Regioisomeric distribution of cholesteryl linoleate hydroperoxides and hydroxides in plasma from healthy humans provides evidence for free radical-mediated lipid peroxidation in vivo

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Abstract **We have previously reported the detection of cholesteryl ester hydroperoxides, consisting mainly of cholesteryl linoleate hydroperoxides (Ch18:2-OOH), at nm levels in plasma from healthy humans (**Y. Yamamoto and E. Niki, **1989.** ***Biochem. Biophys. Res. Commun.*** 165: **988–993). To elucidate their production mechanism** ***in vivo*****, we examined the distribution of Ch18:2-O(O)H regioisomers in blood plasma from nine healthy young subjects using a sequential method consisting of methanol/hexane extraction in the presence of antioxidant, reductant, and internal standard, solid phase extraction to remove unoxidized cholesteryl linoleate, purification by reversed-phase high-performance liquid chromatography (HPLC), and detection by normal phase HPLC. Furthermore, we confirm that little artifactual oxidation of cholesteryl linoleate occurred during analytical procedures indicated by the absence of oxidation products of cholesteryl 11Z,14Z-eicosadienoate (Ch20:2) when provided as an exogenous substrate to the experimental procedure. We detected nm levels of all free radical-mediated oxidation products, 13ZE-, 13EE-, 9-EZ-, and 9-EE-forms of Ch18:2-O(O)H, in blood plasma, whereas the 13ZE-isomer resulting from enzymatic 15-lipoxygenase oxidation was not evident as a major product. These results indicate that free radical chain oxidation of lipids occurs even in healthy young individuals.**—Mashima, R., K. Onodera, and Y. Yamamoto. **Regioisomeric distribution of cholesteryl linoleate hydroperoxides and hydroxides in plasma from healthy humans provides evidence for free radical-mediated lipid peroxidation in vivo.** ***J. Lipid Res.*** **2000.** 41: **109–115.**

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Lipid hydroperoxides are the primary stable products of lipid peroxidation. Owing to the importance of this process in pathophysiological conditions, we have developed an ultrasensitive method for the detection of lipid hydroperoxides (1). Using this method, we found about 3 nm cholesteryl ester hydroperoxides (CE-OOH), mostly cholesteryl linoleate hydroperoxides (Ch18:2-OOH), in blood plasma obtained from healthy individuals (2). It was also an interesting observation that the plasma levels of CE-OOH and the ratios of CE-OOH to cholesteryl esters (CE) increase significantly in the order of humans < Sprague-Dawley rats < Nagase analbuminemic rats (3), and this order may be generally correlated to the comparative life spans of humans and rats, and is consistent with the observation that Nagase analbuminemic rats are more sensitive than Sprague-Dawley rats to carcinogens.

Cholesteryl linoleate (Ch18:2) is a major cholesteryl ester in human blood plasma and **Fig. 1** provides chemical structures of primary autoxidation products: cholesteryl 13-hydroperoxy-9Z,11E-octadecadienoate (13ZE-Ch18:2-OOH), cholesteryl 13-hydroperoxy-9E,11E-octadecadienoate (13EE-Ch18:2-OOH), cholesteryl 9-hydroperoxy-10E,12Z-octadecadienoate (9EZ-Ch18:2-OOH), and cholesteryl 9-hydroperoxy-10E,12E-octadecadienoate (9EE-Ch18:2-OOH). As each isomer has two stereoisomers (S and R forms), there are eight isomeric products formed by Ch18:2 oxidation. Autoxidation proceeds by a free radical chain mechanism as shown in Fig. 1. First, hydrogen atom abstraction from C-11 of the linoleate side chain leads to the pentadienyl radical (Ch18:2·) and addition of oxygen to Ch18:2· yields 13ZE- and 9EZ-peroxyl radical (Ch18:2-OO·). Porter et al. (4) and Kenar et al. (5) demonstrated

Abbreviations: AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); BHT, 2,6-di-*tert*-butyl-4-methylphenol; CE-O(O)H, cholesteryl ester hydro(pero)xides; CE, cholesteryl esters; Ch18:2, cholesteryl linoleate; 13ZE-Ch18:2-O(O)H, cholesteryl 13-hydro(pero)xy-9Z,11E-octadecadienoate; 13EE-Ch18:2-O(O)H, cholesteryl 13-hydro(pero)xy-9E,11E-octadecadienoate; 9EZ-Ch18:2-O(O)H, cholesteryl 9-hydro(pero)xy-10E,12Z-octadecadienoate; 9EE-Ch18:2-O(O)H, cholesteryl 9-hydro(pero)xy-10E,12E-octadecadienoate; Ch18:2-O(O)H, Ch18:2 hydro(pero)xides; Ch20:2, cholesteryl 11Z,14Z-eicosadienoate; Ch20:2-O(O)H, Ch20:2 hydro(pero)xides; Ch18:3, cholesteryl 6Z,9Z,12Z-octadecatrienoate; Ch18:3-O(O)H, Ch18:3 hydro(pero)xides; 6EZZ-Ch18:3-OH, cholesteryl 6-hydroxy-7E,9Z,12Z-octadecatrienoate; HPLC, high performance liquid chromatography.

