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Original Research Communication

Involvement of Reactive Oxygen Species in Cardiac Preconditioning in Rats

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ABSTRACT

To date, the involvement of reactive oxygen species in ischemic preconditioning *in vivo* in rats is not clearly demonstrated. The aim of the present study was to determine whether *N*-(2-mercaptopropionyl)glycine (MPG), a cell-diffusible hydroxyl radical scavenger, and carnosine, a potent singlet oxygen quencher, could block protection afforded by a single cycle of ischemic preconditioning *in vivo* in the rat. An ESR study was first performed to validate in vitro the specific antioxidant properties of carnosine and MPG. In a second set of experiments, open-chest rats were subjected to 30 min of left coronary occlusion followed by 60 min of reperfusion. Preconditioning was elicited by 5 min of ischemia and 5 min of reperfusion. Neither MPG (1-h infusion, 20 mg/kg) nor carnosine injection (bolus, 25 μmol/rat) affected infarct size. The infarct size-limiting effect of preconditioning was completely blunted by MPG, whereas carnosine did not alter the cardioprotection. It is concluded that free radicals and especially hydroxyl radicals could be involved in the adaptive mechanisms induced by a single cycle of preconditioning *in vivo* in rats. *Antioxid. Redox Signal.* 5, 115–122.

INTRODUCTION

BRIEF TRANSIENT EPISODES of nonlethal myocardial ischemia protect the heart by markedly reducing the amount of tissue necrosis induced during a subsequent prolonged ischemic period. This powerful protective adaptation of the myocyte called ischemic preconditioning was first described in dogs by Murry *et al.* (22) and has been reproduced in different species, such as rabbit, rat, and swine (20, 29, 40). The protection afforded by ischemic preconditioning includes the limitation of reperfusion arrhythmias (38), the reduction of infarct size (22), the reduction of apoptotic cell death (21), and the improvement of postischemic contractile recovery (7). Although a large number of studies have attempted to discover the mechanisms responsible for this phenomenon, its cellular basis is still not fully understood.

It is now well established that under conditions of postischemic reperfusion, the stimulated univalent reduction of oxygen leads to the formation of two oxygen-derived free radicals, namely superoxide anion ($O_2^{\cdot -}$) and hydroxyl radical ($^{\cdot}OH$), and two nonradical species, namely hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). These four reactive oxygen species (ROS) have been proposed as possible candidates for mediation of ischemic preconditioning (2, 33). In this view, several authors have tried to replace the intervening ischemia–reperfusion sequence of preconditioning by a mild, artificially induced oxidant stress, in order to test the ability of ROS to trigger endogenous cardioprotective mechanisms (1, 3, 13, 30, 32, 34). Similarly, divers antioxidants have been tested in order to determine whether they could interfere with ischemic preconditioning (1, 3, 11, 13, 16, 21, 23, 24, 28, 31). However, these studies, using the antioxidant approach of preconditioning, have led to apparently conflicting results, possibly because of differences in animal species used, or in the antioxidant administrated. Moreover, Baines *et al.* (3) have proposed that the number of preconditioning episodes applied to the heart might also be of importance in this context. These authors have suggested that the production of

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ROS during a single cycle of preconditioning might be a major contributor to cardioprotection, whereas when several cycles are used, other mediators, such as adenosine or bradykinin, might be released in sufficient quantities to trigger protection.

In this context, the objective of the present study was to determine whether administration of antioxidants specifically directed against ·OH radicals or $^1O_2$ could block protection afforded by a single episode of ischemic preconditioning *in vivo* in the rat.

In the first part of the study, antioxidant properties of *N*-(2-mercaptopropionyl)glycine (MPG) and carnosine have been characterized by electron spin resonance (ESR) spectroscopy by use of the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and 2,2,6,6-tetramethyl-4-piperidone (oxoTEMP).

The second part of the study was designed to test whether the ·OH radical scavenger MPG or the $^1O_2$ quencher carnosine can alter the beneficial effects of a single episode of preconditioning *in vivo* in the rat.

MATERIALS AND METHODS

ESR study

In vitro singlet oxygen quenching activity. Singlet oxygen was generated by photoexcitation of the light-sensitive dye Rose Bengal (RB), which is one of the most efficient sources of $^1O_2$ production (18). Formation of $^1O_2$ was evidenced by ESR spectroscopy using oxoTEMP. OxoTEMP (10 m*M*) and RB (20 μ*M*) were dissolved in phosphate buffer (50 m*M* KH_2PO_4/K_2HPO_4, pH 7.4) containing 1 m*M* desferal. The solution was microfuged and illuminated for 10 min using a xenon-light source (Euromex Illuminator EK-1, Holland) with a filter that removes the emission wavelengths below 500 nm. A sample of the reaction mixture was then rapidly collected in a glass micropipette and kept in the dark until spectra recording.

