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Peroxidase-dependent metal-independent oxidation of low density lipoprotein *in vitro*: A model for *in vivo* oxidation?

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ABSTRACT **Oxidative modification of low density lipoprotein is believed to be an important pathway by which the lipoprotein becomes atherogenic. The *in vitro* systems for oxidative modification of low density lipoprotein thus far described all appear to depend upon the presence in the medium of free transition metal ions (copper or iron). *In vivo*, on the other hand, these metals are present almost exclusively in tightly complexed forms that do not catalyze oxidative modification. The present studies describe oxidation of low density lipoprotein in a simple system that does not depend upon the presence of added free metal ions. It requires the presence of horseradish peroxidase and either hydrogen peroxide or lipid hydroperoxides.**

Oxidative modification of low density lipoprotein (LDL) alters its physical and biological properties in a number of ways, many of which have the potential of increasing its atherogenicity (1–3). A large body of evidence now establishes that oxidative modification of LDL does indeed occur *in vivo* (1–3), and a series of studies in rabbits and in primates show that the appropriate use of antioxidant supplements can markedly suppress the rate of progression of experimental atherosclerosis, both that due to LDL receptor deficiency (4, 5) and that due to cholesterol feeding (6–8). The addition of even low concentrations of serum to cell culture medium prevents oxidative modification of LDL, suggesting that the oxidative modification must occur outside the vascular system. However, very little can be said at this time about exactly where and exactly how oxidative modification of LDL occurs *in vivo*. A particular paradox is presented by the fact that oxidation in *in vitro* systems absolutely requires the presence of metal ions, being completely inhibited by the addition of chelating agents (1, 3). Yet *in vivo*, under normal conditions at least, the transition metals most effective in the oxidation of LDL—copper and iron—are tightly bound to proteins (e.g., ceruloplasmin and transferrin) and their concentration in free form is vanishingly low. Only under pathologic conditions, when there is tissue damage, is the capacity of the binding proteins exceeded, and only then do significant concentrations of metal ion build up (e.g., in hemachromatosis). Under such conditions, hemin or hemoglobin may promote the oxidation of LDL (9, 10).

In this paper, we describe a system independent of added free metal ions for the oxidative modification of LDL. It depends upon the ability of peroxidase—horseradish peroxidase (HRP) in the present studies—to generate free radicals in LDL *pari passu* with the breakdown of hydrogen peroxide or of lipid hydroperoxides.

MATERIALS AND METHODS

Materials. HRP, type VI (P-8375) and type XII (P-8125) (285 units/mg of protein); soybean lipoxygenase (SLO), type V (L-6632; 646,000 units/mg of protein); glucose oxidase, type 11-S (G-6644, 50 units/mg of protein); H_2O_2; fatty acid-free bovine serum albumin; and other reagent chemicals were purchased from Sigma. Cell culture media and supplies were obtained from GIBCO.

Methods. LDL (d = 1.019–1.063 g/ml) was isolated by ultracentrifugation from pooled human plasma, kept always in the presence of EDTA (1 mg/ml) (11). LDL was radioiodinated using Iodo-Gen and carrier-free ^{125}I (12). LDL concentration after isolation was usually 6–8 mg/ml. LDL was used within 2 weeks of isolation. Protein was determined by the method of Lowry *et al.* (13).

For modification of LDL by the H_2O_2/HRP system, labeled LDL was incubated in 60-mm culture dishes in 2 ml of serum-free Ham's F-10 medium or in phosphate-buffered saline (PBS) at 37°C for 16 h. HRP and H_2O_2 were prepared daily and added from fresh sterile solutions. Oxidation of LDL by Cu^{2+} was carried out using 5 μM copper sulfate and LDL protein (100 μg/ml) in PBS.

Washed confluent rabbit aortic endothelial cells or bovine aortic endothelial cells were incubated in serum-free Ham's F-10 medium for 24 h. The concentration of H_2O_2 in the harvested medium (conditioned medium) was determined using a chemiluminescence assay in the presence of luminal (14).

