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Chapter 18

Lipid Peroxidation and Reperfusion Injury in Hypertrophied Hearts

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Additional information is available at the end of the chapter

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1. Introduction

Oxidative stress is characterized by an imbalance between increased exposure to reactive oxygen species (ROS), and antioxidant defenses, comprised of both small molecular weight antioxidants like glutathione, and antioxidant enzymes like superoxide dismutase. ROS cause direct damage to critical biomolecules including DNA, lipids, and proteins. Oxidative stress has been involved in the genesis of hypertension [1, 2] and implicated in the mechanisms of reversible postischemic contractile dysfunction (myocardial stunning), microvascular dysfunction, arrhythmias and cell death [3-6]. In spontaneously hypertensive rats (SHR) there are few reports showing the protective action of antioxidants against ischemia-reperfusion injury [7-9] and specifically in regard to the effects of the scavenger N-(2-mercaptopropionyl)-glycine (MPG) these have not been yet examined.

Ischemic preconditioning (IP) is acknowledged to be an endogenous mechanism of cardioprotection against ischemia and reperfusion injury [10-11]. This intervention is based in that one or more brief periods of ischemia applied previous to a prolonged ischemic period exert beneficial effects on myocardium attenuating the deleterious effects observed in the reperfused myocardium. Although there are some studies showing the beneficial effects of IP in hypertensive animals [12-15], under certain circumstances the effectiveness of that intervention is questioned [16-18]. A recent investigation performed in our laboratory shows that a single cycle of IP attenuated the myocardial stunning produced by 20-min global ischemia in SHR [19] and decreased the lipid peroxidation. Whether this protective action of IP is operating at more extended ischemic period and involves changes in oxidative stress in this rats strain is a point that needs to be clarified.

Therefore, the aim of the present study was to determine if alterations of lipid peroxidation and endogenous antioxidants are linked to myocardial and vascular postischemic damage in ischemic control, preconditioned and MPG treated hearts from SHR.

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2. Material and methods

2.1. Isolated heart preparation

Experiments were performed in SHR of 5-month-old following the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised in 1996). Beginning at 12 weeks of age, systolic blood pressure (SBP) was measured weekly in all animals by the standard tail-cuff method [20] following the modifications detailed in a recent paper by Fritz and Rinaldi [21]. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg body wt). The heart was rapidly excised and perfused by the non-recirculating Langendorff technique with Ringer's solution containing (in mmol/L): 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.35 $CaCl_2$, 20 $NaCO_3H$ and 11.1dextrose. The buffer was saturated with a mixture of 95% O_2-5% CO_2, had a pH 7.4, and was maintained at 37°C. The conductive tissue in the atrial septum was damaged with a fine needle to achieve atrioventricular block, and the right ventricle was paced at 280 ± 10 beats/min. A latex balloon tied to the end of a polyethylene tube was passed into the left ventricle through the mitral valve; the opposite end of the tube was then connected to a Statham P23XL pressure transducer. The balloon was filled with water to provide an end-diastolic pressure (LVEDP) of 8-12 mmHg and this volume remained unchanged for the rest of the experiment. Coronary perfusion pressure (CPP) was monitored at the point of cannulation of the aorta and adjusted to approximately 70 mmHg. Coronary flow (CF), controlled with a peristaltic pump, was 11 ± 2 mL/min. Left ventricular pressure (LVP) and CPP data were acquired by using an analog-to-digital converter and acquisition software (Chart V4.2.3 ADInstruments).

2.2. Experimental protocols

After 10 min of stabilization, hearts from SHR were assigned to the following experimental protocols (Fig. 1):

Non-ischemic control hearts (NIC): Hearts were perfused for 3 hs without any treatment.

Ischemic control hearts (IC): Hearts were subjected to 35 min or 50 min of normothermic global ischemia followed by 2 hours of reperfusion. Global ischemia was induced by stopping the perfusate inflow line and the heart was placed in a saline bath held at 37°C.

Ischemic preconditioning (IP1): A single cycle of 5-min ischemia and 10-min reperfusion was applied previous to the 35-min and 50-min ischemic periods followed by 2-hour reperfusion.

Ischemic preconditioning (IP3): Three cycles of 2-min f ischemia and 5-min reperfusion was applied prior to the 50-min ischemic period followed by 2-hour reperfusion. Previous experiments performed by us showed that three cycles are the fewest for achieving myocardial protection of SHR when global ischemia was extended to 50 min.

MPG: Hearts were treated 10 min before ischemia and during the first 10 min of reperfusion with N-(2-mercaptopropionyl)-glycine (MPG) 2 mM. The administration time for MPG was

chosen to attenuate the ROS production during ischemia and reperfusion. The dose was selected according previous experiments performed in our laboratory [22].

Additional experiments were performed (n = 6 for each protocol) to assess the biochemical parameters.

