Pro-oxidant effects of 7-hydroperoxycholest-5-en-3β-ol on the copper-initiated oxidation of low density lipoprotein

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Abstract In low density lipoproteins (LDL) supplemented with aged cholesterol and oxidized in the presence of Cu^{2+}, an increase of the lipid oxidation parameters was observed compared with pure cholesterol-enriched LDL. A compound, identified as 7-hydroperoxycholesterol (7HPC), isolated from aged cholesterol and added to LDL, reproduced the above effects. The results indicate that the pro-oxidant effect of 7HPC is dependent on the hydroperoxy group since the corresponding alcohol derivative, 7α-hydroxycholesterol, had no such effect. These data suggest that among the LDL-associated lipid peroxides, cholesterol peroxides may have important implications in the susceptibility of this lipoprotein to oxidation.

Key words: Cholesterol hydroperoxide; Aged cholesterol; Oxysterol; Oxidized LDL; Oxidation; Copper

1. Introduction

Modification of low density lipoproteins (LDL) is thought to be a key event in the pathogenesis of atherosclerosis. In particular, oxidative processes are known to transform LDL, inducing the formation of macrophage-derived foam cells through the scavenger receptor pathway [1,2]. The mechanism of initiation of lipid peroxidation in vivo is unclear. Oxidation of LDL in vitro is generally achieved either by incubation with cultured cells, such as endothelial and smooth cells, or in the presence of micromolar concentrations of transition metals such as copper. The nature of the compounds formed during oxidation and their roles in lipoprotein metabolism are the subject of a large number of reports from several laboratories. In particular, fatty acid or phospholipid peroxides produced by different lipoxygenases, such as 12- and 15-lipoxygenase, could be putative candidates [3]. In support of this idea, it has been shown that atherosclerotic lesions from cholesterol-fed rabbits have increased levels of 15-hydroxyeicosanoic acid (15-HETE), resulting from decomposition of 15-hydroperoxyeicosanenoic acid (HPETE) [4]. In the copper-initiated oxidation of LDL the importance of pre-formed peroxides has been documented. In particular, LDL first pre-treated with reducing agents did not oxidize [5], as measured by thiobarbituric acid substances (TBARS). In addition, increased levels of oxygenated products of cholesterol, oxysterols (OS), have also been described in blood and tissues of atherosclerotic patients [4,6]. The formation of these products is most likely due to free radicals, which arise during the oxidation of LDL fatty acids. It has been reported that, at early times of copper-induced oxidation, cholesterol peroxides are formed [7]. They can also be present in aged cholesterol samples which are known to induce atherosclerosis in animal studies [8]. In the present paper, we have addressed the possibility that aged cholesterol, when incorporated in trace amounts into LDL, might have a pro-oxidant effect in the presence of catalytic concentrations of copper. We thus report herein that a peroxidized compound, isolated from aged cholesterol and identified as 7-hydroperoxycholest-5-en-3β-ol, can totally reproduce these effects when incorporated into human LDL.

2. Materials and methods

2.1. Materials

Aged cholesterol was obtained from a nearly 10-year-old cholesterol sample left in the laboratory without particular caution. This aged cholesterol had a yellowish color, a rancid odor and contained about 20% autooxidation products. By contrast, pure cholesterol was white and odorless and was kept in the frozen state at −20°C in the dark under nitrogen or argon. For some studies, cholesterol was repurified by TLC on silica gel (Merck). Authentic oxysterols were obtained from Steraloids or Sigma, and most of the time repurified by TLC. Purity was checked by GLC (see below). Butylated hydroxytoluene (BHT), and tert-butyl-hydroperoxide (TBHP) were from Sigma. 15-Hydroperoxyeicosanenoic acid (HPETE) was prepared [9] by incubation of arachidonic acid with soya bean lipoxidase (Sigma); 15-hydroxyeicosanoic acid (HETE) was from Cascade Biochem. Silylating reagents (Sylon BTZ) were from Supelco.

