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Identification of cholesterol-bound aldehydes in copper-oxidized low density lipoprotein

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Lipid-soluble cholesteryl ester core aldehydes (aldehydes still bound to the cholesterol ring) were identified among the products of copper-catalyzed peroxidation of human low density lipoprotein (LDL). The LDL was exposed to oxygenated buffer and 5 μM $CuSO_4$ for 24 h. The core aldehydes were isolated as the dinitrophenylhydrazones, and were identified by reverse-phase HPLC with mass spectrometry. The major components were the C_4–C_{10} oxoalkanoyl esters of cholesterol and 7-ketocholesterol, and accounted for 1–2% of the cholesteryl linoleate and arachidonate consumed.

Core aldehyde; Oxoalkanoyl ester; 7-Keto cholesterol; Dinitrophenylhydrazone; Liquid chromatography – mass spectrometry

1. INTRODUCTION

There is evidence that oxidatively modified LDL plays a pivotal role in atherogenesis [1]. Although lipid peroxidation is considered to be involved in the modification, limited data are available concerning the specific structures formed in this process. The primary products of lipid peroxidation, lipid hydroperoxides, undergo carbon–carbon bond cleavage in the presence of transition metals. The resulting water-soluble short-chain aldehydes have been extensively investigated [2,3], but the aldehydes still bound to parent lipid molecules have not been studied [4,5].

In this study we have isolated and identified cholesteryl ester core aldehydes from human LDL exposed to in vitro peroxidation conditions believed to mimic those encountered in vivo.

2. MATERIALS AND METHODS

Cholesteryl 5-oxovalerate and cholesteryl 9-oxononanoate were available from a partial synthesis [6], while 7-ketocholesteryl 5-oxovalerate and 9-oxononanoate were available from *t*-butyl hydroperoxide–$FeSO_4$ oxidation of purified cholesteryl arachidonate and linoleate [7]. The 2,4-dinitrophenyl hydrazine was from Aldrich Chemical Co. (Milwaukee, WI) and phospholipase C (*Bacillus cereus*) from Sigma Chemical Co. (St. Louis, MO).

2.1. *Preparation of oxidized LDL*

Whole blood was obtained by venipuncture from a healthy volunteer who had fasted 12 h. Blood samples were collected into tubes containing EDTA (1 mg/ml). Plasma was separated by low speed centrifugation (1,500 × g for 20 min), and butylated hydroxytoluene (20 μM) was added. Plasma LDL (d=1.019–1.063 g/ml) was prepared by sequential ultracentrifugation [8]. LDL was dialyzed for 24 h at 4°C in the dark against vacuum-degassed 0.01 M phosphate buffer, pH 7.4, containing 10 μM EDTA, 0.15 M NaCl and 0.1 mg/ml chloramphenicol [9]. For oxidation [10] the dialyzed lipoprotein solution (1.5 mg LDL protein/ml) was transferred into a dialysis bag and immersed in a 100-fold volume of the dialysis buffer containing 5 μM $CuSO_4$. The system was kept in the dark at room temperature for 24 h, and oxygen was bubbled continuously through the external buffer.

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2.2. *Isolation of core aldehydes*

The oxidized LDL was treated with dinitrophenylhydrazine using a method modified from Esterbauer et al. [11]. To 1 ml of the oxidized LDL solution (1.5 mg protein/ml), 0.1 ml of 1% EDTA, 10 μl of 2% BHT, and 1 ml freshly prepared 2,4-dinitrophenylhydrazine in 1 N HCl (0.5 mg/ml) were added, mixed vigorously, and kept in the dark for 2 h at room temperature and then overnight at 4°C. The reaction mixture was extracted with chloroform–methanol 2:1. The lipid extract was applied to Silica Gel H plates (20×20 cm), which were then subjected to a double development with dichloromethane (to a height of 10 cm) and, after solvent evaporation, with toluene (to a height of 17 cm). The yellow zone corresponding to standard dinitrophenyl hydrazone (DNPH) derivatives of 5-oxovaleroyl (R_F 0.26) and 9-oxononanoyl (R_F 0.33) cholesterol were scraped off and eluted with chloroform-methanol. The core aldehydes of 7-ketocholesteryl esters (R_F 0.10) were recovered in a similar manner.

2.3. *Reverse-phase HPLC*

The DNPH derivatives of the aldehydes were separated by reverse-phase HPLC on a Supelcosil LC-18 column (250 × 4.6 mm ID, Supelco Inc., Mississauga, Ont.) using acetonitrile/2-propanol 4:1 or a linear gradient of 30–90% propionitrile in acetonitrile as the eluting solvents. The column was installed in a Hewlett-Packard Model 1084B Liquid Chromatograph, and was operated at a flow rate of 1.0–1.5 ml/min. The peaks were monitored at 358 nm [6,7].

