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Participation of mitochondrial permeability transition pore in the effects of ischemic preconditioning in hypertrophied hearts: Role of NO and mitoK$_{ATP}$

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ABSTRACT

Background: The mitochondrial permeability transition pore (mPTP) plays an important role in ischemia–reperfusion in normotensive animals. Our study aims to define their participation in the ischemic preconditioning (IP) in hypertrophied hearts and to assess the role played by NO and mitochondrial ATP-dependent K channels (mitoK$_{ATP}$).
Material and methods: Isolated hearts from spontaneously hypertensive rats (SHR) and age-matched normotensive rats Wistar Kyoto (WKY) were subjected to 35-min or 50-min global ischemia (GI) followed by 2-hour reperfusion (R). IP was induced by a single cycle of 5-min GI and 10-min R (IP1) or three cycles of 2-min GI and 5-min R (IP3) applied before to prolonged ischemia. L-NAME (NOS inhibitor) or 5-HD (mitoK$_{ATP}$ blocker) to investigate the role played by NO and mitoK$_{ATP}$, respectively were administered. Infarct size (IS), myocardial function, reduced glutathione (GSH) — as marker of oxidative stress and MnSOD cytosolic activity — as an index of mPTP opening were determined.
Results: IP1 significantly decreased the IS in WKY hearts at both ischemia duration times. In SHR, IP1 decreased the IS observed in GI35 but it did not modify that detected at 50-min GI, which was limited by IP3. IP preserved GSH content and decreased MnSOD cytosolic activity in both rat strains. These protective effects were annulled by L-NAME and 5-HD for both ischemic periods in SHR, whereas in WKY they were only effective for 50-min GI.
Conclusion: Our data demonstrate that the cardioprotection achieved by ischemic preconditioning in hearts from SHR hearts involves an attenuation of mPTP opening NO and mitoK$_{ATP}$-mediated.

1. Introduction

Although coronary flow reperfusion is necessary to save the myocardial cell, it aggravates "per se" ischemic injury producing arrhythmias, myocardial stunning and/or infarct size. In previous studies performed in hypertrophied senile hearts an increased susceptibility to damage due to ischemia and reperfusion was observed [1,2]. However, when adult young rats with moderate hypertrophy were used the response was variable showing a similar [3] or a lesser [4,5] post-ischemic recovery of myocardial function in comparison to age-matched normotensive rats.

Ischemic preconditioning (IP) is acknowledged to be a robust cardioprotective intervention that saves ischemic myocardium attenuating reperfusion injury [6,7]. Although there are numerous studies showing the beneficial effects of IP in hypertensive animals [8–11], under certain circumstances the effectiveness of that intervention is questioned [12–14]. A recent investigation performed in our laboratory shows that a single cycle of IP attenuates the myocardial stunning produced by 20-min global ischemia in adult young SHR in a similar manner to normotensive animals [15].

The application incidence of single or multiple bursts of IP against myocardial necrosis has been examined [16], but the possible relationship between the number of preconditioning cycles and the prolonged ischemia duration time has not been studied yet. Also, it is still unknown the optimum protocol for preconditioning SHR hearts when the ischemic period is prolonged.

Nitric oxide (NO) through different pathways [17,18] some of which include the activation of mitochondrial ATP-dependent K

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channels (mitoK$_{ATP}$) [19] has been involved in the protection IP-mediated in normotensive animals. Although alterations in the NO–cGMP pathway have been considered responsible for the exacerbated postischemic mechanical dysfunction in stroke prone rats [20], the role played by both entities – NO and mitoK$_{ATP}$ – in the effects of IP in SHR has not been explored yet.

It has been reported that principally during reperfusion a burst of reactive oxygen species (ROS) and changes in the enzymatic and non enzymatic antioxidant systems occur [21]. The superoxide dismutases (SODs) represent major enzymatic antioxidant mechanisms and MnSOD specifically has an important role in protection against ROS-induced injury [22]. Under normal physiological conditions, the mitochondrial inner membrane is impermeable and MnSOD between other molecules remains into the mitochondria. However, under conditions of high matrix calcium and oxidative stress, such as those occurring during ischemia and reperfusion, a non-specific pore opens in the inner mitochondrial membrane known as the mitochondrial permeability transition pore (mPTP) [23]. Recent studies suggest that IP protects normal myocardium by attenuation of mPTP opening [24,25]. Whether this mechanism is operating in hypertrophied hearts is still unknown.

Thus, our objective was to study the participation of mPTP in the effects of IP in hypertrophied hearts from adult young SHR assessing the role played by NO and mitoK$_{ATP}$.

2. Material and methods

2.1. Isolated heart preparation

Experiments were conducted in 5-month-old SHR and age-matched normotensive male rats (Wistar Kyoto, WKY), which were originally derived from Charles River Breeding Farms, Wilmington, Mass. All animals were identically housed under controlled lighting and temperature conditions with free access to standard rat chow and tap water. The experiments were conducted 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 in 1996). Beginning at 12 weeks of age, systolic blood pressure (SBP) was measured weekly in all animals by the standard tail-cuff method [26] following the modifications detailed in a recent paper by Fritz and Rinaldi [27]. Left ventricular hypertrophy (LVH) was evaluated by the ratio between heart weight (HW) and body weight (BW).

