Free Rad. Res., Vol. 29, pp. 427–434
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Oxidative Modification of Human Low-Density Lipoprotein by Horseradish Peroxidase in the Absence of Hydrogen Peroxide

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Accepted by Prof. H. Sies

(Received 20 April 1998; In revised form 19 June 1998)

Heme-peroxidases, such as horseradish peroxidase (HRP), are among the most popular catalysts of low density lipoprotein (LDL) peroxidation. In this model system, a suitable oxidant such as H_2O_2 is required to generate the hypervalent iron species able to initiate the peroxidative chain. However, we observed that traces of hydroperoxides present in a fresh solution of linoleic acid can promote lipid peroxidation and apo B oxidation, substituting H_2O_2.

Spectral analysis of HRP showed that an hypervalent iron is generated in the presence of H_2O_2 and peroxidizing linoleic acid. Accordingly, careful reduction of the traces of linoleic acid lipid hydroperoxide prevented formation of the ferryl species in HRP and lipid peroxidation. However, when LDL was oxidized in the presence of HRP, the ferryl form of HRP was not detectable, suggesting a Fenton-like reaction as an alternative mechanism. This was supported by the observation that carbon monoxide, a ligand for the ferrous HRP, completely inhibited peroxidation of LDL.

These results are in agreement with previous studies showing that myoglobin ferryl species is not produced in the presence of phospholipid hydroperoxides, and emphasize the relevance of a Fenton-like chemistry in peroxidation of LDL and indirectly, the role of preexisting lipid hydroperoxides.

Keywords: Hydroperoxides, horseradish peroxidase, low density lipoprotein, linoleic acid

INTRODUCTION

The oxidation by heme-peroxidases is an accepted suitable model for the study of the mechanism of low density lipoprotein (LDL) modification in the early phases of atherosclerosis.[1] Increased peroxidase activity in atherosclerotic lesions supported the concept that a peroxidase activity may actually promote LDL oxidation *in vivo*.[2]

The described and most frequently adopted peroxidase-mediated procedure for LDL oxidative modification requires horseradish peroxidase (HRP) or myeloperoxidase, and H_2O_2 as oxidant.[3–5]

The initiation of peroxidation is attributed to the hypervalent oxoferryl species (Fe(IV) = O). Oxoferryl species can directly attack lipids,[6]

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apo B and/or α-tocopherol,[7,8] or utilize intermediates at low molecular weight, able to convey oxidizing equivalents from the active site of the enzyme to the target of the damage.[1,9] In this study, we report that in HRP-mediated LDL peroxidation, hydrogen peroxide is not necessary and that the initiating radicals are produced from the decomposition of pre-existing lipid hydroperoxides of LDL, *via* redox cycling of heme moiety between ferric and ferrous forms.

MATERIALS AND METHODS

Materials

Horseradish peroxidase type IX, linoleic acid sodium salt, diethylene triamine pentaacetic acid (DETAPAC), superoxide dismutase (SOD), catalase, glutathione (GSH), sodium borohydride, potassium cyanide, fluoresceinamine, chelex-100, phosphate-buffered saline (PBS, containing 0.01 M phosphate buffer, 0.0027 M KCl and 0.138 M NaCl, pH 7.4) and bovine albumin were from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) was from BDH Laboratory Supplies (Poole, UK). Cetyltrimethyl ammonium bromide was from ICN Biomedicals Inc. (Aurora, Ohio, USA). Hydrogen peroxide, chloroform, methyl and ethyl alcohol were from Carlo Erba (Milano, Italy). Paragon lipo electrophoresis system was from Beckman (Fullerton, CA, USA).

Methods

LDL and Apo B Separation

LDL (d 1.019–1.063 g/ml) was isolated from plasma from normal human volunteers after an overnight fast and collected in EDTA (1 mg/ml) by sequential ultracentrifugation in salt solutions, according to Havel *et al.*,[10] using a Beckman T-100 bench-top ultracentrifuge (T-100.3 rotor). LDL solution was flushed with N_2 stored at 4°C and used within one week from the preparation.

LDL was delipidated by extraction of lipids with chloroform/methanol and apo B was resuspended in the solubilization buffer (10 mM PBS, pH 7.4, 1% SDS).[11] Apo B solubilization was verified by measuring the protein content before and after extraction. A 90% recovery was usually obtained.

