Free Rad. Res., Vol. 20, No. 3, pp. 145-163
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LACK OF PROTECTION OF PBN IN ISOLATED HEART DURING ISCHEMIA AND REPERFUSION: IMPLICATIONS FOR RADICAL SCAVENGING MECHANISM

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(Received June 2nd, 1993; in revised form September 6th, 1993)

We evaluated the ability of α-phenyl-*tert*-butyl nitrone (PBN) to trap free radicals and to protect the rat myocardium during ischemia and reperfusion. Isolated bicarbonate buffer-perfused hearts ($n = 8$) were subjected to 20 min global ischemia (37°C) followed by reperfusion with 0.4 to 4.0 mM PBN. Coronary effluent containing the PBN adduct was extracted in toluene. Electron spin resonance analysis of the toluene extract revealed a PBN-hydroxyl adduct. To verify this assignment, a Fenton system was used to generate an authentic PBN-hydroxyl adduct ($n = 8$), which yielded the same ESR spectra as the reperfusion-derived adduct. The structure of the adduct formed in the Fenton system was confirmed by gas chromatography-mass spectrometry. The ESR parameters of the PBN-hydroxyl adduct were exquisitely sensitive to solvent polarity during extraction of the adduct. Extraction of an authentic PBN-hydroxyl adduct into chloroform, chloroform:methanol, and toluene closely matched the ESR parameters obtained during reperfusion of ischemic myocardium in other animal models. To determine whether PBN could confer any protective effect during ischemia or reperfusion, hearts ($n = 8$/group) were subjected to 35 min global ischmia at 37°C with the St. Thomas' II cardioplegic solution followed by 30 min reperfusion. Percent recovery (mean ± SEM) of developed pressure, rate pressure product, and leakage of lactate dehydrogenase during reperfusion in control hearts were 58 ± 3%, 48 ± 4% and 3.2 ± 0.5 IU/15 min/g wet wt. PBN at a concentration of 0.4 mM or 4.0 mM when present either during ischemia alone or reperfusion alone did not exert any effect upon recovery of developed pressure, rate pressure product or post-ischemic enzyme leakage. We conclude that PBN fails to improve contractile recovery and reduce enzyme leakage during reperfusion of myocardium subjected to global ischemia.

KEY WORDS: Free radicals, ischemia, reperfusion, spin trap, hydroxyl radical, cardioprotection, isolated rat heart.

Abbreviations PBN; α-phenyl-*tert*-butyl nitrone, DMPO, 5,5-dimethyl-1-pyrroline N-oxide; PBN/-OH; α-(hydroxybenzyl)-*tert*-butylaminoxyl, ESR, electron spin resonance; RPP, rate pressure product; DP, developed pressure; LDH, lactate dehydrogenase; GC-MS, gas chromatography-mass spectrometry; MSTFA, N-methyl-N-trimethylsilyltrifluoro acetamide.

INTRODUCTION

There is increasing evidence for the generation of reactive oxygen-derived free radicals during reperfusion of the ischemic myocardium.[1-6] Until recently, evidence to support the role of free radicals in myocardial injury has been of an indirect

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nature, inferred from the cardioprotective effects observed by supplementation of cardioplegic solutions with antioxidants (α-tocopherol, glutathione, etc.), antioxidant enzymes (catalase, superoxide dismutase), enzymatic inhibitors of free radicals (allopurinol) and metal-ion chelators, (desferrioxamine), for example.[7-11]

Recently, the technique of electron spin resonance (ESR)-spin trapping has been used to directly detect myocardial free radical generation.[12-21] The spin trapping technique[22-28] is a kinetic technique in which a diamagnetic molecule (spin trap) reacts with a free radical to produce a more stable radical (spin adduct) that is readily detectable by ESR. Spin trapping is, therefore, a very effective approach to increase the lifetime of free radicals such as the hydroxyl (·OH), lipid alkoxyl (LO·), lipid (L·), lipid peroxyl (LOO·) and the superoxide (O_2^-) radical. The spin traps 5,5-dimethyl-1-pyrroline oxide (DMPO) and α-phenyl-*tert*-butyl nitrone (PBN) have been frequently used to detect free radicals in rat,[12,13,15,16] rabbit,[19] and canine[20,21] heart models of ischemia and reperfusion. Whereas ESR spectra of DMPO-derived oxy-radical adducts are distinctly different and well-characterized, the spectra of PBN oxy radical adducts are quite similar and not characterized as well.[22-28]

The lipophilic spin trap PBN was shown to prevent adriamycin-induced cardiotoxicity in rat hearts, whereas the hydrophilic trap DMPO failed to afford a similar kind of protection.[29] Pretreatment of rats with PBN or DMPO showed that PBN, but not DMPO, accumulated in mitochondria, a target organ of toxicity with adriamycin. Selective trapping of oxy-radicals produced during mitochondrial metabolism of adriamycin was suggested to be the reason for cardioprotection by PBN.[29] No attempts were, however, made to detect PBN-derived radical adducts. Administration of PBN during aerobic reperfusion of dog hearts, subjected to regional ischemia, provided significant protection against myocardial stunning.[20,21,30] Although there appeared to be a causal relationship between PBN adduct formation and the functional recovery of stunned myocardium, the exact structures of PBN adducts, however, could not be determined. A rigorous characterization of the PBN adduct generated under well-defined conditions of ischemia and reperfusion will greatly aid in the identification of PBN adducts formed *in vivo*.

The objectives of the present investigation were: 1) To detect the radical species adducted to the spin trap PBN in the coronary effluent during reperfusion of the ischemic myocardium. 2) To characterize the PBN adduct unambiguously using an independent synthesis and by gas chromatography-mass spectrometry (GC-MS). 3) To evaluate the ability of PBN to protect the myocardium during the cycle of ischemia and reperfusion.

MATERIALS AND METHODS

Generation of PBN/·OH adduct

The α-(hydroxybenzyl)-*tert*-butylaminoxyl (PBN/·OH) adduct was generated in an incubation mixture containing 40 mM PBN, 3.6 mM ferrous sulfate and 6.6 mM hydrogen peroxide in 1.5 ml phosphate buffer (20 mM, pH 7.6). The solution was immediately extracted with 1.5 ml toluene and the organic phase was separated, dried over molecular sieve (type 4A, 10–16 mesh, Fisher) and degassed with nitrogen.