All 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 11.1 dextrose. 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 mm Hg 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 mm Hg. 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 WKY and SHR were assigned to the following experimental protocols (Fig. 1):

Non-ischemic control hearts (NIC; n = 4 for each rat strain): Hearts were perfused for 3 h without any treatment.

Ischemic control hearts (IC; n = 13 for WKY and n = 8 for SHR and for 35-min ischemia; n = 7 for each rat strain and for 50-min ischemia): Hearts were subjected to 35 min or 50 min of normothermic global ischemia followed by 2 h 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; n = 9 for WKY and n = 7 for SHR and for 35-min ischemia and n = 6 for each rat strain and for 50-min ischemia): 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; n = 7 for each rat strain): Three cycles of 2-min 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.

IP1 and IP3 groups (n = 6 for each rat strain and for 35-min and 50-min ischemic periods) were repeated in the presence of nitric oxide synthase (NOS) blocker, 1 mM N^G-nitro-L-arginine methyl ester (L-NAME) or mitoK$_{ATP}$ blocker, 5-

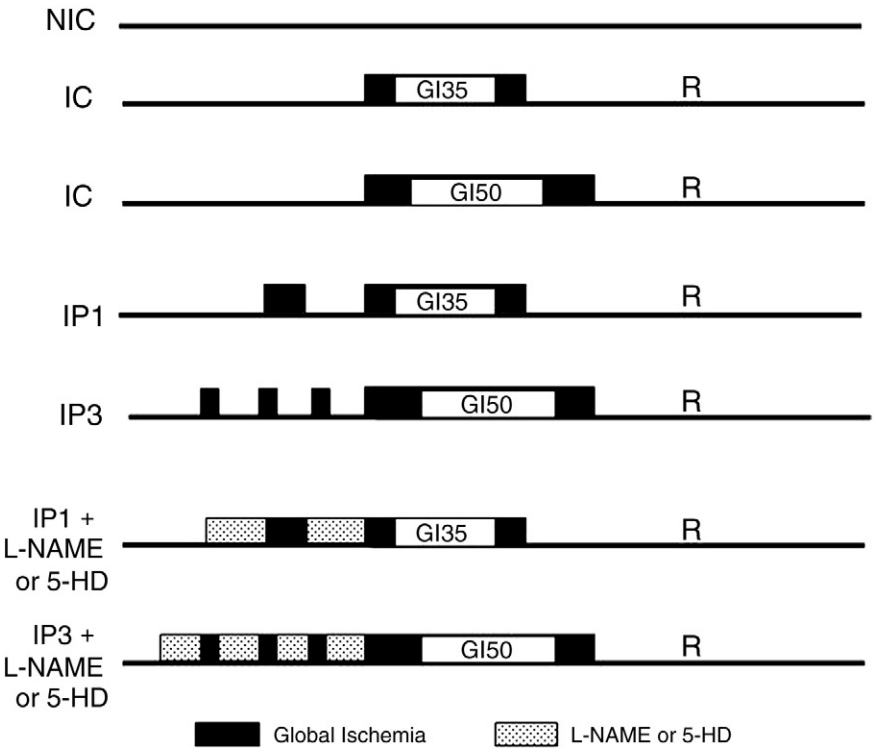

Fig. 1. Scheme of the experimental protocols.

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Table 1
Values of systolic blood pressure (SBP), body weight (BW), heart weight (HW) and hypertrophy index (HI) for SHR and WKY rats.

	WKY (n = 68)	SHR (n = 51)
SBP (mm Hg)	125 ± 4	192 ± 2[a]
BW (g)	316 ± 5	317 ± 6
HW (g)	1.06 ± 0.04	1.49 ± 0.05[a]
HI (mg/g)	3.67 ± 0.12	4.61 ± 0.18[a]

[a] $p<0.05$ with respect to WKY.

hydroxydecanoate (5-HD), and administered 10 min before IP and during the reperfusion phase of preconditioning cycles.

In other hearts the effects of L-NAME or 5-HD alone were examined. All protocols were repeated and the hearts frozen for the biochemical determinations.

2.3. Infarct size determination

Infarct size was assessed by the widely validated triphenyltetrazolium chloride (TTC) staining technique [28]. 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 min 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 $(A1\times W1)+(A2\times W2)+(A3\times W3)+(A4\times W4)+(A5\times W5)+(A6\times W6)$, 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) [29].

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 lipid peroxidation. The remaining homogenate was centrifuged at 12,000 *g* for 5 min at 4 °C and the supernatant stored at −70 °C until superoxide dismutase (SOD) activity was assayed.

