Paradoxical Actions of Antioxidants in the Oxidation of Low Density Lipoprotein by Peroxidases

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Abstract

Oxidation of LDL by peroxidases has been suggested to be a model for in vivo oxidation. The mechanism might involve the generation of an intermediate radical such as a phenoxy radical. We show that, in contrast to the oxidation of LDL by copper, oxidation by peroxidase system (H_2O_2/horse radish peroxidase) showed less resistance. This suggested that either the antioxidants were consumed more rapidly or might have actually participated in the oxidation. Accordingly, addition of vitamin E increased the rate of oxidation of LDL. In contrast, probucol inhibited the oxidation even at low concentrations suggesting ineffective formation of probucol radical or the sterically hindered probucol radical was inefficient in catalyzing subsequent oxidation. The oxidation of LDL by horse radish peroxidase was also enhanced in the presence of diphenylphenylenediamine, an antioxidant that does not have a phenolic $-OH$ group.

Myeloperoxidase was able to oxidize LDL even in the absence of added tyrosine suggesting that it was able to utilize the LDL-associated vitamin E. Addition of free tyrosine inhibited the formation of conjugated dienes.

We suggest that if peroxidases are involved in the initiation of LDL oxidation in vivo, higher concentrations of antioxidants may be indicated to inhibit propagation of oxidation. (*J. Clin. Invest.* 1995. 95:2594–2600.) Key words: atherosclerosis • oxidized low density lipoprotein • vitamin E • myeloperoxidase • probucol

Introduction

Epidemiological and prospective studies have suggested an inverse correlation between antioxidant intake, plasma antioxidant levels, and the incidence of heart disease (1–3). Several animal studies also have established that antioxidants may play a role in the prevention of atherosclerosis (4–9). Human clinical trials are pending but a recent study cautions against dietary supplementation of antioxidants, as an aggravation of several types of cancer in smokers was observed (10) on prolonged antioxidant consumption. The mechanism(s) by which antioxidants may protect against atherosclerosis is yet to be established. However,

several lines of evidence suggest that oxidized LDL is atherogenic (11–15) and prevention of the oxidation of LDL may be one of the mechanisms by which antioxidants may be effective in preventing the disease.

Several mechanisms have been suggested for the oxidation of LDL in vitro (16–20) that require the presence of metals such as iron or copper in the medium. Recently, suggestions have been made that iron-containing proteins such as heme, hemoglobin, and peroxidases may oxidize LDL in the absence of added metals and such oxidations may provide a model for in vivo oxidation (21–25). Accordingly, the participation of vitamin E radical, tyrosine radical, or a protein radical has been suggested (21, 25).

We anticipated that if the formation of such radicals are involved it would pose serious prooxidant consequences in the antioxidant protection of LDL. For example, radicals formed from vitamin E (a phenol, analogous to tyrosine) could adversely affect the oxidation of LDL. The possibility that such radicals themselves are involved in the promotion of oxidation also has to be considered (26, 27).

In this study we show that the oxidation of LDL by peroxidases is actually enhanced by the presence of antioxidants such as vitamin E and diphenylphenylene diamine (DPPD).[1] Ditertiarybutyl phenols, such as, butylated hydroxytoluene (BHT) and probucol, did not increase the rate of oxidation and were effective inhibitors. Incubation of [^{14}C]linoleic acid with H_2O_2/peroxidase, in the presence or absence of vitamin E or tyrosine, did not generate an oxidized fatty acid, suggesting that these radicals themselves do not induce lipid peroxidation. However, their participation in the oxidation of LDL is suggested by their ability to accelerate the oxidation of LDL.

These results would suggest that if peroxidases are involved in the oxidation of LDL, the presence of small amounts of antioxidants present may actually enhance the rate of oxidation. Under these circumstances, the presence of large amounts of antioxidants adequate enough to prevent propagation reactions or a sterically hindered phenol may be more effective in preventing oxidation.

Methods

Materials. Horse radish peroxidase (HRP, type X-lot 82H9535, 250 U/mg), bovine milk lactoperoxidase (LPO; lot 121H3789, 98 U/mg), human leukocyte myeloperoxidase (MPO; lot 73H9483, 185 U/mg), and 1-Palmitoyl-2-linoleoyl phosphatidylcholine were purchased from Sigma Chemical Co. (St. Louis, MO). [1-^{14}C-]Linoleic acid (53 mCi/mmol) was purchased from New England Nuclear (Boston, MA) and was diluted with unlabeled linoleic acid to a specific radioactivity of 1 μCi/μmol.

