UNIVERSITÀ DEGLI STUDI DI TORINO

This Accepted Author Manuscript (AAM) is copyrighted and published by Elsevier. It is posted here by agreement between Elsevier and the University of Turin. Changes resulting from the publishing process - such as editing, corrections, structural formatting, and other quality control mechanisms - may not be reflected in this version of the text. The definitive version of the text was subsequently published in *Penna C, Perrelli MG, Tullio F, Angotti C, Pagliaro P. Acidic infusion in early reperfusion affects the activity of antioxidant enzymes in postischemic isolated rat heart. J Surg Res. 2013 Jul;183(1):111-8. doi: 10.1016/j.jss.2012.12.029.*

You may download, copy and otherwise use the AAM for non-commercial purposes provided that your license is limited by the following restrictions:

(1) You may use this AAM for non-commercial purposes only under the terms of the CC-BY-NC-ND license.

(2) The integrity of the work and identification of the author, copyright owner, and publisher must be preserved in any copy.

(3) You must attribute this AAM in the following format: Creative Commons BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/deed.en), [+ *Digital Object Identifier link to the published journal article on Elsevier's ScienceDirect® platform*]

Acidic infusion in early reperfusion affects the activity of antioxidant enzymes in post-ischemic isolated rat heart

*Penna C, Perrelli MG, Tullio F, Angotti C and Pagliaro P**

Department of Clinical and Biological Sciences, University of Turin, Italy.

Istituto Nazionale per le Ricerche Cardiovascolari, Bologna, Italy

Running head: Antioxidant enzymes in acidic cardioprotection

***Address for the correspondence:**
Dr Pasquale Pagliaro,
Dipartimento di Scienze Cliniche e Biologiche
Università di Torino
Regione Gonzole 10,
10043 ORBASSANO (TO), Italy
Tel: 39-11 6705450
Fax: 39-11 9038639
e-mail: pasquale.pagliaro@unito.it

ABSTRACT

Background. Acidic-perfusion (AP) performed at the onset of reperfusion (*i.e.* acid-postconditioning) is cardioprotective. We investigated the effect of AP on postischemic cardiac function and on the activity of endogenous superoxide-dismutase (SOD), catalase (CAT) and glutathione-peroxidase (GPX). The role of exogenous-CAT or -SOD on AP-cardioprotection was also investigated. Phosphorylation of redox-sensitive survival kinases (PKCε and ERK1/2) was also checked. *Materials and methods.* Isolated rat hearts underwent 30-min ischemia and 120-min reperfusion (I/R). Acidic-perfusion was obtained lowering [HCO_3^-] in the perfusion buffer. Infarct-size and left ventricular pressure were measured. Protocols: I/R only; I/R plus acidic-perfusion in early reperfusion (I/R+AP); I/R plus AP and CAT (I/R+AP+CAT) or SOD (I/R+AP+SOD). I/R+SOD and I/R+CAT additional hearts served as controls. AP and/or antioxidants were given in the initial 3-min of reperfusion. Enzyme activities were studied in postischemic phase (7^{th} min of reperfusion) in I/R or I/R+AP, and in Sham (buffer-perfused) hearts. *Results.* Acidic-perfusion with (I/R+AP+CAT or I/R+AP+SOD) or without (I/R+AP) antioxidant-enzymes resulted in a larger reduction of infarct size compared with I/R, I/R+SOD or I/R+CAT. Compared to I/R, the postischemic systolic and diastolic recovery of cardiac function were markedly improved by the addition of AP and by a lesser extent by AP+SOD or AP+CAT. Acidic-perfusion increased the postischemic activity of CAT and lowered those of SOD and GPX, compared to I/R only. Also the phosphorylation/activity of ERK1/2 and PKCε were increased by AP. *Conclusions.* Acid-postconditioning affects the activity of endogenous-antioxidant-enzymes, activates ERK1/2-PKCε pathways and protects against myocardial I/R injury. The combination of AP and exogenous-SOD or -CAT still provides cardioprotection. It is likely that intracellular (not-extracellular) redox condition plays a pivotal role in acidic protection.

Key words: Cardioprotection; Catalase; Glutathione peroxidase; Ischemia/Reperfusion; Reactive oxygen species; Superoxide dismutase.

INTRODUCTION

It is well known that ischemia and reperfusion (I/R) in the heart induce extensive tissue damage and that a substantial part of myocardial cell death initiated by ischemia occurs at the time of reperfusion [1-13]. I/R injury can be thus prevented by interventions applied at the time of reflow, that is postconditioning (PostC), which is obtained with brief intermittent ischemia or with pharmacological agents at beginning of reperfusion [4,5,8,12]. As such, ischemic and pharmacological PostC clinical application has been rapid for both STEMI (ST-elevation) patients undergoing coronary reperfusion and for patients undergoing on pump cardiac surgery [3-5].

Ischemia will cause tissue acidification which initially protects against tissue damage, but prolonged acidosis associated with limited nutrient level will eventually cease ATP generation [1-5,8,9,13-5]. Nevertheless, a few minutes acidic perfusion (AP) immediately after release of a prolonged coronary occlusion mimics infarct reduction seen with PostC [1-4,15]. In fact, delayed normalization of intracellular pH (pHi) during reperfusion together oxygen delivery plays an important role in the cardioprotective effects of PostC [1,2,4].

The generation of reactive oxygen species (ROS) is believed to be among the major causes of I/R injury. However, recent studies have demonstrated that ROS can also contribute to PostC cardioprotection activating survival kinases, including protein kinase C (PKC) and extracellular-signal-regulated kinases (ERK1/2) [1,2,11,16]. Importantly, either PostC protection or AP protection can be abolished by cell permeant ROS scavengers (N-acetyl-L-cysteine or N-2-mercaptopropionyl glycine) given in early reperfusion [1,2,11,16]. This suggests that pHi may

play a pivotal role in regulating intracellular redox equilibrium in a moment in which oxygen is re-introduced.

