Nitrone spin trap lipophilicity as a determinant for inhibition of low density lipoprotein oxidation and activation of interleukin-1β release from human monocytes

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Abstract One means by which oxidation of low density lipoproteins (LDL) may contribute to atherogenesis is by their ability to induce the release of interleukin-1β from monocytes. In the present study, the effect of the lipophilic nitrone spin trap α-phenyl-*tert*-butylnitrone (PBN) on lipoprotein oxidation and subsequent release of interleukin-1β was examined. The hydrophilic nitrone spin trap α-(4-pyridyl 1-oxide)-N-*tert* butylnitrone (POBN) was also studied to evaluate the importance of spin trap localization within the lipoprotein. PBN inhibited copper-induced modification of low density lipoproteins in a dose-dependent fashion as judged by measurement of thiobarbituric acid reactive substances, electrophoretic mobility, and fluorescence changes, while POBN was relatively ineffective. As demonstrated by electron spin resonance spectrometry, spectra of PBN adducts were highly immobilized, which reflects their presence within the LDL matrix. Experiments using chromium oxalate, a paramagnetic relaxing agent, revealed that the PBN adduct is composed of a mobile component (exposed to the LDL aqueous phase) and an immobilized component, localized in the lipid-protein interface or in the bulk lipid. Cholesteryl ester phospholipid dispersions in which only the core cholesteryl esters are subject to oxidation were also used to compare PBN and POBN. Again, PBN prevented the oxidation of the core lipids while POBN had little effect, further suggesting that PBN is capable of localization within the core of LDL. In agreement, attenuation of low density lipoprotein oxidation by PBN also decreased their ability to induce interleukin-1β release from human monocytes. Conversely, POBN had little effect on the release of interleukin-1β from cells incubated with copper-oxidized lipoproteins. These results suggest that the ability of a nitrone spin trap to prevent low density lipoprotein oxidation and its biologic effect(s) requires its incorporation into the particle.—**Thomas, C. E., G. Ku, and B. Kalyanaraman.** Nitrone spin trap lipophilicity as a determinant for inhibition of low density lipoprotein oxidation and activation of interleukin-1β release from human monocytes. *J. Lipid Res.* 1994. **35:** 610–619.

Supplementary key words lipoprotein • oxidation • atherosclerosis • electron spin resonance

Atherosclerotic plaque formation is the culmination of a number of biochemical and pathological events. Over the years several hypotheses have been put forward that attempt to integrate these events into a unifying theory. Currently, many envision that oxidative modification of LDL plays a central role in atherogenesis (for review, see ref. 1). Ox-LDL have been shown to affect a number of processes that would promote plaque formation including up-regulation of adhesion molecules and enhanced monocyte binding (2, 3), induction of endothelial cell damage (4), and activation of cytokine release (5).

We have focused our attention on the participation of Ox-LDL in the induction of the release of the cytokine IL-1β from human peripheral blood monocytes (HPBM). This phenomenon may be of particular significance to lesion formation as IL-1β has been shown to induce the release of platelet-derived growth factor which stimulates smooth cell proliferation, a hallmark of the atherosclerotic lesion (6). Furthermore, mRNA levels for IL-1β have been shown to be enhanced in the arterial wall of hypercholesterolemic primates (7). We have recently provided evidence (5) that incubation of HPBM with LDL oxidized by exposure to Cu^{2+} or cells results in an elevation in IL-1β mRNA levels and release of the cytokine into the

Abbreviations: CL/CA/POPC, cholesteryl linoleate/cholesteryl arachidonate/palmitoyl oleoyl phosphatidylcholine; CROX, chromium oxalate; ELISA, enzyme-linked immunosorbent assay; ESR, electron spin resonance; HPBM, human peripheral blood monocytes; 9-HODE, 9-hydroxyoctadecadienoic acid; IL-1β, interleukin-1β; LDL, low density lipoproteins; Ox-LDL, oxidized LDL; PBN, α-phenyl-*tert*-butylnitrone; POBN, α-(4-pyridyl 1-oxide)-N-*tert*-butylnitrone; TBARS, thiobarbituric acid reactive substances; TNFα, tumor necrosis factor α.

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medium. A closer examination of the composition of Ox-LDL led us to determine that a specific oxidation product of linoleic acid, 9-HODE, is a potent activator of IL-1β release.

As Ox-LDL formation may promote atherosclerosis via the activation of IL-1β release, as well as by other mechanisms (2–4), minimization of LDL oxidation may represent a means by which disease progression can be slowed or halted. Accordingly, potent antioxidants such as probucol have been shown to provide protection in animal models of atherosclerosis (8). However, probucol possesses multiple pharmacologic properties (9) and the role of Ox-LDL in atherogenesis remains unclear. Kalyanaraman, Joseph, and Parthasarathy (10) have reported that the spin trap PBN can trap LDL-derived lipid radicals. While it is not currently known whether PBN or related nitrones affect lipid metabolism, the use of nitrones allows the study of LDL oxidation by electron spin resonance (ESR) technique. Our demonstration that 9-HODE, generated in LDL by radical reactions, induces IL-1β release from HMDM has led us to more closely examine the effects of PBN on oxidative modification of LDL and the ability of the spin trap to impact on IL-1β release. To further test the hypothesis that trapping of LDL-associated lipid radicals can modulate Ox-LDL formation and its consequences, we have compared the effects of PBN to the more hydrophilic, structurally related nitrone, POBN.

