Enhanced susceptibility of cholesteryl sulfate-enriched low density lipoproteins to copper-mediated oxidation

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Abstract Cholesteryl sulfate (CS) is a minor component of cell membranes, also present in lipoproteins, and its exact function is unknown. Since oxidation of low density lipoproteins (LDL) is thought to be an important determinant of atherogenesis, we investigated the influence of CS enrichment on copper-mediated oxidation of LDL. CS was found to act as a pro-oxidant, as measured by lipid oxidation parameters. The results also suggest that these effects were dependent on the sulfate group since pure cholesterol or cholesteryl acetate did not promote Cu^{2+}-mediated oxidation. Our findings imply that CS may affect the oxidizability and hence the potential atherogenicity of LDL.

Key words: Cholesteryl sulfate; Oxidized LDL; Oxidation; Copper

1. Introduction

Although cholesteryl sulfate (CS) is widely distributed in tissues, its precise function has not been well established [1]. CS is a minor constituent of cells such as erythrocytes [2], spermatozoa [3] and of lipoproteins such as low density lipoproteins (LDL) and chylomicrons [4]. Under certain conditions, CS can be used as a precursor for sulfated steroid hormones without desulfation [5]. Data are scarce about the factors that affect the quantity of CS in cells or lipoproteins. Plasma levels of CS are, however, greatly increased in some clinical conditions such as in microsomal steroid sulfatase deficiency or in recessive X-linked ichthyosis [6]. In these patients, because of abnormal concentrations of CS, the electrophoretic mobility of LDL is greatly increased [7]. In addition, results from this laboratory indicate that hypercholesterolemic patients had higher plasma CS concentrations [8,9].

A key event in the pathogenesis of atherosclerosis is thought to be the formation of macrophage-derived foam cells by scavenger receptor uptake of oxidized LDL [10,11]. In this paper, we report that CS has a pro-oxidant effect when supplemented in LDL as measured by monitoring the formation of conjugated dienes, thiobarbituric acid reactive substances (TBARS) and lipid peroxides after incubation with copper.

2. Materials and methods

2.1. Materials

Sterols and other chemicals were purchased from Sigma or Merck. CS and β-sitosteryl sulfate was synthesized according to the method of Mumma [12]. They were purified by TLC and stored frozen under nitrogen or argon at −20°C. Pyridine and silylating reagent were from Supelco.

2.2. Preparation and oxidation of LDL

After an overnight fast, blood samples taken from volunteers by venipuncture were put into tubes containing disodium EDTA (1.5 mg/ml). Plasma was separated in a cold centrifuge and LDL (1.019–1.055 g/ml) were isolated by sequential ultracentrifugation. Pooled LDL were 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 for no longer than 15 days. Sterols were incubated (0.2–0.4% LDL-cholesterol) with LDL as follows: sterols in CH_2Cl_2/CH_3OH (1/1, by volume) were evaporated to dryness under nitrogen or argon in a glass tube, and 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 [13]. Oxidation of LDL (100 μg/ml) was carried out by exposure to 5 μM Cu^{2+} (using $CuSO_4$) at 37°C for up to 5 or 16 h. Oxidations were also carried out in the presence of the azo initiator, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) obtained from Spiral (France); the final concentration was 8 mM at 37°C under air [14].

2.3. Analytical procedures

CS was measured in LDL using β-sitosteryl sulfate as an internal standard according to the previously published method [8]. Lipid peroxidation was measured by spectrophotometrically (234 nm) monitoring the formation of conjugated dienes [15] in a thermostatted cuvette holder maintained at 37°C (Beckman DU65 or DU640). The absorbance results were adjusted by curve fitting analysis of the initial sigmoid portion of the curve. The oxidation was also followed by measuring TBARS released in the medium and expressed in malondialdehyde (MDA) equivalents as described [16]. Lipid peroxides (LPO) were measured as described [17]. Agarose gel-electrophoresis (0.5%) was performed with a Beckman Paragon lipoprotein electrophoresis kit. Proteins were assayed by the Lowry method [18].