2.2. Isolation and analysis of oxysterols

7α-Hydroperoxycholesterol (7HPC) was isolated from aged cholesterol by TLC on silica gel plates eluted with hexane/ethyl acetate (1:1, v/v) and identified by several procedures: (i) by R_f (≈0.60); (ii) by reaction with a 1% solution of the *N,N*-dimethyl-*p*-phenylenediamine hydrochloride reagent which selectively reveals hydroperoxides forming Wurster's dyes visualized as reddish pink colored zones [10]; (iii) when these zones were scraped off the plate and extracted with ethylacetate, the material was identified as authentic 7α-hydroxycholesterol (7OHC) when analyzed by GLC or by GC-MS after reduction with sodium borohydride.

Sterols and oxysterols were extracted in the presence of an internal standard (19-hydroxycholesterol) for quantification and the analysis carried out after derivatization to trimethylsilyl ether (TMS) for 10 min at 50°C with pyridine/BTZ (70:30, v/v). An aliquot of the mixture was injected in the chromatograph (DI200, Delsi, France) equiped with a falling needle injector, a flame ionisation detector and a bonded capillary column (OV 1701, 0.1 μm thickness, 0.32 mm i.d., 30 m; Spiral, Dijon, France). Conditions were: injector 270°C, detector 300°C, oven set at 260°C and programmed to reach 275°C at a rate of 2°/min with helium velocity set at 20 cm/s. In some cases samples were analyzed by GC-MS using a HP5971A mass detector connected with a HP5890 gas-chromatograph (Hewlett Packard).

2.3. Isolation and oxidation of LDL

After an overnight fast, blood samples were taken from volunteers by venipuncture into tubes containing disodium EDTA (1.5 mg/ml),

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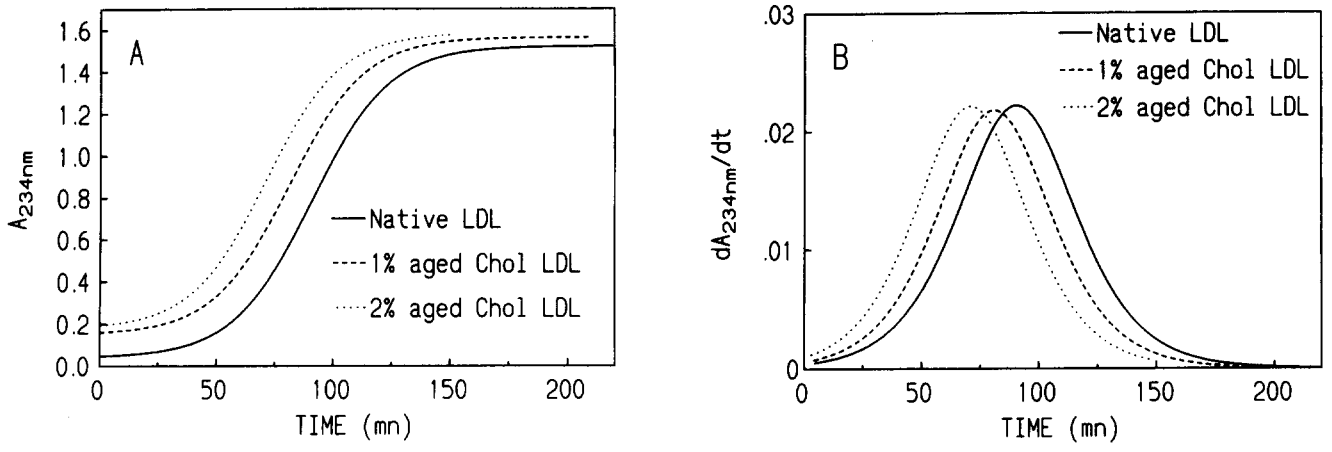

Fig. 1. Monitoring of Cu^{2+}-initiated oxidation of LDL. (A) Lipoproteins (100 μg/ml) oxidation was studied at 37°C by conjugated dienes absorption at 234 nm after addition of 5 μM Cu^{2+}. Native LDL was supplemented with either 1 or 2% aged cholesterol (with respect to total Chol-LDL, 160 μg/ml) as described in section 2. (B) The first derivative, i.e. change of rates of oxidation as a function of time was computed for each curve to obtain the time for half maximum diene formation. The results are representative of two different experiments performed in duplicate.

