Role of apolipoprotein B-derived radical and α-tocopheroxyl radical in peroxidase-dependent oxidation of low density lipoprotein

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Abstract The peroxidation of low density lipoprotein (LDL) may play an important role in the modification of the lipoprotein to an atherogenic form. The oxidation of LDL by peroxidases has recently been suggested as a model for in vivo transition metal ion-independent oxidation of LDL (Wieland, E., S. Pathasarathy, and D. Steinberg. 1993. *Proc. Natl. Acad. Sci. USA.* **90:** 5929–5933). It is possible that in vivo the peroxidase activities of proteins, such as prostaglandin synthase and myeloperoxidase, promote LDL oxidation. We have used horseradish peroxidase (HRP) and H_2O_2 as a model of peroxidase-dependent oxidation of LDL and we observed the following during HRP/H_2O_2-initiated LDL oxidation. *i)* The oxidation of α-tocopherol occurred with the concomitant formation of α-tocopheroxyl radical. This was followed by the production of an apolipoprotein B (apoB)-derived radical. The apoB radical and the α-tocopheroxyl radical were formed under both aerobic and anaerobic conditions. *ii)* Inclusion of *N-t*-butyl-α-phenylnitrone (PBN) did not inhibit α-tocopheroxyl radical formation. The ESR spectrum of a PBN/LDL-lipid derived adduct was observed after prolonged incubation. *iii)* There was formation of conjugated dienes, lipid hydroperoxides and thiobarbituric acid reactive substances. Our data indicate that HRP/H_2O_2 oxidizes both α-tocopherol and apoB to the corresponding radicals and concomitantly initiates lipid peroxidation.—**Kalyanaraman, B., V. Darley-Usmar, A. Struck, N. Hogg, and S. Parthasarathy.** Role of apolipoprotein B derived radical and α-tocopheroxyl radical in peroxidase-dependent oxidation of low density lipoprotein. *J. Lipid Res.* 1995. **36:** 1037–1045.

Supplementary key words horseradish peroxidase • oxidation • vitamin E • apolipoprotein B

There is increasing evidence supporting the hypothesis that oxidized low density lipoprotein (LDL) is atherogenic and that pathways leading to oxidative modification of LDL play a key role in the onset of atherosclerosis (1–4). Although the precise mechanism of LDL oxidation in the arterial wall is not known, there is abundant in vitro evidence for oxidation of LDL by cells present in the vasculature such as endothelial cells (5), smooth muscle cells (6), and monocyte/macrophages (7). The mechanisms involved in cell-mediated oxidation are unclear although it has been shown that LDL oxidation has an absolute requirement for transition metal ions in the cell culture medium. LDL can be chemically oxidized by iron or copper ions (8). In the absence of transition metal ions, LDL can be oxidized by peroxyl radicals, peroxynitrite, hypochlorite, and lipoxygenase (8–10). Hemoglobin has a peroxidase-like activity in the presence of H_2O_2 and has been shown to oxidize LDL (11). Peroxidase-mediated oxidative modification of LDL has recently been reported (12) and an increase in peroxidase activity has been demonstrated in atherosclerotic lesions (13).

Several inflammatory cells contain peroxidases including monocytes which contain myeloperoxidase (14). The myeloperoxidase/H_2O_2 system has been shown to oxidize L-tyrosine in human plasma via a one-electron oxidation mechanism involving the tyrosyl radical (15). Human monocytes/macrophages and mouse macrophages also express an inducible form of prostaglandin synthase that has an intrinsic peroxidase activity (16). The prostaglandin hydroperoxidase/lipid hydroperoxide system has previously been shown to co-oxidize a number of phenolic compounds (17).

Abbreviations: LDL, low density lipoprotein; HRP, horseradish peroxidase; PBN, *N-t*-butyl-α-phenylnitrone; apoB, apolipoprotein B; TBARS, thiobarbituric acid reactive substances; LOOH, lipid hydroperoxide; LOO$^{\cdot}$, lipid peroxyl radical; LO$^{\cdot}$, lipid alkoxyl radical; L$^{\cdot}$, lipid alkyl radical; PBS, phosphate-buffered saline; BHT, butylated hydroxy-toluene; ABAP, 2,2'-azobis(2-amidinopropane hydrochloride); DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance.

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In this study, we have investigated the mechanism of LDL oxidation in a model peroxidase system containing horseradish peroxidase (HRP) and H_2O_2. Our results suggest that *i)* HRP can oxidize lipid, apolipoprotein B (apoB), and α-tocopherol by a H_2O_2-dependent mechanism and *ii)* the oxidation of LDL lipid in the HRP/H_2O_2 system is dependent on the co-oxidation of α-tocopherol and apoB to the corresponding radicals.