METHODS

Isolation of HPBM

Human peripheral blood monocytes were prepared in 10 mM sodium citrate from blood collected from healthy volunteers. Erythrocytes and neutrophils were removed by low speed centrifugation in Leucoprep tubes (Becton Dickinson, Oxnard, CA) according to the manufacturer's suggested protocol. The resulting mixture of platelets and mononuclear cells was incubated in tissue culture dishes (2×10^6 cells per well of 24-well plates, Corning, Corning, NY) for 1 h at 37°C, after which non-adherent platelets and other cells were removed. Fresh medium RPMI-1640 (Gibco, Grand Island, NY) was then added to the adherent cells.

Isolation and oxidation of LDL

Human plasma was obtained from 18–20 normolipidemic fasting donors. Sodium azide (0.01%) EDTA (1 mM), phenylmethylsulfonyl fluoride (0.5 mM), and aprotinin (50 kallikrein inhibitory U/ml) were added immediately and LDL were isolated by ultracentrifugal flotation in KBr between densities 1.019 to 1.063 g/ml (11). After isolation the LDL were stored in 1 mM EDTA and immediately before use the LDL were dialyzed extensively against a total volume of 12 l of deoxygenated phosphate-buffered saline (PBS) with three changes over 24 h at 4°C in the dark.

For determination of the effects of PBN or POBN on Cu^{2+}-dependent oxidation, LDL (2 mg protein/ml) were incubated in PBS with 5 μM Cu^{2+} for 18 h at 37°C. PBN and POBN (100 mM) were prepared in PBS and added to the incubation mixtures 30 min prior to the addition of Cu^{2+} and maintained at 4°C until the oxidation was initiated. For ESR studies the spin traps were prepared in chloroform (100 mM) and taken to dryness under a stream of N_2. LDL (6 mg/ml in PBS) were added to the tubes, lightly agitated, and the tubes were placed at 4°C for 1 h to allow incorporation of the spin traps into the LDL. The soluble LDL was carefully decanted from any insoluble PBN or POBN prior to addition of Cu^{2+}. Oxidation was initiated with 500 μM Cu^{2+} and carried out for 18 h. In preliminary experiments, this procedure was found to yield results on oxidation similar to those observed in experiments using LDL at 2 mg/ml and the spin traps added in PBS. All oxidations were terminated by chelation of Cu^{2+} using a slight excess of EDTA.

Determination of LDL oxidation

Lipid peroxidation was measured as TBARS (12). To 100 μg of LDL were added 0.05 ml of 2% butylated hydroxytoluene and 2 ml of thiobarbituric acid–trichloroacetic acid 2:1 and the mixture was heated at 100°C for 20 min (12). After centrifugation at 3000 rpm for 10 min, the absorbance of the resultant supernatant was read at A_{532} minus A_{580} to account for any turbidity. Quantitation of TBARS was determined by comparison to a standard curve of malondialdehyde equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3-tetraethoxypropane (Sigma).

Assessment of apolipoprotein modification was performed by fluorescence spectroscopy and agarose gel electrophoresis. For fluorescence measurements LDL (2 mg/ml) were diluted 1:9 in PBS and spectra were recorded at 25°C on an SLM-Aminco SPF 500 spectrofluorometer. Maximal excitation was achieved at 360 nm as previously described (13) and emission was monitored over the range of 370–500 nm. Agarose gel electrophoresis was performed on the same samples as those used for fluorescence studies. The samples were applied to a 1% agarose gel and electrophoretic mobility was determined by staining with Fat Red 7B.

Preparation and oxidation of CL/CA/POPC dispersions

Dispersions comprised of cholesteryl esters and phospholipid were prepared essentially as described by Craig et al. (14) with minor modification (15). In LDL, 61% of linoleate and arachidonate are present as cholesteryl esters within the core (16); thus, an effective antioxidant would likely have to be capable of localizing to some ex-

tent in the core. For these studies cholesteryl esters of linoleate and arachidonate were used and POPC was selected as the phospholipid such that only the core lipids in the dispersions could be oxidized. This facilitated the determination of whether PBN or POBN could protect core lipids. Cholesteryl esters were obtained from Nu-Chek Prep (Elysian, MN) and POPC was from Avanti Polar-Lipids (Alabaster, AL). Briefly, CL (0.22 mM), CA (0.04 mM), and POPC (0.24 mM) were dried under N_2 and resolubilized in isopropanol at 37°C in 1% v/v relative to buffer. The buffer (10 mM Tris, 50 mM NaCl, pH 7.4) at 37°C was added to the lipid solution while mixing. Where indicated, PBN and POBN were prepared in ethanol, dried under N_2 with the lipid, and also resolubilized in warm isopropanol.

Oxidation of the dispersions was done in 25-ml beakers under an air atmosphere at 37°C. Histidine-Fe^{3+} (250:50 μM) and Fe^{2+} (50 μM) were added to initiate oxidation as previously described for liposomes (17). One-ml aliquots were removed at varying times and TBARS were determined as described above for LDL.

ESR studies

Each of the LDL samples (6 mg/ml) that were subjected to Cu^{2+}-dependent oxidation in the presence or absence of the spin traps was divided into two aliquots. To one was added 3 volumes of chloroform–methanol 2:1 and the chloroform layer was evaporated to dryness under N_2. For ESR analysis the dried fraction was solubilized in 200 μl ethanol and taken up in an aqueous flat cell. ESR measurements were performed at ambient temperature using a Varian E-109 spectrometer as previously described (10).

For CL/CA/POPC dispersions, 1-ml aliquots were removed immediately after initiation of oxidation with Fe^{2+} (0 time) or at 15 min, and the oxidation was terminated by the addition of 100 μM EDTA. The lipid was extracted with chloroform–methanol as described above and resolubilized in ethanol for ESR analysis.

