Oxidation of low density lipoprotein and plasma by 15-lipoxygenase and free radicals

Hiromasa Yamashita[a], Akio Nakamura[a], Noriko Noguchi[a], Etsuo Niki[a,*], Hartmut Kühn[b]

[a] *Research Center for Advanced Science and Technology, University of Tokyo, 4-6-1 Komaba, Meguro, Tokyo 153, Japan*
[b] *Institute of Biochemistry, University Clinicum Charite, Humboldt University Berlin, Hessische Str. 3-4, D-10115 Berlin, Germany*

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Abstract It is generally accepted that the oxidation of pentadiene structures of polyunsaturated lipids by lipoxygenase (LOX) is regio- and enantio-specific, while the free radical-mediated lipid peroxidation gives stereo-random racemic products. It was confirmed that the oxidation of human low density lipoprotein (LDL) by 15-LOX from rabbit reticulocytes gave phosphatidylcholine (PC) and cholesteryl ester (CE) hydroperoxides regio-, stereo- and enantio-specifically. 15-LOX also oxidized human plasma to give specific PC and CE hydroperoxides in spite of the presence of high concentrations of antioxidants. More CE hydroperoxides were formed than PC hydroperoxides from LDL, but the reverse order was observed for plasma oxidation. The S/R ratio of the hydroperoxides decreased during long time incubation but remained significantly larger than one, while free radical-mediated oxidation of LDL and plasma gave racemic products.

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Key words: Lipoxygenase; Low density lipoprotein; Lipid peroxidation; Plasma

1. Introduction

Oxidative modification of low density lipoprotein (LDL) and its subsequent uptake by macrophages via receptor pathways are thought to play an important role in foam cell formation during early stages of atherosclerosis [1]. However, the nature and sources of oxidative stress responsible for LDL oxidation in vivo have not been elucidated. Several oxidizing enzymes including 15-LOX and myeloperoxidase have been suggested as being involved [2–4]. Evidence for a putative role of 15-LOX includes: (i) 15-LOX are capable of oxidizing human LDL in vitro ([5] and references cited therein), (ii) 15-LOX are expressed in human and rabbit atherosclerotic lesions but not in the normal vessel wall [6,7], (iii) somatic gene transfer of the 15-LOX to the arterial wall induced the appearance of oxidized LDL epitopes [8], (iv) LOX inhibitors are capable of preventing oxidative modification of LDL [9–11], and (v) a recently developed LOX inhibitor, which appears to be specific for mammalian 15-LOX, prevented the development of atherosclerosis in cholesterol fed rabbits [12]. The lipid hydroperoxides formed by LOX have been also proposed to act as source of free radical-mediated secondary reactions [13,14].

LOX are non-haem iron-containing dioxygenases which oxygenate polyenic fatty acids containing a 1Z,4Z-pentadiene system to their 1-hydroperoxyl-2(E),4(Z)-pentadiene product. In contrast to the non-enzymatic, free radical-mediated lipid peroxidation, the LOX reaction is regio- and enantio-specific with respect to the initial hydrogen abstraction and subsequent oxygen insertion [15–17]. Originally, it was believed that LOX can oxidize only free polyenic fatty acid [18]. However, other studies indicated that certain plant and mammalian LOX are capable of specifically oxidizing phospholipids and cholesterol esters in biomembranes and lipoproteins [5,19–23]. Thus, a stereo-specific pattern of oxidation products in cells and tissues may be regarded as indicator for an in vivo action of a LOX [24]. The detection of stereo-specific oxidized polyenic fatty acids in atherosclerotic lesions suggested that 15-LOX may in fact contribute to lipid peroxidation during early stages of atherogenesis [3,4,25].

On the other hand, it was reported that the oxidation of LDL by recombinant human 15-LOX proceeds via a non-enzymatic α-tocopherol-mediated mechanism to give non-specific lipid hydroperoxides [26–28]. Upston et al. concluded that α-tocopherol acted as a prooxidant rather than an antioxidant and they found that LDL oxidation was dependent on the presence of α-tocopherol and that ascorbate, an efficient reducing agent of α-tocopheroxyl radical, completely prevented LDL oxidation [26].