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1. *Abbreviations used in this paper:* BHT, butylated hydroxytoluene; DPPD, diphenylphenylene diamine; HRP, horse radish peroxidase; LPO, lactoperoxidase; MPO, myeloperoxidase; SLO, soybean lipoxygenase.
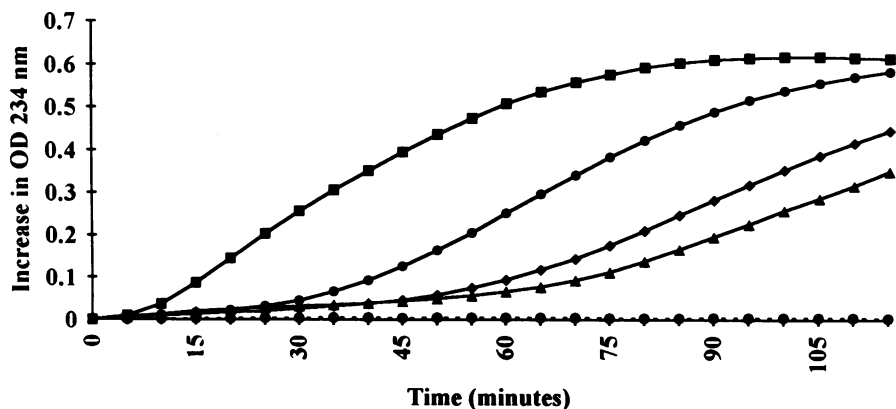
Figure 1. Effect of vitamin E on LDL oxidation by H_2O_2/HRP. 50 μg of human LDL was incubated with 50 μM H_2O_2 and 1 U HRP in the presence of specified concentrations of vitamin E in a total volume of 1 ml PBS. Optical density at 234 nm was monitored continuously. The figure represents a typical experiment from over six individual experiments. ▲, control LDL; ◆, LDL + 1 μM vitamin E; ●, LDL + 2 μM vitamin E; ■, LDL + 3 μM vitamin E; ●, at the x axis, control without LDL.

LDL was isolated from heparinized plasma of normal human donors using a TL-100 table top ultracentrifuge. A single spin gradient isolation was used and the isolated sample was respun at $d = 1.063$ to concentrate and purify LDL from any albumin contamination. The isolation was carried out without any EDTA (to avoid any effect by EDTA) and was complete in < 3 h. The isolated LDL was dialyzed against phosphate-buffered saline (PBS) at 4°C (100 vol) for 6 h. The isolated LDL gave a single band on agarose gel electrophoresis and contained only intact apoprotein B. Isolated LDL was used within 48 h and most of the diene conjugation experiments described in this study were performed immediately after isolation.

The formation of conjugated dienes was measured in a spectrophotometer (model DB-3500; SLM-AMINCO, Urbana, IL) equipped with a 12 position sample changer. 10 samples and 2 references were measured continuously for periods up to 4 h. Typically, 50 μg/ml of LDL was incubated in PBS with 2.5 μM copper or peroxidases (1 U HRP or 1 U LPO or 0.1 U MPO) in the presence of 50 μM H_2O_2. Antioxidants were added in 0.1% ethyl alcohol. Control samples were also treated with ethyl alcohol.

Fluorescence was measured in a spectrofluorometer (model RF-1501; Shimadzu Corp., Tokyo, Japan) using excitation at 330 nm and emission at 390–500 nm. Molecular modeling was done using the Alchemy III program on a 486/66 Compaq Personal Computer.

Results and Discussion

We recently suggested that the oxidative modification of LDL by HRP in the presence of H_2O_2 (21) may provide a ''metal-free'' model for LDL oxidation in vivo. Based on the inability of the system to oxidize simple lipids such as fatty acids, we proposed that the oxidation may involve the formation of an intermediate radical derived from vitamin E or other apoprotein B–derived radicals.

