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Post-Ischemic Early Acidosis in Cardiac Postconditioning Modifies the Activity of Antioxidant Enzymes, Reduces Nitration and Favors Protein S-Nitrosylation

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Short title: Antioxidants and postconditioning-triggering

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Abstract

Postconditioning (PostC) modifies early post-ischemic pH, redox-environment and activity of enzymes. We hypothesized that early acidosis in PostC may affect superoxide-dismutase(SOD) and catalase(CAT) activities, may reduce 3-nitrotyrosine(3-NT)- and may increase S-nitrosylated (SNO)-protein levels, thus deploying its protective effects. To verify this hypothesis, we studied early (7th min) and late (120th min) phases of reperfusion *a*) endogenous-SOD and -CAT activities, and *b*) 3-NT- and SNO-protein levels. Isolated rat hearts underwent 30-min ischemia/120-min reperfusion(I/R) or PostC (5 cycles of 10-s I/R at the beginning of 120-min reperfusion) either with or without exogenous-CAT or -SOD infused during the initial 3-min of reperfusion. The effects of early reperfusion with acidic-buffer(AB, pH 6.8) on endogenous antioxidant-enzymes were also tested. Pressure, infarct-size and lactate-dehydrogenase release were also measured. At the 7th min, PostC induced a significant decrease in SOD-activity with no major change in both Mn- and Cu/Zn-SOD levels, and in CAT-activity and level. PostC also reduced 3-NT and increased SNO levels. Exogenous-SOD, but not -CAT abolished PostC-cardioprotection. In late reperfusion(120-min), I/R increased SOD-activity, but decreased CAT-activity and Cu/Zn-SOD levels; these effects were reversed by PostC; 3-NT was not affected, but SNO was increased by PostC. AB reproduced PostC effects on antioxidant-enzymes. Conclusions: PostC downregulates endogenous-SOD and preserves -CAT activity, thus increasing SNO and reducing 3-NT levels. These effects are triggered by early post-ischemic acidosis. Yet acidosis-induced SOD-downregulation may limit denitrosylation; thus contributing to PostC-triggering. Hence, exogenous-SOD, but not -CAT, interferes with PostC-triggering. Persistent SOD-downregulation and SNO-increase may contribute to PostC and AB beneficial effects.

Key words: Cardioprotection; Infarction; Ischemia; Postconditioning; Reperfusion injury.

Introduction

Clearly the only way to limit ischemia damage is organ reperfusion. However with reperfusion injury amplification (*i.e.*, *reperfusion injury*) occurs. In fact, in early post-ischemic phase, mitochondrial permeability transition pore (mPTP) formation may be the event that leads to irreversible changes in cellular function and cell death [*e.g.*, 4,9,26,27,34]. At the onset of a rapid reperfusion when a large increase in reactive oxygen species (ROS) formation occurs along with pH recovery, and Ca^{2+} overload, mPTP opening is facilitated. This, in turn, favors massive ROS formation by inhibiting the respiratory chain [26,27,34]. Therefore a vicious cycle is likely to be established in the so-called ROS-induced ROS release (RIRR) [26,27,34]; thus inducing ROS stress and *reperfusion injury* [for reviews see 46,50]. Survival mechanisms in the heart can be triggered by short, non-lethal periods of ischemia and reperfusion, applied either before (*preconditioning*) or immediately after (*postconditioning*, *PostC*) the index ischemia [3,10,20,25,33,44-46,62]. Both *in vivo* and *in vitro* ischemic- and pharmacological-preconditioning triggering require protective ROS signaling. In fact, either ROS-generators or ROS-scavengers, given before the index ischemia, trigger or abolish preconditioning [2,7,10,16,23,43,47,48,61]. Therefore, ROS are double edge swords (ROS stress *vs* ROS signaling). Also PostC-protection requires ROS signaling and delayed recovery of intracellular pH during initial reperfusion. The mechanisms by which PostC alters the pathophysiology of reperfusion injury involves molecular and physiological mechanisms, such as delaying re-alkalinization of tissue pH [8,9,17,29,30,55], triggering release of ROS and autacoids [13,46,50-52,54,61], and activation of kinases that impact cellular and subcellular targets or effectors, such as mPTP [*e.g.*, 20,46,50,52]. Importantly acidic buffer (AB) given for the first few minutes of reperfusion is as protective as a PostC protocol [8,9,13,17,29,30,34,55]. This supports the idea that the delayed recovery of intracellular pH during initial reperfusion, is mandatory to limit

infarct size in PostC mainly *via* prevention of mPTP opening and prevention of RIRR [8,9,17,29,30,34,55]. Both *in vitro* and *in vivo* studies demonstrated that *large spectrum ROS-scavengers* such as N-acetyl-L-cysteine (NAC) and/or N-(2-mercaptopropionyl)-glycine (MPG) given either during the PostC maneuvers [8,51,54,63] or during AB infusion [8,9,10,13] prevented their protective effects [13,51,54,63]. Moreover, addition of *alkaline buffer* in early reperfusion abolishes both AB- and PostC-protection [8,9,17,29,55]. Therefore, it is likely that interplays between redox and acid/base conditions in early reperfusion exist. Clearly the PostC cycles (*i.e.*, intermittent re-oxygenation) and the early acidosis may be responsible of modifications of enzyme activities and chemical reactions, which lead to produce a few ROS with signaling role instead of ROS stress [13,51,53].

PostC protection is also influenced by the interplay between endothelial and cardiac function, with a flow of nitric-oxide (NO[•]) from endothelium to myocardium [20,46,50,67]. Indeed, persistence of acidosis is important for non-enzymatic NO[•] production [59,69]. So that ROS in concert with NO[•] and reactive nitrogen species (RNS) may put the heart into a protected state [26,38,46,49-52,63].

Actually, in addition to activating cyclic guanosine monophosphate/protein kinase G-dependent signaling pathways, NO[•] and RNS can modify sulfhydryl residues of protein through *S-nitrosylation (SNO)*, which has emerged as an important post-translational protein modification [11,24,40,56,57]. Nitrosylation has been proposed to be important in the preconditioning cardioprotection, also modifying mitochondrial proteins and limiting oxidative stress [6,36,56,57]. Although PostC requires NO[•] [28,29,33,49,52], nitrosylation has not been studied under this condition [56]. Yet, the studies on nitration of proteins with 3-nitrotyrosine (3-NT) concentration (a marker for ONOO⁻ formation) achieved contradictory results: while Kupai *et al.*[32] reported that PostC increases cardiac 3-NT after 5-min of reperfusion, Iliodromitis *et*

al.[28] observed that PostC reduces myocardial and circulating levels of 3-NT after 10-min of reperfusion. Wang *et al.*[65] also observed a decrease in ONOO⁻ formation after hypoxic PostC. However, we have been unable to reproduce cardioprotection with ROS generator given during early reperfusion [51]. Since ROS-scavengers (NAC or MPG) abolished AB- and PostC-induced protection [8,9,51,54,63], it is likely that the type, the concentration, and/or the compartmentalization of ROS/RNS may play a pivotal role in triggering protection at early reperfusion time. To shed some light on this scenario, we studied antioxidant-enzymes, nitration and S-nitrosylation of proteins.

Many endogenous enzymes regulate the homeostasis of ROS/RNS. In the myocardium, three isoforms of superoxide dismutases (SOD), which convert superoxide (O₂⁻) to hydrogen peroxide (H₂O₂), have been described. Steady-state levels of H₂O₂ by conversion to water are regulated by enzymes such as catalase (CAT) and glutathione peroxidase (GPx) [35,64]. Importantly, the activity and levels of endogenous antioxidant iso-enzymes are differently affected by ischemia/reperfusion (I/R) in the various tissues and compartments [12,37]. Yet little is known about the activity of antioxidant enzymes during PostC in the cardiac tissue.

While the optimal pH is 7.0 for CAT activity, it is 7.8 for SOD. Intriguingly, SOD is also a de-nitrosylating enzyme in different systems [21,42,56]. Therefore, we hypothesized that intracellular acidosis during PostC and AB in early reperfusion may downregulate SOD activity and may lead to modifications of 3-NT and SNO levels (Fig. 1). We also hypothesized that a delicate balance between acidosis, ROS/RNS, the S-nitrosylation and nitration/oxidation may occur in early reperfusion [52,56]. It is, thus, likely that the presence of additional specific antioxidant (SOD or CAT) would alter differently the delicate balance induced by PostC maneuvers in early reperfusion. It is also likely that when post-ischemic pH is recovered in late reperfusion, enzyme activity is also recovered.

