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Role of Reactive Oxygen Species in Cardiac Preconditioning: Study with Photoactivated Rose Bengal in Isolated Rat Hearts

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Oxygen radical scavengers have been shown to prevent the development of ischemic preconditioning, suggesting that reactive oxygen species (ROS) might be involved in this phenomenon. In the present study, we have investigated whether direct exposure to ROS produced by photoactivated Rose Bengal (RB) could mimic the protective effects of ischemic preconditioning.

Methods *In vitro* generation of ROS from photoactivated RB in a physiological buffer was first characterised by ESR spectroscopy in the presence of 2,2,6,6-tetramethyl-1-piperidone (oxoTEMP) or 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). In a second part of the study, isolated rat hearts were exposed for 2.5 min to photoactivated RB. After 5 min washout, hearts underwent 30 min no-flow normothermic ischemia followed by 30 min of reperfusion.

Results and Conclusions The production of singlet oxygen ($^1O_2$) by photoactivated RB in the perfusion medium was evidenced by the ESR detection of the nitroxyl radical oxoTEMPO. Histidine completely inhibited oxoTEMPO formation. In addition, the use of DMPO has indicated that (i) superoxide anions ($O_2^{\bullet-}$) are produced directly and (ii) hydroxyl radicals ($HO^{\bullet}$) are formed indirectly from the successive $O_2^{\bullet-}$ dismutation and the Fenton reaction. In the perfusion experiments, myocardial post-ischemic recovery was dramatically impaired in hearts previously exposed to the ROS produced by RB photoactivation ($^1O_2$, $O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$) as well as when $^1O_2$ was removed by histidine (50 mM) addition. However, functional recovery was significantly improved when hearts were exposed to photoactivated RB in presence of superoxide dismutase (10^5 IU/L) and catalase (10^6 IU/L).

Further studies are now required to determine whether the cardioprotective effects of Rose Bengal in presence of $O_2^{\bullet-}$ and H_2O_2 scavengers are due to singlet oxygen or to other species produced by Rose Bengal degradation.

Keywords: Singlet oxygen, superoxide anion, Rose Bengal, ESR spectroscopy, cardioprotection, isolated rat heart

INTRODUCTION

Exposure to a single or repetitive short period(s) of ischemia/reperfusion protects the heart against a subsequent prolonged ischemia. This

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myocardial adaptation called ischemic preconditioning was first described in dogs by Murry *et al.*[1] and has been reproduced in different species such as rabbit,[2] rat[3] or swine.[4] The protection afforded by ischemic preconditioning includes the limitation of reperfusion arrhythmias,[5] the reduction of infarct size[1] and the improvement of post-ischemic contractile recovery.[6] Although a large number of studies have attempted to identify the mechanisms underlying the beneficial effects of ischemic preconditioning, the intracellular biochemical pathways are still not fully elucidated. It is however well established that ischemic preconditioning can be triggered by different mediators which are released during ischemia/reperfusion.[7] Among these mediators, reactive oxygen species (ROS) have received little attention to date (for review see Ref. [8]).

Murry *et al.*[9] were the first to test the hypothesis that ischemic preconditioning can lead to a generation of ROS that is not sufficient to induce irreversible cellular injury but enough to activate certain cellular functions and trigger endogenous protective mechanisms. Their studies have shown that administration of antioxidants during the preconditioning period in the dog myocardium markedly reduced the protection afforded by ischemic preconditioning in terms of infarct size limitation. Similarly, other authors have shown that various scavengers of oxygen radicals (such as mercaptopropionyl glycine or the enzyme superoxide dismutase) inhibited the beneficial effects of ischemic preconditioning *ex vivo* in the rat[10] as well as *in vivo* in the rabbit.[11] Moreover, Tritto *et al.*[12] and Baines *et al.*[13] have recently shown that, in the absence of any preconditioning ischemia, direct exposure of the rabbit myocardium to a low dose of ROS before prolonged ischemia followed by reperfusion is able to mimic ischemic preconditioning.

Nevertheless, the involvement of ROS in ischemic preconditioning still remains controversial since other studies have led to contradictory results.[14,15]

Most of the studies concerning the role of ROS in ischemia/reperfusion of the myocardium and in ischemic preconditioning have focused on hydroxyl radicals ($HO^{\bullet}$), superoxide anions ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). Singlet oxygen ($^1O_2$), another chemical species which has received little attention in the past, also seems to have an important role in biological systems.[16] This excited state of oxygen has a very short half life of about 10^{-6} s, and a very high reactivity. Its production and its involvement in post-ischemic reperfusion injury of the myocardium has been indirectly shown by Zhai and Ashraf[17] using 5,8-endoperoxide, an oxidation product of β-carotene, as a marker of its generation. However, to our knowledge, no study has tested its possible role as a trigger of ischemic preconditioning to date.

The present study was designed to explore the effects of a direct brief exposure of the rat myocardium to different ROS, and particularly to $^1O_2$, before a prolonged ischemia. ROS were generated by photoactivated Rose Bengal (RB) which provides an excellent tool for *in vitro* studies of oxidant stress in isolated heart preparations.[18,19] An *in vitro* study, by electron spin resonance (ESR) spectroscopy, was first performed to characterise the different ROS produced by photoactivated RB in the perfusion medium. Subsequently, isolated perfused rat hearts were briefly exposed to photoactivated RB before being submitted to 30 min of no-flow normothermic ischemia followed by 30 min of reperfusion. Post-ischemic functional recovery and energy status measured at the end of reperfusion were taken as an index of cardioprotection.