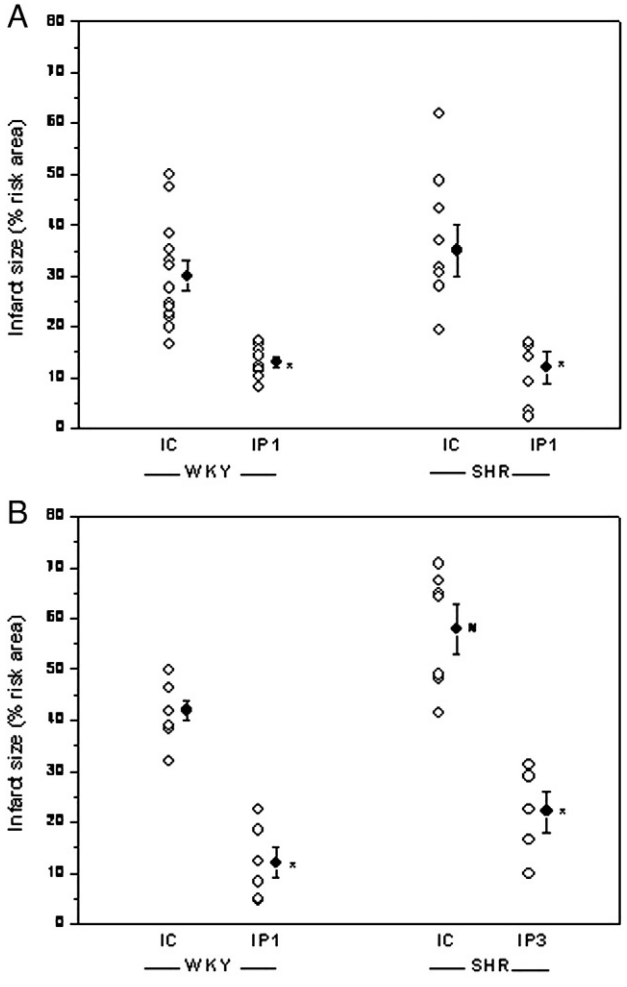

Fig. 2. Infarct size (IS), expressed as percentage of risk area, in ischemic control (IC) and preconditioned hearts (IP) in both ischemic periods (35 and 50 min). Note that hearts from SHR showed a similar IS at 35-min ischemia but significantly higher IS than WKY at 50-min ischemia. Note also that IP one cycle (IP1) decreased the infarct size detected in IC hearts from WKY at the two periods of ischemia. Interestingly, in SHR IP1 diminished the infarct size at 35-min ischemia but it was necessary to apply three cycles (IP3) to protect the hearts when the prolonged ischemia was 50 min. A panel: 35-min global ischemia; B panel: 50-min global ischemia. *$p<0.05$ with respect to IC; #$p<0.05$ with respect to WKY.

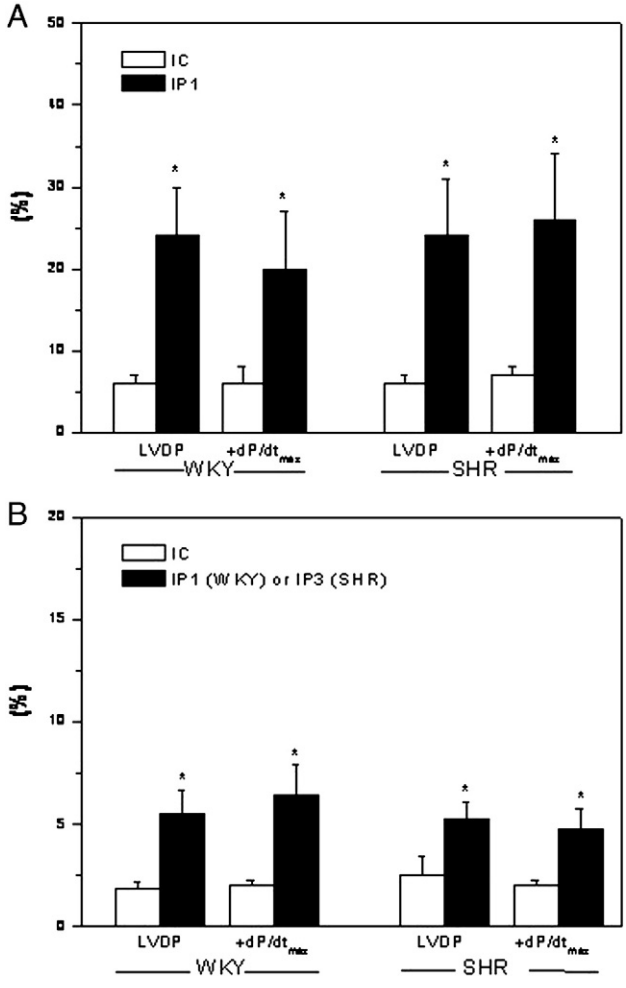

Fig. 3. Values of left ventricular developed pressure (LVDP) and maximal velocity of contraction ($+dP/dt_{max}$) at the end of reperfusion period expressed as percentage of preischemic values, in ischemic control (IC) and preconditioned hearts (IP). Observe that IP significantly improved the postischemic recovery of myocardial systolic function in both rat strains at 35-min and 50-min ischemias (A and B panel). *$p<0.05$ with respect to IC.

