Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2011;27:13-22

Accepted: December 27, 2010

Myocardial Reperfusion Injury: Reactive Oxygen Species vs. NHE-1 Reactivation

Carolina D. Garciarena[1], Juliana C. Fantinelli[1], Claudia I. Caldiz[2], Gladys Chiappe de Cingolani[3], Irene L. Ennis[3], Néstor G. Pérez[3], Horacio E. Cingolani[3] and Susana M. Mosca[3]

Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas, Universidad Nacional de La Plata (UNLP), La Plata, Argentina, [1]Fellows of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), [2]Investigator of UNLP, [3]Established Investigators of CONICET

Key Words

Phosphodiesterase 5A • NHE-1 • Reactive oxygen species • Reperfusion injury

Abstract

Background/Aims: Flow restoration to ischemic myocardium reduces infarct size (IS), but it also promotes reperfusion injury. A burst of reactive oxygen species (ROS) and/or NHE-1 reactivation were proposed to explain this injury. Our study was aimed to shed light on this unresolved issue. Methods: Regional infarction (40 min-ischemia/2 hs-reperfusion) was induced in isolated and perfused rat hearts. Maximal doses of N-(2-mercaptopropionyl)-glycine (MPG 2mmol/L, ROS scavenger), cariporide (10μmol/L, NHE-1 inhibitor), or sildenafil (1μmol/L, phosphodiesterase5A inhibitor) were applied at reperfusion onset. Their effects on IS, myocardial concentration of thiobarbituric acid reactive substances (TBARS), ERK1/2, $p90^{RSK}$, and NHE-1 phosphorylation were analyzed. Results: All treatments decreased IS ~ 50% vs. control. No further protection was obtained by combining cariporide or MPG with sildenafil. Myocardial TBARS increased after infarction and were decreased by MPG or cariporide, but unaffected by sildenafil. In line with the fact that ROS induce MAPK-mediated NHE-1 activation, myocardial infarction increased ERK1/2, $p90^{RSK}$, and NHE-1 phosphorylation. MPG and cariporide cancelled these effects. Sildenafil did not reduce the phosphorylated ERK1/2-$p90^{RSK}$ levels but blunted NHE-1 phosphorylation suggesting a direct dephosphorylating action. Conclusions: 1) Reperfusion injury would result from ROS-triggered MAPK-mediated NHE-1 phosphorylation (and reactivation) during reperfusion; 2) sildenafil protects the myocardium by favouring NHE-1 dephosphorylation and bypassing ROS generation.

Introduction

Following an acute myocardial infarction (MI), early coronary artery reperfusion remains to be the most effective intervention to decrease infarct size (IS), which is a major determinant of the patient's clinical outcome. However, the process of restoring blood flow to the ischemic zone induces cellular injury and cardiomyocyte death, thus increasing IS and reducing the beneficial effect of reperfusion. Reperfusion injury may explain why,

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1015-8987/11/0271-0013$38.00/0

Accessible online at:
www.karger.com/cpb

Dr. Néstor Gustavo Pérez
Centro de Investigaciones Cardiovasculares
Facultad de Ciencias Médicas, UNLP
60 y 120 (1900) La Plata (Argentina)
Tel./Fax (+54-221) 483-4833, E-Mail gperez@atlas.med.unlp.edu.ar

despite early myocardial reperfusion, IS as well as the incidence of heart failure is still elevated, and why several therapeutic strategies are addressed to its decrease.

The reactive oxygen species (ROS) burst at the beginning of reperfusion and cytosolic Ca^{2+} overload are two proposed mechanisms to explain cell injury. It is difficult to separate cause-and-effect relationship between these variables, since ROS by activating kinases upstream the cardiac Na^+/H^+ exchanger (NHE-1) may induce Ca^{2+} overload, and both ROS and Ca^{2+} are known to be mediators in the regulation of mitochondrial permeability transition pore (MPTP) formation [1], a key step in the process of reperfusion injury.

Inhibition of NHE-1 is one of the most powerful interventions known to protect the heart against ischemia/reperfusion injury in experimental animal models [2-8]. Phosphodiesterase 5A (PDE5A) inhibitors also emerged as powerful preconditioning-like, cardioprotective agents that decrease IS [9-11]. PDE5A hydrolyses the phosphodiester bond of cyclic GMP increasing the intracellular concentration of cGMP with the consequent activation of PKG. In a recent study, we reported that chronic treatment with the PDE5A inhibitor, sildenafil (SIL), was able to improve post MI remodeling and function [12]. The beneficial effect of SIL was accompanied by activation of protein kinase G (PKG) and inhibition of NHE-1. The ability of cGMP/PKG to inhibit the NHE was previously demonstrated in tissues other than myocardium [13-16]. Moreover, we recently reported in mammalian myocardium that the effect of PDE5A inhibitors on NHE-1 activity was linked to a PKG-mediated phosphatase activation that dephosphorylates the exchanger [17, 18]. Taken together, these data suggest that the acute beneficial effects of both NHE-1 and PDE5A inhibitors upon ischemia/reperfusion injury would be a linked phenomenon.

Experiments in which IS was measured after ischemia and reperfusion were performed, and the effects of three pharmacological interventions (NHE-1 inhibition, ROS scavenging and PDE5A inhibition) initiated during the early phase of reperfusion were compared. We provide evidence supporting the idea that the three of them reduce IS by converging at a common step the prevention of NHE-1 activation.

Materials and Methods

All procedures followed during this 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.

Isolated heart preparation

Hearts from male Wistar rats were isolated and Langendorff perfused with Ringer's solution containing (in mmol/L) 118 NaCl, 5.9 KCl, 1.2 $MgSO_4$, 1.35 $CaCl_2$, 20 $NaCO_3H$ and 11.0 glucose (gassed with 95% O_2-5% CO_2, pH 7.4, 37°C), as described elsewhere [19].

Experimental protocols

MI was induced after 20 min of stabilization by occluding the left anterior descending coronary artery (LAD) during 40 min followed by 120 min of reperfusion. During the first 20 min of reperfusion the following interventions were applied: (1) preischemic solution (no pharmacological intervention); (2) specific NHE-1 blockade with cariporide (10 μmol/L); (3) ROS scavenging with MPG (2 mmol/L); (4) PDE5A inhibition with SIL (1 μmol/L); (5) combination of SIL (1 μmol/L) + cariporide (10 μmol/L); (6) combination of SIL (1 μmol/L) + MPG (2 mmol/L).