Therefore in the present study we assessed: 1) the ability of two specific antioxidant enzymes (*exogenous*-SOD or -CAT) given during early reperfusion to abolish PostC-triggering; 2) the *activity* and the *level* of endogenous-SOD and -CAT in early and late reperfusion either after PostC or early AB infusion; and 3) the effects of PostC with and without exogenous-SOD on the *levels* of 3-NT and SNO proteins in early and late reperfusion.

Material and Methods

Animals

Male Wistar rats (5-6 months old) (Janvier S.A.S., St Berthevin Cedex, France) received humane care in compliance with Italian law (DL-116, Jan. 27, 1992) and in compliance 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 [45,50-54]. In brief, each animal was anesthetized. Then 10-min after heparin treatment, the heart was rapidly excised, weighed, attached to the perfusion apparatus and retrogradely perfused with oxygenated Krebs-Henseleit buffer (127 mM NaCl, 17.7 mM NaHCO₃, 5.1 mM KCl, 1.5 mM CaCl₂, 1.26 mM MgCl₂, 11 mM D-glucose (Sigma-Aldrich Corp., St. Louis, MO, USA) and gassed with 95% O₂ and 5% CO₂). A constant flow was adjusted with a pump to obtain a perfusion pressure of 80-85 mmHg during stabilization. Thereafter the same flow level (9±1 ml/min/g) was maintained throughout the experiment.

A balloon was placed into the left ventricle and connected to an electromanometer to record left ventricular pressure (LVP). The balloon was filled with saline to achieve an end-diastolic LVP (EDLVP) of 5 mmHg. Coronary perfusion pressure, coronary flow, EDLVP and developed LVP

(DevLVP) were monitored to assess the preparation conditions. The hearts were electrically paced at 280 bpm and kept in a temperature-controlled chamber (37°C).

Experimental protocols (Fig. 2)

After 30-min stabilization, hearts were subjected to a specific protocol, which was the same for all groups and included: 30-min of global, normothermic ischemia, 120-min of reperfusion followed the 30-min ischemia (see below). Pacing was discontinued on initiation of ischemia and restarted after the 3rd minute of reperfusion in all groups [44,50-54]. After stabilization, the hearts of the Control group (I/R, Group 1, n=12) were exposed to 30-min ischemia and then to 120-min reperfusion only. In Group 2 (PostC group; n=12) after the 30-min ischemia the hearts immediately underwent a protocol of PostC (i.e. five cycles of 10-s reperfusion and ischemia) [50-54].

Antioxidants, either CAT or SOD (Sigma-Aldrich Corp, USA), were given at the beginning of reperfusion for 3-min, with and without PostC at doses previously used in isolated rat hearts [1]. In particular, Group 3 (PostC+CAT, n=10) and Group 4 (PostC+SOD, n=10) hearts underwent I/R plus PostC, in the presence of CAT (100 U/ml) or SOD (10 U/ml), respectively; Group 5 (I/R+CAT, n=9) and Group 6 (I/R+SOD, n=9) hearts underwent I/R only in the presence of CAT (100 U/ml) or SOD (10 U/ml), respectively [1].

For comparative purpose, hearts were also perfused in the presence of PostC maneuvers with heat-inactivated CAT (100 U/ml; PostC+CATi, Group 7, n= 6) or heat-inactivated SOD (10 U/ml; PostC+SODi, Group 8, n= 6). The inactivated enzymes were infused at beginning of reperfusion for 3-min. The heat inactivation was obtained as previously described and confirmed by spectrophotometer analysis. Non-heated exogenous enzymes were normally active [14,58].

In other experiments (n= 4 for each group) hearts underwent either I/R or PostC in the presence of SOD (10 U/ml) plus CAT (100 U/ml) co-infused for initial 3-min reperfusion (not shown in Fig. 2).

CAT and SOD activities and levels

Additional hearts (Fig. 2; n= 6 for each group) were subjected to 180-min perfusion only (*i.e.*, Sham), I/R only, PostC, or AB (pH 6.8) in early reperfusion. AB method was identical to that reported by Rodríguez-Sinovas *et al.* [55]. In these hearts the *activities* and *levels* of endogenous-CAT and -SOD were tested at specific time-points (baseline, 7-min and 120-min after the beginning of reperfusion; Sham hearts were tested at corresponding time-points of perfusion only). The 7-min reperfusion time-point was employed on the basis of previous studies on PostC triggering [4,39,62].

CAT and SOD activities by spectrophotometric analysis:

Activity was analyzed according to the manufacturer's directions using Cayman chemical kits (Ann Arbor, MI, USA). Briefly, tissues from heart of rats were homogenized, cell debris was pelleted and the resulting supernatants were used for the enzyme activity assays. CAT and SOD activities were measured at 540 and 450 nm wavelengths, 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 SOD activity was defined as the amount of SOD needed to exhibit 50% dismutation of the produced superoxide radical at 25°C. The final enzymatic activities were calculated by normalizing the results to the total protein concentration of the whole extract.

CAT and SOD levels by Western blot analysis:

Homogenization protocol: in order to check protein levels, samples of Sham, I/R, PostC, and AB hearts (n= 6 for each group) were homogenized on ice in RIPA Lysis buffer (Santa Cruz Biotechnology), using a polytron tissue grinder. The homogenate was centrifuged at 4 °C for 30-

min at 13,000 g, and the supernatant was collected to quantify proteins with Bradford's method [5]. Western blot analyses were performed as previously described [53].

S-nitrosylated and 3-nitrotyrosine myocardial protein levels

Hearts (n= 6 for each group) subjected to I/R only, PostC, PostC+SOD or to perfusion only (*i.e.*, Sham) were used to assess the *levels* of S-nitrosylated and 3-NT at baseline, 7-min after the beginning of reperfusion and at the end of reperfusion; Sham hearts were tested at corresponding time-points of perfusion only.

Biotin switch assay and Western blot for the detection of S-nitrosylated proteins:

All preparative procedures were performed in the dark to prevent light-induced cleavage of nitrosylations [31]. Heart samples, collected at specified time-points, were homogenized and centrifuged on ice in appropriate buffers (20 mmol l⁻¹ Tris pH 7.5, 150 mmol l⁻¹ NaCl, 1% Igepal CA 630, 0.5% Sodium Deoxycholate, 1 mmol l⁻¹ EDTA, 0.1% SDS, 200 mmol l⁻¹ Sodium Orthovanadate, and Protease Inhibitor Cocktail (Sigma-Aldrich Corp.)) using a polytron tissue grinder. Proteins were quantified with Bradford's method [5], and the biotin switch assay with or without ascorbate (1mM) was performed as previously described [31]. In particular, blocking buffer (225 mmol l⁻¹ Hepes, pH 7.7, 0.9 mmol l⁻¹ EDTA, 0.09 mmol l⁻¹ neocuproine, 2.5% SDS, and 20 mmol l⁻¹ MMTS) and HENS buffer (250 mmol l⁻¹ Hepes, pH 7.7, 1 mmol l⁻¹ EDTA, 0.1 mmol l⁻¹ neocuproine, and 1% SDS) were used (Sigma-Aldrich Corp) [31].

To detect biotinylated proteins by Western blot, samples from the biotin switch assay were separated on 12% SDS-PAGE gels, transferred to PVDF membranes (GE Healthcare Biosciences Piscataway, NJ, USA), blocked with non-fat dried milk (Santa Cruz, CA, U.S.A.), and incubated with streptavidin-peroxidase, diluted 1/10000 for 1 h. In order to ascertain that variations of nitrosylation occurs within cells, S-nitrosylation of cardiac electron-transfer-flavoprotein α,β (ETF α,β) and von Willebrand Factor (vWF) were tested in separated assays (2 samples, for 2

hearts for each group): after detection of biotin, the membrane were stripped and reincubated with polyclonal antibodies against ETFA α,β (Santa Cruz, CA, U.S.A.), and vWF (Dako, Denmark) [18,19]. The vWF was tested because of its localization within endothelial cells and ETFA α,β because mitochondrial proteins extracted from whole hearts originate mainly from cardiomyocytes. To confirm equal protein loading, membranes were incubated with an anti- β -Actin antibody (Sigma-Aldrich Corp). The S-Immunoblotted proteins were visualized by using Immuno-Star HRP Substrate Kit (Bio-Rad Laboratories) and quantified by Kodak Image Station 440CF. Image analyses were performed by the Kodak 1D 3.5 software.

