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Isolated Perfused Rat Hearts Release Secondary Free Radicals During Ischemia Reperfusion Injury: Cardiovascular Effects of the Spin Trap α-phenyl *N-tert*-butylnitrone

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Free radicals produced during myocardial post-ischemic reperfusion are aggravating factors for functional disturbances and cellular injury. The aim of our work was to investigate the significance of the secondary free radical release during non ischemic perfusion and post-ischemic reperfusion and to evaluate the cardiovascular effects of the spin trap used. For that purpose, isolated perfused rat hearts underwent 0, 20, 30 or 60 min of a total ischemia, followed by 30 min of reperfusion. The spin trap: α-phenyl *N-tert*-butylnitrone (PBN) was used (3 mM). Functional parameters were recorded and samples of coronary effluents were collected and analyzed using Electron Paramagnetic Resonance (EPR) to identify and quantify the amount of spin adducts produced. During non ischemic perfusion, almost undetectable levels of free radical release were observed. Conversely, a large and long-lasting (30 min) release of spin adducts was detected from the onset of reperfusion. The free radical species were identified as alkyl and alkoxyl radicals with amounts reaching 40 times the pre-ischemic values. On the other hand, PBN showed a cardioprotective effect, allowing a significant reduction of rhythm disturbances and a better post-ischemic recovery for the hearts which were submitted to 20 min of ischemia. When the duration of ischemia increased, the protective effects of PBN disappeared and toxic effects became more important. Our results have therefore confirmed the antioxidant and protective properties of a spin trap agent such as PBN. Moreover, we demonstrated that the persistent post-ischemic dysfunction was associated with a sustained production and release of free radical species.

Keywords: Isolated perfused heart; Free radicals; Spin trapping; Electron paramagnetic resonance (EPR); Ischemia; Reperfusion

INTRODUCTION

The implication of free radicals in the development of myocardial injury during post-ischemic

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reperfusion is now a generally accepted occurrence. A large number of studies have provided direct evidence for a free radical burst starting from the initial minutes of reperfusion using electron paramagnetic resonance (EPR) spectroscopy associated with spin trapping. Primary radical species like superoxide,[1,2] hydroxyl radical[3–5] or ascorbyl free radical,[6,7] which are transiently generated in the early minutes of reperfusion are not considered to be exactly proportional to the severity of myocardial injury[8,9] and their short-lasting release pattern does not fit the characteristics of a long-term myocardial dysfunction. However, in conditions where cellular antioxidative defenses are overwhelmed, these primary radical species may induce radical chain reactions leading to the appearance of secondary radical species and degradation components. Therefore, the detection of secondary free radicals might provide a more suitable index of the oxidative stress and functional injury occurring in the heart during the post-ischemic reperfusion period. The lipophilic spin trap α-phenyl *N-tert*-butylnitrone (PBN) has previously been successfully used in isolated rat heart models,[10–16] open-chest dogs,[17–22] pigs[23] and in humans[24,25] to measure alkyl or alkoxyl radicals in coronary effluents and in the blood during the post-ischemic phase. However, only a few studies[13] have evaluated the effects of increasing the duration of ischemia on the amount of secondary free radical released during reperfusion in the ischemic rat heart.

Increasingly literature has been dealing with the biological effects of spin traps. For instance, PBN was shown to protect against doxorubicin-induced cardiotoxicity,[26] inhibit cytochrome P450,[27] inhibit NF-κB activation,[28,29] expression of cyclooxygenase-2 mRNA,[28] induction of iNOS,[28,30–32] and to increase heme oxygenase-1 expression during kidney ischemia-reperfusion injury.[33] Therefore, the numerous pharmacological properties of PBN may alter the physiological response to ischemia and reperfusion. Furthermore, there is little information concerning the effects of long-term PBN infusion on functional parameters and free radical release of isolated rat hearts during normoxic conditions of perfusion.

The aim of our study was therefore to investigate on isolated perfused rat hearts the cardiovascular effects of PBN administration and the myocardial release of secondary free radicals during non ischemic perfusion and during the reperfusion following increasing duration of global total ischemia.

MATERIALS AND METHODS

Chemicals

The spin trap PBN (Sigma France) was purified by sublimation under argon gas and stocked at −80°C in dark vials. Toluene (HPLC grade) was bought at Fluka, France. 2,2,6,6-tetramethylpiperidine *N*-oxyl (TEMPO) and all other chemicals were purchased from Sigma Chemical, France.

Perfusion Technique and Perfusion Medium

Wistar male rats (331 ± 3 g) were purchased at Depré (France). The rats were anesthetized with sodium thiopental (60 mg per kg, IP) and heparin was intravenously injected (500 IU per kg). After 1 min, the hearts were excised and placed in a cold (4°C) perfusion buffer bath until contractions ceased. Each heart was then immediately cannulated through the aorta and perfused at 37°C by the Langendorff method,[34] at a constant perfusion pressure equivalent to 80 cm of water (8 kPa). The perfusion buffer consisted of a modified Krebs–Henseleit[35] bicarbonate buffer (millimolar concentrations: NaCl 118, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, KCl 4.5, glucose 5.5 and $CaCl_2$ 3). The perfusion fluid was filtered through a 0.8 μm millipore filter to remove any particulate contaminants and gassed with 95% oxygen and 5% carbon dioxide (pH 7.3–7.5 at 37°C). An elastic water-filled latex

balloon (n°4, Hugo Sachs, Germany) was inserted into the left ventricle through the mitral valve and connected to a pressure transducer. The filling pressure was individually adjusted to 12–18 mmHg (1.6–2.5 kPa) left diastolic ventricular pressure (LDVP) to achieve a maximal contractile performance. A Gould TA 240 recorder was used to measure intraventricular pressures (LDVP and left systolic ventricular pressure (LSDP), left ventricular developed pressure (LVDevP) = LSDP−LDVP) and heart rate. The rate-pressure product (RPP) was calculated from the product of LVDevP and heart rate. Coronary flow was measured by the timed collection of the effluent. The spin trap, PBN was dissolved at the concentration of 120 mM in NaCl 0.9%. PBN was administered upstream the coronary bed with a mini pump (Harvard Apparatus), at an infusion rate adjusted to 1/40 of the coronary flow ensuring a final PBN concentration of 3 mM. Samples of coronary effluents were collected at various times for further determination of spin adducts levels with EPR spectroscopy. All procedures using PBN were performed in a dimly lit room and with nitrogen gas bubbling of solutions to avoid light-decomposition and oxidation of the spin trap or its adducts. To reduce the presence of contaminating catalytic metals, all glassware and perfusing apparatus were kept overnight with HCE 0.1 N and carefully rinsed, as recommended by Buettner and Jurkiewicz.[36]