MATERIALS AND METHODS

Materials

Horseradish peroxidase (HRP, type VI), *N-t*-butyl-α-phenylnitrone (PBN) and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co. (St. Louis, MO). 2,2'-Azobis(2-amidinopropane hydrochloride) (ABAP) was obtained from Polyscience, Inc. (Warrington, PA). All other chemicals were of the highest grade available and used without further purification. Human LDL was isolated from plasma as previously described (18). α-Tocopherol-enriched LDL was prepared by isolating LDL by the normal procedure after addition of α-tocopherol to plasma (19).

ESR measurements

A typical incubation for electron spin resonance (ESR) experiments consisted of LDL (6 mg protein/ml), HRP (30 U/ml), H_2O_2 (2 mM), and EDTA (100 μM) in 250 μl of phosphate buffer (pH 7.5, 50 mM). Either EDTA or DTPA was included to prevent the metal-catalyzed oxidation of LDL. For spin-trapping experiments, PBN (100 mM) was included in the buffer. For analysis of spin adducts the incubation mixture was extracted with 750 μl of chloroform–methanol 2:1 mixture. The lipid, aqueous, and protein fractions were separated by centrifugation (2,000 rpm for 10 min). The protein pellet was washed twice with cold acetone, dried under nitrogen, and placed inside a 4-mm cylindrical quartz tube. ESR measurements were carried out using a Varian E-109 spectrometer operating at 9.5 GHz and using 100 kHz field modulation. Samples were placed into a quartz flat-cell for room temperature ESR measurement. Hyperfine couplings were measured directly from magnetic field separations.

Measurement of LDL oxidation

Oxidation of LDL by HRP/H_2O_2 was performed in phosphate-buffered saline (PBS, pH 7.4). The formation of conjugated dienes was monitored continuously at 234 nm as described earlier (20). The reaction was quenched by the addition of BHT (10 mM) and oxidation was monitored. The methods used to determine oxidation were TBARS formation, by reaction with thiobarbituric acid (21), LOOH formation, by the iodometric assay (22), and α-tocopherol, by HPLC using fluorescence detection (23) as previously described.

RESULTS

Depletion of α-tocopherol and formation of conjugated dienes during oxidation of LDL by HRP/H_2O_2

Treatment of LDL (200 μg/ml) with HRP (2.8 U/ml) and H_2O_2 (1 mM) at 37°C resulted in a rapid depletion of the α-tocopherol present within LDL (**Fig. 1**). No depletion of α-tocopherol was observed after incubation of LDL, in the absence of HRP, with or without H_2O_2 (data not shown).

The oxidation of the lipid phase of the LDL particle was monitored continuously by following the formation of

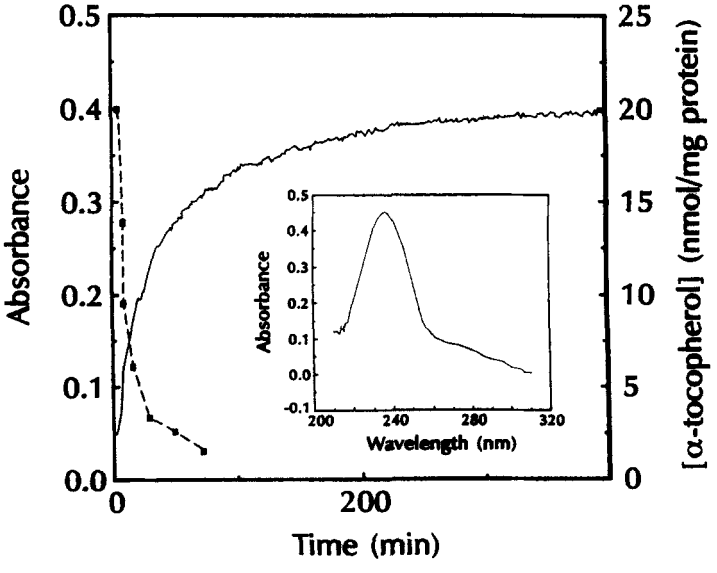

Fig. 1. The depletion of α-tocopherol and the formation of conjugated dienes during the oxidation of LDL by HRP/H_2O_2. LDL (200 μg/ml) was incubated with HRP (2.8 U/ml) and H_2O_2 (1 mM) in PBS at 37°C. The reaction was either monitored continuously at 234 nm (solid line) or quenched with BHT (100 μM) and the α-tocopherol was extracted for estimation by HPLC (-■-). α-Tocopherol data represent the mean of duplicate experiments. Inset: UV spectrum of conjugated dienes formed in the above incubation.

conjugated dienes that have a characteristic absorbance at 234 nm (20). Addition of HRP (2.8 U/ml) and H_2O_2 (1 mM) to LDL (200 μg/ml) resulted in an immediate increase in absorbance at 234 nm (Fig. 1). No such increase was observed after the addition of HRP or H_2O_2 alone (data not shown). Oxidation is unlikely to be due to the effects of free iron, released from the protein, as the metal chelator DTPA had no effect on the rate of conjugated diene formation (data not shown). The UV spectrum of the products formed had a maximum absorbance at 234 nm characteristic of conjugated dienes (Fig. 1, inset). From Fig. 1 we estimated the initial rate of α-tocopherol depletion to be approximately 0.6 μM/min, which is in good agreement with the initial rate of conjugated diene formation (0.51 ± 0.04 μM/min, mean ± SD, n = 4). This result demonstrates that HRP/H_2O_2 is capable of initiating LDL oxidation in the presence of α-tocopherol.