3-nitrotyrosine assay:

Protein concentrations of the samples were quantified with Bradford's method [5]. Nitrotyrosine, as a marker of nitrosative stress, was measured with a competitive enzyme immunoassay: the levels of 3-NT were measured according to the manufacturer's directions using Cell Biolabs kit (Cell Biolabs, Inc., San Diego, CA). The detection sensitivity range was 20 nM to 8 μ M of 3-nitrotyrosine-BSA equivalent.

Assessment of myocardial injury

Since in isolated hearts, pre and postconditioning are known to reduce the release of *lactate-dehydrogenase* (LDH) during reperfusion, the release of LDH was measured during the 2 hours of reperfusion as previously described [45,49,51,53]. *Infarct areas* were also assessed at the end of the experiment with the nitro-blu-tetrazolium technique (Sigma-Aldrich) as described [45,49,51,53,54]. The necrotic mass was expressed as a percentage of total left ventricular mass (*i.e.*, risk area).

Statistical analysis

All data are expressed as means±S.E.M. One-Way ANOVA and Newman–Keuls multiple comparison test (for post-ANOVA comparisons) were used to evaluate the statistical significance among groups. A p value <0.05 was considered statistically significant.

Results

PostC improvement of cardiac function was abolished by exogenous SOD

Table 1 shows I/R (Group 1) having markedly increased CPP and EDLVP, and drastically reduced DevLVP. PostC (Group 2) limited CPP increase and improved the recovery of DevLVP by attenuating the increase in EDLVP during reperfusion. While CAT (Group 3) did not affect PostC-induced improvement of function, SOD (Group 4) abolished these protective effects of PostC. The treatment with either CAT or SOD during the first 3-min of reperfusion in the I/R+CAT or I/R+SOD hearts (Groups 5 and 6, respectively) did not significantly change the deleterious effects of I/R on mechanical function. Moreover, the treatment with inactivated enzymes (CATi or SODi) in the PostC+CATi or PostC+SODi hearts (Groups 7 and 8, respectively) did not significantly change the beneficial effects of PostC on post-ischemic function.

PostC reduction in infarct-size and LDH release were reversed by exogenous SOD

Infarct-size (Fig. 3A), expressed as a percentage of risk area, was 61±5% in Control (Group 1); PostC (Group 2) significantly reduced the infarct-size to 34±5% (p<0.01 vs. Control). PostC+CAT (Group 3) induced a significant reduction of infarct-size to 37±5% (p<0.01 vs. Control; NS vs. PostC; Fig. 3A). In PostC+SOD (Group 4), the infusion of exogenous-SOD abolished the cardioprotection by PostC (Infarct-size 73±8%, p= NS vs. Control).

The treatment with either CAT or SOD in the I/R+CAT or I/R+SOD hearts (Groups 5 and 6, respectively) did not significantly change infarct-size (71±9% and 77±5%, respectively) with

respect to the Control (Group 1). In hearts of Groups 7 and 8 with inactivated enzymes (PostC+CATi and PostC+SODi, respectively), PostC maneuvers still induced cardioprotection. In particular, infarct-size was $36\pm 11\%$ in PostC+CATi and $22\pm 8\%$ of risk area in PostC+SODi ($p = \text{NS}$ vs each other and vs PostC, for both).

Infarct-size data are corroborated by LDH release during reperfusion (Fig. 3 B). In fact, LDH release into the coronary venous effluent was 969 ± 53 U/g wet wt in the Control (Group 1). LDH release was significantly reduced by PostC (Group 2; $p < 0.05$ vs. Control). Also in PostC+CAT (Group 3) a marked reduction of LDH release ($p < 0.01$ vs. Control; $p = \text{NS}$ vs. PostC) was observed. However, in PostC+SOD (Group 4) LDH release was not different from that observed in Control. In I/R+CAT (Group 5) or I/R+SOD (Group 6) LDH release was also similar to that of Control. In hearts of Groups 7 and 8 (PostC+CATi and PostC+SODi, respectively), PostC maneuvers still reduced LDH release ($P = \text{NS}$ vs each other and vs PostC, for both, Fig. 3 B).

In the experiments with the co-infusion of CAT+SOD for initial 3-min, either with or without PostC maneuvers, both infarct-size (78 ± 5 and $69\pm 4\%$, respectively) and LDH release (858 ± 185 or 931 ± 196 U/g, respectively) resulted similar to those of Control group (data not reported in Fig. 3). Notably, once again, the addition of exogenous-SOD in reperfusion abolished PostC protection.

Both PostC and AB reversed the increase in SOD activity induced by I/R, in early reperfusion

In the additional experiments, basal activity of endogenous-CAT and -SOD, detected after 30-min stabilization, was 106 ± 2.4 mol/min/ml and 1.51 ± 0.1 U/ml, respectively. In Fig. 4, data are represented as percent variation with respect to baseline level. No appreciable changes in CAT and SOD activities with respect to baseline level are observed on samples of Sham hearts collected after further 37- or 150-min of perfusion. These time-points in Sham hearts correspond

to the 7th and 120th min of reperfusion in I/R and PostC hearts, *i.e.*, the time-points at which in the I/R, PostC and AB groups the analysis of enzyme activity was also measured.

As can be seen, in the I/R samples, at the 7th minute of reperfusion (Fig. 4 A and B) the CAT activity was 98±2% (p= NS vs. baseline and Sham) and SOD activity was 139±17% (p< 0.05 vs. both baseline and Sham). Yet, in the PostC samples the CAT activity was 126±20% (p= NS vs. its baseline and other Groups) and SOD activity was 78±4% of baseline (p< 0.05 vs. baseline, Sham and I/R). Notably SOD activity resulted about 50% lower than that observed in I/R group. A similar trend in CAT and SOD activity variation was observed after AB infusion (Fig. 4 A and B). In summary, at early reperfusion PostC and AB were associated with significant decreases in total SOD activity with respect to I/R, and with no significant change in catalase activity.

Both PostC and AB prevented the decrease of CAT activity and attenuated the increase in SOD activity induced by I/R, in late reperfusion

At the 120th minute of reperfusion (Fig. 4 C and D), in the I/R samples the CAT activity was 50±10% (p< 0.05 vs. baseline, Sham and the 7th min) and SOD activity 176±6% (p< 0.01 vs. baseline, Sham and the 7th min). That is, in I/R the variations of post-ischemic enzyme activities observed at the 7th min are intensified at the 120th min. Yet, in the PostC samples the CAT activity was 88±9% (p= NS vs. baseline, Sham and the 7th min; p< 0.05 vs. I/R) and SOD activity was 123±5% (p< 0.05 vs. baseline, Sham, I/R and the 7th min); SOD and CAT activities were similarly affected by AB infusion (Fig. 4 C and D). That is, both PostC and AB prevent the decrease of CAT activity and attenuate the increase in SOD activity otherwise induced by I/R in late reperfusion.

Levels of CAT, Cu/Zn-SOD and Mn-SOD were not affected by either I/R, PostC or AB, in early reperfusion

An enzyme level analysis was performed before and after (*i.e.*, at the 7th and 120th min of reperfusion) the index-ischemia in I/R, PostC and AB groups, as well as in corresponding time-points in Sham group (Fig. 5). Notably, Western blotting analysis of tissues revealed that after 7th min of reperfusion there were no significant changes in protein levels in all groups (Fig. 5 A,B and C).

Levels of Cu/Zn-SOD only were reduced in I/R, but not in PostC or AB, in late reperfusion

However, after 120-min reperfusion a significant reduction ($p < 0.01$ vs. Sham) of Cu/Zn-SOD was detected only in I/R; this was reversed by PostC and AB ($p < 0.01$ vs. I/R for both) (Fig. 5 E). That is, in PostC the levels of the two SODs were similar to those of Sham and AB hearts. Catalase levels were stable in all the experimental condition tested. Data suggest that there is a leakage of the cytoplasmatic Cu/Zn-SOD in late reperfusion, which was prevented by PostC and AB. Other enzymes are confined in the organelles and their levels do not change in any relevant way. Alternatively, initial variations in enzyme transcription activated by stress may be responsible of observed differences.