2.4. *LC/MS*

About 1% of the HPLC column effluent was admitted to a Hewlett-Packard Model 5985B quadrupole mass spectrometer via a direct liquid inlet interface [12]. Negative chemical ionization mass spectra were taken every 5 s over the entire chromatogram in the mass range 200–900.

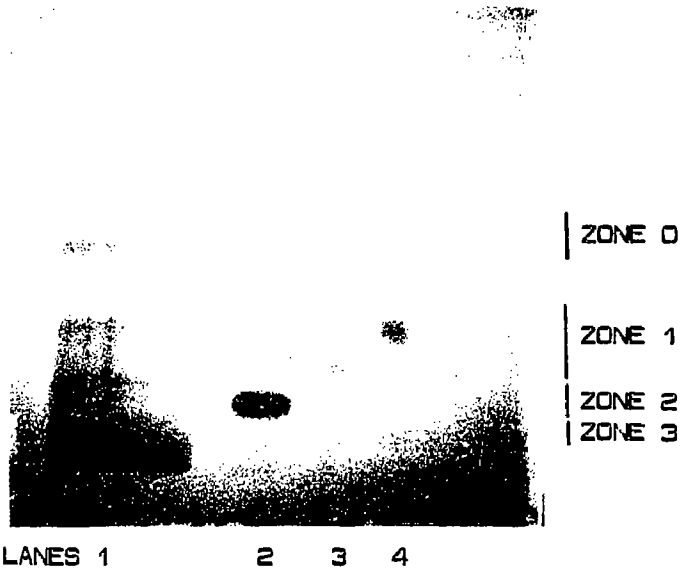

Fig. 1. Thin-layer chromatogram of lipid extract from dinitrophenylhydrazine-treated oxidized LDL. (zone 1) Core aldehydes of cholesteryl esters. (zone 2) Core aldehydes of 7-keto-cholesteryl esters. (zone 3) Core aldehydes of phosphatidylcholines. Lane 1, total lipid extract of DNPH treated oxidized LDL; lane 2, free DNPH reagent; lane 3, DNHP of cholesteryl 5-oxovalerate (R_F 0.26); lane 4, DNPH of cholesteryl 9-oxononanoate (R_F 0.33).

3. RESULTS

Exposure of native LDL to peroxidation in the $CuSO_4$ system was associated with the previously reported [1] increased electrophoretic mobility on agarose gel electrophoresis (results not shown).

Prior to LC/MS the DNPH derivatives of the cholesteryl ester core aldehydes were resolved by TLC (Fig. 1), and the yellow DNPH bands corresponding in R_F values to standard 5- and 9-oxoalkanoyl esters of cholesterol (zone 1) and 7-ketocholesterol (zone 2) were recovered. The core aldehydes in the TLC bands were resolved by reverse-phase HPLC. TLC zone 1 contains the C_5 and C_9 cholesterol core aldehydes as major peaks, along with smaller peaks for the C_4, C_6, C_7, C_8 and C_{10} core aldehydes (Fig. 2A). TLC zone 2 contains the corresponding core aldehydes of 7-ketocholesterol (Fig. 2B). The peak for the C_9 core aldehyde of 7-keto-cholesterol ester overlaps with the peak for the DNPH derivative of 7-ketocholesterol, which was also found to migrate in this TLC zone. The identities of the cholesteryl (Fig. 3A) and 7-ketocholesteryl (Fig. 3B) ester core aldehydes were confirmed by LC/MS with negative chemical ionization. The single ion plots represent the $[M]^-$ ions for the DNPH derivatives of the various core aldehydes found in zones 1 and 2. The full mass spectra of the DNPH derivatives of all major cholesteryl ester core aldehydes isolated from peroxidized LDL agreed completely with the mass spectra of the corresponding synthetic cholesteryl ester core aldehydes [6] and with the mass spectra of the core aldehydes of 7-keto-cholesteryl esters derived by *t*-butyl hydroperoxide–$FeSO_4$ treatment of purified plasma cholesteryl esters [7]. The proportions of the core aldehydes, as recovered from peroxidized LDL, are given in Table I. The overall yield of core aldehydes after 24 h peroxidation was estimated to be 1–2% of each polyunsaturated cholesteryl ester, about equally divided between cholesterol and 7-ketocholesterol derivatives.