3. Results and discussion

In initial experiments, the influence of CS on the formation of conjugated dienes of LDL in the presence of Cu^{2+} was evaluated. As illustrated in Fig. 1, the change in the absorbance at 234 nm of native LDL follows three phases [15]. Firstly, there is a lag phase during which endogenous antioxidants are progressively consumed. Next, there is the propagation phase with a rapid increase in absorbance. Finally, the last phase is the decomposition of hydroperoxides during which no further increase in absorbance occurs. LDL incubated with CS showed a higher initial amount of dienes and a decrease in the lag time. The leftward shift of the progress curves were correlated with increasing concentrations of CS. These results were also obtained with LDL from different donors. The rate vs. time plot of the inset of Fig. 1 is the first derivative dA/dt with a maximum obtained by computer analysis. A value of 90.8 ± 0.6 mn was calculated for native LDL (N-LDL). These peak times

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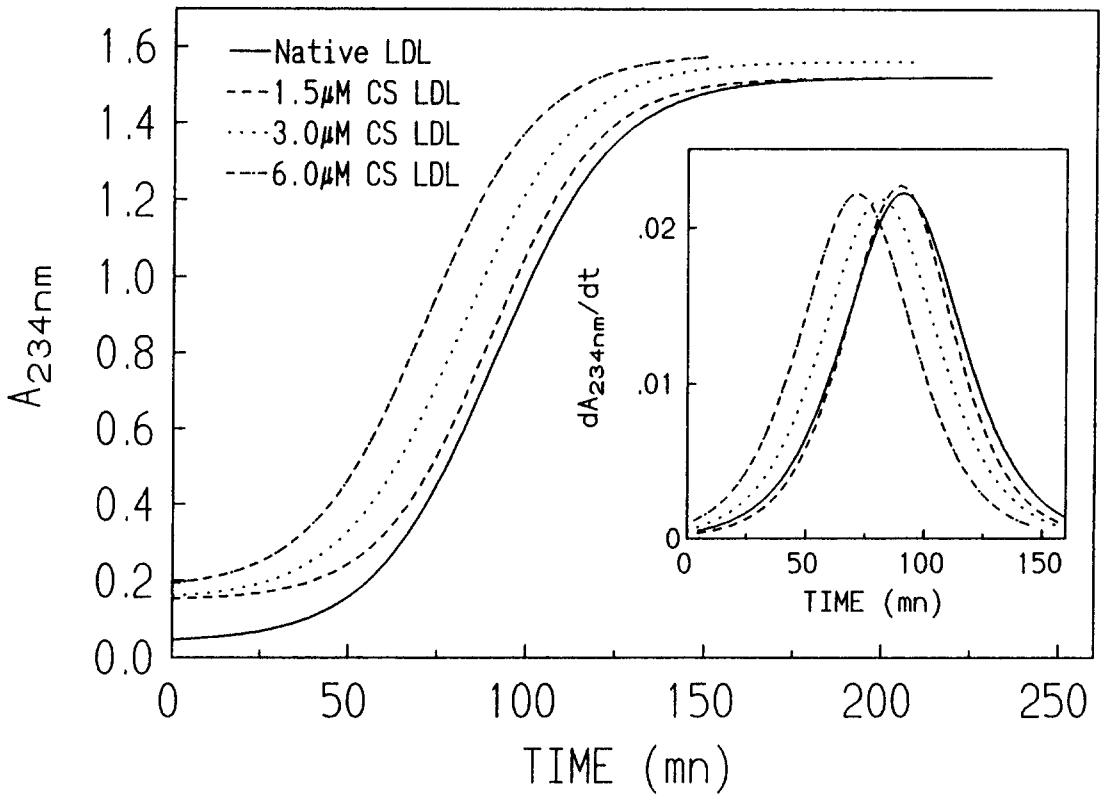