Incubation of LDL with HRP in the presence of H_2O_2 initiated the oxidation of LDL as determined by the formation of conjugated dienes (Fig. 1). In several experiments the lag time was shorter when HRP/H_2O_2 was used as compared to experiments that used copper as the oxidant. For example, in an analysis of 18 subjects, the lag time observed with peroxidase oxidation was 16.6 ± 8.1 vs 55.6 ± 28 min ($P < 0.00001$). This might suggest that endogenous antioxidants in LDL were depleted faster as compared to the copper-mediated oxidation. However, these results by themselves do not suggest that the antioxidants participated in the oxidation process. The enhanced rate of oxidation could be simply due to the depletion of vitamin E in the system. Fig. 1 shows the effect of added vitamin E on the oxidation of LDL by HRP/H_2O_2 system. Addition of increasing amounts of vitamin E further decreased the lag time showing that the antioxidant was clearly acting as a prooxidant under the experimental conditions. When 3 μM vitamin E was added to the system, the lag phase was completely abolished. These results would suggest that the added vitamin E actually participated and enhanced the rate of oxidation. At these concentrations, the prooxidant effect prevailed, suggesting that the amount of free tocopherol was insufficient to act as a chain-breaking antioxidant. The data would suggest that in the HRP system virtually all the tocopherol was converted to its radical form so that little if any vitamin E remained in the active form to prevent lipid peroxidation. At higher concentrations of tocopherol, inhibition of oxidation could be demonstrated (see
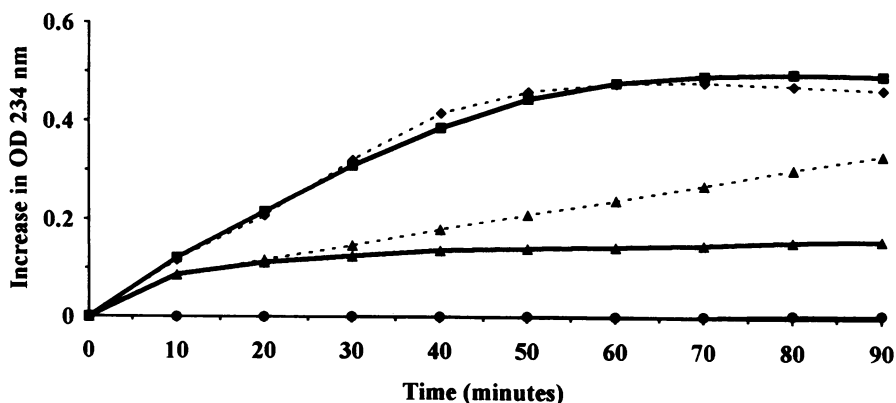

Figure 2. Effect of probucol on LDL oxidation by H_2O_2/HRP. The conditions were as described for Fig. 1. The figure represents results from one of six individual experiments. ■, control LDL; ◆---◆, LDL + 1 μM probucol; ▲---▲, LDL + 2 μM probucol; ▲, LDL + 3 μM probucol; ●, at the x axis, control without LDL.
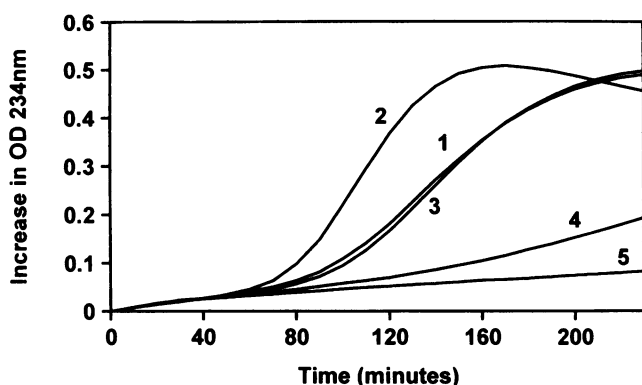
Figure 3. Effect of vitamin E and probucol on the oxidation of LDL by H_2O_2/HRP. Conditions are as described for Fig. 1. Experiment given is a representative experiment from two independent trials. Line 1, LDL only; line 2, LDL plus 0.5 μM vitamin E; line 3, 1 μM vitamin E; line 4, 0.5 μM probucol; line 5, 1 μM probucol.

