Postischemic Free Radical Production in the Venous Blood of the Regionally Ischemic Swine Heart

Effect of Deferoxamine

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Background. We tested the hypothesis that secondarily produced free radicals can be detected in venous coronary effluent without the need for direct exposure of postischemic tissue to the spin trapping agent \( \alpha \)-phenyl-tert-butyl nitronone (PBN).

Methods and Results. The left anterior descending coronary artery (LAD) of pigs was ligated for 15, 30, 40, or 60 minutes, and the tissue was subsequently reperfused for 60 minutes. Venous effluent (6.5 ml) from the risk area was withdrawn sequentially at 1.5-minute intervals during reperfusion. The effluent blood was immediately infused (4.5 ml/min) with an isotonic saline solution containing 120 mM PBN. Preischemic control effluent samples were collected in an identical fashion. Plasma from each sample was extracted in organic solvent and subsequently analyzed by electron spin resonance (ESR) spectroscopy. Another group of pigs received an infusion of the metal chelator deferoxamine mesylate (25 mg/kg/hr) into the right atrium starting 1 hour before the 40-minute ligation and continuing throughout ligation and reperfusion. We were able to demonstrate the postischemic production of ESR signals for PBN adducts from untreated hearts having spectral characteristics similar to an alkoxyl adduct (PBN-RO; hyperfine splitting constants for \( \beta \)-hydrogen \([\alpha_H]=2.0-2.25 \) G; nitrogen \([\alpha_N]=13.5-13.75 \) G). The reperfusion time course of PBN adduct production had a unique pattern: 1) multiple low-level bursts during the initial 15 minutes of reperfusion, and 2) a prominent PBN adduct signal during a relatively late time (20-25 minutes) of reperfusion. Total postischemic PBN adduct production rose with increasing duration (15-60 minutes) of ischemia and was associated with a progressive elevation of total lactate dehydrogenase in the effluent. Infusion of deferoxamine markedly diminished PBN adduct production as well as total release of lactate dehydrogenase.

Conclusions. These data suggest the potential feasibility of using an ex vivo ESR spin trapping technique in blood-perfused models of cardiovascular injury and that chelatable free iron contributes to the production of alkoxyl radicals. (Circulation 1991;84:2079-2090)

The participation of free radicals in the pathobiology of myocardial reperfusion injury has received considerable experimental support in recent years.1-6 Electron spin resonance (ESR) spin trapping studies have successfully demonstrated the production of endogenous free radicals in ischemic and postischemic hearts. Several studies using the spin traps 5,5-dimethyl-1-pyrroline N-oxide (DMPO) and 3,3,5,5-tetramethyl-1-pyrrrole-1-oxide (MPO) have reported bursts of primary oxygen-centered (superoxide anion, hydroxyl) free radical adducts during the initial minutes of postischemic reperfusion.5-9 Coincidental with these bursts of production was the formation of products of lipid peroxidation, such as conjugated dienes,10 lipid hydroperoxides,11 malondialdehyde,8,11 and lipid free radicals (alkyl, alkoxyl, and peroxy). Recent reports using spin trapping techniques1,3,12 have demonstrated formation of secondarily produced lipid free radicals during postischemic reperfusion. In most instances, these were observed over a time frame consistent with the production of primary oxygen-centered free radicals. With few exceptions,12,13 es-
sentially all ESR spin trapping studies conducted in cardiovascular models have involved direct exposure of the tissue to the spin trapping agent. The spin trapping agents most commonly used in these studies were nitrone compounds, some of which may display concentration-dependent toxic1,9,14 or nontoxic1,15,16 effects in cardiovascular systems. Thus, it is quite possible that certain spin trapping agents possess characteristics that could influence the severity of free-radical-mediated injury. To circumvent this potential problem, we have used an approach in which a high concentration of the spin trap α-phenyl-tert-butyl-nitrone (PBN) was introduced to venous effluent rather than to tissue. This technique was performed in the regionally ischemic, reperfused swine heart model, which has minimal collateral flow, allowing us to sample venous effluent selectively from the area at risk. This approach has permitted us to 1) detect and partially identify PBN adducts as secondary alkoxyl radicals, 2) establish time courses of postischemic PBN adduct production, 3) demonstrate a close association between severity of ischemia and total PBN adduct production, and 4) implicate a role for chelatable iron in the cascade leading to formation of alkoxyl radicals.

Methods

Experiments were guided by the principles for the care and use of laboratory animals as recommended by the US Department of Health and Human Services and approved by The George Washington University Animal Care and Use Committee.