Protein was measured by the method of Lowry *et al.*[12] using bovine serum albumin as standard.

LDL Apo B and Linoleic Acid Oxidation

For oxidation experiments, LDL was dialyzed in the dark for 24 h at 4°C against two changes of 1 L each (1000 fold volume) of 0.01 M PBS, pH 7.4.

LDL (200 μg protein/ml) was incubated with 5 U/ml type-IX HRP (5.5 μg/ml) in PBS at 37°C for 16 h in the presence or absence of 200 μM H_2O_2. Apo B (500 μg protein/ml) was incubated with HRP (5 U/ml) and 1 mM commercial not further-purified linoleic acid in PBS at 37°C for 16 h. The SDS final concentration was 0.1% (w/v). At the end of the incubation, samples were immediately processed for the measure of oxidative changes.

In order to verify the formation of ferrous heme, in some experiments PBS and LDL were extensively flushed to equilibrium with carbon monoxide (CO) prior to the incubation with HRP.

Hydroperoxide-containing commercial linoleic acid (500 μM) was incubated with type-IX HRP (5 U/ml) in PBS (0.1% SDS) at 37°C in the presence and absence of 100 μM DETAPAC, 2 mM KCN, 100 U/ml superoxide dismutase (SOD), 100 U/ml catalase, 3 mM reduced glutathione (GSH), 1 μM H_2O_2. In some experiments linoleic acid (0.1 M) was pre-treated with $NaBH_4$ (0.3 M), to reduce endogenous hydroperoxides. The excess of $NaBH_4$ was destroyed by drop-wise addition of concentrated HCl up to pH 4.

SDS, present at concentration 0.1% (w/v) in the reaction mixtures to ease the solubility of both delipidated apo B and linoleic acid, was assessed to not interfere with the activity of the enzymes used in the experiments (HRP, catalase, SOD) (data not shown).

Measure of Oxidative Changes

The time course of conjugated dienes formation was followed by continuously monitoring the absorbance at 234 nm, using a Beckman DU 70 spectrophotometer thermostated at 37°C.

Fluorescence was measured at excitation 360 nm and emission 430 nm, with emission and excitation slit widths at 5 nm (LS-5, Perkin-Elmer).[13] Carbonyl groups were assayed after the reaction with fluoresceinamine.[14] Tryptophan residues were determined by the measure of intrinsic fluorescence in cetyltrimethyl ammonium bromide.[15] LDL electrophoresis was performed at pH 8.6 in 0.05 M barbital buffer on 0.5% agarose gels,[16] using a Beckman Paragon apparatus. The gels were stained with Sudan B black. The increased electrophoretic mobility of LDL was expressed relative to the mobility (REM) of native LDL. The redox transitions of the heme group were followed by monitoring spectra of HRP, using a Beckman DU 70 spectrophotometer thermostated at 37°C, before and after addition of linoleic acid and GSH.

Statistical Analysis

Data were expressed as mean and standard error. Statistical analysis was performed by single factor analysis of variance (ANOVA). The significance between differences was set at $p < 0.01$.

RESULTS

In human LDL (200 μg/ml) incubated in the presence of 5 U/ml type IX HRP and 200 μM H_2O_2, the protein moiety underwent oxidative damage, as indicated by the loss of tryptophan fluorescence and by the increase of REM, carbonyl content and Apo B fluorescence (Table I).

HRP, in the absence of the added oxidant, produced almost the same oxidative effects, while the effect of H_2O_2 alone was negligible. Oxidative damage was dependent on HRP concentration (data not shown).