IS determination

IS was assessed by the tripenyltetrazoliumchloride (TTC) staining technique [20]. At the end of reperfusion, the LAD was occluded again and the myocardium was perfused during 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 5 minutes at 37°C in 1% solution of 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. According to previous data [21], the reperfusion time used in this study is enough to minimize the presence of pink-and-white area patches which difficult the accuracy of IS measurements. 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 × W1) + (A2 × W2) + (A3 × W3) + (A4 × W4) + (A5 × W5) + (A6 × W6), 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.

Systolic and diastolic function

Myocardial contractility was evaluated by the isovolumetric left ventricular developed pressure (LVDP), and calculated by subtracting left ventricular end diastolic pressure (LVEDP) from the LV peak pressure values, and the maximal velocity of rise of LVP ($+dP/dt_{max}$). Data are expressed as

percent of the corresponding pre-ischemic control values. Diastolic function was evaluated by isovolumetric LVEDP.

Assessment of lipid peroxidation

We used the thiobarbituric acid reactive substances (TBARS) spectroscopic technique [22] to evaluate lipid peroxidation as index of ROS formation. Briefly, in separated groups of hearts subjected to the same protocols at the end of the reperfusion period cardiac tissue was homogenized in physiological saline solution and centrifuged at 2500 rpm to allow measuring TBARS in the supernatant. Absorbance at 535nm was measured and TBARS expressed in nmol/g of tissue using an extinction coefficient of $1.56x10^5$ $M^{-1}cm^{-1}$.

Preparation of cardiac slices

Hearts were quickly removed from previously anesthetized animals and perfused through the aorta with Ringer buffer pH 7.4 to eliminate blood. Tissue slices from the left ventricle (1x5 mm) were cut and kept at 4°C until assayed. Assay buffer consisted of Krebs-Hepes buffer of the following composition in mmol/L: 118.3 NaCl; 4.7 KCl; 1.8 $CaCl_2$; 1.2 $MgSO_4$; 1.0 K_2HPO_4; 25 $NaHCO_3$; 11 glucose; 20 HEPES (pH 7.4 after 1.5 h gassed with 95% O_2-5% CO_2, pH 7.4, 37°C). Cardiac tissue was incubated in the assay buffer during 30 min in the presence of different drugs in a metabolic incubator under 95% O_2/ 5% CO_2 at 37 °C before measurements of superoxide anions ($\cdot O_2^-$) production.

Measurements of $\cdot O_2^-$ production

We used lucigenin-enhanced chemiluminescence to measure $\cdot O_2^-$ production by rat cardiac slices in Krebs-Hepes buffer with 5 μmol/L lucigenin. Chemiluminescence (arbitrary units, AU) was recorded with a luminometer (Chameleon, Hidex) during 30 seconds every 4.5-min during 30 min. The lucigenin-containing assay buffer with tissue slices minus background, as well as responses to the different drugs assayed were reported. $\cdot O_2^-$ production was normalized to mg dry weight tissue per minute. Control tissue slices without pharmacological intervention produced low levels of $\cdot O_2^-$ which were only slightly above background. The increase in $\cdot O_2^-$ production was expressed as percent of basal value after 15 minutes of each intervention.

Determination of extracellular signal-regulated protein kinases (ERK1/2) and p90 ribosomal S6 kinase ($p90^{RSK}$)

Cardiac tissue was homogenized in lysis buffer (300 mmol/L sucrose; 1 mmol/L DTT; 4 mmol/L EGTA, protease inhibitors cocktail (Complete Mini Roche); 20 mmol/L Tris-HCl, pH 7.4). After a brief centrifugation the supernatant was kept and protein concentration determined by the Bradford method. Samples were denatured and equal amounts of protein subjected to PAGE and electrotransferred to PVDF membranes. After blocking with non-fat-dry milk, membranes were incubated overnight either with anti-phospho-ERK1/2 or anti-phospho- $p90^{RSK}$ polyclonal antibodies (Santa Cruz Biotechnology). A peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) was used as secondary antibody. Bands were visualized using the ECL-Plus chemiluminescence detection system (Amersham). Autoradiograms were analyzed by densitometric analysis (Scion Image). GAPDH signal was used as a loading control.

NHE-1 phosphorylation

NHE-1 phosphorylation was estimated in NHE-1 immunoprecipitated samples using an anti-P-14-3-3 protein binding motif antibody as previously described [17, 23]. It has been shown that the regulatory Ser703 in the NHE-1 lies within a sequence which creates a binding motif for 14-3-3 proteins upon ERK/$p90^{RSK}$ phosphorylation; thus, the phospho-Ser 14-3-3 binding motif antibody represents a useful tool for estimating NHE-1 Ser703 phosphorylation [24-26]. Briefly, ventricular myocardium was homogenized in lysis buffer at pH 7.5 containing (in mmol/L) Tris-HCl 50, EGTA 5, EDTA 2, NaF 100, and Na_3VO_4 1, as well as 0.05% digitonin and protease inhibitor cocktail (Complete Mini, Roche). The homogenate was then centrifuged at 14,000 × *g* for 30 min at 4 °C and the supernatant discarded. The pellet was then solubilized in ice-cold immunoprecipitation lysis buffer at pH 7.5 containing (in mmol/L) Tris-HCl 20, NaCl 150, EDTA 1, EGTA 1, sodium pyrophosphate 2.5, β–glycerophosphate 1, Na_3VO_4 1, and NaF 100, as well as 1% Triton X-100 and protease inhibitor cocktail (Roche). The samples were centrifuged at 14,000 × *g* for 60 min at 4 °C, after which the supernatant containing the solubilized membranes was removed and protein concentration determined by the Bradford assay. Fixed amounts of protein (400 μg) were then incubated with rabbit polyclonal NHE-1 antibody (4 μL; Millipore) for 3 hs. at 4 ºC . Then, immune complexes were mixed with protein A/G Plus-Agarose (Santa Cruz Biotechnology, Inc.) for 3 h at 4 °C and washed three times with ice-cold immunoprecipitation buffer. The immune complexes were dissociated by the addition of Laemmli sample buffer and heating for 3 min at 60 °C. Proteins were resolved on 7 % SDS-PAGE and analyzed by Western immunoblotting using mouse polyclonal phospho-(Ser) 14-3-3 binding-motif protein antibody (Cell Signaling). Peroxidase-conjugated anti-rabbit (Santa Cruz Biotechnology) was used as secondary antibody and bands were visualized using the ECL-Plus chemiluminescence detection system (Amersham). After stripping, the same membrane was re-probed with anti- NHE antibody, and results shown as the ratio of phosphorylated to total protein. Autoradiograms were analyzed by densitometric analysis (Scion Image).