The initial rate of conjugated diene formation increased as a function of HRP and LDL concentration, but was essentially insensitive to changes in H_2O_2 concentration from 250–1000 μM (data not shown). BHT, a peroxyl radical scavenger, inhibited the rate of conjugated diene formation by HRP in a concentration-dependent fashion (**Fig. 2**).

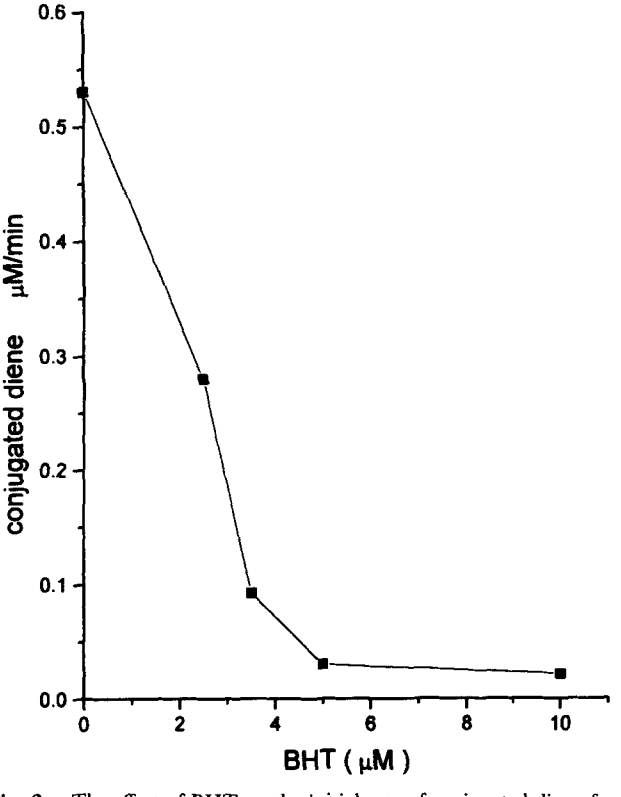

Fig. 2. The effect of BHT on the initial rate of conjugated diene formation. LDL (200 μg/ml) was incubated with HRP (2.8 U/ml) and H_2O_2 (1 mM) in PBS at 37°C in the presence of BHT (2.5–10 μM), and the absorbance at 234 nm was monitored in a UV spectrophotometer. The initial rate was expressed as μM conjugated diene formed/min using an extinction coefficient of 25 mM^{-1} cm^{-1}. The data represent the mean of duplicate determinations.

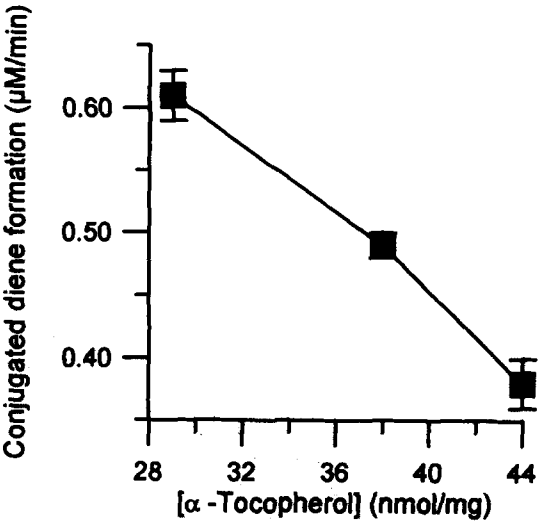

Fig. 3. The effect of α-tocopherol supplementation on the rate of conjugated diene formation. LDL (200 μg/ml) isolated from normal and vitamin E-supplemented plasma was incubated with HRP (2.8 U/ml) and H_2O_2 (1 mM) in PBS at 37°C and the absorbance at 234 nm was monitored in a UV spectrophotometer. The initial rate, expressed as μM conjugated diene formed/min using an extinction coefficient of 25 mM^{-1} cm^{-1}, is shown as a function of vitamin E content. The data represent the mean ± SD (n=3).

To assess the effects of α-tocopherol on LDL oxidation by HRP/H_2O_2 the α-tocopherol content of LDL was artificially enhanced by the addition of α-tocopherol to plasma before isolation of LDL. As shown in **Fig. 3**, increasing the α-tocopherol content resulted in a concentration-dependent decrease in the initial rate of conjugated diene formation.