When oxidation was carried out on delipidated apoprotein (Table II), no oxidative modification of apo B (500 μg/ml) was observed. On the other

TABLE I Oxidative modification of LDL by 5 U/ml HRP (type IX)

Incubation	Apo B fluorescence/mg protein	Tryptophan fluorescence (%)	Carbonyl groups (nmol/mg protein)	Relative Electrophoretic Mobility
LDL alone	57 ± 9^a	100^a	7.4 ± 0.5^a	1^a
+ 5 U/ml HRP IX	194 ± 33^b	68 ± 12^b	$9.8 \pm 1.8^{a,b}$	2.2 ± 0.5^b
LDL + 0.2 mM H_2O_2	66 ± 9^a	87 ± 7^a	6.8 ± 1.0^a	1.4 ± 0.1^a
+ 5 U/ml HRP IX	362 ± 15^c	46 ± 3^b	11.0 ± 0.9^b	2.7 ± 0.3^b

Values with different superscript (a, b, c) are significantly different by 1-factor ANOVA ($p < 0.01$).
LDL (200 μg protein/ml) was incubated with 5 U/ml (5.5 μg/ml) type-IX HRP in PBS (0.01 M) at 37°C for 16 h. Results are mean ± S.E. of at least 3 experiments.

TABLE II Oxidative modification of apo B by 5 U/ml HRP (type IX), in the presence and absence of 1 mM linoleic acid

Incubation	Apo B fluorescence/mg protein	Tryptophan fluorescence (%)	Carbonyl groups (nmol/mg protein)
Apo B alone	11 ± 1^a	100^a	2.9 ± 0.7^a
+ 5 U/ml HRP IX	11 ± 1^a	97 ± 3^a	3.0 ± 0.8^a
Apo B + 1 mM linoleic acid	45 ± 6^a	89 ± 6^a	3.9 ± 0.5^a
+ 5 U/ml HRP IX	379 ± 42^b	55 ± 5^b	9.1 ± 1.3^b

Values with different superscript (a, b, c) are significantly different by 1-factor ANOVA ($p < 0.01$).
Apo B (500 μg protein/ml) was incubated with 5 U/ml (5.5 μg/ml) type-IX HRP in PBS (0.01 M) at 37°C for 16 h in the presence and absence of 1 mM linoleic acid.
Results are mean ± S.E. of at least 3 experiments.

hand, when linoleic acid (1 mM) was added to the reaction mixture containing HRP (5 U/ml), an increase of fluorescence and carbonyl groups and a decrease of tryptophan residues were detected. Also in this case, the rate of modification was dependent on HRP concentration (data not shown).

Since, apparently, apo B was only co-oxidized with linoleic acid, the peroxidation of the latter in the presence of HRP was characterized in more detail. The rate of linoleic acid (500 μM) peroxidation was affected neither by catalase (100 U/ml) nor SOD (100 U/ml), thus ruling out a possible role of H_2O_2 and superoxide anion (Figure 1(a)). A possible role of free iron released from the heme protein was also ruled out since DETAPAC (100 μM) failed to slow the peroxidation rate. Finally, 2 mM cyanide completely inhibited linoleic acid peroxidation, indicating that a reactive heme moiety was necessary to generate the oxidants (Figure 1(b)).

When the spectra of HRP in the presence of linoleic acid undergoing peroxidation were recorded (data not shown), spectral changes of HRP were observed, compatible with the formation of a compound II: shift of the Soret band from 402 to 418 nm, and appearance of new maxima at 527 and 556 nm. This suggested that a fatty acid hydroperoxide, present as a contaminant of linoleic acid solution, can substitute for H_2O_2 in generating the iron hypervalent form in HRP. Linoleic acid hydroperoxides account for 0.03–0.2% of linoleic acid in several commercial preparations tested, supporting this thesis.

In agreement with the formation of an oxoferryl species, GSH (3 mM) inhibited peroxidation of linoleic acid (Figure 1(b)). The ferryl HRP is in fact reduced to the ferric state in the presence of a suitable reductant such as GSH.[17]

The role of traces of lipid hydroperoxides was finally demonstrated by treating the linoleic acid solution with sodium borohydride. This procedure fully prevented spectral changes of HRP observed when using untreated linoleic acid (Figure 2(a)). Accordingly, borohydride treatment largely delayed the onset of lipid peroxidation (Figure 2(b)). Obviously, the addition 1 μM H_2O_2 – reasonably corresponding to the concentration of hydroperoxides present in a 1 mM non-purified 18:2 preparation – restored the rapid peroxidation (Figure 2(b)).