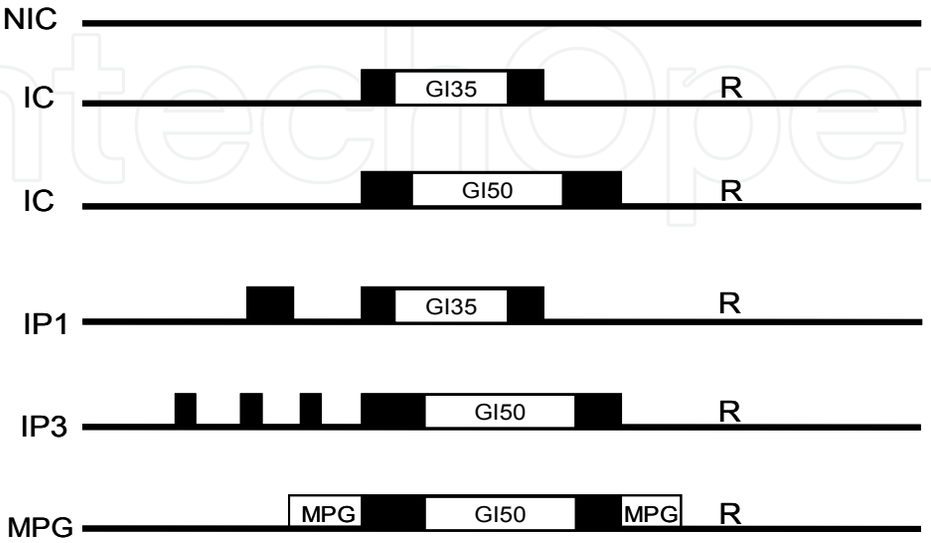


Figure 1. Scheme of the experimental protocols.

2.3. Infarct size determination

Infarct size was assessed by the widely validated triphenyltetrazolium chloride (TTC) staining technique [23]. At the end of reperfusion, atrial and right ventricular tissues were excised and left ventricle (VI) was frozen. The freeze VI was cut into six transverse slices, which were incubated for 5 minutes at 37°C in a 1% solution of triphenyltetrazolium chloride (TTC). To measure myocardial infarction, the slices were weighed and scanned. The infarcted (pale) and viable ischemic/reperfused (red) areas were measured by computed planimetry (Scion Image 1.62; Scion Corp., Frederick, Maryland, USA). Infarct weights were calculated as (A1 × W1) + (A2 × W2) + (A3 × W3) + (A4 × W4) + (A5 × W5) + (A6 × W6), where A is the infarct area for the slice and W is the weight of the respective section. Infarct size was expressed as a percentage of the total area (area at risk, AAR) [24].

2.4. Systolic and diastolic function

Myocardial contractility was assessed by the left ventricular developed pressure (LVDP), obtained by subtracting LVEDP to LVP peak, and maximal velocity of contraction ($+dP/dt_{max}$). The diastolic function was evaluated through LVEDP.

2.5. Assessment of coronary resistance (CR)

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

2.6. Preparation of tissue homogenate

At the end of reperfusion a portion of VI was homogenized in 5 volume of 25 mM PO_4KH_2 - 140 mM ClK at pH = 7.4 with a Polytron homogenizer. Aliquots of homogenate were used to assess reduced glutathione content (GSH) and thiobarbituric acid reactive substances (TBARS) as an index of lipid peroxidation. The remaining homogenate was centrifuged at 12,000 g for 5 min at 4º C and the supernatant stored at -70 ºC until superoxide dismutase (SOD) activity was assayed.

2.6.1. Assessment of reduced glutathione (GSH)

GSH was determined by Ellman's method [25]. This method was based on the reaction of GSH with 5, 5′ dithiobis (2-nitrobenzoic acid) to give a compound that absorbs at 412 nm. GSH levels were expressed as μg/mg of protein.

2.6.2. Assessment of lipid peroxidation

TBARS concentration was determined in the supernatant following the Buege and Aust method´s [26]. 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}.

2.6.3. Measurement of SOD cytosolic activity

SOD activity was measured by means of the nitroblue tetrazolium (NBT) method [27]. Briefly, the supernatant was added to the reaction mixture of NBT with xanthine-xanthine oxidase, and the SOD activity measured colorimetrically in the form of inhibitory activity toward blue formazan formation by SOD in the reaction mixture.

2.6.4. Protein determination

The protein concentration was evaluated by the Bradford method [28] using bovine serum albumin as a standard.

2.6.5. Correlations

The relationships between TBARS, GSH and infarct size and CR were determined by linear regression (equation $y = a + b \cdot x$).

2.7. Statistical analysis

Data are presented as mean ± SE and repeated measures of two-way analysis of variance (ANOVA) with Newman-Keuls test were used for multiple comparisons among groups. Relationships were tested for significance using the Pearson correlation coefficient (r). A P value < 0.05 was considered significant.

3. Results

Fig. 2 shows the infarct size in ischemic control and preconditioned hearts from SHR. In non-ischemic control hearts at the end of the 3-hour perfusion the infarct size was approximately 1 % of risk area. After 35-min global ischemia and 2-hour reperfusion, the infarct size was 35 ± 5 %, which was significantly decreased by one cycle of IP (IP1). When ischemia was extended to 50 min, the infarct size (58 ± 5 %) was not reduced by IP1 indicating that this preconditioning protocol is not adequate for protecting that rat strain against reperfusion injury. However, when a larger number of cycles (three in our case) were applied the hearts were protected and the infarct size diminished. A significant reduction of infarct size was also obtained when MPG was added to the perfusate during 10 min before 50-min ischemia and during the first 10 min of reperfusion.