4. DISCUSSION

In this study we have identified cholesteryl and 7-ketocholesteryl ester core aldehydes as high molecular weight lipid-soluble peroxidation products of cholesteryl esters in LDL. Although the occurrence of ester-bound aldehydes among lipid peroxidation products has been suspected specific lipid ester core aldehydes have not been isolated or identified [13]. The corresponding short-chain aldehydes derived from the

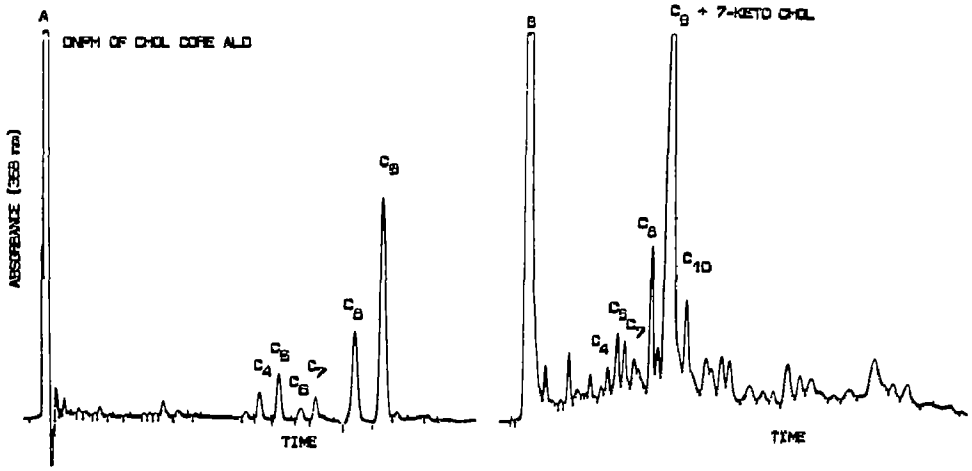

Fig. 2. Reverse-phase HPLC of (A) cholesteryl ester and (B) 7-keto-cholesteryl ester core aldehydes as the dinitrophenylhydrazones. Peak identification is as given in the figure. HPLC conditions are as given in section 2.

