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Lipid ester-bound aldehydes among copper-catalyzed peroxidation products of human plasma lipoproteins

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Abstract: We have isolated the core aldehydes (aldehydes still bound to parent molecules) of phosphatidylcholine (PC) and cholesteryl esters (CE) from copper-catalyzed peroxidation of human plasma low (LDL) and high (HDL) density lipoproteins. The aldehydes were isolated by extraction with acidified chloroform–methanol containing 2,4-dinitrophenylhydrazine. The 2,4-dinitrophenylhydrazone (DNPH) derivatives formed were resolved by reversed phase high performance liquid chromatography (HPLC) and identified by on-line quadrupole mass spectrometry (LC/MS). The major PC core aldehydes from oxidized LDL and HDL were identified as 1-palmitoyl-(1-stearoyl) 2-(9-oxononanoyl)-, 1-palmitoyl-(1-stearoyl) 2-(8-oxooctanoyl)-, and 1-palmitoyl-(1-stearoyl) 2-(5-oxovaleroyl)-*sn*-glycerols after phospholipase C digestion of the DNPH derivatives of the phospholipids. The major aldehydes from peroxidation of cholesteryl esters were the 9-oxononanoyl, 8-oxooctanoyl, and 5-oxovaleroyl esters of cholesterol and 7-ketocholesterol. The core aldehydes were estimated to account for a minimum of 1–2% of the consumed linoleate and arachidonate esters. A relatively smaller yield of the PC core aldehydes from LDL compared to HDL was attributed to the presence of greater amounts of phospholipases in LDL than in HDL. More comparable yields of PC core aldehydes were obtained in the presence of phenylmethylsulfonylfluoride, which inhibits phospholipases. We conclude that peroxidation of LDL and HDL results in formation of detectable amounts of cholesteryl and glycerophospholipid esters containing aldehyde functions. The yield of PC aldehydes varies with the activity of the platelet activating factor (PAF) acetyl hydrolase.—**Kamido, H., A. Kuksis, L. Marai, and J. J. Myher.** Lipid ester-bound aldehydes among copper-catalyzed peroxidation products of human plasma lipoproteins. *J. Lipid Res.* 1995. **36:** 1876–1886.

Supplementary key words cholesteryl ester • 7-keto cholesteryl ester • phosphatidylcholine • core aldehyde • dinitrophenylhydrazones • HPLC • mass spectrometry

Oxidatively modified LDL has been implicated as a factor in the generation of macrophage-derived foam cells (1), which accumulate in the subendothelial space as an early event in atherosclerosis (2). The hypothesis that oxidized LDL is involved in the process of atherogenesis is strengthened by the demonstration that probucol, an inhibitor of LDL oxidation, significantly reduces atherosclerosis in the Watanabe heritable hyperlipidemic rabbit (3, 4). The biological modification of LDL depends on trace amounts of metal ions in the medium (5) and is accompanied by a number of other striking changes in LDL, including hydrolysis of PC to lyso PC by the action of an intrinsic LDL-associated phospholipase A_2 (6) and generation of thiobarbituric acid-reactive substances indicating peroxidation of LDL lipids (5, 7). This oxidative modification can be mimicked in vitro by copper ions (5). However, neither the mechanism of LDL oxidation nor the products have been clearly established. In contrast to LDL, other plasma lipoproteins have been reported not to interact with lipohydroperoxides (8) and apolipoproteins A-I and A-II may have a protective effect on LDL peroxidation (9) and therefore against atherosclerosis.

Despite the widely accepted involvement of lipid peroxidation in the pathophysiological modification of LDL, only a few specific oxidation products have been identified. Thus, Esterbauer et al. (10) have identified a number of low molecular weight aldehydes, and Lenz et al. (11) have isolated several fatty acid hydroperoxy and

Abbreviations: CE, cholesteryl esters; core aldehyde, a lipid ester containing a fatty acid with terminal aldehyde group (e.g., cholesteryl 5-oxovalerate, 1-palmitoyl-2-(9-oxo-nonanoyl)-*sn*-glycerophosphocholine, etc.); DLI, direct liquid inlet interface; DNPH, 2,4-dinitrophenylhydrazone; EDTA, ethylenediamine tetraacetate; GLC, gas–liquid chromatography; HDL, high density lipoprotein; HPLC, high performance liquid chromatography; LC/MS, liquid chromatography with on-line mass spectrometry; LDL, low density lipoprotein; PAF, platelet activating factor; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PMSF, phenylmethylsulfonylfluoride; SPH, sphingomyelins; TLC, thin-layer chromatography; VLDL, very low density lipoprotein; UV, ultraviolet absorption.

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hydroxy derivatives, while others have measured the presence of hydroperoxides in cholesteryl esters (CE) (12) and in glycerophospholipids (13) in plasma and lipid esters (14) in LDL. In a preliminary report, Kamido et al. (15) have reported the isolation of specific cholesterol-bound aldehydes from copper-oxidized LDL.

We wish to report the isolation and identification of both cholesterol- and glycerophospholipid-bound aldehydes from copper-catalyzed peroxidation of LDL and HDL. These aldehydes retain the ester bond of the unsaturated fatty acid from which the aldehydes are formed by cleavage of the oxidized fatty chain. Glycerolipid ester core aldehydes have not been previously isolated from peroxidized plasma lipoproteins or other sources.

MATERIALS AND METHODS

Chemicals and reagents

Standard 5-oxovalerate and 9-oxononanoate of cholesterol and 7-ketocholesterol and the 5-oxovalerate and the 9-oxononanoate esters of monopalmitoyl and monostearoylglycerols were available in the laboratory from previous oxidations (15–17). Synthetic cholesteryl 5-oxovalerate was a gift from Dr. G. Tirzitis, Institute of Organic Synthesis, Riga, Latvia. Phospholipase C (*B. cereus*), and phenylmethylsulfonylfluoride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, MO). 2,4-Dinitrophenylhydrazine was from Aldrich Chemical Co. (Milwaukee, WI), while acetonitrile and 2-propanol (high performance liquid chromatography grade) were from Caledon Laboratories Ltd. (Georgetown, Ontario, Canada) and propionitrile was from Romil Ltd. (Loughborough, England). All other chemicals and solvents were of reagent grade or better quality and were obtained from local suppliers.