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2.6.1. Assessment of reduced glutathione (GSH)

GSH was determined by Ellman's method [30]. 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. Measurement of MnSOD cytosolic activity

SOD activity was measured by means of the nitroblue tetrazolium (NBT) method [31]. Briefly, the supernatant was added to the reaction mixture of NBT with xanthine–xanthine oxidase, and the SOD activity measured colorimetrically in the form

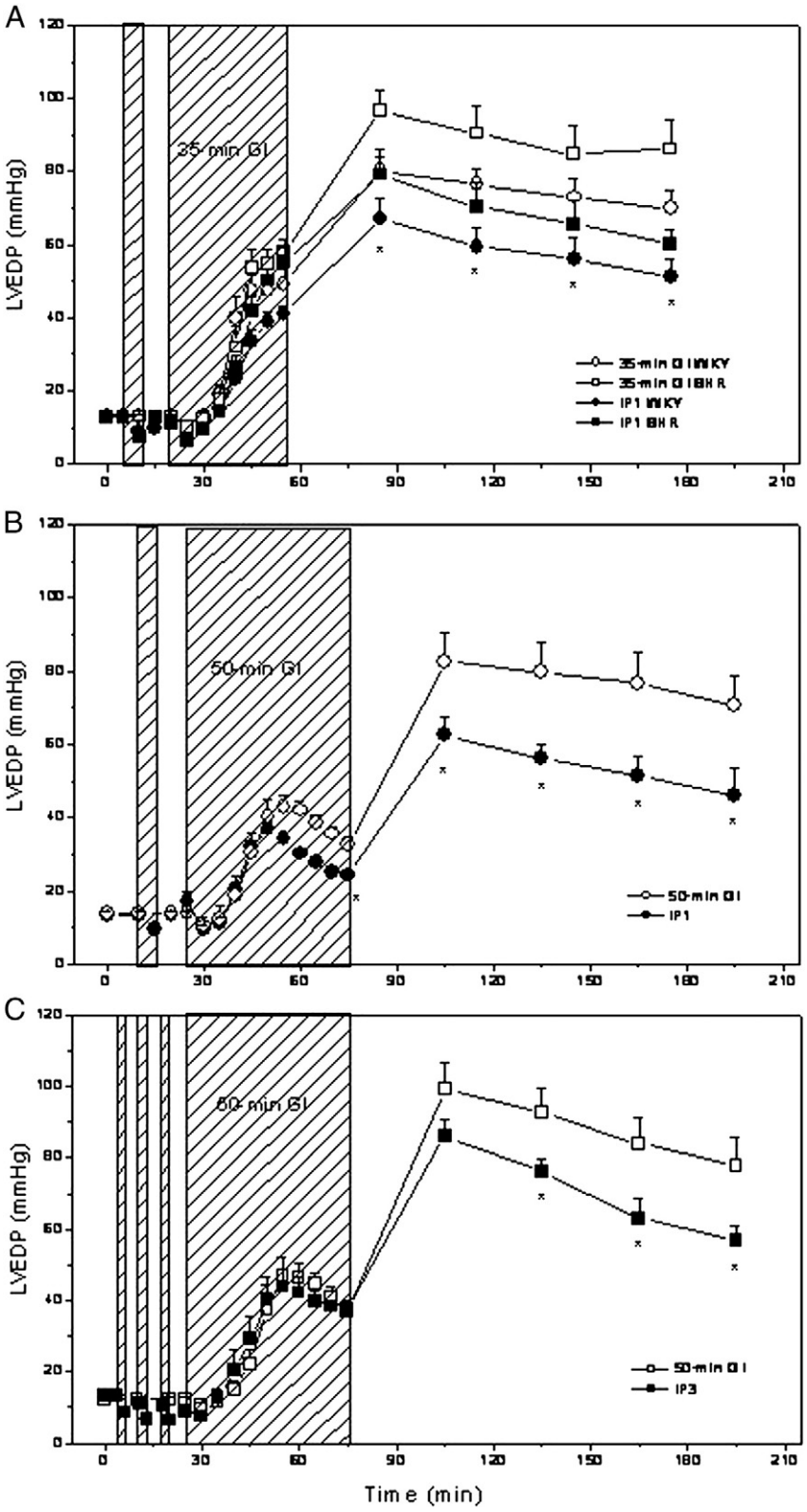

Fig. 4. Time course of left ventricular end diastolic pressure (LVEDP) in ischemic control (IC) and preconditioned hearts (IP) when prolonged ischemia was 35 min (A panel) or 50 min (B and C panels). Note that one cycle of IP (IP1) attenuated the increase of LVEDP detected in IC hearts in both rat strains and that one cycle of IP (IP1) attenuated the increase of LVEDP detected in IC hearts from WKY but it was necessary to apply three cycles (IP3) to obtain the same effect in SHR. *$p<0.05$ with respect to IC.

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of inhibitory activity toward blue formazan formation by SOD in the reaction mixture. For measuring MnSOD activity, 5 mmol/l KCN was added to inhibit Cu-ZnSOD activity.

2.6.3. Protein determination

The protein concentration was evaluated by the Bradford method [32] using bovine serum albumin as a standard.

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. A p value <0.05 was considered significant.

3. Results

As shown in Table 1, 5-month-old SHR have an important LVH and higher values of SBP in comparison to WKY.

Fig. 2 (A panel) shows the infarct size in ischemic control and preconditioned WKY and SHR hearts. In non-ischemic control hearts at the end of the 3-hour perfusion the infarct size was approximately 1% of risk area for both rat strains. After 35-min global ischemia and 2-hour reperfusion, the infarct size was 30 ± 3% and 35 ± 5% for WKY and SHR respectively, which was significantly decreased by one cycle of IP. When ischemia was extended to 50 min, the infarct size in SHR (58 ± 5%) was significantly higher than in WKY (42 ± 2%). IP1 reduced infarct size in WKY but not in SHR 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 from SHR were protected and the infarct size diminished (Fig. 2, B panel).