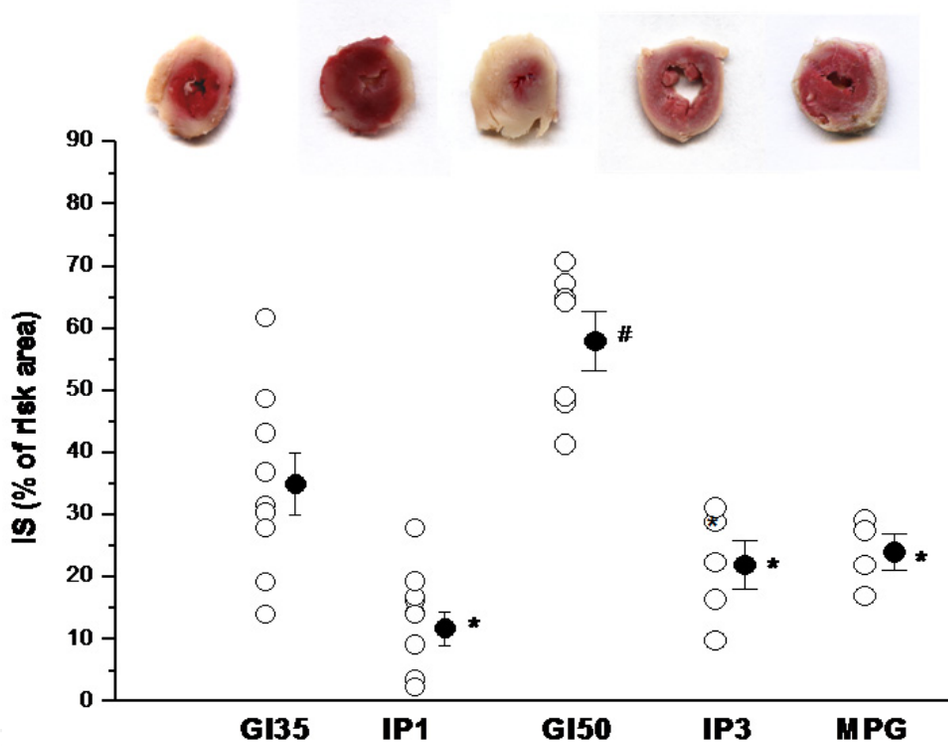


Figure 2. Infarct size (IS), expressed as percentage of risk area, in ischemic control (IC = GI35: 35-min global ischemia; GI50: 50-min global ischemia), preconditioned hearts (IP1= one cycle; IP3= three cycles) and MPG treatment. Note that hearts from SHR showed a higher IS at 50-min compared to 35-min GI. IP1 diminished the IS at 35-min ischemia but it was necessary to apply three cycles (IP3) to protect the hearts when the prolonged ischemia was extended to 50 min and that MPG decreased the IS at a similar value to IP3. * $P < 0.05$ with respect to GI; # $P < 0.05$ with respect to 35-min GI.

At the end of 3-hour non-ischemic hearts exhibited a decrease in contractility of approximately 10 %. After 35-min ischemia and 2-hour reperfusion contractility decreased approximately 90 % with respect to preischemic values. As it is depicted in Fig. 3 the recovery of systolic function was improved by both IP protocols. At the end of the reperfusion period, LVDP and $+dP/dt_{max}$ reached higher values than those obtained in ischemic control hearts. When ischemia was more prolonged (50 min) the postischemic

recovery of contractility was scarce (LVDP and +dP/dt$_{max}$ reached values of approximately 2 %) and it was significantly improved by IP3 and MPG treatment.

The diastolic stiffness characterized by LVEDP increased during 35-min and 50-min global ischemia and acquired greater values during reperfusion. These increases were attenuated by both IP protocols and MPG treatment. Fig. 4 shows the changes of LVEDP occurring at 50-min global ischemia in ischemic control and intervened hearts.

The increase in perfusion pressure at constant coronary flow resulted in an increase of coronary resistance. The increases (4.2 ± 0.4 and 7.0 ± 0.9 mmHg/ml x min^{-1} after 35-min and 50-min ischemia, respectively) were significantly attenuated by both IP protocols and MPG treatment (Fig. 5).