Preparation of oxidized LDL and HDL

LDL and HDL were prepared by ultracentrifugation from fresh human plasma containing EDTA (1 mg/mL) and BHT (20 μM), and were 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 NaCl, and

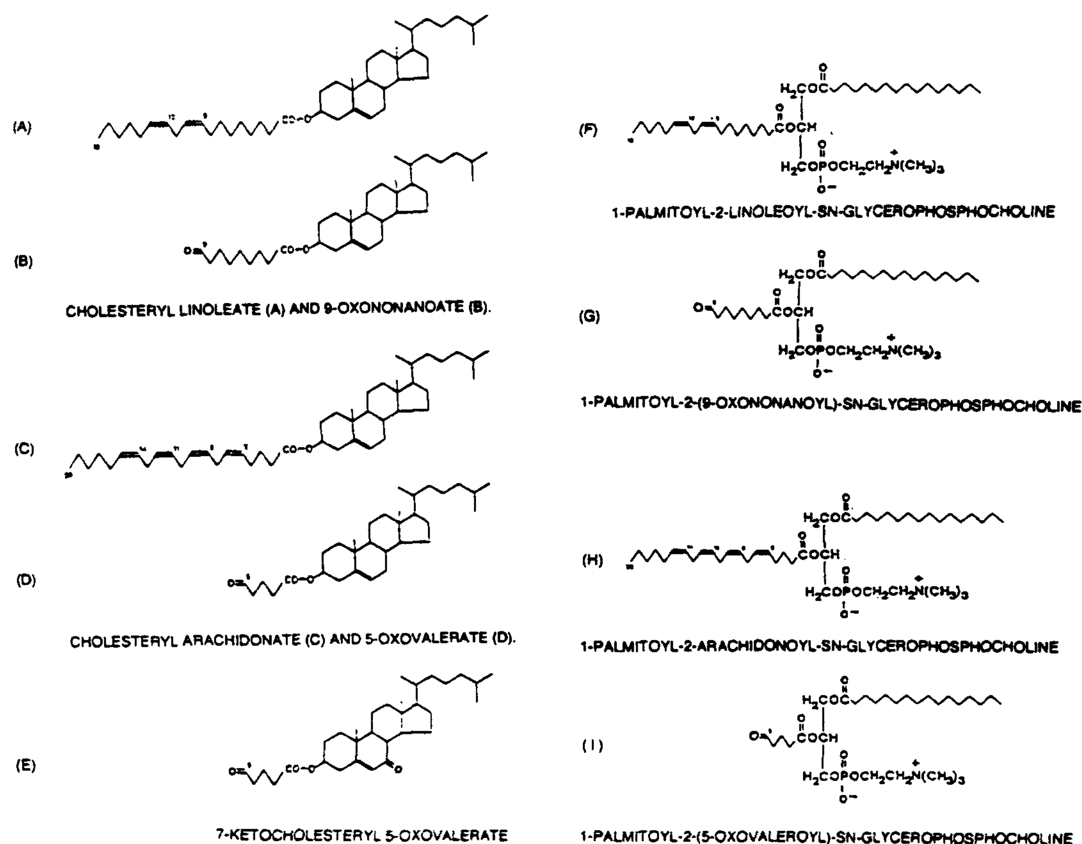

Fig. 1. Chemical structures of precursors and products of lipid peroxidation in LDL and HDL. A: cholesteryl linoleate; B: cholesteryl 9-oxononanoate; C: cholesteryl arachidonate; D: cholesteryl 5-oxovalerate; E: 1-palmitoyl-2-linoleoyl-*sn*-glycerophosphocholine; F: 1-palmitoyl-2-(9-oxononanoyl)-*sn*-glycerophosphocholine; G: 1-palmitoyl-2-arachidonoyl-sn-glycerophosphocholine; H: 1-palmitoyl-2-(5-oxo-valeroyl)-sn-glycerophosphocholine.

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TABLE 1. Relative proportions of unsaturated/saturated fatty acids in native and peroxidized LDL and HDL

Lipid Classes	Fatty Acid Ratio		
	20:4n6/18:0	18:2n6/18:0	18:1n9/18:0
Native LDL[a]			
CE	4.52 ± 0.5	38.51 ± 2.2	15.6 ± 3.3
PC	0.15 ± 0.05	0.45 ± 0.03	0.23 ± 0.1
Oxy LDL[a]			
CE	0.0	0.09 ± 0.03	0.36 ± 0.5
PC	0.0	0.20 ± 0.1	0.20 ± 0.1
Native HDL[a]			
CE	1.6 ± 0.2	16.1 ± 2	8.5 ± 1.0
PC	0.06 ± 0.02	0.2 ± 0.2	0.23 ± 0.1
Oxy HDL[b]			
CE	0.0	0.2	0.56
PC	0.0	0.0	0.30

[a]Means ± SE for three experiments.
[b]Average of two determinations.

0.1 mg/mL chloramphenicol (18). The dialysis bag was Spectra/Por 15.9 diameter tubing that was boiled in distilled deionized water for 30 min prior to use. The lipoproteins were oxidized according to Lenz et al. (11). The dialyzed lipoprotein solution (1.5 mg protein/mL LDL and 4.5 mg protein/mL HDL) in a dialysis bag was 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 while oxygen was bubbled continuously through the external buffer. The oxidized LDL exhibited the well-known higher migration on agarose gel electrophoresis compared to native LDL. Electrophoretic mobility of LDL was determined by standard agarose gel electrophoresis (18). Protein concentration was determined by the method of Lowry et al. (19).

