overload is a determinant of mitochondrial permeability, a diminution of intracellular calcium consequent to NHE-1 blockade could be contributing to a reduction of mPTP opening as previously reported (Toda et al., 2007). This fact could lead to a minor reactive oxygen species release which in small amount acts as signalling molecules triggering cardioprotective pathways (Tsutsumi et al., 2007). It was also demonstrated that the persistent acidosis during the first min of reperfusion could mimic the protection of IPO (Cohen et al., 2007) and that a delayed normalization of intracellular pH by IPO could occur via inhibition of NHE-1 (Inserte et al., 2001). An increase of ERK1/2 and NHE-1 phosphorylation was detected in ischemic control hearts while lower values were found in EMD treated or postconditioned hearts. Taking into account that both interventions attenuate the oxidative damage the decrease of P-ERK1/2 followed by a reduction of P-NHE-1 could be interpreted as a consequence of reactive oxygen species reduction. In other words, we could say that both interventions (EMD and IPO) have a common and crucial point for the cardioprotective action which is the decreased NHE-1 activity through a reactive oxygen species-dependent pathway.

The phosphorylation of specific serine residue (Ser9)/inhibition of GSK-3β plays an important role in different cardioprotective interventions (Zhu et al., 2006). Among the kinases able to activate GSK-3β the PI3K/Akt is found which is involved in amelioration of reperfusion injury. The powerful cardioprotection seen with postconditioning and NHE-1 inhibition has been linked to increased phosphorylation of Akt (Jung et al., 2010; Zhu et al., 2006; Miura and Miki, 2009). In ischemic control hearts we observed a diminution of P-Akt and P-GSK-3β at the end of reperfusion. These data are in disagreement with those recently reported by Inserte et al. (2013) and could be attributed to the differences in experimental design and rat specie. Following the interventions we detected an increase in the expression of phosphorylated forms of those kinases showing EMD treated hearts the highest values. This result can be explained considering that the diminution

obtained by the application of only one cycle of ischemia-reperfusion is an important finding of this study.

What are the mechanisms involved in these cardioprotective actions afforded by EMD and IPO? reactive oxygen species are generated during ischemia and increase significantly within min after reperfusion, contributing to membrane lipid peroxidation, oxidative changes in protein structure/function, and oxidative damage to DNA (Robin et al., 2007). Several reactive oxygen species scavenging enzymes contribute to the antioxidant defences. Glutathione represents the major low molecular weight antioxidant redox recycling thiol in mammalian cells and plays a central role in the cellular defence against oxidative damage. In ischemic control hearts we found an increase of lipid peroxidation and a depletion of GSH content. These changes were significantly attenuated by EMD and IPO indicating a diminution of oxidative stress after interventions. According to recent studies NHE-1 blockade and IPO are able to reduce the superoxide production (Garcia et al., 2008; Inserte et al., 2013; Javdov et al., 2006). Therefore, our data are suggesting that a lesser reactive oxygen species production would take place in intervened compared to non-intervened hearts.