ESR Measurements

ESR measurements were carried out at room temperature on solutions contained in a quartz aqueous flat cell, using a Varian E-109 spectrometer operating at 9.5 GHz and employing 100 kHz field modulation. Magnetic field measurements were made with a Radiopan MJ-110 gaussmeter. For microwave frequency measurements, an EiP 200 counter was used. Hyperfine splittings were measured (to 0.1 G) directly from the magnetic field separation.

GC-MS Analysis

To a 0.5 ml toluene extract of PBN adduct (obtained from the Fenton system) was added 20 μl of 20 mMN-methyl-N-trimethylsilyltrifluoro acetamide (MSTFA).[31] The mixture was left to stand for 30 min at room temperature. A 20 μl aliquot of this derivatized mixture was then injected into a gas chromatograph (Hewlett Packard 5890, equipped with a 5970 manual selective detector) and the components were separated on a Hewlett Packard Ultra-1 glass capillary column (internal diameter, 0.2 mm; column length, 12 meters; and coated with 0.33 μM 100% dimethylpolysiloxane) equipped with temperature programming from 70°C to 210°C at a rate of 10°C per minute.

Organic Extraction of PBN Adduct in Reperfusate

Sequential 2 ml aliquots of the coronary effluents during reperfusion were collected. To each fraction was then added 2 ml of cold (4°C) toluene and vortex-mixed for 30 sec., the top emulsion was removed and centrifuged for 2 min in a microfuge (Eppendorff 5415). The top toluene-containing layer was aspirated into glass vials containing molecular sieve (type 4A, 10–16 mesh, Fisher) to remove water and then degassed with a steady stream of nitrogen gas.

Isolated Heart Model

Adult Sprague-Dawley rats, maintained on a standard diet, were used for this study. Anesthesia was induced and maintained with halothane (4 and 1–2%, respectively), after which the left femoral vein was exposed and heparin (150 IU/kg) was administered intravenously. After 1 min, the heart was rapidly excised and placed in perfusion medium. Within 30 sec, the aorta was attached to a stainless-steel cannula, the pulmonary artery was incised to permit adequate coronary drainage, and the heart was perfused normothermically at 37°C by the method of Langendorff[32] at a perfusion pressure equivalent to 12 kPa (90 mmHg). A three-way tap, located immediately above the site of cannulation, allowed the entire perfusate to be diverted away from the heart to produce global, no-flow ischemia. Reperfusion was achieved by repositioning the tap to allow perfusate to return to the heart. The heart and perfusion fluids were kept in temperature-controlled chambers to maintain the myocardial temperature at 37°C.

Perfusion Media

The standard perfusion medium used was Krebs-Henseleit bicarbonate buffer (pH 7.4 when gassed with 95% O_2 and 5% CO_2) in which the calcium content was reduced to 1.8 mM. To this was added glucose (11.1 mM) as an energy source. During

TABLE 1
Composition of the coronary perfusates

Constituent	Krebs-Henseleit Bicarbonate Buffer	St. Thomas' II Cardioplegic Solution
NaCl mM	118.5	110.0
$NaHCO_3$ mM	25.0	10.0
KCl mM	4.8	16.0
KH_2PO_4 mM	1.2	-
$MgSO_4.7\ H_2O$ mM	1.2	-
$MgCl_2.6\ H_2O$ mM	-	16.0
$CaCl_2.2\ H_2O$ mM	1.8	1.2
Total Fe μM	2 ± 1	2 ± 1
pH*	7.4 ± 0.05	7.8 ± 0.01
O_2 content (ml/dl)	2.31	0.92

* The pH of the cardioplegic solution was manually titrated to 7.8 using HCl.

the preparation of all calcium-containing solutions, the solutions were gassed with 5% carbon dioxide to prevent the precipitation of calcium. St. Thomas' II solution was chosen as an example of a crystalloid cardioplegic solution that has been well characterized experimentally and that is in current clinical use. The composition of the coronary perfusates is shown in Table 1. Before use, all perfusion fluids were filtered through cellulose acetate membranes with pore size 5.0 μm.

Perfusion Sequence

Immediately after mounting on the cannula, hearts (n = 8/per group) were perfused aerobically with bicarbonate buffer at 37°C for a 20 min equilibration period. During this period, left ventricular pressure, coronary flow rate and heart rate were continuously recorded. For the cardioprotective studies hearts were then arrested with a 3 min infusion of the coronary vasculature with St. Thomas' II cardioplegic solution and subjected to normothermic global ischemia for 35 min. PBN was added to the cardioplegic solution at a concentration of either 0.4 or 4.0 mM. After the ischemic duration, hearts were reperfused for 15 min in the Langendorff mode with Krebs buffer containing either 0.4 mM PBN or 4.0 mM PBN with the entire coronary effluent collected for the determination of lactate dehydrogenase activity. Throughout the following 15 min of reperfusion hearts were perfused with PBN-free Krebs buffer.

For the radical adduct characterization studies, two protocols were used: (i) hearts perfused with bicarbonate buffer were subjected to 20 min global normothermic ischemia in the presence of bicarbonate buffer followed by 10 min of aerobic reperfusion with 4 mM PBN, and (ii) hearts perfused with bicarbonate buffer were subjected to normothermic global ischemia for 35 min after cardioplegic arrest followed by 10 min of aerobic reperfusion with Krebs buffer containing 4 mM PBN.