MATERIALS AND METHODS

Characterisation of the ROS Generating System by ESR Spectroscopy

ROS produced in the perfusion buffer by RB photoactivation were first studied *in vitro* by

ESR spectroscopy and spin-trapping in the presence of 2,2,6,6-tetramethyl-1-piperidone (oxo-TEMP) or 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). Care was taken to keep DMPO solutions in the dark to prevent light-induced degradation. DMPO was purified via distillation prior to each use.

Spectra Recording

ESR spectra were recorded on a Bruker ER 100D spectrometer (Wissembourg, France) after illumination of the samples under the following conditions: microwave power: 10 mW; microwave frequency: 9.4 GHz; sweep time: 168 or 100 s; time constant: 200 ms; modulation frequency: 100 kHz; modulation amplitude: 1.25 G.

Procedure

OxoTEMP (300 mM) or DMPO (100 mM) were added to perfusion buffer containing either 0.1 mM or 1 μM RB. Samples were illuminated for 4 or 5 min with 2 optic fibers (Fort EF 150S, France) generating a total light power of 2385 kW/m^2. Superoxide dismutase (SOD: 300 IU/mL) and catalase (CAT: 300 IU/mL) were used for their antioxidant properties. Histidine (HIS: 50 mM) was used as $^1O_2$ quencher. The various products used were mixed in a plugged teat pipette and the reaction volume was adjusted to 1 ml. After illumination, the pipette was placed into the cell of the spectrometer and a recording was performed.

Perfusion

Animals

Male Wistar rats weighing 280–300 g and receiving a standard commercial diet (R20 Extralab, Extralabo, France) were used for all studies. Animals (7 to 12 per group) were cared for according to the guidelines formulated by the European Union for use of experimental animals (L 358.86/609/EEC).

Isolated Heart Preparations

Animals were anaesthetised with an intraperitoneal injection of sodium pentobarbital (Sanofi, France) (40 mg/kg), and heparin (Sigma, France) (100 IU) was injected via a saphenous vein. The heart was rapidly excised and placed in ice-cold perfusion medium until contraction had stopped. The isolated heart was then perfused through the aorta under a constant hydrostatic pressure of 1 m H_2O (9.81 kPa) with a non-recirculating Krebs–Henseleit (K–H) modified buffer containing (in mM): NaCl 118.5; KCl 4.75; $NaHCO_3$ 25; $MgSO_4$ 1.19; KH_2PO_4 1.18; $CaCl_2$ 1.36 and glucose 11. The buffer was maintained at pH 7.4 by bubbling with a mixture of 95% O_2/5% CO_2 at 37°C. A water-filled ultra-thin balloon connected to a pressure transducer (P23ID, Statham, USA) was inserted into the left ventricle and inflated to impose an initial end-diastolic pressure of 4 mmHg. The volume of the balloon was maintained constant throughout the experiment to allow the measurement of left ventricular end-diastolic (LVEDP) and systolic (LVESP) pressures simultaneously. The sinus node was removed by cutting the right atrium and the atrio-ventricular node was isolated by cutting the upper septum. The heart was paced at 360 beats/min (6 Hz) by a monopolar electrode placed on the left atrial wall and connected to a stimulator (Stimulator 6021 SRI, England). Coronary flow was measured by timed-collection of coronary effluent before ischemia and at 5 min intervals during reperfusion.

Exposure of Hearts to ROS

Photoactivated RB was used to generate $^1O_2$ and $O_2^{\bullet -}$.[20] RB was added to the perfusion medium before filtration and all reservoirs containing these solutions as well as all perfusion lines were covered with aluminium foil to prevent interference by ambient light. The hearts were also protected from light throughout experiments.

RB photoactivation was realised by uniformly illuminating the heart through the perfusion

chamber with 2 optic fibers (2385 kW/m^2). Hearts from a control group were illuminated according to the same protocol in order to confirm that illumination, by itself, had no effect on the preparations.

Perfusion Protocol

After 10 min of stabilisation under standard conditions (normoxia, K–H, dark) hearts were perfused for 5 min:

- with K–H only (Cont: $n = 12$)
- with K–H containing RB 250 nM (RB: $n = 7$),
- with K–H containing RB 250 nM and HIS 50 mM (RB + HIS: $n = 8$),
- with K–H containing RB 250 nM, SOD 10^5 IU/L and catalase (CAT) 10^6 IU/L (RB + SOD + CAT: $n = 8$),
- with K–H containing SOD 10^5 IU/L and CAT 10^6 IU/L (SOD + CAT: $n = 7$).

The perfusion was maintained for a 2.5 min during which the hearts were continuously illuminated. The perfusion fluid was switched back to K–H buffer for a further 10 min washout, all experiments then being carried out in the dark. At the end of the washout period, the stimulator was turned off and hearts were submitted to 30 min of total global normothermic ischemia during which hearts were immersed in perfusion medium maintained at 37°C. Hearts were then reperfused for 30 min and pacing was restored after 30 s of reperfusion. At the end of reperfusion, hearts were freeze clamped with Wollenberger tongs cooled to liquid nitrogen temperature,[21] and stored at −80°C for later biochemical analysis.