The $^1O_2$ quenching activity of histidine and carnosine (0.5 or 1 m*M*) was evaluated by adding these compounds to the phosphate buffer/desferal medium before oxoTEMP and RB addition. The reaction mixture was microfuged and illuminated under the same conditions as described above. Quenching effects of histidine and carnosine on $^1O_2$ were compared with a control experiment, in the absence of any quencher. The experiment was repeated three times for each concentration of histidine and carnosine. Relative concentrations of nitroxide in the reaction media were evaluated as the mean amplitude (arbitrary units) of the three bands of the oxoTEMPO signal.

Hydroxyl radicals scavenging activity. Hydroxyl radicals were produced by the Fenton reaction and detected by spin-trapping using DMPO. DMPO (50 m*M*) was dissolved in phosphate buffer (50 m*M* KH_2PO_4/K_2HPO_4; pH 7.4) containing $FeSO_4$ (375 μ*M*). The reaction was initiated by adding H_2O_2 (375 μ*M*). After 3 min of incubation, a sample of reaction mixture was collected for spectra recording.

Effects of MPG (6 m*M*) or carnosine (6 m*M*) on ·OH radical production were determined by adding these compounds to the reaction mixture, before initiation of the reaction with H_2O_2.

ESR recording conditions. ESR spectra were recorded at room temperature on an ESP 300E spectrometer (Brucker, France). Spectra were recorded under the following conditions; X band; microwave frequency, 9.4 GHz; microwave power, 20 mW; modulation amplitude, 1.25 G; sweep time, 100 s; sweep width, 100 G; modulation frequency, 100 kHz.

In vivo study

Animals. Male Wistar rats weighing 280–320 g and receiving a standard commercial diet (R20 Extralab, Extralabo, France) were used for *in vivo* studies. Animals (n = 10 per group) were cared for according to the guidelines formulated by the European Union for use of experimental animals (L 358.86/609/EEC).

Surgical preparation. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg), and anesthesia was maintained by a continuous intravenous infusion (5 μl/min/100 g) of sodium pentobarbital (0.6%). Body temperature was kept constant at 37°C with a heating blanket controlled by a thermostat and connected to a rectal thermocouple (Homeothermic Blanket System, Harvard Apparatus, Holliston, MA, U.S.A.). The animal was intubated through a tracheotomy and mechanically ventilated (tidal volume, 3 ml; ventilation rate, 50 strokes/min). A small Millar Mikro Tip catheter was inserted into the carotid artery to monitor blood pressure. Electrocardiogram was monitored with two limb electrodes. A left thoracotomy was performed, and the heart was exposed. A silk suture (5/0) was passed around the left coronary artery and threaded through a small polyethylene catheter to form a snare. All rats were allowed 10 min after completion of the surgical preparation to reach steady state before the protocol started. The coronary branch was occluded by pulling the snare and the occluded position was maintained by means of a hemostatic clamp. Reperfusion was induced by releasing the snare. Two minutes prior to reperfusion, the rat received 150 U/kg heparin sodium injection *via* the saphenous vein. At the end of the protocol, the heart was removed for postmortem analysis.

Postmortem studies. The heart was cannulated via the aorta and perfused with 15–20 ml of saline at room temperature to wash out the blood. The coronary branch was reoccluded, and a saturated solution of Evans Blue was injected to demarcate the ischemic zone as the tissue area without blue dye.

The heart was then briefly frozen in liquid nitrogen and stored at −20°C. The heart was cut into six or seven transverse slices of 1 mm in thickness each. Slices were weighed before being incubated in triphenyltetrazolium chloride (TTC) in sodium phosphate buffer at 37°C for 20 min. The slices were then immersed in 10% formalin to enhance the contrast between stained and unstained areas. After 4 days, the infarct, risk, and safe zones of each slice were reproduced on a slide, magnified, and the areas determined by planime-

try. The volume of the risk region and the infarct were calculated by multiplying each area by the slice weight and summing them for each heart. The risk zone was expressed as a percentage of the total volume of the heart, and the infarct size was expressed as a percentage of the risk zone.