PostC reversed the reduction in S-nitrosylated protein levels induced by I/R in a SOD-dependent manner, in early reperfusion

Nitrosylated protein levels were also studied before ischemia, at the 7th and 120th min of reperfusion in I/R, PostC and PostC+SOD groups and at corresponding time-points in Sham group (Fig. 6).

At the 7th min post-ischemic levels of S-nitrosylated proteins were different from baseline levels in both I/R and PostC. As can be seen in Fig. 6A, in a broad range (5-70 kDa), a lower amount of S-nitrosylated proteins was present in I/R, whereas in PostC a higher grade of S-nitrosylated

proteins was observed. Their quantification is shown in Fig. 6 B: the amount of S-nitrosylated proteins was low in I/R, markedly increased in PostC, and reduced by the addition of SOD (PostC+SOD). Enhanced S-nitrosylation of the intracellular proteins (mitochondrial ETFA $\alpha\beta$ and endothelial vWF) as a consequence of PostC and the reduction of their levels by SOD addition are shown in Fig 7.

PostC limited the reduction in S-nitrosylated protein levels induced by I/R in a SOD-dependent manner, in late reperfusion

At the end of reperfusion (Fig. 6 C and D), the amount of protein S-nitrosylated was reduced significantly ($p < 0.01$) in the I/R, PostC and PostC+SOD with respect to the Sham group. Although the levels of S-nitrosylated proteins were significantly ($p < 0.05$) lower than those observed at the 7th min, the grade of nitrosylation observed in the PostC group was still significantly ($p < 0.05$) higher than those observed in I/R and PostC+SOD groups ($p = \text{NS}$, vs each other).

PostC reversed the increase in 3-nitrotyrosine levels induced by I/R in a SOD-independent manner, in early reperfusion only

In Fig. 8, 3-NT data are represented as percent variation with respect to baseline level (1653 ± 107 nM). In I/R group 3-NT levels showed a significant ($p < 0.05$) increase at the 7th min of reperfusion with respect to corresponding time-point in Sham (Fig. 8A). As observed by Iliodromitis *et al.* [28], intramyocardial 3-NT levels were significantly ($p < 0.05$) decreased by PostC, being similar to Sham hearts in our experiments, regardless of the presence of exogenous SOD (Fig. 8A).

At the end of reperfusion (Fig. 8B) the levels of 3-NT in the I/R, PostC and PostC+SOD Groups were similar to those observed in Sham hearts.

Discussion

Here we show that postconditioning decreases protein nitration (3-NT) and favors protein S-nitrosylation (SNO) in the *early phase of reperfusion*; this is associated with a significant decrease in total SOD activity with respect to I/R. Intriguingly, cardioprotection was abrogated by SOD addition during PostC supporting the idea that PostC-triggering may be involved in a redox-mechanism, *via* an increase in O_2^- flux. This occurs in a moment in which PostC is prolonging acidosis and favoring NO production [8,9,25,29,62,65]. We suggest that in acidosis the simultaneous increase of the flux of NO and O_2^- favors protein S-nitrosylation and reduces nitration, *via* a secondary reaction between $ONOO^-$ and NO [60,66] (see Fig. 1). Yet, since acidosis downregulates SOD, we also suggest a pivotal role for the limited denitrosylation by SOD-downregulation in PostC (Fig. 1) [41,42,56].

In the *late reperfusion* phase SOD activity is increased and catalase activity decreased in I/R; effects that are reversed by PostC. Our results suggest that enzymes were covalently modified *in situ* either by PostC maneuvers or AB; in fact the variation in activity started during early reperfusion and persisted in late reperfusion, and could be observed also in tissue homogenate when pH was recovered. Hence acidosis and reintroduction of O_2 in early reperfusion permitted ROS/RNS signaling (during PostC maneuvers or AB infusion) which could covalently target enzymes, which in turn modify redox environment (*i.e.*, nitration/nitrosylation at 7th and 120th min).

Our hypotheses and analyses were focused on the PostC-triggering phase at 7th min reperfusion (*early reperfusion*) [4,39,62]. At this time-point, we studied, after I/R and PostC, both the levels and activities of CAT and SOD, and measured the levels of 3-NT and SNO proteins. We also studied these parameters at 120-min reperfusion (*late reperfusion*) to have information on persistence of effects and/or on variations of the compartmentalization of redox-environment in

late reperfusion, when post-ischemic pH variations should be recovered [8,9,17,29,30,55]. The main effects observed in the early and late reperfusion are summarized in Table 2.

Early reperfusion

Importantly, in the *early reperfusion* enzyme levels are similar in I/R and PostC. However, the activity of SOD increases in I/R, but is reduced immediately after PostC maneuvers or acidosis. Hence, whenever we add in early reperfusion an active exogenous-SOD to the perfusate PostC is no longer protective. It is likely that the implementation of this enzyme alters the scenario induced by protective PostC, which *per se* reduces SOD activity. On the contrary, 3-min of an active exogenous-CAT does not limit the cardioprotective effects of PostC, which, in fact, *per se* tends to increase the activity of endogenous-CAT with respect to I/R. These data indicate that specific antioxidant enzymes (SOD or CAT) can or cannot abrogate PostC-triggering. However, large spectrum antioxidants (NAC or MPG) abolish both preconditioning and PostC protection in several settings [8-10,13,22,38,51,54,63]. Although the infused enzymes do not easily enter into the cells, enough SOD enters, at least into endothelial cells, to perturb the redox-environment created by PostC (*i.e.*, SOD down-regulation), thus blocking protection. Indeed PostC attenuates endothelial cell dysfunction by increasing eNOS activity and NO bioavailability in neighboring cells [20,67]. The increased availability of NO may, in turn, contribute to SNO formation and PostC protection. It is likely that exogenous SOD during PostC maneuvers may also alter the necessary crosstalk between endothelium and myocardium. The increased S-nitrosylation of endothelial (vWF) and mitochondrial (ETFA α,β) proteins supports the idea that the mechanism is due, at least in part, to a NO signaling from endothelium to cardiomyocytes, which are the richest cells in mitochondria.

A pivotal cardioprotective role for NO[•] from enzymatic and non-enzymatic origin has been shown for both pre and postconditioning [13,15,25,26,46-50,60,66,69]. In fact, NO[•] is a cardiovascular

protective molecule *via* multiple effects, both in normoxia and particularly during reperfusion [15,50,66]. Accordingly, in preconditioning a central role is played by protein SNO, which provides protection preventing further cellular oxidative and nitrosative stress in *reperfusion* [24,40,56]. Yet, the high reduction potential of NO^\bullet severely limits the formation of peroxynitrite (ONOO^-) in this context [46,60,66]. Actually, ONOO^- has also been proposed to be cardio-protective at very low concentrations [15,32,65,69]. In particular, PostC in the presence of a peroxynitrite decomposition catalyst (FeTPPS, 5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron [III]) failed to reduce infarct size in rat hearts [32]. This is an indirect observation supporting the fact that an early increase in peroxynitrite-induced nitrosative stress is involved in the triggering mechanism of cardioprotection by PostC. To the best of our knowledge no studies have shown that peroxynitrite given at reperfusion may mimic the beneficial effects of PostC. On the basis of our study, we suggest that in protected hearts adequate levels of NO^\bullet can quench the transiently formed ONOO^- *via* a *secondary reaction*, thus forming N_2O_3 and leading to protein SNO [40,66] (Fig. 1). According to Wang *et al.* [65] and to Iliodromitis *et al.* [28], who observed a marked decrease in 3-NT 10-min after the beginning of reperfusion, but in contrast with Kupai *et al.* [32], who observed an increase 5-min after the beginning of reperfusion, we observed a decrease in 3-NT 7-min after the beginning of reperfusion in PostC hearts. To reconcile these apparent opposing results, we can speculate that, after an initial formation of ONOO^- , as reaction product of $\text{O}_2^{\bullet-}$ and NO^\bullet , a further increase in NO^\bullet - *via* enzymatic and non-enzymatic processes [54,60,69] - may allow a *secondary reaction* between NO^\bullet and ONOO^- [59,60,66]. This secondary reaction may be responsible of both ONOO^- and 3-NT level lowering, and SNO augmentation (Fig. 1).