Many different endogenous enzymes regulate the intracellular homeostasis of ROS. For instance, in the myocardium, the main antioxidant-enzymes include: superoxide dismutases (SODs) which convert superoxide (O_2^-) to hydrogen peroxide (H_2O_2), as well as catalase (CAT)) and glutathione peroxidase (GPX) which regulate steady-state levels of H_2O_2 by conversion to water [5,8].

We recently have shown that PostC down-regulates SOD and up-regulates CAT activity [10]. This is in line with the observation that PostC affect the equilibrium of redox conditions in reperfusion [1,2,4,5,8,11,16].

Since all enzymatic activities are strongly influenced by pHi, we hypothesize that an acid buffer perfusion in the early reperfusion phase may affect the activity of anti-oxidant enzymes. To verify this hypothesis we studied the cardioprotective effects of a few minutes of AP in early reperfusion and the influence on endogenous SOD, CAT and GPX levels and activities in an isolated heart model. We also tested if exogenous SOD or CAT, which are *non-cell permeant antioxidants*, co-infused with acidic buffer may affect cardioprotection induced by acid buffer reperfusion. Finally, we checked whether AP may favor the activation/phosphorylation of two redox-sensitive survival kinases, namely ERK1/2 and PKCε.

MATERIALS AND METHODS.

Animals

Male Wistar rats (5-6 month old, body weight 450–550 g) derived from Janvier, Le Genest St Isle - France, received humane care in compliance with Italian law (DL-116, Jan. 27, 1992) and

in accordance with 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).

Isolated heart perfusion

The methods were similar to those previously described [6,7,10,11]. In brief, after anesthesia chest was opened and heart excised. Hearts were constant-flow perfused (9 ml/min/g) with Krebs–Henseleit buffer solution (127 mM NaCl, 17.7 mM $NaHCO_3$, 5.1 mM KCl, 1.5 mM $CaCl_2$, 1.26 mM $MgCl_2$, 11 mM D-glucose (Sigma-Aldrich Corp., St. Louis, MO, USA), and gassed with 95% O_2 and 5% CO_2). The hearts were instrumented to record coronary pressure and left ventricular pressure (LVP), electrically paced at 280 b.p.m. and kept in a temperature-controlled chamber (37°C).

Experimental protocols

Each heart was stabilized for 30 min and subjected to a specific protocol, which included in all groups a 30 min of global, normothermic ischemia, followed by 120 min of reperfusion in all groups. Pacing was discontinued on initiation of ischemia and restarted after the third minute of reperfusion in all groups [6,7,10,11].

After stabilization Control group hearts (I/R, Group 1, n=6) were exposed to 30 min ischemia and then to 120 min reperfusion only. In Group 2 (Acidic Perfusion, AP; n=6) acidic solution was given at the beginning of reperfusion for 3 min. Acidic (pH 6.6) solution was prepared by lowering [$NaHCO_3$] to 4.0mM while maintaining the same gas mixture containing 5% CO_2. [Na^+] was kept constant (144±1 mM) in all solutions by modifying [NaCl] as necessary [3]. In Group 3 (I/R+AP+CAT, n=6) and Group 4 (I/R+AP+SOD, n=6) hearts underwent I/R in the presence of CAT (100 U/ml) or SOD (10 U/ml), respectively. Antioxidants, either CAT or SOD (Sigma-Aldrich, USA), were given at the beginning of reperfusion for 3 min in the presence of the acidic buffer at doses previously used in isolated rat hearts [10,17]. In order to test the effects

of antioxidants given alone for 3 min in early reperfusion, in additional hearts (n=6) I/R protocol was followed by 3 min infusion of CAT (100 U/ml; I/R+CAT) or SOD (10 U/ml; I/R+SOD), at the beginning of 120 min reperfusion.

Assessment of cardiac function

A polyvinyl-chloride balloon was placed into the left ventricle (LV), filled with saline and connected to the electromanometer to obtain an end diastolic LVP (LVEDP) of 5 mmHg. Such a volume of LV was maintained throughout the experiment [6,7,10,11].

Coronary perfusion pressure, coronary flow and LVP were monitored to assess the preparation conditions. LVP was analyzed offline with LabView software (National Instruments).

Assessment of myocardial injury

Infarct areas were assessed at the end of the experiment as previously described [6,7,10,11]. In brief, immediately after reperfusion hearts were rapidly removed from the perfusion apparatus and the LV dissected into 2–3 mm circumferential slices. Following 20 min of incubation at 37°C in 0.1% solution of nitro-blue tetrazolium in phosphate buffer, unstained necrotic tissue was carefully separated from stained viable tissue by an independent observer, who was unaware of the protocols. The necrotic mass was expressed as a percentage of total left ventricular mass which was considered as risk area.

Antioxidant Enzyme Activities and Western Blotting

Hearts (n= 4 for each group) were subjected to 180 min perfusion only (i.e. Sham), or to I/R only or to I/R plus AP, as above described for Groups 1 and 2.

Samples of these hearts were homogenized on ice in RIPA Lysis buffer and processed as previously described for protein quantification with Western blotting [9,10].

In samples of these hearts the activity of CAT, SOD and GPX was tested at 7^{th} min of reperfusion. Sham hearts were tested at corresponding time points (baseline and after 37 min

perfusion only) and after 150 min of buffer perfusion only, in order to have reference points and to verify stability of enzyme activities. The activity of enzymes was analyzed according to the manufacturer's directions using Cayman chemical kits (Ann Arbor, MI, USA) for CAT and SOD and with OXItech chemical kit (Zeptometrix Co., Buffalo, NY;USA) for GPX. Tissues from heart of rats were homogenized, cell debris was pelleted and the resulting supernatants were used for the enzyme activity assays. CAT, SOD and GPX activities were measured spectrophotometrically at 540, 450 and 340 nm wavelength, respectively. A unit of CAT activity was defined as the amount of enzyme that caused the formation of 1.0 nmol of formaldehyde per minute at 25°C. A unit of GPX was defined as the amount of enzyme that will cause the formation of 1 μmol $NADP^+$ from NADPH per min at pH 8.0 and at 25ºC. A unit of SOD activity was defined as the amount of SOD needed to exhibit 50% dismutation of the produced superoxide radical at 25°C. The final enzymes activities were calculated by normalizing the results to the total protein concentration of the whole extract.