Experimental design. Experimental protocols are summarized in Fig. 1. Rats were randomly divided into six groups: control (CONT), preconditioned (PC), control carnosine (CAR), preconditioned carnosine (PC+CAR), control MPG (MPG), and preconditioned MPG (PC+MPG). All rats were subjected to 30 min of coronary occlusion followed by 60 min of reperfusion. Rats from PC, PC+CAR, and PC+MPG groups were preconditioned with 5 min of occlusion followed by 5 min of reperfusion before the 30-min coronary occlusion. The $^1O_2$ quencher carnosine was dissolved in saline (NaCl, 7.5 g/L) to a final concentration of 50 m*M*. A 0.5-ml bolus (25 µmol of carnosine) of this solution was injected into the rats via the saphenous vein, 6 min before ischemia (CAR) or 1 min before reperfusion of ischemic preconditioning cycle (PC+CAR). The ·OH radical scavenger MPG was administered as a 1-h intravenous infusion (20 mg/kg) starting 1 h before the 30-min ischemia (MPG) or 50 min before ischemic preconditioning (PC+MPG).

In PC and CONT groups, a saline infusion was given over the same time frame before the 30-min coronary occlusion.

The rationale for the drug-administration procedures is as follows: MPG was administered according to the protocol previously described (28). As carnosine plasma and tissue levels only transiently increase after injection, due both to the endogenous activity of carnosinase (19) and to the rapid renal clearance (27) of the compound, carnosine was injected just prior to preconditioning reperfusion.

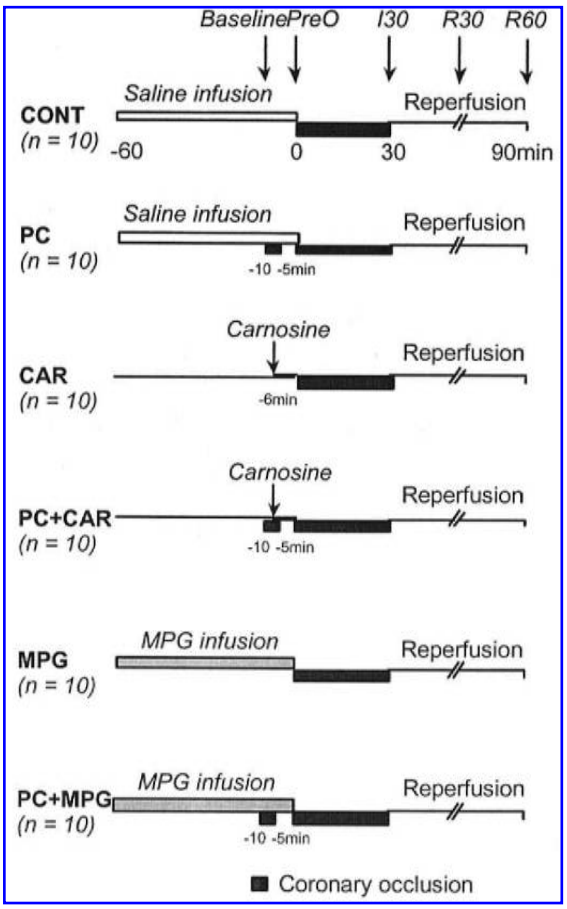

FIG. 1. Experimental protocol. Rats underwent a 30-min left coronary artery occlusion followed by 60 min of reperfusion. Ischemic preconditioning was elicited with a single 5-min episode of ischemia and reflow. In MPG-treated groups, control (MPG) or preconditioned (PC+MPG) animals received an infusion of MPG (20 mg/kg) for 60 min before the 30-min occlusion. In carnosine-treated groups, control (CAR) or preconditioned (PC+CAR) animals received a 0.5-ml bolus injection of saline containing carnosine (25 µmol), 6 min prior to the 30-min occlusion. CONT, control; PC, preconditioned; MPG, control MPG; PC+MPG, preconditioned MPG; CAR, control carnosine; PC+CAR, preconditioned carnosine; Baseline, 10-min equilibration; PreO, immediately before occlusion; I30, occlusion 30 min; R30, reperfusion 30 min; R60, reperfusion 60 min.

Animal exclusion. An initial 60 rats were used in this study (n = 10 per group). One to three animals from each group died before the end of the 60-min reperfusion period, and one heart from the PC+MPG group was excluded after staining because of the absence of risk zone.