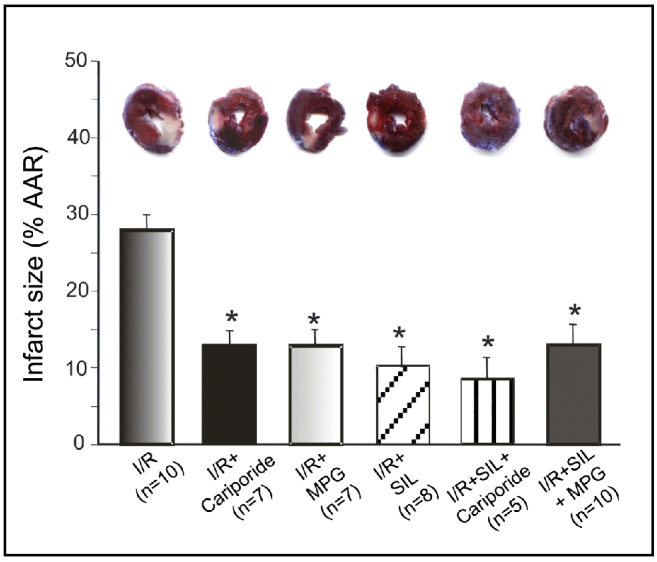
Chemicals

All drugs used in the present study were of analytical grade. Ang II, lucigenin and N-(2-mercaptopropionyl)-glycine (MPG) were purchased from Sigma; cariporide (Aventis); SIL was generously donated by Roemmers Argentina. Either assay buffer or dimethyl sulfoxyde (DMSO) was used to prepare the drugs. For the $\cdot O_2^-$ assay final DMSO concentration was kept <0.5% which did not interfere with the lucigenin signal.

Statistics

Results are presented as mean±SEM. Statistical analysis was performed using the Student's *t*-test or one-way ANOVA

Fig. 1. Forty minutes of regional ischemia followed by 2 hours of reperfusion in the rat myocardium produced an IS of ~30 % of the AAR (I/R). Cariporide, MPG, or SIL reduced IS to a similar extent (~50% less than for non-treated hearts). Combinations of SIL with either cariporide or MPG did not induce further reduction in IS. Original TTC-stained cross sectional slices from a representative heart of each experimental protocol are displayed on top of the corresponding averaged bar graph. * P < 0.05 vs. I/R.



followed by the Student-Newman-Keuls test, when necessary. *P*<0.05 was considered significant.

Results

Forty minutes of regional ischemia followed by 2 hours of reperfusion in rat hearts without any treatment caused an IS of ~30 % of the AAR (Fig. 1). The AAR for all interventions was similar and represented ~32 % of the left ventricle. A significant reduction in IS was obtained when 10 µmol/L cariporide was added to the perfusate during the first 20 minutes of reperfusion (Fig. 1), a result in agreement with previous reports [19, 27-29].

Animal studies have demonstrated an increase in ROS generation following reperfusion, with a burst that peaks 2-3 minutes after reflow and lasts for about 20 minutes [30-32]. However, the beneficial effect of scavengers or antioxidants during reperfusion is still controversial [33-37]. In our hands, when ROS production was mitigated by reperfunding the hearts in the presence of 2 mmol/L MPG (ROS scavenger), a reduction in IS of similar magnitude to that observed after cariporide was achieved (Fig. 1).

Since PDE5A inhibitors were shown to possess powerful cardioprotective properties in ischemia/reperfusion [9-12], we next explored whether SIL could also decrease IS when administered early in the reperfusion. As shown in Figure 1, SIL (1 µmol/L) reduced the IS similarly to the other two pharmacological interventions used. Interestingly, the combination of SIL and cariporide or SIL and MPG did not reduce the IS further (Fig. 1), suggesting that the three interventions probably targeted a common signalling pathway to protect the heart against reperfusion injury.

Fig. 2 shows the effects of the interventions explored on systolic myocardial function. At the end of the

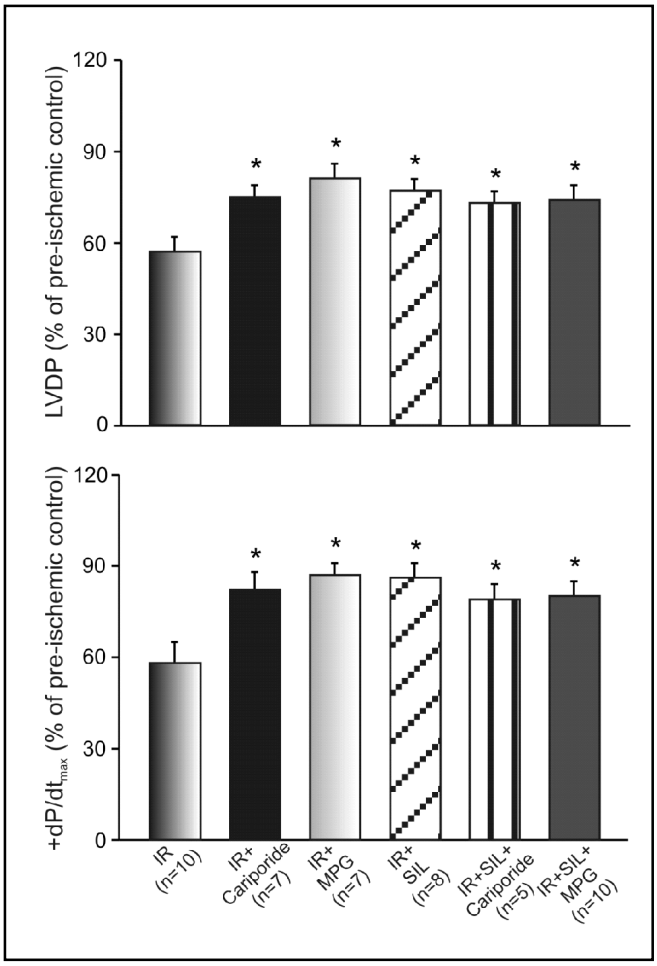


Fig. 2. Left ventricular developed pressure (LVDP) and maximal rise velocity of LVP (+dP/dt$_{max}$) at the end of the reperfusion period (expressed as a percentage of preischemic values) in all experimental groups. All interventions improved postischemic myocardial systolic function to a similar extent compared to non treated I/R. * P < 0.05 vs. I/R.

reperfusion period LVDP decreased to 57 ± 5 % of the pre-ischemic value in non-treated control hearts. Post-ischemic recovery was improved similarly after all pharmacological treatments reaching values of ~ 75 % of the pre-ischemic control (Fig. 2, upper panel). A similar pattern was observed when $+dP/dt_{max}$ values were analyzed (Fig. 2, lower panel).