Assessment of Myocardial Function

Diastolic pressure (LVEDP), systolic pressure (LVESP) and the first positive (+dP/dt) and negative (−dP/dt) derivatives of pressure, monitored via the pressure transducer on a multichannel recorder (Gould 2200, Gould Electronic, Ballainvilliers, France), were measured before ischemia and during reperfusion at 5 min intervals. In all hearts, baseline measurements, taken after 10 min of initial perfusion, were used for normalisation of reperfusion values. Left ventricular developed pressure (LVDevP) was calculated as the difference between LVESP and LVEDP.

Assessment of Cardiac Contracture

Ischemic contracture, or the clinical equivalent "stone heart", is an important complication of severe ischemia, hypothetically developing as a consequence of energy deficit or cytosolic calcium overload. Its measurement can be taken as an index of ischemic injury.

Cardiac ischemic contracture was recorded as the increase in resting pressure following the start of ischemia. After a slow initial rise in resting pressure, there is usually a rapid upstroke which slows to reach a peak value (peak contracture) followed by a plateau or a gradual fall in resting pressure. The indices measured were: (i) time to peak contracture (TTPC expressed in min) from the start of ischemia, and (ii) peak contracture (PC expressed in mmHg).

Heart Samples

Frozen ventricular tissue was ground to a fine powder with a steel pestle and mortar pre-cooled to liquid nitrogen temperature. The resulting powder was then homogenised in perchloric acid (0.6 N) in a glass tube. Samples were kept at 4°C for 15 min before being centrifuged (1000*g*, 8 min, 4°C). The pellet was resuspended in NaOH (1 N) for protein assay. The supernatant was neutralised with KOH (6 N) and placed on ice for 30 min before being centrifuged (1000*g*, 8 min, 4°C). The supernatant containing adenine nucleotides was stored at −80°C until assay.

Assessment of Cardiac Energy Charge

Adenine nucleotides (ATP, ADP, AMP) were assessed by high-performance liquid chromatography

(HPLC). The equipment used was a Waters Millipore (Model 510, Molsheim, France) linked to a UV-detector (model 481 LC Spectrophotometer Waters Millipore; 254 nm). Eluent (sodium pyrophosphate (0.01 M) 95%, methanol 5%) was used over a reverse-phase column (Waters Millipore; Microbondapack C18; diameter = 9.3 mm; length = 150 mm). The peak identification was based on the retention times, which were checked with a mixture of synthetic standards.

The energy charge (EC) was calculated as follows:

$$EC = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}$$

Protein Measurement

The protein content was assayed by the modified method of Lowry *et al.*[22] using bovine serum albumin as a standard.

Chemicals

RB (3′,5′,4′,6′-tetrachloro-2,4,5,7 tetraiodofluorescein), ATP, ADP, AMP, bovine serum albumin, histidine, superoxide dismutase and catalase were purchased from Sigma Chemical, France, DMPO and oxoTEMP from Aldrich Chemical, France.

Statistical Analysis

The data are expressed as mean ± SEM. Statistical significant differences were assessed by analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) *post hoc* test (Stat View, Abacus concepts, Inc., Berkeley, CA, 1992). 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

Spin-Trapping Experiments

As shown in Figure 1, the characteristic ESR spectral pattern of three equal-intensity lines ($^aN = 16.3$ G) of the stable nitroxyl radical oxo-TEMPO produced by the oxidation of oxoTEMP by $^1O_2$ was observed after 4 min of illumination of the perfusion buffer containing RB (100 mM) and oxoTEMP (300 mM) (Figure 1B). Neither incubation of oxoTEMP with RB in the dark (Figure 1A), nor illumination of RB without oxoTEMP (data not shown), produced any signal. Moreover, addition of the $^1O_2$ quencher HIS (50 mM) completely inhibited oxoTEMPO formation (Figure 1C).

Figure 2 shows that a 5 min illumination of the perfusion fluid containing RB (0.1 mM) in presence of DMPO (100 mM) induced the formation of a 1:2:2:1 quartet $^aN = {}^aH = 14.9$ G) characteristic of the DMPO–OH spin-adduct (Figure 2B). The presence of the DMPO–OH spin adduct does not necessarily mean that hydroxyl radicals are formed. Indeed, the spin adduct DMPO–OOH, resulting from the interaction between $O_2^{\bullet -}$ and DMPO, rapidly decomposes to form the

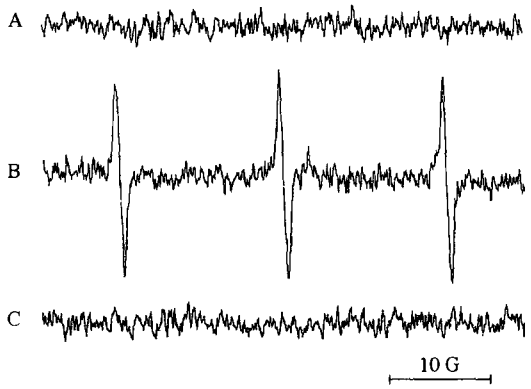

FIGURE 1 ESR spectra of Rose Bengal (RB) in the presence of the spin trap oxoTEMP 2,2,6,6-tetramethyl-1-piperidone). RB (0.1 mM) was dissolved in the perfusion buffer in the presence of oxoTEMP (300 mM). A: in the dark; B: illuminated for 4 min; C: +histidine (50 mM), illuminated for 4 min. The instrument settings are stated in the text.