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 (A panel) the recovery of systolic function was improved by IP. At the end of the reperfusion period, LVDP and $+dP/dt_{max}$ reached higher values than those obtained in ischemic control hearts in both rats strains. When ischemia was more prolonged (50 min) the postischemic recovery of contractility was scarce (LVDP and $+dP/dt_{max}$ reached values of approximately 2% in both rat strains) and it was significantly improved by IP1 in WKY and IP3 in SHR (Fig. 3, B panel).

The diastolic stiffness characterized by LVEDP increased during 35-min and 50-min global ischemia and acquired greater values during reperfusion. The IP attenuated this increase in both rat strains (Fig. 4).

The increase in perfusion pressure at constant coronary flow resulted in increased coronary resistance. These increases (3.1 ± 0.5 and 7.1 ± 1.6 mm Hg/ml × min^{-1} for WKY and 4.2 ± 0.4 and 7.0 ± 0.9 mm Hg/ml × min^{-1} for SHR after 35-min and 50-min ischemia, respectively) were attenuated by IP and restored by L-NAME or 5-HD treatments only in SHR when the ischemic period was 35 min and in both rat strains when the ischemia was 50 min (data not shown).

To assess the role played by NO and mitoK$_{ATP}$ in the cardioprotective effects of IP, L-NAME and 5-HD were used. Both treatments did not modify the infarct size observed in ischemic control hearts but abolished the protection conferred by IP in SHR for both ischemic periods (35 and 50 min). However, in WKY those inhibitors only annulled the beneficial effects of IP when the ischemic period was 50 min (Fig. 5). Similarly, the improvement of postischemic myocardial function achieved by IP was abolished by NOS or mitoK$_{ATP}$ blockade in SHR at both ischemic periods but in WKY only at 50-min global ischemia (Fig. 6).

Given that an increase of ROS generation accompanied by a diminution of antioxidants may be responsible for myocardial reperfusion injury [33,34], we next determined the impact of IP on myocardial GSH content, a marker of oxidative stress. Fig. 7 shows that GSH in non-ischemic hearts from SHR was significantly lower than WKY

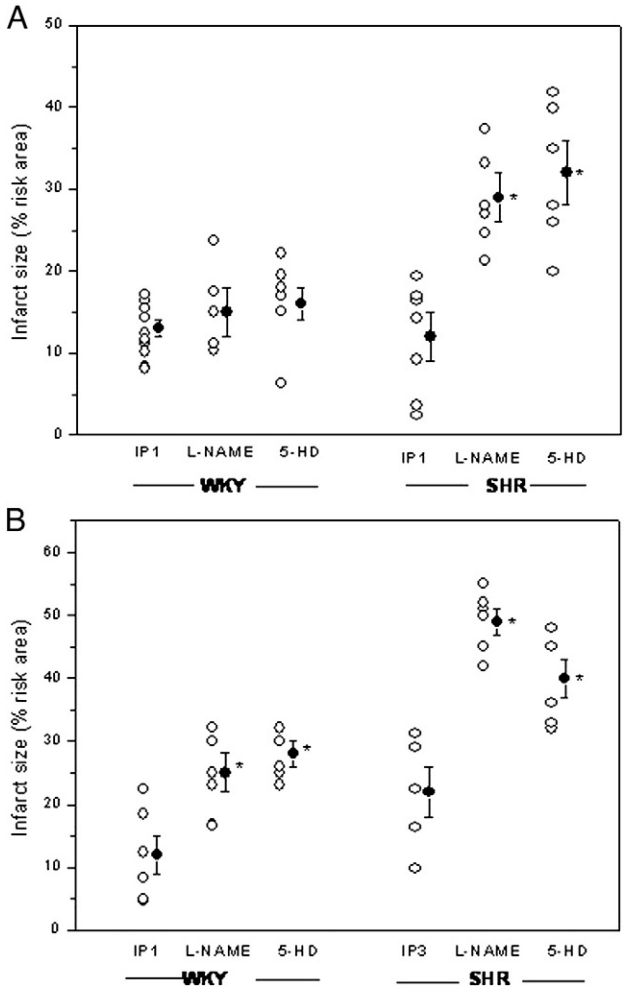

Fig. 5. Infarct size, expressed as percentage of risk area, in untreated and treated with L-NAME preconditioned hearts (IP) from WKY and SHR in both ischemic periods (35 and 50 min). Note that at 35-min global ischemia (GI) the NOS inhibition with L-NAME abolished the effect of IP only in SHR, whereas the treatment was effective in both rats strains when the GI was extended to 50 min. *$p<0.05$ with respect to IP.

(2 ± 0.3 vs. 3.4 ± 0.5 μg/mg prot). Both values were significantly reduced by ischemia and reperfusion. A single or three cycles of IP were able to preserve GSH content in both rat strains. NOS inhibition or mitoK$_{ATP}$ blockade restored the low levels of GSH detected in non-preconditioned hearts in SHR for both ischemic periods and only for 50-min ischemia in WKY rats.