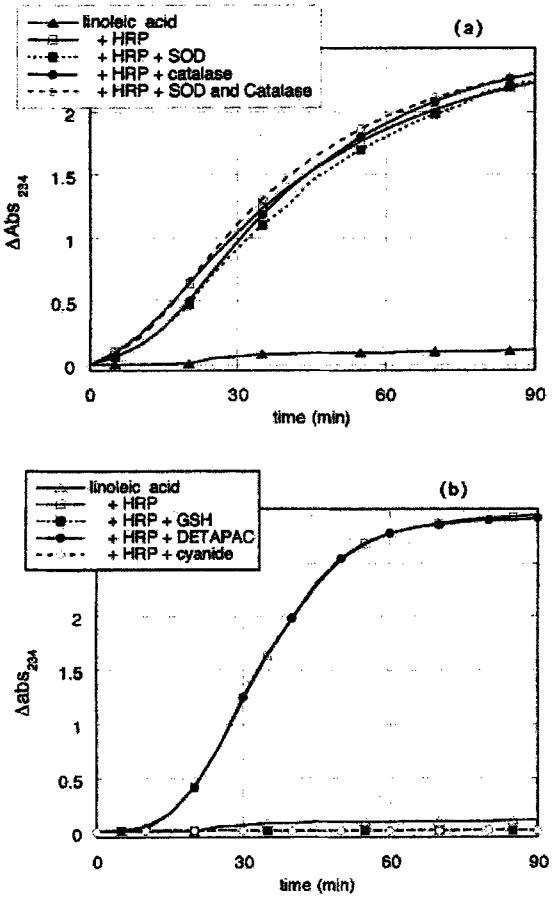

FIGURE 1 Effect of different compounds in the inhibition of linoleic acid peroxidation by HRP: kinetics of conjugated dienes formation. Linoleic acid (500 μM) was incubated in 10 mM PBS (SDS 0.1%) at 37°C with HRP (5 U/ml) in the presence of: (a) SOD (100 U/ml), catalase (100 U/ml), and SOD + catalase (100 U/ml, each); (b) GSH (3 mM), DETAPAC (100 μM) and potassium cyanide (2 mM). Absorbance was continuously monitored at 234 nm.

These results indicate that linoleic acid hydroperoxides can substitute for H_2O_2 in the generation of oxidants eventually damaging Apo B (see Table II).

