Formation of oxysterols during oxidation of low density lipoprotein by peroxynitrite, myoglobin, and copper

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Abstract Oxidation of low density lipoprotein (LDL) in the artery wall leads to the formation of cholesterol oxidation products that may result in cytotoxicity. Different mechanisms could contribute to LDL oxidation in vivo resulting in characteristic and specific modification of the cholesterol molecule. Alternatively, attack on cholesterol by chain propagating peroxyl radicals could result in the same distribution of oxidation products irrespective of the initial pro-oxidant mechanism. To distinguish between these possibilities we have monitored the formation of nine oxysterols during LDL oxidation, promoted by copper, myoglobin, peroxynitrite, or azo bis amidino propane. Regardless of the oxidant used, the pattern of oxysterol formation was essentially the same. The yields of products identified decreased in the order 7-oxocholesterol > 7β-hydroxycholesterol > 7α-hydroxycholesterol > 5,6β-epoxycholesterol > 5,6α-epoxycholesterol except in the case of peroxynitrite in which case a higher yield of 5,6β-epoxycholesterol relative to 7-oxocholesterol was found. No formation of cholestane 3β,5α,6β-triol, or the 24-,25-,27-hydroxycholesterols was seen. Concentration of 7-oxocholesterol levels in LDL was positively correlated with the degree of protein modification. Endogenous α-tocopherol in LDL or supplementation with butylated hydroxytoluene prevented oxysterol formation. Taken together these data indicate that the oxidation of cholesterol and protein in LDL occur as secondary oxidation events consequent on the attack of fatty acid peroxyl/alkoxyl radicals on the 7-position of cholesterol, and with amino acids on apoB. Furthermore, oxidant processes with atherogenic potential, such as peroxynitrite, copper, and myoglobin are capable of producing oxidized LDL containing cytotoxic mediators.—**Patel, R. P., U. Diczfalusy, S. Dzeletovic, M. T. Wilson, and V. M. Darley-Usmar.** Formation of oxysterols during oxidation of low density lipoprotein by peroxynitrite, myoglobin, and copper. *J. Lipid Res.* 1966. **37:** 2361–2371.

Supplementary key words low density lipoprotein • oxysterols • cholesterol • atherosclerosis • peroxynitrite • copper • myoglobin

Evidence has been provided to support the hypothesis that oxidative modification of low density lipoprotein (LDL) is an important event in the development of atherosclerosis (1–3). This includes the detection of oxidized LDL in atherosclerotic lesions in rabbits and humans. LDL oxidized in vitro chemically resembles LDL found in atherosclerotic lesions (4) and is cytotoxic (5). Furthermore, dietary supplementation with antioxidants has been shown to inhibit atherosclerotic lesion formation in animal models (6, 7) and may be linked to a lower incidence of atherosclerosis in humans (8).

One mechanism by which oxidized LDL is pro-atherogenic is due to its unregulated uptake by the macrophage scavenger receptors. This results in high levels of lipid deposits within macrophages endowing them with a foamy appearance, a characteristic feature of atherosclerotic lesions (1). However, other mechanisms have been suggested through which oxidized LDL may contribute to the pathogenesis of atherosclerosis. These include the stimulation and inhibition of cytokine formation (9, 10), stimulation of monocyte chemotactic factor, promoting monocyte and endothelial cell adhesion (11), and cytotoxicity towards fibroblasts (12–14), endothelial (12), and smooth muscle cells (15). All of

Abbreviations: Cu^{2+}, copper; Mb^{3+}, myoglobin; AAPH, 2,2′-azobis(amidino-propane hydrochloride); SIN-1, 3-morpholinosydnonimine-N-ethylcarbamide; $ONOO^-$, peroxynitrite; LDL, low density lipoprotein; BHT, butylated hydroxytoluene; DTPA, diethylenetriaminepentaacetic acid; REM, relative electrophoretic mobility.

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these properties point to the formation of oxidized LDL as an important early step in atherogenesis.

These biological effects are largely mediated by products formed during oxidation of the lipid component of LDL. Recently the cytotoxic fraction of oxidized LDL has been identified as the specific cholesterol oxidation products, 7β-hydroperoxycholesterol, 7-oxocholesterol, and 7β-hydroxycholesterol. These have also been detected in atherosclerotic tissue (14, 16). Apart from the possibility of their formation by oxidation in vivo, they can be present in the diet (17) and an epidemiological study has suggested that the relatively high concentration of oxysterols in the diet of certain Indian immigrant populations in Britain was the cause of the abnormally high incidence of atherosclerosis in such communities (18).

The nature of the oxidant(s) responsible for LDL oxidation in vivo is unknown, although several candidates have been proposed. These include metal ions such as copper and iron (19), the oxygen-carrying heme proteins myoglobin and hemoglobin (20), and metalloproteins such as ceruloplasmin and lipoxygenase (21, 22). These oxidants only oxidize LDL in the presence of hydroperoxides either inserted into the LDL lipid phase or present in the aqueous compartment close to the LDL particle. Oxidants utilizing hydroperoxide-independent mechanisms for LDL oxidation include peroxynitrite ($ONOO^-$), and hypochlorous acid (23–25).

