Biochem. J. (1992) 282, 631–634 Printed in Great Britain

RESEARCH COMMUNICATION

Pro-oxidant effects of lipoxygenase-derived peroxides on the copper-initiated oxidation of low-density lipoprotein

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It has been proposed that lipoxygenases, specifically 15-lipoxygenase, may play an important role in promoting the oxidation of low-density lipoprotein (LDL) in the artery wall. It is well known that peroxides are unstable in the presence of transition metals, decomposing to form the alkoxy and peroxy radicals, and so initiating lipid peroxidation. To test whether lipoxygenase-derived peroxides may promote the oxidation of LDL in the presence of copper, the lipoprotein was enriched with lipid peroxides derived from the enzymic action of 5- and 15-lipoxygenases on either linoleic or arachidonic acid. All of these products were found to promote oxidation, whereas the related hydroxy fatty acids had no effect. This suggests that lipoxygenase-derived peroxides associated with the LDL particle may promote peroxidation in the presence of a suitable transition metal catalyst. This result has implications both for the mechanism of the potential pro-oxidant action of lipoxygenases *in vivo* and for the *ex vivo* assessment of the oxidizability of LDL samples isolated from different donors.

INTRODUCTION

Oxidation of low-density lipoprotein (LDL) in the artery wall is thought to be a key event leading to the formation of macrophage-derived foam cells, and of primary importance in the pathogenesis of atherosclerosis [1–3]. The mechanism of initiation of lipid peroxidation is still unclear, but it has been suggested that lipoxygenases, especially 15-lipoxygenase, may play an important role in this respect [4,5]. In support of this idea, it has been shown that atherosclerotic lesions from both cholesterol-fed and Watanabe Heritable Hyperlipidaemic rabbits have increased levels of the lipoxygenase-derived product 15-hydroxyeicosatetraenoic acid (15-HETE), whereas normal rabbit aorta contains 12-HETE as the principle lipoxygenase metabolite [6,7]. In addition, mRNA for 15-lipoxygenase has been found to be co-localized with epitopes of oxidized LDL in macrophage-rich areas [8]. *In vitro* it has been shown that incubation of LDL with the purified 15-lipoxygenase enzyme from soybean resulted in modification of LDL to a cytotoxic form [9,10]. In contrast, we have shown that the 5-lipoxygenase enzyme is not essential for the macrophage-mediated modification of LDL [11].

Various mechanisms to explain the apparent pro-oxidant effect of lipoxygenase have been proposed. The lipoxygenase enzyme could act directly on the fatty acid side chains of the LDL particle and produce a peroxy or alkoxy radical [10]. Another possibility is that oxidants are generated during the catalytic cycle of the enzyme, and these could be released from the cell and initiate peroxidation [9,12,13]. Here we have addressed the possibility that partition of lipid-derived peroxides, generated by the enzymic action of lipoxygenases, into the LDL molecule may have a pro-oxidant effect in the presence of catalytic amounts of copper.

In vitro, the oxidation of LDL can be initiated by the copper-catalysed decomposition of phospholipid or cholesterol peroxides endogenous to the LDL particle [14]. This observation has formed the basis of an assay used to determine the oxidizability of LDL isolated from different donors or patients [15–19]. Although it is clear that the copper initiation process has an absolute requirement for lipid peroxides, the sensitivity of LDL oxidizability to peroxide content is unknown [14]. In the present study we have attempted to clarify this point by supplementing purified human LDL with lipid peroxides derived from the enzymic action of lipoxygenases, and determining the susceptibility of LDL to oxidation by Cu^{2+} ions.

MATERIALS AND METHODS

Isolation of LDL

Human LDL was isolated from plasma from individual donors by differential centrifugation, using the method described in [20]. After dialysis against calcium- and magnesium-free phosphate-buffered saline (PBS) containing NaCl (140 mM), KCl (2.7 mM), Na_2HPO_4 (8.13 mM), KH_2PO_4 (1.47 mM) and EDTA (10 μM), the LDL was sterilized by filtration through a 0.22 μm filter and stored at 4 °C until used. The protein concentration was measured using the BCA protein assay reagent (Pierce), and was typically 1–2 mg/ml.