Compounds

RB, oxoTEMP, DMPO, histidine, TTC, Evans Blue, heparin, and MPG were purchased from Sigma–Aldrich Chemical (Saint Quentin Fallavier, France); desferal from Novartis (Rueil-Malmaison, France); carnosine from Fluka Chemie AG (Buchs, Switzerland); and sodium pentobarbital from Sanofi Santé et Nutrition Animale (Libourne, France).

Statistical analysis

The data are expressed as mean ± SEM. Statistically significant differences were assessed by analysis of variance followed by Fisher's protected least significant difference *post hoc* test (Stat View, Abacus Concepts, Inc., Berkeley, CA, U.S.A.). Analysis of repeated measures was used for individual comparison within each group and factorial analysis for individual difference between groups. A value of $p = 0.05$ was taken as the limit of significance.

RESULTS

Relative quenching potential of histidine and carnosine

As shown in Fig. 2, the characteristic ESR spectral pattern of the stable nitroxide radical oxoTEMPO (three equal-intensity lines; ^aN = 16.3 G) produced by the oxidation of oxoTEMP by $^1O_2$ was detected after 10 min of illumination of the phosphate buffer containing oxoTEMP (10 m*M*), RB (20 µ*M*), and desferal (1 m*M*) (trace 2A). Addition of the $^1O_2$ quencher histidine (1 m*M*) or carnosine (1 m*M*) to the

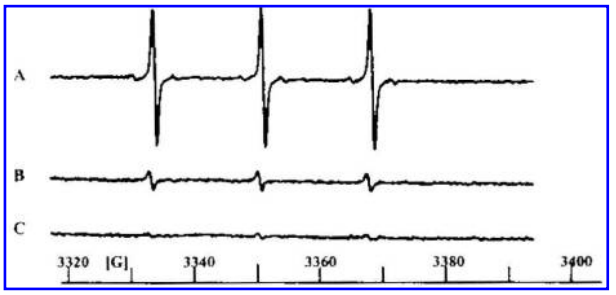

FIG. 2. Comparative triplet ESR spectra of oxoTEMPO signal obtained after 10-min illumination of 10 m*M* oxoTEMP, 20 µ*M* RB, 1 m*M* desferal in 50 m*M* KH_2PO_4/K_2HPO_4 (pH 7.4). Trace A, control conditions; trace B, + 1 m*M* histidine; trace C, + 1 m*M* carnosine. The instrument settings are stated in the text.

reaction mixture reduced the magnitude of the oxoTEMPO signal (traces B and C, respectively). The relative efficiency of histidine and carnosine to quench $^1O_2$ is represented in Fig. 3. At identical concentrations (0.5 or 1.0 m*M*), carnosine appears to be more efficient than histidine in decreasing the ESR signal intensity of the nitroxide oxoTEMPO.

Scavenging properties of MPG

The *in vitro* production of ·OH radicals by the Fenton reaction was evidenced by the ESR detection of the DMPO-OH

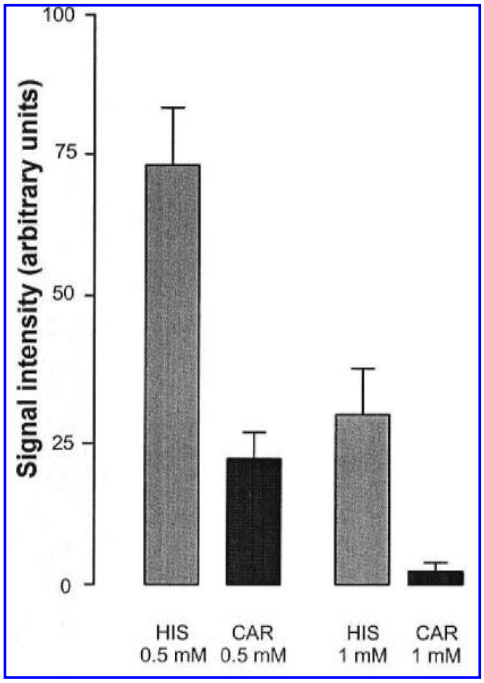

FIG. 3. Comparative ESR signal intensity (in arbitrary units) of the oxoTEMP adducts formed after 10 min illumination of a reaction mixture containing 10 m*M* oxoTEMP, 20 µ*M* RB, 1 m*M* desferal in 50 m*M* KH_2PO_4/K_2HPO_4 (pH 7.4). The graph shows the signal intensity for the reaction mixture with 0.5 or 1 m*M* histidine (HIS) or with 0.5 or 1 m*M* carnosine (CAR).