Determination of PBN incorporation into LDL

PBN (100 mM in $CHCl_3$) was dried under N_2 to a thin film in a 16 × 100 mm glass screw-cap tube. LDL (6 mg protein in 1 ml) were added, the tube was flushed with N_2 and capped and allowed to sit for 1 h at 4°C. The LDL were carefully removed from any insoluble PBN and diluted to a volume of 5 ml with PBS. After concentration to 2 ml with an Amicon centrifuge concentrator with a 30,000 molecular weight cut-off, the samples were extracted with 4 ml $CHCl_3$–MeOH 2:1. The $CHCl_3$ and the MeOH/PBS layers were separated, dried under N_2, and resolubilized in water.

The concentration of PBN in each fraction was determined by HPLC using a Waters 625 system consisting of a Model 625 pump/controller and a Model 996 photodiode array detector. Separation was achieved using a Waters Novapak C-18 (5 micron, 15 cm) column with a Guardpak C-18 precolumn. The samples were eluted with a step gradient as follows: from 0–2 min the mobile phase was 5% acetonitrile, from 2–6 min a linear gradient from 5–25% acetonitrile was run and held for an additional 6 min. The flow rate was maintained at 1.5 ml/min and absorbance at 302.5 nm was used for quantitation based on comparison to areas obtained for a standard curve of PBN. A nitrone synthesized at Marion Merrell Dow was used as an internal standard.

Measurement of IL-1β release from HPBM

Excess Cu^{2+} in Ox-LDL was chelated with 15 μM EDTA. To safeguard against possible endotoxin contamination, Ox-LDL were passed over a pre-packed endotoxin affinity column (Detoxigel, Pierce, Rockford, IL). To the tissue culture dishes was added 350–400 μg of LDL for approximately 24 h. We have previously observed that Ox-LDL induces IL-1β, but not TNFα, thus culture supernatants were assayed for both cytokines to monitor for potential endotoxin contamination. Human IL-1β and TNFα ELISA kits were obtained from Cistron (Pinebrook, NJ) and used in accordance with the manufacturer's suggested protocol.

RESULTS

Effect of PBN and POBN on LDL oxidation and apoB degradation

After incubation for 18 h in the presence of 5 μM Cu^{2+}, LDL underwent extensive oxidation as determined by TBARS (35.1 ± 0.6 nmol/mg LDL protein). The inclusion of 5–20 mM PBN decreased TBARS formation in a dose-dependent fashion to a maximum inhibition of 66%. On the other hand, POBN had no effect on LDL oxidation even at 20 mM.

The effects of the spin traps on oxidative modification were further examined by evaluating apolipoprotein B modification using fluorescence spectroscopy. As shown in **Fig. 1A and B,** Cu^{2+} treatment induced a marked increase in fluorescent products with maximal emission of approximately 430 nm. At this wavelength, 5 mM PBN decreased the formation of fluorescent products by 42%, while POBN diminished fluorescence by 13%. At 1.25 and 2.5 mM, POBN had no effect on the enhancement of fluorescence while PBN decreased fluorescence by 21 and 31%, respectively (Fig. 1B). In control experiments, when added to Cu^{2+}-oxidized LDL at 10 mM, PBN slightly increased fluorescence while POBN gave modest quenching; therefore the protective effect of PBN is even greater than is illustrated in Fig. 1. These data indicate that the spin traps, in particular PBN, trap reactive species that could otherwise form adducts with the apolipoprotein of LDL. This finding is corroborated by the results

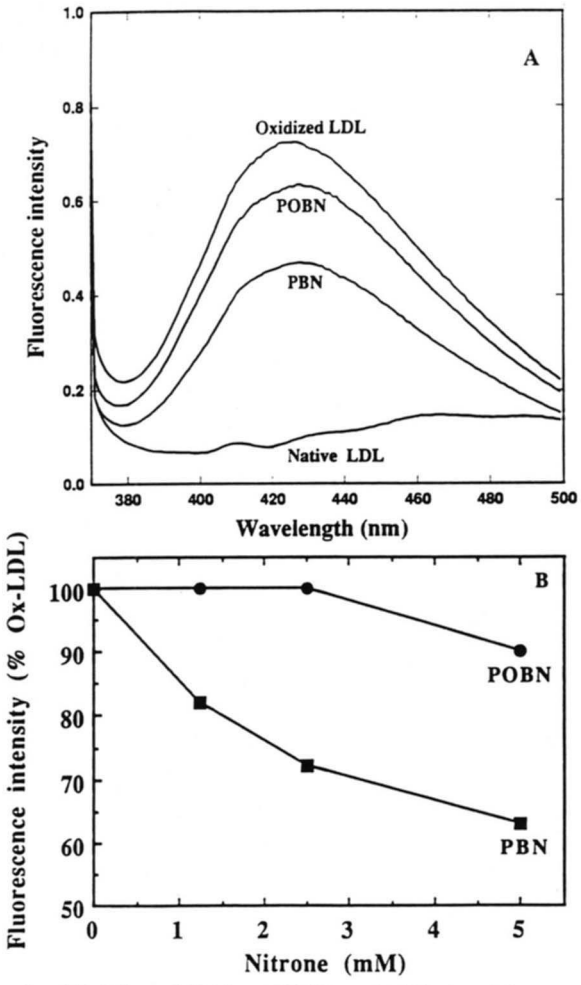

Fig. 1. (A) Effect of PBN or POBN on Cu^{2+}-induced fluorescence changes in LDL. LDL (2 mg/ml) were incubated with 5 μM Cu^{2+} for 18 h in PBS. Where indicated, incubations also contained PBN (5 mM) or POBN (5 mM). (B) Dose-dependent inhibition of Cu^{2+}-induced fluorescence changes in LDL by PBN or POBN. Data points represent the average of two separate incubations with variance of less than 6% for each determination.