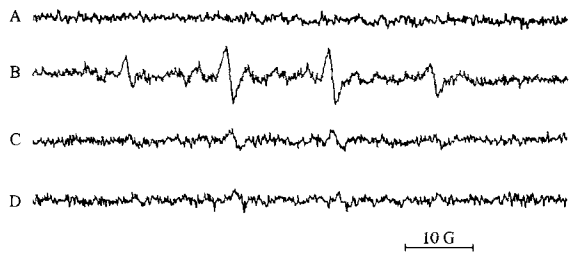

FIGURE 2 ESR spectra of Rose Bengal (RB) in the presence of the spin trap DMPO (5,5-dimethyl-1-pyrroline-N-oxide). RB (1 μmol/L) was dissolved in the perfusion buffer in the presence of DMPO (100 mM) A: in the dark; B: illuminated for 5 min; C: + superoxide dismutase (SOD: 300 IU/mL) illuminated for 5 min; D: + SOD (300 IU/mL) + catalase (300 IU/mL) illuminated for 5 min; The instrument settings are stated in the text.

DMPO–OH adduct. SOD (300 IU/mL), which catalyses $O_2^{\bullet -}$ dismutation, and the mixture SOD (300 IU/mL) plus CAT (300 IU/mL) effectively blunted the DMPO–OH signal (Figure 2C and D) suggesting that $O_2^{\bullet -}$ were originally produced during RB illumination. Finally, no signal was detected when DMPO was incubated with RB in the dark (Figure 2A) or when RB was illuminated in the absence of DMPO (data not shown).

Effects of the Pretreatments on Myocardial Function

Table I shows baseline functional parameters measured after 10 min of perfusion under standard conditions, and their evolution during the pretreatments. No significant difference was observed between control (Cont) and other groups at the end of the stabilisation period. In the control group, perfusion in the dark, followed by illumination and washout (5 min) did not modify the functional variables when compared with stabilisation values.

Coronary Flow

Perfusion in the dark with RB induced a significant vasodilation compared with baseline values ($p < 0.01$). Illumination induced a decrease in coronary flow (CF) in both RB and RB + HIS groups ($p < 0.05$), and this vasoconstrictive effect remained significant after washout ($p < 0.01$). In the RB + SOD + CAT group, CF remained constant during illumination and decreased slightly during washout (13.4 ± 0.4 vs 12.3 ± 0.4, $p < 0.01$). Finally, pretreatment with SOD + CAT induced a persistent vasoconstriction which remained significant after washout ($p < 0.01$).

End Diastolic Pressure

In RB and RB + HIS groups, LVEDP rose slightly during the photoactivation period, although not significantly in the RB group, and dramatically after washout (RB: 16.4 ± 2.7 mmHg vs 4.0 ± 0 mmHg, $p < 0.01$; RB + HIS: 21.1 ± 1.3 mmHg vs 4.0 ± 0 mmHg, $p < 0.01$). Perfusion with RB + SOD + CAT induced an increase in LVEDP which disappeared after washout. In the SOD + CAT group, LVEDP decreased significantly upon illumination ($p < 0.01$) and returned to stabilisation values after washout.

Contractile Function

A positive inotropic effect appeared upon illumination in RB and RB + HIS groups, but this effect vanished after washout. LVDevP, +dP/dt and −dP/dt were significantly reduced throughout pretreatment with RB + SOD + CAT or SOD + CAT.

Ischemic Contracture

As Figure 3A shows, TTPC in RB, RB + HIS and RB + SOD + CAT groups was not different from controls. However, TTPC was significantly delayed in the SOD + CAT group compared to both Cont ($p < 0.01$) and RB + SOD + CAT ($p < 0.01$).

Figure 3B shows that the magnitude of peak contracture was increased in RB and RB + HIS groups (RB: 87 ± 5 mmHg; RB + HIS: 89 ± 1 mmHg vs Cont: 72 ± 5 mmHg, $p < 0.05$ and $p < 0.01$ respectively). Conversely, PC was significantly reduced in the SOD + CAT group