and the plasma was separated in a cold centrifuge. LDL (1.019–1.055 g/ml) were isolated by sequential ultracentrifugation. Pooled LDL was dialyzed against phosphate buffered saline (PBS) containing 100 μM EDTA. They were sterilized by passage through a Millipore filter (0.22 μm) and stored under argon at 4°C in the dark no longer than 15 days. Sterols were incorporated (1–2% cholesterol-LDL) into LDL as followed: sterols in CH_2Cl_2 were evaporated to dryness under nitrogen or argon in a glass tube and the LDL (200 μg/ml) were then added and left in the dark under argon at ambient temperature with gentle swirling for 30 min. Just before the oxidation experiments, LDL were further purified by small column chromatography wrapped in aluminium foil to remove EDTA [11]. Oxidation of LDL (100 μg/ml) was carried out by exposure to 5 μM Cu^{2+} at 37°C over a 5 h time course.

2.4. Analytical procedures

Lipid peroxidation was measured by monitoring spectrophotometrically the formation of conjugated dienes in a thermostatted cuvette holder maintained at 37°C (Beckman DU65 or DU640). The oxidation was also followed by measuring the thiobabituric acid reactive substances (TBARS) released in the medium and expressed in malondialdehyde (MDA) equivalents as described [12]. Agarose gel-electrophoresis (0.5%) was performed with a Beckman's Paragon lipoprotein electrophoresis kit. Proteins were assayed by the Lowry method [13].

3. Results and discussion

The oxidation of LDL was initiated at 37°C by the addition of 5 μM copper. The extent of oxidation was monitored by measuring absorbance at 234 nm, which correlates with the formation of conjugated dienes. A typical curve is shown in Fig. 1A. As described by Esterbauer [14], the oxidizability of an LDL preparation can be measured by the length of the lag phase, namely when absorbance increases slowly and during which antioxidants are consumed. The conjugated dienes increase rapidly and stabilize when the lipid substrates for peroxidation have been consumed. The rate vs. time plot illustrated in Fig. 1B is the first derivative dA/dt with a maximum at a time characterizing the LDL sample.

When native LDL was supplemented with aged cholesterol in different concentrations, the length of the lag phase decreased (Fig. 1A). Also the maximum rate of conjugated diene formation occurred earlier. These changes were statistically significant since, for three experiments, native LDL had a maximum at 90.8 ± 0.6 min whereas values of 81.2 ± 0.9 and 71.3 ± 1.0 mn were obtained for LDL supplemented with 1% or 2% of aged cholesterol (with respect to total cholesterol LDL), respectively (Fig. 2, $P < 0.001$). These effects were not observed in the absence of copper. By contrast, pure cholesterol incorporated in LDL by the same method did not potentiate the Cu-initiated oxidation of LDL, as indicated by the measurement of TBARS (Table 1). The results presented in Table 1 also confirm that an oxidative process took place, since addition of 40 μM BHT in the incubation medium strongly inhibited Cu-mediated TBARS production induced by the incorporation of aged cholesterol into LDL. These results also confirm that in the absence of Cu^{2+}, aged cholesterol did not produce significant peroxidation of LDL. Moreover, the effect of supplement-