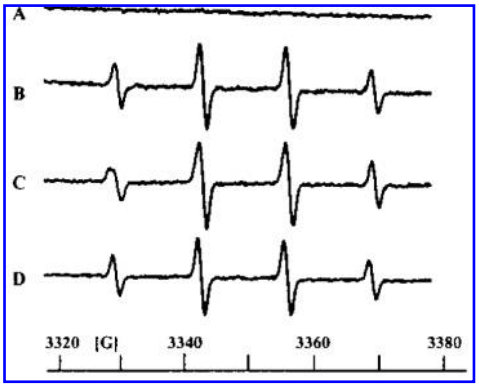

FIG. 4. Effects of carnosine and MGP on the generation of the characteristic ESR spectrum of DMPO-OH. These signals were generated with the Fe/H_2O_2 system (375 µ*M*). Trace A, blank (50 m*M* DMPO without Fe/H_2O_2); trace B, control (50 m*M* DMPO + Fe/H_2O_2); trace C, carnosine (6 m*M* carnosine + 50 m*M* DMPO + Fe/H_2O_2); trace D, MPG (6 m*M* MPG) + 50 m*M* DMPO + Fe/H_2O_2). Instrument settings are given in the text. Decomposition of signal (trace D) is given in Fig. 5.

spin adduct (Fig. 4, trace B). Addition of carnosine (6 m*M*) to the reaction mixture did not alter the DMPO-OH signal (Fig. 4, trace C), indicating that carnosine has no effect on ·OH production.

An ESR spectrum recorded in the presence of MPG (6 m*M*) is shown in Fig. 4 (trace D) and analyzed by computed decomposition in Fig. 5. DMPO and MPG together compete to trap ·OH radicals. MPG scavenges ·OH radical to form a MPG-S· radical, which is, in turn, trapped by DMPO to produce the DMPO-MPG spin adduct (Fig. 5Ba). Meantime, DMPO exerts its scavenging activity on ·OH radical, leading to the formation of DMPO-OH spin adduct (Fig. 5Bb). Thus, ESR spectra obtained with the Fe/H_2O_2 system in the presence of DMPO and MPG (Fig. 5A) represents the sum of two different signals of almost equal proportion: the 1:2:2:1 quartet DMPO-MPG signal ($^aN = {}^aH = 15.2$) (Fig. 5Ba), and the 1:2:2:1 quar-

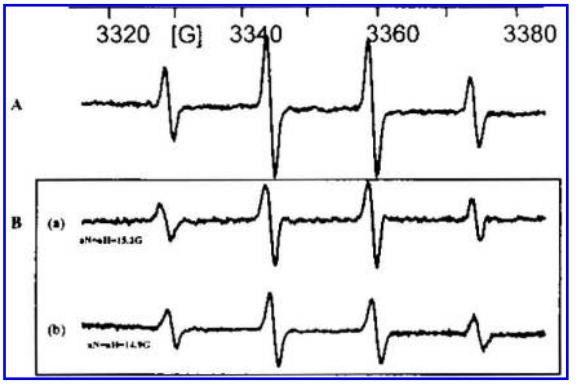

FIG. 5. Analysis of the ESR spectrum obtained with the Fe/H_2O_2 system (375 µ*M*) in the presence of DMPO (50 m*M*) and MPG (6 m*M*). (**A**) Complete ESR signal. (**B**) Decomposition of signal (A), the DMPO spin adducts DMPO-MPG (**a**) and DMPO-OH (**b**) representing 50% of the complete signal. Instrument settings are given in the text.

tet DMPO-OH spin adduct ($^{a}N = {}^{a}H = 14.9$) (Fig. 5Bb) (6). As DMPO (50 m*M*) is much more concentrated than MPG (6 m*M*) in the reaction mixture, these results demonstrate that MPG is a good $^{\cdot}OH$ radical scavenger.

Hemodynamic parameters

Table 1 summarizes the evolution of heart rate and mean blood pressure before and after coronary occlusion and reperfusion. At the end of the equilibration period, hemodynamic parameters were comparable among groups. MPG and carnosine pretreatment did not induce any significant changes in these parameters. Besides, heart rate and mean blood pressure decreased progressively during ischemia and reperfusion. This last phenomenon was almost equivalent among groups.

Risk zone and infarct size

Figure 6A shows that the size of the risk zone (expressed as a percentage of total ventricular volume) was equivalent in the six experimental groups.