TABLE I Functional parameters after stabilisation and during pretreatments

	Experimental groups				
	Cont ($n=12$)	RB ($n=7$)	RB + HIS ($n=8$)	RB + SOD + CAT ($n=8$)	SOD + CAT ($n=7$)
CF (mL/min)					
Stab	13.6 ± 0.3	14.4 ± 0.7	14.1 ± 0.2	13.4 ± 0.4	14.0 ± 0.4
Dark	13.5 ± 0.3	15.7 ± 1.0**	14.7 ± 0.4	13.2 ± 0.4	11.9 ± 1.0**
Lux	14.1 ± 0.4	13.3 ± 0.8*	13.4 ± 0.3*	13.1 ± 0.3	11.7 ± 1.1**
W-o	14.2 ± 0.4	11.6 ± 1.0**	12.3 ± 0.4**	12.3 ± 0.4**	11.5 ± 0.9**
LVEDP (mmHg)					
Stab	4.0 ± 0	4.0 ± 0	4.0 ± 0	4.0 ± 0	4.0 ± 0
Dark	4.8 ± 0.5	4.1 ± 0.1	4.0 ± 0	6.8 ± 1.0**	3.7 ± 0.2
Lux	4.9 ± 0.6	6.0 ± 0.8	7.0 ± 0.7*	6.6 ± 0.7**	3.0 ± 0.4**
W-o	3.9 ± 0.2	16.4 ± 2.7**	21.1 ± 1.3**	5.0 ± 0.9	3.9 ± 0.1
LVDevP (mmHg)					
Stab	126 ± 3	131 ± 4	131 ± 3	126 ± 5	124 ± 3
Dark	121 ± 4	138 ± 3	131 ± 2	99 ± 7**	100 ± 5**
Lux	122 ± 4	146 ± 3**	149 ± 4**	97 ± 6**	94 ± 6**
W-o	123 ± 3	135 ± 4	125 ± 3	107 ± 6**	102 ± 7**
+dP/dt (mmHg/s)					
Stab	6201 ± 237	6906 ± 206	6697 ± 180	6343 ± 385	5543 ± 189
Dark	5958 ± 244	7169 ± 187	6692 ± 140	5000 ± 383*	4300 ± 256**
Lux	5974 ± 224	7816 ± 231*	7854 ± 268**	5129 ± 170**	4114 ± 272**
W-o	6027 ± 232	7253 ± 263	6650 ± 201	5643 ± 379	4643 ± 378**
−dP/dt (mmHg/s)					
Stab	4366 ± 128	4626 ± 168	4552 ± 88	4471 ± 291	4414 ± 152
Dark	4149 ± 151	4754 ± 160	4575 ± 85	3343 ± 292**	3386 ± 278**
Lux	4172 ± 140	5285 ± 149	4665 ± 111	3500 ± 232**	3357 ± 267**
W-o	4159 ± 141	5252 ± 174	4373 ± 123	3657 ± 250**	3643 ± 304**

Cont = control; RB = Rose Bengal; HIS = histidine; SOD = superoxide dismutase; CAT = catalase. Stab: after 10 min stabilisation under standard conditions; Dark: after the 5 min perfusion in the dark; Lux: after 2.5 min illumination; W-o: after 5 min washout in the dark; CF: coronary flow; LVDevP: left ventricular developed pressure; +dP/dt and −dP/dt: positive and negative first derivatives of pressure. *$p < 0.05$, **$p < 0.01$ vs corresponding stabilisation value.

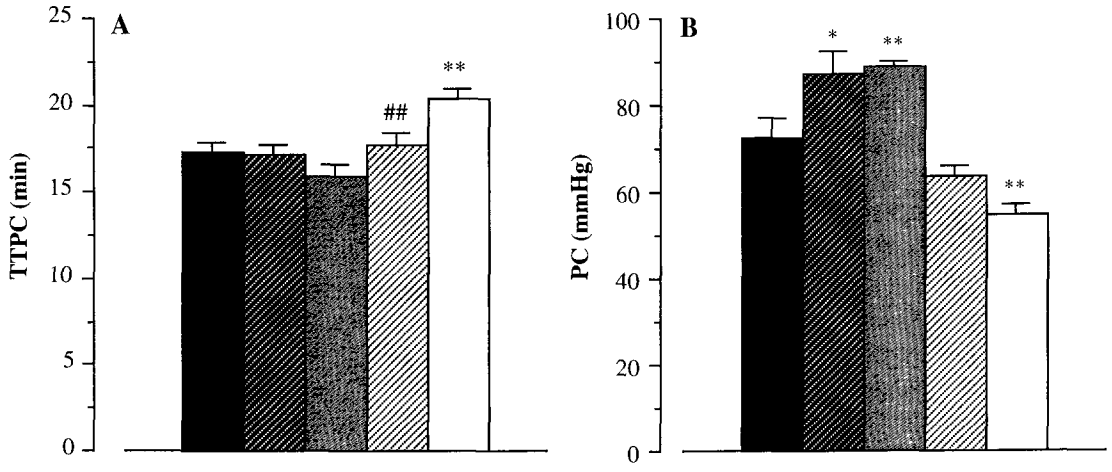

FIGURE 3 Effects of the pretreatments on cardiac contracture developed by isolated rat hearts during the 30 min ischemia. A: time to peak contracture (TTPC); B: magnitude of peak contracture (PC). ■ control; ▨ Rose Bengal; ▩ Rose Bengal + histidine; ▨ Rose Bengal + superoxide dismutase + catalase; □ superoxide dismutase + catalase. *$p < 0.05$, **$p < 0.01$ vs control, ##$p < 0.01$ vs superoxide dismutase + catalase.

($p < 0.01$). Thus, these results suggest that ischemic injury was more pronounced in both RB and RB + HIS groups whereas ischemic contracture appeared less severe in hearts pretreated with SOD + CAT.

Post-Ischemic Functional Recovery

Figure 4 illustrates the evolution of LVDevP during the 30 min of reperfusion. Post-ischemic recovery of LVDevP was markedly altered in RB and RB + HIS groups throughout reperfusion ($p < 0.01$). Within the first 10 min of reperfusion, LVDevP was higher in the SOD + CAT group compared with controls (SOD + CAT: 21 ± 2% vs Cont: 15 ± 2%, $p < 0.05$) but was thereafter equivalent in both groups except at 30 min of reperfusion, when recovery of LVDevP was significantly higher in SOD + CAT group. On the other hand, RB + SOD + CAT induced a protective effect on post-ischemic contractile recovery ($p < 0.01$ vs Cont throughout reperfusion; $p < 0.05$ vs SOD + CAT at 15 and 20 min of reperfusion).