The second question to resolve is about the mechanisms involved in the reduction of reactive oxygen species and its consequences. In this sense, being the mitochondria the main site of reactive oxygen species production our attention was directed to agents able to modify the activity of that organelle. The mitochondrial inner membrane is usually impermeable to all but a few selected metabolites and ions. However, when mitochondria are exposed to high calcium concentration this permeability barrier is lost. The loss of internal mitochondrial membrane impermeability leads to the release of mitochondrial matrix components, as MnSOD to cytosol. Thus, an increase of cytosolic MnSOD activity was detected in ischemic control hearts indicating an increase of mitochondrial permeability. Its attenuation in EMD and postconditioned hearts allows us to speculate that both interventions are preserving mitochondrial integrity. These results are in agreement with previous reports (Javdov et al., 2006, 2008; Garcia et al., 2008; Arcand et al., 2005). On the other hand, it has been reported by Sabri et al. (1998) and Snabaitis et al. (2008) that reactive oxygen species can activate NHE-1, and by Rothstein et al. (2002) that approximately 50% of myocardial Ca^2+ overload after reperfusion was due to reactive oxygen species-mediated NHE-1 activation through an ERK1/2 MAP kinase signalling pathway. Considering that calcium overload is a determinant of mitochondrial permeability, a diminution of intracellular calcium consequent to NHE-1 blockade could be contributing to a reduction of mPTP opening as previously reported (Toda et al., 2007). This fact could lead to a minor reactive oxygen species release which in small amount acts as signalling molecules triggering cardioprotective pathways (Tsutsumi et al., 2007). It was also demonstrated that the persistent acidosis during the first min of reperfusion could mimic the protection of IPO (Cohen et al., 2007) and that a delayed normalization of intracellular pH by IPO could occur via inhibition of NHE-1 (Inserte et al., 2001). An increase of ERK1/2 and NHE-1 phosphorylation was detected in ischemic control hearts while lower values were found in EMD treated or postconditioned hearts. Taking into account that both interventions attenuate the oxidative damage the decrease of P-ERK1/2 followed by a reduction of P-NHE-1 could be interpreted as a consequence of reactive oxygen species reduction. In other words, we could say that both interventions (EMD and IPO) have a common and crucial point for the cardioprotective action which is the decreased NHE-1 activity through a reactive oxygen species-dependent pathway.

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![Graph showing ERK1/2 and 14-3-3 Protein Phosphorylation](image.png)

**Fig. 8.** Effects of NHE-1 blocker, EMD-87580 (EMD, n=6) and ischemic postconditioning (IPO, n=5) on SODMn cytosolic activity, expressed as percentage of inhibition per mg of protein. EMD treatment significantly attenuated the increase of cytosolic activity of SODMn detected in ischemic control hearts (IC, n=6). In non-ischemic control (NIC, n=6) and postconditioned hearts (IPO) the activity of that enzyme did not change. *P< 0.05 vs. NIC; **P< 0.05 vs. IC.
of mitochondrial calcium consequent to NHE-1 blockade is an additional factor to those promoted by IPO. Anyway the data suggest that the infarct-limiting effect afforded by both interventions is linked in a cause-effect relationship to GSK-3β-dependent mechanism.

NO bioavailability is hampered either through a decrease in its production by the presence of endogenous NOS inhibitors, by uncoupling of NOS due to tetrahydrobiopterin oxidation or by its oxidative inactivation via its reaction with superoxide. A relevant pathway that decreases NO levels and therefore inactivates its signalling activities is the diffusion-controlled reaction with superoxide that gives rise to the powerful oxidizing and nitrating agent, peroxynitrite (ONOO−), which is phosphorylated, is modulated by the balanced actions of protein kinases (Akt, PKA, PKC, AMPK), protein phosphatases, and protein-protein interactions. Regulation of eNOS by phosphorylation is relatively complex because eNOS activity is increased by phosphorylation at Ser1177 but decreased by Thr495 phosphorylation (Mount et al., 2007). Importantly, regulation of eNOS phosphorylation by specific kinases and phosphatases can vary depending on the activating stimulus and/or the tissue bed (Kukreja and Xi, 2007). In the current study we detected a diminution of P-eNOS in hearts submitted to 40-min regional ischemia and 1-h reperfusion. This result could be consequent of a diminution of P-Akt and/or an ONOO− mediated inactivation (Chen et al., 2010). Hearts treated with EMD or postconditioned showed increase of P-eNOS probably mediated by reactive oxygen species reduction and increase of P-Akt. These data suggest that NO participates in the cardioprotective pathways making it possible the attenuation of mitochondrial permeability.

5. Conclusions

The present study demonstrates that one cycle of ischemia-reperfusion and EMD-87580 treatment at the beginning of reperfusion protects the myocardium against alterations subsequent to ischemia and reperfusion. Our data also provide evidence on the participation of ERK1/2/Akt/GSK-3β/eNOS reactive oxygen species-mediated pathways in the cardioprotective effects being the NHE-1 blockade a common and crucial event of both interventions (Fig. 9). Moreover, our findings reinforce the beneficial effects of NHE-1 blockers at experimental level previously reported.

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