The LVEDP (an index of diastolic stiffness) was approximately 10 mmHg at the end of the stabilization period in the different experimental groups. This parameter significantly increased reaching a value of 26 ± 5 mmHg after 2 hs. of reperfusion. However, this increase was not detected in any of the treated groups (data not shown). In other words, myocardial diastolic stiffness of treated hearts did not significantly change during reperfusion.

Given that ROS generation and the consequent tissue damage may be responsible for myocardial reperfusion injury [38, 39], we next determined the impact of the three pharmacological interventions on myocardial TBARS concentration, an index of lipid peroxidation. Fig. 3 shows that TBARS were ~10 nmol/g in control ischemic hearts, a value that was significantly reduced by 10 μmol/L cariporide or 2 mmol/L MPG, but was unaffected by 1 μmol/L SIL. When SIL was combined with either cariporide or MPG, similar reduction in TBARS to that obtained with each drug alone was observed (Fig. 3). These results show that though the final effect (IS reduction) of cariporide, MPG and SIL was of a similar magnitude, cariporide and MPG decreased myocardial impact of ROS whereas SIL did not. These data reinforce the idea of an "antioxidant property" of cariporide, which we [19, 40] and others [41] previously reported. In order to further analyze the effect of these compounds on ROS production, we used exogenous Ang II (1 nmol/L) as a tool to stimulate $\cdot O_2^-$ production in myocardial tissue slices [42]. Figure 4 shows that whereas cariporide and MPG blunted the Ang II-induced increase in $\cdot O_2^-$ production, SIL did not, which is coincident with the lack of effect of SIL upon myocardial TBARS (Fig. 3).

Given that the increase in ROS production may stimulate the ERK1/2- $p90^{RSK}$ kinase pathway leading to NHE-1 phosphorylation and activation [43-47], we next analyzed the phosphorylation state of the NHE-1and its upstream kinases at the end of the reperfusion period. NHE-1 phosphorylation at Ser703 was estimated by quantifying levels of P-14-3-3 binding motif present on immunoprecipitated samples, as explained in Methods. The three pharmacological interventions (cariporide, MPG,

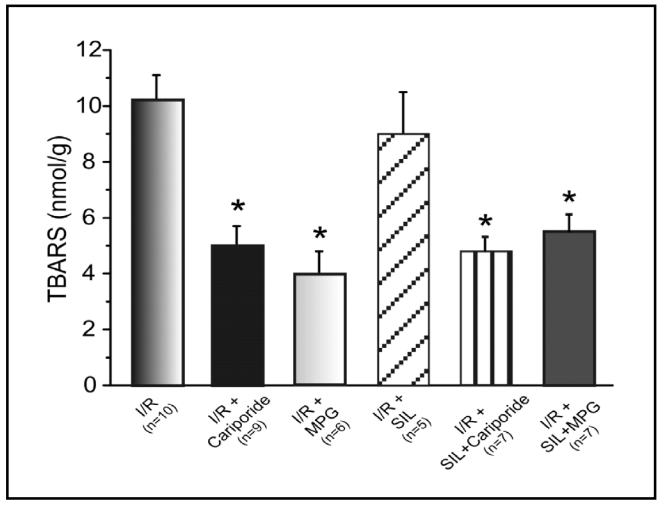


Fig. 3. Myocardial TBARS concentration was determined under different experimental conditions as an index of ROS-mediated lipid peroxidation. TBARS were ~10 nmol/g in control post-ischemic hearts, a value that was significantly reduced by cariporide or MPG, but unaffected by SIL. As expected, the combination of SIL with either cariporide or MPG also reduced TBARS. These results suggest a miscorrelation between ROS production and IS and reinforce the notion that cariporide has an "antioxidant property" not observed after SIL alone. * $P < 0.05$ vs. I/R.

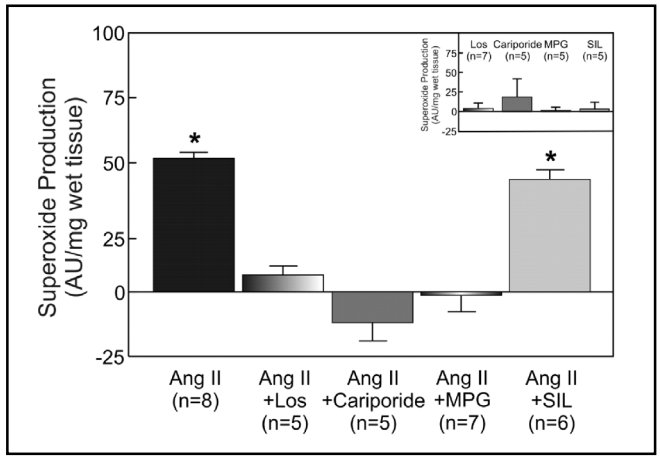


Fig. 4. Effect of cariporide, MPG, and SIL on $\cdot O_2^-$ production induced by 1 nmol/L Ang II in isolated myocardial tissue slices. Incubation with Ang II for 15 minutes caused a significant increase in $\cdot O_2^-$ production that was, as expected, blunted by specific AT1 receptor blockade with Losartan (Los), but also by cariporide and MPG. However, SIL did not exert this effect. These results clearly agree with the TBARS measurements after ischemia/reperfusion (Fig. 3) and reinforce the notion of the "antioxidant property" of the NHE-1 inhibitor, which was not detected after PDE5A inhibition. Since $\cdot O_2^-$ production stabilized after 15 minutes as previously reported,[37] the data obtained at this time point were expressed as percent of control values without additions. *Inset* shows the lack of effect of all drugs used upon the basal measurement. * $P < 0.05$ vs. control.