Oxidation of cholesterol can lead to the formation of a variety of different oxysterols which could be formed from the secondary reaction of fatty acid-derived peroxyl radicals abstracting a hydrogen atom from the cholesterol molecule or direct abstraction by a primary oxidant formed during the atherosclerotic process. Transition metal or lipoxygenase-dependent mechanisms for the oxidation of LDL are unlikely to oxidize the cholesterol molecule directly as they either require lipid hydroperoxides for activation or preferentially utilize fatty acids as a substrate. The co-oxidation of cholesterol in a model lipid oxidation system by myoglobin in the presence of hydrogen peroxide and salicylate has been described (26) and this has raised the possibility that ferryl heme species may be capable of direct oxidation of cholesterol in LDL. Furthermore, the recent finding that the rapid reaction between nitric oxide and superoxide may occur in human atherosclerotic lesions to form the powerful oxidant $ONOO^-$ suggests a mechanism by which direct oxidation of cholesterol could occur (23, 24). Previous studies have demonstrated the formation of oxysterols during the oxidation of LDL in cell and in vitro systems but a direct comparison of different mechanisms of oxidation has not been reported. In this study we have oxidized LDL by a variety of oxidants, having different mechanisms of action, and have examined the pattern of formation of nine oxysterols using isotope dilution mass spectrometry. Our results show that the pattern of oxysterols is independent of the oxidant used, and that 7-oxo-cholesterol and 7β-hydroxycholesterol are formed in substantial quantities. It has been suggested that the parent lipid hydroperoxide (7β-hydroperoxycholesterol) is the cytotoxic mediator (15) whereas other experimental evidence implicates its decomposition products 7-oxocholesterol and 7β-hydroxycholesterol (16). The results are discussed in terms of a model in which the initial event is to oxidize polyunsaturated fatty acids, the products of which, in turn, attack cholesterol and protein in LDL.

MATERIALS AND METHODS

Materials

AAPH was obtained from Polysciences, Warrington, PA. Horse heart myoglobin and superoxide dismutase were obtained from Sigma and SIN-1 was a generous gift from Casella A.G. All other chemicals were from Sigma and used without further purification.

Isolation and purification of LDL

Human LDL was isolated from plasma of healthy donors by differential centrifugation using the method previously described (27). The LDL thus obtained was dialyzed against Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) containing 10 μM EDTA and then sterilized by filtration through a 0.2 μM pore size filter. LDL was stored at 4°C and used within 1 week of preparation. The concentration of LDL protein was determined using the BCA protein assay reagent (Pierce).

Preparation of peroxynitrite

Peroxynitrite was prepared in a quenched flow reactor as previously described and stored at pH 11–12 until used (28). The concentration was measured by its absorbance at 302 nm and using an extinction coefficient of 1670 M^{-1} cm^{-1}.

Oxidation of LDL

Stock solutions of Cu^{2+} were made up in distilled water and those of Mb^{3+}, SIN-1, and AAPH in PBS (Ca^{2+}/Mg^{2+}-free). SIN-1 and AAPH stock solutions were made just before use. LDL (500 μg/ml) was incubated with each of the oxidants and aliquots were taken at the specified times. Reactions were stopped by addition of BHT (50 μM). In some incubations with SIN-1, superoxide dismutase (200 U/ml) was also added.

For incubation with $ONOO^-$ solutions, LDL was diluted into PBS supplemented with 100 mM potassium

phosphate buffer, pH 7.4. Peroxynitrite (10–20 μl) was added onto the cap of an Eppendorf tube containing 1 ml LDL (500 μg/ml). Reactions were started by vortex mixing. To avoid additional LDL oxidation by contaminating metal ions present in the buffers, DTPA (100 μM) was added to all oxidation experiments except those involving copper. After oxidation, samples were snap frozen in liquid nitrogen and stored at −80°C before being shipped on dry ice for analysis.

Measurement of REM and α-tocopherol

The relative electrophoretic mobility of LDL was determined on agarose gels using the lipoprotein electrophoresis system supplied by Beckman. α-Tocopherol was extracted into heptane and quantitated by HPLC using fluorescence detection (29).

Measurement of oxysterols

The oxysterols cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol), cholest-5-ene-3β,7β-diol (7β-hydroxycholesterol), 3β-hydroxycholest-5-ene-7-one (7-oxocholesterol), 5,6α-epoxy-5α-cholestan-3β-ol (cholesterol-5α,6α-epoxide), 5,6β-epoxy-5β-cholestan-3β-ol (cholesterol-5β,6β-epoxide), cholestane-3β,5α,6β-triol, cholest-5-ene-3β,24-diol (24-hydroxycholesterol), cholest-5-ene-3β,25-diol (25-hydroxycholesterol), and cholest-5-ene-3β,27-diol (27-hydroxycholesterol) were measured by a method based on isotope dilution–mass spectrometry as previously described (30). In this study only the total oxysterol was determined as described previously (31). To determine the fate of 7α-hydroperoxycholesterol during analysis of free oxysterols (i.e., when omitting alkaline hydrolysis), cholesterol was converted into 5α-hydroperoxycholest-7-en-3β-ol by photosensitized oxidation as described (32). The 5α-hydroperoxycholest-7-en-3β-ol was allowed to isomerize to 7α-hydroperoxycholesterol by standing overnight in chloroform (33) and was subsequently purified by thin-layer chromatography (TLC) using toluene–ethyl acetate 3:7 (v/v). The 7α-hydroperoxycholesterol was carried through the analytical procedure for oxysterols and product analysis (**Table 1**) indicated approximately 90% conversion of the cholesterol peroxide to the corresponding 7α-hydroxy and 7-oxo analogs with little formation of the 7β-isomer. This result shows that the analytic method can be used for determination of unesterified oxysterols with reasonable accuracy.