To generate H_2O_2 enzymatically, glucose oxidase (16 units/ml) was incubated with Ham's F-10 for specified periods of time. Glucose present in the medium (1100 mg/liter) served as substrate. H_2O_2 was determined as described (14).

Lipid peroxidation was determined using the thiobarbituric acid-reactive substance (TBARS) assay and expressed as malondialdehyde equivalents. Acetyl-LDL was prepared as described by Goldstein *et al.* (15). Resident mouse peritoneal macrophages were used for lipoprotein degradation studies.

Agarose gel electrophoresis was performed using lipoprotein samples of similar radioactivity at 30 mA and 300 V for 2 h in barbitol buffer. The gel was fixed in 5% (vol/vol) acetic acid/70% (vol/vol) ethyl alcohol for 1 h, dried, and subjected to autoradiography (16). SDS/PAGE analysis of apoprotein B was carried out as described by Fong *et al.* (17) on 3–14% gradient gels. Lysophosphatidylcholine was determined after thin layer chromatography (TLC), as described by Steinbrecher *et al.* (18). TLC for polar fatty acids was carried out using silica gel G plates and the solvent system chloroform/methyl alcohol/acetic acid/water 90:10:0.5:0.5 (vol/vol).

Modification of LDL by Sequential Treatment with SLO and HRP. LDL (1 mg/ml) in PBS was treated with SLO (15,000 units/ml) at 37°C for 16 h to generate lipid hydroperoxides. Control incubations were performed without SLO. After 24 h, aliquots of the LDL (usually 100 μg) were incubated with 0.5 unit of HRP for an additional 24 h in 1 ml of PBS and then

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Abbreviations: LDL, low density lipoprotein; HRP, horseradish peroxidase; SLO, soybean lipoxygenase; TBARS, thiobarbituric acid-reactive substance.

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evaluated for oxidative modification. The samples were filtered using a 0.45-μm (pore size) filter to ensure sterility and that no aggregated LDL was added. More than 90% of the initial ^{125}I radioactivity was recovered as trichloroacetic acid-precipitable radioactivity in the filtered solution.

RESULTS

Modification of LDL by Incubation with H_2O_2 and HRP. As shown in Table 1, the simultaneous incubation of ^{125}I-labeled LDL with H_2O_2 and HRP resulted in significant LDL oxidation, whereas incubation of LDL with either one alone did not. Assessed in terms of the increase in rate of degradation by macrophages, the oxidative modification induced by H_2O_2 plus HRP was almost as great as that induced by incubation with Cu^{2+}. Assessed in terms of reactivity with TBARS, the oxidation, while very significant, was only about 50% as great as that induced by incubation with Cu^{2+}. Boiled HRP was without effect. The possibility that some of the heme iron had been released was considered. However, the addition of even a very high concentration of EDTA (10 mM) had very little effect on H_2O_2/HRP-induced oxidation (see Table 5). However, nonheme peroxidases such as glutathione peroxidase appeared to be ineffective in this system (data not shown), suggesting that the heme-bound iron may well be involved.

Early in the course of this investigation, type VI HRP was used, as in Table 1, experiment A. We then found that affinity-purified HRP, type XII, was considerably more active and could be used at much lower concentrations. As shown in Table 1, experiment B, type XII could be used at 10% the concentration of type VI with comparable results.

In some of the initial studies using the less-purified preparation of HRP, incubation of LDL with HRP alone did result in some degree of oxidative modification but with the affinity-purified HRP that was not seen. In retrospect, and as brought out by the experiments described below, the effects of HRP alone were probably obtained when older preparations of LDL were used, preparations that may already have contained some significant amount of lipid hydroperoxide.

The increased uptake and degradation of LDL previously incubated with HRP/H_2O_2 were shown to be attributable to macrophage scavenger receptors; i.e., unlabeled Cu^{2+}-oxidized LDL was an effective competitor (data not shown). Acetyl-LDL competed to a significant extent whereas unlabeled native LDL showed no competition.