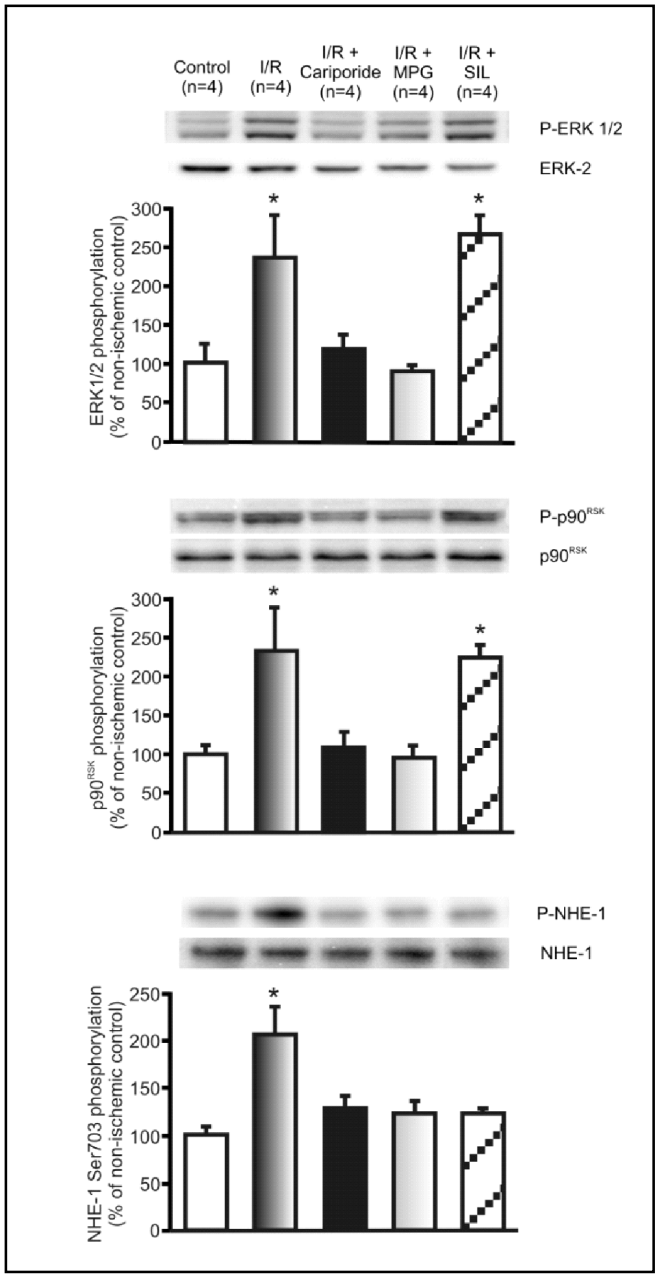


Fig. 5. Increased phosphorylation of ERK1/2 (upper panel), $p90^{RSK}$ (middle panel), and the 14-3-3 binding motif (lower panel) was detected in post-ischemia non-treated hearts, effects that were all blunted by MPG or cariporide. Interestingly, SIL did not affect the increase in phospho-ERK1/2 and phospho-$p90^{RSK}$, but fully prevented the increase in the phospho-Ser 14-3-3 binding motif signalling. Therefore, in spite of a similar endpoint (reduction in NHE-1 phosphorylation), SIL did not act through the inactivation of ROS-sensitive kinases upstream the exchanger, result that agrees with the lack of prevention of myocardial lipid peroxidation by SIL shown in Fig. 3.* $P < 0.05$ vs. non-ischemic control.

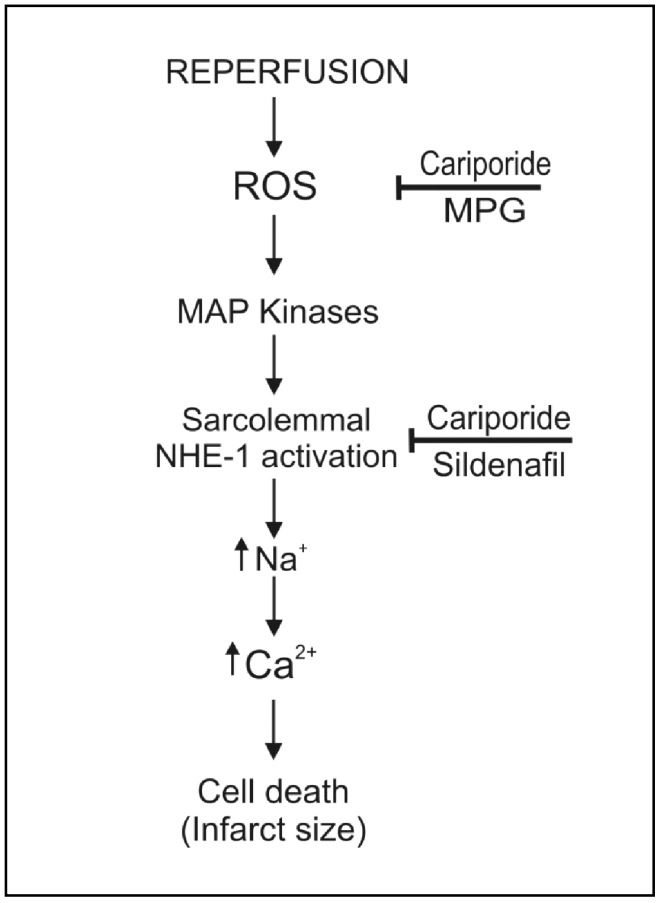


Fig. 6. Proposed chain of events triggered by reperfusion where both a burst of ROS and Ca^{2+} overload are not mutually exclusive events, but steps in the same pathway. In this regard, the ROS burst appears to be just a triggering factor of the pathologic process by promoting MAPK-mediated NHE-1 phosphorylation, which is responsible for myocardial tissue damaging. Thus, preventing ROS formation either by MPG or cariporide would prevent cell injury by avoiding NHE-1 activation, while PDE5A inhibitors would directly target sarcolemmal NHE-1 phosphorylation without affecting oxidative stress.

and SIL) induced similar reductions in NHE-1 phosphorylation (Fig. 5). However, while cariporide and MPG decreased phospho-ERK1/2 and phospho- $p90^{RSK}$ -probably by preventing ROS formation- these kinases were unaffected by SIL (Fig. 5). Therefore, though the three interventions reached the similar endpoint of reducing NHE-1 phosphorylation while MPG and cariporide prevented activation of the ROS-sensitive MAPK, SIL directly targeted NHE-1.

Discussion

In the present study, we provide evidence that three different pharmacological interventions -cariporide, MPG,

and SIL- applied during the early reperfusion phase (first 20 minutes of reflow) decreased IS to a similar extent (~50 % of that seen in non-treated infarcted hearts) and that combination of treatments did not induce further reduction of IS suggesting a common mechanism of protection. Postischemic systolic and diastolic function was equally improved by each treatment. Given that all interventions converged to a reduction in the estimated NHE-1 phosphorylation at Ser703, and that phosphorylation of the exchanger at this site is one of the main pathways to activate NHE-1 [26, 45], we suggest that the exchanger inhibition would be the underlying common mechanism of protection for the three compounds tested.

A large burst of ROS during the first minutes of reperfusion has been consistently shown to occur [30-37]. This increased ROS production could lead to an extensive oxidative damage resulting in cell death by apoptosis, necrosis and/or autophagy [48]. However, the beneficial effects of scavengers or antioxidants in ischemia/reperfusion are still a matter of controversy (see ref. [49] for review). It has been proposed that ROS production early at reperfusion lead to MPTP opening with a consequent loss of cell viability [50]. Actually, recent reports in animals [51] and patients [52] have shown protection against reperfusion injury when MPTP formation is inhibited with cyclosporine. Herein, we present evidence that myocardial damage by lipid peroxidation (estimated by TBARS) is blunted by MPG and cariporide, but not by SIL, which would suggest that ROS may not have deleterious effects *per-se,* but through kinase activation leading to NHE-1 phosphorylation. The concept that the burst of ROS at the beginning of reperfusion acts through NHE-1 activation deserves further research directed to analyze the effects of MPTP inhibitors on the exchanger activity.