Assessment of Myocardial Injury

A saline-filled latex balloon was inserted into the left ventricle during the 20 min equilibration period through the mitral valve in the left atrium, and secured in place with a ligature. The balloon was connected via a rigid saline-filled catheter to a pressure transducer (Deseret Medical, Model 8148) for the measurement of left

ventricular pressure and heart rate. The transducer output was amplified using a universal signal conditioner (Model 20-4615-58, Gould, Cleveland, OH) and recorded on an analog chart recorder (Astromed 9500, Astromed, Providence, RI). The intraventricular balloon was inflated until end-diastolic pressure was 3-6 mmHg. Rate pressure product (RPP) was determined as a product of heart rate per second and developed pressure (mmHg/sec). The coronary flow rate was measured throughout the experiment by collecting the effluent from the right side of the heart into a graduated cylinder and is expressed as ml per min. The entire coronary effluent during the 15 min reperfusion period was collected in a graduated cylinder kept in ice. After thoroughly mixing the contents, an aliquot of the coronary effluent was used for the determination of total lactate dehydrogenase activity.[33]

Ethical Considerations

Animals used in this study received humane care in compliance with "Guiding Principles in the Care and Use of Animals," approved by the Council of the American Physiological Society, and the "Guide for the Care and Use of Laboratory Animals," prepared by the National Academy of Science and published by the National Institutes of Health (NIH publication No. 85-23, revised 1985).

RESULTS

Detection of PBN Adduct in Coronary Effluents During Reperfusion

The ESR spectra of the toluene extract of the coronary effluents collected during aerobic reperfusion are shown in Figure 1a-1e. The signal intensity increased during the early minutes of reperfusion and then declined. The ESR hyperfine parameters were calculated to be ca. $a_N = 14.0 \pm 0.1$ G and $a_H^\beta = 2.0 \pm 0.1$ G. No ESR spectra were detected from the aqueous coronary effluents under otherwise identical conditions. Extraction with toluene was, therefore, absolutely crucial to detect an ESR signal from the PBN-adduct. At lower temperatures ($\approx -100°C$), the ESR signal intensity was increased with a slight loss in spectral resolution. Similar results have previously been reported.[16]

Detection of PBN-OH Adduct Formed in a Fenton System

A rapidly-decaying six-line ESR spectrum ($a_N = 15.6 \pm 0.1$ G, $a_H^\beta = 2.7 \pm 0.1$ G) was obtained following the addition of Fe^{2+} to phosphate buffer containing PBN and H_2O_2 (Figure 2a). Based on the literature data,[26] this adduct was assigned to PBN/·OH, formed from trapping of ·OH by PBN. Upon extraction of the Fenton mixture with toluene followed by degassing with nitrogen, a more persistent ESR spectrum ($a_N = 14.0 \pm 0.1$ G, $a_H^\beta = 2.0 \pm 0.1$ G) was obtained (Figure 2b). The spectral intensity was again increased at lower temperatures (Figure 2c). The close similarity between the ESR spectrum of the Fenton-derived PBN adduct (Figure 2c) and reperfusion-derived PBN adduct suggests that the structure of PBN adduct (Figure 1e) formed in these systems is the same.

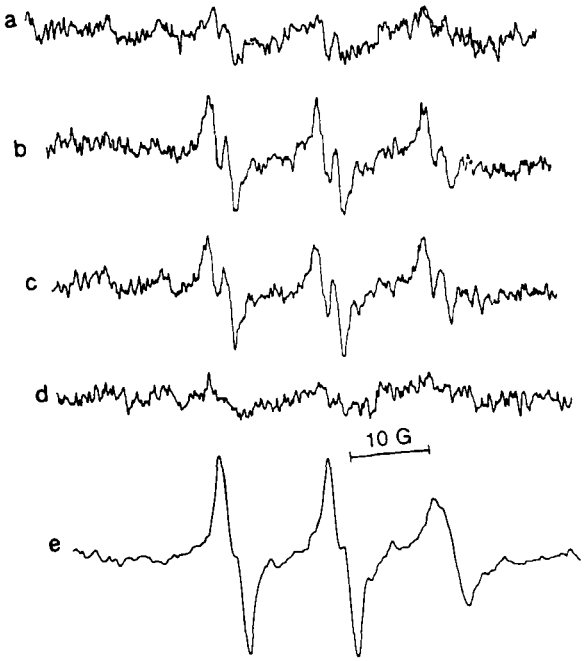

FIGURE 1 ESR spectra of PBN adducts present in coronary effluent extracted into toluene after (a) 1 min, (b) 2 min, (c) 3 min, and (d) 4 min of post-ischemic aerobic reperfusion. (e) The same as (c) except that the spectrum was obtained at −100°C. Spectrometer conditions: scan range, 100 G; modulation amplitude, 1 G; microwave power, 20 mW; scan time, 4 min; time constant, 0.5 sec.

Solvent Effects on ESR Parameters of PBN/·OH

The toluene extracts of the Fenton mixture (*cf.* Figure 2) were bubbled with dry nitrogen until dryness. To the dried white residue was added 0.5 ml of either chloroform, methanol, benzene, chloroform: methanol (2:1) or ethanol. The resulting solution was then degassed with nitrogen and the ESR spectra recorded at room temperature. Table II shows that the ESR hyperfine coupling parameters of PBN/·OH adduct in various solvents. Re-extraction of these solutions with toluene gave the initial ESR spectrum of PBN/·OH in toluene (Figure 2), indicating that this adduct has not undergone decomposition during the extraction process (data not shown).

Mass Spectral Analysis of PBN Adduct

Because of its instability, the PBN/·OH adduct decomposed in the GC column preventing direct analysis by GC-MS. Therefore, it was converted into thermally stable and volatile trimethylsilyl derivatives, which are amenable for analysis by GC-MS.

The toluene extract of the Fenton mixture containing PBN was trimethylsilylated using MSTFA (N-methyl-N-trimethylsilyltrifluoro acetamide). This mixture containing PBN, derivatized products, and MSTFA was then separated by GC (Figure 3).