In order to analyze the effect of IP on the mitochondrial integrity we measured the cytosolic activity of MnSOD as an index of mitochondrial permeability transition pore (mPTP) opening as considered in previous work [35]. Fig. 8 shows that a significant level of MnSOD cytosolic activity was only detected in non-ischemic hearts from SHR. The ischemia and reperfusion produced an increase of MnSOD cytosolic activity in both rat strains, which was diminished by IP protocols. The NOS inhibition and mitoK$_{ATP}$ blockade annulled the effects of IP in SHR for both ischemic periods being only effective in WKY when the ischemic period was 50 min.

4. Discussion

This study shows that hypertrophied hearts from adult young SHR suffer higher irreversible damage at more prolonged ischemic period than its normotensive control WKY. In this condition a single cycle of IP failed to limit the infarct size, becoming necessary the application of three cycles.

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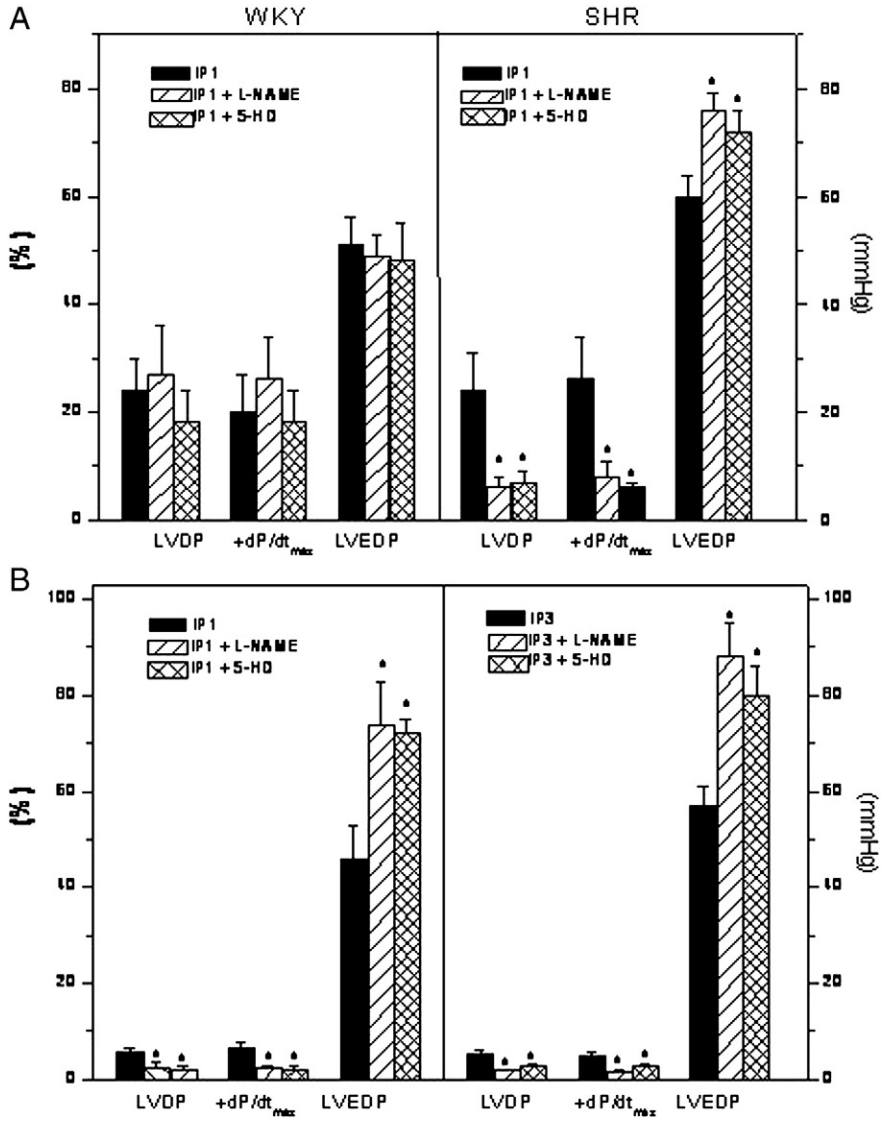

Fig. 6. Values of left ventricular developed pressure (LVDP), maximal velocity of contraction ($+dP/dt_{max}$) and left ventricular end diastolic pressure (LVEDP) at the end of reperfusion period in untreated and treated with L-NAME preconditioned hearts (IP) from WKY and SHR in both ischemic periods (A panel: 35 min; B panel: 50 min). Observe that L-NAME treatment abolished the improvement of cardiac function at 35-min ischemia only in SHR, whereas it was effective for both rat strains when the ischemic period was 50 min. *$p<0.05$ with respect to IP.

In a recent study we demonstrated that adult young SHR developed a similar degree of myocardial stunning (20-min GI) in comparison to WKY rats [15]. When the ischemic period is more prolonged cell death occurs. It has been reported that hypertensive animals compared to normotensive develop a higher [5] or similar infarct size [8,12]. In our current experimental preparation hearts from young adult SHR developed a similar infarct size to control animals when the ischemic period was 35 min. However, when the ischemia was extended to 50 min SHR showed a higher infarct size than WKY.