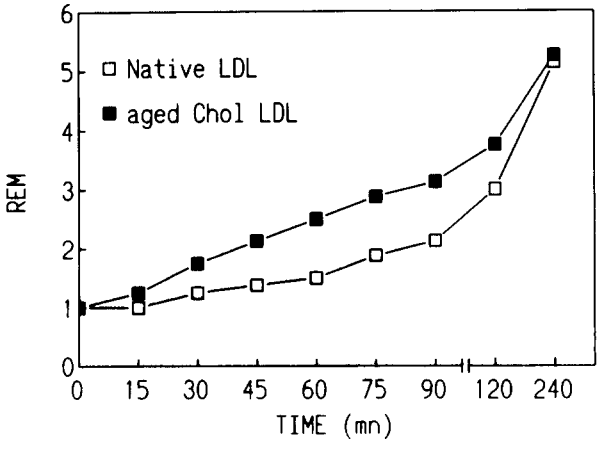

Fig. 2. Effect of aged cholesterol on the electrophoretic mobility of Cu^{2+}-oxidized LDL. Native LDL and 1% aged cholesterol LDL were oxidized as in Fig. 1. After addition of EDTA and BHT (100 and 50 μM, respectively) to stop the reaction at the times shown, the samples were subjected to agarose gel-electrophoresis. Results were expressed relative to the native LDL without Cu^{2+} (REM). The maximum changes in mobility were 20.5 mm for N-LDL and 21.0 mm for AC-LDL. Pure cholesterol-supplemented LDL (1%) behaved as N-LDL and was not represented for the clarity of the figure. Results are the means (±S.D., smaller than the symbol height) of three samples from one experiment carried out with pooled LDL from healthy volunteers.

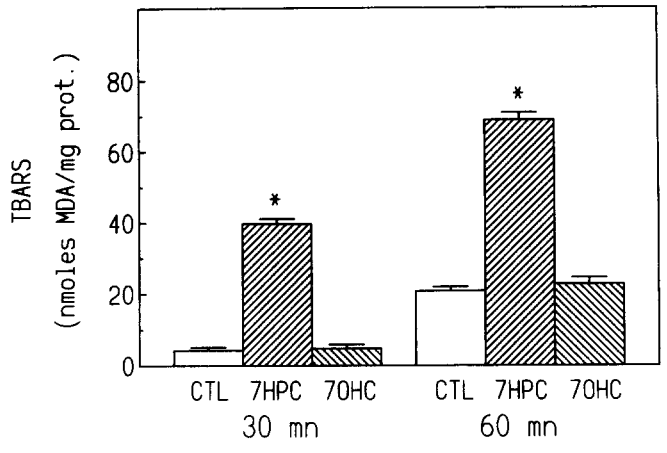

Fig. 3. Effect of 7HPC and 7OHC on the Cu^{2+}-initiated LDL oxidations. Native LDL (CTL) were supplemented with 4 μM 7HPC or 4 μM 7OHC as described in section 2 and oxidized in the presence of 5 μM Cu^{2+}. The reaction was stopped at different times (30 and 60 min) as in Fig. 2 and TBARS measured as described [12]. Results of three experiments are expressed (mean ± S.D., $n = 3$) as nmol MDA per mg protein.

ing LDL with aged cholesterol was determined by agarose gel-electrophoresis (Fig. 2) after the reaction was stopped at various time points up to 240 mn by the addition of EDTA and BHT (100 and 50 μM, respectively). Consistent with the increase in both conjugated dienes and TBARS, the electrophoretic mobility of aged cholesterol-enriched LDL increased significantly more rapidly than native LDL (Fig. 2) or LDL supplemented with pure cholesterol (not shown).

These results indicate that aged cholesterol potentiates the pro-oxidant effect of copper. It has been demonstrated that the oxidation of LDL in vitro by Cu^{2+} requires a trace amount of pre-formed lipid hydroperoxide to be present in the isolated LDL. Indeed, treatment of LDL by a glutathione peroxidase-like activity totally prevented its oxidative modification by copper [5].

Having established that aged cholesterol can act as a pro-oxidant in the presence of Cu^{2+}, the compound responsible for these effects was tentatively identified. Aged cholesterol was analyzed by TLC and eluted with hexane/ethyl acetate (1/1, by volume). Spraying with 50% sulfuric acid revealed that numerous oxidation products of cholesterol were present. They could be identified by GLC, after scraping them off the gel and elution, as oxycholesterols oxidized on the side chain (25-hydroxycholesterol, 25-OH) but also on the ring (cholestane-3β,5α,6β-triol) and mainly in C7 (7α-hydroxy-, 7β-hydroxy- and 7-oxo-cholesterol). When these pure compounds were incorporated into LDL and subsequently oxidized under the same conditions as above by copper ions, no significant pro-oxidant activity was detected. Results of such experiments are represented in Fig. 3 for LDL supplemented with 7α-hydroxycholesterol (7OHC).