Considering these inconsistent observations, the present study was carried out to re-examine and confirm the regio-, stereo- and enantio- specificity in the oxidation of LDL induced by mammalian 15-LOX, because such specificity is the best criterion to distinguish enzymatic and free radical-mediated oxidation. Phosphatidylcholine hydroperoxides which received less attention than cholesteryl ester hydroperoxides were also analyzed. Furthermore, as an extension, the oxidation of plasma by 15-LOX was also studied and the products were carefully analyzed.

2. Materials and methods

2.1. Materials

The mammalian 15-LOX was purified from reticulocytes of rabbits with bleeding-induced anemia as reported previously [22]. LDL was isolated from human plasma of healthy donors by ultracentrifugation as described previously [29] within a density cut-off of 1.019 to 1.063 g/ml and then dialyzed with cellulose membrane in phosphate buffered saline (PBS, pH 7.4) containing 0.1 mM EDTA. Blood samples from healthy volunteers were centrifuged at 1000×*g* for 5 min to obtain

*Corresponding author. Fax: +81 (3) 3481 4574.
E-mail: eniki@oxygen.rcast.u-tokyo.ac.jp

Abbreviations: CE, cholesteryl ester; CEOH, cholesteryl ester hydroxide; CEOOH, cholesteryl ester hydroperoxide; EDTA, ethylenediaminetetraacetic acid; HPLC, high pressure liquid chromatography; HODE, hydroxyoctadecadienoate; LDL, low density lipoprotein; LOX, lipoxygenase(s); MeO-AMVN, 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile); PBS, phosphate buffered saline; PC, phosphatidylcholine; PCOH, phosphatidylcholine hydroxide; PCOOH, phosphatidylcholine hydroperoxide; TMAH, tetramethylammonium hydroxide

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plasma, which was used immediately for oxidation. 2,2′-Azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN), used as a lipophilic radical initiator [30], was obtained from Wako Pure Chemical Ind. (Osaka, Japan) and used as received.

2.2. Assay systems

Whole human plasma and LDL were oxidized and analyzed in a similar way. The oxidation was initiated by the addition of LOX or MeO-AMVN to the reaction medium. The rate of oxidation was measured from the rates of oxygen uptake and formation of lipid hydroperoxides with an oxygen electrode (Model YSI 5300, Yellow Springs Instrument Co., OH, USA) and HPLC respectively.

The enzyme activity of rabbit reticulocyte 15-LOX used in this study was measured from the rate of oxygen uptake and hydroperoxide formation at the initial stage of the oxidation of linoleic acid in aqueous solution. During this phase of oxidation, the amounts of oxygen uptake and hydroperoxide formation agreed well.

2.3. Analytical procedures

After the incubation period, the lipids were extracted with chloroform/methanol (2:1 by vol) and analyzed for the oxidation products from PC and CE with an HPLC. CE hydroperoxide (CEOOH) and CE hydroxide (CEOH) were analyzed as described before [30]. For analysis of PC hydroperoxide (PCOOH) and PC hydroxide (PCOH) in some experiments, an ODS column (TSK ODS-80Ts, Tosoh Co., Tokyo, Japan; 25 cm×4.6 mm; 5 μm particle size) was used. The eluent was acetonitrile/methanol/water (75:21:4 by vol) containing 10 mM choline chloride at a flow rate of 1.5 ml/min. The column temperature was maintained at 40°C. For analysis of the regio- and stereoisomers of the hydroperoxides, the oxidized lipids were fractionally collected by HPLC and subsequently treated with sodium borohydride and then transmethylated with TMAH to obtain methyl ester of hydroxy fatty acids. Aliquots of the transmethylated mixture were injected to chiral phase HPLC on a Chiralcel OD column (Daicel Chemical Ind., Tokyo, Japan; 25 cm×4.6 mm; 5 μm particle size) with a solvent system of hexane/2-propanol/acetic acid (100:5:0.1 by vol) and a flow rate of 1 ml/min. α-Tocopherol was analyzed by reverse phase HPLC with an electrochemical detector on a LC-18 column (Supelco, Tokyo, Japan) using methanol/*tert*-butyl alcohol (9:1) containing 50 mM $NaClO_4$ as an eluent at a flow rate of 1.0 ml/min.

The oxidation of LDL and plasma was repeated more than five times and substantially the same results were obtained for specificity and relative oxidizabilities of PC and CE. A typical example is shown in the figures.