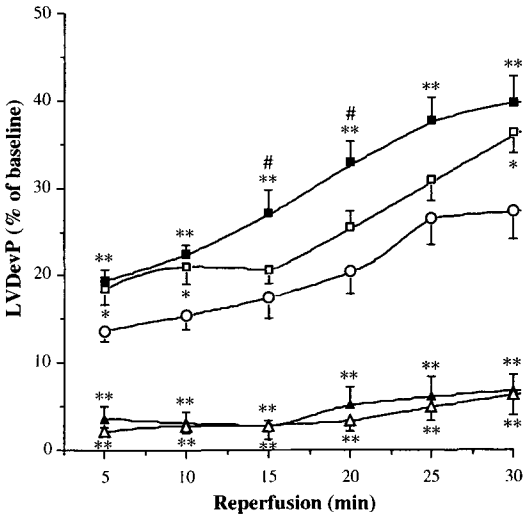

FIGURE 4 Effects of the pretreatments on post-ischemic left ventricular developed pressure (LVDevP) of isolated rat hearts during the 30 min reperfusion. ○ control; ▲ Rose Bengal; △ Rose Bengal + histidine; ■ Rose Bengal + superoxide dismutase + catalase; □ superoxide dismutase + catalase. $^{*}p < 0.05$, $^{**}p < 0.01$ vs control, $^{\#}p < 0.05$ vs superoxide dismutase + catalase.

Figure 5 shows the evolution of +dP/dt and −dP/dt during reperfusion. Recovery of +dP/dt was only transitory enhanced within the first 10 min of reperfusion in SOD + CAT group ($p < 0.05$ vs Cont) and became equivalent to Cont thereafter. No statistical difference was observed between SOD + CAT and Cont groups for −dP/dt recovery. While a dramatic impairment was observed in RB and RB + HIS groups ($p < 0.01$), post-ischemic recovery of +dP/dt and −dP/dt was markedly improved in the RB + SOD + CAT group throughout reperfusion (+dP/dt: RB + SOD + CAT: 44 ± 6% vs Cont: 28 ± 4%, $p < 0.01$; −dP/dt: RB + SOD + CAT: 42 ± 4% vs Cont: 29 ± 4%, $p < 0.01$ after 30 min of reperfusion). When compared to SOD + CAT, the recovery of + and −dP/dt was also improved in RB + SOD + CAT although this effect was only significant at 20 min of reperfusion.

Energy Charge

Cardiac energy charge (EC) was dramatically depressed in both RB and RB + HIS groups compared to controls ($p < 0.01$). In contrast, EC was preserved in the RB + SOD + CAT group despite the increased contractile recovery, indicating a better preservation of ATP turnover in this group of hearts. Finally, EC appeared to be slightly (although non-significantly) increased in SOD + CAT (EC = 0.63 ± 0.02) compared to both Cont (EC = 0.57 ± 0.02; ns) and RB + SOD + CAT (EC = 0.57 ± 0.02; ns). Nevertheless, this metabolic preservation was not accompanied by any improvement of cardiac function.

Coronary Flow, Diastolic Function and Edema

As shown in Table II, coronary flow was dramatically lower in RB and RB + HIS groups compared with controls after 30 min of reperfusion ($p < 0.01$). In RB + SOD + CAT and SOD + CAT groups, recovery of coronary flow was also less

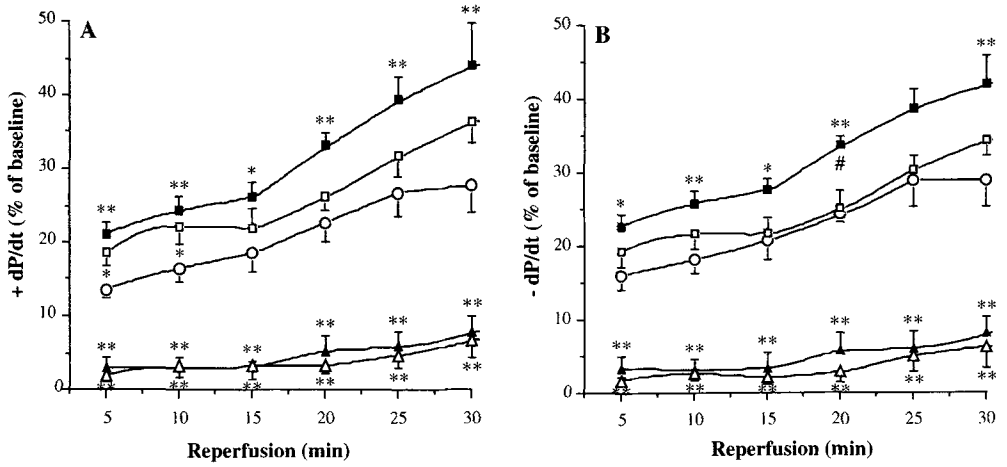

FIGURE 5 Effects of the pretreatments on post-ischemic recovery of the first derivative of left ventricular pressure (dP/dt) of isolated rat hearts during the 30 min reperfusion. A: +dP/dt; B: −dP/dt. ○ control; ▲ Rose Bengal; △ Rose Bengal + histidine; ■ Rose Bengal + superoxide dismutase + catalase; □ superoxide dismutase + catalase. $^{*}p < 0.05$, $^{**}p < 0.01$ vs control, $^{\#}p < 0.05$ vs superoxide dismutase + catalase.