Oxidation of LDL

15-Hydroperoxyeicosatetraenoic acid (15-HPETE), 5-HPETE, 13-hydroperoxyoctadecadienoic acid (13-HPODE), 9-HPODE, 15-HETE, 5-HETE and 13-hydroxyoctadecadienoic acid (13-HODE) were purchased from Cascade Biochem Ltd., and were supplied as ethanolic solutions free of antioxidants. Peroxide samples were kept under nitrogen at −20 °C prior to use. Additions were made such that the ethanol content of each

Abbreviations used: LDL, low-density lipoprotein; 15-HPETE 15(*S*)-hydroperoxy-*cis*-5,8,11-*trans*-13-eicosatetraenoic acid; 5-HETE, 15(*S*)-hydroxy-*cis*-5,8,11-*trans*-13-eicosatetraenoic acid; 5-HPETE, 5(*S*)-hydroperoxy-*trans*-6-*cis*-8,11,14-eicosatetraenoic acid; 5-HETE, 5(*S*)-hydroxy-*trans*-6-*cis*-8,11,14-eicosatetraenoic acid; 13-HPODE, [9*Z*,11*E*,13(*S*)]-13-hydroperoxyoctadecadien-1-oic acid; 13-HODE [9*Z*,11*E*,13(*S*)]-13-hydroxy-octadecadiene-1-oic acid; 9-HPODE, [9(*S*),10*E*,12*Z*]-hydroperoxyoctadecadien-1-oic acid; BHT, butylated hydroxytoluene; DTPA, diethylenetriamine-penta-acetic acid; PBS, phosphate-buffered saline; TBARS, thiobarbituric-acid-reactive substances; REM, relative electrophoretic mobility.

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sample was kept constant at 1% (v/v). H.p.l.c. analysis of these samples conformed with the supplier's specification and showed no detectable decomposition if used within 1 week. Samples of native LDL (1 mg/ml) were diluted to 125 μg/ml with PBS in the cuvettes and incubated at 37 °C. Oxidation was initiated by the addition of 10 μM-$CuSO_4$, and was monitored spectrophotometrically by measuring the formation of conjugated dienes in the LDL particle in a thermostatted cuvette holder maintained at 37 °C. The final concentration of EDTA in the experiment was 1.25 μM, giving a Cu^{2+}/EDTA ratio of 8:1. The lag phase, which was used as a measure of oxidizability, was determined as described in [21]. The oxidation was also followed by measuring the electrophoretic mobility of samples incubated with and without 15-HPETE relative to native LDL. Thiobarbituric-acid-reactive substances (TBARS) were measured in the same samples, using the spectrophotometric method described in [22]. The TBARS values were then converted to malondialdehyde equivalents by reference to a standard curve prepared by the hydrolysis of known amounts of tetraethoxypropane. Measurement of the lipid hydroperoxide content of LDL was carried out as described in [23].

The amount of peroxide product bound to LDL was measured under the conditions used to measure the oxidizability of LDL. Samples of LDL (125 μg/ml) were mixed with 13-HPODE (3 μM) and incubated at room temperature for 10 min. The samples were then transferred to Centricon-30 microconcentrators (Amicon) and centrifuged for 15 min at 4 °C at 2500 *g*. Under these conditions approx. 10% of the sample passed through the ultrafiltration membrane, ensuring only a minimal perturbation of the equilibrium distribution of compound between LDL and the aqueous phase. The concentration of 13-HPODE in the filtrate was measured by h.p.l.c. [24].