Isolation of lipid ester-bound aldehydes from oxidized LDL and HDL

The ester-bound fatty aldehydes (**Fig. 1**) were isolated as previously described for synthetic lipid ester core aldehydes (15–17) using a method modified from Esterbauer et al. (10). To 1 mL of the oxidized lipoprotein solution (1.5 and 4.5 mg protein/mL of LDL and HDL, respectively), 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 allowed to stand 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 of the reaction mixture was applied to Silica gel H plates and the dinitrophenylhydrazone (DNPH) derivatives were separated by 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 zones corresponding to standard DNPH derivatives of 5-oxovaleroyl (R_f 0.26), 9-oxononanoyl (R_f 0.33), cholesterol, and the aldehyde esters of 7-ketocholesterol (R_f 0.10) were scraped off and eluted with methanol. Similarly recovered were the DNPH derivatives of the phospholipid-bound aldehydes from the origin of the TLC plate (R_f 0.0–0.05). Aliquots of the lipid extracts of the total peroxidation mixture and of the extracts of the origin of the TLC plate were digested with phospholipase C (*Bacillus cereus*) for 2 h at 37°C, and the released DNPH derivatives of the diacylglycerol core aldehydes were purified by TLC using chloroform–methanol 95:5 (v/v) as the developing solvent (17). The DNPH derivatives (R_f 0.20) were resolved from residual diacylglycerols (R_f 0.30) and ceramides (0.25) and were recovered separately by extraction with chloroform–methanol 2:1 (v/v) after location by UV absorption and fluorescein spraying.

Alternatively, the oxidized LDL and HDL lipids were extracted with chloroform–methanol in absence of the dinitrophenylhydrazine and an aliquot of the extract was

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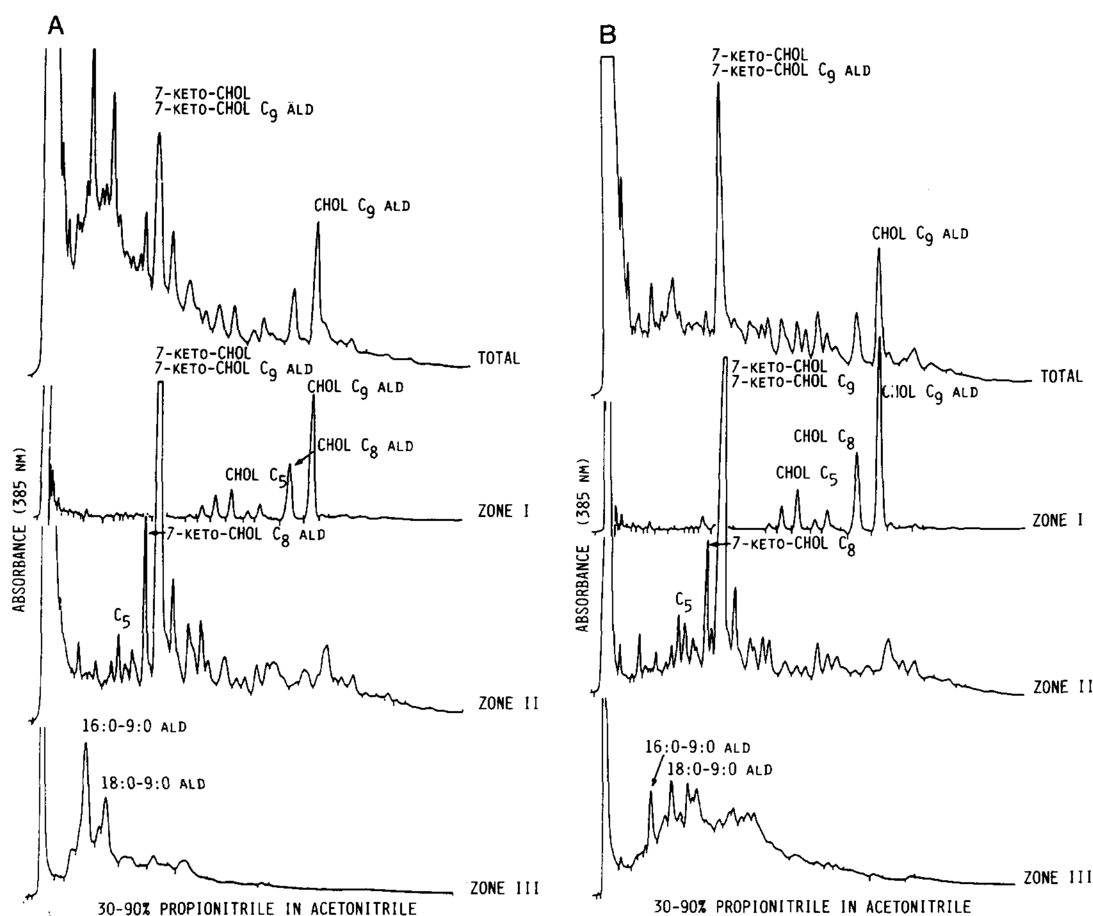

Fig. 2. Reversed phase HPLC of TLC fractions of the DNPH derivatives of lipid ester core aldehydes from oxidized human LDL (A) and HDL (B). Peak identification: Total, DNPH of total lipid extract; Zone I, DNPH of cholesteryl ester core aldehydes; Zone II, DNPH of 7-ketocholesteryl ester core aldehydes; Zone III, DNPH of diacylglycerol core aldehydes derived from glycerophospholipids. HPLC conditions: Supelco LC-18 column (250 mm × 4.6 mm, Supelco Inc., Mississauga, Ontario); eluant, linear gradient of 30–90% propionitrile in acetonitrile in 30 min; detector, UV (358 nm).

resolved by TLC into various neutral lipid classes, which were quantitated by high temperature GLC using tridecanoylglycerol as an internal standard (15–17). The remainder of the extract was converted to the DNPH derivatives, which were treated as described above.

GLC of fatty acids

The fatty acids of the various lipid classes of the original and oxidized LDL and HDL were determined by GLC of the methyl esters after acidic or alkaline transmethylation (20). The methyl esters were resolved on a 15 m RTx 2330 polar capillary column (Restek Corp., Port Matilda, PA) using a 7:1 split injection system (Model 5880A gas chromatograph, Hewlett-Packard, Palo Alto, CA). The carrier gas was hydrogen at 2 psi, and the column temperature was programmed from 80 to 130°C at 20°C/min and then to 220°C at 5°C/min.