Infarct size expressed as a percentage of area at risk (Fig. 6B) was significantly ($p < 0.01$) reduced in PC (34.4 ± 3.6%) compared with CONT (55.6 ± 5.9%). Infarct/risk zone was also markedly decreased in the preconditioned group with carnosine (PC+CAR: 35.5 ± 4.5%) compared with the corresponding control group (CAR: 67.9 ± 6.4) ($p < 0.01$). In contrast, MPG infusion completely blunted the infarct size-limiting effect of preconditioning (PC+MPG: 61.4 ± 7.6 versus MPG: 66.2 ± 7.8, NS; PC+MPG: 61.4 ± 7.6 versus PC: 34.4 ± 3.6, $p < 0.01$).

DISCUSSION

The present study shows that ischemic preconditioning with a single episode of ischemia–reperfusion *in vivo* in rats limits infarct size, therefore confirming the results of other groups (40). The cardioprotective effect of ischemic preconditioning was not affected by the $^{1}O_2$ quencher carnosine. However, the $^{\cdot}OH$ radical scavenger MPG completely blunted the infarct-size limitation induced by preconditioning. These data support the hypothesis that among the different ROS, $^{\cdot}OH$ is a potential candidate that could contribute to the cardioprotective mechanisms of preconditioning induced by a single cycle of ischemia–reperfusion in rats.

Carnosine and MPG

Carnosine (β-alanyl-L-histidine) is an endogenous dipeptide physiologically present in animal tissues (10). The complete biological role of carnosine has not yet been clearly elucidated but it has been described both for its metabolic function as a buffering agent (14) and for antioxidant properties (10, 12).

It has been previously reported that the quenching of RB by oxygen in aqueous solutions leads to the production of singlet oxygen ($^{1}O_2$) and superoxide anion ($O_2^{\cdot -}$) (18). For this reason, RB, dissolved in an oxygenated phosphate buffer solution, was used as an artificial $^{1}O_2$ generating system in our ESR *in vitro* studies. Moreover, the iron chelator desferal was added to the buffer in order to limit the prooxidant activity of metals through Fenton and Haber–Weiss reactions that might attenuate $^{1}O_2$ production. Our ESR study with oxoTEMP demonstrates that carnosine quenches $^{1}O_2$ more efficiently than equimolar histidine, which is usually taken as a standard for $^{1}O_2$ quenching. Moreover, the use of DMPO as a spin trap has allowed us to demonstrate that the $^{\cdot}OH$ scavenging properties of carnosine are very poor because they cannot be evidenced under our experimental conditions.

MPG is a cell-diffusible compound usually used as a free radical scavenger. In our ESR study, the *in vitro* $^{\cdot}OH$ radical scavenging properties of MPG have been investigated using

TABLE 1. EVOLUTION OF HEART RATE AND MEAN BLOOD PRESSURE DURING EXPERIMENTAL PROTOCOL

Group	*Baseline*	*PreO*	*I30*	*R30*	*R60*
Heart rate (beats/min)					
CONT ($n = 9$)	443 ± 8	443 ± 8	413 ± 9*	399 ± 11*	370 ± 12*
PC ($n = 9$)	432 ± 10	433 ± 11	387 ± 15*	364 ± 11*	351 ± 18*
CAR ($n = 8$)	426 ± 13	434 ± 14	404 ± 10*	388 ± 14*	368 ± 14*
PC+CAR ($n = 8$)	453 ± 5	449 ± 9	419 ± 11†	408 ± 6*	384 ± 12*
MPG ($n = 7$)	452 ± 14	439 ± 16	414 ± 21	399 ± 20†	393 ± 23†
PC+MPG ($n = 6$)	455 ± 8	438 ± 10	410 ± 14†	380 ± 27*	375 ± 29*
Blood pressure (mm Hg)					
CONT ($n = 9$)	111 ± 5	111 ± 5	94 ± 8†	80 ± 5*	77 ± 5*
PC ($n = 9$)	114 ± 5	106 ± 5	83 ± 7†	76 ± 5*	73 ± 4*
CAR ($n = 8$)	117 ± 5	112 ± 6	92 ± 5	83 ± 7*	76 ± 9*
PC+CAR ($n = 8$)	109 ± 5	104 ± 6	78 ± 11†	73 ± 8*	68 ± 8*
MPG ($n = 7$)	100 ± 6	96 ± 6	83 ± 7	82 ± 5	80 ± 6
PC+MPG ($n = 6$)	107 ± 8	107 ± 7	101 ± 9	100 ± 8	99 ± 7

Baseline, after 10-min equilibration; PreO, immediately before the 30-min occlusion; I30, after 30-min occlusion; R30, after 30-min reperfusion; R60, after 60-min reperfusion; CONT, control; PC, preconditioned; CAR, control carnosine; PC+CAR, preconditioned carnosine; MPG, control MPG; PC+MPG, preconditioned MPG.