Surgical Preparation

Postischemic free radical production was evaluated in 34 pigs (Archer Farms, Belcamp, Md.) with a mean weight of 41 kg. Pigs were anesthetized with halothane (5% by mask), and the trachea was intubated after administration of vecuronium (10 mg i.v.). Ventilation with an Engström ventilator was adjusted to maintain the PCO2 between 36 and 40 mm Hg, and the PO2 was kept between 90 and 110 mm Hg. After a loading dose of 50 μg/kg fentanyl, anesthesia was maintained with 0.5% halothane and fentanyl (50 μg/kg/hr). Through a median sternotomy, the left anterior descending coronary artery (LAD) was approached and isolated with a vessel loop two thirds down from the root of the aorta. In selective studies, the LAD was cannulated with a 24-g angiocatheter (Insyte-W ¾ in., Deseret Medical Inc.) just below the vessel loop for direct spin trap infusion. For effluent venous blood sampling, the left anterior interventricular vein was cannulated with a 24-g angiocatheter, making sure that the tip of the catheter was distal to the LAD ligature. A transducer-tipped catheter (Millar) was placed in the left ventricular cavity via the left atrium to measure left ventricular end-diastolic pressure (LVEDP). The right carotid artery was cannulated for pressure measurements in the ascending aorta. A pulmonary artery catheter was advanced into the pulmonary artery from the right external jugular vein for the measurement of cardiac output, pulmonary artery pressure, central venous pressure, and temperature. All pressures, except the LVEDP, were measured with fluid-filled transducers (Gould Transol P23XL) calibrated with a mercury manometer and recorded on paper (Gould 2800S). The temperature of the pigs was maintained between 37 and 38°C by heat lamps and warming blankets. Lactated Ringer's solution was infused to keep the LVEDP at 8±2 mm Hg.

Experimental Model and ESR Spin Trapping

Our initial assessments of secondary free radical production in the postischemic swine heart were performed by the direct spin trap infusion technique and sample extraction method described by Bolli et al1 in studies with the stunned canine myocardium. All spin trap (Aldrich Chemicals) preparations and experiments were performed in room darkness to prevent photolytic degradation of the trap. Isosmotic PBN/saline solutions were prepared (30 minutes stirring, N2 gassed, 24°C) just before use, and concentrations were verified by UV spectroscopy (ε=17,087 M⁻¹·cm⁻¹ at 295 nm).14 After hemodynamic parameters were stable for 20 minutes, the LAD was ligated for 15 minutes, and the tissue was subsequently reperfused for 60 minutes. Hemodynamic data were also collected during ligation (5 and 15 minutes) and at 5, 25, and 60 minutes of reperfusion. Direct trapping was performed by infusing a 60-mM PBN solution (5.8 ml/min; final venous blood level at 5–8 mM) into the LAD during the presischemic control period and during the initial 15 minutes of reperfusion. Venous blood samples from the interventricular vein draining the risk area were withdrawn (Harvard pump; 4.3–4.5 ml/min) sequentially at 1.5-minute intervals during various times of reperfusion. Samples were collected in siliconized glass tubes to which beef heparin (100 units/tube) was added and were then centrifuged at 425g for 5 minutes at 24°C. Plasma samples were extracted with chloroform:methanol (2:1) before ESR spectroscopy. Although encouraging results were obtained, these studies were discontinued because of the high degree of ventricular irritability experienced during LAD cannulation.

The relative intolerance of swine hearts to LAD cannulation prompted our use of an ex vivo spin trapping technique. Following the stabilization period, the LAD was ligated for 15-, 30-, 40-, or 60-minute periods and the tissue was reperfused for 60 minutes. Venous blood samples were withdrawn during various times of reperfusion as described above. Immediately as the blood exited the vein, a second pump was used to infuse a 120-mM PBN solution into the venous blood (final 50 mM) for 1 minute at a rate of 4.5 ml/min. This was accomplished by inserting a 25-gauge needle into the rubber hub of a T-connector (Abbot Hospitals Co., Extension Set with T, #4612) that was inserted into the angiocatheter previously placed into the left anterior interventricular vein (dead space volume, 50–70 μl; efflu-
ent/trap mixing time, ~0.7 seconds). Preischemic control effluent samples were obtained according to the above protocol. Samples were collected as described and effluent plasma was extracted with toluene before ESR spectroscopy. No red blood cell hemolysis was detected because of the spin trapping or sample collection protocols used.

In parallel ex vivo spin trapping studies, hearts were treated with the metal chelator deferoxamine (Sigma Chemical) 60 minutes before ischemia, as well as during the 40-minute ligation and 60-minute reperfusion periods. Deferoxamine was infused into the right atrium at 25 mg/kg/hr. This dose was selected because it was the highest concentration that caused no overt effects on cardiac hemodynamics or blood components in this animal model. Venous effluent collection and spin trapping were performed as described above.

Control Experiments to Assess the ESR Spin Trapping Technique

We examined the possibility that the PBN adducts detected in our ex vivo trapping studies were formed predominantly in collection tubes over time, rather than in the circulating myocardial venous effluent. Since deferoxamine has been reported to have effective antiradical properties, either this agent (0.5 mM final) or an equivalent volume of saline was added to one of two collection tubes before the parallel withdrawal of duplicate venous blood samples (6.5 ml each) at various times of reperfusion. The PBN infusion was performed as described, in 40 minutes ischemic, 60 minutes reperfused swine hearts. The above protocol permitted us to compare the effect of deferoxamine on adduct formation in duplicate effluent samples from the same heart.

