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The spin trap, α-phenyl *N-tert*-butylnitrone, inhibits the oxidative modification of low density lipoprotein

B. Kalyanaraman[1], Joy Joseph[1] and Sampath Parthasarathy[2]

[1]*Department of Radiology, Medical College of Wisconsin, Milwaukee, WI 53226, USA* and [2]*Department of Medicine, University of California at San Diego, LaJolla, CA 92930, USA*

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The lipophilic spin trap, *N-tert*-butylnitrone (PBN) inhibits the formation of the oxidatively-modified low density lipoprotein (LDL) by endothelial cells and by cupric ions. The LDL incubated in the presence of PBN with cells or cupric ions was less readily degraded by macrophages than the LDL incubated in the absence of PBN. A lipid-derived radical formed during oxidation of LDL was detected by spin trapping with PBN. It is likely that PBN inhibits the oxidative and biological modification of LDL by scavenging the LDL-lipid-derived radical.

Atherosclerosis; Lipid-derived radical; Electron spin resonance (ESR); Lipid peroxidation

1. INTRODUCTION

Several clinical and experimental studies have firmly established a correlation between elevated levels of low density lipoprotein (LDL) and the onset of atherosclerosis (reviewed in [1,2]). There are fewer native LDL receptors in macrophages; consequently, LDL undergoes little degradation by these cells [1,2]. However, the LDL that has been modified by trace metals [3,4], lipoxygenase [5] and endothelial cells [6,7] is rapidly degraded in macrophages by an alternate scavenger receptor which ultimately leads to the formation of 'foam cells' [2]. Results from studies using antioxidants have shown a definite involvement of free radicals during the conversion of the 'native' LDL to the more atherogenic 'oxidatively-modified' LDL [2,8,9].

Recently, we have shown that an LDL-associated α-tocopheroxyl radical is formed during oxidation of LDL by Cu^{2+} and lipoxygenase [10]. This study has also shown that the LDL-lipid-derived radicals are responsible for formation of the α-tocopheroxyl radical. The study also leads to the formulation of the following hypothesis: 'If oxidative modification of LDL is mediated by lipid-derived radicals, then trapping them with the lipophilic spin trap, α-phenyl *N-tert*-butylnitrone (PBN), should decrease the modification of the LDL and the subsequent degradation by macrophages'.

Correspondence address: B. Kalyanaraman, Department of Radiology, Medical College of Wisconsin, Milwaukee, WI 53226, USA

The purpose of the study reported in this paper was (i) to investigate the effect of PBN on the oxidation of LDL by Cu^{2+} and the endothelial cells, and (ii) to demonstrate directly the formation of free radicals in LDL particles by electron spin resonance (ESR).

2. EXPERIMENTAL

2.1. *Chemicals*

PBN was obtained from Sigma and used as received. All other chemicals were of reagent grade and used as received. Agarose electrophoresis gels were from Corning Medical Co. (Palo Alto, CA).

2.2. *Isolation of LDL*

LDL (ϱ = 1.019–1.063) was isolated by ultracentrifugation from pooled normal human plasma collected in EDTA (1 mg/ml) [3]. EDTA was present throughout the isolation and dialysis to prevent oxidation of LDL. All lipoprotein samples were dialyzed against phosphate-buffered saline (PBS) containing 1 mg/ml EDTA at 4°C. Protein was determined by the Lowry method using bovine serum albumin as a standard.

2.3. *ESR sample preparation*

A typical incubation contained 6–8 mg/ml of LDL protein, Cu^{2+} 500 μM and the spin trap (PBN) in a 0.25 ml PBS buffer (pH 7.8, 10 mM NaCl) saturated with air. PBN (100 mM) was dissolved in the buffer. After incubating for 16 h, the incubation was extracted with 750 μl of chloroform:methanol (2:1) mixture, evaporated to dryness over N_2 gas, dissolved in 200 μl of ethanol and taken up in an aqueous flat cell for ESR analysis. ESR measurements at ambient temperature were carried out in a Varian E-109 spectrometer operating at 9.5 GHz and employing 100 kHz field modulation.

2.4. *Synthesis of PBN-alkyl adducts*

PBN-pentyl, -decyl and -tetradecyl adducts were prepared from the respective Grignard reagent as follows [12]: to a solution of 15 mg of PBN in 2 ml dry benzene under N_2 was added 0.3 ml of a 1 M solution of the respective Grignard reagent and stirred at room temperature

for 5 min. The reaction was quenched by adding 1 ml of a 1:1 acetonitrile/water mixture. After drying over anhydrous Na_2SO_4, the organic layer was removed and diluted to 3 ml with benzene. The organic extracts were mixed with 200 mg of lead dioxide and stirred for 30 min at room temperature. After the solution turned orange-yellow, the solution was filtered to remove the lead oxide and used in ESR studies.