The mechanism of NO-conferred prevention of peroxynitrite-dependent damage has recently been reported and is based on formation of a nitrosating species in this process [11]. We can argue that

the simultaneous presence of elevated levels of NO[•] and reduced activity of SOD may favor the appropriate amount of protein SNO (Fig. 6A). Actually, our data are in accordance with the reported denitrosylase activity of SOD [21,42,56]. In fact, beside an increased nitrosylating activity, an increased level of S-nitrosylated protein in PostC hearts may be due to a decreased rate of denitrosylation by SOD-downregulation. Indeed, also preconditioning delays the denitrosylation that is favored by the high oxygen availability [56,57], typically of reperfusion. Accordingly, preconditioning also delays the normalization of tissue pH and requires ROS signaling in early reperfusion after the index ischemia [22,38].

Of note, *persistence of acidosis* is important for both non-enzymatic NO[•] production [60,69] and PostC cardioprotection [8,9,17,29,34,55]. In fact a common mechanism by which ischemic PostC protects the heart is by delaying the normalization of tissue pH, and, in fact, early reperfusion with an acidotic buffer reduces infarct-size to the same extent as PostC [8,9,17,29,34,55 and *unpublished observations of the authors*]. It is likely that low pH buffers act primarily on the endothelial cells, supporting the importance of cross-talk between endothelium and cardiomyocytes. Full reperfusion quickly restores the intracellular pH and initiates several adverse effects, which are collectively known as “pH paradox”. PostC delays re-alkalinization of the heart during early reperfusion, *i.e.* tissue pH remains acidic longer after PostC compared with an abruptly reperfused heart [8,9,17,29,34,55]. For instance, intracellular acidosis during early reperfusion inhibits *calpain* activity and contributes to PostC-protection [29,30]. Since the enzyme activities are strongly influenced by the intracellular pH, we suggest that the acidic intracellular environment maintained by PostC in the early reperfusion play an important role in the observed variation of SOD (optimal pH 7.8) and CAT (optimal pH 7.0) activities. In fact, enzyme activities are similarly influenced by AB infusion and PostC (Fig. 4). Notably, protection by early reperfusion with an acidotic buffer is also redox-dependent [8,9, and *unpublished*

observations] and keeps lower post-ischemic SOD activity for at least 2 hours (present study, Fig. 4). However, it is not easy to study how the pH varies in different organelles during I/R and/or PostC. For this reason, in a perspective of future investigations on the role of pH, here we have opted for a study of the total activity of SOD rather than a study of individual isoforms confined in organelles. Nevertheless, in the early reperfusion there are no significant changes in iso-enzyme levels in I/R and PostC (Fig. 5). We suggest that the observed changes in *activities* of antioxidant enzymes contribute to PostC-triggering.

Overall, in the *early reperfusion*, the observed changes in enzyme activities, nitration and nitrosylation may represent modification of the redox-environment in a delicate moment for the triggering of protection. This redox-mechanism includes an increase in NO[•] and derivative production [9,15,20,34,49,56] that increases the amount of protein SNO, which is also maintained by reduced nitrosylation breakdown by SOD downregulation (as hypothesized, Fig. 1). The high protein S-nitrosylation may provide “protection preventing further cellular oxidative and nitrosative stress” as well as channel opening [56]. Since the main sources of ROS are mitochondria, we can argue that mitochondrial proteins can be among the main targets for either SNO or 3-NT. Actually, several mitochondrial SNO proteins have been seen to be protective in preconditioning and reperfusion [11,36,56,57]. In fact N₂O₃ has an increased stability in the hydrophobic milieu of the mitochondria, where the high levels of reactive cysteines would favor SNO formation [56]. Whether these occur in PostC deserve future studies.

Late reperfusion

In the *late reperfusion* the activity of SOD is still reduced and that of CAT increased by both PostC and AB, if compared with their activities in I/R group (different covalent modifications (?), see below). The increased activity of CAT by PostC or AB in this phase may be important to prevent the further reduction of H₂O₂ to hydroxyl radical (OH[•]), which represents a dangerous

step because an increase in toxicity can occur. Yet, protection with PostC and AB prevents the reduction of the levels of the cytosolic Cu/Zn-SOD, which are otherwise reduced after I/R. The dichotomy on *activities* and *levels* between cytosolic and organelle confined enzymes [68], supports a role for compartmentalization of redox-environment in late reperfusion [52]. Importantly, SNO proteins are reduced by I/R, but this reduction is limited by PostC and not by PostC+SOD. Therefore, also in late reperfusion SNO proteins are still higher in PostC hearts. Whether the compartmentalization plays a role on both enzyme activity and protein nitrosylation needs to be studied further.

Preliminary data obtained with two hearts for each group suggest that both the prolonged SOD-upregulation by I/R and the -downregulation by PostC are likely due to different covalent oxidations; in fact a large spectrum scavenger (MPG) prevents these modifications in activity. Preliminary data obtained with three hearts for each group also show that the *activity* of the cytoplasmatic enzyme GPx does not change either after I/R, PostC or AB (activity ranging between 90 and 110% of baseline level in all hearts). Yet, GPx *levels* tend to decrease (about – 20% vs baseline level) in late reperfusion after I/R, but not after PostC or AB (n=3 for each group). Also the dichotomy of effect (activity/level) on GPx supports a role for compartmentalization of redox-environment [52], which deserves future studies. In fact, we argue that in different compartments, where pH, enzyme activities and/or levels may be differently influenced, the redox-environment may be subtly/patchy varied. Nevertheless, overall reduced activity of SOD by PostC is evident.

Methodological problems

We did not measure radicals, but assessed SOD and CAT activities and the levels of 3-NT and SNO proteins *in vitro*. These may give information about the results of changing ROS/RNS production, and help to overcome the difficulties in measuring the levels of changing radicals *in*

situ. In fact, the various radicals react reciprocally with unpredictable outcome in their levels. For example, we could measure O_2^- and NO^* , but cannot easily predict whether $ONOO^-$ or N_2O_3 formation will prevail (Fig. 1). Thus studying 3-NT- and SNO-proteins, which are more stable products, we could simultaneously have reliable indices of redox environment and of reaction direction. However, enzyme activities were studied at V_{max} , thus we cannot have information on the efficacy of changed activities *in situ*. Moreover, we have studied these redox effects in the isolated hearts and could not predict whether the effects can be replicated *in vivo*. Nevertheless, we have shown a redox signaling in PostC in isolated heart [54] and this observation has been confirmed *in vivo* in different laboratories [8,9,63]. Also these new results need to be confirmed *in vivo*. Moreover, due to variable amount of necrosis and protein leakage from different compartments, the comprehensive interpretation of results requires future *systems biology* studies, which should clarify the role of compartmentalization in enzyme/proteins their specific nitration/nitrosylation, and the consequent effects on their structure, activity and/or response to oxidative/nitrosative stress.

Finally, 3-min of CAT and/or SOD in our I/R setting are not sufficient to limit *per se* infarct-size and the recovery of cardiac function, as more prolonged infusions can do in some experimental settings [41]. Here, we specifically tested the possibility to alter PostC-triggering with these two different antioxidants and did not test longer infusions which have already been largely studied.

In summary: in *early reperfusion* PostC induces SOD downregulation, which together with the persistence of acidosis [8,9,17,29,30,34] and the NO^* augmentation (enzymatic and non-enzymatic production [49,60,65,69]) may favor nitrosylation and/or may limit protein denitrosylation; these together with limited nitrosative stress and preserved CAT activity accompany PostC-triggering. Intriguingly, exogenous-SOD prevents PostC-triggering, whereas exogenous-CAT does not interfere with PostC protection. That is, the addition of exogenous-SOD

does not allow the early reduction in SOD activity, normally induced by PostC or AB. In *late reperfusion*, SOD activity is still reduced and that of CAT increased by PostC and AB, with respect to I/R. However, levels of cytoplasmatic enzymes (*e.g.* Cu/Zn-SOD) are reduced by I/R and preserved by PostC, whereas levels of enzymes confined in the organelles (*e.g.* CAT, Mn-SOD) are not significantly effected by either I/R or PostC. Yet levels of nitrosylated proteins are still higher in PostC.

In conclusion: variations in activity of redox-enzymes, reduced levels of 3-NT and increased levels of SNO proteins may contribute to cardioprotection.

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Figure Legends

Fig. 1. Suggested schematic chemical relationship among different reactive oxygen species and reactive nitrogen species in PostC and early acidotic reperfusion.