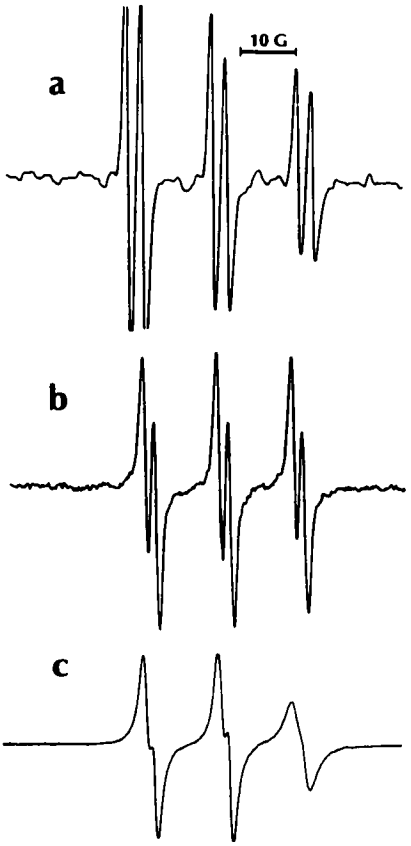

FIGURE 2 ESR spectra of authentic PBN adduct formed in the Fenton system, (a) in phosphate buffer at room temperature, (b) in toluene extracts of (a) at room temperature, and (c) same as (b) but spectrum recorded at -100°C. Spectrometer conditions: scan range, 100 G; modulation amplitude, 1.0 G; microwave power, 5 mW; scan time, 4 min; time constant, 0.25 sec. Note that the spectrometer gain was different in (a), (b), and (c).

Mass spectral analysis of the GC peaks corresponding to 7.1 min in Figure 3 revealed the molecular ion at m/z 177 for PBN.

Figure 4a shows mass spectra of the peak at 7.8 min using PBN. Figure 4a is tentatively attributed to fragments formed from the PBN-OSi$(CH_3)_3$ (formed by trimethylsilation of PBN-·OH). The molecular ion peak at m/z 266 (M^+) was not detected. The peak at m/z 265 (M-1) results from the loss of a proton and the peak at m/z 250 (M-16) is assigned to the loss of a proton and the methyl cation. The peak at m/z 73 is characteristic of the fragment $(CH_3)_3Si^+$ and the peak at m/z 193 (M-73) probably arises from the loss of $(CH_3)_3Si^+$ from the parent ion. Figure 4b shows mass spectra of the peak obtained between 9 and 10 min. Figure 4b is tentatively attributed to fragments formed by *bis*-trimethylsilation of the hydroxylamine of PBN/·OH adduct. The molecular ion peak at m/z 339 (M^+) was not detected. The peak at 338 (M-1) is assigned to the loss of a proton from the molecular ion and the peak at m/z 282 (M-57) probably results from the loss of the *tert*-butyl group.

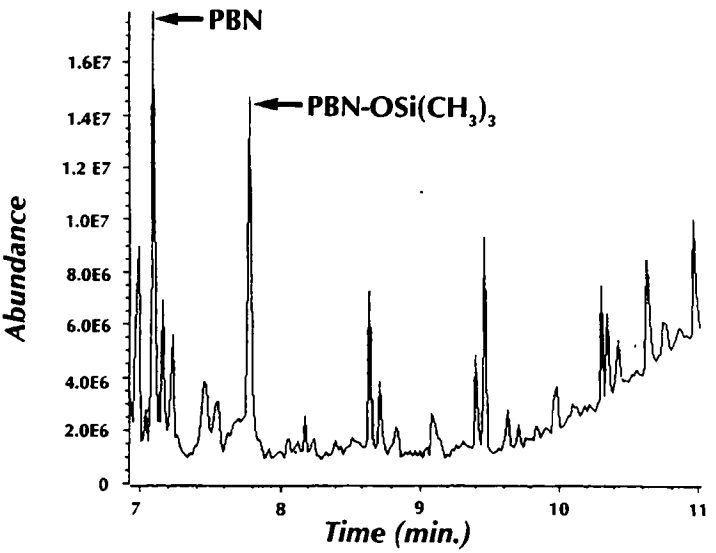

FIGURE 3 A total-ion chromatogram obtained from the toluene extract of an authentic PBN/·OH adduct formed in a Fenton system containing phosphate buffer (pH 7.5, 20 mM), PBN (40 mM), H_2O_2 (6.6 mM), and Fe^{2+} (3.6 mM).

The characteristic fragment $(CH_3)_3Si^+$ at m/z 73 was also evident. The structures of the remaining peaks between 7 and 11 min could not be assigned confidently at this time.

Effect of PBN on Reperfusion-Induced Myocardial Injury

We have previously investigated the dose-dependent effect of PBN on cardiac function, and determined that PBN is cardiotoxic at concentrations in excess of 5 mM.[34] Similar results have also been obtained in previous studies.[20,35,36] Accordingly, we have used a concentration of 0.4 and 4 mM PBN in studies designed to assess the effect of PBN on post-ischemic contractile recovery. To determine whether PBN could confer any protective effect, hearts were subjected to 35 min of global ischemia (37°C) in St. Thomas' II cardioplegic solution followed by 30 min of aerobic reperfusion. PBN was present in the cardioplegic solution (Figure 5) or during reperfusion (Figuer 6). PBN did not exert any significant effect on the recovery of developed pressure, rate pressure product, or post-ischemic leakage of lactate dehydrogenase (Figure 7) in comparison with PBN-free control hearts.

DISCUSSION

This study demonstrates that in the isolated bicarbonate-buffer perfused heart model of myocardial ischemia and reperfusion, the PBN adduct generated during reperfusion of the ischemic rat myocardium is derived predominantly from the hydroxyl radical as determined by ESR spectroscopy. The technique of GC-MS has been used, for the first time, in conjunction with ESR spectroscopy to definitively characterize