Further analysis of TLC plates with a reagent specific for hydroperoxidized compounds, *N,N*-dimethyl-*p*-phenylenediamine hydrochloride [10], revealed that cholesterol peroxides were present between the 7-oxocholesterol and cholesterol. This zone ($R_f \approx 0.60$) was scraped off, and the compounds eluted from the gel with ethylacetate. After reduction with sodium borohydride, extraction and derivatization to TMS, this spot was essentially identified as 7HPC since GLC analysis indicated the presence of 7OHC. Furthermore, when 7HPC was incorporated into LDL, its Cu-induced oxidation markedly increased with respect to control LDL by the assay of TBARS as indicated in Fig. 3. These results have also been confirmed by the change in the electrophoretic mobility of the different LDL preprations (Fig. 4). It is interesting to note that AC-LDL and 7HPC-LDL had similar increased migrations after incubation with Cu^{2+} for 75 min as compared to control LDL or cholesterol-incorporated LDL (not shown).

Fig. 5 illustrates the same type of experiment performed with various hydroperoxides. The results show that TBHP and HPETE, when added to LDL, potentiated the oxidation induced by Cu^{2+} in a similar manner as 7HPC for two different incubation times. Thus the pro-oxidant effect of 7HPC was not specific and could be achieved by incubation of a number of peroxide products with LDL. These results confirm the data obtained by O'Leary et al. [15] in their work about the effect of fatty acid peroxides on LDL oxidation. They have reported that several fatty acid peroxides have little difference in potency. As in our work, their results support the pro-oxidant property of peroxide products being dependent on the hydroperoxy group. This is clearly indicated since the corresponding alcohol derivatives, HETE or 7OHC, did not show any effect.

Table 1
The effect of aged cholesterol on Cu^{2+}-initiated oxidation of LDL

	Cu^{2+}	TBARS (nmol MDA/mg LDL)	
		30 min	60 min
N-LDL	−	1.6 ± 0.5	1.8 ± 0.6
	+	4.3 ± 0.8	20.8 ± 1.2
AC-LDL	−	1.8 ± 0.7	2.1 ± 0.6
	+	48.4 ± 1.1	71.5 ± 1.9
C-LDL	−	1.8 ± 0.6	2.0 ± 0.9
	+	4.6 ± 0.9	21.2 ± 1.2
AC-LDL + BHT	−	1.6 ± 0.6	1.8 ± 0.7
	+	4.1 ± 0.8	4.3 ± 0.9

Native LDL (N-LDL), pure cholesterol-LDL (C-LDL) and aged cholesterol LDL (AC-LDL), were oxidized (100 μg/ml) in triplicate for 30 and 60 min at 37°C with or without Cu^{2+} (5 μM). AC-LDL was also incubated in the presence of 40 μM BHT. The samples were assayed for TBARS as described in section 2 [12]. Results (mean ± S.D., $n = 3$) are expressed as nmol MDA/mg protein LDL.

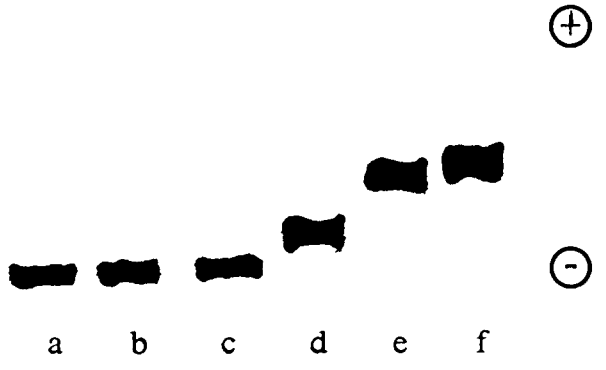

Fig. 4. Agarose gel-electrophoresis of LDL enriched with aged cholesterol and 7HPC. LDL (a,d) was supplemented with either 1% aged cholesterol (b,e) or 4 μM 7HPC (c,f) as described in section 2 and incubated in the presence of 5 μM Cu^{2+}. The reaction was stopped as in Fig. 2, before (lanes a, b, c) and 75 min after oxidations (lanes d, e, f) and the samples subjected to agarose gel-electrophoresis.