Table 1. Modification of LDL incubated in the presence of H_2O_2 plus HRP

Condition of incubation	TBARS	Macrophage degradation
Experiment A		
Unincubated LDL	4.8	1.4
LDL incubated without additions	6.4	1.3
LDL + H_2O_2 alone (0.2 mM)	6.6	1.4
LDL + HRP alone (5 units, type VI)	6.6	0.8
LDL + H_2O_2 + boiled HRP	6.6	0.8
LDL + H_2O_2 + HRP	28.4	8.2
LDL + Cu^{2+}	57.9	11.6
Experiment B		
Unincubated LDL	0.7	2.1
LDL + H_2O_2 alone (0.2 mM)	4.0	1.2
LDL + HRP alone (0.5 unit, type XII)	2.8	2.3
LDL + H_2O_2 + HRP	22.3	7.3
LDL + Cu^{2+}	53.5	9.8

^{125}I-labeled LDL (100 μg/ml) was incubated for 16 h in a final volume of 2 ml of Ham's F-10 medium at 37°C. Results are averages of duplicates from a representative experiment. Three or more individual experiments were performed in each set. Experiments A and B were performed with 5.0 units (type VI) and 0.5 unit (type XII) of HRP, respectively. TBARS is reported in nmol of malondialdehyde per mg of LDL and macrophage degradation is reported in μg of LDL per 5 h per mg of cell protein.

LDL preincubated with HRP/H_2O_2 showed the same physical/chemical changes that accompany oxidation of LDL induced by cells or by incubation with Cu^{2+}—an increased electrophoretic mobility, fragmentation of apoprotein B, an increase in lysophosphatidylcholine, and an increase in more polar (oxidized) fatty acids (data not shown).

Modification of LDL by Pretreatment with SLO Followed by Treatment with HRP. Earlier studies from this laboratory (19) showed that LDL treated with SLO alone, despite evidence of some degree of oxidation, was not degraded more rapidly by mouse peritoneal macrophages. However, addition of phospholipase A_2 along with the SLO resulted in full oxidative modification of the LDL. It was inferred that the SLO treatment generated some level of lipoperoxides but that propagation was not sufficiently rapid or extensive to result in an LDL recognized by the scavenger receptor. In the present experiments, we again treated LDL with SLO alone, under conditions that did not convert it to a ligand for the scavenger receptors, and then treated this product with HRP. As shown in Table 2, this sequential treatment generated a modified LDL that was taken up and degraded by macrophages at a rate three times that of control LDL. This did not depend on the presence of hydrogen peroxide, since preincubation of the SLO-treated LDL with catalase did not alter the result of the subsequent incubation with HRP. Nor was the HRP effect due to contamination with phospholipase A_2 activity; direct assay revealed no detectable phospholipase A_2 activity in the HRP. Incubation of LDL with SLO alone or with HRP alone did not change its electrophoretic mobility. However, when the LDL was first incubated with SLO and then with HRP, the product showed both increased electrophoretic mobility and increased degradation by macrophages. The simplest interpretation of these results is that treatment with SLO alone generates some level of lipid hydroperoxides in the LDL but that, in the absence of propagation and decomposition of lipid hydroperoxides, there is no detectable change in electrophoretic mobility, nor is the LDL converted to a ligand for the scavenger receptor. The subsequent incubation with HRP must generate initiating free radicals, either from SLO-generated lipid hydroperoxides or from other LDL components. Then, propagation yields a more fully oxidized LDL, as recognized by changes in charge and changes in recognition by scavenger receptors.

Table 2. Oxidative modification of LDL by pretreatment with SLO followed by incubation with HRP

Condition of incubation	Macrophage degradation
Preincubated without SLO for 24 h	1.62 ± 0.30 (7)
Followed by a 24-h incubation with	
HRP (0.5 unit)	2.01 ± 0.43 (7)
Boiled HRP (0.5 unit)	1.90 ± 0.31 (3)
HRP (0.5 unit), preceded by a 15-min incubation with catalase (1.0 unit)	1.80 ± 0.27 (3)
Preincubated with SLO for 24 h	1.81 ± 0.42 (7)
Followed by a 24-h incubation with	
HRP (0.5 unit)	5.73 ± 1.36 (7)
Boiled HRP (0.5 unit)	1.41 ± 0.19 (3)
HRP (0.5 unit), preceded by a 15-min incubation with catalase (1.0 unit)	6.12 ± 1.40 (3)
Unincubated LDL	1.43 ± 0.21 (7)

^{125}I-labeled LDL (100 μg/ml) was incubated in PBS with or without 1250 units of SLO for 24 h. Aliquots of these samples were then further incubated for 24 h with the additions indicated. Degradation by macrophages was measured as described (16) and reported as μg per 5 h per mg of cell protein. Numbers in parentheses are *n*.