TABLE 1. Recovery of 7α-hydroperoxycholesterol as 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-oxo-cholesterol

Conditions	7α-OH-Chol	7β-OH-Chol	7-Oxo-Chol	Total
		ng oxysterol		
With hydrolysis	1040	33	928	2001
	688	31	1204	1923
	420	23	1125	1567
	1252	45	1147	2444
Mean	850	33	1101	1984
Without hydrolysis	285	21	1301	1606
	557	25	1555	2136
	212	20	1329	1560
	242	20	1405	1666
Mean	324	21	1398	1742

Authentic cholesterol hydroperoxide was prepared as described in Materials and Methods and 2 μg of 7α-hydroperoxycholesterol processed through the analytical procedure for oxysterols either including or omitting alkaline hydrolysis.

RESULTS

Table 2 shows the production of the different oxysterols during oxidation of LDL by Cu^{2+}, Mb^{3+}, SIN-1, and AAPH. The first two of these oxidants require the presence of endogenous "seeding" lipid hydroperoxides which are present in human LDL preparations at varying concentrations and are not introduced during isolation (19, 20, 34). In contrast, SIN-1 has been shown to oxidize LDL via its decomposition to form nitric oxide and superoxide (23) and AAPH decays to yield amphipathic peroxyl radicals (35). Both SIN-1 and AAPH represent mechanisms of peroxidation in which initiation occurs independent of the presence of lipid peroxides.

Regardless of the oxidant used, only the 7-oxo-cholesterol, 7α- and 7β-hydroxycholesterols, 5,6α- and 5,6β-epoxycholesterols were formed in significant amounts. Little or no production of 3,5,6-triol, 24, 25, 27-hydroxyl cholesterols was seen during Cu^{2+}-mediated oxidation, with no enhanced formation relative to controls seen with Mb^{3+}, SIN-1, or AAPH. Furthermore, the relative concentrations of oxysterols formed were also oxidant-independent, with the concentrations decreasing in the order: 7-oxo-cholesterol > 7β-hydroxycholesterol > 7α-hydroxycholesterol > 5,6β-epoxycholesterol > 5,6α-epoxycholesterol. This suggests that the oxysterols are formed through a common mechanism. Similar results were observed with 6 other LDL preparations, although the absolute concentrations of oxysterols varied, presumably due to the well-established differences in the intrinsic oxidizability of LDL isolated from different donors (36).

Figure 1 shows that formation of 7-oxo-cholesterol during Cu^{2+}-, Mb^{3+}-, SIN-1-mediated oxidation occurs after a lag phase that corresponded to the consumption of α-tocopherol. This is consistent with previously reported antioxidant effect of α-tocopherol during LDL oxidation (29, 35, 36). Essentially identical data was obtained for the other 4 oxysterols formed (data not shown). The rates of oxidation of LDL for these oxidants is significantly different, as can be seen from both

TABLE 2. Formation of oxysterols on incubation of LDL (500 μg/ml) with either Cu^{2+} (20 μM), myoglobin (5 μM), SIN-1 (1mM), or AAPH (1 mM)

Oxidant	Oxysterol								
	7-Oxo	7β-OH	7α-OH	5,6β-Epoxy	5,6α-Epoxy	3,5,6-Triol	24-OH	25-OH	27-OH
	ng/ml								
None	24	19	30	46	25	18	13	19	25
Copper	32,300	22,890	19,650	12,830	2,855	214	190	495	143
Myoglobin	9,600	5,870	4,680	4,060	665	88	132	114	24
SIN-1	2,135	1,875	1,525	1,242	210	38	10	27	14
AAPH	1,320	438	297	532	96	19	13	18	23

Experiments were carried out in PBS at 37°C for 8.5 h. Reactions were stopped by addition of BHT (50 μM). All values represent an average of duplicate samples from a single LDL preparation.

the rate of depletion of α-tocopherol and accumulation of the cholesterol oxidation products. The relative electrophoretic mobility (REM) of LDL was also determined as an indication of the extent of protein modification that occurs upon reaction between amino acid residues (such as lysine and arginine) on apoB and lipid-derived oxidation products. Over these short time courses only where Cu^{2+} was used as an oxidant was a significant change in REM detected consistent with the hypothesis that protein modification is consequent on achieving a threshold level of lipid oxidation products.

In agreement with previous reports (23), in experiments where SIN-1 was used, addition of superoxide dismutase (220 U/ml) inhibited oxysterol formation by 98% (**Table 3**), indicating that $ONOO^-$ was the prooxidant species. Furthermore, BHT (50 μM), a lipoprotein antioxidant that scavenges peroxyl/alkoxyl radicals, inhibited oxysterol formation completely (Table 3).