In other studies, we investigated the possibility that PBN adducts similar to those observed after ex vivo trapping in the postischemic swine heart might be generated from analogues of lipid hydroperoxides (LOOH). Spin trapping was performed at 37°C in a 3-mM aqueous reaction mixture containing (final): 50 mM PBN, 8.3 μM FeSO₄·7H₂O, and 0.13% (vol/vol) cumene hydroperoxide (Cu-OOH; Sigma). An aliquot (0.3 ml) of the aqueous sample was analyzed by ESR spectroscopy and the remaining reaction mixture was extracted with either toluene or chloroform: methanol before ESR analysis.

A recent study has suggested that the hydroxyl adduct of PBN (PBN-OH) may be the dominant adduct of spectra obtained after toluene extraction of postischemic coronary effluent samples. In order to assess its possible contribution in our studies, ESR spectroscopy was performed on toluene extracts of an aqueous Fenton system mixture (3.0 mM H₂O₂, +0.3 mM Fe²⁺ and 50 mM PBN); however, the hyperfine splitting constants (β-hydrogen[αH]=2.5 G; nitrogen[αN]=14.5 G) of the PBN-OH adduct obtained under these conditions were not consistent with the spectral parameters observed in the swine heart studies. Furthermore, addition of swine plasma (>100 μl) to the aqueous Fenton system mixture (1 ml final) completely abolished the PBN-OH signal, presumably because of the presence of endogenous antioxidants. Interestingly, similar studies performed with an aqueous mixture of Cu-OOH plus Fe²⁺ (PBN-RO⁻ generation) demonstrated 20–30 times less sensitivity to the antioxidant effects of plasma. Thus, it is unlikely that PBN-OH contributes significantly to the spectra obtained in toluene-extracted samples from the blood-perfused heart.

Sample Extraction, ESR Spectroscopy, and Adduct Quantification

Plasma/spin-trap samples (6 ml) obtained following ex vivo spin trapping were extracted (45 minutes, 24°C) with nitrogen-gassed, high-performance liquid chromatography (HPLC)-grade toluene (1 vol sample:2 vol toluene) and then centrifuged (24°C) at 170g for 5 minutes. The extracted volumes were reduced under nitrogen gas (24°C) and reconstituted in 0.3 ml toluene before ESR spectroscopy. Extraction efficiency for the spin trap was 85–90% as determined by UV spectroscopy, and no significant ESR signal artifact was noted with toluene extraction. In initial studies involving direct spin trap infusion of the LAD, plasma from effluent samples was extracted (1 vol sample:3 vol solvent) with chloroform:methanol (2:1) and reduced under N₂ gas before ESR spectroscopy. ESR was performed at 24°C with a Bruker ER 100 series, X-band spectrometer using a magnetic field modulation frequency of 100 kHz. ESR spectra were obtained with a quartz flat cell (60×10×0.25 mm). Microwave power was 10 mW to avoid saturation, and scans were performed with a modulation amplitude of 1 G or lower. Unless otherwise stated, the gain was 1.25×10⁶ and sweep time was 200 seconds. Hyperfine coupling constants were measured from the field scan using 10-G marker signals for calibration, and measurements of signal intensity were performed as described.

In addition to signal intensity measurements, values shown for PBN adduct time courses were also quantified with an EPR Data Acquisition System (Version 2.41; Scientific Software Services, Bloomington, Ill.) by comparing the double integral of the observed signal with that of known concentrations of TEMPO free radical. Quantified values were reported as micromolar concentrations in the text and normalized to area at risk in appropriate figures. Data for total postischemic PBN adduct production were corrected for area at risk and expressed as relative units in figures and quantified values (μM/g area at risk) in text.

Measurement of Lactate Dehydrogenase and Lactic Acid

In some studies, additional venous effluent samples (2 ml) were collected during reperfusion for use in measurements of both myocardial lactic dehydrogenase (LDH) and lactic acid release into venous effluent. LDH was assayed by a coupled-enzyme spectrophotometric technique using Sigma assay kits. Lactate release was measured by a spectrophotomet-
pheric method using Boehringer-Mannheim kits. Data presented for total LDH released during reperfusion were corrected for area at risk to account for any differences between hearts.

Delineation of the Risk Area

After reperfusion, hearts were excised, and the LAD was ligated again. Catheters were secured in the left and right coronary ostia with a purse-string suture and injected with white silicone rubber (Microfil®; Canton Bio-Medical Products, Inc.) at 120 mm Hg. The area delineated by the Microfil® represented the area at risk or ischemic area. After the Microfil® had hardened, atria were removed, the heart was sectioned into five equal slices, and each slice was weighed. Photographs of slices were taken and color slide projections were made at constant magnification to permit digitization of the left ventricular risk area. The area at risk was expressed both in grams wet weight and as a percent of left ventricular weight (gram percent).

Statistical Analysis

Analysis of variance was used for comparison of several means and the Tukey test for all paired comparison of means. Significance was considered as p<0.05; unless otherwise stated, values are mean±SEM of multiple hearts.