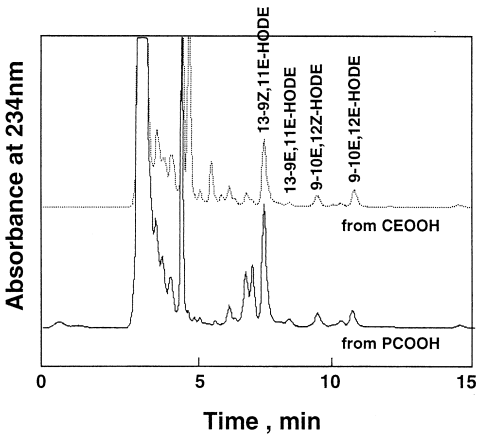

Fig. 1. Regio- and stereo-specificity in the oxidation of LDL induced by 15-LOX. Human LDL (0.75 mg protein/ml) was incubated with rabbit reticulocyte 15-LOX (10.5 nkat/ml) for 20 min at room temperature in air, and phosphatidylcholine and cholesteryl ester hydroperoxides (PCOOH and CEOOH respectively) were collected using HPLC and converted to methyl hydroxylinoleate (HODE) as described in Section 2.

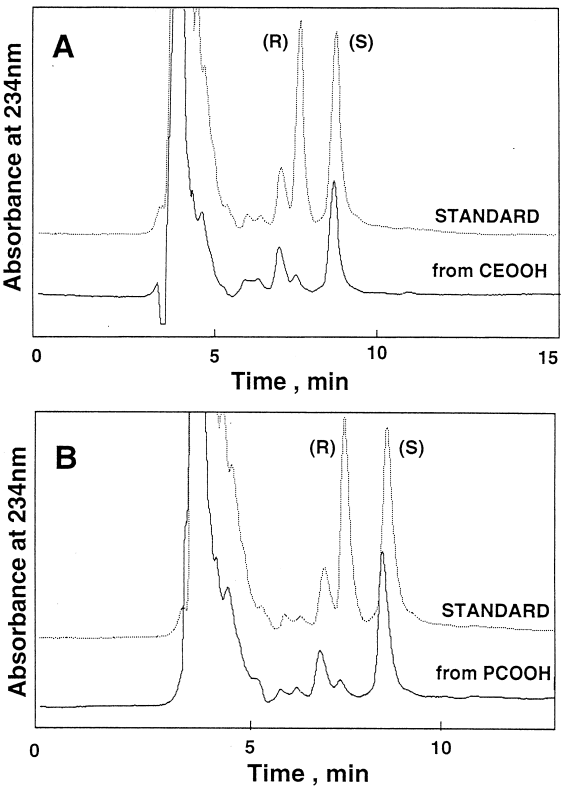

Fig. 2. Enantio-specificity in the oxidation of LDL induced by 15-lipoxygenase. The reaction conditions are the same as in Fig. 1. 13-9Z,11E-HODE was collected and analyzed with a chiral phase HPLC as described in Section 2. The standard PC and CE hydroperoxides were prepared by the oxidation of methyl linoleate with a radical initiator.

3. Results

3.1. Oxidation of LDL by 15-lipoxygenase

Firstly, human LDL was oxidized with rabbit reticulocyte 15-LOX in aqueous suspensions and the oxidation products were carefully analyzed in order to ascertain the specificity of the oxidation process. As observed previously [5], both PC and CE hydroperoxides were formed, the latter being in higher yield than the former. They were fractionally collected with an HPLC and converted to methyl esters of hydroxy fatty acids by reduction with sodium borohydride followed by transmethylation with TMAH. As shown in Fig. 1, 13-hydroxy-9Z,11E-octadecadienoate(13-9Z,11E-HODE) was the major product and the other three isomers 13-9E,11E-HODE, 9-10E,12Z-HODE and 9-10E,12E-HODE, were much smaller from both PC and CE hydroperoxides. Furthermore, this 13-9Z,11E-HODE derived from PC and CE hydroperoxides was fractionally collected and analyzed with a chiral phase HPLC. It was found that they were exclusively S form (Fig. 2). These results demonstrate that the peroxidation of CE as well as PC of LDL by 15-LOX proceeds enzymatically, not via a free radical-mediated mechanism.