Western blotting

About 50 μg of protein extracts were separated by SDS-PAGE on 8% (PKCε and phospho-PKCε), 10% (SOD, CAT and β-actin) or 12% (for ERK1/2 and phospho-ERK1/2) gel and transferred to PVDF membrane (GE-Healthcare). The membranes were incubated overnight with the following primary antibodies: anti ERK1/2, anti–phospho-(Thr-202/Tyr-204)-ERK1/2; anti-PKCε, anti-phospho-(Ser-729)-PKCε, anti SOD and anti-CAT, (St. Cruz, and BD Biosciences), all were diluted according to the manufacturer's instructions. Immunoblotted proteins were visualized by using Immuno-Star HRP Substrate Kit (BioRad) and quantified by Kodak Image Station 440CF. To confirm equal protein loading, blots were stripped with 0.4 M NaOH and then re-blotted with an anti-actin antibody (Sigma). Image analyses were performed by the Kodak 1D 3.5 software [9,10]. Western blot densiometric data are normalized with respect to the mean value

of single value loading of β-actin, and data have been presented as the normalized ratio of phospho to total kinases.

Statistical analysis

All data are expressed as means±SEM. One-way ANOVA and Newman–Keuls multiple comparison test (for post-ANOVA comparisons) have been used to compare infarct size. Functional data (Figs 2- 3) were compared with the analysis of area under the curve during reperfusion. A *t* test with Bonferroni correction was also used to compare the last-time points of functional data (Figs 2-3). Postischemic enzymatic activity was related to the mean value of the baseline level. Differences with $p<0.05$ were regarded as statistically significant.

RESULTS

Infarct size

Total infarct size (Fig 1), expressed as a percentage of risk area, was 61±5% in Control Group 1. The early reperfusion with acidic buffer (I/R+AP) significantly reduced the infarct size to 25±5% ($p<0.05$ *vs* I/R Control Group).

The infusion of SOD and CAT during the early reperfusion with Acidic buffer (I/R+AP+CAT Group 3, I/R+AP+SOD Group 4) did not abolish the cardioprotection by Acid buffer (infarct size 28±7%, and 27±4%; $p<0.05$ *vs*. I/R Control Groups, p=NS *vs* I/R+AP for both).

The treatment with antioxidant-enzymes only (I/R+SOD or I/R+CAT) during the first 3-min of reperfusion did not significantly change infarct size (71±7% and 76±7%, respectively) with respect to the I/R Control Group. The infarct size in these two groups resulted significantly higher ($p< 0.05$ for all) than I/R+AP, I/R+AP+SOD and I/R+AP+CAT groups.

Cardiac functional parameters

Baseline values of dLVP, dP/dt_{max}, LVEDP and dP/dt_{min} were similar among groups (data not shown).

Systolic function

In Fig 2, dLVP and dP/dt_{max} (panels A and B, respectively) are reported as percent variation with respect to baseline level. The hearts of the I/R Control Group displayed a marked limitation of dLVP recovery; in fact at the end of reperfusion dLVP was about 35% of baseline level ($p<0.05$ *vs* baseline). In I/R+AP a significant improvement of the dLVP recovery was observed during reperfusion period ($p<0.05$ *vs* I/R group); in particular, at the end of reperfusion the recovery was about 110% ($p< 0.05$ *vs* I/R) of baseline levels. Yet, in I/R+AP+CAT or I/R+AP+SOD, the dLVP recovery was significantly ($p<0.05$ *vs* Control group) improved. Nevertheless, the recovery of dLVP in these two groups was significantly lower that that of I/R+AP. A similar trend was observed for dP/dt_{max} recovery. Of note, in the additional hearts in which CAT or SOD was used alone the recovery of dLVP and dP/dt_{max} was similar to that of I/R Control Group (data not shown).

Diastolic function

Diastolic function is represented by the level of LVEDP and by dP/dt_{min} percent recovery during reperfusion (Fig. 3 panels A and B). In I/R Control Group, it was observed a marked increase of LVEDP, an index of hypercontracture. In I/R+AP group a striking limitation of contracture development was observed during reperfusion (Fig. 3 panel A), though LVEDP remains higher than baseline level (*i.e.* in the contracture range). Yet, in I/R+AP+CAT and in I/R+AP+SOD groups contracture development was somewhat intermediate between I/R and I/R+AP groups. Similarly dP/dt_{min} recovery was improved by acidic perfusion, even in the presence of non-cell permeant exogenous SOD or CAT. In the additional hearts in which CAT

or SOD was used alone the recovery of LVEDP and by dP/dt_{min} was similar to that of I/R Control Group (data not shown).

Antioxidant enzyme activities

Basal activity of SOD CAT, and GPX detected after 30-min stabilization was 1.49±0.5 U/ml, 1.03±0.3 U/ml and 1.98±0.3 U/ml, respectively. No appreciable changes with respect to these baseline activities were observed on samples of Sham hearts collected after stabilization and further 37 or 120 min of perfusion (data not shown). These two latter time points in Sham hearts correspond to the 7^{th} and 120^{th} min of reperfusion in I/R and I/R+AP hearts, and were collected in order to verify the stability of activities during the period of observation in the absence of experimental interventions.

In hearts subjected to I/R (I/R or I/R+AP) the analysis of enzyme activity was measured at the 7^{th} of reperfusion because after triggering of protection at this time point there are relevant modifications of protective signaling pathways [10,18]. In Fig 4, data are represented as percent variation with respect to the mean value of baseline level. As can be seen, in the I/R samples at the 7^{th} minute of reperfusion SOD activity increased to 140±15% ($p< 0.05$ *vs* baseline and Sham). Yet, the activities of CAT and GPX were not changed by I/R. In the I/R+AP samples, the activity of SOD resulted lower than that of I/R, but similar to that of Sham; CAT activity resulted higher than those of Sham and I/R; yet GPX activity resulted lower than those of Sham and I/R.