HPLC and LC/MS

The lipids recovered from the TLC plates were reanalyzed by reversed phase HPLC and LC/MS. The DNPH derivatives of the aldehydes were separated by reversed phase HPLC on a Supelcosil LC-18 column (250 × 4.6 mm ID), Supelco Inc., Mississauga, Ontario) using a linear gradient of 30–90% propionitrile in acetonitrile as the eluting solvent. The column was installed in a Hewlett-Packard Model 1084B liquid chromatograph, and was operated at a flow rate of 1–1.5 ml/min. The peaks were monitored at 358 nm (17) and the components were quantitated using the 2,4-dinitrophenylhydrazone of 5-oxovaleroyl cholesterol as external standard. About 1% of the HPLC column effluent was admitted to a Hewlett-Packard Model 5985B quadrupole mass spectrometer via a direct liquid inlet interface (16). Negative chemical ionization (electron capture) mass spectra were taken every 5 s over the entire chro-

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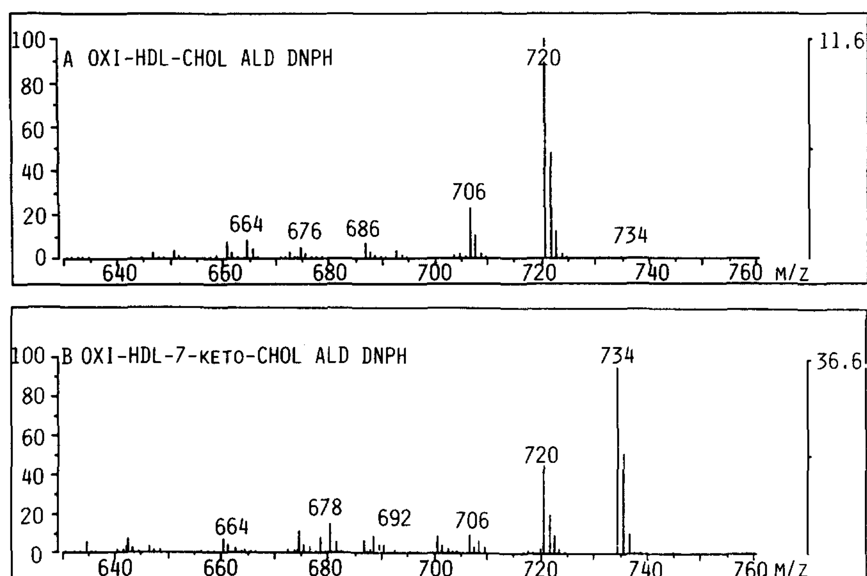

Fig. 3. Mass spectra of the cholesteryl (A) and 7-ketocholesteryl (B) ester core aldehydes averaged over the elution range. [M]· ion identification (A): *m/z* 650, Chol 4:0ALD; *m/z* 664, Chol 5:0ALD; *m/z* 676, Chol 6:1ALD; *m/z* 678, Chol 6:0ALD; *m/z* 692, Chol 7:0ALD; *m/z* 706, Chol 8:0ALD; *m/z* 720, Chol 9:0ALD; Ion identification (B): *m/z* 664, 7-ketochol 4:0ALD; *m/z* 678, 7-ketochol 5:0ALD; *m/z* 692, 7-ketochol 6:0ALD; *m/z* 706, 7-ketochol 7:0ALD; *m/z* 720, 7-ketochol 8:0ALD; *m/z* 734, 7-ketochol 9:0ALD. LC/MS conditions are as given in text.

matogram in the mass range 200–900. The single ion plots were obtained by recalling the data stored in the computer.

RESULTS

General characteristics of oxidized LDL and HDL lipids

TLC examination of the total lipid extracts of oxidized LDL and HDL using a neutral lipid solvent revealed the presence of native, hydroperoxy and hydroxy cholesteryl and 7-ketocholesteryl esters similar to those seen among the products of peroxidation of total lipid extracts of human plasma lipoproteins (15–17). In contrast, TLC of the oxidized phospholipids showed little change in the lipid class proportions because the polar lipid solvent failed to resolve native and oxidized phospholipids. There was, however, a significant increase in the amount of lysophosphatidylcholine (results not shown).

The oxidation of LDL and HDL resulted in an extensive destruction of the polyunsaturated fatty acid esters in all lipid classes in both lipoproteins. **Table 1** gives the ratios of 20:4/18:0, 18:2/18:0 and 18:1/18:0 for the various lipid classes of native and oxidized LDL and HDL. The 20:4 is consumed completely and 18:2 nearly completely in all lipid ester classes. This indicates the involvement of both surface monolayer (PC) and the particle interior (CE) in the peroxidation reaction. However, the 18:1 content was also extensively affected in both PC and CE. In addition, over 50% of free cholesterol and cholesteryl esters were converted into the corresponding 7-ketocholesterol derivatives (see below).

Isolation of lipid ester core aldehydes

In order to effect a complete recovery of the lipid ester core aldehydes, the oxidized LDL and HDL were directly treated with 2,4-dinitrophenylhydrazine, which displaced any protein bound aldehydes from their Schiff base complexes and converted them, along with the free aldehydes, into the dinitrophenylhydrazone (DNPH) derivatives. The hydrazones were extracted with chloroform–methanol 2:1 and resolved by TLC into three zones corresponding to synthetic standards. Zone I (R_f = 0.23–0.33) contained the DNPH derivatives of C_5–C_9 cholesteryl ester core aldehydes. Zone II (R_f = 0.11–0.23) contained the DNPH derivatives of 7-ketocholesterol and the C_5–C_9 7-keto cholesteryl ester core aldehydes. Zone III (R_f = 0.00–0.11) contained the DNPH derivatives of the C_5–C_9 core aldehydes of the choline and ethanolamine glycerophospholipids. All DNPH derivatives were recovered by extraction of the silica gel with chloroform–methanol 2:1 and analyzed further.

The total lipid extract and the extracts of Zone III were digested with phospholipase C in order to remove the polar head groups of the phospholipids. Prior to HPLC, the digestion products were resolved by TLC