Fig. 1. Monitoring of Cu^{2+}-initiated oxidation of LDL. Native LDL (100 μg/ml) oxidation was studied at 37°C by conjugated dienes absorption at 234 nm with 5 μM Cu^{2+}. Native LDL was incubated with either 1.5, 3 or 6 μM CS as described in section 2. The inset shows the first derivative, i.e. change of rates of oxidation as a function of time, which was computed to obtain the time for half maximum diene formation.

decreased to 89.5 ± 1.0, 82.7 ± 1.3 and 72.8 ± 1.0 mn for LDL incubated with 1.5, 3 and 6 μM CS, respectively.

The results of time–course studies dealing with the release of TBARS and LPO in the medium of Cu^{2+}-oxidized LDL were comparable to previous studies. In our conditions, the production of TBARS and LPO leveled off or decreased after about 2 h and 3 h, respectively. Consequently, maximum differences between samples could be defined at shorter incubation times. We thus decided to perform our investigations with incubation times of 30 or 60 min for TBARS and of 60 or 180 min for LPO. CS-enriched LDL showed an increased production of TBARS (Fig. 2A) and LPO (Fig. 2B) as compared to control LDL. This enhanced lipid oxidation was concentration-dependent, since at 30 min the increase in TBARS reached about +350% and +720% with respect to controls for 3 and 6 μM CS, respectively. Similar enhancements were obtained for LPO (at 60 min: +196 and +450% for 3 and 6 μM CS, respectively). As reported previously [7,19], we confirmed that enrichment of LDL with CS increased the electrophoretic mobility of these lipoproteins. Although in the present work we did not measure the precise CS concentration that went into LDL, these data support idea that an effective incorporation took place. Furthermore, in the presence of Cu^{2+}, the data presented in Fig. 3 indicate that supplementing LDL with CS resulted in an increase in the electrophoretic mobility relative to controls (REM) for the different times studied.

The mechanism by which CS enhances lipid oxidation is

Table 1
Effects of EDTA and BHT on Cu^{2+}-mediated oxidation of native LDL (N-LDL) and CS-enriched LDL (CS-LDL)

	TBARS (nmol MDA/mg)
N-LDL	22.6 ± 1.6
+EDTA	4.3 ± 1.1
+BHT	4.5 ± 1.8
CS-LDL	71.3 ± 4.8*
+EDTA	6.8 ± 2.2
+BHT	7.4 ± 3.1

The reactions were initiated by addition of 5 μM copper, stopped at 60 min, and TBARS assayed as described in section 2 (difference from N-LDL: $P < 0.001$) for comparison between N-LDL and CS-LDL.

Table 2
Effect of cholesterol, cholesteryl acetate and sulfate on Cu^{2+}-mediated LDL oxidation

	N-LDL	CS-LDL	CA-LDL	C-LDL
Dienes (μmol/mg)	101.7 ± 11.8	413.6 ± 22.6*	129.9 ± 12.4	106.4 ± 14.1
TBARS (nmol MDA/mg)	20.8 ± 1.4	63.5 ± 3.8*	24.6 ± 2.9	21.2 ± 1.2
LPO (nmol mg)	25.4 ± 0.6	26.8 ± 2.1*	7.2 ± 0.9	6.8 ± 1.0
REM	1.50	3.35	1.55	1.65

Native LDL (N-LDL) were incubated with 6 μM of either cholesteryl sulfate (CS), cholesteryl acetate (CA) or pure cholesterol (C), and oxidized with 5 μM Cu^{2+}. Dienes, thiobarbituric acid reactive substances (TBARS), lipoperoxides (LPO) and electrophoretic mobility relative to N-LDL (REM) were measured as described in section 2 at 80 min, 60 min, 180 min and 60 min, respectively. Results of triplicate assays are given as means ± S.D., except for REM which are the average of duplicate values (difference from N-LDL: $P < 0.001$).