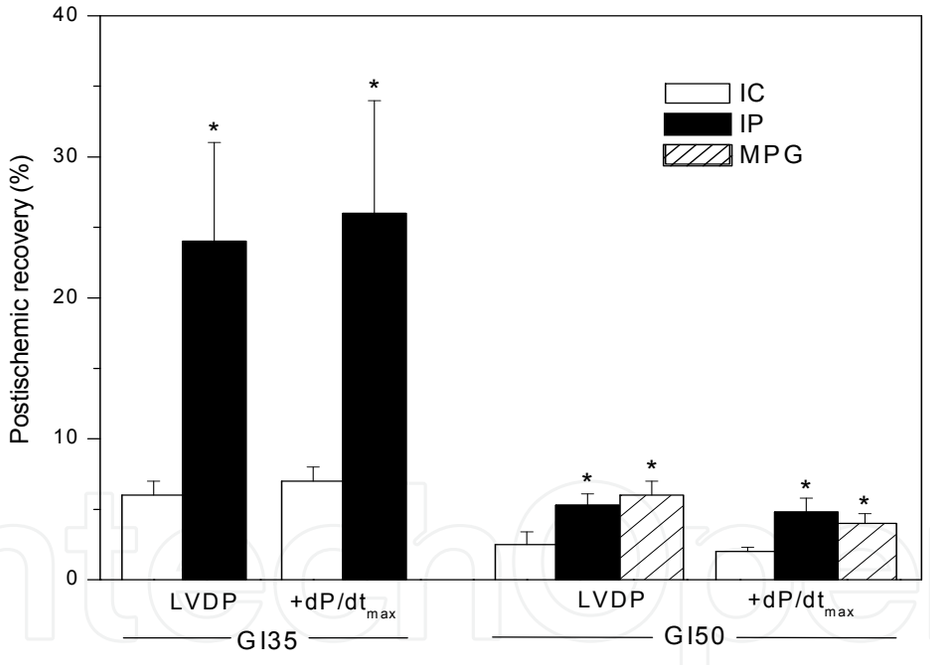


Figure 3. Values of left ventricular developed pressure (LVDP) and maximal velocity of contraction (+dP/dt$_{max}$) at the end of reperfusion period expressed as percentage of preischemic values, in ischemic control (IC = GI35: 35-min global ischemia; GI50: 50-min global ischemia), preconditioned hearts (IP) and MPG treatment. Observe that IP and MPG significantly improved the postischemic recovery of myocardial systolic function at 35-min and 50-min GI. * $P < 0.05$ with respect to IC

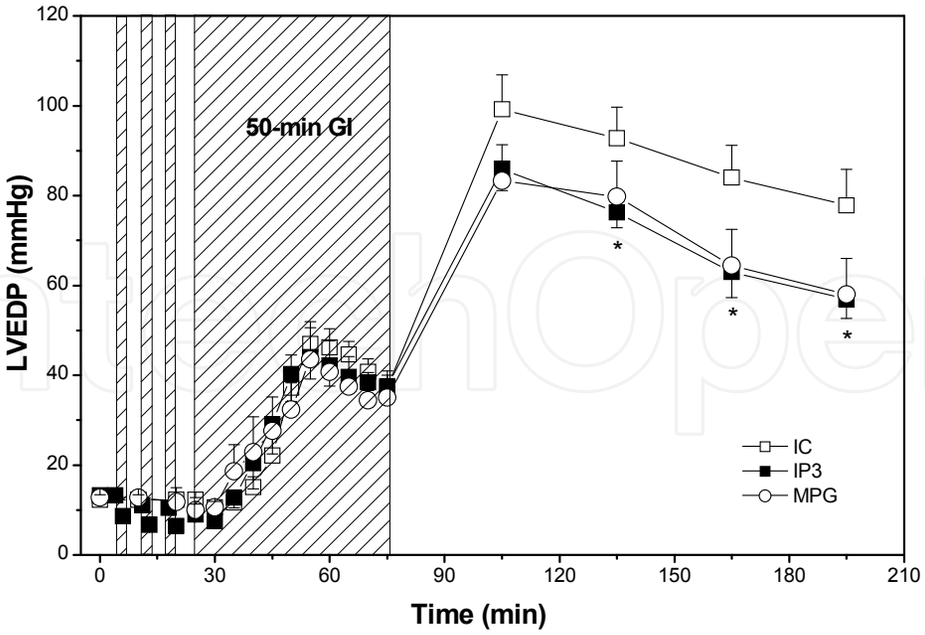


Figure 4. Time course of left ventricular end diastolic pressure (LVEDP) in ischemic control (IC = GI50: 50-min global ischemia), preconditioned hearts (IP) and MPG treatment. The three cycles of IP (IP3) and MPG attenuated in a similar manner the increase of LVEDP detected in IC hearts. * $P < 0.05$ with respect to IC.

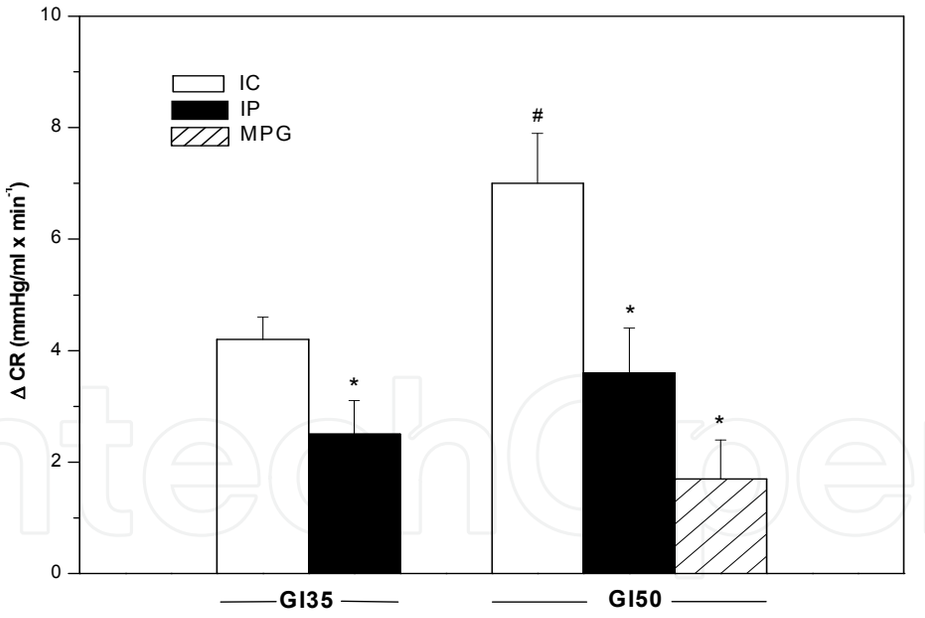


Figure 5. Changes of coronary resistance (CR) at the end of reperfusion in ischemic control (IC = GI35: 35-min global ischemia; GI50: 50-min global ischemia), preconditioned hearts (IP) and MPG treatment. The interventions attenuated the increase of CR detected in IC hearts being MPG the most effective. * $P < 0.05$ with respect to IC; # $P < 0.05$ with respect to GI35.