Perfusion Protocols

Eight groups, each composed of eight rat hearts, were subjected to different ischemia-reperfusion protocols (Fig. 1).

After a stabilization phase of 15 min, isolated hearts were perfused under controlled conditions for 10 min (pre-ischemic control period). Non ischemic hearts were perfused for an additional 30 min period. For the three ischemic groups, global total normothermic ischemia was induced by clamping aortic inflow for 20, 30 or 60 min during which a thermoregulated chamber maintained the heart temperature at 37°C. After ischemia, aortic inflow was resumed for 30 min (reperfusion period).

For non ischemic hearts, PBN or vehicle (NaCl 0.9%) were infused during 20 min and during the last 5 min of the perfusion protocol. For ischemic groups, PBN or vehicle were infused 5 min before the onset of ischemia and during the initial 15 min and the last 5 min of reperfusion. Aliquots of coronary effluent samples (5 mL) were collected every 5 min in non ischemic hearts and sequentially before ischemia (3 and 1 min before starting ischemia) and during reperfusion: 1, 3, 5, 7, 10, 12, 14 and 29 min after the beginning of reperfusion (see arrows in Fig. 1). Effluents were immediately extracted with N_2-gassed ice-cold toluene (0.75 mL) as previously described,[10,11,13,16] frozen and kept in liquid nitrogen until EPR measurement.

EPR Spin Trapping

Toluene extracts were thawed and bubbled with N_2 for 20 s. EPR spectra were recorded at 293°K with a Bruker ESP 300E-X band spectrometer using a TM_{110} cavity and an aqueous flat cell. The spin adduct concentration was shown to be stable during the time for EPR measurement (data not shown). The following parameters were selected for optimal detection of PBN spin adducts in coronary effluents: microwave power = 20 mW, microwave frequency = 9.74 GHz, modulation amplitude = 1.6 G, modulation frequency = 100 kHz, gain = 1.6–$3.2\,10^6$, scan rate = 0.95 G s^{-1}, time constant = 163.84 ms, conversion time = 82 ms.

The signal intensity which is proportional to the concentration of spin adducts was measured directly from the field scan and expressed as spin adduct concentration (nM) by double integration of the experimental spectra using TEMPO nitroxide as an integration standard. The amount of myocardial PBN spin adduct liberation (picomol/min/g of heart) at each perfusion

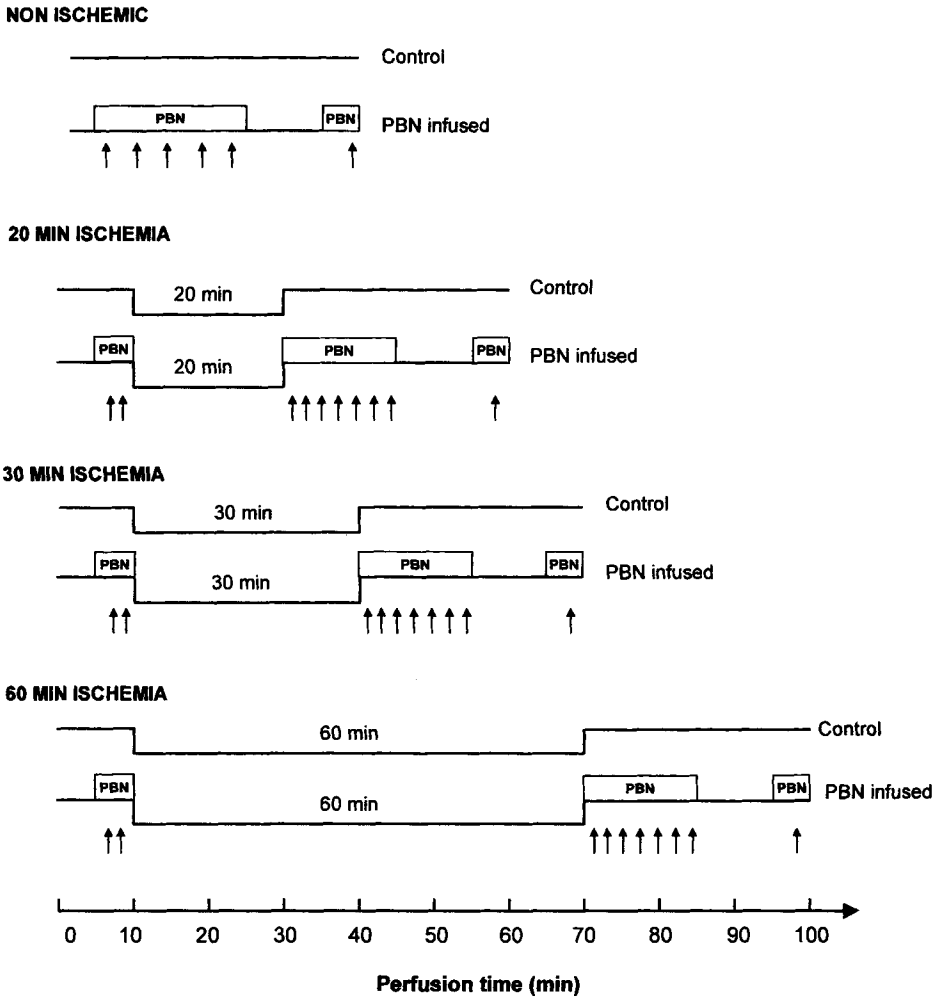

FIGURE 1 Perfusion protocols of isolated rat hearts. Eight groups, each composed of 8 hearts, were undergoing 0, 20, 30 or 60 min of global total ischemia followed by 30 min of reperfusion. The lipophilic spin trap PBN was infused as indicated in the frame. Arrows (↑) indicate time for coronary sample collection.

time was obtained by multiplying the adduct concentration by the respective coronary flow.