*$p < 0.01$, †$p < 0.05$, versus PreO.

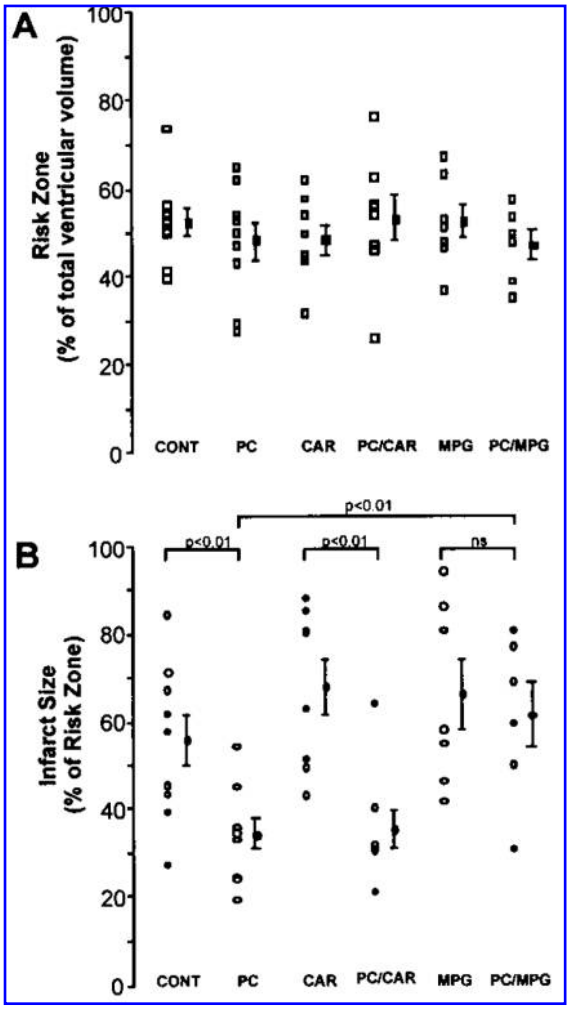

FIG. 6. Scatter plots of risk zone and infarct size. (**A**) Risk zone is expressed as % of total ventricular volume in individual animals (□). Results are also expressed as means ± SEM for each experimental group (■). (**B**) Infarct size expressed as % of risk zone in individual animals (◯). Results are also expressed as means ± SEM for each experimental group (●). CONT, control; PC, preconditioned; MPG, control MPG; PC/MPG, preconditioned MPG; CAR, control carnosine; PC/CAR, preconditioned carnosine; ns, not significant.

DMPO spin-trapping. Our results indicate that MPG is ~10 times more efficient than DMPO in traping ·OH. Moreover, Bolli *et al.* (5) have shown that MPG has no significant effect on $O_2^{\cdot -}$, H_2O_2, or non-·OH initiated lipid peroxidation.

In summary, our ESR study confirms that carnosine and MPG are powerful antioxidants and demonstrate that carnosine is a relatively pure $^1O_2$ quencher, whereas MPG is a good ·OH scavenger.

Role of ROS in cardiac preconditioning: single cycle versus multiple cycles

According to Richard *et al.* (28), the use of MPG *in vivo* in the rat has no effect on the cardioprotective effects of ischemic preconditioning with three episodes of intervening ischemia–reperfusion. Similar *in vivo* experiments in the rabbit have shown that whereas preconditioning with several episodes of intervening ischemia–reperfusion is not affected by antioxidant administration (3, 16), the cardioprotective action of a single episode of preconditioning appears to be dramatically attenuated by administration of either superoxide dismutase or MPG (3, 13, 31). To explain these contradictory results, Baines *et al.* (3) have suggested that the negative results observed *in vivo* in the rat by Richard *et al.* (28) could be due to their preconditioning protocol. In fact, three cycles of ischemia–reperfusion may be responsible for the release of other possible mediators of preconditioning (such as bradykinin or adenosine). In this case, the elimination of the free radical production by MPG would not be sufficient to prevent the development of the cardioprotection.

Reinforcing Baines' hypothesis, the present work shows that, when a single cycle of preconditioning is applied to the heart, antioxidant administration can effectively blunt the beneficial effect of ischemic preconditioning *in vivo* in rats.