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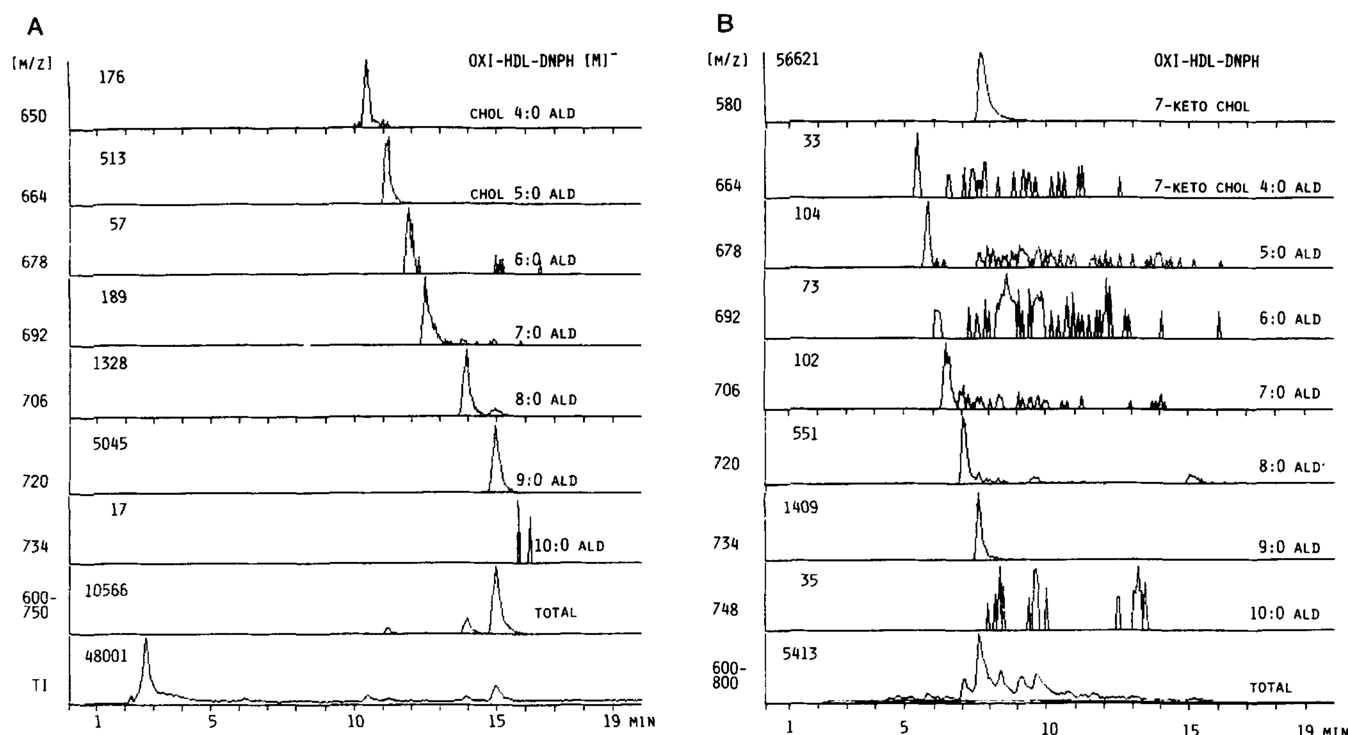

Fig. 4. Total negative ion current profiles (TI) and single ion plots (*m/z*) of the DNPH derivatives of the major cholesteryl (A) and 7-ketocholesteryl (B) ester core aldehydes as obtained by LC/MS for oxidized human HDL. Peak identification is given in figure. HPLC conditions are given in Fig. 2. The numbers inside the mass chromatograms indicate ion counts for full scale peaks.

into separate fractions for the DNPH derivatives of the diacylglycerol core aldehydes (R_f 0.25), residual diacylglycerols (R_f 0.15) released from the glycerophospholipids, and the ceramides (R_f 0.05) released from the sphingomyelins. **Figures 2A and 2B** show the UV profiles of reversed phase HPLC separation of the DNPH derivatives of the lipid esters of oxidized LDL and HDL, respectively, as obtained for the total hydrazone preparation and the hydrazone subfractions of cholesteryl and 7-ketocholesteryl ester core aldehydes and of the core aldehydes of diacylglycerols. The UV profiles of the total hydrazones of LDL and HDL differ greatly because of the difference in the neutral lipid composition and in the relative proportions of neutral and polar lipids. The oxidized HDL contains a higher proportion of the 7-ketocholesterol and a lower proportion of the phospholipid core aldehydes than oxidized LDL (see below). However, the proportions of the hydrazones within the core aldehyde classes are closely similar. The diacylglycerol core aldehydes possess comparable front parts

TABLE 2. Relative proportions of cholesteryl (Chol) and 7-ketocholesteryl (Oxo-Chol) ester core aldehyde in peroxidized LDL and HDL (mole %)

	LDL		HDL	
Molecular Species	Chol	Oxo-Chol	Chol	Oxo-Chol
Chol 4:0ald	1.7 ± 0.1	2.8 ± 0.2	2.0 ± 0.5	1.6 ± 0.5
Chol 5:0ald	8.5 ± 0.5	8.4 ± 0.5	6.3 ± 1.0	3.7 ± 1.0
Chol 6:0ald	1.3 ± 0.1	1.4 ± 0.1	0.6 ± 0.1	2.4 ± 0.5
Chol 7:0ald	2.9 ± 0.1	6.0 ± 0.2	2.6 ± 0.5	4.3 ± 1.0
Chol 8:0ald	16.2 ± 1.0	24.5 ± 1.5	17.8 ± 1.0	25.8 ± 2.5
Chol 9:0ald	69.1 ± 3.0	55.7 ± 2.5	70.6 ± 3.0	60.9 ± 5.0
Chol 10:0ald	0.3 ± 0.1	1.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1

Values, estimated from peak areas in mass chromatograms, are given as means ± SE of three or four determinations.

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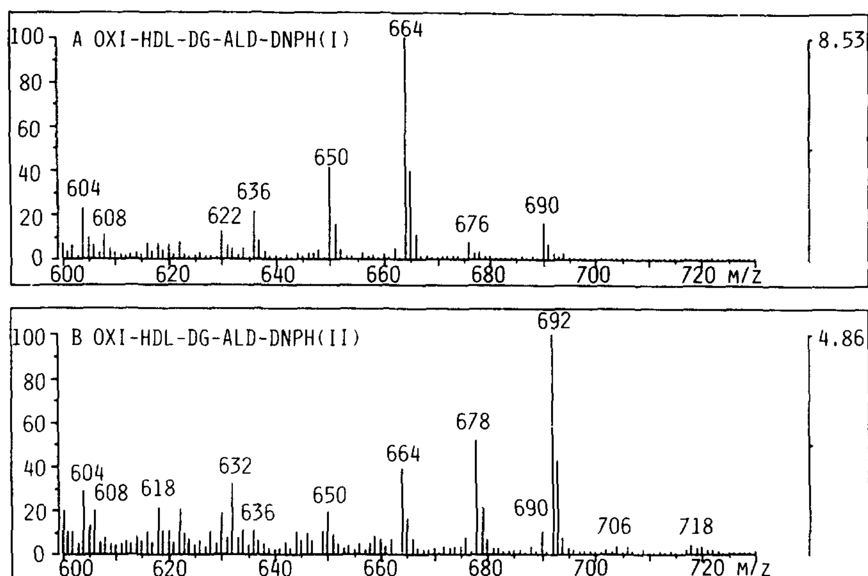