RESULTS AND DISCUSSION

It has been shown that the oxidation of LDL initiated *in vitro* by Cu^{2+} requires a trace amount of lipid hydroperoxide to be present in the LDL particle [14]. In good agreement with estimations from other laboratories using the iodometric assay, freshly prepared native LDL was shown to contain 22.3 ± 3.8 nmol of lipid peroxides per mg of LDL protein (mean ± S.D. of six LDL preparations) [23]. The oxidation of LDL (125 μg/ml) was initiated by the addition of 10 μM-Cu^{2+} with incubation at 37 °C, and monitored by measuring the absorbance at 234 nm for 240 min. The absorbance at this wavelength correlates to the formation of conjugated dienes [16,21]. A typical progress curve for this reaction is shown in Fig. 1 and can be divided into three phases. Firstly there is a lag phase, when absorbance due to conjugated diene formation increases slowly and during which endogenous antioxidants are consumed [16]. The length of this lag phase was measured as described by Esterbauer *et al.* [21], and used as a measure of the oxidizability of the LDL sample. The next phase is characterized by a rapid increase in the levels of conjugated dienes and is generally regarded as the propagation phase of the lipid peroxidation reaction. The decomposition phase occurs when the lipid substrates for peroxidation have been consumed and, consequently, there is no further increase in absorbance due to conjugated diene formation [21].

The effect of lipid hydroperoxides on the Cu^{2+}-initiated oxidation was investigated by supplementing LDL with peroxides derived from the catalytic action of lipoxygenases on arachidonic and linoleic acids. As illustrated in Fig. 1, increasing concentrations of 15-HPETE decreased the length of the lag phase, as indicated by the leftwards shift of the progress curve for conjugated diene formation. 15-HPETE added to LDL in the absence of Cu^{2+} did not promote any significant peroxidation during the course of the experiment (result not shown). 13-HPODE, the major 15-lipoxygenase-derived product from linoleic acid, had a very similar effect to that found with 15-HPETE. The results (Table 1) show that with increasing concentrations of 13-HPODE, as with 15-HPETE, there was a decrease in the lag phase for LDL oxidation. We found that in both cases the pro-oxidant effect of these peroxides was maximal under these conditions at concentrations of 12 μM and above (result not shown). It is known that LDL isolated from the plasma of different donors shows variability in the rate of lipid peroxidation in the presence of copper [16]. To test whether such variability

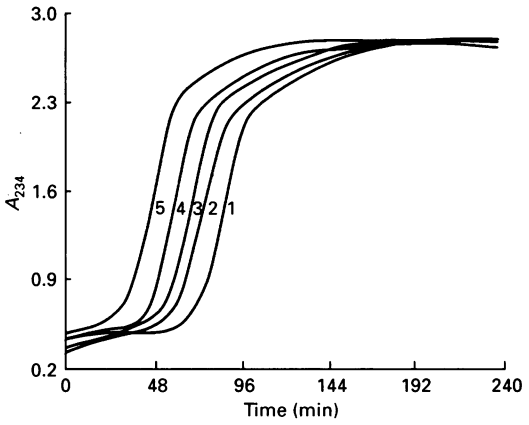

Fig. 1. Effect of 15-HPETE on the Cu^{2+}-initiated oxidation of LDL

Samples of native LDL (125 μg/ml) were incubated at 37 °C with the following concentrations of 15-HPETE: 1, control (1% ethanol); 2, 1 μM; 3, 2 μM; 4, 3 μM; 5, 6 μM. Oxidation was initiated by the addition of 10 μM-$CuSO_4$ and monitored by the measurement of absorbance at 234 nm.

Table 1. Effect of lipoxygenase products on the oxidizability of LDL

Samples of native LDL were incubated with 3 μM of each lipoxygenase product and with a range of 13-HPODE concentrations. Oxidation was initiated by the addition of 10 μM-$CuSO_4$, and samples were incubated at 37 °C. The oxidation was followed by continuous measurement of the absorbance at 234 nm for 360 min. The experiments reported were conducted with a single LDL preparation, except for one set of data using 15-HPETE, marked *, which represents the mean of results with three preparations of LDL isolated from the plasma of three individuals. The results are expressed as the means ± S.D. taken from three experiments performed in duplicate.