Western blotting analysis showed that levels of antioxidant-enzymes were stable after 7 min reperfusion in all the experimental condition tested (data not shown).

Phosphorylation levels of Por-Survival Kinases (Fig 5) were differently affected by the different protocols. In particular, the AP treatment induced a significant increase of phosphorylation/activation of PKCε and ERK1/2 with respect to I/R protocol.

DISCUSSION

Clearly the effectiveness of ischemic postconditioning protocols in limiting infarct size depends on their ability to delay normalization of intracellular acidosis during the first minutes of reperfusion [1,4,5,8].

Here we show that acidic perfusion limits myocardial injury at least in part through modification of the activity of endogenous antioxidant-enzymes at the onset of reperfusion. We also show that the acidic early reperfusion not only reduces infarct size but also improves postischemic cardiac function. It seems that acidic perfusion prevents the stunning and limits hypercontracture. These beneficial effects of acidosis in early reperfusion have been correlated to the prevention of calpain activation and to PKG activation [3,4,6]. Here we show that PKCε and ERK1/2 are activated by AP in early reperfusion. Actually, both PKC and PKG activation are followed by phospholamban phosphorylation [19, and unpublished observations of the authors], which may explain, at least in part, diastolic and systolic cardiac function improvement. Most notably, it has been suggested that limitation of infarct size and limitation of contracture are more robust indicators of protection than systolic functional recovery [20].

The exogenous co-infusion for three minutes of CAT or SOD does not avoid beneficial effects of acidic reperfusion against infarct size, but only slightly blunts the improvement of cardiac function induced by early acidic reperfusion. It is likely that exogenous-enzymes (CAT and SOD) do not penetrate the cardiomyocytes and only slightly interferes with cardiac mechanical function. This interference might occur affecting the second-messenger signaling of ROS; specifically H_2O_2, which is cell permeable, has longer half-life, can diffuse across longer distances and can regulate cardiac function [5,8].

The protective effect of acidosis during the first minutes of reperfusion has been demonstrated in different models, including isolated cardiomyocytes and intact hearts [*e.g.* 1-3,10,13]. This protective effects is the consequence of the combined action of different systems, including Na^+/H^+ exchanger (NHE), Na^+/HCO_3^{-2} synport and H^+-coupled lactate efflux [1-5,21], as well as slowed ATP hydrolysis and closure of closed the gap-junctions, which may prevent propagation of necrosis [4,22,23]. In particular, it seems that cell death or survival depends on the correlation between the time of *pHi recovery* and of *Ca^{2+} overload reduction*: if the former occurs first, lethal reperfusion injury may occur, if it is the latter which occurs first, reperfusion injury is attenuated [4,22,23]. Also PostC maneuvers (brief occlusions and reperfusions after ischemia) are effective because they delay the recovery of postischemic pHi, which inhibits mitochondrial permeability transition pores (mPTP) and proteolitic enzymes calpain [1-5,8]. Moreover, early acidosis and ROS signaling keeping closed mPTP give to the heart enough time to activate protective signaling pathways [1,2,5,8,11,18], including ROS-dependent activation of pro-survival kinase, such as PKCε and ERK1/2. In fact, it is well known that PKCε and ERK1/2 are activated by ROS signaling, with a relevant role of inactivation of phosphatases [1,2,4,5,8].

Importantly, the ROS signaling, which we demonstrated to be of pivotal importance in PostC [11] has been documented to be mandatory also in acidic perfusion in early reperfusion [1,2]. This suggests that the slow recovery of postischemic pHi modifies the balance between ROS production and ROS scavenging in early reperfusion in a favorable cardioprotective balance. Of note, acidosis is also responsible of non-enzymatic nitric oxide formation [24], which has been suggested to be involved in PostC-triggering [6,10]. Here we demonstrate that the cardioprotection induced by acidosis modulates the activity of anti-oxidant enzymes (see Fig 4). How these effects on antioxidant-enzymes modify the ROS balance in favor of a species or another can not be inferred from our experiments. However, the lowering of SOD activity with

respect to I/R, in one hand suggest a lower level of dismutation, whereas the different effects on CAT and GPX activities (increase and decrease, respectively) in the other hand suggest a compartmentalization of conversion to water and oxygen of H_2O_2. Of note, hydrogen peroxide may exert a protective signaling role, but may be also a potent oxidizing agent that can be converted to hydroxyl radical in the presence of ferric compounds [5]. Thus a compartmentalization of its conversion might be beneficial limiting its indirect strong oxidizing capacity. Actually, the nature, the site and the moment of production of protective ROS are matter of intense debate [*e.g.* 1,2,5,6,8,10,24]. Nevertheless, as said ROS may activate pro-survival kinases [1,2,4,5,8]. Here, we show that AP activates pro-survival kinases, likely *via* the modulation of redox-environment [1,2, and present study].

Importantly, the optimal pH for SOD, GPX and CAT activity is reported to be around 7.8, 7.4 and 7.0, respectively [25]; see also Sigma-Aldrich Product Information and references therein (www.sigma-aldrich.com). The optimal pH may explain why only CAT increases its activity when acidosis (pH lower than 7.4) persists in early reperfusion [1,2,4].

In conclusion, here we confirm that acidic early reperfusion protects against myocardial I/R injury and affects the activity of endogenous antioxidant-enzymes, thus influencing intracellular redox conditions as well as ERK1/2 and PKCε upregulation, in early reperfusion. Contrary to cell permeant ROS scavengers [1,2], exogenous CAT or SOD are not able to counteract the protective effects of early acidic reperfusion.