Fig. 3). The presence of vitamin E would have no effect on oxidation parameters if they are measured after several hours. Consequently, there is no net change in the total amounts of dienes or thiobarbituric acid reactive substances produced after several hours. The concentration at which vitamin E showed pro- and antioxidant effects varied with the LDL preparation.

The effect of probucol on the oxidation of LDL by HRP is shown in Fig. 2. In contrast to vitamin E, probucol (and BHT,

data not shown) inhibited the oxidation of LDL. There was virtually no lag phase in this experiment and even in the presence or absence of probucol, which might suggest that the tocopherol in the LDL was the target of oxidation by HRP and probucol acted primarily as an inhibitor of propagation. The net amount of conjugated diene formed was decreased in the presence of probucol, showing that the endogenous antioxidant (for example vitamin E) might have participated in the oxidation process whereas the added probucol acted as a chain-breaking antioxidant. The differences in the effects of vitamin E and probucol were not due to differences in the antioxidant efficiencies of these two antioxidants. Addition of probucol even at substantially lower concentrations did not show an enhancement of oxidation (Fig. 3), whereas vitamin E in the same experiment showed an enhancement of oxidation at a concentration of 1 μM and began to inhibit at higher concentrations. Molecular modeling of these antioxidants revealed interesting differences in the conformation of the phenoxy radicals derived from these antioxidants (Fig. 4). Vitamin E–and tyrosine-derived radicals were readily accessible, whereas the phenoxy radical of probucol appeared to be shielded by the neighboring, bulky, t-butyl groups. Therefore, it is proposed that the radicals derived from vitamin E or tyrosine may be able to generate radicals in the lipoprotein whereas those derived from probucol or BHT may not be accessible for propagation. However, since probucol radicals have been suggested to be reduced by ascorbate (28) to the parent compound, it is likely that amino acid or fatty acid residues of LDL that interact with these radicals are larger in
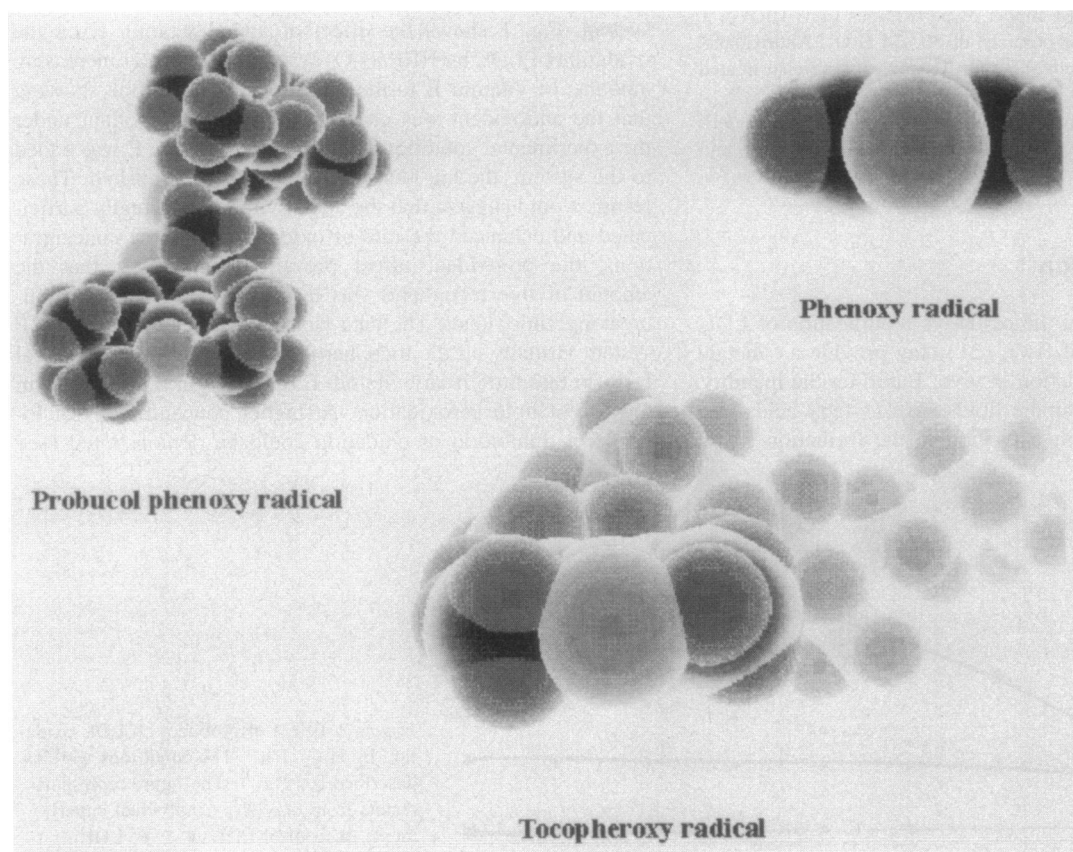