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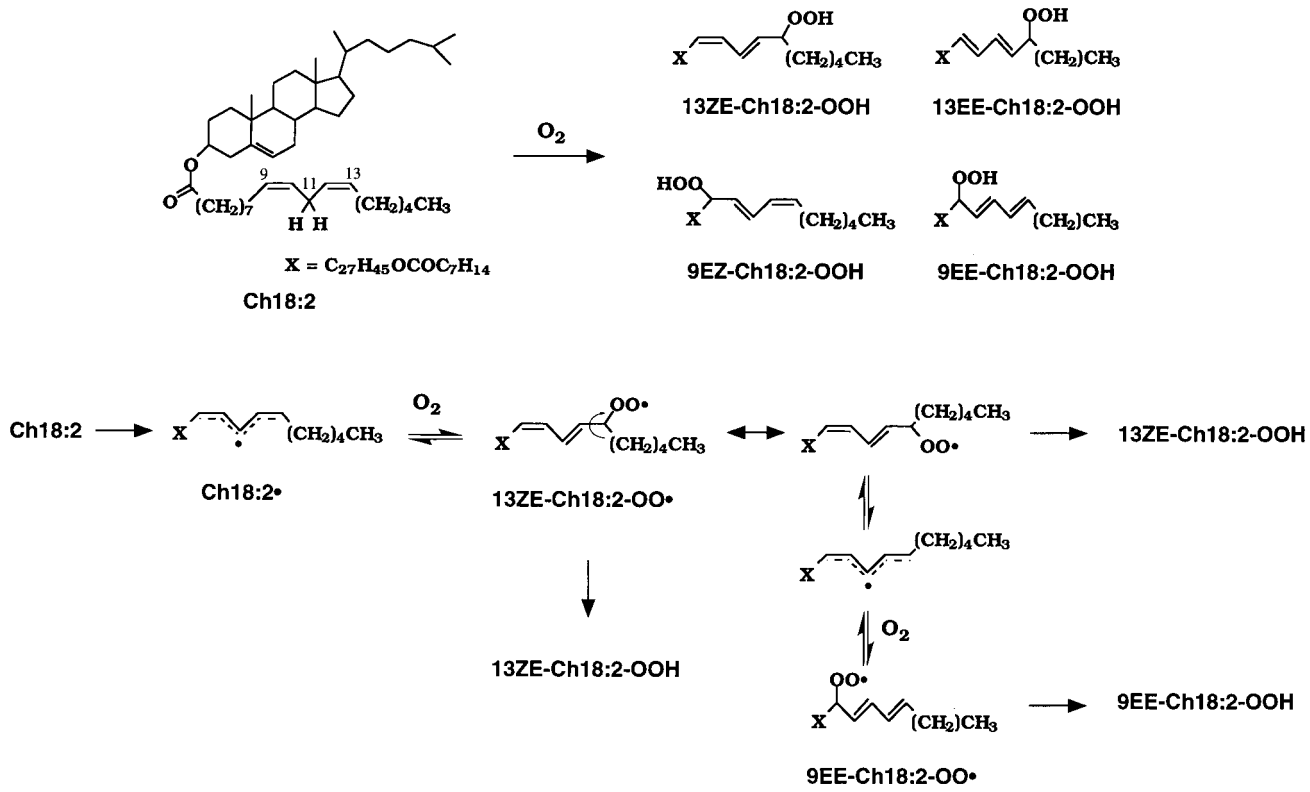

Fig. 1. Products and reaction mechanism of the autoxidation of cholesteryl linoleate (Ch18:2).

the conversion of 13ZE-peroxyl radical to 9EE-peroxyl radical through the rotation of the C9–C10 bond, removal of oxygen from Ch18:2-OO·, and addition of oxygen to Ch18:2· to give the 9EE peroxyl radical. Likewise, transformation of the 9EZ peroxyl radical to the 13EE peroxyl radical proceeds by a similar manner. The ratio of (13ZE-Ch18:2-OOH + 9EZ-Ch18:2-OOH) to (13EE-Ch18:2-OOH + 9EE-Ch18:2-OOH) is therefore a good indicator for the presence of active hydrogen donors such as antioxidants and polyunsaturated lipids as hydrogen donation diminishes the yields of EE forms during autoxidation.