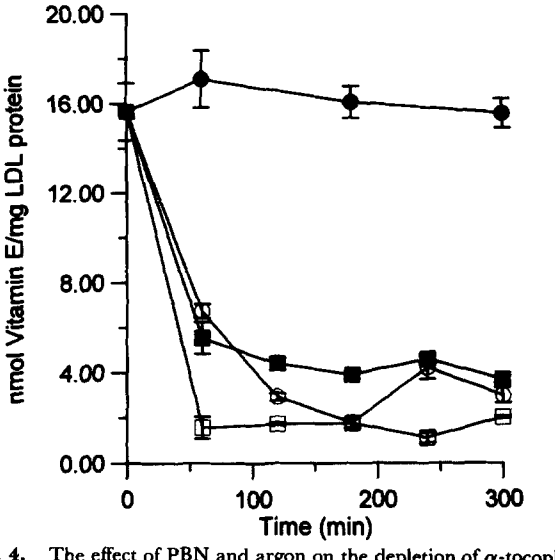

Fig. 4. The effect of PBN and argon on the depletion of α-tocopherol by HRP/H_2O_2. LDL (200 μg/ml) was incubated in PBS at 37°C (●), with HRP (2.8 U/ml) and H_2O_2 (1 mM, ■) in an argon atmosphere (○) or in the presence of PBN (2 mM, □). Aliquots were quenched with BHT (10 mM) and the α-tocopherol content was measured. Data represent the mean ± SD for three experiments.

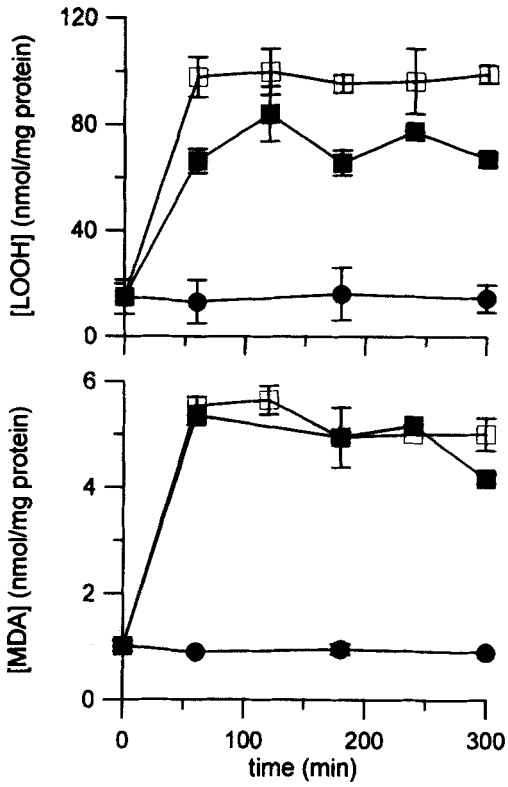

Fig. 5. The effect of PBN on LOOH and TBARS formation by HRP/H_2O_2. LDL (200 μg/ml) was incubated in PBS at 37°C (●) with HRP (2.8 U/ml) and H_2O_2 (1 mM) either alone (□) or in the presence of PBN (10 mM, ■). Aliquots were quenched with BHT (10 mM) and the LOOH (top) and TBARS (bottom) contents were measured. Data represent the mean ± SD for three experiments.

Effect of PBN on LDL oxidation by HRP/H_2O_2 and HRP alone

The effect of the spin trap PBN and argon on the depletion of α-tocopherol in LDL treated with HRP/H_2O_2 is shown in **Fig. 4.** HRP/H_2O_2 caused a rapid depletion of α-tocopherol during the early stage of oxidation. Neither PBN nor argon inhibited the HRP/H_2O_2-induced depletion of α-tocopherol (Fig. 4).

The effects of HRP/H_2O_2 on LDL lipid were detected by measuring both LOOH and TBARS (**Fig. 5**). In the presence of HRP/H_2O_2, there was an increase in both LOOH and TBARS formation during the first 60 min and no further increase was observed upon prolonged incubation. PBN marginally inhibited LOOH formation and did not inhibit TBARS formation in LDL treated with HRP/H_2O_2.

HRP alone oxidized LDL to a significant extent after prolonged incubation (**Fig. 6**). This process is probably dependent upon the endogenous lipid hydroperoxide of LDL. The effect of PBN on the oxidation of LDL by HRP was monitored by measuring both TBARS (Fig. 6A) and LOOH (Fig. 6B). PBN greatly inhibited both TBARS and LOOH formation. PBN also inhibited the depletion of α-tocopherol observed under these conditions (data not shown).