The effect of $ONOO^-$ was also examined by its direct addition to LDL. As with SIN-1 and the other oxidants examined, $ONOO^-$ caused a concentration-dependent increase in 7-oxocholesterol, 7α- and 7β-hydroxycholesterol, 5,6α- and 5,6β-epoxycholesterols (**Fig. 2**) that was complete in less than 5 min (data not shown). At the lower concentration of $ONOO^-$ (0.5 mM) a greater yield of the 5,6β-epoxycholesterol was found relative to 7-oxocholesterol. This could reflect $ONOO^-$-dependent effect on the formation of secondary oxidation products from the decomposition of the 7β-cholesterol hydroperoxide. On addition of decomposed $ONOO^-$ (equivalents to 5 mM $ONOO^-$) to LDL, no increase in oxysterols was seen, indicating that the observed changes were due to the oxidant and not its decomposition products. Relative to SIN-1, however, direct addition of $ONOO^-$ caused only a modest increase in oxysterol concentrations, values increasing up to approximately 100–300 ng/ml compared to 2000–3000 ng/ml when SIN-1 was used.

As noted from the data in Fig. 1 it is clear that the oxidants utilized in this study show intrinsically different rates of oxidation, which is to be expected when diverse mechanisms of promoting lipid peroxidation are used. To test the hypothesis that the formation of cholesterol oxidation products is independent of the mechanisms of oxidation, it was then necessary to normalize oxysterol concentrations with respect to the level of protein modification as measured by an increase in REM. This may then be taken as an index of the extent of oxidation that is independent of the efficiency of the initiating mechanism of oxidation. Data obtained from 6 LDL preparations are shown in **Fig. 3A** which compares the levels of 7-oxocholesterol formed with increase in REM during Cu^{2+}-, Mb^{3+}-, SIN-1, and AAPH-mediated oxidation in which the reactions were allowed to proceed for different periods of time (0–24 h). This allowed preparation of samples with the different oxidants to the same degree of protein modification despite the fact that the intrinsic efficiency for promoting oxidation varied. A linear relationship between increase in 7-oxocholesterol and REM is seen up to REM values of around 3. Above this value, which in the experiments conducted here was only achieved by Cu^{2+}, the levels of cholesterol oxidation are enhanced with respect to protein modification. Essentially similar data were obtained for the REM versus 7α/7β-hydroxycholesterol and 5,6α/5,6β-epoxycholesterols (data not shown). A similar trend has been noted by others when comparing 7β-hydroxycholesterol and REM during macrophage and Cu^{2+}-dependent LDL oxidation, although in that case the linear relationship started to deviate at REM values of approximately 1.5 (37).

Figure 3B directly compares the effect of adding a $ONOO^-$ solution and its in situ formation from the simultaneous generation of superoxide and nitric oxide by SIN-1. A greater extent of lipid peroxidation relative to protein modification occurs with SIN-1 compared to the direct addition of $ONOO^-$, which appears to result in protein modification but relatively little cholesterol oxidation.