When LDL was incubated with HRP, lipid peroxidation was observed after a lag of

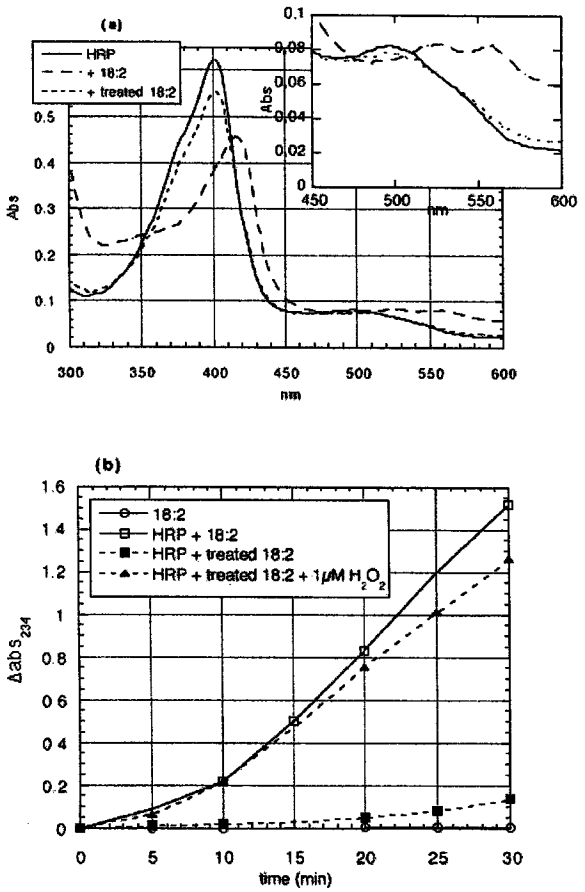

FIGURE 2 Effect of the reduction of fatty acid hydroperoxides in the oxidation of linoleic acid by HRP. (a) Spectral changes of HRP (100 U/ml) in the presence and absence of linoleic acid (2 mM) treated or untreated with $NaBH_4$. The spectrum of HRP in 10 mM PBS (0.1% SDS, 100 μM DETAPAC) was recorded at time 0 and after the addition of treated and untreated linoleic acid (time 5 min). The insert shows the magnification of the spectral changes from 600 to 450 nm. (b) Kinetics of conjugated dienes formation. 500 μM linoleic acid (treated or untreated with $NaBH_4$) was incubated in 10 mM PBS (0.1% SDS, 100 μM DETAPAC) at 37°C with HRP (5 U/ml). Treated linoleic acid was also oxidized in the presence of 1 μM H_2O_2. Absorbance was continuously monitored at 234 nm.

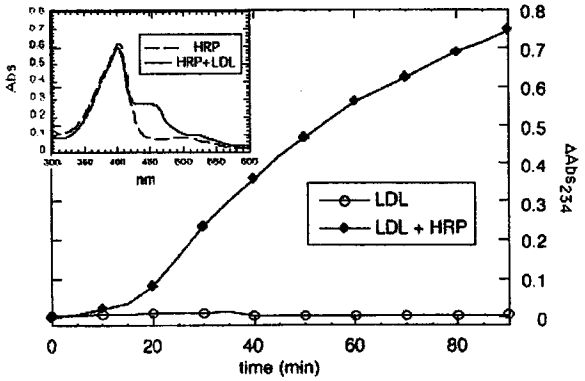

FIGURE 3 Low-density lipoprotein oxidation by HRP: kinetics of conjugated dienes formation. LDL (100 μg/ml) was incubated in 10 mM PBS (100 μM DETAPAC) at 37°C with HRP (100 U/ml). Absorbance was continuously monitored at 234 nm. The insert shows the spectral changes of HRP after 1 h incubation with LDL.

approximately 15 min, but not spectral changes compatible with the formation of the above oxoferryl species were observed (Figure 3).

This lack of hypervalent species in the spectrum suggested an alternative mechanism for the generation of the initiating oxidant. A reasonable mechanism, already suggested to take place in myoglobin-catalyzed decomposition of phosphatidyl choline hydroperoxides,[18] was an organic Fenton-chemistry, where peroxyl and alkoxyl radicals are produced while heme iron shuttles between the ferric and the ferrous form.

The involvement of the ferrous HRP was actually demonstrated by the dramatic antioxidant effect of CO, a specific ligand of ferrous heme (Figure 4). In the presence of CO, HRP was completely unable to peroxidize LDL. Notably only minor inhibition by CO (only of the extent but not the rate of peroxidation) was observed when LDLs were peroxidized with HRP and H_2O_2, accordingly with the fact that ferryl-HRP is the true initiator and that some Fenton-like reaction could have some role, when enough lipid hydroperoxides are built up to compete with hydrogen peroxide for the ferric HRP.

DISCUSSION

In heme-peroxidase reactions, HRP reacts with H_2O_2 to give compound I, a two-electron oxidized species in which the heme is oxidized to a ferryl porphyrin radical action. Sequential electron abstraction from two substrate molecules reduces the porphyrin radical action first to the ferryl

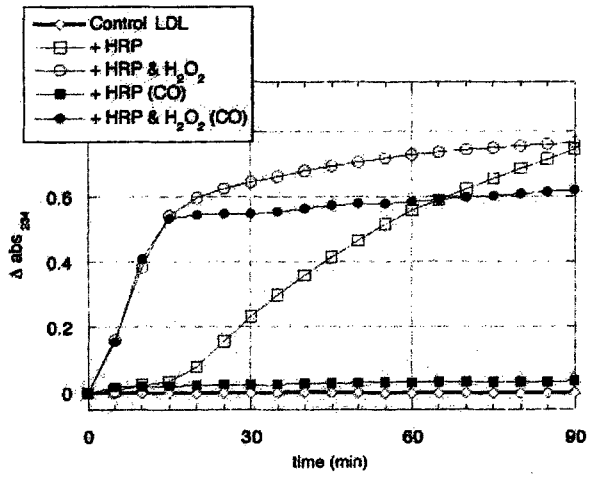

FIGURE 4 Effect of the trapping of ferrous heme by CO on the LDL oxidation by HRP: kinetics of conjugated dienes formation. LDL (100 μg/ml) was incubated in 10 mM PBS (100 μM DETAPAC) at 37°C with HRP (100 U/ml) in the presence and absence of CO and/or H_2O_2 (100 μM). Absorbance was continuously monitored at 234 nm.