Formation of α-tocopherol radical during oxidation of LDL by HRP/H_2O_2

Incubation of LDL with HRP/H_2O_2 in the presence of either EDTA or DTPA immediately produced a seven-line ESR spectrum characteristic of the α-tocopheroxyl radical (24) as shown in **Fig. 7.** Formation of this radical was not inhibited when the incubation mixture was purged with argon before the addition of H_2O_2. Formation of the α-tocopheroxyl radical did not occur in the presence of H_2O_2 alone and was detectable at low levels in the presence of HRP alone (Fig. 7).

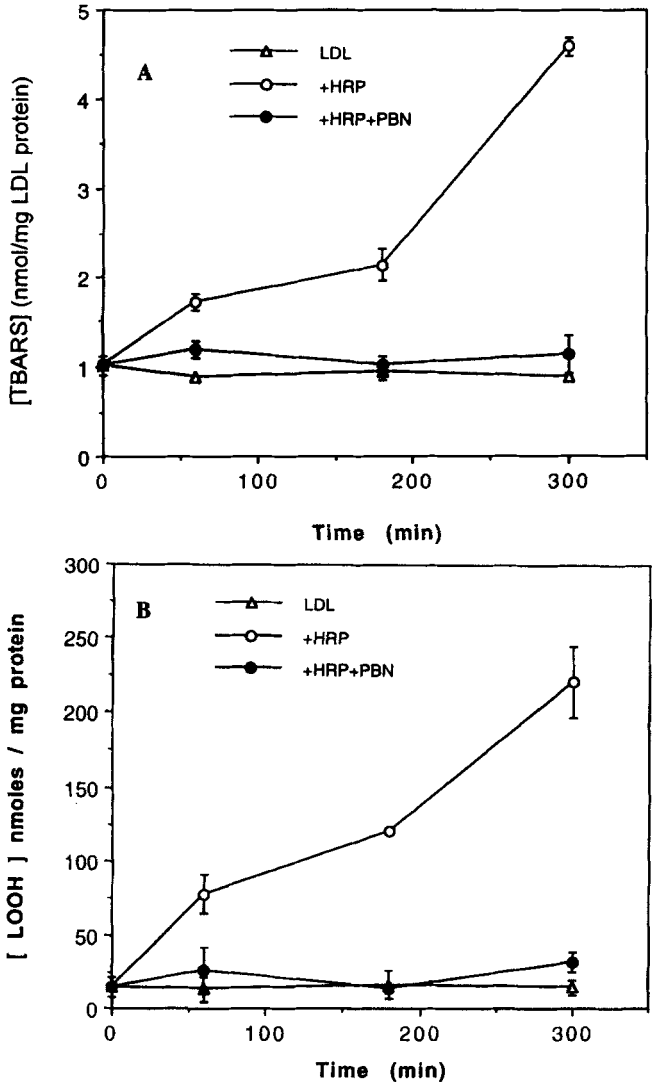

Fig. 6. The effect of PBN on LOOH and TBARS formation by HRP alone. LDL (200 μg/ml) was incubated in PBS at 37°C (△) with HRP (2.8 U/ml) either alone (○) or in the presence of PBN (10 mM, ●). Aliquots were quenched with BHT (10 mM) and the TBARS (A) and LOOH (B) contents were measured. Data represent the mean ± SD for three experiments.

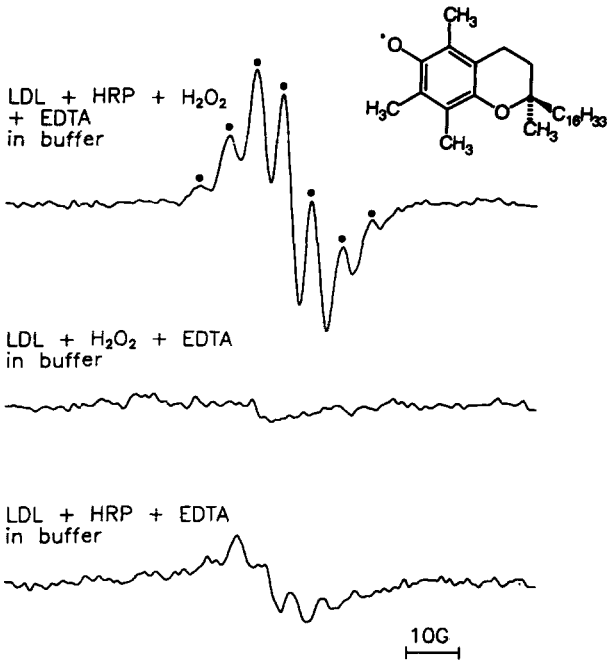

Fig. 7. The ESR spectrum of the α-tocopheroxyl radical (●) formed during the oxidation of LDL by HRP and H_2O_2. (Top) LDL (3 mg/ml) was incubated with HRP (30 U/ml) and H_2O_2 (2 mM) in phosphate buffer containing EDTA (100 μM). The spectrum was obtained immediately after the addition of HRP, (middle) as above except that HRP was omitted from the incubation, and (bottom) as above, but without H_2O_2. Spectrometer conditions: scan range, 100 G; modulation amplitude, 3.2 G; microwave power, 100 mW; scan time, 4 min; and time constant 0.25 s. EDTA was included in the incubation mixture to prevent metal-catalyzed oxidation of LDL.