Lipoxygenase product		Lag phase (% of control)
Control (no added product)		100
13-HPODE	1 μM	86 ± 5
	2 μM	67
	3 μM	69 ± 6
	6 μM	61 ± 5
	12 μM	56
15-HPETE	3 μM	76 ± 3
15-HPETE	3 μM	71 ± 6*
5-HPETE	3 μM	78 ± 5
9-HPODE	3 μM	68 ± 2
15-HETE	3 μM	107 ± 2
5-HETE	3 μM	102 ± 4
13-HODE	3 μM	103 ± 3

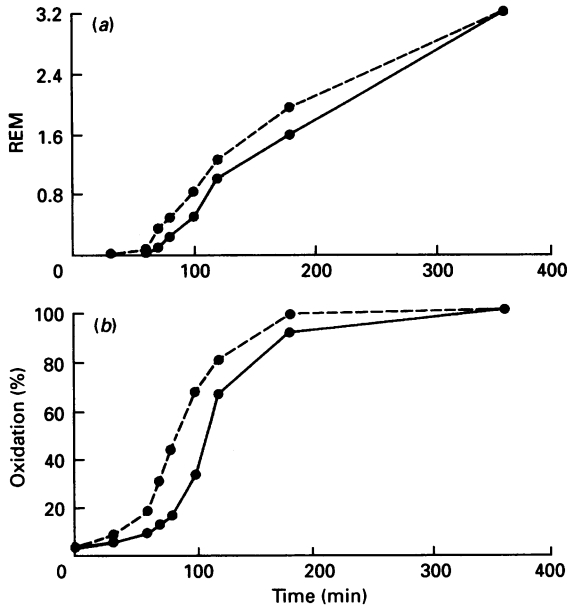

Fig. 2. Effect of 15-HPETE on the rate of LDL oxidation

Oxidation of LDL (125 μg/ml) was initiated by the addition of 10 μM-$CuSO_4$. Samples were incubated at 37 °C for the times shown, and the reaction was stopped by the addition of BHT (100 μM) and DTPA (100 μM). Samples were incubated with (---) or without (—) 15-HPETE (5.9 μM). (*a*) The REM of the samples was measured relative to that of native LDL. The maximum change in mobility was 22 mm, and the variation between triplicate samples of the same experiment was less than 8%. Results are the means of three samples from one experiment. (*b*) The TBARS in each sample were measured. The maximum TBARS content was 112 nmol/mg of protein. Results are expressed as a percentage of the maximum, and are the means of three samples from one experiment.

could influence the pro-oxidant action of 15-HPETE, we measured the lag phase in three preparations of LDL isolated from the plasma of different donors. The pro-oxidant effect of this peroxide was not dependent on the donor (Table 1).

Having established that 15-HPETE and 13-HPODE can act as pro-oxidants in the presence of Cu^{2+}, the specificity of this response was assessed by incubating LDL under similar conditions with products derived by the action of other lipoxygenases on arachidonic acid and linoleic acid. As shown in Table 1, both 5-HPETE and 9-HPODE were pro-oxidant in the presence of Cu^{2+} with little difference in potency, suggesting that this effect could be achieved by incubation of a number of peroxide products with LDL. It is likely that an association of the fatty acid peroxides with LDL is required to initiate oxidation in the lipid phase of the particle. Consistent with this hypothesis, ultrafiltration studies showed that approx. 40% of 13-HPODE was bound to LDL under identical conditions to those used for the conjugated diene assay.

To determine whether the pro-oxidant property of the lipoxygenase products was dependent on the hydroperoxy group, the corresponding alcohol derivatives, 5- and 15-HETE and 13-HODE, were also tested for their effects on the oxidizability of LDL at a concentration of 3 μM. As can be seen in Table 1, none of these products showed a pro-oxidant effect when added to LDL, suggesting that such an action is due specifically to the increase in the lipid hydroperoxide content of LDL on addition of peroxides.