2.5. *Cells*

Rabbit aortic endothelial cells are used from a line established and characterized by Buonassisi and coworkers [13]. These cells are grown in Ham's F-10 medium/15% fetal bovine serum containing epidermal growth factor at 10 ng/ml in 60 mm plastic culture dishes and are used for experiments at confluence. Resident peritoneal macrophages were obtained from female Swiss mice by peritoneal lavage with ice-cold Dulbecco's PBS. The cells were suspended in alpha MEM with 10% fetal bovine serum and plated in 12-well plastic culture plates (10^6 cells/dish). Non-adherent cells were removed after 1 h by medium exchange and the adherent macrophages cultured overnight prior to use in experiments [3].

2.6. *Oxidative modification of LDL*

LDL was oxidized in a cell-free system in the presence of Cu^{2+} in Ham's F-10 at 37°C. Typically, ^{125}I-labeled LDL (100 μg/ml) was incubated in Ham's F-10 media containing 5 μM cupric sulfate for 24 h. In cell-induced modification of LDL, ^{125}I-labeled LDL (100 μg) was incubated with washed endothelial cells in Ham's F-10 media at 37°C for 24 h [3,11].

2.7. *Lipid peroxidation measurement*

The extent of lipid peroxidation was measured as thiobarbituric acid-reactive substances (TBARS) and expressed as malondialdehyde equivalents [3,11]. LDL (50 μg of protein) in 0.5 ml was mixed with 0.3 ml of 6 N hydrochloric acid and 1.5 ml of 0.67% thiobarbituric acid in 0.05 N NaOH. After heating in boiling water for 30 min, the samples were centrifuged at 2000 rpm and the optical density read at 532 nm. Fresh tetramethoxypropane, which produces malondialdehyde, was used as standard.

2.8. *Agarose gel electrophoresis*

The electrophoretic mobility of LDL was measured in a 0.7% gel at pH 8.6 in 0.05 M barbital buffer, as reported previously [14]. LDL was identified either by oil Red-O staining or autoradiography when labeled LDL was used.

2.9. *Macrophage degradation of LDL*

The uptake and degradation of ^{125}I-LDL by mouse peritoneal macrophages was measured as the appearance of trichloroacetic acid-soluble radioactivity. This non-iodide radioactivity was formed by the cells and excreted in the medium following a 5 h incubation of oxidized ^{125}I-LDL with the cells [3].

2.10. *Cytotoxicity of spin traps*

The cytotoxicity of the spin trap was determined by microscopic observation of the endothelial cells as well as by cell protein measurements before and after the 24 h incubation period.

3. RESULTS

3.1. *Effect of PBN on the oxidative modification of LDL*

PBN was found to be toxic to the endothelial cells at concentrations greater than 5 mM. At non-toxic concentrations, PBN inhibited the biological modification of LDL as determined by the macrophage degradation in a dose-dependent manner (Fig. 1). Addition of 5 mM PBN at the end of the incubation period did not show

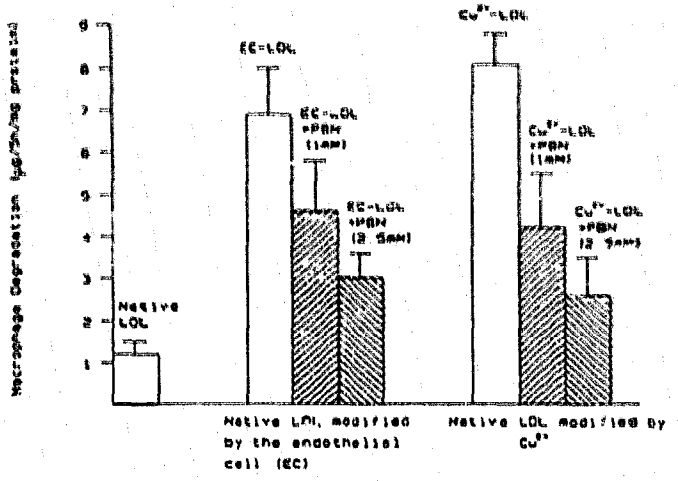

Fig. 1. Inhibition of Cu^{2+}- and endothelial cell (EC)-induced oxidation of LDL by PBN. ^{125}I-labeled LDL (100 μg of protein/ml) was incubated with cultured EC or with Cu^{2+} (5 μM) in serum-free Ham's F-10 media with or without PBN for 24 h, as indicated. After the incubation, the rate of degradation of LDL by macrophages was determined. Values represent the mean ± SD from 6 experiments.

any inhibitory effect. This should exclude any non-specific effect of PBN on macrophage degradation.