Clinical considerations

A few studies have analyzed the effect of transient acidosis during reperfusion in large mammalians (dogs [26,27] and pigs [13]) confirming a protective action against infarction. However, some concerns derive from the possibility to increase arrhythmias [13]; although this observation might be species-specific and at variance with the strong anti-arrhythmic effect of

PostC (*i.e*. intermittent ischemia/reperfusion). In fact, acidic reperfusion reduces the incidence of ventricular fibrillation in rat heart models of regional ischemia [28,29], and there are early reports which propose intermittent ischaemia/reperfusion as a maneuver against ventricular arrhythmias in reperfusion in different species [30-32], including pigs [33] and humans [34]. Nevertheless, NHE inhibitors (eniporide or cariporide) were used as an adjunct to therapy in patients in order to delay pHi recovery during reperfusion in ESCAMI, GUARDIAN and EXPEDITION trials and gave largely disappointing results [36-37]. Further research is needed to investigate the relationship between acidosis, ROS signaling and other mechanisms of PostC cardioprotection and to identify potential ways to translate this knowledge to the treatment of patients undergoing reperfusion.

ACKNOWLEDGMENTS

The authors were supported by Compagnia di S. Paolo, Regione Piemonte, MIUR (PRIN) and University of Turin (ex 60%). We thank Prof. D. Gattullo for insightful suggestions and advice and Miss Deborah Giancotti for the excellent technical assistance.

REFERENCE

1. Cohen MV, Yang XM, Downey JM. The pH hypothesis of postconditioning: staccato reperfusion reintroduces oxygen and perpetuates myocardial acidosis. Circulation 2007;115: 1895.
2. Cohen MV, Yang XM, Downey JM. Acidosis, oxygen, and interference with mitochondrial permeability transition pore formation in the early minutes of reperfusion are critical to postconditioning's success. Basic Res Cardiol 2008;103:464.
3. Inserte J, Barba I, Hernando V, et al. Effect of acidic reperfusion on prolongation of intracellular acidosis and myocardial salvage. Cardiovasc Res 2008;77: 782.
4. Inserte J, Ruiz–Meana M, Rodrìguez–Sinovas A, et al. Contribution of delayed intracellular pH recovery to ischemic postconditioning protection. Antioxid Redox Signal 2011;14:923.
5. Pagliaro P, Moro F, Tullio F, et al. Cardioprotective pathways during reperfusion: focus on redox signaling and other modalities of cell signaling. Antioxid Redox Signal. 2011;14:833.
6. Penna C, Cappello S, Mancardi D, et al. Post-conditioning reduces infarct size in the isolated rat heart: role of coronary flow and pressure and the nitric oxide/cGMP pathway. Basic Res Cardiol 2006;101:168.
7. Penna C, Mancardi D, Tullio F, et al. Intermittent adenosine at the beginning of reperfusion does not trigger cardioprotection. J Surg Res 2009;153:231.
8. Penna C, Perrelli MG, Pagliaro P. Mitochondrial pathways, permeability transition pore, and redox signaling in cardioprotection: therapeutic implications. Antioxid Redox Signal. 2012 (in press).

9. Penna C, Perrelli MG, Raimondo S, et al. Postconditioning induces an anti-apoptotic effect and preserves mitochondrial integrity in isolated rat hearts. Biochim Biophys Acta. 2009;1787:794.
10. Penna C, Perrelli MG, Tullio F, et al. Post-ischemic early acidosis in cardiac postconditioning modifies the activity of antioxidant enzymes, reduces nitration, and favors protein S-nitrosylation. Pflugers Arch 2011;462:219.
11. Penna C, Rastaldo R, Mancardi D, et al. Post-conditioning induced cardioprotection requires signaling through a redox-sensitive mechanism, mitochondrial ATP-sensitive K^+ channel and protein kinase C activation. Basic Res Cardiol 2006;101:180.
12. Perrelli MG, Pagliaro P, Penna C. Ischemia/reperfusion injury and cardioprotective mechanisms: Role of mitochondria and reactive oxygen species. World J Cardiol. 2011;3:186.
13. Rodríguez-Sinovas A, Cabestrero A, García del Blanco B, et al. Intracoronary acid infusion as an alternative to ischemic postconditioning in pigs. Basic Res Cardiol 2009;104:761.
14. Avkiran M, Ibuki C, Shimada Y, et al. Effects of acidic reperfusion on arrhythmias and Na^+-K^+-ATPase activity in regionally ischemic rat hears. Am J Physiol. 1996;270:H957.
15. Fujita M, Asanuma H, Hirata A, et al. Prolonged transient acidosis during early reperfusion contributes to the cardioprotective effects of postconditioning. Am J Physiol Heart Circ Physiol 2007;292:H2004.
16. Tsutsumi YM, Yokoyama T, Horikawa Y, et al. Reactive oxygen species trigger ischemic and pharmacological postconditioning: In vivo and in vitro characterization. Life Sci 2007;81:1223.

17. Altug S, Demiryürek AT, Cakici I, et al. The beneficial effects of peroxynitrite on ischaemia–reperfusion arrhythmias in rat isolated hearts. Eur J Pharmacol 1999;384:157.
18. Tsang A, Hausenloy DJ, Mocanu MM, et al. Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. Circ Res 2004;95:230.
19. Colyer J. Phosphorylation states of phospholamban. Ann N Y Acad Sci. 1998;853:79.
20. Lochner A, Genade S, Moolman JA. Ischemic preconditioning: infarct size is a more reliable endpoint than functional recovery. Basic Res Cardiol. 2003;98:337.
21. Karwatowska-Prokopczuk E, Nordberg JA, Li HL, et al. Effect of vacuolar proton ATPase on pHi, Ca^{2+}, and apoptosis in neonatal cardiomyocytes during metabolic inhibition/recovery. Circ Res 1998;82:1139.
22. Yano Y, Riggs TR, Milam DF, et al. Calcium-accentuated ischemic damage during reperfusion: the time course of the reperfusion injury in the isolated working rat heart model. J Surg Res 1987;42:51.
23. Swietach P, Camelliti P, Hulikova A, et al. Spatial regulation of intracellular pH in multicellular strands of neonatal rat cardiomyocytes. Cardiovasc Res. 2010;85:729.
24. Zweier JL, Samouilov A, Kuppusamy P. Nonenzymatic nitric oxide synthesis in biological systems. Biochim Biophys Acta 1999;1411:250.
25. Shulgin KK, Popova TN, Rakhmanova TI. Isolation and purification of glutathione peroxidase. Appl Biochem and Microbiol 2008;44:247.
26. Kitakaze M, Takashima S, Funaya H, et al. Temporary acidosis during reperfusion limits myocardial infarct size in dogs. Am J Physiol 1997;272: H2071.