The decrease in TBARS and IS induced by MPG and cariporide were previously reported by us [19]. The results obtained with cariporide and MPG preventing the activation of the kinases that phosphorylate the NHE-1 are in line with the previously reported action of ROS on MAPK activation [44, 47]. NHE-1 inhibition would decrease intracellular Na^+ concentration and consequently intracellular Ca^{2+} thus preventing mitochondrial Ca^{2+} overload.

The experiments performed under PDE5A inhibition with SIL allowed us to demonstrate a similar protection in terms of IS reduction but without interfering with ROS formation at the onset of reperfusion. This conclusion was based on the fact that SIL did not prevent the increase in TBARS detected after ischemia/reperfusion (Fig. 3), and it was further supported by the results obtained in isolated tissue slices where SIL was unable to prevent the Ang II-induced increase in $\cdot O_2^-$ production (Fig. 4).

The activation of the redox-sensitive upstream-NHE-1 kinases (ERK1/2 and $p90^{RSK}$) during ischemia/reperfusion has been previously reported [53]. However, the ability of the PDE5A inhibitor SIL to prevent NHE-1 phosphorylation under these circumstances without affecting kinase activation but limiting IS represents a novel finding of the present work. It seems possible that the increase in ROS production only triggers ERK1/2-$p90^{RSK}$ activation which in turn promotes NHE-1 phosphorylation influencing IS with no deleterious effects *per se*. In other words, in our current experimental conditions NHE-1 seems to be the sole culprit of myocardial tissue damage during reperfusion.

The observed decrease in NHE-1 phosphorylation (Fig. 5) suggests that PDE5A inhibition exerts an action upon NHE-1 that is independent of ERK1/2-$p90^{RSK}$ activation. This finding agrees with recent reports from our laboratory suggesting that PDE5A inhibition induces NHE-1 dephosphorylation through a PKG-mediated protein phosphatase activation [18].

The effectiveness of SIL treatment started at the beginning of reperfusion to reduce myocardial damaging without evidence of an "antioxidant effect" constitutes a novel piece of information. Figure 6 summarizes the proposed chain of events triggered by reperfusion and leading to myocardial tissue damage, together with the potential sites of action of MPG, cariporide and SIL.

Yet, possible limitations of the present study should be considered. First, it is known that measurement of IS by the TTC staining technique is affected by the duration of reperfusion. In this regard, though the same protocol was used for the three pharmacological interventions, if one of the interventions retards the manifestation of necrosis more than the others, we could have arrived at erroneous conclusions. Second, neither the source nor the exact type of ROS was determined in the present work. Mitochondria are a potential source of ROS through their ability to produce $\cdot O_2^-$ in the electron transport chain. Third, NHE-1 phosphorylation state was estimated through a phosphospecific antibody which recognizes the Ser703-phosphorylated in the 14-3-3 binding motif of the immunoprecipitated NHE-1. Therefore, we have estimated NHE-1 phosphorylation at Ser703, but not at other regulatory sites such as Ser770 or Ser771 [54].

In conclusion, we demonstrate in isolated rat heart that three different pharmacological interventions -

cariporide, MPG, or SIL- applied at the beginning of reperfusion decrease IS and improve postischemic cardiac function to a similar extent. All interventions converge to a reduction in NHE-1 phosphorylation at Ser703, suggesting that the exchanger inhibition would be the underlying common mechanism of protection. However, the protective effect exerted by SIL cannot be attributed to an attenuation of oxidative damage.

Finally, and from a clinical point of view, we would like to emphasize, that the reported distinctive property of PDE5A inhibitors of only affecting NHE-1 activity after an acidic challenge [12, 17, 18] may confer a potential therapeutic advantage, compared to other known NHE-1 inhibitory compounds, in the prevention of myocardial ischemia/reperfusion injury.

Acknowledgements

This work was supported in part by grants PIP 5141 and 1386 from Consejo Nacional de Investigaciones Científicas y Técnicas to Drs. Horacio E. Cingolani and Néstor G. Pérez respectively and PICT 25475, 12480, 01031 and 01046 from Agencia Nacional de Promoción Científica of Argentina to Drs. Horacio E. Cingolani, Gladys Chiappe de Cingolani, Néstor G. Pérez and Susana Mosca respectively. We specially thank Rubén Feldman from Roemmers Argentina for kindly providing Sildenafil.

References

1 Javadov S, Karmazyn M: Mitochondrial permeability transition pore opening as an endpoint to initiate cell death and as a putative target for cardioprotection. Cell Physiol Biochem 2007;20:1-22.

2 Karmazyn M, Gan XT, Humphreys RA, Yoshida H, Kusumoto K: The myocardial Na^+-H^+ exchange: Structure, regulation, and its role in heart disease. Circ Res 1999;85:777-786.

3 Karmazyn M: Amiloride enhances postischemic ventricular recovery: Possible role of Na^+-H^+ exchange. Am J Physiol 1988;255:H608-615.

4 Avkiran M, Marber MS: Na^+/H^+ exchange inhibitors for cardioprotective therapy: Progress, problems and prospects. J Am Coll Cardiol 2002;39:747-753.

5 Karmazyn M, Sostaric JV, Gan XT: The myocardial Na^+/H^+ exchanger: A potential therapeutic target for the prevention of myocardial ischaemic and reperfusion injury and attenuation of postinfarction heart failure. Drugs 2001;61:375-389.

6 Chen L, Chen CX, Gan XT, Beier N, Scholz W, Karmazyn M: Inhibition and reversal of myocardial infarction-induced hypertrophy and heart failure by nhe-1 inhibition. Am J Physiol Heart Circ Physiol 2004;286:H381-387.

7 Gumina RJ, Buerger E, Eickmeier C, Moore J, Daemmgen J, Gross GJ: Inhibition of the Na^+/H^+ exchanger confers greater cardioprotection against 90 minutes of myocardial ischemia than ischemic preconditioning in dogs. Circulation 1999;100:2519-2526.

8 Rupprecht HJ, vom Dahl J, Terres W, Seyfarth KM, Richardt G, Schultheibeta HP, Buerke M, Sheehan FH, Drexler H: Cardioprotective effects of the Na^+/H^+ exchange inhibitor cariporide in patients with acute anterior myocardial infarction undergoing direct ptca. Circulation 2000;101:2902-2908.