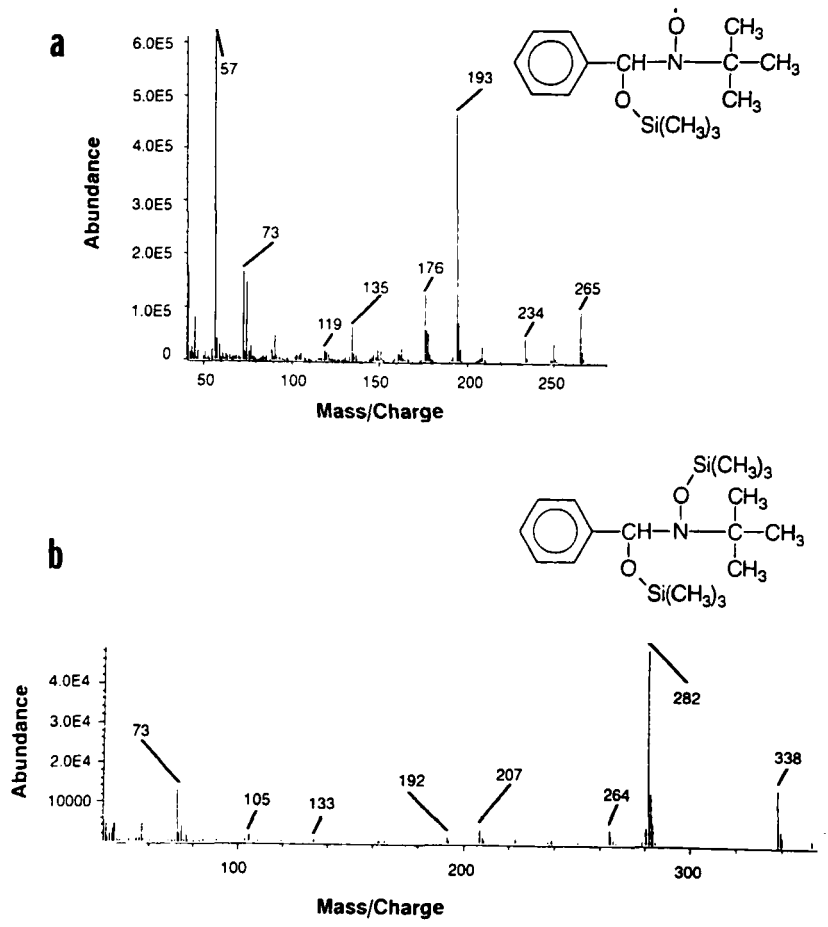

FIGURE 4 Mass spectra of (a) mono-trimethylsilylated and (b) *bis*-trimethylsilylated hydroxyl adduct of PBN. The PBN/·OH adduct was obtained from a Fenton system, as described under Materials and Methods.

the structure of an authentic PBN-hydroxyl radical spin adduct formed in a model system. PBN however failed to confer protection against post-ischemic contractile dysfunction and enzyme leakage.

PBN as the Spin Trap of Choice for Myocardial Ischemia-Reperfusion Studies

We previously assessed the effect of a wide variety of structurally related nitrone and nitroso spin traps upon aerobic function in the isolated perfused rat heart.[34] Our study demonstrated that the spin traps exerted a dose-dependent effect of progressively depressing aerobic cardiac function. In our model all spin traps studied also exerted a potent vasodilatory effect at high concentrations. For hearts perfused with PBN there was no significant increase in coronary flow rate or depression of rate pressure product at a concentration of 4 mM. In addition, the highly lipophilic nature

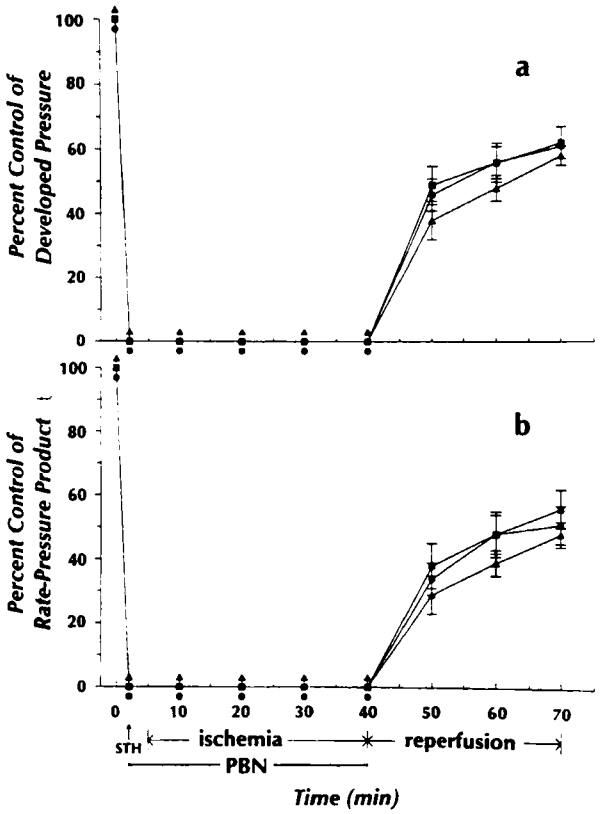

FIGURE 5 Effect of PBN on post-ischemic recovery of developed pressure and rate pressure product. PBN was present in the cardioplegic solution. Closed triangles represent data from PBN-free hearts. Closed circles represent data from hearts infused with 0.4 mM PBN. Closed squares represent data from hearts infused with 4.0 mM PBN. STH - St. Thomas' II cardioplegic solution.

of PBN, as determined from its octanol/water partition coefficient, in comparison with other spin traps, permits its access into the intracellular compartment. Thus PBN has the ability to trap radicals generated in both the extracellular and intracellular compartments without the toxic effects associated with other lipophilic spin traps such as 2-methyl-2-nitroso propane and nitrosobenzene.[34] Based upon these properties we selected PBN at a concentration of 4 mM for use in our ischemia-reperfusion studies.

Trapping of Free Radicals with PBN during Myocardial Ischemia and Reperfusion

Highly reactive and short-lived free radicals can react with spin traps to form a stable spin adduct. The resulting spin adduct can be detected and identified by ESR spectroscopy. In our model, the radical adduct exits the coronary vasculature via the right heart in an aqueous bicarbonate-buffered media. Under these conditions, the radical adducted to the spin trap PBN rapidly degrades to products that are undetectable by ESR-spin trapping.