27. Preckel B, Schlack W, Obal D, et al. Effect of acidotic blood reperfusion on reperfusion injury after coronary artery occlusion in the dog heart. J Cardiovasc Pharmacol 1998;31:179.
28. Avkiran M, Ibuki C. Reperfusion-induced arrhythmias. A role for washout of extracellular protons? Circ Res 1992;71:1429.
29. Ibuki C, Hearse DJ, Avkiran M. Mechanisms of antifibrillatory effect of acidic reperfusion: role of perfusate bicarbonate concentration. Am J Physiol 199;264:H783.
30. Na HS, Kim YI, Yoon YW, et al. Ventricular premature beat driven intermittent restoration of coronary blood flow reduces the incidence of reperfusion-induced ventricular fibrillation in a cat model of regional ischaemia. Am Heart J 1996;132:78.
31. Galagudza M, Kurapeev D, Minasian S, et al. Ischemic postconditioning: brief ischemia during reperfusion converts persistent ventricular fibrillation into regular rhythm. Eur J Cardiothorac Surg 2004;25:1006.
32. Kloner RA, Dow J, Bhandari A. Postconditioning markedly attenuates ventricular arrhythmias after ischemia-reperfusion. J Cardiovasc Pharmacol Ther. 2006; 11: 55–63.
33. Iliodromitis EK, Georgiadis M, Cohen MV, et al. Protection from post-conditioning depends on the number of short ischemic insults in anesthetized pigs. Basic Res Cardiol. 2006;101:502.
34. Grech ED, Ramsdale DR. Termination of reperfusion arrhythmia by coronary artery occlusion. Br Heart J 1994;72:94.
35. Zeymer U, Suryapranata H, Monassier JP, et al. The Na(+)/H(+) exchange inhibitor eniporide as an adjunct to early reperfusion therapy for acute myocardial infarction. Results of the evaluation of the safety and cardioprotective effects of eniporide in acute myocardial infarction (ESCAMI) trial. J Am Coll Cardiol 2001;38:1644.

36. Theroux P, Chaitman BR, Danchin N, et al. Inhibition of the sodiumhydrogen exchanger with cariporide to prevent myocardial infarction in highrisk ischemic situations. Main results of the GUARDIAN trial. Guard during ischemia against necrosis (GUARDIAN) Investigators. Circulation 2000;102:3032.
37. Mentzer RM, Jr., Bartels C, Bolli R, et al. Sodium-hydrogen exchange inhibition by cariporide to reduce the risk of ischemic cardiac events in patients undergoing coronary artery bypass grafting: results of the EXPEDITION study. Ann Thorac Surg 2008;85:1261.

FIGURE LEGENDS

Fig. 1. Infarct size following 30 min of global ischemia and 30 min reperfusion. Infarct size is expressed as the percent of left ventricle, which is considered the risk area. I/R = 30 min ischemia and 120 min reperfusion; AP = acid perfusion given for 3 min in early reperfusion; CAT= catalase, SOD= superoxide dismutase given for 3 min in early reperfusion.

*p < 0.05 *vs.* I/R group, #p < 0.05 *vs.* groups treated with AP with and without antioxidant enzymes (CAT or SOD). n=6 for each group.

Fig. 2. Heart contractile function during 120 min reperfusion following 30 min global ischemia (vertical dashed lines). (A) Percent temporal change in developed left ventricular pressure (dLVP) and (B) in maximum rate of increase in LVP during systole (dP/dt_{max}). I/R = 30 min ischemia and 120 min reperfusion; AP = acid perfusion given for 3 min in early reperfusion; CAT= catalase, SOD= superoxide dismutase given for 3 min in early reperfusion.

*p < 0.05 *vs.* I/R group, #p < 0.05 *vs.* groups treated with acid perfusion (AP) plus antioxidant enzymes (CAT or SOD). n=6 for each group.

Fig. 3. Heart diastolic function during 120 min reperfusion following 30 min global ischemia (vertical dashed lines). (A) Temporal change in left ventricular end diastolic pressure (LVEDP) and (B) percent change in maximum rate of decrease in LVP during diastole (dP/dt_{min}). I/R = 30 min ischemia and 120 min reperfusion; AP = acid perfusion given for 3 min in early reperfusion; CAT= catalase, SOD= superoxide dismutase given for 3 min in early reperfusion. *p < 0.05 *vs.* I/R group, #p < 0.05 *vs.* groups treated with acid perfusion (AP) plus antioxidant enzymes (CAT or SOD). n=6 for each group.

Fig. 4. Antioxidant enzyme activity in Sham, ischemia/reperfusion (I/R), and acid buffer perfusion (I/R+AP). Panels from top to bottom are superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities at the 7th min of reperfusion. Data are presented as percent variation of baseline level. *p < 0.05 *vs.* Sham group, #p < 0.05 *vs.* I/R group. n=4 for each group.

Fig. 5. Western blot analysis for two Pro-Survival Kinases (PKCε and ERK1/2) at the 7th min of reperfusion. Representative Western blots and relative densitometry showing that AP given in early reperfusion results in an increased phosphorylation of PKCε and ERK1/2, with respect to I/R group. Total kinases and phospho-kinases for each heart are normalized to β-actin, and data are presented as the normalized ratio of phospho to total kinases. Values are mean ± SEM. *p<0.05 with respect to I/R; #p<0.05 with respect to Sham. n=4 for each group.