Results

Characteristics of the Postischemic Swine Heart Model

A main advantage of the in situ swine heart model compared with the canine model is its consistently low collateral flow; however, pig hearts are also prone to ventricular fibrillation (VF). This was most evident during LAD cannulation. In studies where LAD cannulation was not applied, most episodes of VF occurred during the ligation period between 14 and 32 minutes (n=11). Two pigs developed VF before 14 minutes and one before any ligature was applied. Only three pigs had a second episode of VF during the reperfusion phase. Three pigs (9.6%) experienced fatal VF, and the overall incidence of fatal and nonfatal VF was 13 of 31 pigs (41.9%). Most surviving pigs were easily defibrillated. The hematocrit of the pigs ranged from 27% to 33% and is considered normal for young pigs.

Hemodynamic Parameters and Area at Risk Measurements

Hemodynamic data indicated very stable, well-anesthetized animals (Table 1). Heart rates during the preischemic control period ranged between 70 and 105 beats/min. Compared with the untreated group, the deferoxamine-treated 40-minute-ischemic swine showed a significant transient increase in heart rate but only at 5 minutes’ reperfusion. Significant differences in LVEDP values were found only upon comparing the untreated 40- and 60-minute groups at the 25-minute ischemic time point. Mean arterial pressures ranged from 85 to 100 mm Hg during the control period and averaged around 80 mm Hg throughout the experiment. Cardiac outputs and mean pulmonary arterial pressures did not change significantly. The size of the mean area at risk was reasonably constant, displaying no statistically significant difference between any of the experimental groups except the 60-minute-ischemic group (Table 2); the risk area of this group was significantly (p<0.05) smaller (10.4 g%, 8.77 g) than the other four groups.

Spin Trapping, Lactic Acid, and Total Lactate Dehydrogenase Release in Untreated Hearts

Because of ventricular irritability, only a limited number of studies (n=3) involving direct PBN infusion of the LAD were conducted in the swine. Figure 1A demonstrates the absence of a PBN adduct signal in chloroform-extracted effluent taken during the nonischemic control period; by contrast (Figure 1B), a reasonably strong adduct signal was detected at 3 minutes of reperfusion following 15 minutes of ligation, but no significant signals were seen beyond 12 minutes. The dominant component of the spectrum shown in Figure 1B had hyperfine splitting constants (aH=2.75-3.0 G; αN=14.75 G) consistent with that reported by Bolli et al in similarly extracted samples from the stunned canine heart. These investigators described the mixed spectra as containing secondary alkoxyl and/or alkyl radicals.

The ex vivo PBN spin trapping technique was used in subsequent studies (Figures 2, 3, and 5–8) to circumvent both the potential effects of spin traps and the ventricular irritability experienced during LAD cannulation. Figure 2 depicts representative ESR spectra obtained following toluene extraction of venous plasma samples from 40-minute-ischemic, reperfused hearts: the spectrum in Figure 2A is from venous blood taken during the control phase before ligation of the LAD and shows no discernible signal; the spectrum in Figure 2B was obtained after 20 minutes of reperfusion, a time when maximal spin adduct signals were detected. PBN adduct signals were not apparent in the unextracted plasma; extraction and volume reduction were required to allow signal resolution. The dominant component of the signal described in Figure 2B has spectral characteristics similar to those reported for a PBN-alkyl radical adduct (PBN-LOX; αN=2.0–2.25 G; αH=13.5–13.75 G; half-life, ~3.3 hours). The typically higher nitrogen splitting constants (αN>14.2 G; solvent dependent) of carbon-centered adducts (alkyl, PBN-LR) and the relative instability of PBN-alkylperoxyl adducts (PBN-LOXX) make these unlikely as the dominant contributor(s) to the observed spectra.

Potential precursors for the PBN-alkoxyl adduct observed in the postischemic swine heart studies may be lipid hydroperoxides. The spectral parameters (αH=3.5 G; αN=15.0 G; half-life, ~6.7 minutes) measured in the current study for an aqueous solu-
TABLE 1. Hemodynamic Data

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Control</th>
<th>Ligation (minutes)</th>
<th>Reperfusion (minutes)</th>
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<tr>
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<tr>
<td>40</td>
<td>96±5</td>
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<td>86±6</td>
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<tr>
<td>40-D</td>
<td>86±5</td>
<td>78±6</td>
<td>80±9</td>
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<tr>
<td>60</td>
<td>86±11</td>
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<td>84±11</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
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<tr>
<td>15</td>
<td>85±9</td>
<td>93±13</td>
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<td>60</td>
<td>4.3±0.1</td>
<td>4.1±0.1</td>
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<td>Left ventricular end-diastolic pressure (mm Hg)</td>
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Mean±SEM of 3–8 swine preparations. *p<0.05. Experimental group as minutes of ischemia; 40-D, deferoxamine-treated 40-minute ischemia.

N/A, not available.

tion of a lipid hydroperoxide analogue (Cu-OOH) and Fe2+ was essentially identical to those values ($\alpha_1=3.52$ G; $\alpha_{N}=15.08$ G) reported for a PBN-alkoxy adduct in the aqueous phase; moreover, we obtained a PBN adduct signal (Figure 2C) nearly identical to that described for the swine heart, after tolune extraction of this aqueous solution ($\alpha_1=2.25$ G; $\alpha_{N}=13.5–13.75$ G; half-life, ~3.6 hours).