The scheme represents a summary of 1) established facts, 2) well known flow of chemical reactions and 3) hypotheses; two dotted vertical lines divide the scheme in these three elements. The scheme is built starting from the evidences that in *early reperfusion* postconditioning (PostC) is characterized by intermittent re-introduction of O₂, reactive oxygen species signaling, persistence of acidosis and nitric oxide (NO[•]) production by endothelial nitric oxide synthase (eNOS) and non-enzymatic processes [8,9,17,20,28-30,46,50,55,56,65]. Based on superoxide (SOD) optimal pH (7.8) and catalase (CAT) optimal pH (7.0), scheme suggests the hypotheses of SOD down-regulation (arrow down) and CAT up-regulation (arrow up) by the early acidosis, which may be a key event for PostC cardioprotection [8,9,17,29,30,55]. In the excess of NO[•], the secondary reaction between ONOO⁻ and NO[•] may increase [60,66] and this might favor S-nitrosylation; SOD down-regulation may limit de-nitrosylation. Therefore, it is also suggested a reduction in tyrosine nitration (arrow down) and an increase in S-nitrosylation (arrow up). In the scheme, the processes, the enzyme activities and the reactions hypothetically decreased/reduced by PostC/acidosis are in dashed lines. GSNO: S-nitrosoglutathione. See text for further explanation.

Fig. 2. Experimental design

The isolated, Langendorff-perfused hearts were stabilized for 30-min (Stab), and then subjected to 30-min of normothermic, global ischemia (I) followed by 120-min of reperfusion (R). Postconditioning (PostC) protocol (5 cycles 10-s ischemia/reperfusion) is indicated by vertical lines at the beginning of reperfusion period. Treatment with exogenous active or inactivated

antioxidant enzymes: either catalase (CAT, CATi) or superoxide dismutase (SOD, SODi) has been infused for 3-min during early reperfusion, as indicated by horizontal black lines.

In *additional experiments* (Sham, I/R, PostC and Acid Buffer (AB)) the activity of CAT and SOD was tested at specific time-points (baseline, 7th min and 120th min after the beginning of reperfusion; Sham hearts were tested at corresponding time-points of perfusion only as indicated by the arrows). For further explanation see text.

Fig. 3. Analysis of ischemia/reperfusion injury and cardioprotective effects on infarct size and LDH release

A: Infarct size (percent of risk area). The amount of necrotic tissue is expressed as percent of the left ventricle, which is considered the risk area.

B: LDH release. The amount of LDH released during reperfusion is expressed as international unit for grams (wet weight) of hearts. Groups are as in Fig. 1. * p< 0.05, **p< 0.01 vs. Control groups. NS= non significant. Groups 1 and 2, n= 12 each; Groups 3 and 4, n= 10 each; Groups 5 and 6, n= 9 each; Groups 7 and 8, n= 6 each.

Fig. 4. Antioxidant enzyme activity in Sham, ischemia/reperfusion (I/R), postconditioning (PostC) and Acid Buffer (AB) hearts.

Panels A and B: catalase and superoxide dismutase (SOD), respectively, at the 7th min of reperfusion.

Panels C and D: catalase and superoxide dismutase (SOD), respectively, at the 120th min of reperfusion.

Data are presented as percent variation of baseline level. § p < 0.05, §§ p < 0.01 vs. baseline level; * p < 0.05, ** p < 0.01 vs. Sham; # p < 0.05 vs. I/R; \$ p < 0.05, \$\$ p < 0.01 vs. corresponding group at the 7th min of reperfusion. NS= non significant. n= 6 for each group.

Fig. 5. Antioxidant enzyme protein levels analyzed by Western blot in Sham, ischemia/reperfusion (I/R), postconditioning (PostC) and Acid Buffer (AB) hearts.

Panels A, B and C are Mn/SOD, Cu-Zn/SOD and Catalase, respectively, at 7th min of reperfusion. Panels D, E and F are Mn/SOD, Cu-Zn/SOD and Catalase, respectively, at 120th min of reperfusion. Data in bargraph are mean (\pm SE) and are presented as percent variation of baseline level. ** p < 0.01 vs. Sham; ## p < 0.01 vs. I/R. n= 6 for each group. For further explanation see text.

Fig. 6. S-nitrosylated proteins in Sham, ischemia/reperfusion (I/R), postconditioning (PostC) and PostC+SOD hearts.

Panels A and C: representative blots of S-nitrosylated proteins in rat heart homogenates at 7th and 120th min of reperfusion, respectively. To control on specificity of the biotin-switch-assay, ascorbate has been omitted in Sham samples (-Asc).

Panels B and D: bargraph are mean (\pm SE) of band concentrations at 7th and 120th min of reperfusion, respectively. Data are presented as percent variation of baseline level. * p < 0.05, ** p < 0.01 vs. Sham; # p < 0.05 vs. I/R; § p < 0.05 vs. PostC; \$ p < 0.05 vs. corresponding group at the 7th min of reperfusion. NS= non significant. n= 6 for each group.

Fig. 7. S-nitrosylation of ETFA α,β and vWF in Sham, ischemia/reperfusion (I/R), postconditioning (PostC) and PostC+SOD hearts.

Protein bands show an increase in S-nitrosylation during PostC and a reduction during PostC+SOD at 7th min of reperfusion.

Fig. 8. Levels of 3-nitrotyrosine in Sham, ischemia/reperfusion (I/R), postconditioning (PostC) and PostC+SOD hearts.

Panels A and B are mean (\pm SE) of 3-NT levels in the ventricular tissue after 7 and 120 min of reperfusion, respectively. Data are percent variation of baseline level. §§ $p < 0.01$ vs. baseline level; ** $p < 0.01$ vs Sham; # $p < 0.05$ vs. I/R. NS= non significant. $n = 6$ for each group.

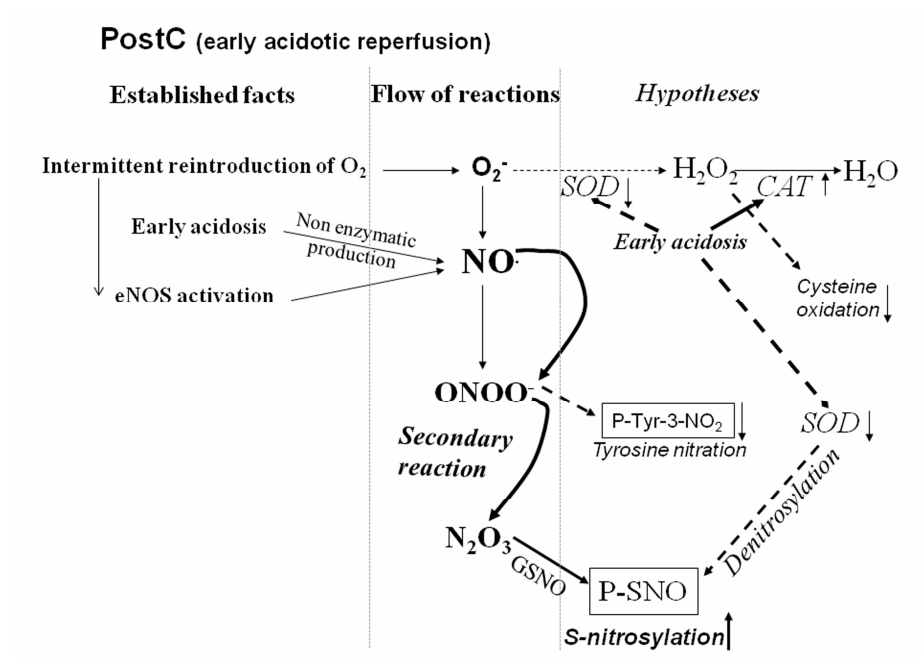


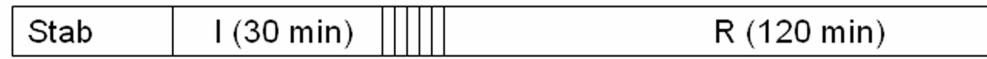
Fig.1

Experimental Design

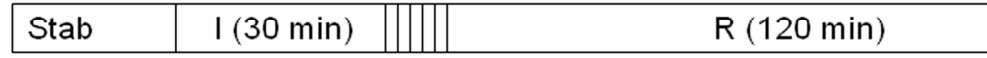
I/R, Group 1



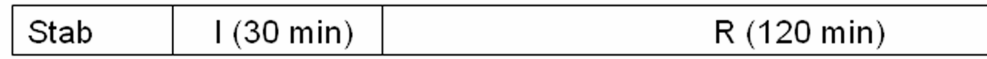
PostC, Group 2



PostC+CAT, Group 3; PostC+SOD, Group 4;



I/R+CAT, Group 5; I/R+SOD, Group 6.