Formation of a protein-derived radical during oxidation of LDL by HRP/H_2O_2

Figures 8A–D show the time-dependent changes in the ESR spectra of radicals formed during oxidation of LDL by HRP/H_2O_2. The initially formed α-tocopheroxyl radical (Fig. 8A) decayed over 30 min (Fig. 8B). After the disappearance of the α-tocopheroxyl radical, a different ESR spectrum of a secondary radical was detected, at a higher spectrometer gain, that subsequently grew in intensity (Figs. 8C–D). The hyperfine couplings and the spectral width (51 ± 1.0 G) of the secondary radical were completely different from those of the α-tocopheroxyl radical (36 ± 1.0 G). Similar spectral changes were also observed under anaerobic conditions (data not shown), indicating that molecular oxygen was not required for radical formation. A Folch extraction of the incubation mixture revealed that the secondary radical spectrum was associated with the protein fraction of LDL but not the aqueous or the lipid fractions (data not shown).

Formation of an LDL-lipid-derived radical adduct of PBN during oxidation of LDL by HRP/H_2O_2

To determine whether the HRP/H_2O_2 system is capable of oxidizing the lipid component of LDL via a radical mechanism, PBN (100 mM) was included in the reaction mixture containing LDL (3 mg/ml), HRP (30 U/ml), and H_2O_2 (2 mM). The ESR spectral changes are shown in **Fig. 9.** Even in the presence of PBN, the ESR spectrum of the α-tocopheroxyl radical appeared immediately. This implies that LDL-lipid-derived radicals are not required for formation of α-tocopheroxyl radical during HRP/H_2O_2-mediated oxidation. This is in contrast to the lipoxygenase or copper-catalyzed oxidation of LDL-lipid, where PBN inhibited formation of α-tocopheroxyl radical (24).

An ESR spectrum corresponding to a PBN-lipid adduct appeared after 60 min. Figures 9B and C consist of ESR spectra of the α-tocopheroxyl radical as well as the PBN-LDL-lipid adduct. After prolonged incubation the ESR spectrum is predominantly derived from the PBN-LDL-lipid adduct (Fig. 9D). The lipid extraction of the incubation mixture with chloroform–methanol 2:1 showed that the PBN adduct is associated with LDL-lipid

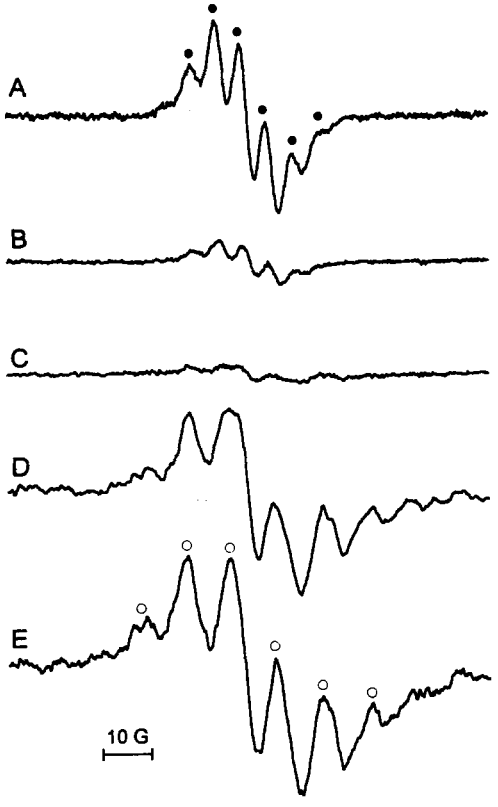

Fig. 8. Time-course ESR spectra of radicals formed during the oxidation of LDL by HRP and H_2O_2. (A) The ESR spectrum of the α-tocopheroxyl radical (●) obtained immediately after the addition of H_2O_2 (2 mM) to a mixture containing LDL (3 mg/ml), HRP (30 U/ml), and EDTA (100 μM) in phosphate buffer. Spectrum taken (B) after 30 min; (C) after 100 min; and (D) after 240 min. Note the spectrum in (D) is distinctly different from the α-tocopheroxyl radical spectrum, (E) the same as (D), but recorded under a higher spectrometer gain. The spectrum denoted (○) is tentatively attributed to an apoB-derived radical. Spectrometer conditions: scan range, 100 G; modulation amplitude, 3.2 G; microwave power, 100 mW. Except for the spectrometer gain, conditions for spectra A–D were identical with E. The spectra were the average of four scans.