Figure 3 A, B, and C depicts the reperfusion time courses of PBN adduct production following 40 minutes (panel A), 30 minutes (panel B), and 15 minutes (panel C) of LAD occlusion. Since ESR signal intensity is directly proportional to free radical adduct content, and signal line widths were found to be relatively constant for well-resolved spectra, we were able to quantify (normalized to area at risk in figure: $[\mu M/g$ area at risk$] \times 10$) adduct production based on known levels of a standard (TEMPO); however, in view of the indiscriminate reactions of several free radical species, as well as the presence of antioxidants in tissue and blood, the quantified values presented should be considered merely estimates of PBN adduct content. The time course of 40-minute ischemic hearts (Figure 3A) differed from those reported in other animal models by the occurrence of 1) multiple low-level bursts of PBN adduct formation during the initial 15 minutes of reperfusion and 2) a prominent peak in adduct signal intensity ($9.77 \pm 1.6 \mu M$) at a relatively late time (20–25 minutes) of reperfusion. Signal intensity declined to very low levels by 30 minutes and remained at these levels until the end of reperfusion. The time course of signal production from 30-minute ischemic hearts (Figure 3B) was similar to that of the 40-minute group, except for substantially lower signal intensi-

TABLE 2. Area at Risk of Left Ventricle

<table>
<thead>
<tr>
<th>Duration of ligation</th>
<th>Gram percent</th>
<th>Grams</th>
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<tr>
<td>15 Minutes</td>
<td>19.2±3.0</td>
<td>19.2±0.6</td>
</tr>
<tr>
<td>30 Minutes</td>
<td>15.2±2.7</td>
<td>14.8±2.3</td>
</tr>
<tr>
<td>40 Minutes</td>
<td>14.8±2.5</td>
<td>13.7±2.4</td>
</tr>
<tr>
<td>40 Minutes+deferoxamine</td>
<td>16.6±3.6</td>
<td>16.9±1.8</td>
</tr>
<tr>
<td>60 Minutes</td>
<td>10.4±1.0*</td>
<td>8.8±1.7*</td>
</tr>
</tbody>
</table>

Mean size±SEM of 3–8 swine preparations. *p<0.05 vs. the other four groups.
ties. Maximal PBN adduct production (6.37±0.7 μM) occurred at 20–25 minutes of reperfusion but was only two thirds of that observed from the 40-minute-ischemic group. The 15-minute-ischemic group (Figure 3C) demonstrated only low levels (3.45±0.4 μM; 4.5–6.0 minutes) of adduct production with no discernible peak after 15 minutes of reperfusion. Because of the occurrence of the late PBN adduct peak during reperfusion, we investigated the possibility that washout of ischemic tissue (area at risk) was also a later event in this reperfused model. Venous effluent lactic acid was measured at various times of reperfusion (30 and 40 minutes of ischemia), and a representative time course is depicted in Figure 4. These data demonstrate that peak lactic acid release occurred early in reperfusion (5–10 minutes) and subsequently declined, approaching baseline levels during 20–25 minutes of reperfusion. Thus, delayed washout of the ischemic risk area was not a likely cause of the late PBN adduct peak in our postischemic swine heart model.

A close association between duration of ischemia, with both total postischemic production of PBN adducts and LDH release, was observed in the swine heart model. Data in Figure 5 were normalized to the size of the area at risk. By integrating the area under each individual time course (15, 30, 40, and 60 minutes of ischemia), we found that prolonged ischemic episodes resulted in greater total adduct production during reperfusion. The increase in total PBN adduct production (8.78±0.98 μM/g area at risk) was small following 15 minutes of ischemia but was significantly different (p<0.05) from control values (2.17±0.23 μM/g area at risk). When ischemic duration was prolonged from 15 minutes to 30, 40, and 60 minutes, total levels of PBN adduct were 2.6, 7.1, and 13.4 times greater (22.58±4.7, 62.56±15.3, and 117.93±19.7 μM/g area at risk), respectively.

**Figure 1.** Representative electron spin resonance (ESR) spectra obtained from extracted (chloroform: methanol) effluent following direct α-phenyl-tert-butylnitrone (PBN) infusion into the left anterior descending coronary artery (LAD) of 15-minute-ischemic, reperfused swine hearts. Panel A: Preischemic control spectrum. Panel B: PBN-radical adduct at 3 minutes of reperfusion. Conditions for ESR spectroscopy were as described in "Methods," except gain was 3.2×10^4.

**Figure 2.** Representative electron spin resonance (ESR) spectra obtained from toluene-extracted effluent after ex vivo trapping in 40-minute-ischemic, reperfused swine hearts. Panel A: Control obtained before ligation. Panel B: Alkoxyl radical adduct obtained at 20 minutes of reperfusion. Panel C: Chemical production of spectrum B using extracted alkoxyl radical adduct generated from Cu-OOH and Fe^{2+}. Spectroscopy conditions used to obtain spectrum C were as described in "Methods," except gain was 5.0×10^4.
Total LDH released into the venous effluent during reperfusion was also monitored in tissue exposed to various ischemic durations (Figure 5). The control LDH values determined before ischemia averaged 122±7.3 IU/l (n=20). Mean values determined for postischemic hearts were relative units (control, 1.0) obtained by integrating the areas under the reperfusion time courses for each individual ischemic heart. Hearts exposed to 15 minutes of ischemia experienced an insignificant rise in total LDH release. However, total LDH progressively increased (p<0.05) with longer durations (30–60 minutes) of ischemia in a manner resembling that seen for total PBN adduct formation.