The cardioprotective effects of IP against reperfusion injury on hypertrophied hearts were previously reported [8–14]. However, the possibility of a correlation between the number of IP cycles and duration time of prolonged ischemia represents a novel finding of the present work. Thus, we demonstrate that it was necessary to apply three cycles of IP to protect SHR hearts against the irreversible damage when the ischemic period was 50 min. Then, it seems to be possible that the number of IP cycles appears as other key factor for determining the efficacy of IP. Therefore, the optimum protocol of IP must be selected to protect the hypertrophied hearts according to the duration time of prolonged ischemia.

Hypertension is associated with an elevation of ROS and frequently with an impairment of endogenous antioxidant mechanisms [36]. Although we did not measure ROS production, non-ischemic control hearts from SHR exhibited a lesser GSH content compared to WKY which would be associated to the greatest oxidative stress present in the hypertensive animals. Oxidative stress also plays an important role in ischemia and reperfusion injury. During the first minutes of reperfusion an increase of ROS generation and a depletion of endogenous antioxidants occur [37,38]. In our experimental conditions, the ischemia and reperfusion similarly decreased the GSH level in both rat strains which was preserved by a single or three cycles of IP. It has been also proposed that ROS contribute to mPTP opening with a consequent loss of impermeability of internal mitochondrial membrane and cell viability [39]. According to data reported by Jin et al. [35], the significant increase of MnSOD cytosolic activity in ischemic control hearts found in this study could be an indication of mitochondrial damage associated to mPTP formation. Interestingly, there was an inverse relationship between changes in GSH and MnSOD. Thus, in ischemic control hearts a decrease in GSH content was accompanied by an increase of MnSOD cytosolic activity. These changes are

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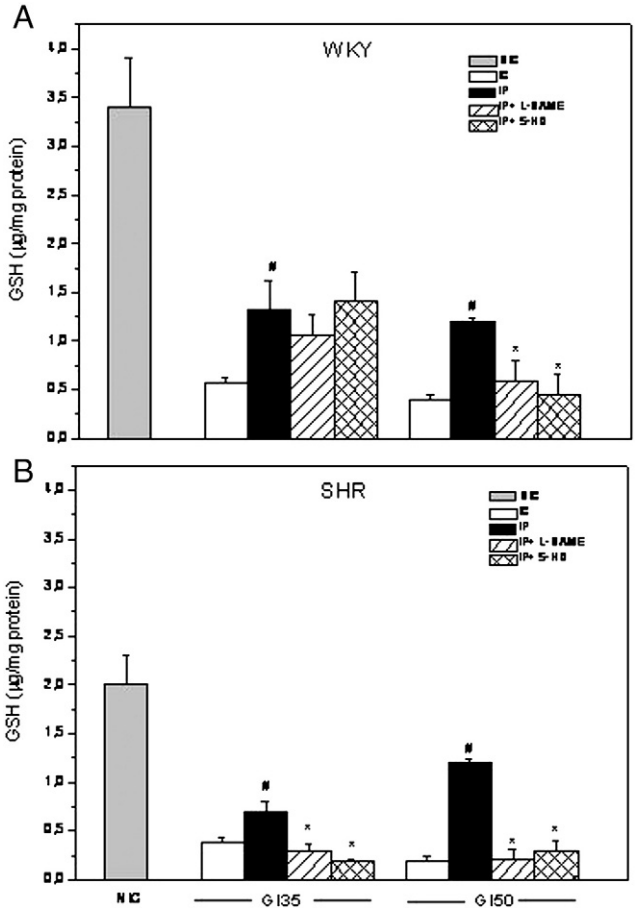

Fig. 7. Myocardial reduced glutathione content (GSH, µg/mg protein) in all experimental situations for WKY (A panel) and SHR (B panel). Observe that non-ischemic control hearts (NIC) from SHR show a lesser GSH content than WKY. GSH levels decreased after ischemia and reperfusion and were preserved by IP. These beneficial effects of IP were canceled by treatment with L-NAME and 5-HD only in SHR when prolonged ischemia was 35 min while they were effective in both rat strains when the ischemia was 50 min. #p<0.05 with respect to IC; *p<0.05 with respect to IP.