Fig. 5. Mass spectra of DNPH diacylglycerol core aldehydes derived from glycerophospholipids of oxidized HDL. I: spectra averaged over the first peak of total ion current profile; II: spectra averaged over second peak of total ion current profile. [M]⁻ ion identification: *m/z* 608, 16:0-5:0ALD; *m/z* 636, 18:0-5:0ALD; *m/z* 650, 16:0-8:0ALD; *m/z* 664, 16:0-9:0ALD; *m/z* 676, 18:0-8:1ALD; *m/z* 678, 18:0-8:0ALD; *m/z* 690, 18:1-9:0ALD; *m/z* 692, 18:0-9:0ALD. LC/MS conditions are given in Fig. 2.

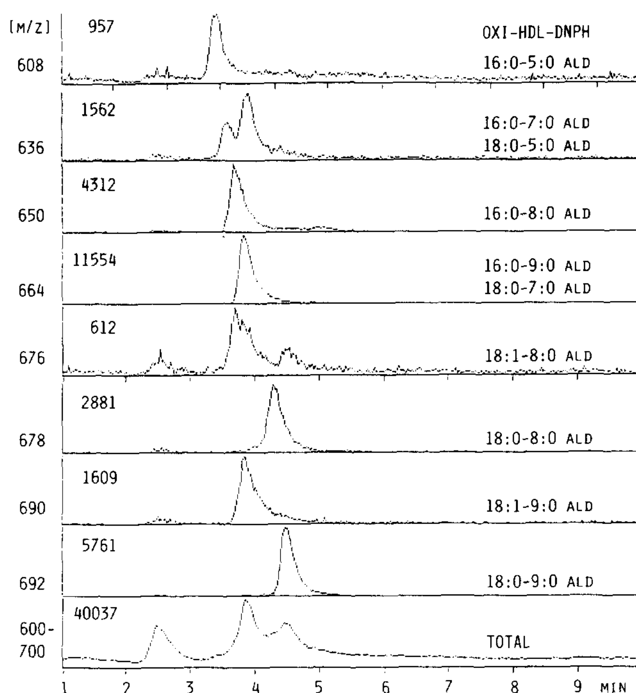

Fig. 6. Total negative ion current profiles (Total) and single ion plots (*m/z*) for the DNPH derivatives of the major diacylglycerol core aldehydes derived from the glycerophospholipids of oxidized human HDL. Peak identification is given in figure. LC/MS conditions are given in Fig. 2, except that the gradient of the eluent was completed over a period of 10 min. MS conditions are given in text. Numbers inside the mass chromatograms indicate intensities of the full scale peaks.

of the UV profiles, which correspond to similar mass distributions. The rear parts of the UV elution profiles differ greatly, but these discrepancies are not accompanied by comparable mass differences. The LC/MS spectra showed little mass in the region beyond 18:0-9:0ALD (see below). On the basis of the UV extinction coefficient of the DNPH moieties, the yields of the core aldehydes account for a minimum of 1-2% of the destroyed linoleoyl and arachidonoyl esters of cholesterol and glycerophosphocholine.

Identification and quantitation of lipid ester core aldehydes

Figure 3 shows the LC/MS NCI spectra of the DNPH derivatives of the core aldehydes of cholesterol and 7-ketocholesteryl esters isolated from peroxidized HDL (TLC Zones I and II, respectively, Fig. 2). The core aldehydes of cholesteryl esters (Fig. 3A) contain the 9-oxononanoyl (*m/z* 720) and the 8-oxooctanoyl (*m/z* 706) esters as major components, and 5-oxovaleroyl (*m/z* 664) ester as a minor component. The 7-ketocholesteryl ester core aldehydes (Fig. 3B) were made up of a similar series of homologues 14 mass units higher, e.g., 7-ketocholesteryl 9:0ALD (*m/z* 734) and 7-ketocholesteryl 8:0ALD (*m/z* 720) as the major components. Both cholesteryl and 7-ketocholesteryl ester core aldehydes contained additional masses corresponding to some minor components, the presence of which was confirmed by the correct retention times and Gaussian peak

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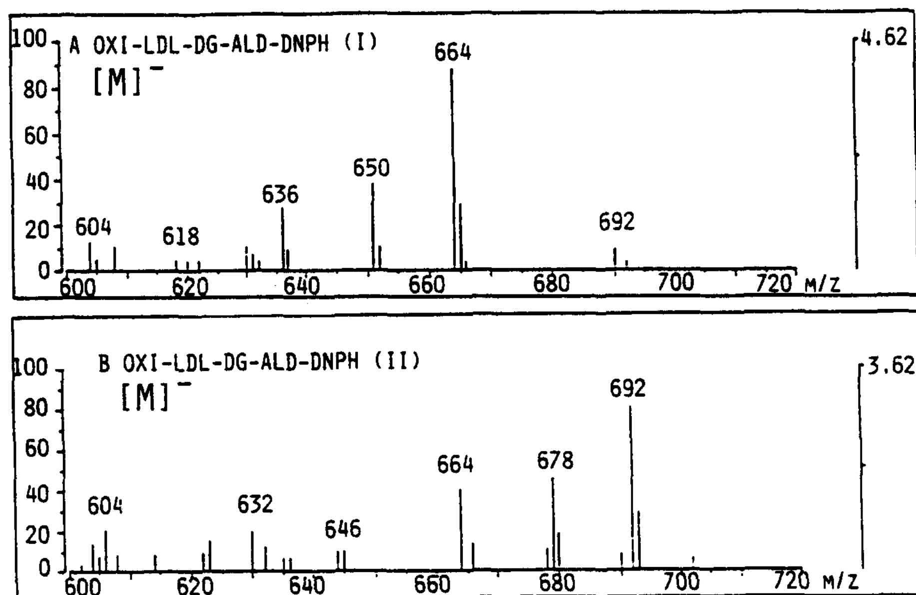