Electron Spin Resonance Spin Trapping and Total Lactate Dehydrogenase Release in Deferoxamine-Treated Hearts

We used the metal chelator deferoxamine to test the hypothesis that iron plays a role in the production of secondary free radicals in the postischemic swine heart model. Deferoxamine (25 mg/kg/hr) was infused into the right atrium 1 hour before and during 40 minutes of ligation and 60 minutes of reperfusion.
The postischemic time courses of PBN adduct formation from deferoxamine-treated and untreated hearts are shown as relative ESR signal intensities along with the corresponding quantified values ([μM/g area at risk]×10) (Figure 6). Infusion of deferoxamine resulted in a substantial reduction in the formation of PBN adducts during the entire reperfusion period; most notable was the loss of the burst of PBN adduct signal typically observed during late reperfusion (maximum 3.49±0.2 μM at 26 minutes). The effect of deferoxamine on total PBN adduct formation and total LDH release is described in Figure 7. As before, values were obtained by time-course area integration and were normalized to areas at risk (Table 2). Deferoxamine infusion led to significant reductions (p<0.05) in both total PBN adduct production (83% less; 10.97±3.2 μM/g area at risk) and total LDH release (52% less) during 60 minutes of reperfusion.

To exclude the possibility that PBN adducts might actually be formed artificially in the collection tube over time, rather than in the circulating myocardial venous effluent at the time of withdrawal, deferoxamine (0.5 mM) was added to one of two sample collection tubes before spin trapping and simultaneous sample withdrawal. This concentration of deferoxamine was found to be effective in previous studies. The time courses of PBN adduct production in duplicate effluent samples (with or without deferoxamine) taken from representative 40-minute-ischemic, reperfused hearts were superimposed in Figure 8. The profiles of PBN adduct production from the duplicate samples were nearly identical with respect to time of signal detection and intensity; this suggests that PBN adduct formation had a limited production time, which was not sustained in the collection tubes.
but which was a pathobiological event occurring in situ in circulating myocardial blood.

Discussion

Methods of Free Radical Detection

Whole blood contains a number of natural antioxidant defense systems (catalase, superoxide dismutase, glutathione, and \( \alpha \)-tocopherol) that may effectively compete with infused spin trapping agents for endogenously produced free radicals. Despite this drawback, most ESR spin trapping studies in blood-perfused models as well as buffer-perfused tissue systems continue to involve direct exposure to the spin trapping agents. Our initial applications of direct LAD infusion of PBN in the postischemic swine heart enabled us to detect PBN adduct(s) in venous effluent (Figure 1), which had spectral characteristics similar to those described for an alkoxyl and/or alkyl free radical. However, a more thorough investigation into the nature and characteristics of production of these adducts have been restricted because of the relative intolerance of the swine heart preparation to this method of spin trapping.

Although a commonly used approach, interpretation of data from direct spin trapping studies could be confounded by several factors, including 1) alteration of spin trap concentrations resulting from tissue uptake or subsequent release, 2) metabolism of the spin adducts, and 3) concentration-dependent toxic or protective effects of spin traps. Thus, it is quite possible that certain spin trapping agents may actually influence the natural progression of ischemia- and reperfusion-associated events. We overcame these concerns in the present study by using a spin trapping approach in which the agent was introduced rapidly into venous effluent of the heart (ex vivo trapping). Important advantages of this technique include 1) assessing the efficacies of antiradical agents without concerns for potential cardiotoxic properties of the trapping agent and 2) its potential applicability to clinical studies, because patients would not be directly exposed to the spin trap.