Figure 4. Conformation of vitamin E, tyrosine, and probucol phenoxy radicals. Modeling was performed using the Alchemy III program and the images were captured using a screen capture utility.
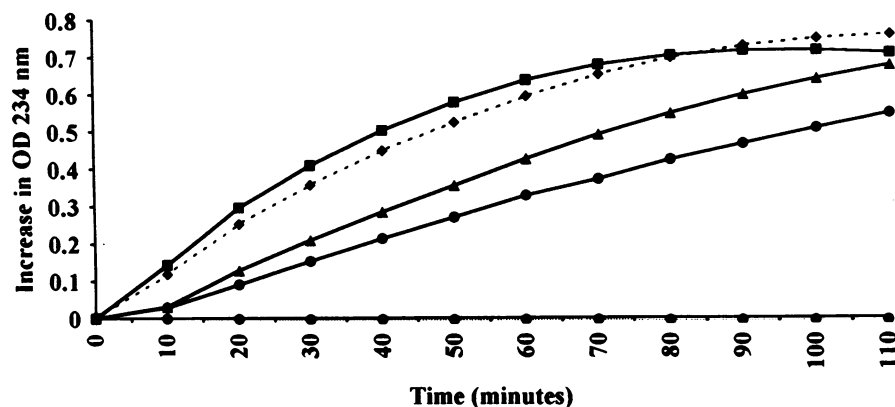
Figure 5. Effect of DPPD on LDL oxidation by H_2O_2/HRP. The figure represents results from one of six individual experiments. ●, control LDL; ▲, LDL + 1 μM DPPD; ◆---◆, LDL + 2 μM DPPD; ■, LDL + 3 μM DPPD; ●---●, control without LDL.

size than ascorbate. The formation of probucol radical by HRP has not yet been demonstrated.

DPPD, an antioxidant that does not have a phenolic function, has been suggested to inhibit the oxidation of LDL and reduce atherosclerosis in cholesterol-fed animals (4). The antioxidant effect of this compound in copper catalyzed oxidation of LDL was also shown in this study. We tested the effect of DPPD in the oxidation of LDL by HRP and observed that the addition of DPPD at 1–3 μM concentrations increased the rate of oxidation as observed for vitamin E (Fig. 5). These results would suggest that compounds other than simple phenols may be able to form radicals with HRP and such radicals may be able to propagate the oxidation of LDL. Indeed, molecular modeling of DPPD radicals reveals the ready accessibility of the radical.

HRP is a plant peroxidase and has been used extensively as a model system. To extend the above observations to a mammalian peroxidase system, we used LPO in the presence of H_2O_2. Figs. 6 and 7 show the effect of vitamin E and probucol on the oxidation of LDL by LPO. Addition of vitamin E at a concentration of 1 μM increased the rate of oxidation and decreased the lag time of LDL oxidation, whereas higher concentrations were gradually inhibitory. Probucol, as was the case with HRP, inhibited the formation of conjugated dienes. Since the lag time was not affected, from the shape of the curves, it would appear that probucol acted as a chain-breaking agent and prevented the generation of conjugated dienes. Accordingly, there was a decrease in the net amounts of dienes formed.

The oxidation of lipoproteins by MPO reaction has been

suggested to be important (25, 29, 30). It was suggested that free tyrosine, and not protein tyrosine, is capable of the formation of the tyrosyl radical and such radicals were capable of inducing further oxidation (25, 31, 32). It was, therefore, concluded that the presence of large amounts (> 100 μM) free tyrosine is essential for the oxidation of lipoproteins. The oxidation of LDL by MPO is shown in Fig. 8. Addition of free tyrosine was not necessary, as oxidation could be demonstrated even in the absence of added tyrosine, suggesting that either MPO formed lipid peroxides directly or a LDL radical (apo B or vitamin E) could be formed readily. Analogous to the HRP system, the addition of vitamin E at low concentrations increased and at higher concentrations decreased the oxidation. The addition of probucol again inhibited the oxidation.