Singlet oxygen and ischemic preconditioning

Singlet oxygen has been shown to play important roles in biological systems (39). This excited state of oxygen has a very short lifetime of $\sim 10^{-6}$ and a very high reactivity. The production and the involvement of $^1O_2$ in postischemic reperfusion injury of the myocardium have been indirectly shown by Zhai and Ashraf (41) using 5,8-endoperoxide, an oxidation product of ß-carotene, as a marker of its generation. Perfusion with $^1O_2$ quenchers has also been shown to improve postischemic functional recovery (17). In a recent study, we have shown that direct exposure of isolated rat hearts to chemically generated $^1O_2$ induced protective effects against subsequent ischemia–reperfusion (32), suggesting that $^1O_2$ might be involved in the transduction signal of ischemic preconditioning. Conversely, in the present study, quenching of $^1O_2$ by carnosine, contrary to ·OH scavenging by MPG, failed to inhibit preconditioning *in vivo*. MPG is known to penetrate and to accumulate into cardiomyocytes after 1 h of infusion. In contrast to MPG, the carnosine tissue level only transiently increases after injection, due to the endogenous activity of plasma and tissue carnosinase (19) and to rapid renal excretion (27). For this reason, in the present study, carnosine was injected as a bolus, just before the intermittent reperfusion of preconditioning, so that the compound reaches a peak plasma and tissue level in the critical period of ROS generation. Nevertheless, although reoxygenation upon postischemic reperfusion has been shown to be the most favorable period for ROS production (15, 41, 42), some authors have suggested that ROS might also be generated during ischemia itself (37). In this respect, Becker *et al.* (4) have recently shown a production of $O_2^{\cdot -}$ from ubisemiquinone oxidation in the mitochondrial electron transport chain during ischemia. Therefore, we cannot exclude that production of ROS (such as $^1O_2$) during preconditioning ischemia, in our experiments, could have occurred before carnosine administration. Nonetheless, as $^1O_2$ has been shown to be mainly produced during postischemic reperfusion (41), our results suggest that $^1O_2$ production during preconditioning stimulus is unlikely to play a dominant role in triggering preconditioning *in vivo*.

Study limitations

In the present study, a single dose of carnosine was tested *in vivo*. Our ESR experiments showed that 1 m*M* carnosine inhibited *in vitro* production of $^1O_2$. We therefore assumed that the concentration of carnosine injected *in vivo* in the rat (~1.5 m*M*) would be sufficient to overcome any $^1O_2$ production during preconditioning. Further experiments testing the effects of different carnosine concentrations may, however, be necessary to confirm this hypothesis.

Besides its $^{\cdot}OH$ scavenging properties, MPG has also been recognized as displaying other potentially beneficial activities. As a thiol-containing agent, it has been shown to interact with peroxynitrite ($ONOO^-$) (8), and this nitric oxide derivative has been proposed to participate in ischemic preconditioning in an isolated rat heart model (1). Moreover, MPG scavenges hypochlorous acid (26), and it has also been used as a metal chelator (35). Although peroxynitrite may be a possible candidate as an ischemic preconditioning trigger, to our knowledge, there is no evidence in the literature suggesting that hypochlorous acid and/or metal ions could play a decisive role in the development of preconditioning. Nevertheless, in the present study, we cannot exclude that *in vivo*, the inhibitory effects of MPG on a single cycle of preconditioning may be related to one or several of its properties.

Conclusion

In summary, we have demonstrated that the free radical scavenger MPG, but not specific $^1O_2$ oxygen quenching by carnosine, abolishes the infarct size limitation afforded by ischemic preconditioning *in vivo* in rats. These results suggest that, among the different ROS, $^{\cdot}OH$ radical is the most likely to play a decisive role in single-cycle preconditioning-induced cardioprotection.

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ABBREVATIONS

CAR, control carnosine; CONT, control; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; ESR, electron spin resonance; H_2O_2, hydrogen peroxide; MPG, *N*-(2-mercaptopropionyl) glycine; $^1O_2$, singlet oxygen; $O_2^{\cdot -}$, superoxide anion; $^{\cdot}OH$, hydroxyl radical; oxoTEMP, 2,2,6,6-tetramethyl-4-piperidone; PC, preconditioned; PC+CAR, preconditioned carnosine; PC+MPG, preconditioned MPG; RB, Rose Bengal; ROS, reactive oxygen species; TTC, triphenyltetrazolium chloride.

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