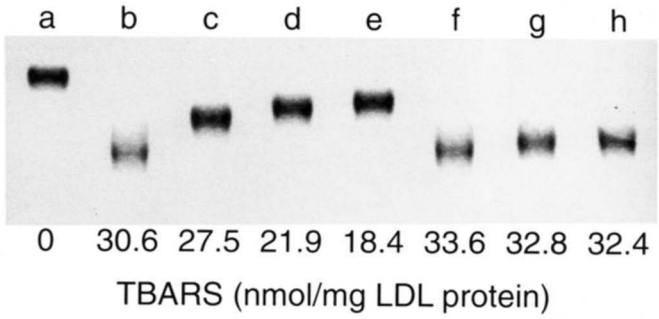

Fig. 2. Effect of PBN or POBN on agarose gel electrophoretic mobility. LDL (2 mg/ml) were incubated with 5 μM Cu^{2+} for 18 h in PBS. Lanes: a, no Cu^{2+}; b, +Cu^{2+}; c–e, +Cu^{2+} and 2.5, 5, and 10 mM PBN, respectively; f–h, +Cu^{2+} and 2.5, 5, and 10 mM POBN, respectively. TBARS determinations were done on the same incubations.

presented in **Fig. 2** which demonstrate that, in a dose-dependent fashion, PBN prevents the oxidation-induced increase in electrophoretic mobility, while POBN was without effect. These results confirm and extend those recently reported (18).

ESR spectra of PBN and POBN adducts in LDL

The greater ability of PBN to prevent TBARS formation, fluorescent products, and increased electronegativity indicates that the spin trap reacts more effectively with lipid-derived species than does POBN. The trapping of radicals by PBN and POBN was examined by ESR. It has been previously demonstrated that the nitroxide adduct of PBN is rotationally restricted in LDL and that a better-resolved ESR spectrum is obtained by lipid extraction and solubilization in ethanol. As seen in **Fig. 3A,** the nitroxide adduct of PBN is rotationally restricted in LDL and, indeed, a more well-resolved ESR spectrum is obtained by lipid extraction and solubilization in ethanol (Fig. 3B). No signal was observable in the absence of Cu^{2+} (Fig. 3C) and these results are consistent with the previous report (10).

When PBN is added to the LDL at 100 mM some of the spin trap precipitates as insoluble material as the LDL are not vigorously mixed to minimize aggregation. The LDL were removed from the unincorporated spin trap

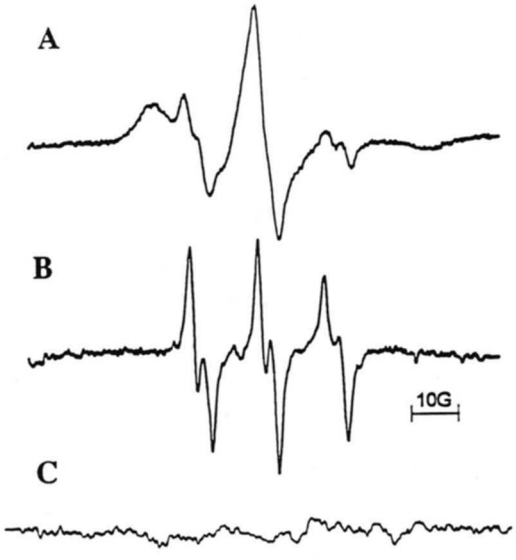

Fig. 3. The ESR spectra of PBN adduct in Cu^{2+}-oxidized LDL. In (A), (6 mg/ml) were incubated with 0.5 mM Cu^{2+} and PBN (100 mM) in PBS for 18 h. Spectrometer conditions: modulation amplitude, 2.5 G; microwave power, 20 mW; scan range, 100 G; time constant, 0.25 sec; scan time, 4 min. In (B) the ESR spectrum was obtained from the incubation after extraction. The incubation mixture was extracted in chloroform–methanol 2:1, dried under N_2, and taken up in 0.2 ml of N_2-purged ethanol. Spectrometer conditions: modulation amplitude, 1 G; microwave power, 5 mW; scan range, 100 G; time constant 0.128 sec; scan time, 4 min. (C) As in (A), but in the absence of Cu^{2+}. Spectrometer settings were identical to that of (B).

TABLE 1. Incorporation of PBN into LDL

PBN in $CHCl_3$ (mg)	8.1 ± 0.4
PBN in MeOH/PBS (mg)	0.6 ± 0.2
Total recovery (%)	49.1 ± 3.5
% in $CHCl_3$ phase	93.0 ± 1.8

PBN (100 mM) in $CHCl_3$ was dried under N_2 and 1 ml of LDL (6 mg protein) was added. After sitting 1 h at 4°C, the LDL were extracted with $CHCl_3$–MeOH and the amounts of PBN in each fraction were determined by HPLC. Values represent mean ± SD; n = 3.

and the actual amount of recovered protein was determined as was the concentration of PBN. Approximately half of the added PBN was associated with the LDL and, after extraction with $CHCl_3$–MeOH 2:1, 93% of the incorporated PBN was found associated with the lipid ($CHCl_3$) fraction (**Table 1**). The recovered protein level was 5.4 ± 0.8 mg.

When LDL incubated with POBN and Cu^{2+} were examined by ESR spectroscopy a different pattern was observed. A well-resolved spectrum was obtained without extraction (**Fig. 4A**). This suggests that the more water-soluble POBN traps radicals which have diffused into the aqueous phase. In the lipid extract (Fig. 4B), a spectrum was observed indicating that a POBN adduct is also partially lipid-soluble. In both solution and in the extract, no signal was observed in the absence of Cu^{2+} (Fig. 4C).