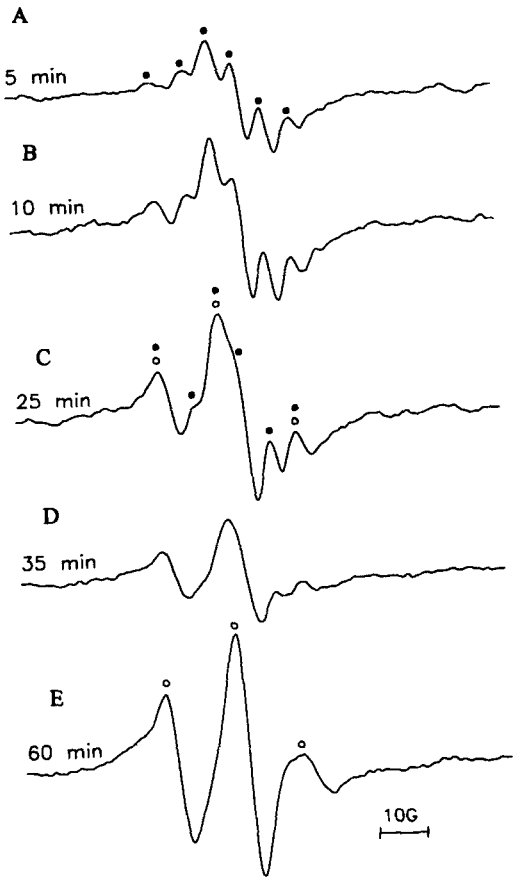

Fig. 9. Time-course ESR spectra of radicals formed during the oxidation of LDL by HRP/H_2O_2 in the presence of PBN. (A) The ESR spectrum of the α-tocopheroxyl radical (●) obtained immediately after the addition of H_2O_2 (2 mM) to a mixture containing LDL (3 mg/ml), HRP (30 U/ml), and PBN (100 mM); B, C, D, and E denote spectra obtained after the corresponding time interval as shown in the figure. The spectrum denoted (○) is assigned to the PBN-LDL-lipid-derived adduct.

(data not shown). The spectrum (Fig. 9E) is consistent with restricted rotation of the PBN adduct in the LDL particle, similar to that of a PBN adduct obtained by oxidation of LDL with Cu^{2+} or lipoxygenase (24).

These results show that HRP/H_2O_2 is capable of indirectly oxidizing the lipid component of LDL in the presence of vitamin E and apoB.

DISCUSSION

Oxidation of LDL by HRP/H_2O_2

We have shown that the incubation of LDL with HRP/H_2O_2 results in a depletion of α-tocopherol with a concomitant production of conjugated dienes, LOOH, and TBARS. These processes are all typical of lipid peroxidation and suggest that HRP/H_2O_2 causes the oxidation of unsaturated fatty acids within LDL. This is emphasized by the observation that BHT, an inhibitor of lipid peroxidation, decreased the initial rate of conjugated diene formation in this system (Fig. 2). The kinetics of oxidation differ from most other systems in that no lag-time is evident. The lag-time observed during copper oxidation is a complex phenomenon that is, in part, a result of the α-tocopherol content of LDL. Here we report that increasing the α-tocopherol content decreases the initial rate of conjugated diene formation in HRP/H_2O_2-mediated oxidation. In general, in vitro oxidation of LDL is thought to occur by the direct action of the initiating agent (for example copper ions or ABAP) on the lipid molecules; however, there is no evidence that the HRP/H_2O_2 system is able to oxidize lipid directly (12). This implies that oxidation occurs indirectly and is mediated by another component of the LDL particle. Two possible candidates for this role are α-tocopherol and apoB, both of which we show are oxidized to radical forms by HRP/H_2O_2 (Figs. 7 and 8).

Evidence for α-tocopherol mediated oxidation

It has been shown that HRP/H_2O_2 directly oxidizes α-tocopherol in solution as shown in equation 1 (25). Although antioxidant roles of α-tocopherol have been demonstrated within LDL, it has also been suggested that α-tocopherol may in fact be a pro-oxidant in certain conditions (26).

$$\alpha\text{-tocopherol} \xrightarrow{\text{HRP/H}_2\text{O}_2} \alpha\text{-tocopheroxyl radical} \quad \textit{Eq. 1)}$$

The pro-oxidant behavior was rationalized by implicating a mechanism whereby α-tocopheroxyl radical is able to abstract a hydrogen from the bis-allylic position of an unsaturated fatty acid and thus initiate lipid peroxidation (equation 2).

$$\alpha\text{-tocopheroxyl radical} + LH \rightarrow \alpha\text{-tocopherol} + L^{\cdot} \quad \textit{Eq. 2)}$$

We show here that α-tocopheroxyl radical is formed when LDL is incubated with HRP/H_2O_2 and therefore this represents a plausible route for lipid oxidation.