Oxidative Modification of LDL by HRP by Using Cell-Conditioned Medium as the Source of H_2O_2. Previous studies have shown that rabbit aortic endothelial cells can effectively oxidize LDL in culture but that conditioned medium from such cells is without effect (1). To test whether the amounts of H_2O_2 released by such cells might be sufficient to support HRP-induced oxidative modification, we incubated rabbit aortic endothelial cells in Ham's F-10 medium for 24 h (in the absence of LDL) and then added that conditioned medium plus HRP to a control preparation of LDL. As shown in Table 3, after a 24-h incubation with both HRP and conditioned medium, the oxidative modification was as extensive as that obtained by Cu^{2+}-induced oxidation or that induced when both H_2O_2 and HRP were incubated simultaneously with LDL. Incubation of the LDL with conditioned medium alone resulted in no oxidative modification (data not shown). When the conditioned medium was first treated with catalase and then incubated with HRP, there was no oxidative modification. When conditioned medium obtained from bovine aortic endothelial cells ($BAEC_{11}$), a cell line that poorly oxidizes LDL, was used, there was very little modification. In other studies, H_2O_2 was generated during the incubation with HRP and LDL by including glucose (1100 mg/liter) and glucose oxidase (16 units/ml) in the medium. This system yielded similar results.

Effects of HRP and of SLO on Conjugated Diene Formation from LDL or from Linoleic Acid. As discussed above, particularly in our early studies using a less-purified HRP preparation, some oxidative modification was induced by HRP alone and this appeared to be associated with the use of LDL preparations that had been stored for longer periods of time. We attribute those results to the presence of lipoperoxides in the LDL sample. Fresh LDL preparations showed no such effect. In Table 4, we show the results of an experiment consistent with this interpretation. Incubation of linoleic acid with SLO led to the expected increase in diene conjugation. In contrast, incubation of linoleic acid with HRP, either in the presence or absence of H_2O_2, did not change diene conjugation at all, consistent with the fact that SLO can act on linoleic acid whereas HRP cannot.

In contrast, the incubation of LDL with HRP alone showed a distinct increase in diene conjugation (from A_{234} = 0.12 to A_{234} = 1.12). The incubation with SLO alone showed somewhat greater effect, but the HRP effect is clearly significant. The magnitude of the HRP effect was quite variable, possibly depending on the levels of preformed lipoperoxides in the various LDL preparations. Addition of HRP to the SLO-preincubated LDL did not increase the dienes further but appears to have actually decreased the net dienes in the subsequent incubation.

Table 3. Modification of LDL by HRP using cell-conditioned medium as the source of hydrogen peroxide

Medium	Macrophage degradation
Fresh F-10 medium + H_2O_2/HRP	8.9
F-10 medium conditioned by incubation with rabbit endothelial cells + HRP	10.7
F-10 medium conditioned by incubation with bovine endothelial cells + HRP	0.9
DMEM conditioned by incubation with bovine endothelial cells + HRP	0.9

Conditioned medium from the cells was obtained by overnight incubation of washed cells with 2 ml of the appropriate medium. LDL (100 μg/ml) was then incubated in the conditioned medium at 37°C for 24 h. Results are duplicates from a representative experiment. When the LDL preparation used in this experiment was oxidized by addition of 5 μM Cu^{2+} to fresh F-10 medium, the resulting modified LDL was degraded by macrophages at a rate of 11.9 μg per 5 h per mg of cell protein. Type VI HRP was used. Macrophage degradation is reported as μg of LDL degraded per 5 h per mg of cell protein. DMEM, Dulbecco's modified Eagle's medium.