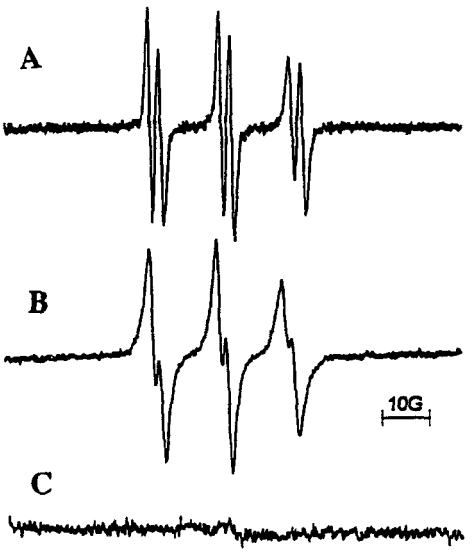

Fig. 4. The ESR spectra of POBN adduct in Cu^{2+}-oxidized LDL. The LDL were oxidized in the presence of 100 mM POBN as described for Fig. 3. In (A), an adduct was observable in aqueous solution. In (B), the reaction mixture was extracted with chloroform–methanol 2:1 and resolubilized in ethanol. (C) As for (A), but in the absence of Cu^{2+}. Spectrometer settings were identical to that of Fig. 3B, except for the spectrometer gain.

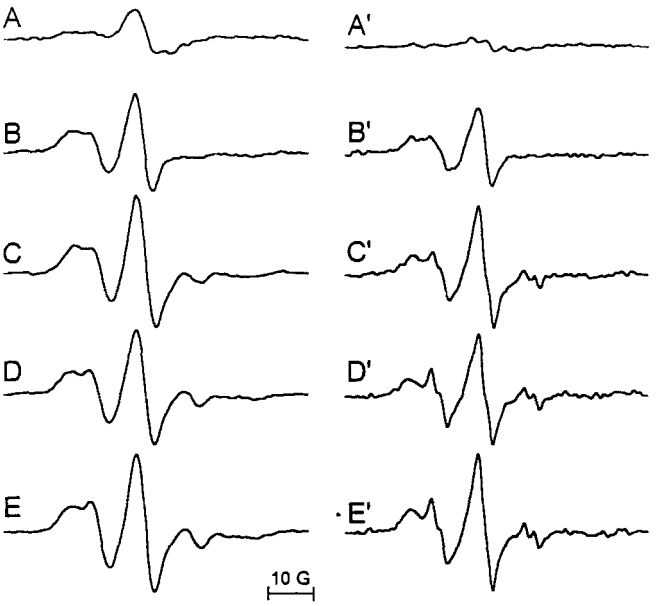

Fig. 5. Time-course of formation of PBN-LDL-lipid adduct. LDL (6 mg/ml) were incubated with 0.5 mM Cu^{2+} and PBN (100 mM) in PBS. (Left) A, spectrum obtained immediately after mixing Cu^{2+} with LDL and PBN; B, C, D, and E spectra obtained after 2, 6, 10, and 18 h, respectively. Spectrometer conditions: modulation amplitude, 5 G; microwave power, 5 MW; scan range, 100 G; time constant, 0.25 sec; scan time, 2 min. (Right) A', B', C', D', and E' show ESR spectra obtained under the same conditions as above, except for the modulation amplitude of 2 G. Note that at lower modulation amplitude, the low and high field absorption lines of the fast tumbling component clearly show a doublet splitting from the β-hydrogen of the spin adduct.

Time-course of formation of PBN adduct in LDL

To find out whether the ESR spectrum of the PBN adduct (Fig. 3A) obtained after 16 h is due to trapping of a primary or secondary radical, we monitored the time-course of PBN-LDL adduct formation. **Figs. 5A–E** show time-dependent ESR spectra obtained at higher modulation amplitude, and clearly the spectra observed after 2 h (Fig. 5B) and after 18 h (Fig. 5E) are very similar, although the intensity of the ESR spectrum is greater after 18 h. This suggests that the PBN adduct is formed from trapping of a primary radical and apparently no decomposition of the spin adduct occurred over the prolonged incubation period ($\cong$ 18 h). Figs. 5A'–E' show a similar series of ESR spectra obtained at lower modulation amplitude. At lower modulation amplitude, the doublet feature characteristic of a β-hydrogen coupling was resolved.

Localization of PBN-LDL-lipid adduct

The ESR spectrum of the PBN-LDL-lipid adduct (Fig. 3A) is characterized by an immobilized component (indicated by the left arrow in **Fig. 6A**) and a fast-tumbling component (indicated by the right arrow in Fig. 6A). Similar ESR spectral patterns have been obtained previ-

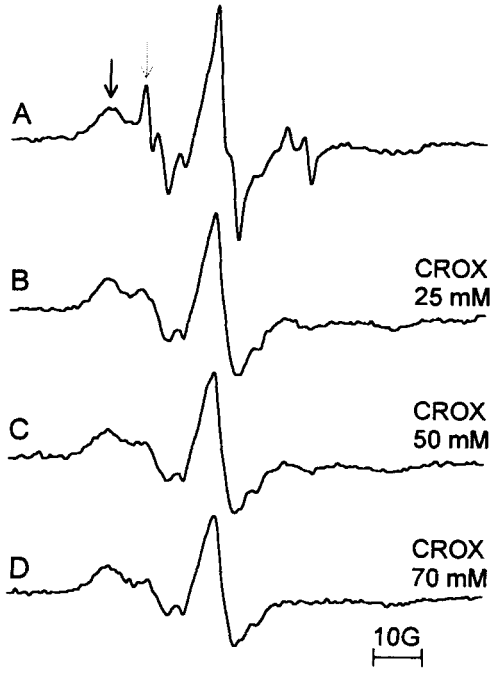

Fig. 6. Effect of chromium oxalate on the ESR spectrum of the PBN-LDL-lipid adduct. A, ESR spectrum obtained after 18 h, after the addition of Cu^{2+} (0.5 mM) to a mixture containing LDL (6 mg/ml) and PBN (100 mM) in PBS; B, the same as above, but in the presence of 25 mM CROX; C and D, in the presence of 50 and 70 mM CROX, respectively. Solutions of CROX were added to the incubation mixture in (A) after 18 h. Arrows indicate the line-positions of the immobilized (left arrow) and the fast tumbling (right arrow) components of PBN-LDL adduct.