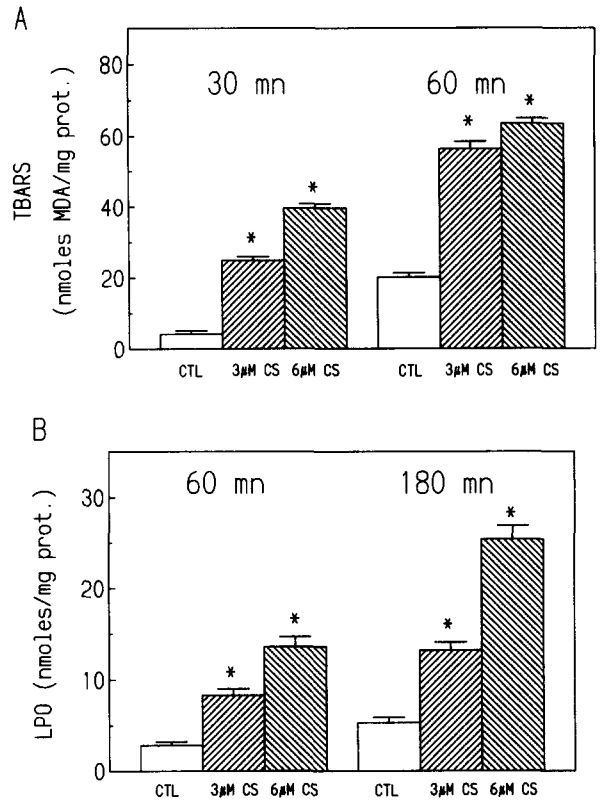

Fig. 2. Effect of CS on Cu^{2+}-initiated LDL oxidation. Native LDL (CTL) were incubated with 3 or 6 μM CS as described in section 2 and oxidized with 5 μM Cu^{2+}. The reaction was stopped at different times by the addition of EDTA and BHT (100 and 50 μM, respectively) and analyzed for TBARS (A) and LPO (B). The results are representative of 3 experiments performed in triplicate. *$P < 0.001$ when CTL was compared with CS.

unknown but results presented in Table 1 suggest that it could be by stimulating oxidative processes. When LDL supplemented with 6 μM CS were incubated with either EDTA or BHT, the Cu^{2+}-initiated oxidation was inhibited. To determine whether the pro-oxidant property of CS was dependent on the sulfate group, pure cholesterol and cholesteryl acetate were tested for their effects on the oxidizability of LDL at the same concentration (6 μM). As can be seen in Table 2, none of these compounds showed a pro-oxidant effect when added to LDL, suggesting that oxidation was specifically due to the presence of the sulfate group.

The results presented in Table 3 suggest that interactions between copper ions and the sulfate group may exist. These data indicate that no supplementary oxidation was obtained with CS when carried out using the azo initiator AAPH. This oxidation system is not dependent on the decomposition of lipid peroxides.

The presence of a sulfate group on the cholesterol molecule provides important biological properties such as membrane stability [2,3], regulation of cholesterol biosynthesis [7,19] or inhibition of lecithin: cholesterol acyltransferase [20]. CS has also been found to display a strong ability to trigger the activation of factor XII [20] and thus to be involved in the intrinsic coagulation system. CS accumulates in X-linked ichthyosis patients deficient in aryl sulfatase [6,7], and CS is implicated in the skin scaling abnormalities of these subjects [21]. Increased plasma CS concentrations have also been found in hypercholesterolemic and cirrhotic patients, although plasma levels were less elevated than in the genetic disorder [8,9,22].