TABLE II Energy charge, coronary flow (CF), left ventricular end-diastolic pressure (LVEDP) and tissue edema measured after 30 min of reperfusion

Experimental group	Energy charge	CF (% of baseline)	LVEDP (mmHg)	Edema (mg protein/g wet weight)
Cont	0.57 ± 0.02	61 ± 3	80.0 ± 5.7	71 ± 4
RB	0.45 ± 0.03**	32 ± 3**	95.7 ± 2.4**	76 ± 5
RB + HIS	0.45 ± 0.01**	35 ± 1**	96.3 ± 1.7**	69 ± 3
RB + SOD + CAT	0.57 ± 0.02	50 ± 2*	68.3 ± 3.7*#	76 ± 3
SOD + CAT	0.63 ± 0.02	52 ± 6*	53.4 ± 3.2**	68 ± 2

Cont = control; RB = Rose Bengal; HIS = histidine; SOD = superoxide dismutase; CAT = catalase. Energy change calculation is described in the text. Edema is expressed as protein weight per heart wet weight. $^{*}p < 0.05$, $^{**}p < 0.01$ vs Cont, $^{\#}p < 0.05$ vs SOD + CAT.

pronounced than controls. Reperfusion contracture, assessed by the rise in LVEDP, was more severe in RB and RB + HIS groups ($p < 0.05$). Conversely, it was reduced in the RB + SOD + CAT and SOD + CAT groups (RB + SOD + CAT: 68.3 ± 3.7 mmHg; SOD + CAT: 53.4 ± 3.2 mmHg vs Cont: 80.0 ± 5.7 mmHg, $p < 0.05$ and $p < 0.01$ respectively). Finally, the edema, expressed in mg of protein per gram wet weight was equivalent among groups.

DISCUSSION

It has been previously reported that the quenching of RB by oxygen in aqueous solutions leads to the production of singlet oxygen ($^1O_2 = 75\%$) and superoxide anion ($O_2^{\bullet -} = 25\%$).[20] Our *in vitro* ESR studies have shown that, in the perfusion buffer, photoactivated RB generated a cocktail of $^1O_2$ and $O_2^{\bullet -}$ indirectly producing H_2O_2 and $HO^{\bullet}$ through the Fenton and Haber–Weiss reactions.

Exposure of rat hearts to this ROS generating system before a sequence of ischemia/reperfusion impaired post-ischemic functional recovery and altered metabolic status. Addition of histidine (HIS) did not improve post-ischemic recovery, suggesting that $O_2^{\bullet -}$, H_2O_2 and/or $HO^{\bullet}$ could be directly responsible for the deleterious effects of the RB pretreatment. In contrast, administration of a mixture of SOD and CAT completely inhibited the toxic effects of photoactivated RB and conferred to the generating system a cardioprotective action against ischemia and reperfusion.

Since the description of ischemic preconditioning by Murry *et al.*[1] and the demonstration of its existence in various mammalian species[2–4] numerous studies have been designed to elucidate the cellular mechanisms involved in this phenomenon. Among the different hypotheses formulated to date[7], the ROS theory of ischemic preconditioning still remains controversial.[8] It is now widely accepted that ROS produced during post-ischemic reperfusion are involved in the etiology of reperfusion injury.[23] Nevertheless, although strong oxidant stresses have obvious deleterious effects on the myocardium, it has been suggested that low concentrations of ROS may be able to modulate certain cellular functions without exhibiting any toxic effect. On this basis, some authors have proposed that the ROS produced upon reperfusion after the preconditioning ischemia, may participate in the triggering mechanisms of ischemic preconditioning.[9–11] Murry *et al.*[9] who were the first to test this hypothesis, have shown that the addition of SOD and CAT after the first period of preconditioning ischemia, significantly attenuated the cardioprotective effects of ischemic preconditioning in terms of infarct size limitation. Administration of free-radical scavengers also completely inhibited the protective effects of ischemic preconditioning in most experimental studies, either in terms of infarct size[11] or in terms of reduction of reperfusion-induced arrhythmias.[10] However, some other studies have led to negative results[14,15] rendering the involvement of ROS in ischemic preconditioning uncertain. Besides these studies designed to explore the role of the ROS that are produced during ischemic preconditioning, some have consisted in directly exposing the heart to chemically-generated ROS before submitting it to a prolonged ischemia and reperfusion sequence. Thus, Tritto *et al.*[12] have shown that direct exposure of isolated perfused rabbit hearts to $O_2^{\bullet -}$ artificially generated by the purine/xanthine oxidase system induced a protective effect against regional ischemia and improved post-ischemic functional recovery. Moreover, using a similar procedure in rabbit isolated heart preparations, Baines *et al.*[13] have shown that this superoxide-induced cardioprotection was probably dependent on protein kinase C, which plays a key role in the cellular transduction pathway of ischemic preconditioning.[24,25] The purine/xanthine oxidase system, which is the only ROS-generating system that has been tested in this context to date, produces mainly $O_2^{\bullet -}$ and H_2O_2. H_2O_2 probably reacts with transition metals, such as iron or copper, to produce indirectly $HO^{\bullet}$ in the perfusion buffer. To our knowledge, no study reported in the literature has been devoted to the possible role that $^1O_2$ may play in ischemic preconditioning.