ously, using spin-labeled lipid probes (19, 20). To understand further whether the PBN-LDL-lipid adduct is located within the core lipid, lipid-protein, or lipid-water interphase, we determined the effect of chromium oxalate (CROX) on the spectral line-shape of the PBN-LDL adduct. Chromium oxalate is a charged water-soluble and membrane-impermeable paramagnetic relaxing agent (21). Typically, CROX shortens the effective spin-lattice relaxation time and broadens the ESR spectrum of a nitroxide upon collision (21). CROX is not expected to enter the LDL particle and will undergo collision only with the PBN adduct component exposed to the aqueous phase. Figs. 6A–D show the ESR spectra of PBN-LDL-lipid adduct obtained in the absence of CROX and in the presence of various concentrations of CROX. Clearly, addition of CROX completely broadens the fast-tumbling component of PBN-LDL-lipid adduct and the immobilized, broad component is affected only to a minor extent in the presence of CROX. This implies that only a part of the PBN-LDL-adduct is localized near the aqueous phase and a major portion of the spin adduct (exhibiting immobilized spectral features) is either buried in the LDL core lipid or is embedded near the lipid-protein interface, such that collision between this component and CROX does not occur.

Effect of PBN and POBN on oxidation of CL/CA/POPC dispersions

The data obtained in the LDL experiments suggested that PBN is a more effective protectant because it associates with or incorporates into the LDL to a greater degree than POBN. To ensure that this hypothesis was correct, additional experiments were performed with dispersions prepared from cholesteryl esters and phospholipid. Both PBN and POBN were solubilized with the lipids to allow the nitrones equal opportunity to partition within the cholesteryl ester, phospholipid, or aqueous phases. As shown in **Fig. 7A,** PBN inhibited iron-dependent oxidation of the dispersions in a dose-dependent fashion. POBN (Fig. 7B) provided only marginal protection when tested to a concentration of 20 mM. The difference between PBN and POBN in their effectiveness in these dispersions suggests that PBN more readily partitions into the dispersion. Furthermore, the TBARS are derived from the cholesteryl esters that are in the core, thus PBN can access this region of the dispersion.

ESR spectra of PBN and POBN in dispersions

When chloroform–methanol extracts of the dispersions were examined by ESR, adducts were detected for both

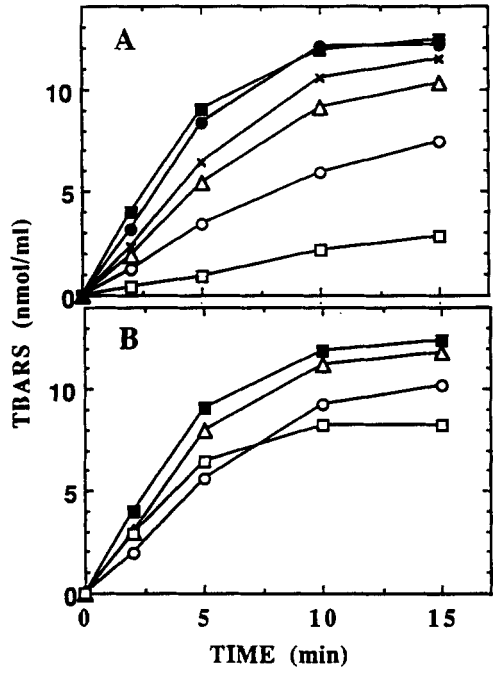

Fig. 7. Effect of PBN (A) or POBN (B) on TBARS formation in CL/CA/POPC dispersions. Dispersions were prepared as described in Methods and oxidized with histidine-Fe^{3+} (250:50 μM) and $FeCl_2$ (50 μM) for the times indicated. TBARS were determined on 1-ml aliquots as described in Materials and Methods. In (A), the concentrations of PBN were: no PBN (■), 1 mM (●), 3 mM (X), 5 mM (△), 10 mM (○), and 20 mM (□). In (B), the concentrations of POBN were: no POBN (■), 5 mM (△), 10 mM (○), and 20 mM (□).

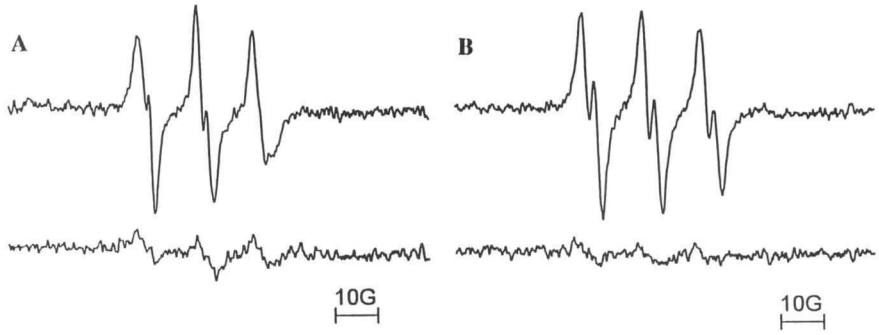

Fig. 8. The ESR spectra of adducts of PBN and POBN in oxidized CL/CA/POPC dispersions. The dispersions were oxidized with histidine-Fe^{3+} and $FeCl_2$ in the presence of PBN (50 mM) or POBN (50 mM). The incubation mixtures were stopped at 0 or 15 min by addition of EDTA, extracted in chloroform–methanol 2:1, dried under N_2, and taken up in 0.2 ml of N_2-purged ethanol. In (A), the dispersions contained PBN with oxidation terminated at 15 min (upper trace). Spectra in (B) are similar to those in (A), but for POBN-containing dispersions. The lower traces represent the 0 time samples. Spectrometer settings were: modulation amplitude, 1 G; microwave power, 5 mW; time constant, 0.25 sec; scan time, 2 min; scan range, 100 G. All spectra are the averages of five scans.