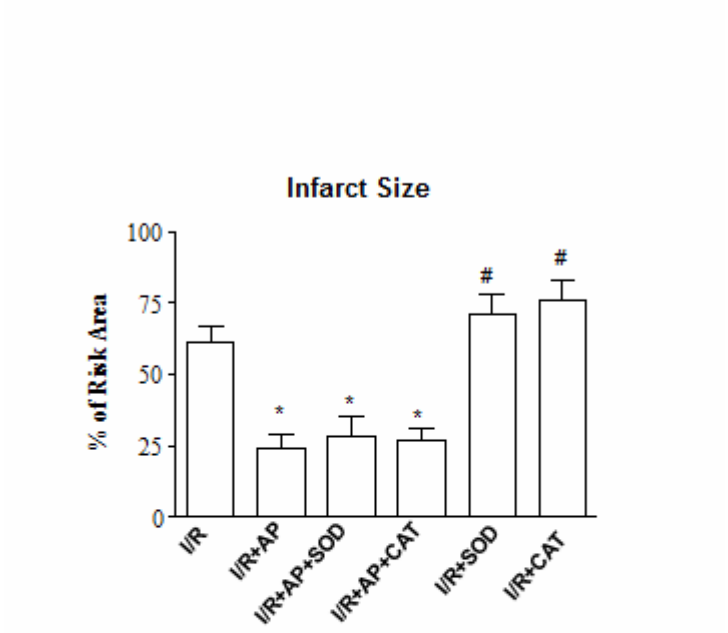
Infarct Size
% of Risk Area
100
75
50
25
0
*
*
*
#
#
I/R
I/R+AP
I/R+AP+SOD
I/R+AP+CAT
I/R+SOD
I/R+CAT

Fig.1

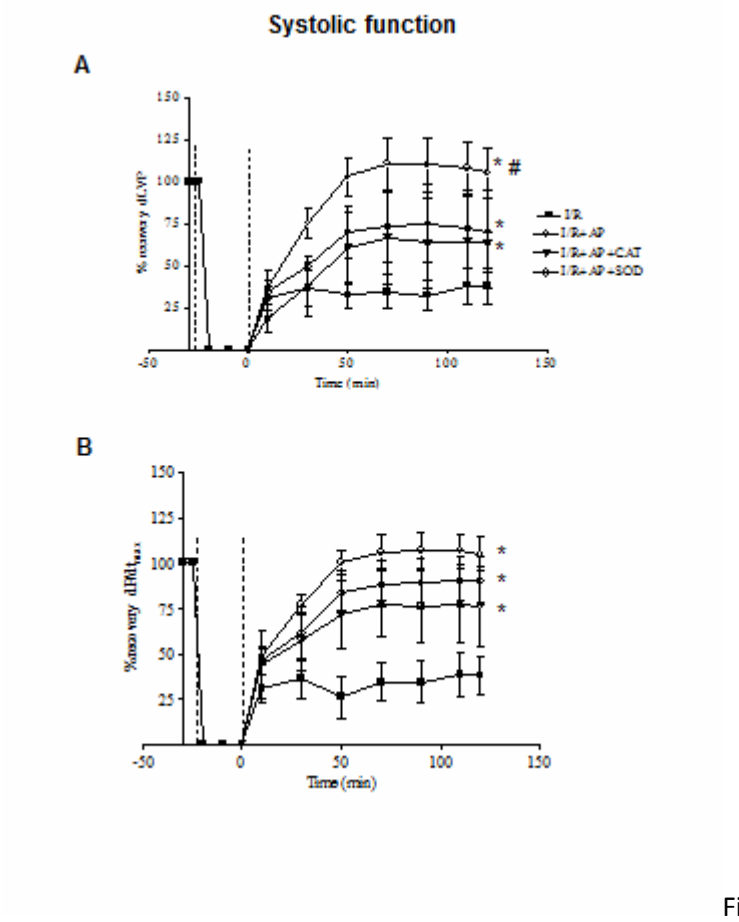
Systolic function
A
% recovery dLVP
Time (min)
I/R
I/R+ AP
I/R+ AP +CAT
I/R+ AP +SOD
B
%recovery dP/dt max
Time (min)

Fig.2

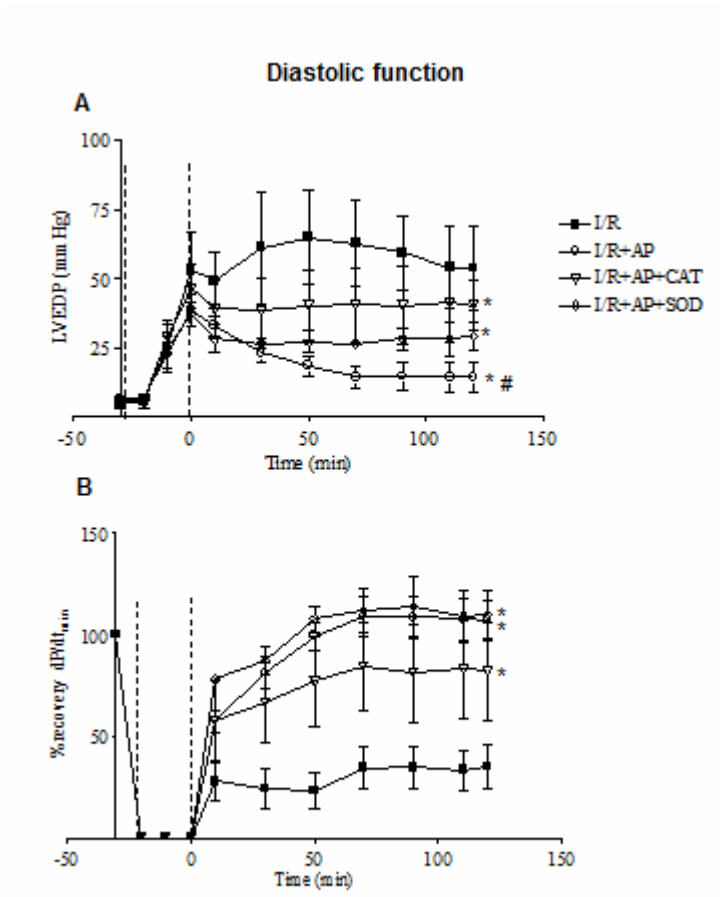
Diastolic function
A
LVEDP (mm Hg)
100
75
50
25
-50
0
50
100
150
Time (min)
I/R
I/R+AP
I/R+AP+CAT
I/R+AP+SOD
*
*
* #
B
%recovery dP/dt max
150
100
50
-50
0
50
100
150
Time (min)
*
*
*