Table 4. Generation of conjugated dienes during the incubation of LDL and linoleic acid with HRP and SLO

Condition of incubation	Increase in A_{234}, units
Linoleic acid only	0.20
Linoleic acid + SLO	2.66
Linoleic acid + HRP	0.22
LDL only	0.12
LDL + HRP	1.12
LDL + SLO	2.12
LDL + SLO + HRP	1.72

LDL (100 μg) or linoleic acid (200 nmols) in 1 ml of PBS was incubated with 1500 units of SLO or 1 unit of HRP (type XII) for 16 h. A_{234} was followed continuously. Results given are the values from one of four similar studies. Linoleic acid oxidation in the presence of both SLO and HRP was not separately studied due to the very rapid oxidation of the fatty acid by SLO alone.

Effects of Inhibitors on Modification of LDL by H_2O_2 and HRP. As shown in Table 5, neither mannitol, an hydroxyl radical trapping agent, nor superoxide dismutase showed any inhibitory effect. EDTA, even at 10 mM, had only a marginal effect. Catalase inhibited by almost 90% but boiled catalase had no effect. Butylated hydroxytoluene inhibited strongly, indicating participation of free radicals.

DISCUSSION

These studies show clearly that LDL can be oxidatively modified by incubation with both H_2O_2 and HRP but not by incubation with either of them alone. Modification by H_2O_2/HRP was just as great in PBS as it was in F-10 medium and EDTA inhibited only marginally. Thus, the mechanism involved does not appear to require added free metal ions. The inability of boiled HRP to catalyze the oxidation of the lipoprotein and the lack of inhibition by EDTA also suggest that release of free iron from HRP is not required for the oxidation. The failure of superoxide dismutase to inhibit appears to rule out the generation of superoxide anion as the radical intermediate and yet the effectiveness of butylated hydroxytoluene points to involvement of some free radical. Mannitol, a hydroxyl radical trapping agent, did not inhibit

Table 5. Effects of inhibitors on the modification of LDL by H_2O_2 plus HRP

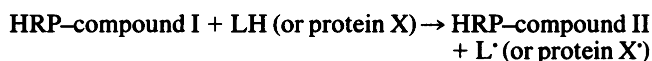
Treatment of LDL	Macrophage degradation rate, %
No addition	13.5
H_2O_2/HRP alone	100.0
H_2O_2/HRP + mannitol (10 mM)	107.0
H_2O_2/HRP + EDTA (10 mM)	73.0
H_2O_2/HRP + catalase (100 μg/ml)	12.3
H_2O_2/HRP + boiled catalase (100 μg/ml)	130.0
H_2O_2/HRP + BHT (40 μmol/liter)	22.0
H_2O_2/HRP + SOD (110 μg/ml)	106.0
Cu^{2+} (5 μM)	140.0

LDL (100 μg/ml) was incubated in Ham's F-10 medium for 16 h at 37°C under the indicated conditions. H_2O_2 was added to a final concentration of 200 μM; type VI HRP was added at 5 units/ml. Results are averages of duplicate incubations from representative experiments. The rate of degradation of LDL incubated with H_2O_2 and HRP alone was set equal to 100%. The absolute values ranged from 6.0 to 8.2 μg of LDL degraded per 5 h per mg of cell protein. Data are reported as the percent of LDL incubated with H_2O_2/HRP alone. BHT, butylated hydroxytoluene; SOD, superoxide dismutase.

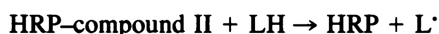
(Table 5), but that negative result should be interpreted cautiously. In some systems, mannitol cannot find access to the hydroxyl radicals because they are generated at a specific localized site and react very quickly near that site. For example, if hydroxyl radical were generated at the surface of HRP (in the vicinity of the iron), mannitol might not be effective. On the other hand, if the hydroxyl radical were generated in such close proximity to the HRP, it seems unlikely that it would be able to diffuse away and have any effect on the LDL particle. We are driven to conclude that H_2O_2/HRP is generating a free radical from one or more components of LDL.