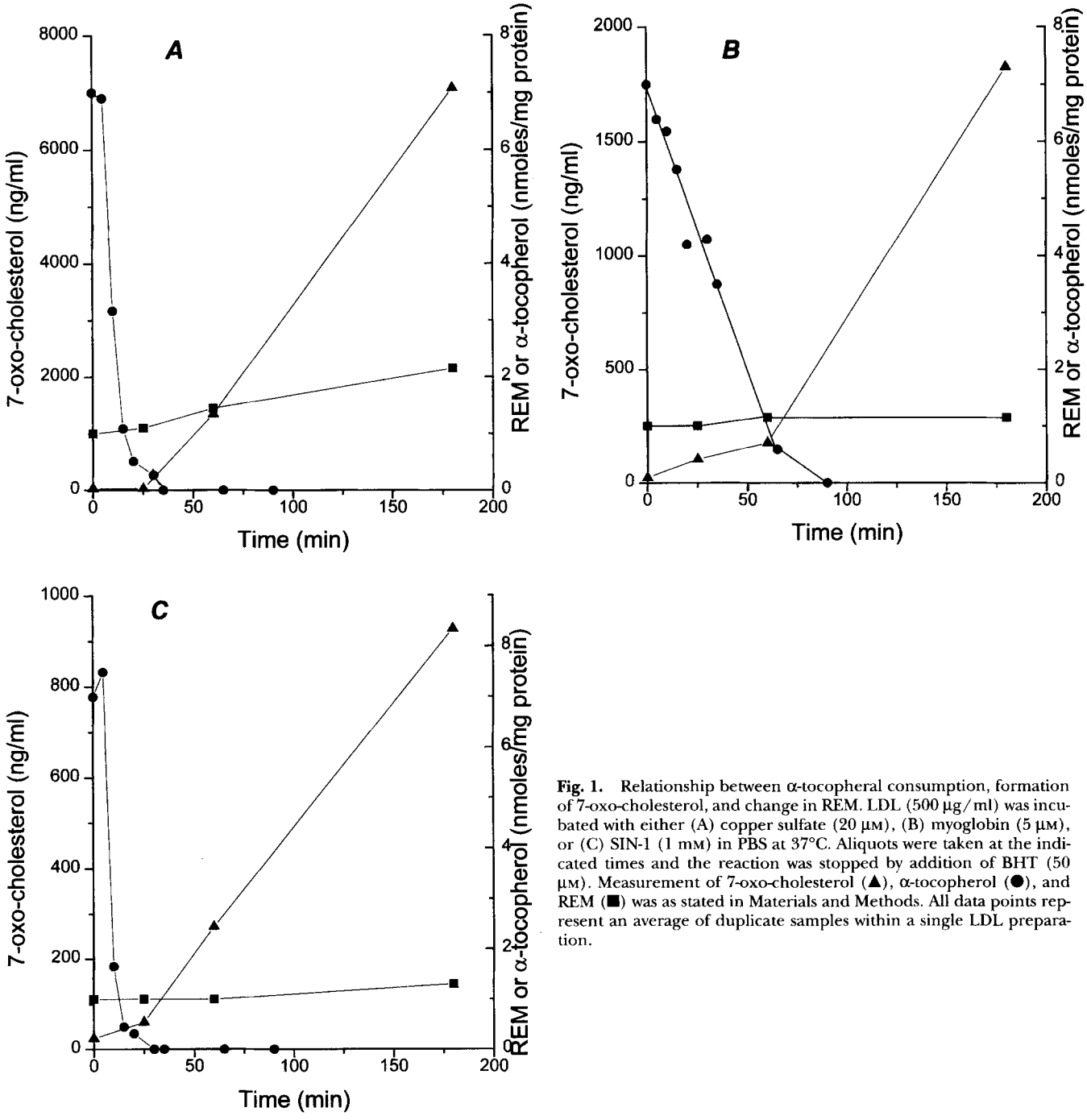


Fig. 1. Relationship between α-tocopheral consumption, formation of 7-oxo-cholesterol, and change in REM. LDL (500 μg/ml) was incubated with either (A) copper sulfate (20 μM), (B) myoglobin (5 μM), or (C) SIN-1 (1 mM) in PBS at 37°C. Aliquots were taken at the indicated times and the reaction was stopped by addition of BHT (50 μM). Measurement of 7-oxo-cholesterol (▲), α-tocopherol (●), and REM (■) was as stated in Materials and Methods. All data points represent an average of duplicate samples within a single LDL preparation.

DISCUSSION

Oxysterols, formed during oxidation of LDL, possess cytotoxic properties that may contribute to the pro-atherogenic effect of oxidized LDL. The 7-oxygenated sterols (i.e., 7-oxocholesterol, 7α- and 7β-hydroxycholesterol) are derived from the reduction and subsequent dehydration of the respective 7-hydroperoxycholesterols. The 5,6-epoxides are believed to be formed by the reaction of 7-peroxyl radicals with the C-5 double bond in cholesterol (38). The 7β-hydroxy derivative was always formed in greater amounts than the 7α-hydroxy derivative, a fact which may be explained by the spontaneous unidirectional epimerization of 7α- to 7β-hydroperoxycholesterol (31). Due to the presence of abstractable hydrogen atoms at the 7 position of the

TABLE 3. Effect of superoxide dismutase (SOD) and butylated hydroxytoluene (BHT) on SIN-1-mediated formation of oxysterols during oxidation of LDL (500 μg/ml)

	Oxysterol				
	7-Oxo	7β-OH	7α-OH	5,6β-Epoxy	5,6α-Epoxy
	ng/ml				
Control	45	56	32	41	9
SIN-1 (1 mM)	4755	3025	2520	2110	420
SIN-1 (1 mM) + SOD (200 U/ml)	67	81	80	130	50
SIN-1 (1 mM) + BHT (50 μM)	58	75	54	165	41

Experiments were carried out in PBS at 37°C for 16 h. Reactions were stopped by addition of BHT (50 μM). All values represent an average of duplicate samples from a single LDL preparation.

cholesterol molecule, this is the site most susceptible to oxidation and it is likely that attack by fatty acid peroxyl/alkoxyl radicals at this position will yield the hydroperoxide (reactions 1 and 2, **Scheme 1**). In the presence of redox active agents such as Cu^{2+} or Mb^{3+}, hydroperoxides can be decomposed (reaction 3, Scheme 1), eventually forming the hydroxy derivatives. Exactly how these oxysterols arise in the lipid hydroperoxide-independent oxidation systems is not known, although recently a reductase enzymatic activity, intrinsic to LDL, has been proposed (39).

During the analytical procedure used to monitor oxysterols, cholesterol 7-hydroperoxides were quantitatively converted into 7-hydroxycholesterol and 7-oxocholesterol (31). Reduction of fatty ester hydroperoxides to the corresponding alcohols has been shown to occur during alkaline hydrolysis under conditions com-

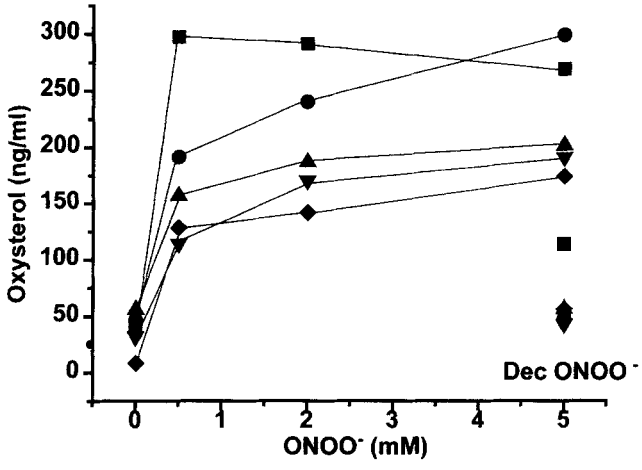


Fig. 2. Peroxynitrite-mediated formation of oxysterols in LDL. LDL (500 μg/ml) was incubated with varying concentrations of $ONOO^-$ for 30 min. Reactions were stopped by addition of BHT (50 μM). In control experiments decomposed $ONOO^-$ (Dec $ONOO^-$) at a concentration of up to 5 mM was incubated with LDL for 30 min. All reactions were carried out in PBS at 37°C and each data point represents an average of two separate determinations; key: 7-oxo-cholesterol (●); 7β-hydroxycholesterol (▲); 7α-hydroxycholesterol (▼); 5,6β-epoxycholesterol (■); 5,6α-epoxycholesterol (◆).