Fig. 7. Mass spectra of the DNPH diacylglycerol core aldehydes derived from the glycerophospholipids of peroxidized human LDL. I: spectra averaged over the first peak of the total ion current profile; II: spectra averaged over the second peak of the total ion current profile. $[M]^-$ ions are identified in Fig. 5. LC/MS conditions are given in Fig. 3.

shape shown in the single ion plots. **Figures 4A and 4B** show the mass chromatograms of the HDL CE and 7-keto CE core aldehydes, respectively, as the DNPH derivatives. The core aldehydes are eluted in order of increasing molecular weight and range from C_4 to C_{10} as already anticipated from the averaged full mass spectra (Fig. 3). The major species correspond to the core aldehydes formed from the linoleoyl esters, which are major components of CE of HDL. From 45–50% of the CE core aldehydes were in the form of the corresponding 7-keto CE. The DNPH derivatives of the core aldehydes of LDL cholesteryl and 7-ketocholesteryl esters gave mass chromatograms similar to those shown elsewhere (15) and corresponding mass spectra (results not shown). **Table 2** gives the mole % composition of the identified and quantitated core aldehydes of CE and 7-keto CE in the LDL and HDL samples. We have previously shown (15) that the proportions of CE core aldehydes estimated by LC/MS closely approximately those derived from UV absorption. The finding of 7-ketocholesterol as the major oxysterol in oxidized LDL and HDL agrees with the results of peroxidation of liposomal cholesteryl esters (17).

Figure 5 gives the total NCI mass spectra of the DNPH derivatives of the diacylglycerol core aldehydes derived from the glycerophospholipids of oxidized HDL as averaged over the two peaks of the total ion current profile (**Fig. 6**). The spectra averaged over the first peak in the total ion current profile show the C_7 (*m/z* 636), C_8 (*m/z* 650), and C_9 (*m/z* 664) aldehydes combined with palmitic and the C_9 (*m/z* 690) aldehyde combined with oleic acid in the glycerol molecule. The spectra averaged over the second peak of the doublet show the C_7 (*m/z* 664), C_8 (*m/z* 678), and C_9 (*m/z* 692) aldehydes combined with stearic acid in the glycerol molecule as the major species. In addition, both total ion current peaks show masses for minor components, the identity of which was further confirmed by the single ion plots shown in Fig. 6. In addition to the major components, the plots indicate the presence of the C_5 and C_6 species of the core aldehydes of the monopalmitoyl, monostearoyl and monooleoylglycerols in oxidized HDL.

Similar mass spectra were obtained for the core aldehydes of the glycerophospholipids of oxidized LDL. **Figure 7** shows the total LC/MS spectra averaged over the two DNPH peaks of the diacylglycerols derived from LDL. They are made up of essentially the same molecular species as those in oxidized HDL. There were somewhat smaller amounts of the C_5 aldehyde in the oxidized LDL than in oxidized HDL. Double aldehyde derivatives were not found in either LDL or HDL. However, the DNPH derivatives of the diacylglycerol core aldehydes overlapped with the DNPH derivatives of the simple long chain aldehydes released from the plasmalogens under the acid conditions (1 N HCl) used for the preparation of the hydrazones. **Figure 8** shows that the DNPH of palmitoyl (*m/z* 420) and oleoyl (*m/z* 446) aldehydes partly overlap with the 16:0–9:0ALD (*m/z* 664),

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18:0-8:0ALD (*m/z* 678), and 18:0-9:0ALD (*m/z* 692). The DNPH derivatives of the corresponding monoacylglycerols carrying the C_5 (*m/z* 370) and C_9 (*m/z* 418) core aldehydes were not detected as the expected minor amounts were probably lost in the aqueous phase during organic solvent extraction.

As LDL is known to contain more of the platelet-activating factor acetyl hydrolase than HDL, which is known to hydrolyze PC possessing 5-oxovaleric and to a lesser extent 9-oxononanoic acid in the *sn*-2-position (21-23), it was of interest to determine whether the differences in the core aldehyde profiles between LDL and HDL (compare Figs. 1A and 1B) were possibly due to the activity of this enzyme. **Table 3** shows the effect of inclusion PMSF (10 mM) in the oxidation mixture. This reagent is a general inhibitor of serine type of phospholipases (23). It is seen that in the presence of the inhibitor relatively more of the C_5 core aldehyde is recovered than in its absence, and that the total aldehyde profile of PC of oxidized LDL becomes more like that of oxidized HDL.

DISCUSSION

The present study demonstrates the formation of lipid soluble CE, 7-keto CE, and glycerophospholipid core aldehydes during the peroxidation of human LDL and HDL using the commonly used 10 mM copper catalysis (11, 24, 25). The core aldehydes were derived mainly from peroxidation of the linoleic and arachidonic acid esters. The aldehydes were not readily formed from oleic acid esters. This result is consistent with the general finding that LDL rich in oleic acid is resistant to peroxidation and may be protective against oxidative modification (26). On the basis of previous work with *tert*-butylhydroperoxide, the oleic acid esters yielded less than 10% of the core aldehyde obtained from linoleic acid esters (17). As the core aldehydes of both cholesterol and 7-ketocholesteryl esters possessed closely similar profiles, it can be concluded that a prior oxidation of the steroid ring did not facilitate the peroxidation of the unsaturated fatty chains in the ester molecules (27).

The mechanism of peroxidation of both 18:2n6 and 20:4n6 esters has been described by Esterbauer, Zollner, and Schaur (28), who have predicted the formation of the C_5 and C_9 aldehyde esters as the major components of terminal oxidation of arachidonic and linoleic acid esters, respectively. They have also proposed the formation of unsaturated longer chain aldehydes (e.g., 13:2), which we were able to detect only occasionally.