On the other hand, enzymatic oxidation of Ch18:2 with 15-lipoxygenase gives predominantly only one stereospecific product (S form of 13ZE-Ch18:2-OOH) (6). It has been shown that 15-lipoxygenase can also oxidize Ch18:2 in low density lipoprotein to give the S form of 13ZE-Ch18:2-OOH as the major product (7). These results indicate that the site and the orientation of oxygen addition are restricted by enzymatic oxidation and that the Ch18:2-OO· intermediate produced during enzymatic oxidation is not free to stereo-isomerization.

To elucidate the mechanism of Ch18:2 oxidation in vivo, we have developed a method to determine the regioisomeric composition of Ch18:2-O(O)H. We analyzed for both Ch18:2-OOH and its hydroxide (Ch18:2-OH) because apolipoproteins A and B-100 (8–10) in human plasma can reduce Ch18:2-OOH to Ch18:2-OH. We also demonstrate that little artifactual oxidation occurs during our analytical procedure by using cholesteryl 11Z,14Z-eicosadienoate (Ch20:2) as an exogenous probe; Ch20:2 is not present in human plasma and the oxidizabilities for both substrates are identical as described in this report.

MATERIALS AND METHODS

Reagents

Ch18:2 was purchased from Sigma (Tokyo, Japan). Ch20:2 and cholesteryl 6Z,9Z,12Z-octadecatrienoate (Ch18:3) were obtained from Nu-Chek-Prep (Elysian, MN). Other reagents were of the highest grade available. 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) was obtained from Wako (Osaka, Japan).

To remove oxidation products, Ch18:2 and Ch20:2 were purified on a semipreparative octadecylsilyl column (Superiorex ODS, 20 × 250 mm, Shiseido, Tokyo) using methanol–2-propanol 25:75 (v/v) as the mobile phase delivered at a flow rate of 8.0 ml/min (11). Then, Ch18:2-OOH was prepared by spontaneous autoxidation of hydroperoxide-free Ch18:2 (80 mol) in 1 ml of hexane at room temperature for 10 days. Addition of triphenylphosphine reduced Ch18:2-OOH to Ch18:2-OH. After exchanging the solvent to the above mobile phase, Ch18:2-OOH and Ch18:2-OH were fractionated using the same HPLC conditions described above. To separate the four regioisomers, the Ch18:2-OOH and Ch18:2-OH fractions were injected onto a semipreparative silica gel column (Supelcosil LC-Si, 10 × 250 mm, Supelco, Tokyo) using hexane–2-propanol 1000:5 (v/v) as the mobile phase (5.0 ml/min) (5). Ch20:2 hydro(pero)xides (Ch20:2-O(O)H) were prepared similarly. Cholesteryl 15-hydroxy-11Z,13E-eicosadienoate (15ZE-Ch20:2-OH), cholesteryl 15-hydroxy-11E, 13E-eicosadienoate (15EE-Ch20:2-OH), cholesteryl 11-hydroxy-

12E,14Z-eicosadienoate (11EZ-Ch20:2-OH), and cholesteryl 11-hydroxy-12E,14E-eicosadienoate (11EE-Ch20:2-OH) were separated by using an identical, normal phase HPLC procedure.

Ch18:3 was oxidized in hexane in the presence of 5 mole % of α-tocopherol at room temperature for 3 days. α-Tocopherol was added to decrease the formation of the ZEE isomers of Ch18:3 hydroperoxides (Ch18:3-OOH) and to accelerate autoxidation (12). Amongst the four ZZE isomers of Ch18:3 hydroxides (Ch18:3-OH), cholesteryl 6-hydroxy-7E,9Z,12Z-octadecatrienoate (6EZZ-Ch18:3-OH) was selected as an internal standard in the following experiments. The HPLC elution profiles for the Ch18:2-O(O)H, Ch20:2-O(O)H, and Ch18:3-O(O)H isomers and characterization by gas chromatography/mass spectrometry have been determined and will be published elsewhere. Concentrations were determined photometrically by absorbance at 234 nm using the molar absorption coefficient for a conjugated diene, 28,000 $cm^{-1}M^{-1}$ (13).

Oxidation of Ch18:2 and Ch20:2 in 2-propanol

To compare the oxidizability, we initiated the oxidation of Ch18:2 and Ch20:2 (1 mm each) with 100 mm AMVN in 2-propanol at 37°C under aerobic conditions.

Aliquots (5 μl) from the reaction were injected onto an octadecylsilyl column (Supelcosil LC-18, 4.6 × 250 mm, Supelco) to determine rates of Ch18:2-OOH and Ch20:2-OOH production. Methanol–2-propanol 1:1 (v/v) was used as the mobile phase delivered at a flow rate of 1.0 ml/min and peaks were detected at 234 nm.