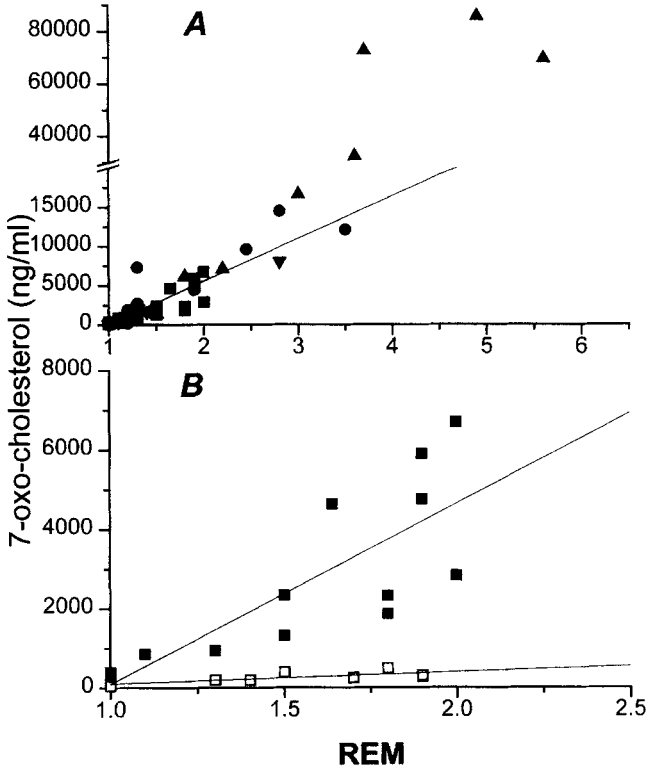
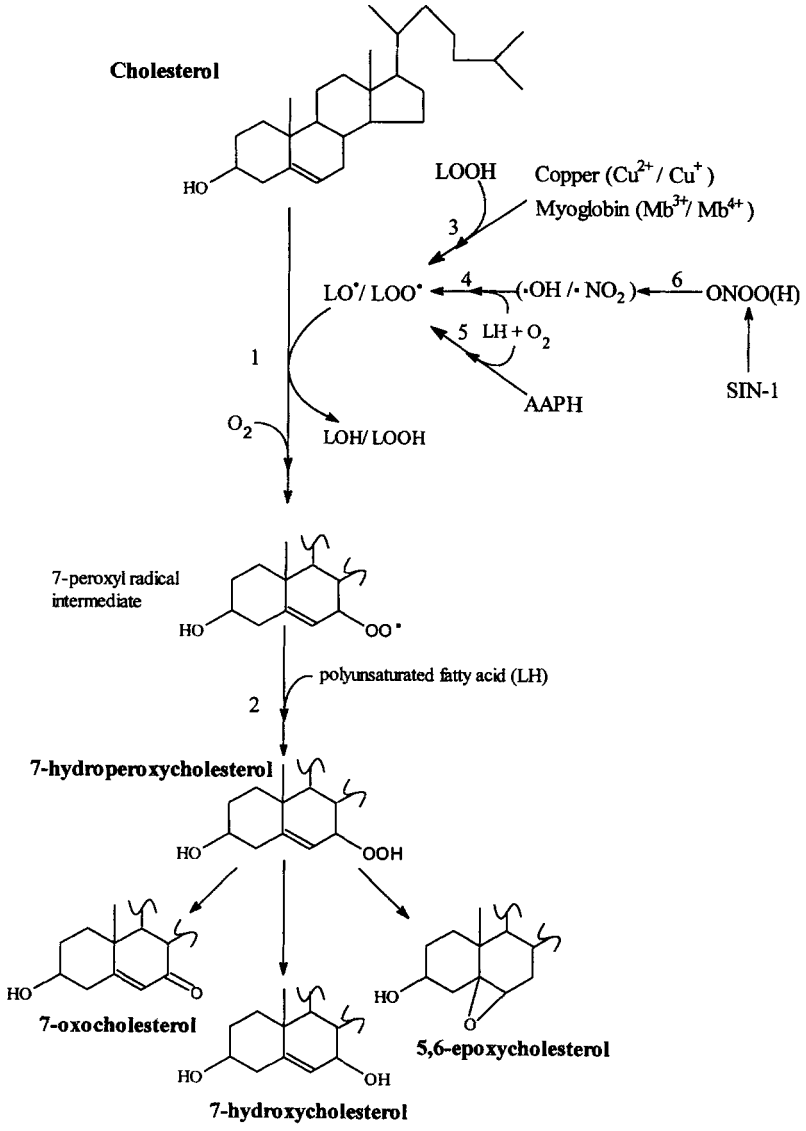


Fig. 3. Correlation of 7-oxocholesterol formation and increase in REM. LDL (500 μg/ml) was oxidized using copper (▲), myoglobin (●), SIN-1 (■), $ONOO^-$ (□), or AAPH (▼). Each point represents an average of duplicate measurements and was obtained from 6 different LDL preparations incubated for varying periods (0–24 h) with the different oxidants before the reaction was stopped by addition of BHT (50 μM). Solid lines represent best fit line calculated by linear regression analysis. Only data points up to REM 3 were used for fitting on Fig. 3A; $r^2 = 0.893$). All data points were used for fitting on Fig. 3B; $r^2 = 0.832$ (SIN-1, ■) and $r^2 = 0.704$ ($ONOO^-$, □). All reactions were carried out in PBS at 37°C and were stopped prior to oxysterol and REM measurement.