PBN (**Fig. 8A,** upper trace) and POBN (Fig. 8B, upper trace). As observed with LDL, the PBN adduct is reasonably well resolved when extracted and resolubilized in ethanol, and the POBN adducts have sufficient lipophilic character to be extracted in this manner. Although the structures of the radical adducts cannot be assigned at this time, based on ESR parameters (a_N = 16.0 ± 0.25 G, a_H = 3.0 ± 0.25 G), it appears that both adducts are derived from trapping of carbon-centered radicals. When oxidation was terminated immediately, the concentration of the spin adducts was very much reduced (Fig. 8A and B, lower traces).

Effect of PBN- and POBN-treated Ox-LDL on the induction of IL-1β

The ability of PBN to prevent oxidation of LDL and dispersions led us to examine the effect of PBN and POBN on the induction of IL-1β from monocytes. After incubation with Cu^{2+} and the spin traps, the LDL were then added to HPBM in culture and the release of IL-1β was determined by ELISA. In **Fig. 9A,** LDL oxidized in the presence of PBN showed a dose-dependent loss in capacity to induce IL-1β release. Conversely, POBN had no effect on IL-1β levels. These results correlate positively with TBARS determinations done on these LDL preparations.

As IL-1β release can be induced by a number of factors and varies greatly among individuals, this experiment was repeated with a separate batch of oxidized LDL and a different donor. In Fig. 9B, the magnitude of IL-1β release by oxidized LDL was less for this donor and PBN again inhibited its release dose-dependently. In this experiment, POBN showed some inhibitory effects, although in comparison to PBN the inhibition was relatively modest.

In control experiments, with two donors, the addition of PBN or POBN (2.5–10 mM) to cells in the absence of an inducer of IL-1β did not affect the basal levels of IL-1β (data not shown). When added to cells exposed to oxidized LDL, PBN, and to a lesser extent POBN, enhanced the release of IL-1β. While the mechanism for this effect remains unknown, it clearly indicates that the inhibitory effect of PBN is mediated via inhibition of LDL oxidation and not a direct compound effect.

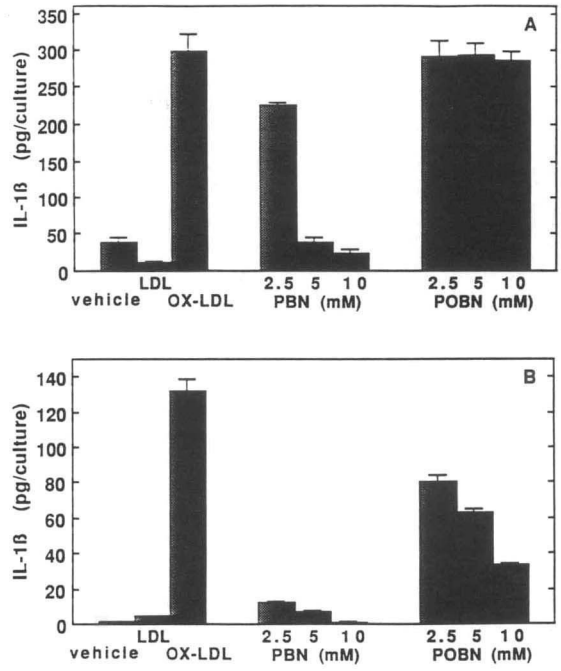

Fig. 9. Induction of IL-1β Release From HMDM by LDL oxidized with Cu^{2+} in the presence or absence of PBN or POBN. LDL (2 mg/ml) were treated with 5 μM Cu^{2+} for 18 h in the presence of 1.25–10 mM PBN or POBN. LDL were detoxified and added to HMDM for 22 h at a concentration of 350 μg/ml (A) or 24 h at 400 μg/ml (B). Determination of IL-1β in the culture medium was determined by ELISA. IL-1β was determined in triplicate for each of two LDL samples.

DISCUSSION

Our results clearly indicate that the lipophilicity of a nitrone spin trap determines its efficacy against oxidation of LDL. LDL modification was evaluated both for lipid oxidation (TBARS) and for protein modification (fluorescence spectroscopy and electrophoretic mobility), although changes in the apoprotein likely result from reaction with lipid-derived species (22, 23). In each instance, PBN was markedly better than POBN at preventing oxidative modification of the LDL. These results confirm the findings recently reported by Parthasarathy and colleagues (18) who suggested that the incorporation of PBN into LDL was necessary for its protective activity. Disparate results between laboratories have often been reported when spin traps are used in biological systems. Thus, the similar results obtained herein with totally different LDL preparations and means for incorporation of PBN are important confirmatory data to extend the earlier hypothesis (18).

PBN has an octanol:buffer partition coefficient of 16.6 compared to 0.2 for POBN (24, 25), thus it is much more likely to incorporate into the LDL and, indeed, we found an approximate 20:1 ratio of PBN in the lipid phase relative to the aqueous after extraction with $CHCl_3$–MeOH 2:1. This is important as it has previously been demonstrated (26) for other LDL protective antioxidants like probucol, that the concentration of the compound within the LDL is a critical determinant in the prevention of its oxidation. Cu^{2+}-mediated oxidation appears to require the presence of LDL-associated peroxides as peroxide removal prevents (27, 28) and peroxide addition promotes (29) LDL oxidation. Thus, the presence of a spin trap at the site of peroxide cleavage reactions (i.e., within the LDL) would be expected to more efficiently prevent propagation reactions and the spread of lipid oxidation throughout the particle.