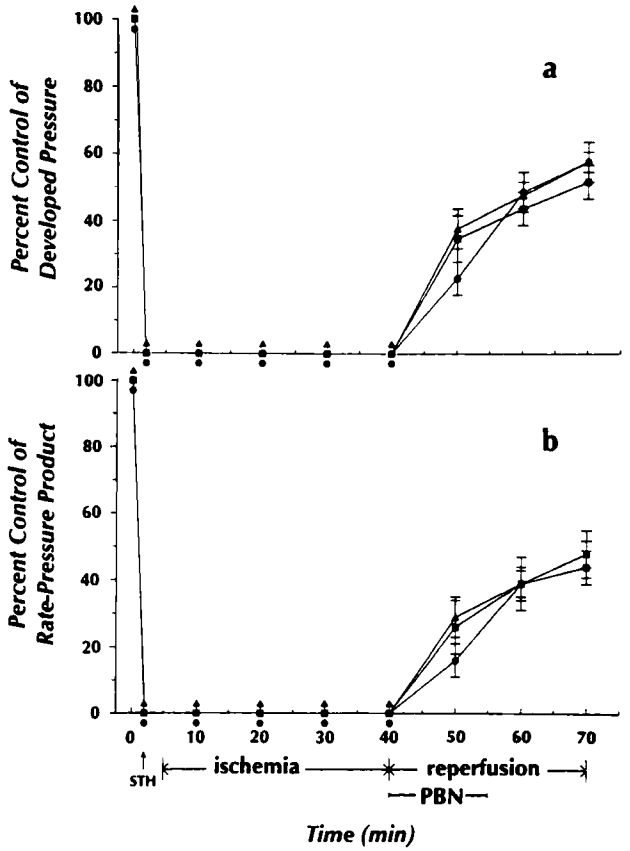

FIGURE 6 Effect of PBN on post-ischemic recovery of developed pressure and rate pressure product. PBN was present during the reperfusion phase only. Closed triangles represent data from PBN-free control hearts. Closed circles represent data from hearts perfused with 0.4 mM PBN. Closed squares represent data from hearts reperfused with 4.0 mM PBN. STH - St. Thomas' II cardioplegic solution.

Kotake and Janzen [37] have recently reported that the lifetime of PBN/·OH adduct is dependent on the pH of the media; the halflife of PBN/·OH adduct at pHs 6 and 8 were 90 and 10 s, respectively. The PBN/·OH adduct was found to decay via the unimolecular dissociation mechanism, forming the benzaldehyde and *tert*-butylhydroaminoxyl as major products.[37]

The intracellular pH in the myocardial tissue during ischemia reportedly falls to 6.4; therefore, the PBN/·OH should be relatively more persistent under ischemic conditions as opposed to reperfusion. In aqueous solution, the decomposition of PBN/·OH is facilitated due to the ease with which the acidic β-proton is dissociated. In contrast, the unimolecular decomposition of PBN/·OH becomes energetically difficult in non-protic solvents such as toluene and benzene. This may well account for the increased stability of PBN/·OH adduct in these solvents.

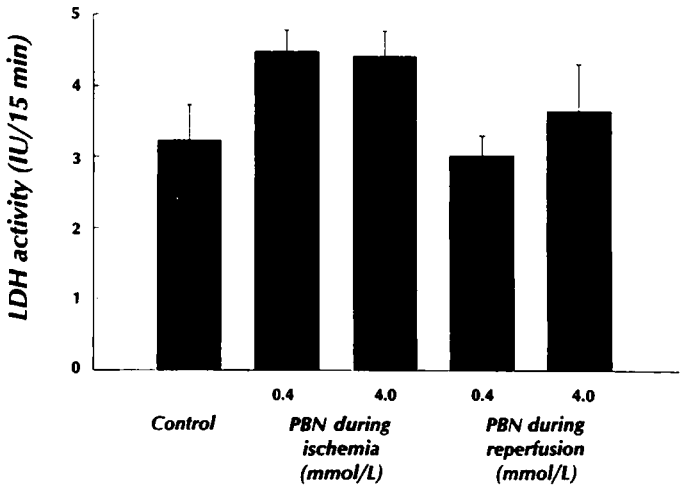

FIGURE 7 Effect of PBN on lactate dehydrogenase release during 15 min reperfusion. Each bar represents the mean ± standard error of the mean from six experiments.

TABLE 2
Solvent effect on the hyperfine coupling of PBN/·OH adduct

Solvent system	a_N (G)	a_H^β (G)
Phosphate Buffer	15.6 ± 0.1	2.7 ± 0.1
Ethanol	14.9 ± 0.1	2.8 ± 0.1
Methanol	15.0 ± 0.1	3.0 ± 0.1
Chloroform/Methanol	15.0 ± 0.1	3.0 ± 0.1
Chloroform	14.8 ± 0.1	2.7 ± 0.1
Toluene	14.0 ± 0.1	2.0 ± 0.1
Benzene	14.2 ± 0.1	2.1 ± 0.1

ESR Parameters of PBN Adducts Formed during Myocardial Ischemia and Reperfusion

Because of their instability in aqueous solution, PBN adducts have been generally extracted in organic solvents prior to ESR analysis. A number of investigators[14-16,20,21,41-44] have used this extraction procedure in various models of myocardial ischemia and reperfusion. Table III shows the list of ESR parameters of PBN adducts and the tentative structures assigned for these adducts. Closer examination of Table III reveals that with the exception of one study reported by us, no other investigator has previously proposed the PBN/·OH structure to PBN adducts formed during myocardial ischemia and reperfusion. In all of these studies, the PBN adducts were presumed to be formed from trapping of either a carbon-centered lipid radical or an oxygen-centered lipid alkoxyl, or a mixture of both. A major rationale for this interpretation may be that the hyperfine coupling values of nitrogen and β-hydrogen for PBN-oxygen centered adducts (a_N = 14–14.5 G; a_H = 2–2.5 G) reported in the literature are typically smaller than those corresponding to PBN-carbon-centered adducts (a_N = 15.5–16 G; a_H = 3–3.5 G).[23] However, very little is known about the

TABLE 3
ESR parameters of PBN Adducts formed in various heart models of myocardial ischemia and reperfusion