The lack of requirement for tyrosine was further evident as added tyrosine actually inhibited the formation of conjugated dienes (Fig. 9). In the same experiments, the presence of tyrosine generated substantial dityrosine adduct as measured by the large increase in fluorescence (Fig. 10). Thus, the addition of large amounts of free tyrosine actually acted as an inhibitor of lipid peroxidation as would be predicted from the effects of large amounts of added antioxidants in this system.

The inability of peroxidases to oxidize free fatty acids directly, in the presence or absence of vitamin E or tyrosine, is shown in Table I. When [^{14}C]linoleic acid was incubated with peroxidases in the presence of H_2O_2, no oxidized labeled products could be demonstrated. Regardless of the concentration of the fatty acid or whether it was presented as free acid or sodium salt, no oxidized fatty acids could be generated in the system.
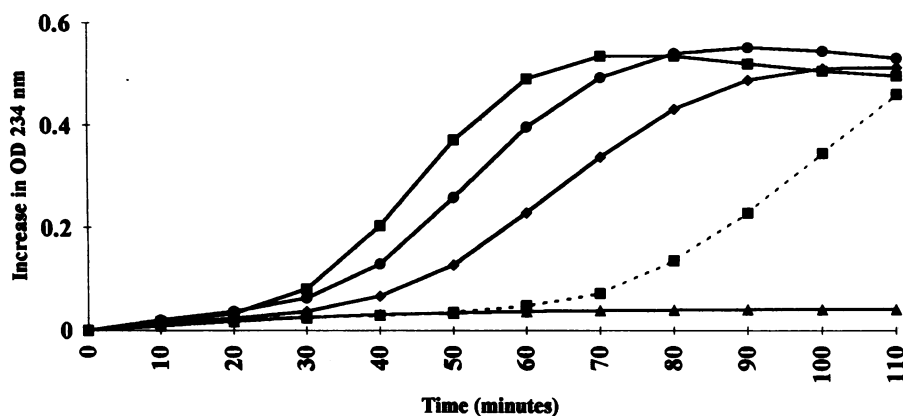

Figure 6. Effect of vitamin E on LDL oxidation by H_2O_2/LPO. 50 μg of human LDL was incubated with 50 μM H_2O_2 and 1 U LPO in the presence of specified concentrations of vitamin E in a total volume of 1 ml PBS, pH 7.4. OD at 234 nm was monitored continuously. The figure represents results from a typical experiment. ■---■, control LDL; ■—■, LDL + 1 μM vitamin E; ●, LDL + 2 μM vitamin E; ◆, LDL + 3 μM vitamin E; ▲, control without LDL.
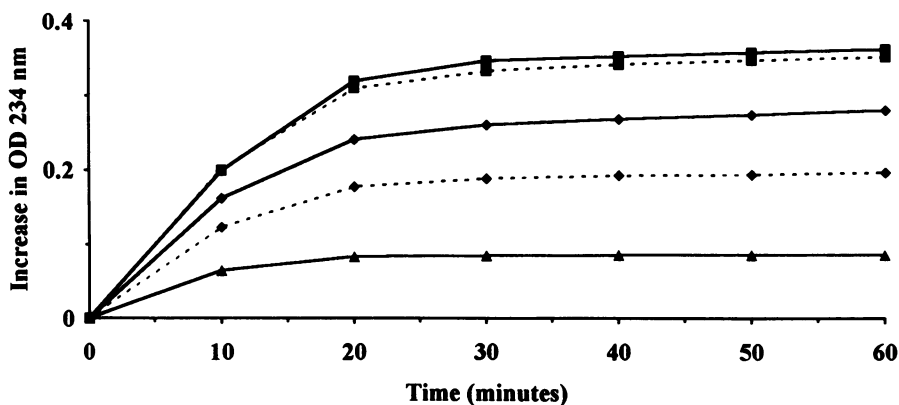
Figure 7. Effect of probucol on LDL oxidation by H_2O_2/LPO. The figure represents results from a representative experiment. ■—■, control LDL; ■---■, LDL + 1 μM probucol; ◆—◆, LDL + 2 μM probucol; ◆---◆, LDL + 3 μM probucol; ▲, LDL + 5 μM probucol.