Statistical Analysis

All data are presented as means ± SEM. For functional parameters, statistical analysis was performed with a *t*-test, determining differences between control and PBN infused rat hearts for each ischemia-reperfusion protocol. For spin adduct release, tests of significance yielding statistical comparisons were performed with one factor ANOVA test followed by inter-group pair wise comparisons with Tukey HSD multiple comparisons.

RESULTS

Functional Parameters

Effects of PBN during non Ischemic Perfusion

The effects of PBN infusion (3 mM) on myocardial parameters were determined on non

ischemic rat hearts during 40 min, in conditions of normoxic perfusion (Fig. 2). 20 min of PBN administration did not modify the evolution of coronary flow or LDVP. From the onset of PBN infusion, LVDevP rapidly increased to a peak by about 30 mmHg, but this effect was only transitory since LVDevP decreased slowly and returned to control values after 10 min of PBN administration. The second administration of PBN during the last 5 min of perfusion was unable to increase LVDevP again. In addition, PBN was shown to exert a negative chronotropic effect, with heart rate lessened by about 15% throughout PBN infusion and returning to control levels when PBN infusion was stopped. The second infusion of PBN induced the same effect on the heart rate.

Effects of PBN on Myocardial Post-ischemic Recovery

Myocardial functional recovery was tested during the 30 min of reperfusion following 20,

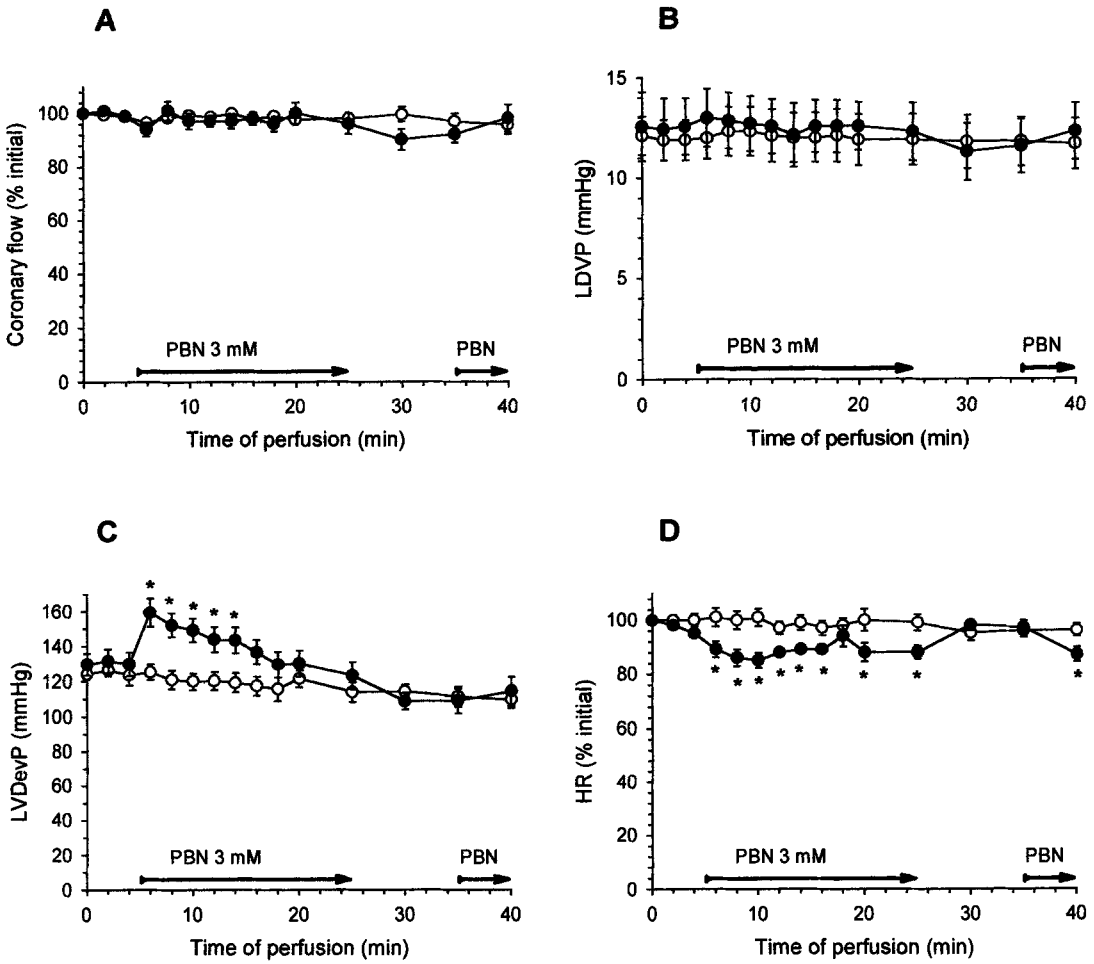

FIGURE. 2 Effect of 3 mM PBN infusion on the evolution of functional parameters of isolated rat hearts during 40 min of non ischemic perfusion. Vehicle (○, $n = 8$) or PBN (●, $n = 8$) were perfused as indicated with the arrows. A: Evolution of coronary flow (CF, initial level 16.3 ± 0.8 mL/min); B: Evolution of left diastolic ventricular pressure (LDVP); C: Evolution of left ventricular developed pressure (LVDevP), D: Evolution of heart rate (HR, initial level 297 ± 11 bpm). Results are presented as means ± SEM. Significantly different from control hearts: $^*p < 0.05$

30 or 60 min of a global total ischemia. Coronary flow (Fig. 3) was only partially restored during reperfusion with a level of recovery reaching only 50% of the pre-ischemic values for 20 or 30 min of ischemia and reduced to 30% after 60 min of ischemia. PBN infusion did not modify the evolution of coronary flow during reperfusion in the three groups.