Identity of Free Radical Adducts Detected by Ex Vivo Trapping

Using the ex vivo procedure described, we were able to demonstrate endogenous production of PBN adducts in venous effluent exiting the postischemic swine heart (Figure 2). The dominant spin adduct detected had spectral characteristics (\( \alpha_R = 2.0-2.25 \) G; \( \alpha_S = 13.5-13.75 \) G) similar to those reported for an alkoxyl radical. This result does not preclude the production of alkyl or peroxyl radicals in the postischemic heart but may merely define the relative instability of peroxyl radicals and the relatively low quantity of alkyl radicals detected under our experimental conditions. Other investigators have partially identified secondary free radical species in postischemic heart models using ESR and direct PBN spin trapping. Garlick et al. reported production of alkyl or alkoxyl radicals in postischemic rat hearts using direct PBN trapping. They described spectral parameters (\( \alpha_R = 1.56 \) G; \( \alpha_S = 13.6 \) G) of toluene-extracted samples that were similar to our findings (alkoxyl adduct) obtained by ex vivo trapping. Boll et al. reported the production of alkoxyl and/or alkyl radicals in the stunned canine heart. However, the hyperfine splitting constants reported for these radical species (\( \alpha_R = 2.67-2.79 \) G; \( \alpha_S = 14.75-15.00 \) G), though similar to our values obtained by direct spin trapping (Figure 1), were somewhat different from those we had measured using the ex vivo method. Dissimilarities in animal models, experimental protocol, and PBN adduct extraction procedures (chloroform:methanol versus toluene in our study) may explain these differences. Interestingly, when an aqueous mixture of Cu-OOH and Fe\(^{2+}\) was extracted with chloroform:methanol (spectra not presented), the resulting PBN adduct was identified as an alkoxyl radical and had spectral parameters (\( \alpha_R = 2.05-2.30 \) G; \( \alpha_S = 14.2 \) G) that were also different from those determined for Figure 1 and in the stunned canine model. This suggests that the PBN adduct spectra shown in Figure 1 and in the previous study contained a mixture of at least two adduct components (alkyl and alkoxyl), and was different from the alkoxyl adduct spectra obtained by ex vivo trapping (Figure 2). It is known from MNP (2-methyl-2-nitroso-propane) spin trapping studies in vitro that alkoxyl radicals can undergo \( \beta \)-scission to form methyl radicals, and perhaps this mechanism can account for some of the differences observed. Alternatively, it has been reported that PBN-RO\(^-\) in the aqueous phase was much less susceptible to superoxide anion-mediated destruction than was PBN-R\(^*\), and this may explain the dominance of PBN-RO\(^-\) during ex vivo trapping. Moreover, this latter study also showed that sample aeration of a Fenton system (H\(_2\)O\(_2\)+Fe\(^{2+}\)+DMSO) resulted in the predominant detection of PBN-RO\(^-\), whereas identical samples exposed to continuous oxygen-free conditions displayed a spectrum identified as an alkoxyl adduct. When we used an aqueous Cu-OOH+Fe\(^{2+}\) system, variations in oxygen exposure yielded similar findings, as well as mixed spectra of alkyl and alkoxyl adducts (spectra not shown). Thus, it appears that graded changes in oxygen content of the environment (hypoxic or oxygen-repleted tissue and blood) in which spin trapping was performed may conceivably dictate ESR spectral characteristics and lead to detection of either mixed- or single-adduct (Figure 2) spectra. In any event, since the full chemical nature of the trapped secondary radicals (or their precursors) was not reported in the previous studies, we cannot conclusively determine whether or not the free radical(s) detected using ex vivo trapping were the same as those reported earlier.

Free Radical Production Time Course by Ex Vivo Trapping

Production of secondary free radical species during reperfusion of the ischemic swine heart follows a unique time course (Figure 3) and appears to depend
on the severity of ischemia (Figure 5), as well as on the presence of chelatable iron (Figures 6 and 7). Other investigators have demonstrated bursts of PBN adduct signals during the initial 10 minutes of reperfusion with only low-level detection thereafter in both 15-minute-ischemic, reperfused rat and canine hearts. It is interesting to note that the relatively low level of PBN adduct production in our 15-minute-ischemic, reperfused group (Figure 3C) does resemble both the detection time and relative signal intensity reported in these other animal models. The late PBN adduct peak (20–25 minutes) observed during reperfusion of the swine heart occurred only when ischemic duration was increased beyond 15 minutes. We have previously documented the accumulation of lipid radicals (alkyl and alkoxyl) in tissue during prolonged regional ischemia (not reperfused) of the canine heart using ESR and direct tissue exposure to both PBN and MNP (2-methyl-2-nitrosopropane). Thus, it is possible that the late adduct peak may have resulted from the delayed or uneven washout of accumulated lipid radicals during reperfusion of the ischemic vascular bed. If this were the case, then release of tissue lactate that normally occurs early during reperfusion should also be delayed; such a delay was not observed (Figure 4). These data support the contention that the PBN adducts were formed during reperfusion and were not simply the result of washout of previously generated free radicals from the ischemic tissue. The fact that relatively high levels of free radical production were observed only when ischemia was extended beyond 15 minutes suggests the involvement of other factors that may be associated with longer durations of ischemia. Though not fully explored, a progressive depletion of tissue antioxidants or gradual reduction of mitochondrial electron transport chain carriers with longer durations of ischemia could further predispose reperfused myocardium to primary oxidative injury and subsequent formation of secondary free radicals. Although a controversial issue, the involvement of activated polymorphonuclear leukocytes in in vivo mechanisms of free radical injury also requires mention. Activated polymorphonuclear leukocytes may represent a prominent source of primary oxygen free radical production, which can induce tissue lipid peroxidation and secondary free radical production and trigger the arachidonic acid cascade. Myocardial infiltration of polymorphonuclear leukocytes is generally associated with prolonged ischemia and tissue necrosis (>20 minutes), and this could explain our failure to observe higher levels of postischemic secondary radical production in the 15-minute-ischemic group. Moreover, the appearance of the massive burst of secondary PBN adducts late in reperfusion of 30- and 40-minute-ischemic hearts may reflect the time for chemotactic attraction of tissue-resident polymorphonuclear leukocytes to the sites of damaged vascular endothelium, where they can adhere and become activated. Nevertheless, our current findings do not distinguish between the influences of these potential mediators of the free radical response. We also considered the possibility that the unique time course observed in our swine heart model was strictly a manifestation of the spin trapping technique. However, in separate studies using the same trapping method, we have demonstrated time-course profiles of PBN adduct(s) in buffer-perfused, postischemic rat hearts and in oxidatively stressed adult myocytes that resembled those reported by others. Therefore, there was no adduct production beyond 10–12 minutes of oxygen repletion in these polymorphonuclear leukocyte–free models. Thus, we suggest that the unusual pattern of PBN adduct formation in the current study is a reflection of both the experimental animal model and durations of ischemia, rather than of the spin trapping methodology.