Addition of vitamin E or tyrosine to the system had no effect on the formation of oxidized fatty acids. In contrast, the fatty acid was readily oxidized by soybean lipoxygenase (SLO). Phospholipid liposomes prepared with increasing amounts of vitamin E were also not oxidized. However, phospholipid-deoxycholate micelles were oxidized by the peroxidase system and the inclusion of vitamin E prevented the formation of conjugated dienes (results not given). These studies may indicate that the physical state of lipids and their proximity to the radical generated in lipoproteins may be important in the oxidation by peroxidases. However, the intermediate formation and participation of a deoxycholate radical cannot be ruled out, as the peroxidase system is capable of oxidizing a variety of substrates.

The mechanism(s) of in vivo oxidation of LDL could only be speculated at present. If peroxidases are involved, there are several candidates to be considered. There is no HRP in the system. However, MPO, LPO, prostaglandin H synthase, and thyroid peroxidase are present in a variety of tissues. In addition, lipoxygenases, particularly 15-lipoxygenase reaction has an associated peroxidase activity (33). Recently, it was suggested that MPO is associated with macrophages in the atherosclerotic lesion, and the participation of this enzyme in the oxidation of LDL was suggested (25, 34). However, as mentioned before,

the presence of free tyrosine was suggested to be obligatory for the oxidation of lipoproteins by MPO. The present findings that LDL was oxidized readily by MPO in the absence of added tyrosine and such additions actually inhibited the oxidation of LDL by MPO are in direct contradiction to the findings of Heinecke and co-workers (25, 29, 31). This discrepancy can be readily explained by the nature of LDL used. The LDL samples used in this study were isolated by a rapid isolation technique and were used within hours after isolation. Usually < 24 h elapsed between the collection of plasma and the completion of the experiment. Under these conditions, one would expect that vitamin E is preserved and is readily available for oxidation by the enzyme. In contrast, the LDL used by Savenkova et al. (25) was obtained from another laboratory at Seattle and may have already been at least partially depleted of its vitamin E content. Under such conditions, the formation of tyrosyl radical may be needed to initiate the oxidation of LDL lipids. It is interesting that these studies also lend support for our hypothesis that the peroxidases themselves do not initiate the oxidation of LDL lipids as MPO itself was ineffective in the absence of added tyrosine.

In these studies, direct demonstration of the formation of vitamin E radical during the initiation of oxidation was not feasible. However, ESR studies carried in parallel demonstrated a rapid formation of the tocopheroxy radical after the addition of HRP to the system (35). The tocopheroxy radical disappeared over the course of oxidation and the formation of new protein-derived radicals could be seen.

Despite vast interest in the oxidative processes that may be involved in atherogenesis, the specific nature of the involvement is poorly understood. On the one hand there is an increased
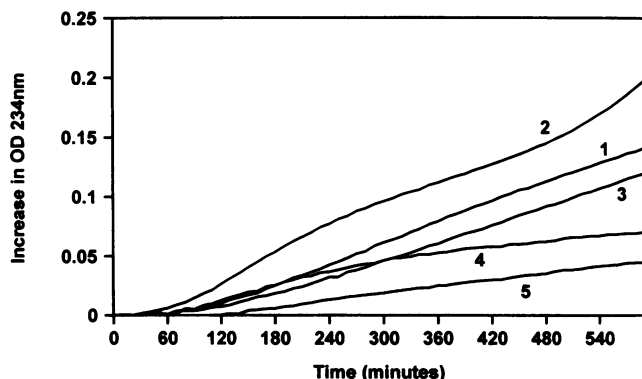

Figure 8. Effect of vitamin E on LDL oxidation by H_2O_2/MPO. 50 μg of human LDL was incubated with 50 μM H_2O_2 and 0.1 U MPO in the presence of specified concentrations of vitamin E in a total volume of 1 ml PBS, pH 7.4. OD at 234 nm was monitored continuously. The figure represents results from a representative experiment. Line *1*, control LDL; line *2*, LDL + 0.25 μM vitamin E; line *3*, LDL + 1 μM vitamin E; line *4*, LDL + 0.5 μM probucol; line *5*, LDL + 1.0 μM probucol.