Heart Model	Time Course of Product of PBN Adduct		Solvent Used in Extraction	ESR Parameters (G) a_N	a_H^β	Nature of Trapped Radical (R·)	Ref
Open-Chest Dog	Aerobic Reperfusion	3 min	Chloroform	14.75	2.69	Mixture of alkoxy and alkyl	20
		30 min	Chloroform	15.00	2.78	radicals	
Open-Chest Dog	Occlusion		Chloroform	14.63	2.35	Unknown (major)	21
				14.90	3.20	Carbon-centered (minor)	
	Aerobic Reperfusion	4 min	Chloroform	14.8	2.5	Mixture (unknown adducts)	21
		3 h	Chloroform	14.9	3.2	Carbon-centered (major)	
Open-Chest Dog	Aerobic Reperfusion		Chloroform	15.31	2.94	Alkyl (major)	30
					5.10	Unknown (minor)	
		5 min	Chloroform	15.37	2.94	Alkyl (major)	
				-	4.72	Unknown (minor)	
		20 min	Chloroform	15.30	2.89	(major)	
				-	4.72	(minor)	
Open-Chest Dog	Regional Ischemia		Chloroform: Methanol (2:1)	15.20	3.85	Lipid	14
				13.60	1.56	Lipid alkoxy	
Conscious Dog	Aerobic Reperfusion	3 min	Chloroform	15.20	6.0	Phospholipid	57
				14.60	3.0	Alkyl	
Swine	Aerobic Reperfusion	3 min	Chloroform: Methanol (2:1)	14.75	2.75.30	Alkyl or alkoxyl	42
	PBN Adduct formed *ex vivo*	20 min	Toluene	13.5–13.75	2.00–2.25	Alkoxyl	
Isolated Rat Heart	Aerobic Reperfusion		Toluene	13.60	1.56	Alkoxyl or carbon-centered	15, 16
				13.63	1.88	Hydroxyl radical	41
				13.60	1.56	Not assigned	43
PTC Angioplasty in Humans	Reperfusion		Toluene	13.75	1.88	Carbon-centered	44

ESR parameters of PBN-oxygen centered adducts in different solvents.[23,45] The present study clearly indicates that both the nitrogen and β-hydrogen values of PBN/·OH adduct range from 15 and 3 G in ethanol to 14 and 2 G in toluene. From comparing the ESR parameters between Tables II and III, we infer that most of the PBN adducts reported in Table III could be assigned to PBN/·OH. Given such solvent-dependent variations in ESR parameters of PBN/·OH adduct, it is very likely that some of the PBN adducts derived in earlier studies may be PBN/·OH. A comparison of Tables II and III clearly bears this out.

However, it must be pointed out that most of the studies cited in Table III were performed in blood-perfused models. In buffer-perfused models, PBN adduct formed in coronary effluents could be extracted into toluence within seconds. On the contrary, in blood-perfused models, the whole-blood samples require centrifugation to obtain plasma fractions that are then extracted into solvents. The processing of blood samples involves a delay time of several minutes. Therefore, it can be argued that PBN/·OH would not survive the delay time for sample processing. The actual half-life of PBN/·OH in the blood is, however, not known.

It is conceivable that the PBN-lipid alkoxyl and PBN/·OH adducts have similar ESR parameters in various solvents.[45] The PBN-lipid alkoxyl adduct, because it is more lipophilic, should be associated with the myocardial tissue and is unlikely to be released into the perfusate. The present study shows that PBN/·OH adduct is also fairly nonpolar. It is clear that other strategies to differentiate between the PBN/·OH and PBN-lipid alkoxyl adduct should be undertaken, such as isotopic substitution and mass spectrometry.

Isotopic substitution in ESR is frequently used to distinguish carbon-centered from oxygen-centered radical adducts of PBN.[47,48] Substitution of [$^{16}O_2$] oxygen (nuclear spin = 0) with [$^{17}O_2$] oxygen (nuclear spin = 5/2) should give additional hyperfine structures for oxygen-centered adducts. Considerable differences exist for ^{17}O-coupling value in PBN/·OH ($a^{17}O = 3.36$ G) and PBN-alkoxyl ($a^{17}O = 5.05$ G) adducts. Although in principle, this technique is feasible under *in vitro* conditions, in practice, it may be nearly impossible to adopt this technique *in vivo* using animals because of the enormous cost involved.

GC-MS of Derivatized PBN Adducts

The use of mass spectrometry is clearly the most unambiguous approach to elucidate the structures of small molecular weight PBN adducts. Abe *et al.*[49] have previously analyzed the structures of trimethylsilyl derivatives of adducts of PBN and POBN. Of interest was the detection of the bis-trimethylsilylated hydroxyl adduct of POBN. From the mass spectral data, the POBN/·OH adduct was found to be trimethylsilylated both on the hydroxyl group and on the oxygen atom of the aminoxyl group. Janzen *et al.*,[31] using deuterated PBN, have recently analyzed in detail the fragmentation pattern and the mechanisms of formation of various fragments of trimethylsilyl derivative of PBN/·CH_3 adduct. The oxygen atom of the aminoxyl group was determined to be the site of trimethylsilation.

It should be pointed out that a clear distinction between the trimethylsilyl derivative of the aminoxyl oxygen and the hydroxyl oxygen is difficult, based on the mass spectral fragmentation pattern alone. However, trimethylsilylation of the aminoxyl oxygen should make the adduct diamagnetic and, thus, cause the disappearance of the ESR signal. Addition of MSTFA to the PBN/·OH adduct in toluene did not result in the total loss of ESR signal (data not shown). This further confirms our

assignment in Figure 4a. Although, formation of *bis*-trimethyl derivative of PBN/·OH adduct should also cause a decrease in the ESR signal, the mass spectrum (Figure 4b) shows that the *bis*-trimethylsilylated derivative is not formed in great abundance. As mentioned earlier, the bis-trimethylsilated derivative is presumably formed from the corresponding hydroxylamine of PBN/·OH adduct. In blood-perfused systems, where formation of PBN-alkoxyl and peroxyl adducts is more likely, one has to also consider the reagent-induced cleavage of the oxygen-oxygen bond.

The relative instability and heat-sensitive nature of PBN-lipid or PBN-lipid alkoxyl adducts have so far precluded the use of GC-MS to identify these structures.[31, 50] More recently, Albro *et al.*[51] had determined the structure of reduced α-pyridyl-*tert*-N-butyl nitrone (POBN)-linoleic acid carbon-centered adduct using electron impact mass spectrometry. The authors[51] had found that the trimethylsilylated product decomposed at the temperatures employed.