Collection of blood plasma

Blood was collected from nine fasted healthy volunteers (nonsmoking Japanese males, 21–29 years old) and placed in a heparin-containing vacutainer. Plasma was separated from blood by centrifugation at 1500 *g* for 10 min and was stored at −80°C until analysis.

This study was approved by the Ethics Committee of the Research Center for Advanced Science and Technology, University of Tokyo.

Analyses of Ch18:2-O(O)H regioisomers in human plasma

Analytical procedures are outlined in **Fig. 2.** Briefly, 10 ml of methanol containing 325 μm 2,6-di-*tert*-butyl-4-methylphenol (BHT), 70 ml of hexane containing 1.4 μm triphenylphosphine, and 5 μl of a hexane solution of 10.9 μm 6EZZ-Ch18:3-OH (corresponding to 21.8 nm in plasma) were added to 2.5 ml of heparinized plasma and were mixed vigorously for 5 min. After centrifugation at 1500 *g* for 1 min, 60 ml of the hexane phase was collected and the solvent was removed on a rotary vacuum evaporator.

The dry residue was dissolved in 3 ml of hexane–ethyl acetate 50:1 (v/v) containing 10 μm BHT and was applied to a solid phase extraction column constructed of glass (inner diameter = 25 mm) having a 60 ml volume and containing 2.5 g of silica with an aminopropylsilyl surface-bonded phase (LC-NH_2, Supelco). The column was prewashed with 100 ml of the above hexane–ethyl acetate before applying 3 ml of the reconstituted extract was added. After washing with 50 ml of the above hexane–ethyl acetate mixture, Ch18:2-OH was eluted with 50 ml of hexane–ethyl acetate 9:1 (v/v) containing 10 μm BHT.

After removing eluted solvent on a rotary vacuum evaporator, the collected Ch18:2-OH was dissolved in 500 μl of methanol–2-propanol 9:1 (v/v) and an aliquot (350 μl) was injected onto an octyl column (Capcell Pak C8, 10 × 250 mm, Shiseido) to remove contaminants; methanol–2-propanol 9:1 (v/v) was used as the mobile phase delivered at a flow rate of 4.0 ml/min with detection at 234 nm. The fraction containing Ch18:2-OH and 6EZZ-Ch18:3-OH was collected.

After removing the solvent and redissolving the residue in 500 μl of hexane–2-propanol 1000:5 (v/v), an aliquot (100 μl: corresponding to 300 μl of plasma) was injected onto a silica gel column (Supelcosil LC-Si, 4.6 × 250 mm, Supelco) to determine the regioisomeric composition of Ch18:2-OH using hexane–2-propanol 1000:5 (v/v) as the mobile phase delivered at a flow rate of 1.0 ml/min with detection at 234 nm.

For a control comparison, distilled water was substituted for plasma and treated exactly as described above.

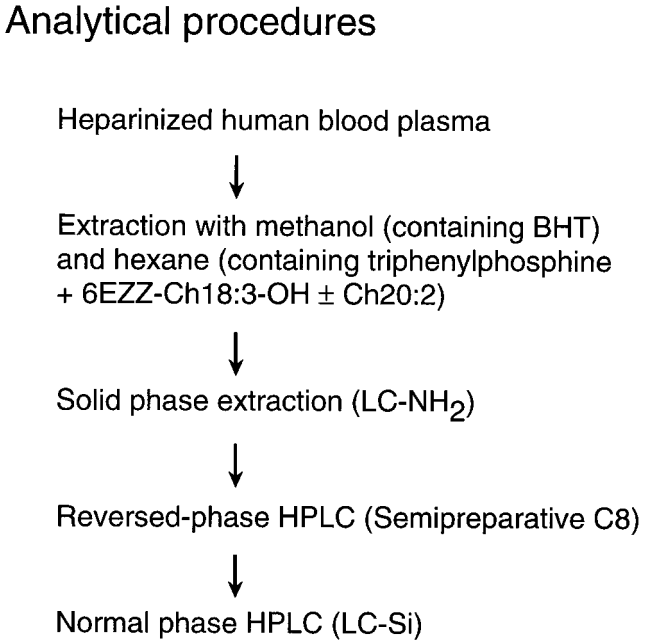

Fig. 2. Analytical procedure for the measurement of regioisomers of cholesterol linoleate hydro(pero)xides.

Assessment of artifactual oxidation during analytical procedure

In addition to 10 ml of methanol containing 325 μm BHT, 70 ml of hexane containing 1.4 μm triphenylphosphine, and 5 μl of a hexane solution of 10.9 μm 6EZZ-Ch18:3-OH, 1 ml of a hexane solution of 4 mm freshly purified, oxidation products-free Ch20:2 (corresponding to 1.6 mm in plasma) were added to 2.5 ml of human plasma and identical analytical procedures were used with the exception that the fraction containing Ch20:2-OH was collected on the purification step using a reversed-phase octyl column. Regioisomers of Ch20:2-OH were then analyzed by the same normal phase HPLC method as described above. Separately, 10 μl of a hexane solution of 3.1 μm Ch20:2-OH (corresponding to 12.4 μm in plasma) was added instead of hexane solution of Ch20:2 and the sample was similarly analyzed to demonstrate that nm levels of added Ch20:2-OH can be measured by our method.