PostC+CATi, Group 7; PostC+SODi, Group 8



Additional experiments

Sham



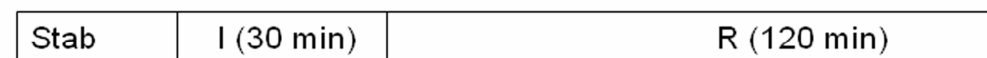
I/R



PostC



AB



↑
baseline

↑
7th

↑
120th min

Fig.2

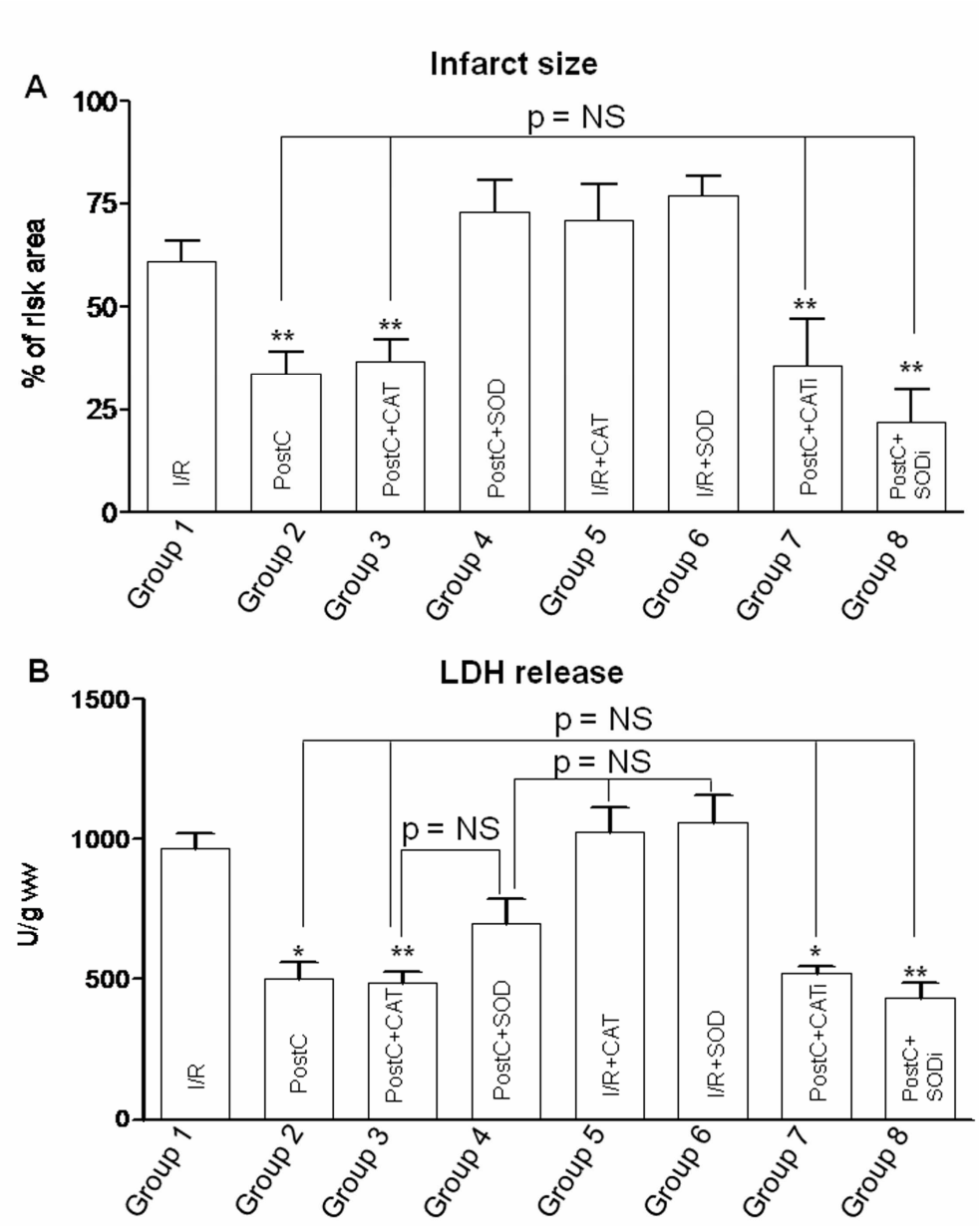


Fig.3

Antioxidant Enzyme Activity