Lipid peroxidation products decompose to aldehydes, which in turn result in the modification of the lysine residues of the apo B lipoprotein, rendering the particle more electronegative. This change in net charge of the LDL particle is required for recognition by the scavenger receptor [25] and hence for unregulated uptake by macrophages. Consequently, we have determined whether the increased oxidizability is also reflected in an increased rate of change in electrophoretic mobility and aldehyde content. Samples of native LDL (125 μg/ml) were incubated at 37 °C with or without 15-HPETE (5.9 μM). This concentration of 15-HPETE was calculated to approximately double the lipid hydroperoxide content of the LDL sample. Oxidation was initiated by the addition of 10 μM-Cu^{2+} and the reaction was stopped at various time points up to 360 min by the addition of butylated hydroxytoluene (BHT; 100 μM) and diethylenetriamine penta-acetic acid (DTPA; 100 μM). The electrophoretic mobility of LDL incubated with 15-HPETE increased more rapidly than that of LDL incubated in its absence (relative to native LDL), thus confirming the pro-oxidant effect of incubation with 15-HPETE (Fig. 2*a*).

Under the same conditions, the TBARS assay also confirmed the pro-oxidant effect of 15-HPETE (Fig. 2*b*), aldehyde products being detected at higher concentrations earlier in the time course in the samples containing 15-HPETE. Maximum concentrations of TBARS were obtained in the presence and absence of 15-HPETE by 360 min. Interestingly, both significant formation of TBARS and the change in the relative electrophoretic mobility (REM) of LDL treated with copper occurs after the maximum formation of conjugated dienes (Figs. 1 and 2). This is consistent with the hypothesis that peroxide decomposition to aldehydes is required prior to the oxidative modification of LDL [16]. The kinetics of the aldehyde-dependent modification of primary amino groups on LDL are poorly understood, but the experiment here demonstrates that the pro-oxidant properties of peroxides persist during this phase of the reaction, although the effect is less pronounced. For example, 6 μM-15-HPETE decreased the lag phase for conjugated diene formation in the presence of copper by approx. 50 min (Fig. 1), whereas the time to onset of LDL oxidation measured by TBARS and REM decreased by approx. 30 min and 20 min respectively (Fig. 2).

In summary, the addition of lipoxygenase-derived products to LDL has a pro-oxidant effect, as measured by monitoring the formation of conjugated dienes, TBARS and electronegativity of the LDL particle, when oxidation is initiated by the addition of copper ions. This effect of added hydroperoxides was concentration-dependent over the range investigated. It is interesting to note that LDL prepared from human plasma by rapid ultracentrifugation contains few or no peroxides, suggesting that the peroxides measured in our samples, prepared after extended ultracentrifugation (total time 5 h) may be formed during isolation [26]. However, peroxide formation and association with LDL in the artery wall remain a possibility which has yet to be explored, and would not necessarily be associated with increased peroxide levels in LDL isolated from plasma. In the experiments described here we have used passive diffusion to incorporate peroxides into the LDL molecule, and this may differ significantly from the biological process whereby cholesterol and phospholipid peroxides are formed within LDL. For example, a direct interaction between the lipoxygenase enzyme and the LDL molecule cannot be ruled out. Furthermore, it is important to recognize that the relevance of our findings to the process of peroxidation occurring in the artery wall is open to question, particularly with respect to the use of copper as a pro-oxidant. However, the recent finding that high plasma copper levels are associated with an increased risk for developing coronary heart disease suggests that such transition-metal-dependent reactions could be important *in vivo* [27]. It is also possible that oxidized haem proteins such as myoglobin, released after cell damage, can

also promote a similar reaction to the one described here for copper [28]. Recently, Cu^{2+} has been used to determine the oxidizability of LDL isolated from different donors [17–19]. The results presented here suggest that the endogenous peroxide content of the isolated LDL may be an important determinant of the oxidizability of LDL when assessed using systems dependent on the decomposition of these peroxides.

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Received 12 December 1991/13 January 1992; accepted 15 January 1992