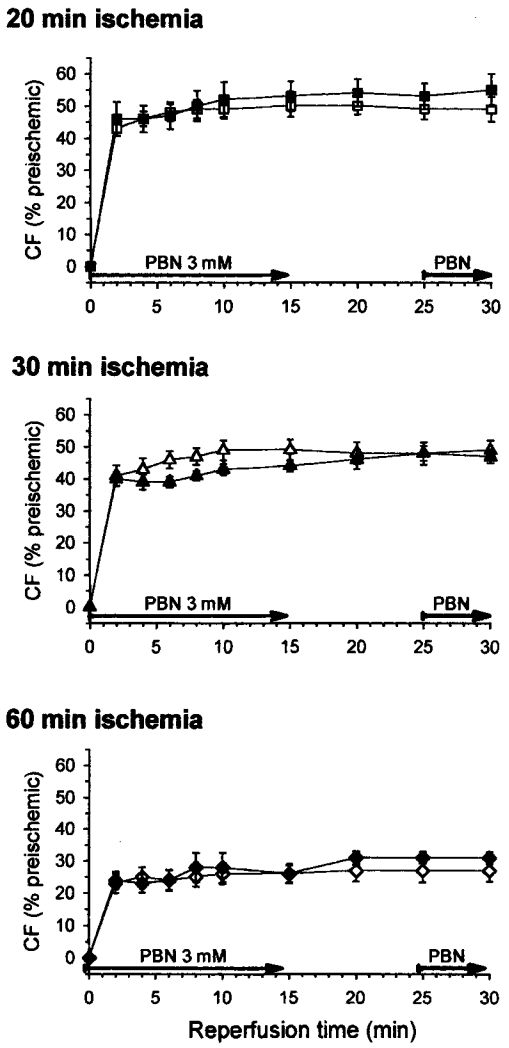

FIGURE 3 Evolution of coronary flow (CF) during the 30 min of reperfusion period following, 20, 30 or 60 min of global total ischemia. Results correspond to the percentage of pre-ischemic coronary flow after □: 20 min of ischemia ($n = 8$); ■: 20 min of ischemia + PBN ($n = 8$); △: 30 min of ischemia ($n = 8$); ▲: 30 min of ischemia + PBN; ◇: 60 min of ischemia ($n = 8$); ◆: 60 min of ischemia + PBN ($n = 8$). PBN or vehicle was perfused as indicated with the arrows. Results are expressed as means ± SEM.

From the onset of reperfusion, LDVP rose rapidly to a peak value that was obtained in 5 min and was increased when the duration of ischemia was augmented (Fig. 4). LDVP then steadily decreased but remained at a high level during 30 min of reperfusion. This feature which is characteristic of post-ischemic contracture was lessened by PBN treatment in all groups of hearts, whichever was the duration of the previous ischemic period.

With reperfusion, LVDevP (Fig. 5) recovered very slowly and remained at a low level by about 20% of its pre-ischemic value at the end of the reperfusion period. After 20 min of ischemia, LVDevP of PBN-treated hearts recovered significantly better ($p < 0.05$) and reached 40% of its pre-ischemic value at the end of reperfusion. This effect became evident when the first infusion of PBN was stopped. No differences in LVDevP recovery between vehicle and PBN infused hearts were observed when the length of ischemia was increased to 30 min. After 60 min of ischemia, PBN was shown to impair markedly the recovery of LVDevP at the end of reperfusion.

The evolution of the rate pressure product (RPP) during reperfusion was found to show almost the same pattern as described for LVDevP (Fig. 6), with a significantly better recovery of RPP, observed for PBN infused hearts as compared with vehicle after 20 or 30 min of ischemia, that became observable when the first infusion of PBN was stopped. However, this beneficial effect of PBN was reduced or removed with the second infusion of PBN. No effect of PBN on RPP recovery was observed for a longer duration of ischemia.

Rhythm disturbances (Fig. 7) are frequently observed after a global normothermic ischemia, and are mostly represented by ventricular tachycardia and fibrillation after 20 or 30 min

of ischemia and by the absence of cardiac contractions (asystole) after 60 min of ischemia. The average duration of ventricular tachycardia and fibrillation was significantly reduced by about 86 and 75% for PBN treated hearts, compared with control hearts undergoing 20 or 30 min of ischemia. No effects of PBN infusion on the occurrence of rhythm disturbances was observed after 60 min of a global total ischemia.