Cholesteryl ester/phospholipid dispersions were also used in these studies to corroborate the apparent localization of PBN within the LDL particle. Adding lipophilic compounds to an aqueous medium containing LDL may not promote efficient incorporation of the compound into the LDL or may artifactually locate it in very specific regions of the particle. With the CL/CA/POPC dispersions, both PBN and POBN were solubilized in isopropanol to allow the compounds full access to either the lipid or aqueous phases of the dispersions. We further simplified the system by preparing dispersions in which only the core lipids (linoleate and arachidonate) were susceptible to oxidation. This facilitates a determination of the ability of PBN to localize within core lipids. In the dispersions, PBN had an IC_{50} of 11.5 mM as compared to 23.3 mM for POBN. This disparity indicates that a greater percentage of the PBN orients itself in the core than does POBN. PBN can also localize within phospholipids as dispersions prepared from cholesteryl oleate and soybean phosphatidylcholine were also protected from oxidation by PBN (results not shown). The ESR experiments with CROX provide further evidence that the majority of the PBN adduct is buried within the LDL.

Alternatively, it could be argued that PBN is simply a more effective radical trap than POBN. However, the rate constants for radical trapping with PBN and POBN are, in general, very similar. The difference in efficacy between PBN and POBN against oxidation of LDL or cholesteryl ester phospholipid dispersions is best explained by differences in concentration and/or location within the lipids. While POBN can trap alkyl-type radicals derived from secondary reactions of lipid hydroperoxides, the primary lipid-derived radicals are likely to be trapped by the more lipophilic PBN. The studies with the dispersions and LDL examining lipid oxidation clearly show PBN to be more effective than POBN.

The ESR studies demonstrate that PBN traps radicals and is buried within the lipid phase. This is the most likely reason for its ability to inhibit TBARS formation and IL-1β release. It cannot be entirely discounted that PBN does not affect oxidation by physically altering the LDL structure. Very recent work by Noguchi, Gotoh, and Niki (30) indicates that the kinetic chain length for the oxidation of cholesteryl ester is greater than that of phosphatidylcholine, probably due to the aggregate nature of the esters. However, physical changes induced by PBN would likely affect only the kinetics of oxidation and would not be expected to account for the protective effects observed during the 18 h oxidation used herein.

The ability of a spin trap to minimize LDL oxidation, as does PBN, is significant in view of the numerous reports implicating LDL oxidation in atherogenesis. The induction of IL-1β release from monocytes/macrophages is a potential means by which oxidized LDL might promote atherosclerosis. The formation of oxygenated lipids, in particular 9-HODE, within oxidized LDL promotes up-regulation of IL-1β mRNA and release of the cytokine (5). A lipid-soluble spin trap such as PBN could react directly with linoleate-derived radicals to minimize the formation of HODE and thus decrease IL-1β release by Ox-LDL. In agreement, PBN was much more effective at preventing the release of IL-1β from HPBM after their incubation with Ox-LDL.

That PBN is, in fact, trapping LDL-lipid-derived radicals is supported by the ESR spectra which indicate rotationally restricted motion that is characteristic of spin labels possessing 14 or more carbon atoms. LDL incubated with POBN exhibited more well-resolved spectra with less restriction suggesting, as expected, that it favors trapping of species that have been cleared from the LDL particle. This renders POBN ineffective at preventing IL-1β release for two reasons. First, it is unlikely to significantly affect the formation and concentration of

LDL-associated lipids with biologic activity such as 9-HODE. Second, POBN does not prevent the increase in electronegativity of the LDL when incubated with Cu^{2+} and it has been shown that this correlates well with macrophage uptake and degradation of LDL (10). Therefore, if uptake of oxidized LDL is required for IL-1β release, POBN would not be expected to ameliorate this response. By the same reasoning, the ability of PBN to prevent IL-1β release may be partially attributable to its prevention of extensive LDL modification which would limit uptake through the scavenger receptor. Whether cellular uptake of oxidized LDL is involved in this process has not been fully examined, although we have determined that acetylated LDL increases the responsiveness to 9-HODE (31).

From our results, it can be concluded that effective prevention of LDL oxidation by nitrone spin traps is dependent upon the ability of the nitrone to associate with the LDL. PBN is clearly capable of trapping LDL-lipid-derived radicals and can markedly diminish oxidation relative to POBN. Importantly, the capacity for PBN to prevent LDL oxidation also served to prevent the ability of these modified LDL to induce the release of IL-1β from HPBM, which may represent an event playing a causal role in atherogenesis. These events would likely be relevant to the early stages of atherosclerosis wherein the cells are primarily monocytic in nature and are not lipid-laden. Whether macrophage-derived foam cells elicit IL-1β in response to oxidized LDL remains to be determined. A recent communication (32) reported that foam cells isolated from atherosclerotic lesions were refractory to 9-HODE, thus the phenomenon reported herein may best represent events relevant to the development of lesions.

While clearly more effective than POBN, the protection afforded by PBN occurs at relatively high concentrations. Based on the results of this study, the assumption can be made that incorporation of more lipophilic spin traps into LDL may yield greater protection against LDL oxidation and the activation of IL-1β release. Strong affinity of the spin trap with the LDL may allow the use of ESR in the study of LDL isolated from animal models of atherosclerosis. Such an approach could provide a new class of compounds which not only possess anti-atherosclerotic activity but may also further our understanding of the role of LDL oxidation in atherogenesis.

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