CE-OOH and CE assay

The levels of CE-OOH, α-tocopherol (VE), free cholesterol (FC), and CE in human plasma were determined by a method previously described (1, 2). We used Ch18:2-OOH as a standard instead of methyl linoleate hydroperoxide (2).

RESULTS AND DISCUSSION

Procedure for the analysis of regioisomers of Ch18:2-O(O)H

Our analytical procedure (Fig. 2) consisted of methanol–hexane extraction in the presence of antioxidant (BHT),

reductant (triphenylphosphine), and internal standard, solid phase extraction, purification by reversed-phase HPLC, and analysis by normal phase HPLC separation. Antioxidant (BHT) was always present until unoxidized Ch18:2 was removed by solid phase extraction to minimize autoxidation of Ch18:2 during sample treatment. Triphenylphosphine was added to reduce Ch18:2-OOH to Ch18:2-OH as normal phase HPLC (5) separates 13EZ-, 13EE-, 9ZE-, and 9EE-forms of Ch18:2-OH, but not 9EZ-Ch18:2-OOH and 9EE-Ch18:2-OOH. We selected 6EZZ-Ch18:3-OH as an internal standard because it coelutes with Ch18:2-OH on reversed-phase HPLC at the purification step and elutes separately from the regioisomers of Ch18:2-OH on normal phase analysis. Solid phase extraction was used to separate Ch18:2-OH from unoxidized, endogenous Ch18:2 to prevent artifactual substrate oxidation. It was necessary to remove interfering impurities by reversed-phase HPLC.

Co-oxidation of Ch18:2 and Ch20:2

Figure 3 shows that oxidation of Ch18:2 and Ch20:2 (1 mm each) initiated with 100 mm AMVN at 37°C under aerobic conditions affords near identical rates in the production of 18:2-OOH and Ch20:2-OOH, respectively. Both substrates are similar having two reactive bisallylic hydrogens, thus providing an equivalent degree of oxidizability.

Artifactual oxidation

To assess artifactual oxidation during sample processing and analysis, purified Ch20:2 was added to the plasma extract as an internal control and subjected to identical procedures. Ch20:2 was selected as the oxidation probe because Ch20:2 is not normally present in human plasma and the oxidizabilities of Ch18:2 and Ch20:2 are demonstrated to be identical (above). As the plasma levels of Ch18:2 in our volunteers was 1.4 mm (data not shown), we added an equivalent concentration of Ch20:2 (1.6 mm) to the methanol–hexane extract obtained from 2.5 ml of plasma. Separately, Ch20:2-OH (31.1 pmol: corresponding 12.4 nm in plasma) was added to the methanol/hexane extract obtained from 2.5 ml of plasma. Both extracts were taken to the solid phase extraction stage to remove unoxidized Ch18:2 and Ch20:2.

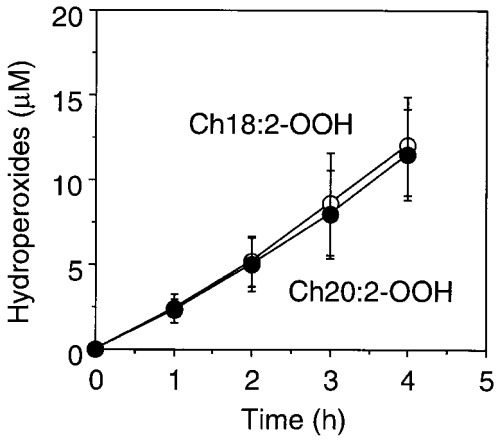

Fig. 3. Formation of Ch18:2-OOH and Ch20:2-OOH during the oxidation of Ch18:2 and Ch20:2 (1 mm each) initiated with 100 mm AMVN in 2-propanol at 37°C under aerobic conditions.

Next, we moved to a purification step using reversed-phase HPLC. Fraction 1 which may contain Ch20:2-OH was collected from the extract of human plasma with added Ch20:2 (**Fig. 4A**). Fraction 2 containing Ch20:2-OH was collected from the extract of human plasma with added Ch20:2-OH (Fig. 4B). Finally, we analyzed fraction 1 using normal phase HPLC and could not detect any significant peaks of four regioisomers (15ZE-, 15EE-, 11EZ-, and 11EE-Ch20:2-OH) (**Fig. 5A**). On the other hand, four regioisomers were detected in the analysis of fraction 2 obtained from the extract of plasma spiked with prepared Ch20:2-OH isomers (Fig. 5B), demonstrating that low nm levels of CE-OH in plasma can be measured by our method. From these data we concluded that artifactual oxidation products of Ch18:2 are not formed during our analytical treatment.