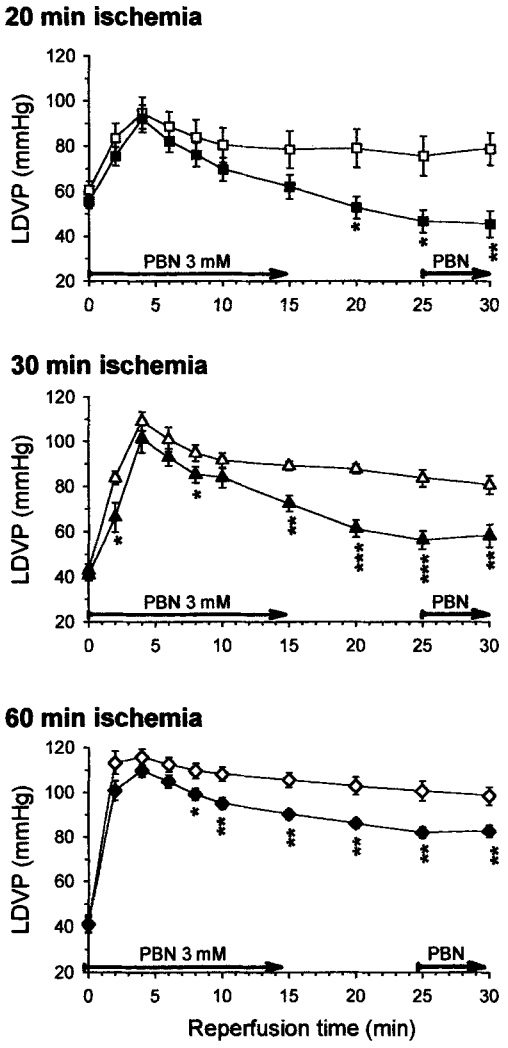

FIGURE 4 Evolution of left diastolic ventricular pressure (LDVP) during the 30 min of reperfusion period following □: 20 min of ischemia ($n = 8$); ■: 20 min of ischemia + PBN ($n = 8$); △: 30 min of ischemia ($n = 8$); ▲: 30 min of ischemia + PBN; ◇: 60 min of ischemia ($n = 8$); ◆: 60 min of ischemia + PBN ($n = 8$). PBN or vehicle was perfused as indicated with the arrows. Results are expressed in mmHg as means ± SEM. Significantly different from control hearts: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

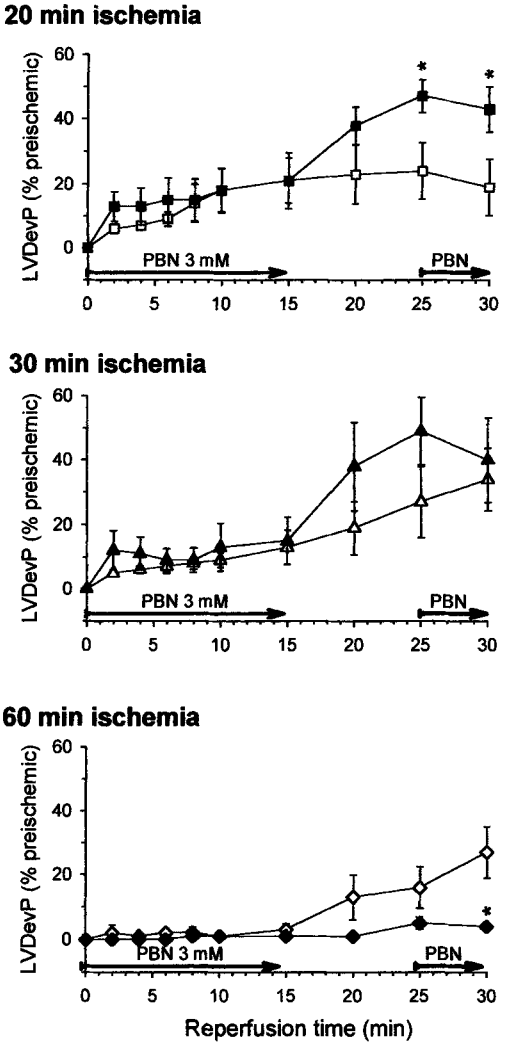

FIGURE 5 Evolution of left ventricular developed pressure (LVDevP) during the 30 min reperfusion period following: □: 20 min of ischemia ($n = 8$); ■: 20 min of ischemia + PBN ($n = 8$); △: 30 min of ischemia ($n = 8$); ▲: 30 min of ischemia + PBN; ◇: 60 min of ischemia ($n = 8$); ◆: 60 min of ischemia + PBN ($n = 8$). PBN or vehicle was perfused as indicated with the arrows. Results are expressed percentage of pre-ischemic LVDevP as means ± SEM. Significantly different from control hearts: $^{*}p < 0.05$

EPR Spin Trapping

Experiments performed with EPR on coronary effluents showed that in non ischemic hearts, the basal spin adduct signal was virtually undetectable (Fig. 8). The presence of a PBN adduct became very evident in coronary effluents under post-ischemic conditions. The spectral analysis showed the presence of a sextuplet signal (a_N = 13.5 G, a_H = 2.1 G) with coupling constants that could be attributed to alkyl/alkoxyl spin adducts and of a triplet (a_N = 7.9 G) that might be related to the presence of a PBN oxidation product. The concentration of the alkyl/alkoxyl species in the coronary effluent was evaluated (Fig. 9A) during non ischemic perfusion and under post-ischemic reperfusion. Under non ischemic conditions, a very low spin adduct concentration (~0.5 nM) was observed in coronary effluents. When PBN was infused throughout 20 min and during the last 5 min of perfusion, no modification of this basal spin adduct release was observed. Conversely, after an ischemic period, a large release of alkyl/alkoxyl species occurred in the vascular bed of reperfused hearts starting from the onset of reperfusion and remaining at a high level during 30 min of reperfusion with no return to basal pre-ischemic values. When the duration of ischemia was lengthened, the peak concentrations of spin