Given that an increase of ROS generation accompanied by a diminution of antioxidants may be responsible for myocardial reperfusion injury [29, 30], we next determined the impact of IP and MPG on myocardial GSH content, a marker of oxidative stress. Fig. 6 shows that

GSH content in non-ischemic hearts (2 ± 0.3 μg/mg prot) was significantly reduced by ischemia and reperfusion. A single or three cycles of IP and MPG treatment were able to preserve part of the GSH content.

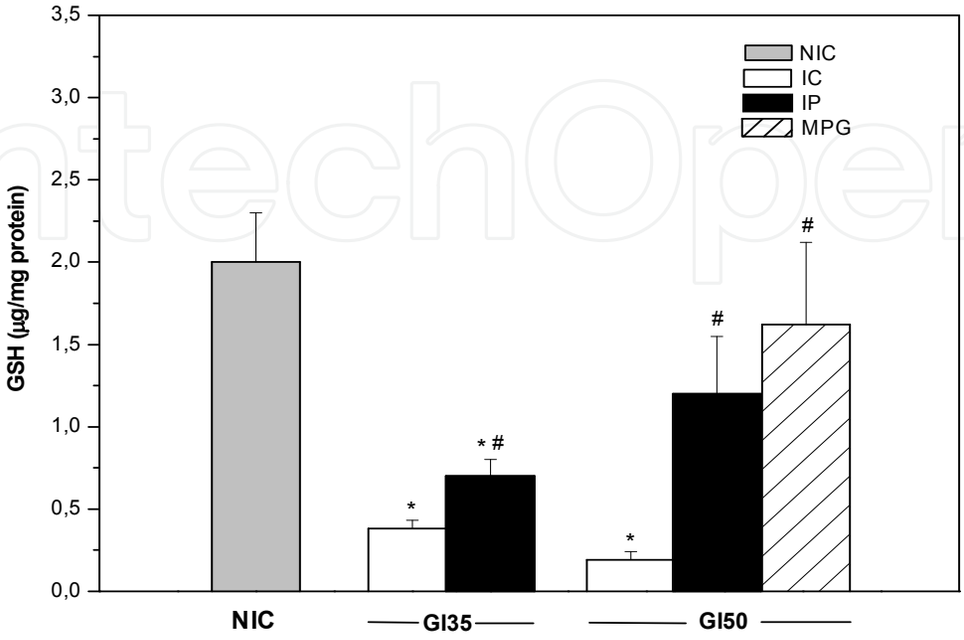


Figure 6. Myocardial reduced glutathione content (GSH, μg/mg protein) in non-ischemic control (NIC), ischemic control (IC = GI35: 35-min global ischemia; GI50: 50-min global ischemia) and preconditioned (IP) and MPG treated hearts. Observe that GSH levels decreased after ischemia and reperfusion in both ischemic periods and were partially preserved by IP and MPG. * $P < 0.05$ with respect to NIC; # $P < 0.05$ with respect to IC.

Moreover, the SOD cytosolic activity increased in ischemic controls hearts and significantly decreased in all intervened hearts (Fig. 7). Both parameters (GSH and SOD) are indicating the presence of oxidative stress caused by ischemia-reperfusion which may be attenuated by IP and MPG treatment.

Since ROS induce membrane lipid peroxidation [29], we determined TBARS content of untreated and treated ischemic-reperfused hearts. Although TBARS determination suffers from potential artifacts associated with sampling, storage and problems caused by the complexity of the biological systems, being easy and reproducible, it is one of the most widely used indexes for assessing oxidative stress. There was an increase in myocardial TBARS content in hearts submitted to ischemia and reperfusion detecting a higher value at 50-min compared to 35-min global ischemia. Preconditioned and MPG treated hearts exhibited lower TBARS levels (Fig. 8).

The analysis of data of the different interventions showed the presence of significant positive correlations TBARS vs IS (Fig. 9, A panel; r = 0,47) and TBARS vs CR (Fig. 9, B panel; n = 0,45) and negative correlations GSH vs IS (Fig. 10, A panel; n = 0,41) and GSH vs CR (Fig. 10, B panel; n = 0,40) in isolated hearts from SHR.