The present results show that the lipid ester core aldehydes are readily formed during the peroxidation of both LDL and HDL in agreement with recent measurements of malonaldehyde (29), although earlier studies (8, 9) had suggested that HDL might be more resistant to peroxidation than LDL. Bowry, Stanley, and Stocker (25) have reported that the HDL fraction carries most (85%) of the detectable lipid hydroperoxides, with LDL lipids being relatively peroxide free. In vitro, the LDL lipids were peroxidized more rapidly than HDL lipids after the ubiquinol-10 (major endogenous antioxidant) present was consumed. Preferential accumulation of lipid peroxides in the HDL compared to LDL reflected the lack of antioxidants in nascent HDL particles

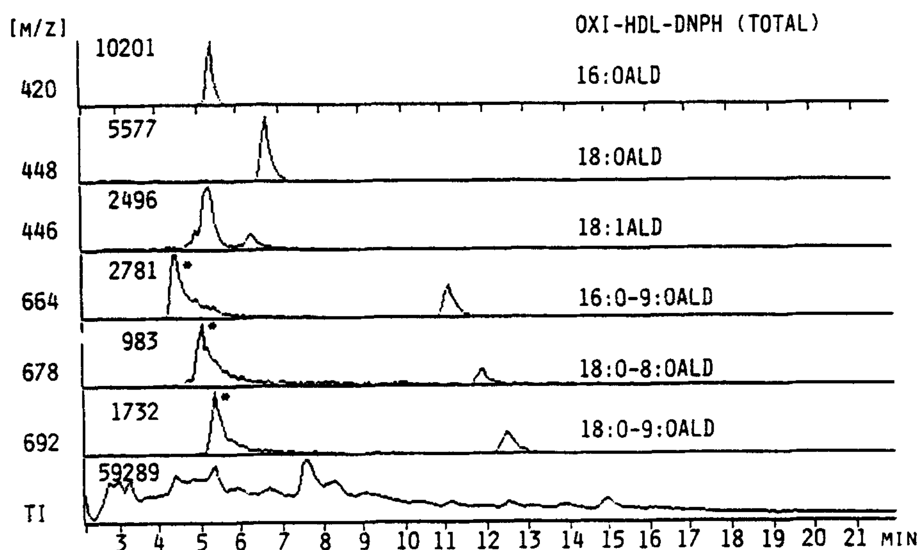

Fig. 8. Total negative ion current profiles (TI) and single ion plots (*m/z*) of the DNPH derivatives of the major long chain aldehydes released from plasmalogens of oxidized LDL during preparation of hydrazones. Peak identification as is given in figure. LC/MS conditions are given in Fig. 3.

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TABLE 3. Relative proportions of phosphatidylcholine core aldehydes recovered from peroxidized LDL and HDL in presence and absence of phenylmethylsulfonylfluoride (PMSF) (mole %)

Molecular Species	Mass [M]⁻	LDL		HDL
		-PMSF	+PMSF	-PMSF
16:0-5:0ald	608	ND	4.1 ± 0.5	3.4 ± 0.2
16:0-7:0ald	636]	ND]	17.3 ± 1.5]	7.1 ± 0.5
18:0-5:0ald	636]			
16:0-8:0ald	650	9.0 ± 1.0	14.1 ± 1.5	13.5 ± 0.7
16:0-9:0ald	664	32.6 ± 3.0	34.1 ± 2.5	36.1 ± 1.5
18:1-8:0ald	676]		1.0 ± 0.1]	2.4 ± 0.1
18:0-8:1ald	676]			
16:0-10:0ald	678]			
18:0-8:0ald	678]	21.2 ± 2.0]	7.1 ± 1.0]	11.2 ± 1.0
18:1-9:0ald	690		3.0 ± 0.5	6.2 ± 0.5
18:0-9:0ald	692	36.5 ± 2.5	18.2 ± 1.0	19.0 ± 0.5
18:0-10:0ald	706			0.5 ± 0.1

Values, estimated from peak areas in mass chromatograms, are given as means ± SE of three or four determinations.

compared to LDL (25).

The present study demonstrates that after a 24-h peroxidation the LDL contains relatively less of the PC-derived diacylglycerol core aldehydes than HDL. This difference is largely due to a lower proportion of the C_5 aldehyde species, although native LDL and HDL possessed comparable amounts of 20:4n6 in their PC. We have attributed this discrepancy to the greater activity of the PAF acetyl hydrolase in LDL than in HDL (24). This enzyme has been shown to release the 5-oxovaleroyl to a greater extent than the C_9 aldehyde group from the *sn*-2-position of synthetic 1-palmitoyl-2-(omega-oxoalkanoyl)-*sn*-glycerophosphocholine (21, 22). In the presence of PMSF, a general inhibitor for serine type of phospholipases (23), relatively more of the C_5 core aldehyde was recovered from LDL than in the absence of the inhibitor. The possibility that the LDL fraction contained other phospholipase A_2-types of activities, which could have destroyed the hydroperoxide or core aldehyde containing lipids, appears to have been excluded (21, 23).

The formation of lipid ester core aldehydes during in vitro peroxidation of LDL and HDL suggests that the core aldehydes could also be formed in vivo. Previous work (5, 30) has shown that copper- and iron-catalyzed lipid peroxidation closely resembles lipoprotein peroxidation in vivo. Although cholesteryl or glyceryl ester core aldehydes have not been previously reported in tissues or plasma, the presumed precursors have been isolated by several laboratories. Thus, Yamamoto and Niki (12) reported CE hydroperoxide values of 34 nM in human blood plasma. Miyazawa (13) reported plasma PC hydroperoxide values of 10–500 nM for healthy and 500–9000 nM for unhealthy donors. Using an automated determination, Akasaka et al. (31) detected 4–24 nM PC hydroperoxide in fresh human plasma, but PE hydroperoxide was not detected. More specifically, Miyazawa, Fujimoto, and Oikawa (14) estimated the PC hydroperoxide concentration in LDL as about 466 ± 154 nM for patients with atherosclerosis and hyperlipidemia, while for normal subjects a value of about 65 ± 43 nM was obtained.

The physiological and pharmacological effects of any of the lipid ester core aldehydes are not known, but the potential interference of PC core aldehydes with membrane structure and function has been the subject of early theoretical speculation (32). Furthermore, the potential formation of Schiff-base complexes between the lipid ester core aldehydes and the amino groups of proteins suggests far-reaching effects upon protein association with lipid membranes (30, 33). Clearly, a more complete knowledge of the peroxidation products of plasma lipoproteins should permit a better management of the benefits to be derived from antioxidants, including vitamins and drugs, and a better protection against the side effects of lipid peroxidation.

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