Regioisomers of Ch18:2-O(O)H in human plasma

We applied our method to the analyses of human plasma using distilled water as a plasma control blank. Detailed analytical procedurs are described in Materials and Methods section. **Figure 6** shows the collected fractions at the purification step using reversed-phase HPLC separation where Ch18:2-OH and the internal standard (6EZZ-Ch18:3-OH: corresponding to 21.8 μm in plasma) elute between 7.5–9 min. Fractions 3 and 4 were collected from the extracts of plasma from a young healthy individual and control samples, respectively. The normal phase HPLC chromatogram of fraction 3 (**Fig. 7A**) shows that

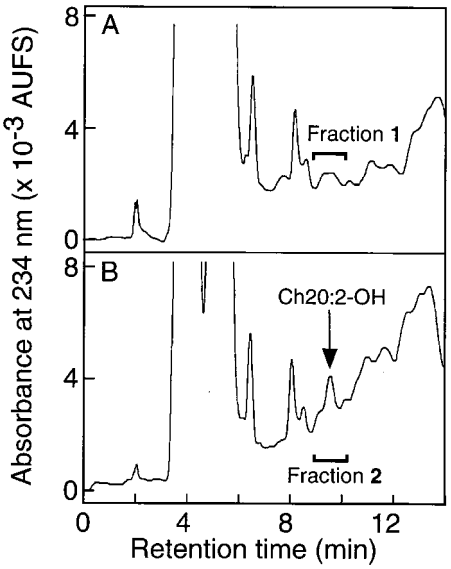

Fig. 4. (A) Collection of fraction 1 which may contain Ch20:2-OH from the extract of human plasma with added Ch20:2 (corresponding to 1.6 mm in plasma) and (B) collection of fraction 2 containing Ch20:2-OH from the extract of human plasma with added Ch20:2-OH (corresponding to 12.4 nm in plasma). Fractionation indicated by the collection brackets was achieved on an octyl column (10 × 250 mm) with methanol–2-propanol 9:1 (v/v) as the mobile phase (4.0 ml/min). Elution time of Ch20:2-OH is as indicated.

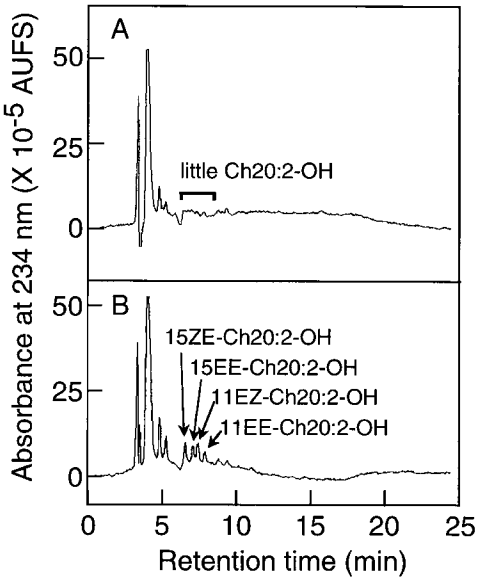

Fig. 5. Normal phase HPLC analysis of (A) fraction 1 (Fig. 4A) and (B) fraction 2 (Fig. 4B) containing Ch20:2-OH. A silica gel column (4.6 × 250 mm) was used with hexane–2-propanol 1000:5 (v/v) as the mobile phase (1.0 ml/min). 15ZE, 15EE, 11EZ, and 11EE are indicated as the four designated regioisomers of Ch20:2-OH.

four regioisomers of Ch18:2-OH are present at low nm levels in plasma from a healthy human. Identification of Ch18:2-OH regioisomers was confirmed by spiking of prepared Ch18:2-OH isomers (total 3.8 pmol: corresponding to 10.9 nm in plasma) to fraction 3 (Fig. 7B). It is noteworthy that there was no significant peak, other than the