When LDL was incubated with Cu^{2+} and the endothelial cells, thiobarbituric acid-reactive substance (TBARS) formation increased (Fig. 2). Although not very specific, TBARS measurements have been shown to parallel the other oxidative changes (e.g. Apo-B degradation, electrophoretic mobility, etc.) occurring during LDL modification [3]. Oxidation of LDL in the presence of PBN inhibits formation of the TBARS to a small but significant extent (Fig. 2). It has been previously shown that only a small portion of lipid hydroperoxides actually decompose into TBARS [16]. This may in part explain the reduced inhibitory effect of PBN.

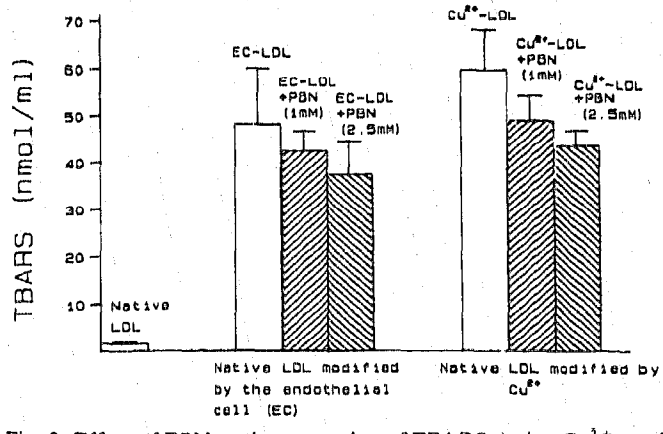

Fig. 2. Effect of PBN on the generation of TBARS during Cu^{2+}- and EC-induced oxidation of LDL. The incubation conditions are identical to that of Fig. 1, except that the medium was analyzed for TBARS at the end of the incubation. Values represent the mean ± SD from 6 experiments.

Fig. 3. Effect of PBN on agarose gel electrophoretic mobility of oxidized LDL. Lanes: (1) unmodified LDL; (2) EC-LDL; (3) EC-LDL in the presence of 1 mM PBN; (4) EC-LDL in the presence of 5 mM PBN; (5) Cu^{2+}-LDL (6) Cu^{2+}-LDL in the presence of 1 mM PBN; and (7) Cu^{2+}-LDL in the presence of 5 mM PBN. The arrow indicates the point of application.

PBN also decreased the electrophoretic mobility of EC- and Cu^{2+}-modified LDL in a dose-dependent manner (Fig. 3). Addtion of PBN at the end of the incubation period did not decrease the electrophoretic mobility. PBN alone did not have any effect on the migration of unmodified LDL. Since PBN is not likely to react with the unsaturated aldehydes (i.e. malondialdehyde, 4-hydroxynonenal, etc.) to an appreciable extent, the observed inhibitory effort of PBN may be attributed to its radical scavenging ability.

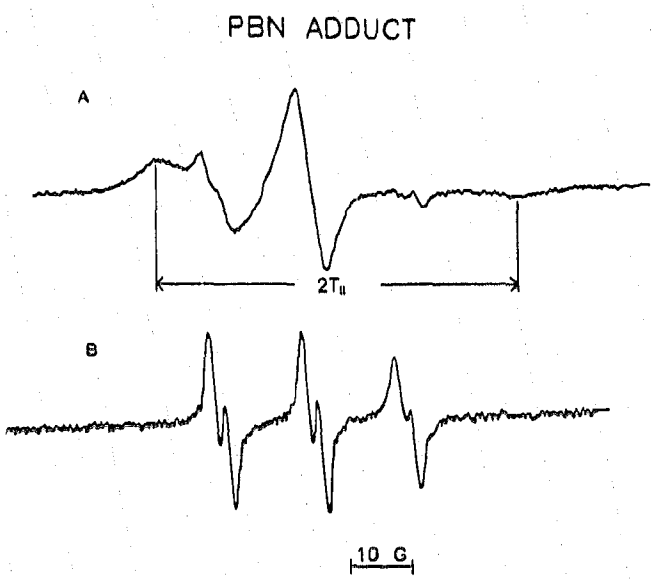

Fig. 4. The ESP spectra of PBN adduct in the LDL particle (A) and in ethanol. Conditions: (A) the incubation mixture contained 6 mg/ml DLD, 0.5 mM Cu^{2+}, and PBN (100 mM) in a 0.25 ml of PBS buffer (pH 7-8, 150 mM). Spectrometer conditions: modulation amplitude, 2.0 G; microwave power, 5 mW; scan range, 100 G; time constant, 0.25 s. (B) The incubation mixture in (A) was extracted in chloroform/methanol (2:1), dried under N_2, and taken up in a 0.2 ml of ethanol purged with N_2. Spectrometer conditions: modulation amplitude, 0.4 G; microwave power, 5 mW.