porphyrin species, known as compound II, and subsequently to the ferric resting state.[19] The reaction mechanism requires a hydroperoxide to generate the hypervalent iron species able to start the chain reaction. Oxidants able to form the oxyferryl species are H_2O_2 and fatty acid hydroperoxides. A similar reaction has been suggested for other hemeproteins and hematin.[17,20–23] Both homolytic and heterolytic cleavage of O–O bond of the hydroperoxide have been suggested and the homolytic cleavage has been demonstrated for cytochrome C, in a EPR spin-trap study.[24]

The role of the ferryl species is well documented also in the case of HRP/H_2O_2-induced LDL oxidation. There is no agreement on the radical species responsible for the initiation of the peroxidative chain. Witting and co-workers[8] recently demonstrated the occurrence of a tocopherol-mediated LDL peroxidation, without any involvement of the protein radical, as previously proposed by Kalyanaraman *et al.*[7] However, in these studies, the presence of the ferryl species was granted by the reaction between HRP and H_2O_2.

Our results indicate that H_2O_2 can be substituted by free fatty acid hydroperoxides in generating the ferryl species which initiate the peroxidation chain. However, our data also suggest a different reaction mechanism when intact LDL are used, i.e. when hydrogen peroxide or free fatty acid hydroperoxides are not available. In fact, the lipid hydroperoxides – about 1 nmol/mg cholesterol[25] – in intact LDL, separated by the rapid procedure described in the methods section, are esterified, as the homologous fatty acids.

In our experiments with LDL, in the absence of free hydroperoxides, no spectral changes of HRP were detected (although lipid peroxidation was going on, as indicated by the conjugated dienes formation), suggesting that the hypervalent iron form is not produced by LDL hydroperoxides. The lack of observation of the ferryl species can be due to: (i) its rapid reduction to the ferric form by some LDL components, corresponding to an 'apparent' absence; (ii) an alternative redox cycling of the ferric heme, corresponding to a 'real' absence.

In vitro, in the presence of transition metal ions, lipid hydroperoxides decompose to produce free radicals.[26] On this basis, we hypothesized that in the model HRP/LDL a Fenton-like reaction takes place leading to the generation of lipid radicals initiating lipid peroxidation:

$$LOOH + HRP\text{-}Fe^{III} \rightarrow LOO^{\bullet} + HRP\text{-}Fe^{II} + H^{+}$$

$$LOOH + HRP\text{-}Fe^{II} \rightarrow LO^{\bullet} + HRP\text{-}Fe^{III} + OH^{-}$$

This hypothesis was supported by the experiments where HRP- mediated LDL oxidation was inhibited by the presence of CO, a specific ligand for ferrous heme.

An organic Fenton-reaction – suggested for other heme protein[27–29] – cannot be excluded to take place also when the spectrum of oxoferryl species is observed, i.e. in the presence of hydrogen or fatty acid peroxide (See Figure 4). In fact, the presence of CO reduced the extent of conjugated dienes formation (delta absorption at

plateau) in HRP/H_2O_2 mediated LDL oxidation, where the ferryl species is the initiator.

Oxoferryl species are reported to play a relevant role in the oxidative modification of LDL by other heme proteins (myoglobin and hemoglobin).[30,31] These studies, however, report oxoferryl species (as sulfomyoglobin complex) after a very long incubation time (5 h), when LDL was likely to be fully oxidized. Reasonably, the discrepancy between our results and previously published data could be attributed to the release of fatty acid hydroperoxides after such a long incubation. In agreement with our data, Maiorino *et al.*[18] showed no formation of oxoferryl species when myoglobin decomposes phospholipid hydroperoxides, thus starting lipid peroxidation.

However, the mechanism of different heme proteins in LDL peroxidation does not necessarily have to be the same; in fact, CO does not affect oxidation of lipids by hemoglobin, excluding the activity of the ferrous heme in this model.[32]

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