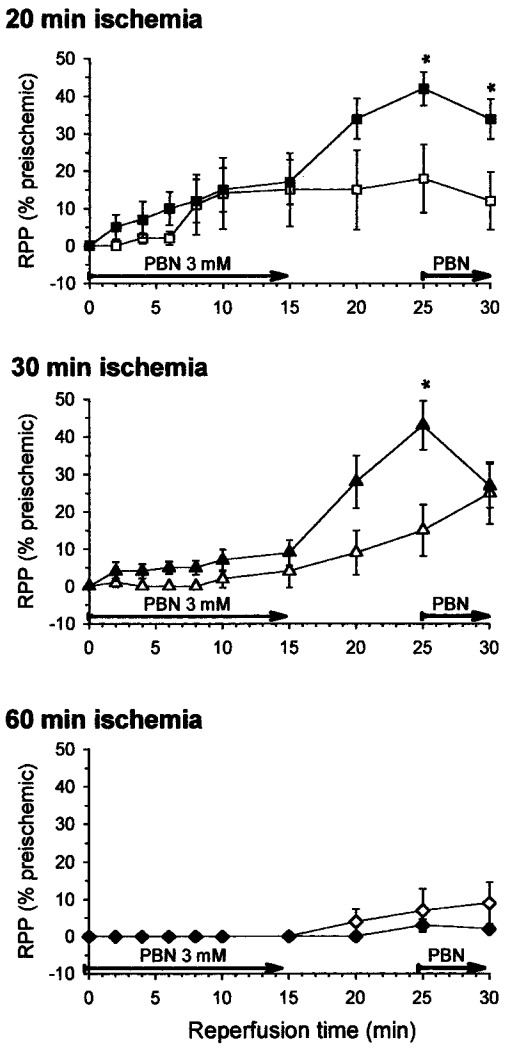

FIGURE 6 Evolution of rate pressure product (RPP) during the 30 min of reperfusion period following: □: 20 min of ischemia (n = 8); ■: 20 min of ischemia + PBN (n = 8); △: 30 min of ischemia (n = 8); ▲: 30 min of ischemia + PBN; ◇: 60 min of ischemia (n = 8); ◆: 60 min of ischemia + PBN (n = 8). PBN or vehicle was perfused as indicated with the arrows. Results are expressed percentage of pre-ischemic RPP as means ± SEM. Significantly different from control hearts: *$p < 0.05$

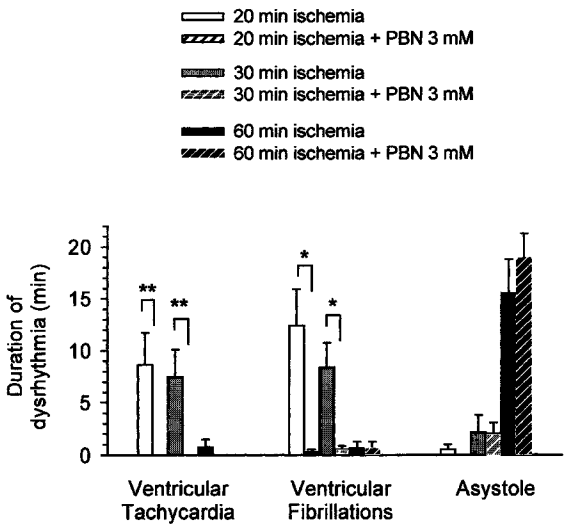

FIGURE 7 Occurrence of rhythm disturbances during the 30 min or reperfusion period following 20, 30 or 60 min of global total ischemia. Results are expressed as means ± SEM. Significantly different from control hearts: *$p < 0.05$; **$p < 0.01$

adducts in the coronary effluents was increased, reaching at least 15, 20 and 40 times their pre-ischemic values after 20, 30 and 60 min of ischemia, respectively. At the end of the reperfusion period, the concentration of spin adducts in the coronary effluent of 60 min-ischemic hearts was significantly 40 times higher than non ischemic hearts ($p<0.001$) and twice that of the other two groups of post-ischemic hearts ($p<0.05$). Spin adduct release rate of isolated hearts was calculated by taking into account the coronary flow and weight of each heart (Fig. 9B). Non ischemic hearts were shown to release a steady state of spin adducts throughout the perfusion protocol that was close to 1 pico-mole/min/g of heart. The pre-ischemic release of spin adducts was identical to that observed in non ischemic hearts, but was dramatically increased during reperfusion, increasing gradually during the first 5–10 min of reperfusion to levels up to 13 times the basal levels ($p < 0.001$). Spin adduct release rate maintained a steady state during 30 min of reperfusion, and no significant differences were observed among the three post-ischemic groups.

DISCUSSION

The first aim of our work was to describe the effects of PBN infusion on the functional parameters of isolated perfused rat hearts.

Our study has shown the absence of any vasoactive effect of 3 mM PBN infusion in isolated perfused rat hearts. This absence of effect of PBN on coronary flow had been previously described[37–39] for PBN concentrations between 2.5 and 5 mM. However, vasodilatory effects of PBN have been described in some other experimental studies[14,40] for the same range of concentrations (2–6 mM) or in open-chest dogs.[38] The vasodilatory effects of PBN are usually proposed to be due to the trapping of superoxide anion which allows larger amounts of nitric oxide to exert relaxation on smooth muscle cells.[38,41] However, PBN is