3.2. Oxidation of human plasma by 15-lipoxygenase and MeO-AMVN

Human plasma was then used as a substrate and incubated with rabbit reticulocyte 15-LOX. Upon addition of 15-LOX to human plasma, a rapid oxygen uptake was observed for

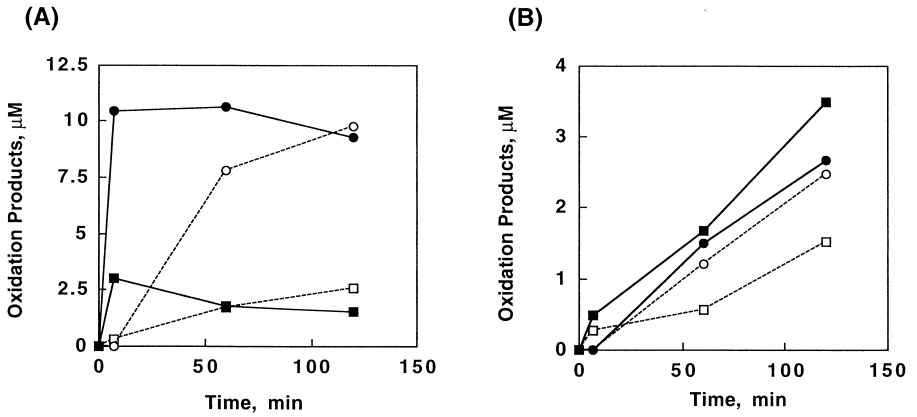

Fig. 3. Oxidation of human plasma with (A) rabbit reticulocyte 15-lipoxygenase (50 nkat/ml) and (B) MeO-AMVN (2.0 mM) at 37°C in air. ●: PC hydroperoxide; ○: PC hydroxide; ■: CE hydroperoxide; □: CE hydroxide.

only 5 min and then oxygen was consumed at a much slower but constant and steady rate (data not shown). In agreement with the oxygen uptake, PC and CE hydroperoxides were formed significantly at the earliest stage in spite of the presence of many antioxidants and then their formation was markedly decreased (Fig. 3). The formation of free linoleic acid hydroperoxide was quite small. In contrast to the oxidation of LDL, more PC hydroperoxide was formed than CE hydroperoxide. Both PC and CE hydroxides increased with time. Ubiquinol was oxidized rapidly to ubiquinone but neither ascorbate nor α-tocopherol was consumed appreciably even after 300 min incubation (data not shown). To clarify the mechanism, the geometric and optical isomers of the linoleate hydroperoxides were examined as described above. It was found that 13(S)-9Z,11E-HODE was formed predominantly from both PC and CE (Fig. 4), again showing that the LOX-catalyzed oxidation of plasma lipids proceeds enzymatically and specifically. The ratio of S and R form of the PC hydroperoxides decreased with time: S/R being 10.5, 6.98, and 6.01 at 5, 200, and 300 min, respectively.