Scheme 1. Mechanism(s) through which lipid alkoxyl ($LO^{\cdot}$)/peroxyl ($LOO^{\cdot}$) radicals, formed via the reaction Cu^{2+}, myoglobin (Mb^{3+}), AAPH, or $ONOO^{-}$ with polyunsaturated fatty acids (LH), react with cholesterol to form oxysterols. Lipid hydroperoxide, LOOH; lipid hydroxide, LOH; hydroxyl radical like species, [$^{\cdot}OH/NO_2$].

parable to those in the present investigation (40). When 7α-hydroperoxycholesterol was carried through the analytical procedure omitting the hydrolysis step, it was recovered as 7α-hydroxycholesterol and 7-oxo-cholesterol in 88% yield (Table 1). No formation of 7β-hydroxycholesterol was observed. The absence of a readily abstractable hydrogen in the side chain of the cholesterol molecule renders it relatively insensitive to oxidation at these positions by peroxyl radicals. Peroxynitrite or ferryl heme could, in principle, abstract a hydrogen atom at this position but no formation of the side chain oxysterols (24, 25 or 27-hydroxycholesterol) was seen. The cholestane-3β,5α,6β-triol is the product of hydration of the epoxides and is normally detected during the auto-oxidation of cholesterol. However, no significant amounts were formed during LDL oxidation with the pro-oxidants used in this study.

Both Cu^{2+} and Mb^{3+} require the presence of lipid hydroperoxides, reacting with them via a redox cycle producing lipid-based peroxyl and alkoxyl radicals (reaction 3, Scheme 1) which further propagate the peroxidation process (19, 20). With Mb^{3+} an additional process involving ferryl myoglobin-dependent hydrogen abstraction can also occur (20). Both peroxynitrite and AAPH react through lipid hydroperoxide-independent mechanisms (reactions 4 and 5, Scheme 1). Peroxynitrite, when protonated to form peroxynitrous acid (reaction 6, Scheme 1), decays to a hydroxyl radical-like species sufficiently reactive to abstract an allylic hydrogen atom, and AAPH decomposes to peroxyl radicals

that can also directly initiate lipid peroxidation reactions (23, 35, 41). Under the experimental conditions used, α-tocopheral acted as an antioxidant (Fig. 2). The rates of α-tocopheral consumption for both copper- and SIN-1-dependent oxidation were similar. However, copper was a more potent oxidant as illustrated by the elevated levels of oxysterol formation and protein modification. Both Cu^{2+} and $ONOO^-$ react directly with α-tocopherol (42, 43), but the autocatalytic nature of Cu^{2+}-dependent oxidation, resulting from redox cycling, leads to a more efficient oxidation.

With the exception of $ONOO^-$, the major oxysterols formed during oxidation of LDL with different oxidants were 7-oxo-cholesterol, 7β-hydroxycholesterol, 7α-hydroxycholesterol, and cholesterol 5,6-epoxides in descending order. In the present investigation total oxysterols (free + esterified) were determined. These oxysterols are the same as those found by Zhang, Basra, and Steinbrecher (44) who oxidized LDL with Cu ions and made a semiquantitative determination of the oxysterols formed. These authors analyzed free oxysterols but showed by TLC that esterified oxysterols were also formed. They found 7-oxo-cholesterol to be the most abundant oxysterol. However, in their gas chromatographic analysis, a dehydration product of 7-oxo-cholesterol, probably cholest-3,5-dien-7-one, was the most abundant component. They showed that this compound was generated from 7-oxo-cholesterol during analysis of underivatized samples, but disappeared when the samples were silylated prior to analysis (44). Bhadra et al. (45) also found cholest-3-5-dien-7-one to be the dominant oxysterol after copper oxidation of LDL. In this investigation the oxidized LDL samples were subjected to alkaline hydrolysis and thus total oxysterols were determined. It is likely that cholest-3,5-dien-7-one was artifactually formed from 7-oxo-cholesterol by dehydration as discussed by the authors. We have previously shown that alkaline hydrolysis of LDL lipids for more than 2 h at 22°C results in degradation of 7-oxocholesterol (30). Jialal, Freeman, and Grundy (46) identified 7-oxo-cholesterol as the major oxysterol in copper-oxidized LDL but did not report the presence of 7α-, 7β-, or 25-hydroxycholesterol. The reason for this is not clear but the resolution of the gas chromatographic analysis used may have been a limiting factor. Both free and total oxysterols were determined and the authors found that free sterol was only slightly more susceptible to oxidation than esterified cholesterol (46). The primary 7-oxygenated oxysterols, 7α- and 7β-hydroperoxycholesterol, have been identified in oxidized LDL where they were the first detectable cholesterol oxidation products (47). The 7-hydroperoxycholesterol isomers were isolated from the fraction containing free oxysterols. We have determined 7-hydroperoxycholesterol qualitatively by TLC in copper-oxidized LDL where it appeared in time together with other oxysterols (31). The reason for this is most probably the relatively insensitive detection system used in our study. Recently, the unstable cholesterol hydroperoxide 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide was identified in oxidized human LDL (48). The hydroperoxide easily undergoes allylic rearrangement to cholesterol 7α-hydroperoxide, which in turn can epimerize to the 7β-hydroperoxide (49). The presence of 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide indicates singlet oxygen oxidation of cholesterol. The 5-hydroperoxide has earlier been detected in photooxidized erythrocyte membranes (50).