Our ESR study confirms that under our experimental conditions, photoactivated RB produces $^1O_2$ and $O_2^{\bullet -}$ as already reported by Lee and Rodgers.[20] Moreover, our experiments with the spin trap DMPO have led to the conclusion that $HO^{\bullet}$ is also produced indirectly from the successive dismutation of $O_2^{\bullet -}$ and H_2O_2 through the Fenton reaction.

Thus, the use of RB in isolated perfused rat hearts (RB group) has allowed us to observe the effects of an oxidant stress mediated by a cocktail of $^1O_2$, $O_2^{\bullet -}$, H_2O_2 and $HO^{\bullet}$. The addition of histidine to RB, in the RB + HIS group of hearts, has avoided the formation of $^1O_2$ without affecting the production of $O_2^{\bullet -}$, H_2O_2 and $HO^{\bullet}$. Moreover, addition of SOD and catalase, in the RB + SOD + CAT group of hearts, has allowed to produce mainly $^1O_2$.

Effects of RB on Pre-Ischemic Myocardial Function

Pretreatment with RB under our experimental conditions induced an inotropic effect and a progressive vasoconstriction followed by a rise in end-diastolic pressure upon illumination. Shattock *et al.*[26] have previously shown, in isolated papillary muscles, that exposure to photoactivated RB induced a transient inotropic effect. This has subsequently been proposed to be the consequence of the release of Ca^{2+} from the sarcoplasmic reticulum.[27] Kusama *et al.*[18] have suggested that a progressive calcium overload in vascular smooth muscle cells and the development of vascular compression as a consequence of the contracture of the myocytes surrounding coronary vessels may be responsible for the constrictor effect of RB in isolated perfused hearts.

In our study, the washout perfusion following RB treatment was carried out in the dark to avoid any artefact related to the potential adsorption of RB to the vasculature. In spite of this precaution, LVEDP continued to rise markedly during this period of perfusion. This observation suggests that the brief period of RB photoactivation (2.5 min) is sufficient to induce profound and durable cellular modifications to the myocardium. $^1O_2$ is not involved in these phenomena since its quenching by HIS had no significant effect on diastolic function. In contrast, the addition of SOD and CAT to RB completely abolished the rise in LVEDP after washout. The latter result clearly demonstrates that the deleterious effects of RB responsible for the development of diastolic dysfunction are, at least in part, due to $O_2^{\bullet -}$, H_2O_2 and/or $HO^{\bullet}$.

Effects of RB on the Subsequent Ischemia/Reperfusion Sequence

Pretreatment with RB alone exacerbated the deleterious effects of ischemia and reperfusion in terms of functional recovery under our experimental conditions.

Addition of HIS to the ROS-generating system had no significant effect on the subsequent ischemia/reperfusion sequence suggesting that the observed potentiation of reperfusion injury by RB pretreatment is not caused by $^1O_2$. These data indicate moreover, that the ROS that are generated under these conditions, mainly $O_2^{\bullet -}$, H_2O_2 and $HO^{\bullet}$ are unable to exert any cardioprotective effect. This conclusion apparently contradicts that of the studies of Tritto[12] and Baines[13] with the purine/xanthine oxidase system, which was shown to exhibit preconditioning effects on the rabbit myocardium. However, these conflicting results could be due to differences of concentration or proportion of the ROS produced in the different studies.

Addition of SOD and CAT to photoactivated RB significantly improved post-ischemic functional recovery of the hearts. This cardioprotective effect of the system RB + SOD + CAT on LVDevP upon reperfusion did not induce any further energy deficit since energy charge remained equivalent to controls. We can therefore suggest that the energy metabolism of the hearts might have been protected through a preservation of ATP turnover.

Since SOD and CAT are able to scavenge $O_2^{\bullet -}$ and H_2O_2 during RB photoactivation, $^1O_2$ could be reponsible for the beneficial effects of RB + SOD + CAT pretreatment against ischemia/reperfusion injury on the rat myocardium.

Finally, these preconditioning effects should not be related to the addition of the mixture of SOD and CAT itself since these enzymes only exerted minimal effects in the absence of RB. Indeed, only a slight and transient improvement in post-ischemic functional recovery was observed in the SOD + CAT group.

Reservations to Study

This study was performed using an isolated rat heart perfused with a crystalloid solution. It is well established that circulating phagocytes play a major role *in vivo* in ischemia and reperfusion.

Blood-perfused or *in vivo* models would therefore be required to elucidate more exactly the role that oxygen reactive species could play in these pathophysiological situations.

Our results suggest that $^1O_2$ might be cardioprotective under our experimental conditions of perfusion. However, we can not exclude that this phenomenon could be indirectly due to other mediators of ischemic preconditioning such as bradykinine, catecholamines, angiotensine II, adenosine or endothelin which release might be initiated by ROS.

Finally, the present study demonstrates that photoactivated RB has cardioprotective effects only when SOD and catalase are added to the perfusate. Nevertheless, it remains unclear whether these effects are to be attributed to $^1O_2$ or to other species derived from RB degradation.

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