Fig.3

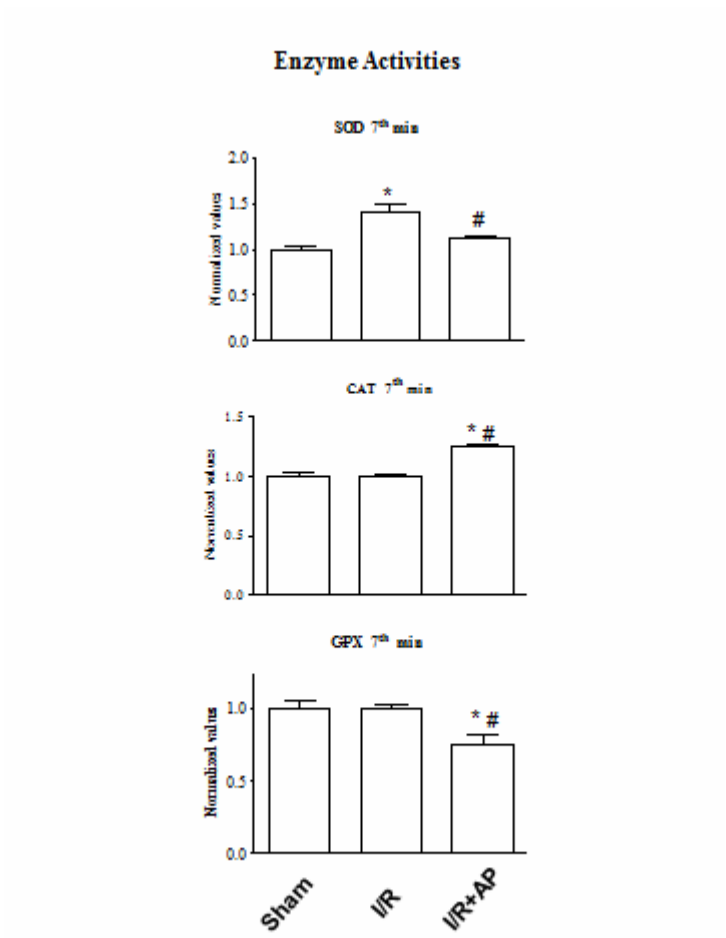
Enzyme Activities
SOD 7th min
Normalized values
2.0
1.5
1.0
0.5
0.0
*
#
CAT 7th min
Normalized values
1.5
1.0
0.5
0.0
* #
GPX 7th min
Normalized values
1.0
0.5
0.0
* #
Sham
I/R
I/R+AP

Fig.4

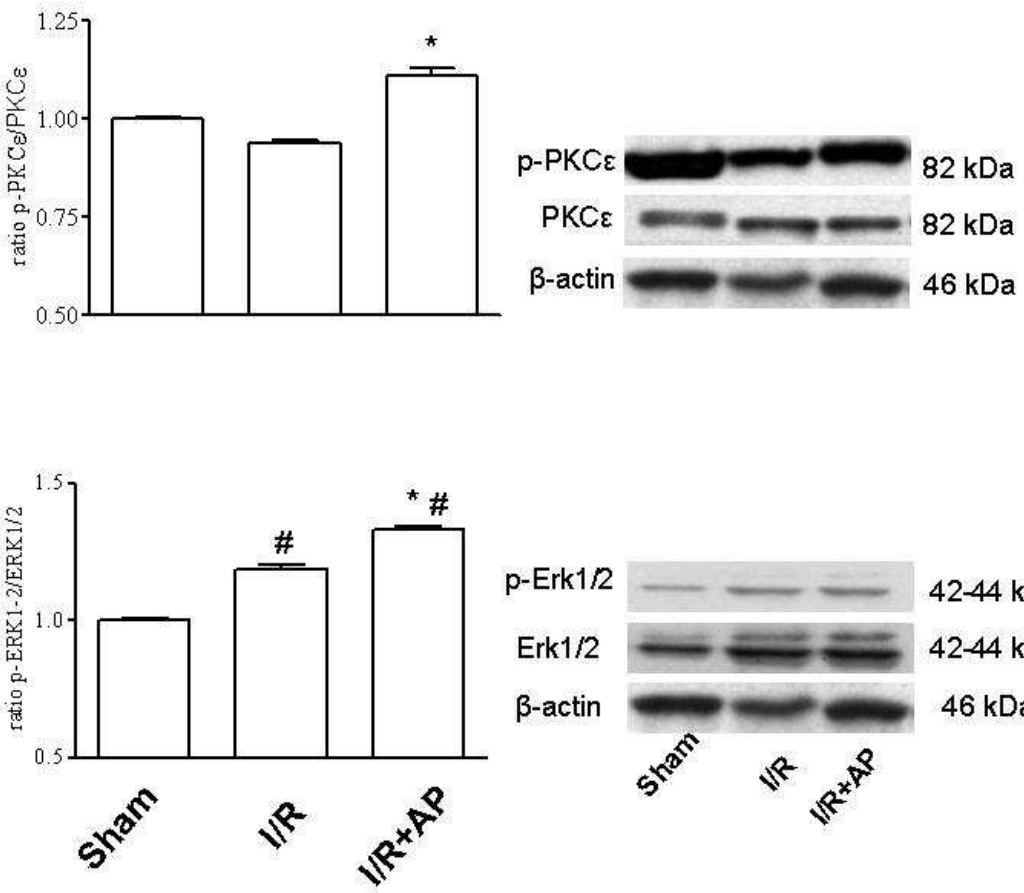
Pro-Survival Kinases
ratio p-PKCε/PKCε
1.25
1.00
0.75
0.50
*
p-PKCε
PKCε
β-actin
82 kDa
82 kDa
46 kDa
ratio p-ERK1-2/ERK1/2
1.5
1.0
0.5
#
* #
p-Erk1/2
Erk1/2
β-actin
42-44 kDa
42-44 kDa
46 kDa
Sham
I/R
I/R+AP

Fig.5