It is difficult to reconcile this mechanism (equation 2) with the data shown in Figs. 1 and 3 for the following reasons. *1)* HRP/H_2O_2-mediated oxidation of LDL is inhibited by α-tocopherol supplementation (Fig. 3). *2)* Equation 2 predicts that α-tocopherol depletion would not occur concomitantly with lipid peroxidation as α-tocopherol is regenerated. Our data however show a clear synchronicity between α-tocopherol depletion and the formation of both conjugated dienes and LOOH (Figs. 1 and 3). *3)* No apoB radical is observed upon oxidation of LDL by copper ions (B. Kalyanaraman, unpublished data) indicating that lipid peroxidation alone is not sufficient to form this radical. It is plausible but rather unlikely that α-tocopheroxyl radical is able to directly oxidize apoB to apoB radical.

Evidence for apoB-mediated oxidation

An alternative hypothesis is that the HRP/H_2O_2 system generates a free radical on the protein that is able to initiate lipid peroxidation (equations 3 and 4).

$$\text{apoB} \xrightarrow{\text{HRP/H}_2\text{O}_2} \text{apoB radical} \qquad \textit{Eq. 3)}$$

$$\text{apoB radical} + \text{LH} \rightarrow \text{L}^{\cdot} + \text{apoB} \qquad \textit{Eq. 4)}$$

$L^{\cdot}$ will react with oxygen to form $LOO^{\cdot}$ which will then abstract a hydrogen from α-tocopherol to give the α-tocopheroxyl radical (equations 5 and 6).

$$\text{L}^{\cdot} + \text{O}_2 \rightarrow \text{LOO}^{\cdot} \qquad \textit{Eq. 5)}$$

$$\text{LOO}^{\cdot} + \alpha\text{-tocopheroxyl} \downarrow \alpha\text{-tocopheroxyl radical} + \text{LOOH} \qquad \textit{Eq. 6)}$$

$LOO^{\cdot}$ can also remove α-tocopheroxyl radical as shown in equation 7.

$$\text{LOO}^{\cdot} + \alpha\text{-tocopheroxyl radical} \downarrow \text{LOO-}\alpha\text{-tocopherol} \qquad \textit{Eq. 7)}$$

Each initiation event would result in the formation of at least one conjugated diene molecule before termination occurs by equations 6 and 7. This is supported by the observation that the initial rate of conjugated diene formation is similar to the initial rate of α-tocopherol depletion. The observation that α-tocopherol can reduce the initial rate of conjugated diene formation suggests that α-tocopherol, at higher concentrations, can react directly with the oxidant responsible for lipid oxidation. Radical formation occurs in the absence of oxygen suggesting that under these conditions α-tocopherol is directly oxidized by HRP/H_2O_2.

Structure of apoB radical

Radicals are present as intrinsic components in the catalytic cycle of several enzymes such as ribonucleotide reductase (27). Most protein-derived radicals are formed from the oxidation of tyrosine (28), tryptophan (29), or histidine (30). Tyrosyl radicals formed in PG-synthase/arachidonic acid (31) and myoglobin/H_2O_2 (32) systems have been detected by ESR. However, the ESR spectral parameters are very different in each case. Depending upon the conformation of the side-chain C–H bond with respect to the π-orbital of the phenoxyl-radical group, the α-proton couplings vary significantly (33, 34). As a result, either a two-line spectrum or a multi-line spectra have been observed for tyrosyl radicals (33, 34).

The ESR spectrum observed for the apoB radical may be attributed to a mixture of tyrosyl radicals, however, clear proof for this assignment is lacking. The protein radical in the HRP/H_2O_2 system is persistent at room temperature in marked contrast to the tyrosyl radicals associated with other proteins. Another possibility is a radical derived from oxidation of tryptophan (29). This radical, however, would be expected to show a three-line spectrum with 1:2:1 intensity. The apoB radical spectrum clearly lacks this feature, thus negating the above possibility. Recently the histidine derivative, 2-oxo-histidine, has been detected during oxidation of LDL by Cu^{2+} (35). Amino acid analysis of apoB had previously shown that histidine is disproportionately degraded in LDL during oxidation (36). It is conceivable that the apoB radical is formed from oxidation of a mixture of amino acids, including tyrosine and/or histidine.

HRP/H_2O_2 has previously been used to generate an oxidized form of LDL for use in biological systems (37, 38), however, the mechanism underlying this oxidation has not been established. Our data show that HRP/H_2O_2 appears to initiate oxidation of LDL-lipid indirectly in the presence of apoB and α-tocopherol. It is proposed that apoB radical formed in the HRP/H_2O_2 system may play a role in initiating lipid peroxidation of LDL-lipid. Recently it has been reported that addition of tyrosine enhances macrophage-dependent oxidative-modification of LDL by a mechanism involving the oxidation of lipid by the tyrosyl radical (39). However, lipid oxidation by the myoglobin/H_2O_2 system has recently been shown to occur in the absence of tyrosyl radical formation (40). The role of tyrosyl radical as an initiator of LDL-lipid peroxidation is clearly controversial and further studies are required to resolve this issue.

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