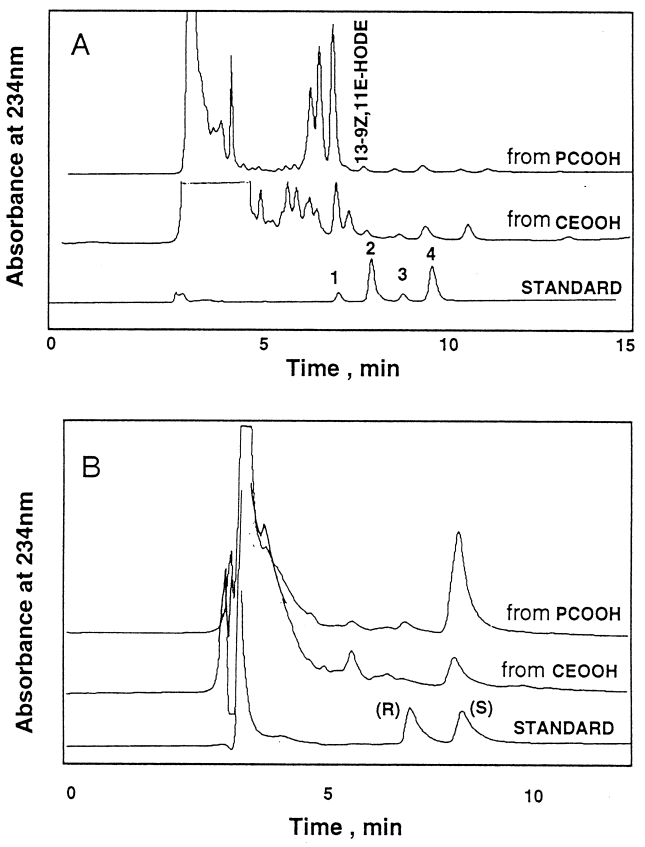

Fig. 4. Geometrical and optical specificities in the oxidation of plasma by 15-lipoxygenase. Human plasma was incubated with rabbit reticulocyte 15-lipoxygenase (49 nkat/ml) at room temperature in air for 20 min. A: Phosphatidylcholine and cholesteryl ester hydroperoxides (PCOOH and CEOOH respectively) were collected and converted into methyl hydroxylinoleate (HODE) and analyzed with an HPLC. 1: 13-9Z,11E-HODE; 2: 13-9E,11E-HODE; 3: 9-10E,12Z-HODE; 4: 9-10E,12E-HODE. B: 13-9Z,11E-HODE from PCOOH and CEOOH were collected and analyzed with chiral phase HPCL as described in Section 2. The standard products were obtained from the oxidation of methyl linoleate with AMVN and analyzed similarly.

In order to compare the oxidation of plasma by LOX and by free radicals, human plasma was oxidized with MeO-AMVN. The results are included in Fig. 3. In contrast to the oxidation induced by 15-LOX, hydroperoxides and hydroxides from both PC and CE increased with time and the S/R ratio was close to one for both PC and CE hydroperoxides, showing that the oxidation gave random products.

4. Discussion

The results of the present study confirm the previous observation that CE in LDL particle is oxidized by rabbit reticulocyte 15-LOX in a regio-, stereo- and enantio-specific manner, and show that PC in LDL is also oxidized specifically and that plasma lipids are oxidized similarly to give regio- and enantio-specific products. The selective formation of 13(S)-9Z,11E-HODE from both PC and CE clearly demonstrates that the oxidation of lipids in LDL and plasma by 15-LOX proceeds by an enzymatic mechanism, not by free radical- or α-tocopherol-mediated mechanism. It is interesting to note that more CE hydroperoxides were formed than PC hydroperoxides from LDL, while the reverse order was observed in the oxidation of plasma.

Another characteristic of 15-LOX-induced lipid peroxidation is that the rate of oxidation decreased rapidly probably due to its inactivation. As mentioned above, the fast oxidation of plasma proceeded only about 5 min, after which the rate of

oxygen uptake became quite small and both PC and CE hydroperoxides decreased.

The results obtained in the present study are inconsistent with those by Upston et al. [26,27] on the oxidation of LDL by a recombinant human 15-LOX. They observed a stereo-random formation of PC and CE hydroperoxides and concluded that the oxidation proceeded by a tocopherol-mediated random mechanism. The tocopherol content in the present study was between 5–10 molecules per each LDL particle, which is similar to those of Upston et al. [26,27]. The reason for such a discrepancy is not clear but there may be two possible explanations: (i) It may be possible that the native rabbit and the recombinant human LOX behave differently, although an earlier study on LDL oxidation by the human recombinant 15-LOX revealed a specific pattern of oxidation products [31]. (ii) The duration of incubation period in the present study and that of Upston et al. [26,27] is quite different. The oxidation for a short period employed in this and previous studies gave specific products selectively, while those for long term incubation yielded more stereo-random oxidation products [5,32]. It was also observed in the present study that the S/R ratio of 13-9Z,11Z-HODE derived from either PC or CE hydroperoxides decreased with increasing incubation time. It may be stated that the 15-LOX-induced lipid peroxidation proceeds by a regio-, stereo- and enantio-specific mechanism but that the enzyme undergoes suicidal inactivation rapidly and then the specific primary oxidation products may initiate free radical-mediated secondary reactions or undergo isomerization which lead to more or less unspecific oxidation products. It is not clear, however, whether and how LOX may contribute to the secondary reactions of hydroperoxides.

In conclusion, the present study confirmed that the oxidation of PC and CE in LDL and, more importantly, in plasma by rabbit 15-LOX, proceeds enzymatically and gives specific products.

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