9 du Toit EF, Rossouw E, Salie R, Opie LH, Lochner A: Effect of sildenafil on reperfusion function, infarct size, and cyclic nucleotide levels in the isolated rat heart model. Cardiovasc Drugs Ther 2005;19:23-31.

10 Salloum FN, Takenoshita Y, Ockaili RA, Daoud VP, Chou E, Yoshida K, Kukreja RC: Sildenafil and vardenafil but not nitroglycerin limit myocardial infarction through opening of mitochondrial K(ATP) channels when administered at reperfusion following ischemia in rabbits. J Mol Cell Cardiol 2007;42:453-458.

11 Kukreja RC, Ockaili R, Salloum F, Yin C, Hawkins J, Das A, Xi L: Cardioprotection with phosphodiesterase-5 inhibition-a novel preconditioning strategy. J Mol Cell Cardiol 2004;36:165-173.

12 Perez NG, Piaggio MR, Ennis IL, Garciarena CD, Morales C, Escudero EM, Cingolani OH, Chiappe de Cingolani G, Yang XP, Cingolani HE: Phosphodiesterase 5a inhibition induces Na^+/H^+ exchanger blockade and protection against myocardial infarction. Hypertension 2007;49:1095-1103.

13 Schulte EA, Hohendahl A, Stegemann H, Hirsch JR, Saleh H, Schlatter E: Natriuretic peptides and diadenosine polyphosphates modulate ph regulation of rat mesangial cells. Cell Physiol Biochem 1999;9:310-322.

14 Fidzinski P, Salvador-Silva M, Choritz L, Geibel J, Coca-Prados M: Inhibition of NHE-1 Na^+/H^+ exchanger by natriuretic peptides in ocular nonpigmented ciliary epithelium. Am J Physiol Cell Physiol 2004;287:C655-663.

15 Caramelo C, Lopez-Farre A, Riesco A, Olivera A, Okada K, Cragoe EJ, Jr., Tsai P, Briner VA, Schrier RW: Atrial natriuretic peptide and cgmp inhibit Na^+/H^+ antiporter in vascular smooth muscle cells in culture. Kidney Int 1994;45:66-75.

16 Gill RK, Saksena S, Syed IA, Tyagi S, Alrefai WA, Malakooti J, Ramaswamy K, Dudeja PK: Regulation of NHE3 by nitric oxide in caco-2 cells. Am J Physiol Gastrointest Liver Physiol 2002;283:G747-756.
17 Yeves AM, Garciarena CD, Nolly MB, Chiappe de Cingolani GE, Cingolani HE, Ennis IL: Decreased activity of the Na^+/H^+ exchanger by phosphodiesterase 5a inhibition is attributed to an increase in protein phosphatase activity. Hypertension 2010;56:690-695.
18 Díaz RG, Nolly MB, Massarutti CD, Casarini MJ, Garciarena CD, Ennis IL, Cingolani HE, Pérez NG: Phosphodiesterase 5a inhibition decreases NHE-1 activity without altering steady state phi: Role of phosphatases. Cell Physiol Biochem 2010;26:531-540.
19 Fantinelli JC, Cingolani HE, Mosca SM: Na^+/H^+ exchanger inhibition at the onset of reperfusion decreases myocardial infarct size: Role of reactive oxygen species. Cardiovasc Pathol 2006;15:179-184.
20 Fishbein MC, Meerbaum S, Rit J, Lando U, Kanmatsuse K, Mercier JC, Corday E, Ganz W: Early phase acute myocardial infarct size quantification: Validation of the triphenyl tetrazolium chloride tissue enzyme staining technique. Am Heart J 1981;101:593-600.
21 Schwarz ER, Somoano Y, Hale SL, Kloner RA: What is the required reperfusion period for assessment of myocardial infarct size using triphenyltetrazolium chloride staining in the rat? J Thromb Thrombolysis 2000;10:181-187.
22 Buege JA, Aust SD: Microsomal lipid peroxidation. Methods Enzymol 1978;52:302-310.
23 Garciarena CD, Caldiz CI, Portiansky EL, Chiappe de Cingolani GE, Ennis IL: Chronic NHE-1 blockade induces an antiapoptotic effect in the hypertrophied heart. J Appl Physiol 2009;106:1325-1331.
24 Snabaitis AK, D'Mello R, Dashnyam S, Avkiran M: A novel role for protein phosphatase 2a in receptor-mediated regulation of the cardiac sarcolemmal Na^+/H^+ exchanger nhe1. J Biol Chem 2006;281:20252-20262.
25 Lehoux S, Abe J, Florian JA, Berk BC: 14-3-3 binding to Na^+/H^+ exchanger isoform-1 is associated with serum-dependent activation of Na^+/H^+ exchange. J Biol Chem 2001;276:15794-15800.
26 Maekawa N, Abe J, Shishido T, Itoh S, Ding B, Sharma VK, Sheu SS, Blaxall BC, Berk BC: Inhibiting p90 ribosomal s6 kinase prevents Na^+/H^+ exchanger-mediated cardiac ischemia-reperfusion injury. Circulation 2006;113:2516-2523.
27 An J, Varadarajan SG, Camara A, Chen Q, Novalija E, Gross GJ, Stowe DF: Blocking Na^+/H^+ exchange reduces Na^+_i and Ca^{2+}_i load after ischemia and improves function in intact hearts. Am J Physiol Heart Circ Physiol 2001;281:H2398-2409.
28 Hurtado C, Pierce GN: Inhibition of Na^+/H^+ exchange at the beginning of reperfusion is cardioprotective in isolated, beating adult cardiomyocytes. J Mol Cell Cardiol 2000;32:1897-1907.
29 Xiao XH, Allen DG: Activity of the Na^+/H^+ exchanger is critical to reperfusion damage and preconditioning in the isolated rat heart. Cardiovasc Res 2000;48:244-253.
30 Bolli R, Jeroudi MO, Patel BS, Aruoma OI, Halliwell B, Lai EK, McCay PB: Marked reduction of free radical generation and contractile dysfunction by antioxidant therapy begun at the time of reperfusion. Evidence that myocardial "Stunning" Is a manifestation of reperfusion injury. Circ Res 1989;65:607-622.
31 Bolli R, Patel BS, Jeroudi MO, Li XY, Triana JF, Lai EK, McCay PB: Iron-mediated radical reactions upon reperfusion contribute to myocardial "Stunning". Am J Physiol 1990;259:H1901-1911.
32 Sekili S, McCay PB, Li XY, Zughaib M, Sun JZ, Tang L, Thornby JI, Bolli R: Direct evidence that the hydroxyl radical plays a pathogenetic role in myocardial "Stunning" In the conscious dog and demonstration that stunning can be markedly attenuated without subsequent adverse effects. Circ Res 1993;73:705-723.
33 Matsuki A, Igawa A, Nozawa T, Nakadate T, Igarashi N, Nonomura M, Inoue H: Early administration of fluvastatin, but not at the onset of ischemia or reperfusion, attenuates myocardial ischemia-reperfusion injury through the nitric oxide pathway rather than its antioxidant property. Circ J 2006;70:1643-1649.
34 Tanaka M, Stoler RC, FitzHarris GP, Jennings RB, Reimer KA: Evidence against the "Early protection-delayed death" Hypothesis of superoxide dismutase therapy in experimental myocardial infarction. Polyethylene glycol-superoxide dismutase plus catalase does not limit myocardial infarct size in dogs. Circ Res 1990;67:636-644.
35 Onogi H, Minatoguchi S, Chen XH, Bao N, Kobayashi H, Misao Y, Yasuda S, Yamaki T, Maruyama R, Uno Y, Arai M, Takemura G, Fujiwara H: Edaravone reduces myocardial infarct size and improves cardiac function and remodelling in rabbits. Clin Exp Pharmacol Physiol 2006;33:1035-1041.
36 Andreadou I, Tasouli A, Iliodromitis E, Tsantili-Kakoulidou A, Papalois A, Siatra T, Kremastinos DT: Reduction of myocardial infarct size in rabbits by a novel indole derivative with antioxidant and free radical scavenging properties. Eur J Pharmacol 2002;453:271-277.
37 Nakajima H, Ishizaka N, Hangaishi M, Taguchi J, Itoh J, Igarashi R, Mizushima Y, Nagai R, Ohno M: Lecithinized copper, zinc-superoxide dismutase ameliorates prolonged hypoxia-induced injury of cardiomyocytes. Free Radic Biol Med 2000;29:34-41.
38 Ambrosio G, Flaherty JT, Duilio C, Tritto I, Santoro G, Elia PP, Condorelli M, Chiariello M: Oxygen radicals generated at reflow induce peroxidation of membrane lipids in reperfused hearts. J Clin Invest 1991;87:2056-2066.
39 Zweier JL: Measurement of superoxide-derived free radicals in the reperfused heart. Evidence for a free radical mechanism of reperfusion injury. J Biol Chem 1988;263:1353-1357.
40 Garciarena CD, Caldiz CI, Correa MV, Schinella GR, Mosca SM, Chiappe de Cingolani GE, Cingolani HE, Ennis IL: Na^+/H^+ exchanger-1 inhibitors decrease myocardial superoxide production via direct mitochondrial action. J Appl Physiol 2008;105:1706-1713.
41 Javadov S, Choi A, Rajapurohitam V, Zeidan A, Basnakian AG, Karmazyn M: NHE-1 inhibition-induced cardioprotection against ischaemia/reperfusion is associated with attenuation of the mitochondrial permeability transition. Cardiovasc Res 2008;77:416-424.
42 Caldiz CI, Garciarena CD, Dulce RA, Novaretto LP, Yeves AM, Ennis IL, Cingolani HE, Chiappe de Cingolani G, Perez NG: Mitochondrial reactive oxygen species activate the slow force response to stretch in feline myocardium. J Physiol 2007;584:895-905.
43 Wang H, Silva NL, Lucchesi PA, Haworth R, Wang K, Michalak M, Pelech S, Fliegel L: Phosphorylation and regulation of the Na^+/H^+ exchanger through mitogen-activated protein kinase. Biochemistry 1997;36:9151-9158.
44 Sabri A, Byron KL, Samarel AM, Bell J, Lucchesi PA: Hydrogen peroxide activates mitogen-activated protein kinases and Na^+/H^+ exchange in neonatal rat cardiac myocytes. Circ Res 1998;82:1053-1062.
45 Takahashi E, Abe J, Gallis B, Aebersold R, Spring DJ, Krebs EG, Berk BC: P90(rsk) is a serum-stimulated Na^+/H^+ exchanger isoform-1 kinase. Regulatory phosphorylation of serine 703 of Na^+/H^+ exchanger isoform-1. J Biol Chem 1999;274:20206-20214.