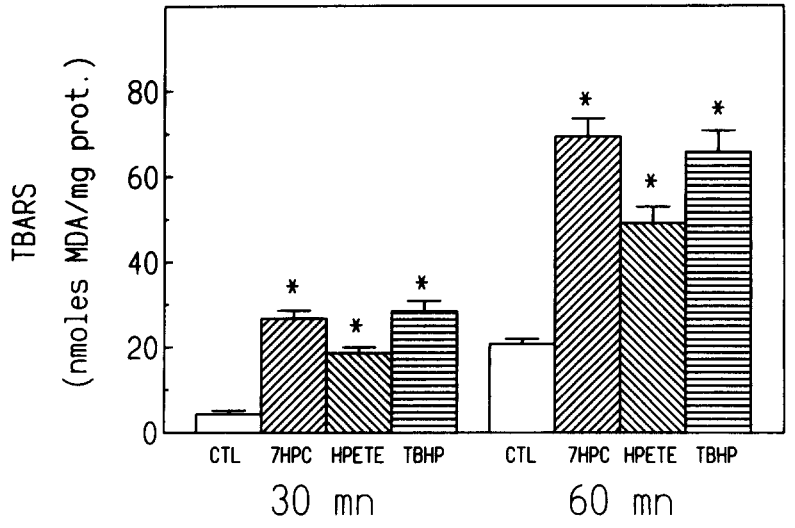

Fig. 5. TBARS of Cu^{2+}-induced oxidation of LDL supplemented with various peroxides. LDL were enriched with either 4 μM 7HPC or 15-HPETE or tert-butylhydroperoxide (TBHP) and incubated with 5 μM Cu^{2+}. Reactions were stopped and TBARS analyzed as in Fig. 3.

Oxysterols (OS) can have various origins [6]. They have been identified in different biological fluids and tissues. In particular, OS are present in oxidized LDL [16], and human atheroma samples contain larger amounts than normal aorta [4]. In vivo OS may partly be formed by reduction of cholesterol peroxides, as has been observed for hydroxy fatty acids. OS were first demonstrated to induce atherosclerosis in animals and to be toxic for vascular cells [16–19]. Epidemiological studies were conducted in Indian migrants in England who have a high incidence of atherosclerosis [19]. The large amount of OS found in their particular diet and the absence of commonly accepted risk factors suggested that OS might be involved in the atherogenic process. The exact mechanism is not yet clearly established. In particular, specific OS markedly influenced cellular cholesterol metabolism since an inhibition of 3-hydroxy-3-methylglutaryl-CoA:reductase and an enhanced cholesterol esterification associated with an increase in acyl CoA: cholesterol acyltransferase have been reported in their presence [20,21].

In summary, we found that in vitro addition of cholesterol peroxide to LDL has a pro-oxidant effect as measured by several lipid oxidation parameters, such as conjugated diene formation, TBARS release, and changes in the electrophoretic mobility of LDL. These data emphasize the role of pre-formed peroxides in the LDL fate. Peroxides are probably formed in LDL during the lengthy isolation procedure. In addition, they could be present in vivo arising from food lipids or resulting from cell-mediated oxidation through oxygenase activity. While little is known about absorption and transport of dietary lipid peroxides during fat digestion, the effect of lipoxygenases and metals on isolated LDL is under intensive studies. The passive mechanism used in our study to incorporate peroxides in LDL may be different from the process by which phospholipids or cholesterol peroxides are formed within LDL. It is interesting to point out that 7HPC has already been detected in oxidized LDL [7]. However, the exact mechanism whereby copper ions lead to the formation of lipid radicals is still being debated. Although it is known that metal ions catalyse the decomposition of peroxides, the occurrence of a finite number of copper binding sites on the LDL particle has recently been reported [22]. In addition, for a still unknown reason, this number can vary considerably among LDL prepared from different donors. This might be important in vivo since high plasma copper levels have been associated with an increased risk for premature cardiovascular diseases [7]. Consequently, the results presented in this work further suggest that, among lipid peroxides, those originating from cholesterol may be relevant for the susceptibility of LDL to oxidation.

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