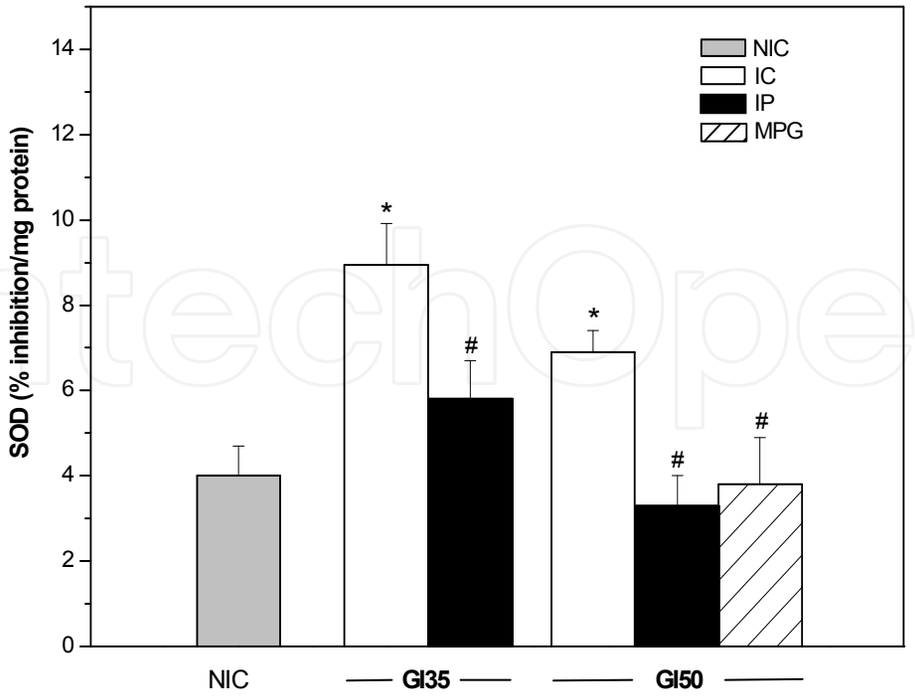


Figure 7. Myocardial SOD cytosolic activity (SOD, % inhibition/mg protein) in non-ischemic control (NIC), ischemic control (IC = GI35: 35-min global ischemia; GI50: 50-min global ischemia), preconditioned (IP) and MPG treated hearts. Note that SOD cytosolic activity increased after 35-min or 50-min GI in comparison to NIC. These increases were attenuated by both interventions (IP and MPG). * $P < 0.05$ with respect to NIC; # $P < 0.05$ with respect to IC.

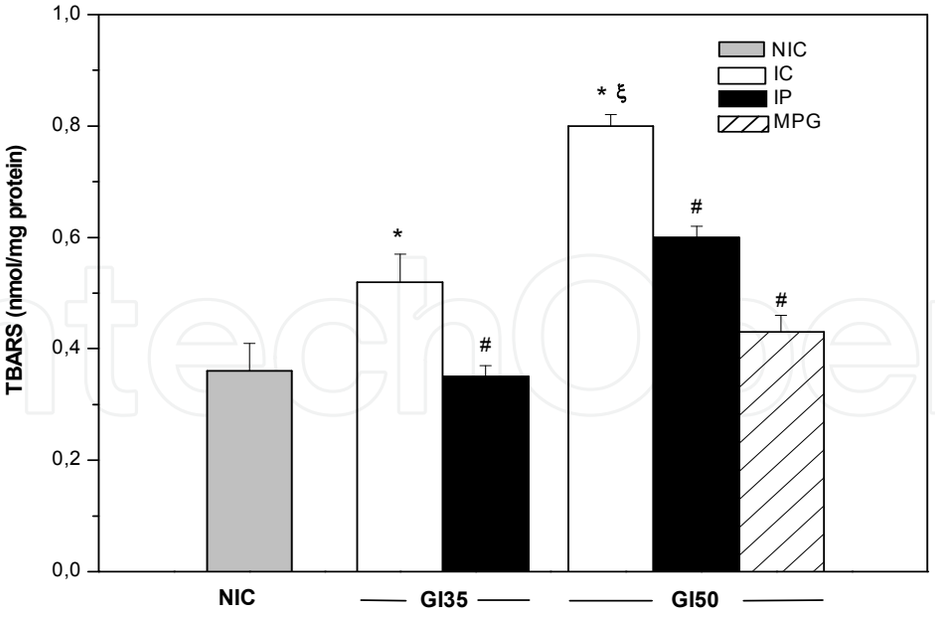


Figure 8. Myocardial thiobarbituric acid reactive substances (TBARS) concentration, expressed in nmol/mg protein in non-ischemic control (NIC), ischemic control (IC = GI35: 35-min global ischemia; GI50: 50-min global ischemia), preconditioned (IP) and MPG treated hearts. An increase of TBARS occurred at the end of reperfusion after the two ischemic periods which were attenuated by IP and MPG. * $P < 0.05$ with respect to NIC ; # $P < 0.05$ with respect to IC; ς $P < 0.05$ with respect to GI35.