Since, CS concentration measured in the LDL prepared from three different normolipidemic volunteer donors, was 2.99 ± 0.16 nmol/mg protein, CS enrichments of LDL of 1.5, 3 and 6 μM would represent increases of about 1.5, 2 and 6 times the endogenous concentration of CS in these subjects. In normal LDL, the ratio of CS to total cholesterol (TC) is less than 0.1% [22]. The various preparations tested in this study resulted in a ratio of about 0.5–2%, which is in the reported range for patients with recessive X-linked ichthyosis [19]. Although calculated in total plasma, the CS/TC ratio for cirrhotics was roughly 0.33% and close to normal (0.14%) in hypercholesterolemics [8,9,22]. Therefore these findings may have important implications in the pathophysiology of these diseases.

The mechanism by which CS influences LDL oxidative susceptibility has not been defined. Although the sulfate group seems to play a role, its precise involvement has not been defined. One possibility is that Cu^{2+} ions bind more to the CS-enriched LDL because of the presence of the negatively charged sulfate groups. It has been recently demonstrated that a finite number of saturable pro-oxidant copper binding sites exist within the LDL particle [23]. In addition, this number may vary from one subject to another. However, the nature of these binding sites and the reason for their variation is not clear. In the procedures used in the present paper, the endogenous peroxide content of the isolated LDL is an important determinant of the oxidizability of LDL for the copper-mediated oxidation, whereas using AAPH the mechanism in less dependent on the lipid peroxides. Besides fatty acid peroxides [24,25], cholesterol peroxides potentiated the Cu^{2+}-initiated LDL oxidation [26]. However, these latter compounds did not account for the effects observed with CS in the present study since TLC-repurified CS was as pro-oxidant as stock CS.

To assess whether the in vitro data presented in this paper are relevant in human pathology, studies are now in progress in our laboratories to investigate the susceptibility to oxidation

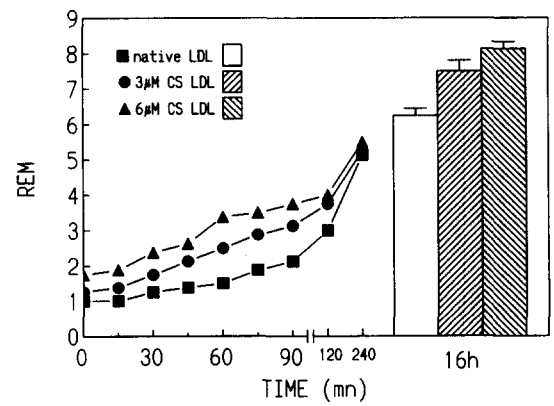

Fig. 3. Effect of CS on the electrophoretic mobility of Cu^{2+}-initiated oxidation of LDL. Native LDL and LDL incubated with 3 or 6 μM CS were oxidized with Cu^{2+}. The reaction was stopped for the times shown as in Fig. 2 and the samples were subjected to agarose gel-electrophoresis. Results are expressed relative to native LDL without Cu^{2+} (REM). The maximum changes in mobility obtained for 16 h were 25 mm for native LDL and 30 and 32.5 mm for 3 μM and 6 μM CS-enriched LDL, respectively. Results are representative of two experiments performed in duplicate or triplicate over a 16 h time-course.

Table 3
Effect of CS on AAPH-induced LDL oxidation

	Time (min)	N-LDL	CS-LDL
TBARS	2 h	7.8 ± 1.2	8.1 ± 1.4
	4 h	16.4 ± 2.3	17.4 ± 2.6
REM	0 h	1.0	1.0
	2 h	1.85	1.90
	4 h	2.25	2.20

AAPH-induced oxidations were carried out on native LDL (N-LDL) and CS-incubated LDL (CS-LDL) at 37°C under air and the reactions stopped by adding 100 μM EDTA and 50 μM BHT. Final LDL and AAPH concentrations were 0.2 mg/ml and 8 mM, respectively. TBARS, expressed in nmol MDA/mg protein ($n = 3$), and electrophoretic mobility relative to N-LDL (REM) were measured as described in section 2.

of LDL from patients with endogenous high levels of CS such as those with X-linked ichthyosis and hypercholesterolemia.

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