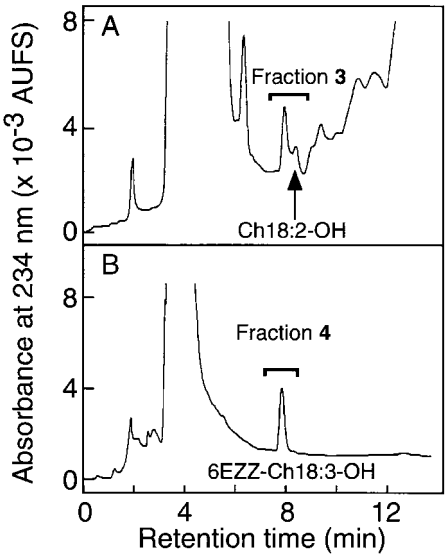

Fig. 6. (A) Collection of fraction 3 containing Ch18:2-OH and the internal standard (6EZZ-Ch18:3-OH) from the extract of human plasma with added 6EZZ-Ch18:3-OH (corresponding to 21.8 nm in plasma) and (B) collection of fraction 4 from the extract of water control with added 6EZZ-Ch18:3-OH (corresponding to 21.8 nm in water). HPLC conditions were as described in Fig. 4. Fractionation of Ch18:2-OH and 6EZZ-Ch18:3-OH is indicated by the collection brackets. Their elution times are as indicated.

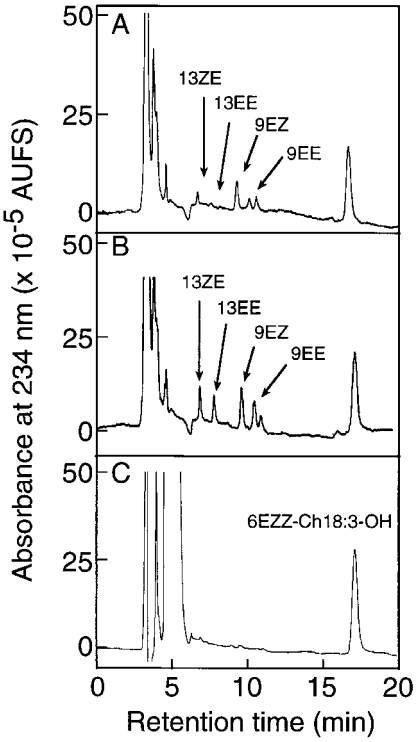

Fig. 7. Normal phase HPLC analysis of (A) fraction 3 from the extract of human plasma (Fig. 6A) (B) fraction 3 spiked with 3.8 pmol Ch18:2-OH (corresponding to 10.9 nm in plasma), and (C) fraction 4 (Fig. 6B) containing 6EZZ-Ch18:3-OH from the water control. HPLC conditions are as described in Fig. 5. 13ZE, 13EE, 9EZ, and 9EE are the four designated regioisomers of Ch18:2-OH and their elution times are as indicated.

internal standard, in the analysis of control fraction 4 obtained from distilled water (Fig. 7C).

Table 1 summarizes the plasma levels of Ch18:2-O(O)H, CE-OOH, VE, FC, and CE obtained from nine young healthy volunteers. Recovery of the internal standard was 76% on an average, indicating that most of plasma Ch18:2-O(O)H isomers were detected without a significant loss in our assay. Plasma samples were repetitively analyzed (n = 3–6) and the results were found to be reproducible. All four regioisomers of Ch18:2-O(O)H were always present and the formation of 13ZE-Ch18:2-O(O)H isomer with contribution of enzymatic oxidation was not observed as the major product (Fig. 7A and Table 1). These results offer substantial evidence of free radical-mediated oxidation of lipids in vivo.

Our conclusion is further verified by the presence of isoprostanes, nonenzymatic oxidation products of arachidonic acid, in plasma and urine from healthy humans (14–18). Plasma levels of isoprostanes are significantly elevated in smokers (15) and in patients with renal failure (17) in comparison to suitable controls. It was also found that urinary isoprostane levels in patients treated with coronary reperfusion via percutaneous transluminal coronary angioplasty were markedly increased from baseline in the first 6 h returning to preprocedural levels within 24 h (16). We are also planning to apply our method to patients in pathophysiological distress. Levels of isopros-

TABLE 1. Levels of the four cholesteryl linoleate hydro(pero)xides (Ch18:2-O(O)H) regioisomers, cholesteryl ester hydroperoxides (CE-OOH), α-tocopherol (VE), free cholesterol (FC), and cholesteryl esters (CE) in plasma from healthy human donors