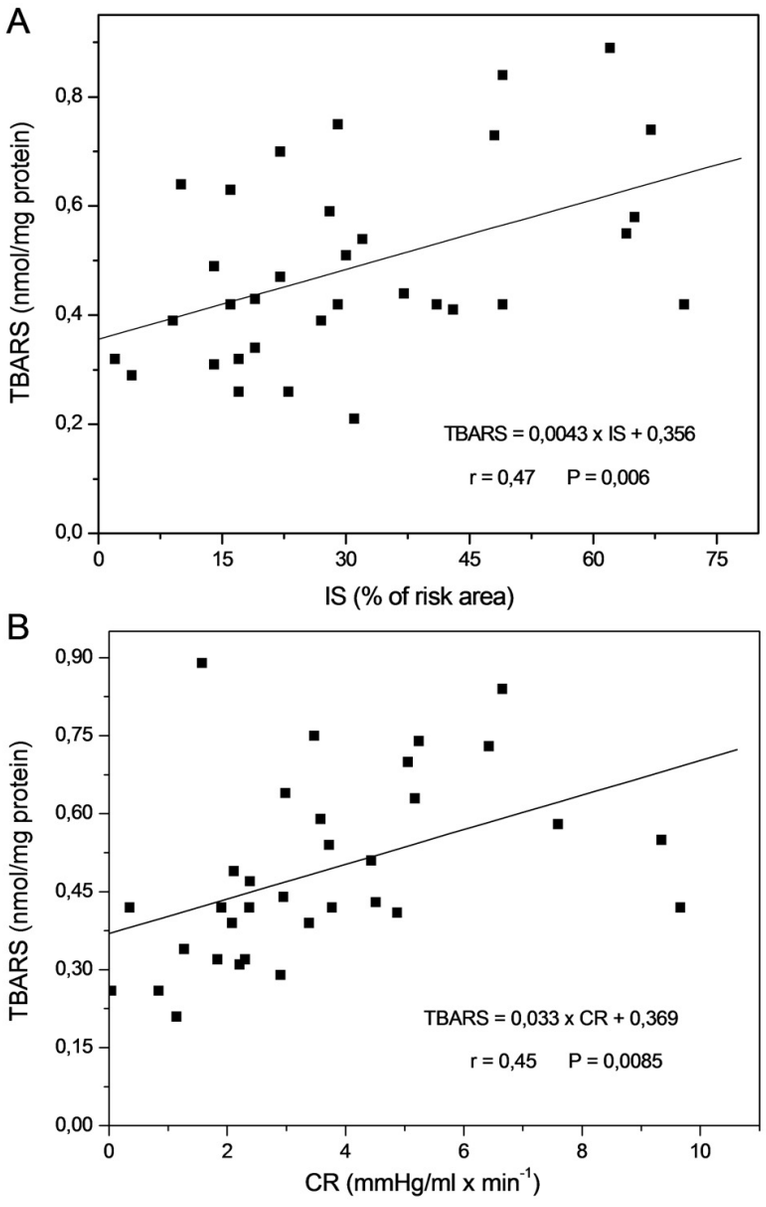


Figure 9. Relationship between TBARS and infarct size (IS, A panel) and TBARS and coronary resistance (CR, B panel) in all experimental situations. The resulting data were fitted to straight line by linear regression. Significant positive correlations between TBARS and IS and CR were found.

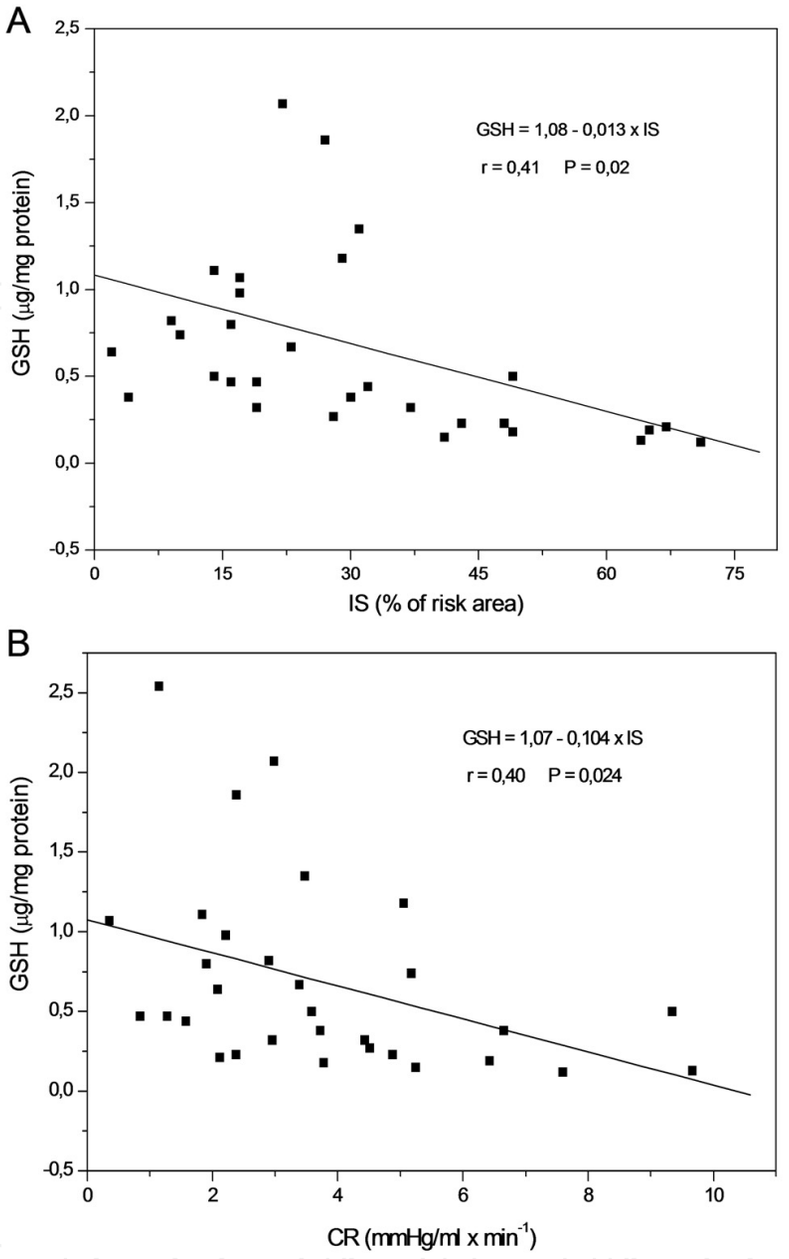


Figure 10. Relationship between GSH and infarct size (IS, A panel) and TBARS and coronary resistance (CR, B panel) in all experimental situations. The resulting data were fitted to straight line by linear regression. Significant negative correlations between GSH and IS and CR were found.