Because of the different mechanisms of action of the oxidants used in this study, a direct comparison of their effects on oxysterol production could only be made by normalizing the data with respect to the level of protein modification. At REM values below 3, and irrespective of the oxidant used, a linear relationship between REM and oxysterol concentration was seen. This, taken together with the fact that the pattern of oxysterol formation was the same regardless of the oxidation regime used, suggests that both oxysterol formation and protein modification reflect a common oxidation event, namely the attack of fatty acid peroxyl/alkoxyl radicals on cholesterol, and either directly or through other intermediates on the protein (Scheme 1). The inhibitory effects of the lipophilic antioxidants further supports this notion. The oxysterol levels increased dramatically when the REM of the LDL particle went above 3, and a similar trend has been reported during Cu^{2+}- and macrophage-mediated LDL oxidation (37). This could represent a shift between oxidation of unesterified cholesterol, present in the outer monolayer of the LDL particle, to oxidation of esterified cholesterol present in the neutral core. However, during Cu^{2+}- and lipoxygenase-mediated LDL oxidation, consumption of both esterified and free cholesterol was seen to occur concomitantly (31).

Although SIN-1 oxidizes LDL via $ONOO^-$, it causes a greater level of lipid peroxidation than does the direct addition of a $ONOO^-$ solution that results in the same degree of protein modification. We have seen similar results when monitoring the formation of the arachidonic acid oxidation products, the F_2-isoprostanes (51). The preferential partitioning of nitric oxide in the hydrophobic compartment of LDL and subsequent reaction with superoxide would form $ONOO^-$ in close proximity to the lipids in the lipoprotein particle. The high reactivity of the hydroxyl radical-like species implies that initiation of lipid peroxidation reactions is likely to occur. This is corroborated by the similarity of the relationship between formation of oxysterols and REM,

for SIN-1, Cu^{2+} and Mb^{3+} which are known to oxidize LDL lipids. For exogenously added $ONOO^-$ to initiate lipid peroxidation, not only must it migrate into the hydrophobic phase, but do so rapidly before its complete decomposition to nitrate (51). In addition, our data would also imply that pre-formed $ONOO^-$ reacts directly with the protein, thereby increasing the REM of the LDL particle without significantly causing lipid peroxidation. However, once oxidation was initiated by authentic $ONOO^-$, the relative proportion of 7-oxocholesterol as an oxidation product was approximately 26% (Fig. 2) which was not significantly different from SIN-1 (approximately 30%). In turn, this is essentially similar to the extent of formation of 7-oxocholesterol by Cu^{2+} where this oxidation product was approximately 35% of the total. From these data it is evident that $ONOO^-$ is one of the few oxidants reported that can simultaneously modify both protein and lipid and may be formed in the atherosclerotic lesion (23, 24). A similar process, which does not require initial oxidation of LDL lipids, has been described for hypochlorite-mediated oxidation (52).

The pattern of oxysterol formation found here is similar to the pattern found in human atherosclerotic femoral arteries (7-oxo > 7β-OH > 7α-OH > 5α,6α-epoxide/5β,6β-epoxide/3,5,6-triol > 25-OH > 24-OH) (16). However, in that study the oxysterol found in largest quantities was 27-hydroxycholesterol. This was not formed in the studies carried out here, further corroborating the hypothesis that 27-hydroxycholesterol is not formed by a nonspecific oxidative mechanism but by the enzymatic action of sterol 27-hydroxylase. It has been suggested that its formation represents a protective mechanism by which macrophages can remove excess cholesterol (16).

The agent in oxidized LDL cytotoxic to cultured fibroblasts has been identified as 7β-hydroperoxycholesterol (14). However, 7-oxocholesterol and 7β-hydroxycholesterol have been reported to be cytotoxic towards aortic smooth muscle cells (15). Decomposition of the hydroperoxy derivative to alkoxyl and peroxyl radicals probably accounts for the cytotoxicity of 7β- hydroperoxycholesterol (53, 54). The exact mechanism(s) through which 7-oxo and 7β-hydroxycholesterol mediate cell death is not known, although 7β-hydroxycholesterol has been shown to induce apoptosis (55). In such studies of cytotoxicity, LDL was oxidized by either macrophages or Cu^{2+} ions. As other potential in vivo oxidants such as $ONOO^-$ and Mb^{3+} also resulted in the formation of a similar pattern of oxysterols as Cu^{2+}, it is presumed that they will also form oxidized LDL with cytotoxic properties. Oxysterols have a wide variety of biological effects with both pro- and anti-atherogenic effects being reported (17). In vivo, a mixture of different oxysterols is likely to result and it is their cumulative effect which will, therefore, determine their contribution to the atherogenic process.

R. P. thanks BBSRC for financial support (CASE award); U.D. for support from the Marianne and Marcus Wallenberg Foundation, Karolinska Institutets Stiftelser, and the European Community (PL 931790); and V.M.D.U. for National Institutes of Health Grant HL 48676-04.

Manuscript received 12 April 1996 and in revised form 31 July 1996.

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