Figure 9. Effect of tyrosine on LDL oxidation by H_2O_2/MPO. 50 μg of human LDL was incubated with 50 μM H_2O_2 and 0.1 U MPO in the presence of 100 μM tyrosine in a total volume of 1 ml PBS, pH 7.4. OD at 234 nm was monitored continuously. The figure represents results from a representative experiment.
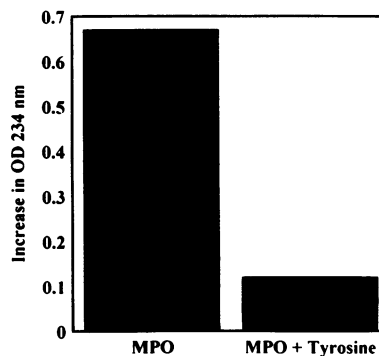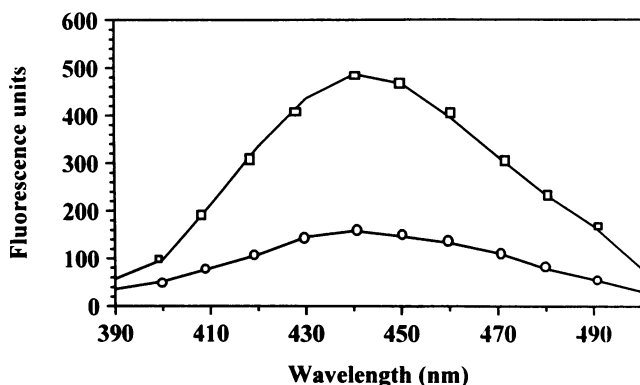
Figure 10. Effect of tyrosine on LDL oxidation by H_2O_2/MPO. 50 μg of human LDL was incubated with 50 μM H_2O_2 and 0.1 U MPO in the presence or absence of 100 μM tyrosine in a total volume of 1 ml PBS, pH 7.4. Fluorescence spectrum was monitored with excitation wavelength 330 nm and emission wavelength 390–500 nm.

urgency to validate the hypothesis in humans. On the other hand, a detailed understanding of the mechanism(s) of the oxidative pathways is warranted, as such an understanding would lead to the development of more specific inhibitors. Furthermore, an understanding of the malfunctioning of the oxidative pathway would help to devise preventive strategies. The findings of the epidemiological studies and prospective studies may suggest beneficial effects of long-term consumption of antioxidants in normal humans (1–3). On the other hand, the deleterious effects of beta carotene and the lack of effect of vitamin E observed in smokers in preventing cancer deaths (10) would suggest that small doses of antioxidants may be ineffective in oxidatively challenged humans. The results presented in our study confirm the findings of Bowry et al. (26) which suggest that, when antioxidant radicals are generated, they may participate and promote further oxidation. Adequate availability of free antioxidants to inhibit the propagation of lipid peroxidation or

Table I. Lack of Oxidation of Linoleic Acid by Peroxidases in the Presence or Absence of Vitamin E and Tyrosine

Incubation conditions	Linoleate oxidation
	nmol/h
Linoleic acid alone	1.5
Linoleic acid + SLO	36
Linoleic acid + H_2O_2/HRP	2.3
Linoleic acid + H_2O_2/HRP + tyrosine	1.6
Linoleic acid + H_2O_2/HRp + vitamin E	2.2
Linoleic acid + tyrosine	1.6
Linoleic acid + MPO/H_2O_2	1.5
Linoleic acid + MPO/H_2O_2 + tyrosine	1.5

100 nmol of [^{14}C]linoleic acid was incubated with the corresponding oxidizing system (HRP, MPO, or SLO) in the presence or absence of tyrosine (100 μM) or vitamin E (2 μM) for 1 h at 37°C. Total lipids were extracted and separated on thin layer chromatography using chloroform:methanol:water (90:10:0.5; vol/vol/vol) as the solvent system. Fatty acid spots were identified by exposure to iodine vapors and counted in a scintillation counter. At least three separate experiments were performed and the results represent one of the three experiments.

the choice of an antioxidant that does not generate a propagating radical may afford better protection.

Acknowledgments

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