The duration and severity of ischemia influences total secondary free radical adduct formation (Figure 5). This is consistent with our earlier DMPO spin trapping study in the postischemic rat heart model, which has been confirmed by others. The association between total adduct production and severity of injury is suggested by the coincidental increases in LDH release. Deferoxamine infusion led to significant reductions in PBN adduct signal intensity, total adduct formation, and total LDH release (Figure 6). This suggests a link between secondary radical production and severity of injury; however, since production of secondary radicals should typically occur after oxidative attack in tissue by primary free radicals, it might be reasonable to expect that alkoxyl radical formation was a consequence rather than a cause of the initial cell injury.

**Origin of Free Radical Adducts Detected by Ex Vivo Trapping**

Because of the relatively short lifetimes of most endogenous free radicals (milliseconds), the use of an ex vivo trapping procedure makes it less likely that the secondary radicals being trapped were formed initially in the myocardial membranes before release into the circulating blood. Alternatively, we propose that the free radicals detected were secondarily generated from membrane lipid hydroperoxides (LOOH), which were formed as a consequence of primary oxygen free radical attack and subsequently released into the extracellular compartment from myocardial cells. In the presence of trace Fe2+ or Fe3+, alkoxyl or peroxy radicals can be produced from the released LOOH. This proposal is supported by our ability to produce nearly identical PBN-alkoxyl signals (Figure 2C) after toluene extraction of an aqueous reaction mixture of a lipid hydroperoxide analogue (Cu-OOH) and Fe2+. Our ability to detect secondary free radicals using the ex vivo trapping method in swine hearts may also depend on a more sustained production of these labile species in circulating blood, and the assumption that released LOOH may not always react immediately with cata-
lytic iron (i.e., dependent on availability of extracellular free iron) to form these radicals. Thus, we propose that the alkoxyl radical detected in our studies may be secondarily formed at a relatively sustained rate in the extracellular space, rather than in the myocardial cell. The importance of this mechanism of free radical production rests with the potential of these reactive secondary radicals, if formed in close proximity to myocardial membranes, to propagate lipid peroxidative injury further. The observation that hepatocytes can be protected from hydroperoxide-induced injury by free radical antioxidants lends support to the possible occurrence of this cytotoxic mechanism.

Recent evidence has suggested that deferoxamine, in addition to its metal chelation properties, may also function as a primary oxygen free radical scavenger in vitro. However, its scavenging properties in vivo were deemed to be insignificant because typically used doses of the drug (10–80 mg/kg/hr) give maximum plasma levels (20–320 μM) that would appear to be too low for significant scavenging of •OH or •O₂⁻ in blood. Thus, we agree with others who also concluded that deferoxamine’s ability to inhibit free radical reactions in vivo is probably a result of its iron-chelation properties. Deferoxamine may afford protection to the postischemic heart by inhibiting iron-catalyzed formation of hydroxyl radicals and/or secondary free radicals (alkoxyl or peroxyl). Although the importance of iron in free radical reactions is evident, the endogenous source of this chelatable metal is uncertain. It has been suggested that superoxide anion and/or hydrogen peroxide may mobilize cellular iron from nonhaem proteins such as ferritin, or from the haem nucleus of myoglobin or hemoglobin. Nevertheless, our ability to demonstrate production of identical secondary radical species in blood-free tissue and isolated cell systems challenges the concept of hemoglobin as the only source of this catalytic trace iron.

Summary

To circumvent potential complications associated with direct spin trapping approaches, we have used an ex vivo technique using PBN to demonstrate production of secondary free radicals in venous effluent from postischemic swine hearts. The dominant free radical species was identified as an alkoxyl radical, and its postischemic production was directly influenced by ischemic duration and the availability of chelatable iron. In view of the relatively short lifetimes of endogenous free radicals, it is likely that the free radical(s) detected by the ex vivo technique were secondarily generated from the interaction of LOOH, released by the oxidatively stressed myocardium, with iron. Infusion of the metal chelator deferoxamine significantly reduced PBN adduct signal intensity, total adduct production, and loss of tissue LDH; this suggests an association between the magnitude of secondary radical production and severity of tissue injury. This study demonstrates the potential feasibility of using a non-invasive ESR spin trapping method in clinically relevant models of cardiovascular injury.

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