PBN Adduct Formation in Other Organs during Ischemia and Reperfusion

Using PBN as spin trap, Pincemail *et al.*[52] reported the first direct evidence of *in vivo* free radical generation during ischemia/reperfusion in rabbit kidneys. ESR spectra were detected in the chloroform extracts of blood samples. Based on the hyperfine coupling parameters (a_N = 14.75–15.0 G; a_H^β = 2.5–3 G), the authors interpreted their ESR spectra as being a mixture of PBN-carbon centered and PBN-alkoxyl or peroxyl adducts. Again, the solvent-dependency of ESR parameters of the PBN/·OH adduct was not taken into consideration. The hyperfine parameters reported in that study are well within the range reported for PBN/·OH (Table III) in chloroform.

In a recent study, Connor *et al.*[53] have used PBN to detect radicals formed as a consequence of reperfusion during orthotopic liver transplantation. Results from this study also showed that PBN adduct formation was related to graft failure. The reported hyperfine parameters of PBN adduct formation were similar to those obtained by Bolli and coworkers.[20,21,30] Of interest is the detection of a PBN adduct with large β-hydrogen (> 4 G) coupling constant, similar to that reported in canine model during reperfusion. A large β-hydrogen coupling constant is usually typical of a PBN-carbon dioxide anion adduct.[53]

Protective Effect of PBN on Ischemic-Reperfused Myocardium

Conflicting views exist in the literature on the cardioprotective effect of PBN.[36,38–40] By far the most detailed investigation on this subject came from pioneering studies by Bolli and coworkers.[20,21,30] These investigators reported a causal relationship between production of PBN adducts and the post-ischemic functional recovery of stunned myocardium afforded by PBN in canine models. However, another report on the lack of cardioprotection by PBN in a canine model has recently appeared.[38] Hearse and Tosaki[39] have previously shown that PBN inhibits the development of arrhythmias in a rat model; however, the concentrations of PBN used in that study were too low to be acting as a radical scavenger. It is plausible that some hitherto unknown pharmacologic property of PBN[34,54] could have been responsible for the observed antiarrhythmic effect.

Several lipophilic hydroxyl radical scavengers inhibited formation of PBN adducts.[30] By virtue of its amphophilic nature, PBN could trap radicals formed in both intra- and extra-cellular compartments. Consistent with this, several studies

have reported only partial inhibition of PBN adducts in the presence of catalase, superoxide dismutase and water-soluble iron chelators.[15]

Cova *et al.*[29] have recently investigated the intracellular distribution of PBN in rat myocardium. The authors have found that a large fraction of PBN accumulates in the cytosolic compartment as compared with the nuclear and mitochondrial compartments in rat hearts perfused for 60 min with PBN. PBN levels in the sarcolemma and sarcoplasmic reticulum were below the detection limit. Recently, Bradamante *et al.*[36] have reported that PBN did not confer any protective effect against myocardial damage in isolated rat hearts subjected to a cycle of global *no flow* ischemia and aerobic reperfusion. However, PBN showed a marked protective effect in rat hearts subjected to *low-flow* ischemia. It was rationalized that the continuous loading of the myocardial tissue with PBN throughout low-flow ischemia is crucial for observing the protective effect. It is also conceivable that the toxic by-products of PBN/·OH, such as benzaldehyde, are being washed out from the cells during low-flow ischemia. This may also explain the positive and negative data on myocardial protection obtained using PBN in different animal models.[20,21,30,38,55,56]

PBN failed to exert a cardioprotective role in our model of ischemia and reperfusion. There are a number of possibilities that may explain the absence of any protective effect. First, PBN may not have gained access to the site where the hydroxyl radical is generated. Failure to deliver PBN to this locus of radical production would exclude any possible protective effect for this spin trap. Second, the trapping efficiency between a radical species and the spin trap is poor, due to lower concentrations of the spin trap employed. Thus PBN may trap only a small fraction of the total amount of the hydroxyl radical produced. Third, in the aqueous environment of the myocardial cell, the PBN/·OH adduct formed is unstable and spontaneously decomposes into benzaldehyde and the *tert*-butylhydroaminoxyl radical. These toxic breakdown products are more stable than the parent molecule and may diffuse from their site of production to other areas resulting in an extension of tissue injury. Fourth, the pharmacological properties of PBN (i.e., calcium channel inhibition, etc.) may play a role in myocardial protection. Finally, we must acknowledge the possibility that the hydroxyl radical contributes only minimally to myocardial cell injury in this model.

SUMMARY AND CONCLUSIONS

1. PBN adduct generated during reperfusion in our model is derived predominantly from the PBN·OH adduct.

2. The ESR parameters of the PBN/·OH adduct are extremely sensitive to the solvent polarity. Within experimental error, the ESR parameters of PBN/·OH adduct in chloroform, chloroform/methanol, and toluene closely match the ESR parameters of PBN adducts obtained during myocardial reperfusion in other animal models. This is an important consideration that should be borne in mind before making structural assignments for PBN adducts formed in the myocardium. Consistent with ESR interpretations, Sun *et al.* had recently provided evidence for ·OH production using aromatic hydroxylation of phenylalanine in the stunned myocardium of dogs.[57]

3. The GC-MS technique has been used, for the first time, in conjunction with ESR to characterize the structure of a PBN-derived spin adduct formed in a Fenton system.

4. A detailed analysis of PBN adducts obtained in various heart models and their solvent dependency as reported in the present study may be useful in structural elucidation of PBN adducts formed in other organs.

5. In the present model, PBN, failed to improve contractile recovery and reduce enzyme leakage during reperfusion of ischemic myocardium. However, this does not necessarily imply that free radicals do not contribute to the ischemia/reperfusion induced injury to myocardium. As previously discussed, a proper choice of heart model in which the lipophilic spin trap is continuously loaded into the target site of myocardial injury may be critically important for ablation of the free radical-mediated injury. Clearly, additional experiments using PBN in blood-perfused rodent models are warranted.

Acknowledgements

This research was supported by NIH grants HL-45048, HL-43040 and RR-01008.

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