		Ch18:2-O(O)H										
Sample	Age	13ZE	13EE	9EZ	9EE	Total	ZE/EE	CE-OOH	Recovery	VE	FC	CE
	yr			*nM*				*nM*	*%*	*μM*	*mM*	*mM*
A (n = 5)	28	3.3 ± 1.0 [28]	0.5 ± 0.2 [5]	5.7 ± 1.0 [49]	2.2 ± 0.5 [19]	11.8 ± 2.2	3.3	8.0 ± 1.1	72 ± 4	31.1 ± 3.8	1.30 ± 0.10	2.37 ± 0.19
B (n = 6)	28	1.6 ± 0.2 [16]	0.8 ± 0.3 [8]	4.8 ± 0.7 [48]	2.8 ± 0.5 [28]	10.0 ± 1.4	1.8	5.5 ± 0.6	76 ± 10	28.1 ± 1.4	1.08 ± 0.04	2.05 ± 0.09
C (n = 5)	21	1.3 ± 0.2 [31]	0.4 ± 0.1 [9]	1.8 ± 0.3 [44]	0.7 ± 0.2 [17]	4.1 ± 0.6	2.9	2.9 ± 0.7	83 ± 4	24.7 ± 0.8	1.10 ± 0.02	2.13 ± 0.15
D (n = 3)	29	3.4 ± 0.7 [18]	0.7 ± 0.6 [4]	10.5 ± 3.3 [53]	4.7 ± 0.6 [25]	19.4 ± 4.4	2.6	12.9 ± 1.9	67 ± 3	23.9 ± 0.9	1.09 ± 0.16	1.65 ± 0.24
E (n = 3)	27	9.6 ± 1.6 [36]	0.5 ± 0.1 [2]	11.5 ± 1.5 [43]	4.9 ± 0.3 [18]	26.7 ± 1.0	3.9	5.9 ± 0.4	80 ± 3	28.9 ± 1.5	1.28 ± 0.29	2.03 ± 0.13
F (n = 3)	27	2.5 ± 0.9 [23]	0.3 ± 0.1 [3]	6.0 ± 2.3 [52]	2.6 ± 0.7 [22]	11.4 ± 3.3	3.0	8.5 ± 1.7	104 ± 3	21.7 ± 1.6	0.93 ± 0.18	1.72 ± 0.10
G (n = 3)	21	6.5 ± 2.3 [32]	0.9 ± 0.4 [5]	9.0 ± 1.0 [46]	3.6 ± 0.9 [18]	20.1 ± 3.8	3.4	17.2 ± 0.1	51 ± 10	34.9 ± 1.1	1.91 ± 0.40	2.45 ± 0.25
H (n = 3)	23	2.1 ± 0.9 [22]	0.4 ± 0.2 [4]	4.5 ± 1.0 [51]	2.2 ± 0.7 [23]	9.1 ± 2.4	2.5	6.3 ± 1.9	86 ± 6	18.3 ± 0.2	1.06 ± 0.18	1.46 ± 0.09
I (n = 3)	23	2.9 ± 1.6 [28]	0.3 ± 0.2 [3]	6.0 ± 3.5 [57]	1.0 ± 0.3 [11]	10.1 ± 5.4	7.0	4.7 ± 1.2	69 ± 14	25.2 ± 0.8	1.43 ± 0.21	1.67 ± 0.12

Values are expressed as mean ± SD. Numbers in brackets show percentages. Recovery is based on measurement of the internal standard; CE, cholesteryl arachidonate + cholesteryl linoleate + cholesteryl oleate.

tanes in plasma from healthy humans are reported to be 0.1–0.3 nm (15, 17, 18), which are significantly lower than our plasma Ch18:2-O(O)H levels; 13.6 nm. This is not surprising as CE-OOH is a major product while isoprostanes are minor products during the copper-induced oxidation of low density lipoprotein (18).

Plasma Ch18:2-O(O)H levels were always greater than CE-OOH, indicating the formation of Ch18:2-OH. Plasma glutathione peroxidase does not reduce CE-OOH (19), however, it has been recently reported that apolipoproteins A-I and A-II can reduce CE-OOH to CE-OH (8, 9). We found that apolipoprotein B-100 can also reduce CE-OOH (10).

Plasma CE-OOH levels observed in this study were in the range of 8.0 ± 4.5 nm (mean ± SD, n = 9) which is higher than previously reported values (3.4 ± 1.9 nm, mean ± SD, n = 23) (2). We do not know the reason for this difference but this may be due to the selection and limitation of our sampling.

As described above, the ratio of ZE/EE is dependent on the concentration of hydrogen donors in the reaction media. The ratios observed in the autoxidation of neat dilinoleoyl phosphatidylcholine and its liposomal dispersion at 37°C (without added hydrogen donors) were 1.25 and 1.26, respectively (4). These numbers are significantly lower than our observed values (3.4 ± 1.5, mean ± SD), indicating that active hydrogen donors, such as α-tocopherol, are present at the location where Ch18:2 is oxidized.

In summary, we demonstrate a reliable method for evaluating the regioisomeric composition of Ch18:2-O(O)H in human plasma without affecting artifactual oxidation during sample treatment and analyses. We have detected all four regioisomers of Ch18:2-O(O)H in blood plasma obtained from healthy young subjects. Furthermore, the 13ZE-Ch18:2-OOH isomer did not predominate as a product attributed to an enzymatic oxidation of Ch18:2. Our data suggest that free radical-mediated oxidation of polyunsaturated lipids is an ongoing process within normal healthy individuals.

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