4. Discussion

To our knowledge, this is the first demonstration that the beneficial action of ischemic preconditioning and MPG against ischemia-reperfusion injury is similar in hearts from SHR and is associated with a mitigation of oxidative stress. Thus, our data show the existence of a positive correlation between TBARS concentration-used as an index of lipid peroxidation- and infarct size indicating that it will be found more infarct size when TBARS acquire higher values.

Simultaneously an inverse correlation was detected between GSH content and infarct size indicating that higher levels of GSH are associated to minor infarct size. Both variables (TBARS and GSH) suffered opposite changes due to a possible cause-effect relationship.

These results were also accompanied by changes of SOD cytosolic activity which showed lesser values in preconditioned and MPG treated hearts. Taken together, these data provide evidence to suggest that formation of lipoperoxides is a significant cause of ischemia and reperfusion injury and that the mechanism whereby IP and MPG confer cardioprotection involves, at least in part, an attenuation of those nocive products through a diminution of ROS release and/or production and an improvement of the endogenous antioxidants.

This study clearly shows that hearts from SHR suffer higher irreversible damage at 50-min compared to 35-min global ischemia accompanied with greater impairment of postichemic myocardial function. Thus, at the end of reperfusion the recovery of systolic function was scarce and diastolic stiffness significantly increased in ischemic control hearts. These alterations were attenuated by IP being one cycle of IP (IP1) effective when the ischemic period was 35 min and three cycles (IP3) when the ischemia was extended to 50 min. Thus, although the cardioprotective action of IP in hypertrophied hearts was previously reported [12-15] our study demonstrates that the optimum protocol of IP to protect SHR hearts must be selected according to the duration time of prolonged ischemia. Then, it seems to be possible that the number of IP cycles appears as other key factor for determining the efficacy of IP. Moreover MPG treated hearts in the same way that the preconditioned showed lesser infarct size and improved postischemic recovery of myocardial function in comparison to ischemic control hearts.

Hypertension is associated with an elevation of ROS and frequently with an impairment of endogenous antioxidant mechanisms [30]. These alterations have also been described during ischemia and reperfusion [3, 4, 31-33]. In this study, at the end of reperfusion after ischemic period cardiac tissue showed lesser GSH content, higher TBARS concentration and SOD cytosolic activity in comparison to non-ischemic control hearts. Major changes of GSH and TBARS were detected at 50-min compared to 35-min global ischemia. However, SOD cytosolic activity showed higher increase at 35 min of ischemia. This result may explain the lesser lipid peroxidation found in this experimental group. All these changes were partially reversed by both IP protocols and MPG treatment. Thus, GSH content was higher and SOD cytosolic activity was lower than the values observed in untreated hearts. The favorable changes in GSH and SOD cytosolic activity were reflected in the lower lipid peroxidation (decreased TBARS concentration) observed in preconditioned hearts and in those treated with MPG in comparison to ischemic control hearts. In other words the improvement of the antioxidant systems (SOD and GSH) by IP and MPG treatment were enough to attenuate the oxidative damage detected in untreated hearts. These results suggest that changes of lipid peroxidation and antioxidant systems would be sufficient to promote differences in the cell death and the attenuation of oxidative stress would be considered as a factor contributing to the cardioprotection by IP and MPG treatment in hearts from SHR.

On the other hand, a balance between the production of nitric oxide (NO) and ROS controls the endothelial function [34, 35]. When the NO production is normal its bioavailability may

be reduced because of the oxidative inactivation by an excessive production of superoxide ($O_2^{-\cdot}$) in the vascular wall. The available data on the NO system in SHR are limited and apparently contradictory. Increased ROS in SHR have been demonstrated to enhance NO inactivation and reduce NO bioavailability [36], which contributes to the maintenance of hypertension. According to a previous study the peroxynitrite- product of NO and $O_2^{-\cdot}$ combination- may also be involved in maintenance of the high levels of blood pressure in SHR [37. Furthermore in this rats strain was reported that the activity and/or expression of the different nitric oxide synthase (NOS) isoforms would be altered [38-40] which might act as a compensatory mechanism to maintain the production of bioactive NO in the face of increased oxidant stress [41]. In our study, ischemic control hearts showed an increase of coronary resistance at the end of reperfusion compared to pre-ischemic period which was greater after 50-min than 35-min global ischemia. These increases were attenuated by IP and MPG treatment being this last intervention the most effective. Thus, the beneficial effect of IP and MPG on coronary resistance would be attributed to a greater NO availability mediated by an attenuation of oxidative stress. This mechanism could explain the significant correlations between TBARS, GSH and coronary resistance found in this study and reinforces the idea that changes of oxidative stress constitute the basis of myocardial and vascular postischemic alterations.

5. Conclusions

We can conclude that the level of lipid peroxidation and antioxidant defenses are linked to reperfusion injury in hypertrophied hearts from SHR. The finding that IP and MPG reduce the postischemic myocardial and vascular injury as well as levels of TBARS and improve the endogenous antioxidants suggest that the decrease in ROS levels would be the common mechanism of cardioprotection of both interventions.

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