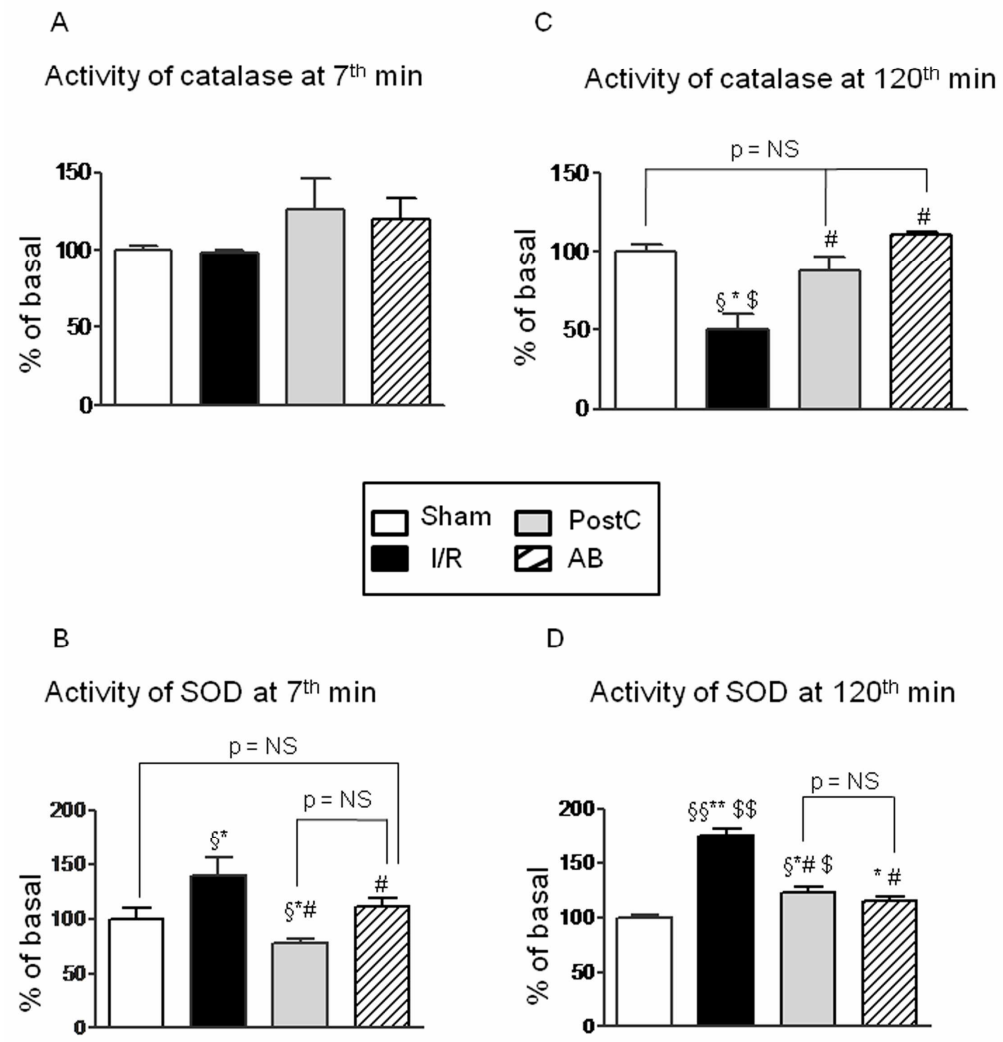


Fig.4

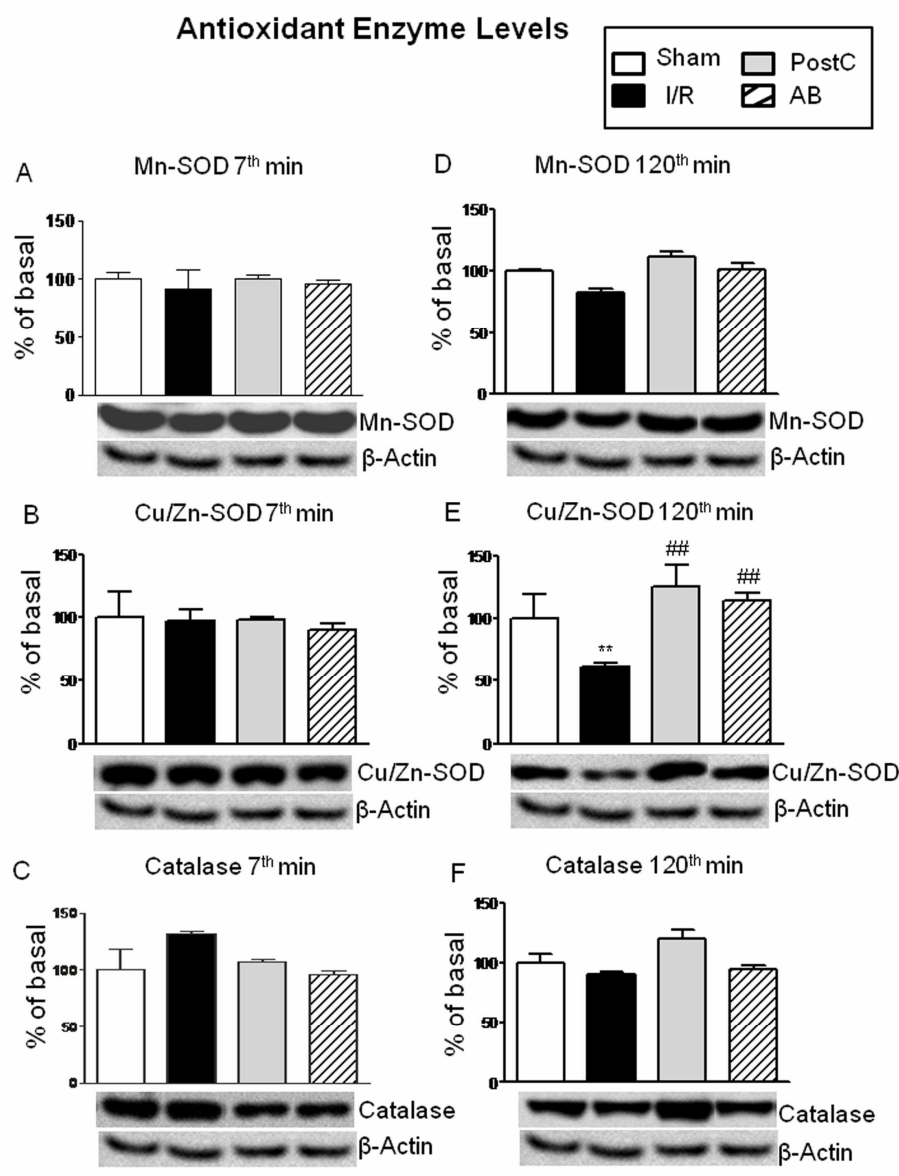


Fig.5

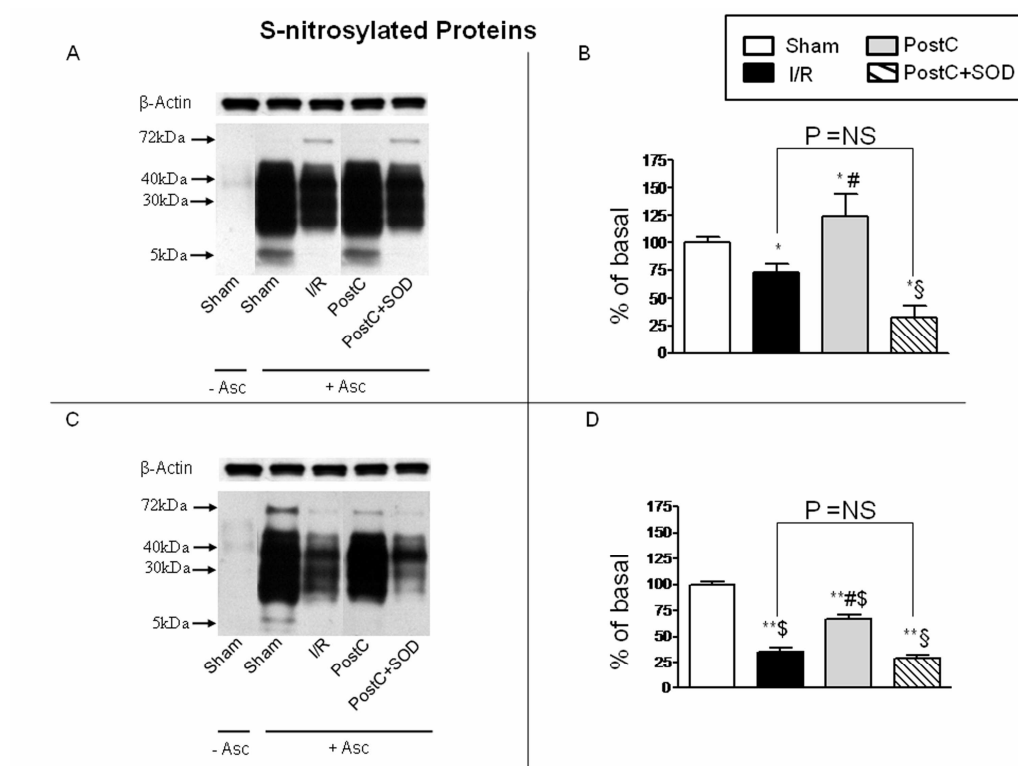


Fig.6

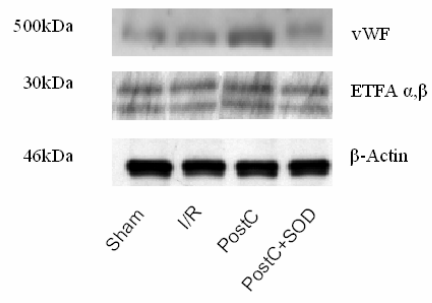


Fig.7

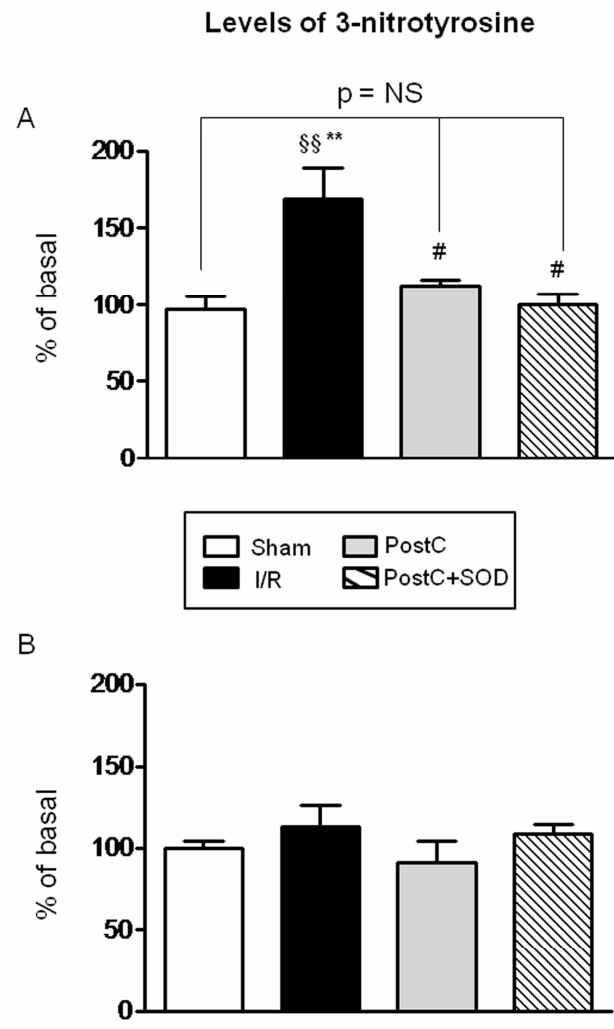


Fig.8