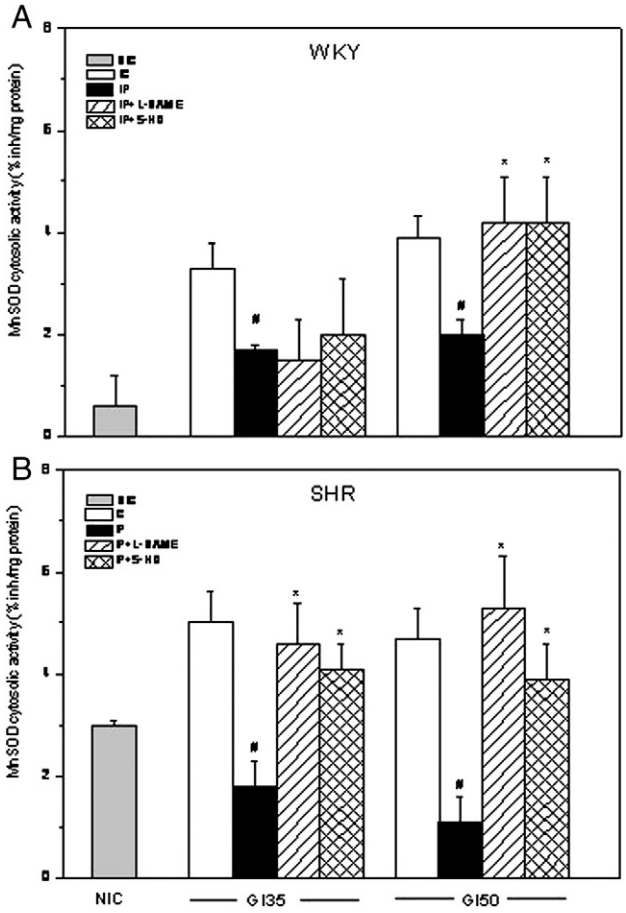

Fig. 8. MnSOD cytosolic activity (% inhibition/mg protein) in all experimental situations for WKY (A panel) and SHR (B panel). Note that a significant activity of MnSOD was only detected in non-ischemic control hearts (NIC) from SHR. After ischemia and reperfusion SODMn cytosolic activity increased and this increase was attenuated by IP. This beneficial effect of IP were canceled by treatment with L-NAME and 5-HD only in SHR when prolonged ischemia was 35 min while they were effective in both rat strains when the ischemia was 50 min. #p<0.05 with respect to IC; *p<0.05 with respect to IP.

indicating the presence of oxidative stress. Opposite changes of both parameters were obtained in preconditioned hearts highlighting the decrease of oxidative stress and associated attenuation of mPTP opening events that may be involved in the cardioprotective mechanism of IP.

Although the intermittent ischemic periods of IP activate pathways that involve to NO [17,18], its role in reperfusion injury is still controversial [40]. Our data in SHR show that NOS blockade abolished the IP-mediated cardioprotection at the two ischemic periods suggesting that NO, independently of ischemia duration time, is involved in IP. Also, the increase of coronary resistance produced by both ischemia protocols was reverted by NOS inhibition, demonstrating the importance of NO in the regulation of vascular tone in hypertensive rats. However, these effects were not observed in WKY, in which the cardioprotection afforded by IP was only annulled by L-NAME treatment at 50-min ischemia. The difference can be explained taking into account that an increased NOS expression was detected in hypertensive rats [41] and that its activation by IP cycles could provide sufficient NO availability to achieve cardioprotective effect. Therefore, in normotensive rats, only a more prolonged ischemia could provide the necessary NO amount to mediate protective signaling pathways.

The mitoK$_{ATP}$ [42] or/and mPTP [43] are recognized NO targets in normotensive animals. In our experimental conditions, IP-mediated effects were abolished by mitoK$_{ATP}$ blockade with 5-HD indicating that these channels are participating in the cardioprotective pathway. Also our data show that MnSOD cytosolic activity increased and GSH content decreased when IP was applied in presence of mitoK$_{ATP}$ blockade suggesting that mitochondrial permeability deteriorated probably due to mPTP formation and/or opening. Taken together these data constitute an evidence about the possible relationship between NO and mitoK$_{ATP}$ as a pathway leading to the diminution of mPTP opening which could attenuate ROS release thus providing an explanation of the observed lesser myocardial damage afforded by IP in hypertrophied hearts.

5. Conclusions

Our data support the conclusion that hearts from young adult SHR suffer a higher irreversible damage than normal myocardium at more extended ischemic period; and in these conditions a single cycle of IP was insufficient, and multiple cycles were necessary for the efficacy of IP. In all cases a diminution of mPTP opening through NO-mitoK$_{ATP}$-dependent pathways appears to be as possible mechanism (Fig. 9). Thus, it seems to be possible that pharmacological targeting of the mPTP may be beneficial for preventing or slowing the reperfusion injury in hypertrophied hearts.

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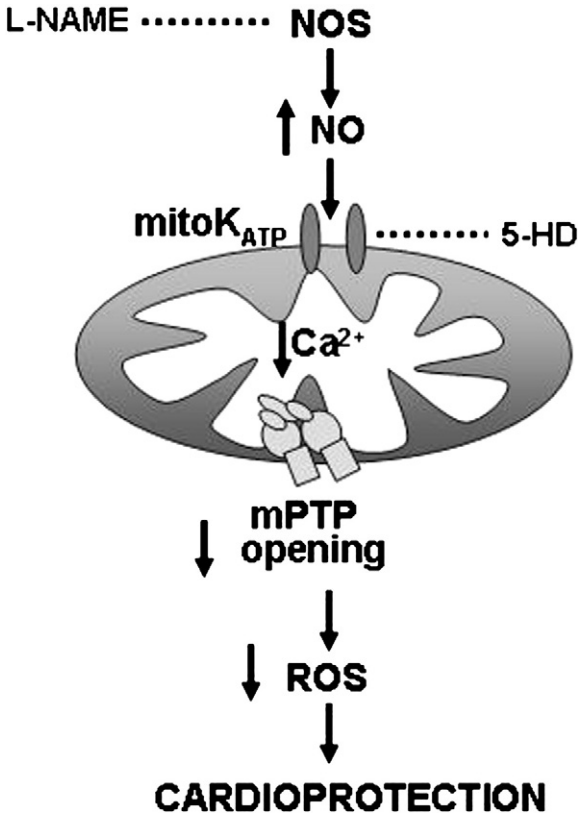

Fig. 9. Scheme of proposed mechanism for cardioprotection by ischemic preconditioning in hypertrophied hearts from SHR.

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The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [44].

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