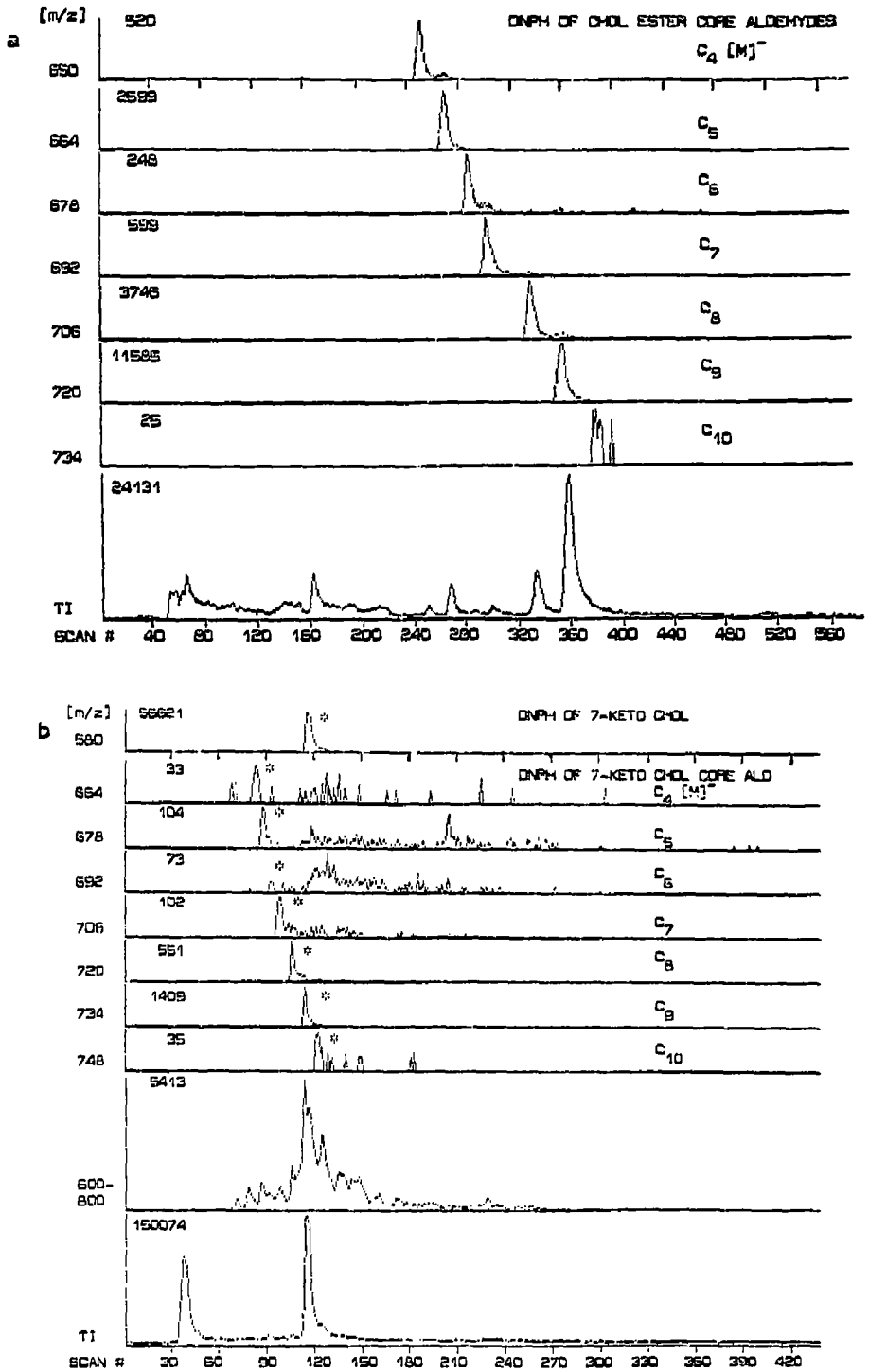

Fig. 3. Reverse-phase LC/MS of the DNPH derivatives of (A) cholesteryl ester and (B) 7-keto cholesteryl ester core aldehydes. Peak identification is as given in the text. Numbers in the left hand column inside the chromatograms indicate peak intensity. TI, total negative ion current; *m/z* 600–800, restricted mass range profile of DNPH derivatives, excluding DNPH of 7-ketocholesterol; *m/z* values. $[M]^-$ ions of C_4 to C_{10} core aldehydes. LC/MS conditions are as given in section 2.

methyl termini of various LDL lipids have been previously identified and their properties investigated [14]. Likewise, 7-ketocholesterol [15] and its dehydration product [16] have been recovered from LDL peroxidized in vitro. The isolated aldehydes presumably represent both free and Schiff base-bound species, since the dinitrophenylhydrazine used in the extraction is known to displace them from proteins [17]. The ratio of C_9/C_5 cholesteryl ester core aldehydes in oxidized LDL was 7.1, and approximated the ratio of the original [18:2(n-

Table I

Composition of cholesteryl ester core aldehydes from copper-peroxidized low density lipoproteins

Core aldehydes	Chol. esters (% total)		7-keto chol. esters (% total)	
	UV*	LC/MS**	UV	LC/MS
C_4	4.4	1.7	3.0	2.3
C_5	9.8	8.5	8.4	8.4
C_6	2.2	1.3	5.6	1.4
C_7	4.4	2.9	2.5	6.0
C_8	21.7	16.2	18.5	24.5
C_9	57.0	69.1	59.0	55.7
C_{10}	0.5	0.3	3.0	1.2

* Peak areas at 358 nm; average of 2-3 determinations
**Peak areas for $[M]^-$ ions

6) + 18:3(n-3)]/[20:4(n-6)] fatty acids, which was 7.9. The minor proportions of the C_4 and C_8 aldehydes could have arisen from double-bond migration during peroxidation [18] and from the presence of small amounts of 22:6(n-3) and 20:3(n-6) in the cholesteryl esters. The overall yields of the core aldehydes estimated at 1–2% of the destroyed cholesteryl ester compare favourably to the yields of the short chain aldehydes (4%) determined under comparable conditions [17].

The copper-oxidized LDL is believed to have essentially all the properties of so-called oxidatively modified LDL [1]. The cytotoxicity of oxidized LDL has been reported to be located in the lipid fraction, and oxidized forms of cholesterol have been suggested to be one of the toxic agents [19]. The toxicity of the core aldehydes of cholesterol of oxocholesterol has not been investigated. Since the cholesteryl ester core aldehydes are hydrolyzed by bacterial cholesteryl ester hydrolase [7] it is possible that they are also subject to endogenous degradation. Proportionally more 7-ketocholesterol was associated with the core aldehydes than with saturated cholesteryl esters, which suggested that the polyunsaturated fatty acid moieties promote peroxidation of cholesterol ring as already proposed [20].

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