46 Khaled AR, Moor AN, Li A, Kim K, Ferris DK, Muegge K, Fisher RJ, Fliegel L, Durum SK: Trophic factor withdrawal: P38 mitogen-activated protein kinase activates NHE1, which induces intracellular alkalinization. Mol Cell Biol 2001;21:7545-7557.

47 Rothstein EC, Byron KL, Reed RE, Fliegel L, Lucchesi PA: H_2O_2-induced Ca^{2+} overload in nrvm involves ERK1/2 MAP kinases: Role for an nhe-1-dependent pathway. Am J Physiol Heart Circ Physiol 2002;283:H598-605.

48 Teshima Y, Akao M, Jones SP, Marban E: Cariporide (HOE642), a selective Na^+/H^+ exchange inhibitor, inhibits the mitochondrial death pathway. Circulation 2003;108:2275-2281.

49 Murphy E, Steenbergen C: Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. Physiol Rev 2008;88:581-609.

50 Kim JS, Jin Y, Lemasters JJ: Reactive oxygen species, but not Ca^{2+} overloading, trigger ph- and mitochondrial permeability transition-dependent death of adult rat myocytes after ischemia-reperfusion. Am J Physiol Heart Circ Physiol 2006;290:H2024-2034.

51 Hausenloy DJ, Duchen MR, Yellon DM: Inhibiting mitochondrial permeability transition pore opening at reperfusion protects against ischaemia-reperfusion injury. Cardiovasc Res 2003;60:617-625.

52 Piot C, Croisille P, Staat P, Thibault H, Rioufol G, Mewton N, Elbelghiti R, Cung TT, Bonnefoy E, Angoulvant D, Macia C, Raczka F, Sportouch C, Gahide G, Finet G, Andre-Fouet X, Revel D, Kirkorian G, Monassier JP, Derumeaux G, Ovize M: Effect of cyclosporine on reperfusion injury in acute myocardial infarction. N Engl J Med 2008;359:473-481.

53 Moor AN, Gan XT, Karmazyn M, Fliegel L: Activation of Na^+/H^+ exchanger-directed protein kinases in the ischemic and ischemic-reperfused rat myocardium. J Biol Chem 2001;276:16113-16122.

54 Malo ME, Li L, Fliegel L: Mitogen-activated protein kinase-dependent activation of the Na^+/H^+ exchanger is mediated through phosphorylation of amino acids ser770 and ser771. J Biol Chem 2007;282:6292-6299.