There is ample precedent for a mechanism of this kind. HRP and other heme-containing peroxidases can react with H_2O_2 and generate intermediates that oxidize a wide array of cosubstrate compounds (20), including free fatty acids (21) and phospholipids (22). The direct oxidation of free fatty acids by HRP is, however, very unlikely (Table 4). Mason and coworkers have recently elucidated the intimate mechanisms of H_2O_2/HRP oxidation of several phenolic compounds (23), deoxyribose (24), and N-substituted aromatic amines (25). The present findings with respect to H_2O_2/HRP oxidation of LDL could be explained by an analogous mechanism. Let L represent a polyunsaturated fatty acid—free or in phospholipids or cholesterol esters—and X represent any other candidate cosubstrate in LDL (e.g., amino acid residues, minor lipids, or even α-tocopherol). HRP compounds I and II represent the postulated enzyme intermediate complexes generated during HRP catalysis of H_2O_2 degradation.

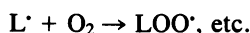
$$\text{HRP} + H_2O_2 \rightarrow \text{HRP–compound I} + H_2O$$



$$\text{HRP–compound I} + \text{LH (or protein X)} \rightarrow \text{HRP–compound II} + \text{L}^{\cdot} \text{(or protein X}^{\cdot}\text{)}$$



$$\text{HRP–compound II} + \text{LH} \rightarrow \text{HRP} + \text{L}^{\cdot}$$



$$\text{L}^{\cdot} + O_2 \rightarrow \text{LOO}^{\cdot}\text{, etc.}$$

Although HRP did not oxidize native LDL unless H_2O_2 was also present, it did oxidize LDL that had been pretreated with SLO to generate lipid hydroperoxides. This may be explained by the ability of heme-containing proteins or of hematin to generate free radicals from lipid hydroperoxides (26–28), a reaction not necessarily dependent on the catalytic site of the heme-containing enzyme. For partially oxidized LDL in the present studies, we would propose generation of peroxy or alkoxy radicals followed by propagation or oxidation to an epoxide of an adjacent double bond (29). We have reported that HRP greatly increases the generation of fluorescent products from bovine serum albumin incubated with linoleic acid hydroperoxide (30). Here again HRP may work via its heme iron to degrade lipid hydroperoxide (LOOH) to $\text{LOO}^{\cdot}$, initiating a chain reaction. In analogous fashion, when LDL is pretreated with lipoxygenase alone, there is an increase in diene conjugation (A_{234}) but no fluorescence (data not shown).

Whatever the precise molecular mechanisms involved, these studies demonstrate that LDL oxidation can occur independently of the availability of free metal ions. H_2O_2 is produced by most animal cells and passes freely across membranes so that it can be released to the extracellular fluid. Whether it plays a major role in LDL oxidation is not known with certainty, but there is some evidence that it may. For example, Montgomery *et al.* (31) have reported the secretion of H_2O_2 by endothelial cells. Studies by Steinbrecher (32) and unpublished results from our laboratory suggest that catalase may have unexplained inhibitory effects on the oxidation of LDL by cells in culture. Moreover, many cells are known to contain heme-peroxidases that can utilize lipid hydroperoxides. For example, prostaglandin H synthase, which has an intrinsic peroxidase activity, can oxidize a variety of cosubstrates in the presence of lipid peroxide (33, 34). One attractive aspect of this hypothesis is that there would be no need to postulate a microenvironment *in vivo* containing free metal ions. The lipid peroxides generated within the cell could then translocate across the plasma membrane and transfer to the lipoproteins in the intercellular space.

There are undoubtedly a number of different mechanisms by which cells can catalyze the oxidative modification of LDL. Free radicals are produced by cells at several sites, including the mitochondrial electron transport chain, the several cytochrome P-450 systems, the NADPH oxidase system, lipoxygenases, cyclooxygenases, etc. "Leakage" of one or another free radical from any of these systems could in principle contribute to the oxidation of extracellular LDL—either by release of reactive oxygen itself or by release of peroxidized cell constituents that enter and confer instability on the LDL particle. The most likely candidate in this latter category would be lipoperoxides generated by the action of lipoxygenases or other lipid-oxidizing systems in the cell. Whether or not the metal-independent mechanism suggested by the present studies is operative *in vivo* remains to be determined.

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