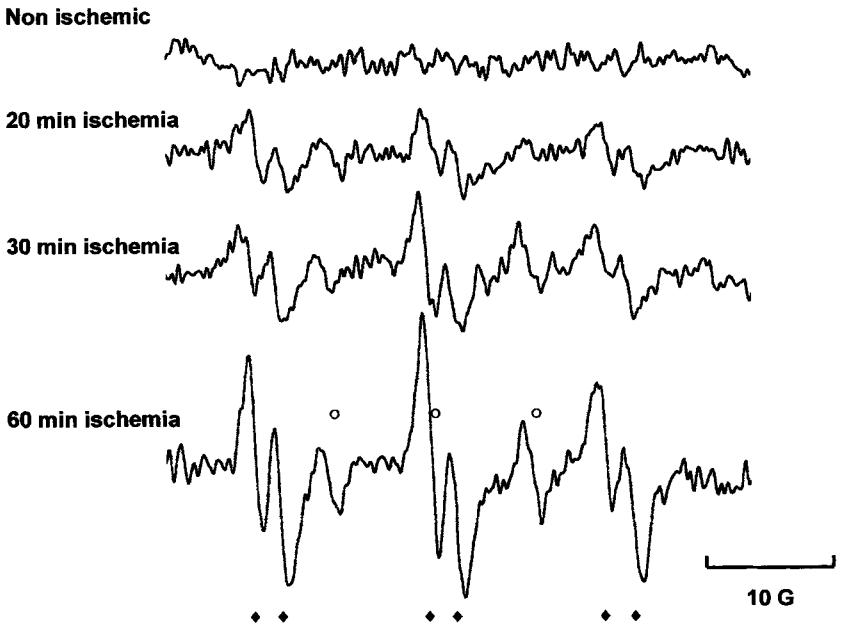

FIGURE 8 EPR representative spectra of coronary effluent extracts obtained 10 min after beginning of PBN infusion under non ischemic conditions (upper spectrum) and after 5 min of reperfusion following 20, 30 or 60 min of global total ischemia. Spectral analysis showed the presence of two distinct signals (◆) $a_N = 13.5$ G, $a_H = 2.1$ G, and (○) $a_N = 7.9$ G.

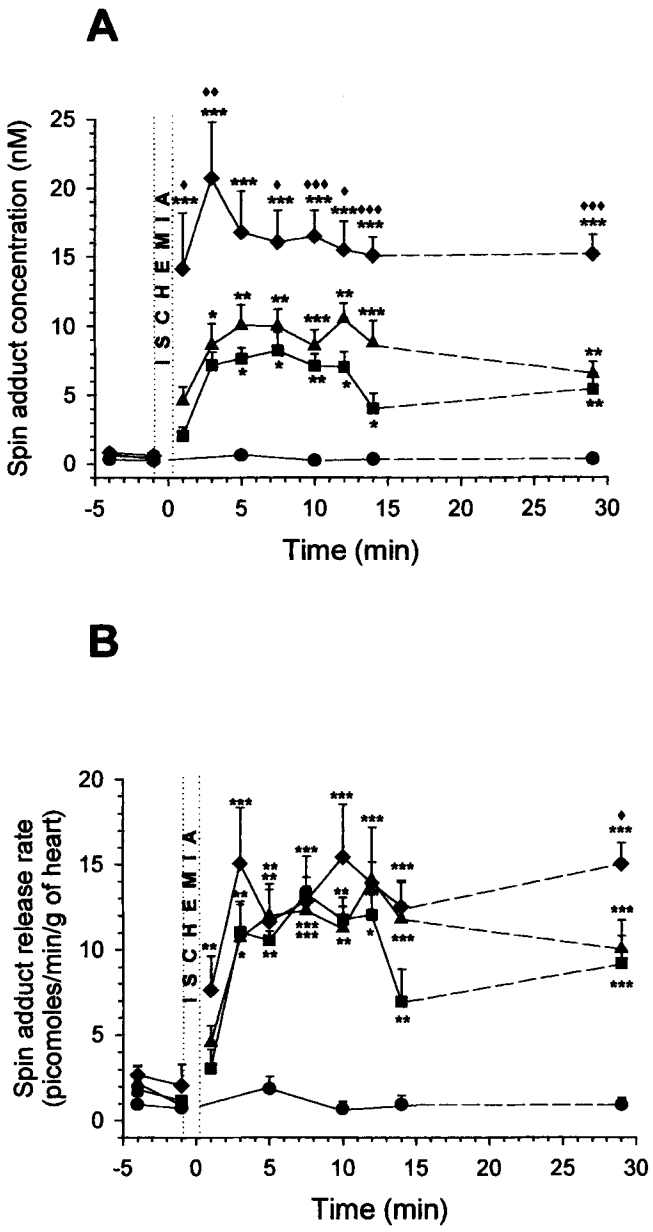

FIGURE 9 Free radical release of isolated perfused rat hearts during non ischemic perfusion (●, $n = 8$), pre-ischemic period and reperfusion following 20 (■, $n = 8$), 30 (▲, $n = 8$) or 60 min (◆, $n = 8$) of global total ischemia. A: Spin adduct concentration in coronary effluent. B: Spin adduct release rate during 30 min of reperfusion. Results are expressed as means ± SEM. Significantly different from non ischemic hearts: $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$. Significantly different from hearts reperfused after 20 or 30 min of ischemia: ◆$p < 0.05$; ◆◆$p < 0.01$, ◆◆◆$p < 0.001$

not a very good superoxide scavenger,[42] and the vasodilatory effects of nitrones might rather be due to their transformation to nitric oxide by the enzymatic cleavage of the spin trap, as is observed for other NO or NO_2-containing compounds such as sodium nitroprussate, sidnonimines and glycerol trinitrate. Moreover, some authors have shown that PBN was able to

release nitric oxide when subjected to light-decomposition.[43-45] The vasodilatory effects of PBN might therefore be rather due to nitric oxide release from the nitrone during light-induced, oxidative or biological decomposition of the trap. As our model is buffer-perfused and care was taken to avoid light or oxygen degradation of the trap, these vasodilatory effects were prevented. More recently, patch-clamp studies[46] have revealed a reversible calcium blockade with PBN at 1 mM concentration that may account for the relaxation observed in isolated pulmonary artery ring preparations. The high calcium concentration of our perfusion buffer (3 mM) may impair the responsiveness to PBN-induced calcium current blockade.

In our experimental model, we were able to detect a rapid but transitory positive inotropic effect of 3 mM PBN infusion as was previously observed by Blasig *et al.*[14] who reported that the positive inotropic effect was associated with an increase in myocardial oxygen consumption. However, the reasons for this inotropic effect, that is not observed by all authors,[38,39] still remains unclear. The negative chronotropic effect of PBN observed in our study had been previously noticed by Li *et al.*[15] for concentrations close to ours, but no significant variation had been found. This effect might not be related to an auto-regulation of the heart rate as its feature does not fit the variations of ventricular developed pressure. The reasons for this effect remain to be elucidated.