3.2. *Detection of PBN-LDL-lipid derived adduct during Cu^{2+}-catalyzed oxidation of LDL*

Addition of Cu^{2+} to LDL particles in the presence of PBN produced an ESR spectrum characteristic of a nitroxide exhibiting a rotationally-restricted motion (Fig. 4). The spectral intensity of this adduct increased slowly over a period of several hours, suggesting radical formation through lipid peroxidation. A better resolved ESR spectrum of this adduct was obtained upon extraction of the LDL-associated PBN adduct in a chloroform/methanol (2:1) mixture followed by concentration to dryness, and solubilization in ethanol (Fig. 4). The ESR parameters of the PBN-LDL adduct in ethanol are: a_H = 14.9 G; a_H = 2.2 G.

Although the exact structure of the PBN-LDL radical adduct still remains to be determined, we feel that this adduct contains a lipid-derived moiety with 14 or more carbon atoms based on the following observation: of the several fatty acid spin labels, only those with 14 or more carbon atoms exhibit a rotationally-immobilized nitroxide spectrum in the LDL matrix - similar to that of the PBN-LDL radical adduct. The PBN-LDL radical adduct is, therefore, tentatively attributed to a primary adduct obtained from trapping of a lipid radical. Additional mass spectrometric analyses are clearly needed in order to fully characterize this adduct.

4. DISCUSSION

The cardioprotective effects of PBN have previously been demonstrated [17–19]. PBN has been shown to partially reverse the reperfusion-induced damage in intact myocardium of dogs [17,18]. This protective mechanism has been attributed to the scavenging of lipid or lipid-oxy radicals by PBN [17,18]. PBN has also been shown to afford protection against reperfusion-induced arrhythmias in isolated heart models [19]. Again, radical scavenging by PBN has been proposed to account for the observed effects.

Previous studies have implicated formation of the PBN-lipid adduct during lipid peroxidation induced by xenobiotics [20–22]. However, the structure of this adduct has not been determined. Using the denterated forms of PBN (e.g. PBN-d_9 and PBN-d_{14}), the PBN-lipid adduct has been characterized as a secondary PBN-alkyl adduct [23]. This is also consistent with the finding that alkyl radicals (e.g. ethyl and pentyl radicals) are formed from the fragmentation reactions of lipid hydroperoxides [24]. However, the spectral parameters of the PBN-LDL-lipid derived adduct (Fig. 4) reported in the present study are not consistent with those of PBN-pentyl (a_N = 15.0 G; a_H = 3.70 G), PBN-decyl (a_N = 15.0 G; a_H = 3.75 G) and PBN-tetradecyl (a_N = 15.25 G; a_H = 3.75 G) adducts in alcohol. Based on this, we conclude that the PBN-LDL-lipid derived adduct is not a secondary adduct formed from trapping of the alkyl-type radicals by PBN.

Polyunsaturated fatty acids constitute about one-half of the total fatty acids present in the LDL particle [25]. Linoleic acid is present in more abundance than arachidonic acid in the LDL-particle [25]. One of the major lipid peroxidation products formed during Cu^{2+}-catalyzed oxidation of LDL has been determined to be the linoleic acid hydroperoxide, and only a small portion of the hydroperoxide has been shown to decompose to form TBARS [16]. It is plausible that the PBN-LDL-lipid adduct produced during Cu^{2+}-catalyzed oxidation of LDL is derived from trapping of the linoleic acid-derived radical. Therefore, it may be more appropriate to correlate the linoleic acid hydroperoxide rather than TBARS, with the LDL degradation by macrophages in the presence of PBN.

In conclusion, we have shown in this paper that the spin trap PBN inhibits the oxidative modification of LDL induced by Cu^{2+} and endothelial cells. PBN also inhibits the formation of TBARS under these conditions, albeit to a smaller extent. Taken together, the mechanism of inhibition may involve trapping of the LDL-lipid-derived radical by PBN.

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