The second aim of our study was to investigate the effects of PBN infusion on the post-ischemic myocardial recovery after increasing periods of global total ischemia. In our experimental conditions, PBN dramatically improved the recovery of the myocardial function after 20 min of ischemia, decreasing LDVP, and the occurrence of arrhythmia and increasing LVDevP and RPP. Hearse and Tosaki[47] had also previously described that 30 μM PBN was the optimal dose to reduce the vulnerablility of the myocardium to reperfusion-induced fibrillation after 10 min of a transitory regional ischemia. This beneficial effect of PBN on the insults of reversible regional ischemia has been described in other models.[17,19] Due to this lipophilic nature, as determined from its chloroform/water partition coefficient ($Kp = 199$ at 24°C), PBN is allowed to enter the intracellular compartment and to accumulate in cellular organelles such as the mitochondria, cytosol and nucleus[48] where it can exert its properties.

After 30 min of ischemia, the effect of 3 mM PBN on myocardial recovery was milder, reducing post-ischemic contracture and arrhythmia, but only transiently increasing RPP. Our results are in agreement with those of Vrbjar *et al.*[16] who have shown a protective effect of PBN on the occurrence of ventricular fibrillation and on post-ischemic recovery of heart rate and LVDevP after 30 min of a global normothermic ischemia. However, Vrbjar *et al.*[16] were unable to see protective effects on left diastolic ventricular pressure (LDVP) and they have provided evidence for negative effects on the recovery of coronary flow. Bradamante *et al.*[37] were unable to detect any effect of 5 mM PBN on the recovery of RPP after 30 min of a global total normothermic ischemia. After 35 min of global total ischemia at 37°C, Baker *et al.*[39] showed that PBN, at a concentration of 0.4 or 4 mM, when present either during ischemia alone or reperfusion alone did not exert any effect upon the recovery of developed pressure, RPP or post-ischemic enzyme leakage. These discrepancies might be due to differences in the timed-sequence of PBN administration and in buffer calcium concentration which can influence the basal level of myocardial recovery during reperfusion, but also might be due to the fact that 30 min duration of ischemia represents a transition between reversible and irreversible injury and that the protective effects of PBN are observable on reversible injury.

In fact, when the duration of ischemia was increased to 60 min, PBN-treated hearts still

had a lower level of post-ischemic contracture but the recovery of LVDevP was markedly impaired. Myocardial toxic effects of PBN have been depicted elsewhere in the literature for coronary levels over 10[38] or 15 mM,[17,49] but concentrations usually used for spin trapping procedures (3–5 mM) may be considered as sub-toxic concentrations. The induction of an irreversible injury to the myocardium might therefore reveal the toxic effects of PBN that are not observable under milder conditions of ischemia.

Our study has shown the presence of PBN spin adducts in the coronary effluents of post-ischemic hearts. The characteristics of the splitting constants observed in our experimental conditions ($a_N = 13.5$ G, $a_H = 2.1$ G) are in agreement with those depicted in the literature[15,16] and with positive control experiments done in our laboratory aimed to generate alkyl radicals. However, due to the poor resolution of the signals observed *in vivo*, we assume that the spectra observed might be composed of alkyl and alkoxyl spin adducts. We have also observed a triplet signal ($a_N =$ 7.9 G) that might be related to the presence of a PBN oxidation product. This signal has previously been observed by other authors during spin trapping experiments[50–52] and can correspond to benzoyl *tert*-butyl nitroxide. The conditions of the oxidative burst that occurs during post-ischemic reperfusion might therefore induce the production of primary and secondary free radicals trapped by PBN to produce alkyl/alkoxyl spin adducts, but also lead to the oxidation of the trap.

By controlling the conditions of spin trap administration, the amount of spin adducts detected in the coronary effluents during non ischemic perfusion were just above detection limits, i.e. 0.3 ± 0.2 nM concentration and 1.0 ± 0.5 picomoles/min/g. Other authors have observed higher pre-ischemic radical adduct concentrations from 6[14] to 12.8 nM [15] and observed higher peak release (18[14] to 25 nM[15]) after 30 min of global total ischemia than those observed in our study (10.0 ± 1.5 nM). These differences could be explained by a lower toluene extraction efficiency (70%) obtained in our study compared with that reported in other experimental conditions (90% efficiency).[15] When PBN was directly dissolved in Krebs–Henseleit buffer,[10,11,53] basal amounts of spin adducts under pre-ischemic conditions could reach 0.5 μM.[53] Therefore, variations in spin adduct release due to pathological conditions like ischemia were more difficult to observe. Experiments with PBN must therefore be carefully processed to avoid the presence of artifactual spin adducts due to: insufficient purification of the trap, insufficient purity-grade of the solvent, light or oxygen-induced decomposition of the trap, catalytic metal over-contamination of buffers and perfusion apparatus. In our experiments, decreasing the basal level of artifactual spin adducts in the perfusate, allowed us to detect a free radical release during at least 30 min of reperfusion with no return to the basal pre-ischemic levels. This long-lasting release of alkyl/alkoxyl radicals might be due to either a rapid and short-lived burst of radical formation followed by a slow release of the adducts from heart tissue, or an ongoing long-term generation of radicals released slowly from the tissue.

In conclusion, our study has shown that 3 mM PBN infusion induced transitory positive inotropic and lasting negative chronotropic effects on isolated rat hearts perfused under non ischemic conditions. After 20 min of global total ischemia, PBN markedly increased post-ischemic recovery, but this effect was weaker after 30 min of ischemia. When the duration of ischemia was increased to 60 min, PBN exerted a deleterious effect on the recovery of the myocardial function during reperfusion. In spite of these double-edged sword properties, the use of these sub-toxic concentrations of PBN allowed us to measure a large release of secondary free radicals during reperfusion and

almost undetectable levels during non ischemic perfusion.

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