TITLE PAGE

Title:

Identification of molecular species of phosphatidylcholine hydroperoxides in native and copper-oxidized triglyceride-rich lipoproteins in humans

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DECLARATIONS:

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Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study was carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was obtained from all human volunteers and study was approved by the Ethical Committee of Faculty of Health Sciences, Hokkaido University, Sapporo, Japan (Approval Number 08-57-2).

Guarantor

SPH

Contributorship

RS, SPH, and HC researched literature and conceived the study. RS, YM, YM, and TS were involved in sample collection, lipoprotein separation, lipid measurements, and data analysis. RS and ZC did the mass spectrometric analysis. RS wrote the first draft of the manuscript. All the authors have made a substantial contribution to the conception and design of the study, and acquisition, analysis, and interpretation of data. The manuscript has been reviewed and approved by all authors.

ABSTRACT

Background: Triglyceride-rich lipoproteins (TRL) are considered to be independent predictors of atherosclerotic cardiovascular disease. The molecular basis of its atherogenicity is uncertain. Here, we aim to identify molecular species of phosphatidylcholine hydroperoxides (PCOOH) in TRL. For comparison, copper-oxidized TRL were investigated as well.

Methods: A fasting EDTA blood sample was collected from six healthy human volunteers to isolate two major TRL fractions – very low-density lipoproteins (VLDL) and intermediate-density lipoproteins (IDL) using sequential ultracentrifugation. TRL and plasma samples were studied for PCOOH by liquid chromatography (LC) coupled with Orbitrap mass spectrometry.

Results: Twelve molecular species of PCOOH species in TRL and/or plasma were identified using the criterion consisting of 1) high-resolution mass spectrometry (MS) with mass accuracy within 5 ppm, 2) retention time in LC, and 3) fragmentation pattern in MS2 and MS3. PC36:4-OOH was most often detected in VLDL, IDL, and plasma. The ratio of total PCOOH to phosphatidylcholine progressively increased with the duration of oxidation in both VLDL and IDL.

Conclusion: This study demonstrated the presence of 12 molecular species of PCOOH in native TRL. The frequent detection of PCOOH in TRL provides a molecular basis of the atherogenicity of TRL. PCOOH in TRL might serve as a molecular basis of the atherogenicity of TRL.

Keywords: Lipid hydroperoxide, oxidation, mass spectrometry, phospholipids, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL)

Introduction

Plasma triglycerides is a promising candidate for the risk stratification of coronary heart disease (CHD), which is predominantly carried by triglyceride-rich lipoproteins (TRL). However, the specific role of TRL towards the progression of CHD is uncertain and has long been controversial.[1] Alterations of TRL are linked with endothelial dysfunction and considered to be atherogenic.[2] Hence, detection of lipid oxidation products in TRL might provide a basis of the its atherogenicity. The surface of lipoproteins is primarily covered by phosphatidylcholine and are susceptible to oxidative modification in exposure to reactive oxygen species (ROS). Phosphatidylcholine hydroperoxides (PCOOH) are one of its oxidation products and are linked with the pathogenesis of various diseases including atherosclerosis, Alzheimer's disease, Parkinson's disease, multiple sclerosis, rheumatoid arthritis, diabetes, oxidative stress and chronic alcoholism.

In the present study, we aimed to identify and characterize molecular species of PCOOH in TRL and plasma. Moreover, we also observed a time course generation of PCOOH during its copper-catalyzed oxidation.

Materials and methods

EDTA plasma was collected from 6 healthy human volunteers after fasting for 14-16 h. Sequential ultracentrifugation was carried out to isolate VLDL and IDL from the plasma as described earlier.[3] Oxidized VLDL and oxidized IDL were prepared by incubating the isolated lipoprotein fraction with Cu^{2+}. For this, EDTA free native VLDL and IDL were incubated with $CuSO_4$ in PBS (Final concentration- 0.5 mg lipids, 5 μM $CuSO_4$) at 37°C for 0, 0.5, 1, 2, 3, 4, and 8 h. Oxidation of lipoproteins was evaluated by agarose gel electrophoresis (Helena Laboratories, Beaumont, TX, USA). The oxidized lipoproteins were also examined for thiobarbituric acid reactive substances (TBARS) (Cayman Chemical Company, MI, USA).

For the LC/LTQ Orbitrap mass spectrometer (Thermo-Fisher Scientific Inc., San Jose, CA, USA) analysis, total lipids were extracted from native or oxidized lipoproteins, and plasma samples as described earlier.[4] The lipid extracts were injected to a reversed-phase Atlantic T3 C18 HPLC column [150 mm × 2.1 (i.d.) mm; particle size 3.0 μm] (Waters, Milford, MA, USA), maintained at 40°C. Gradient elution was performed with a mobile phase composed of 5 mmol/L aqueous ammonium acetate, isopropanol, and methanol. Synthetic standards of PCOOH were used for assignment of equivocal peaks. PCOOH in TRL and plasma samples were identified based on retention time (RT) on LC, elemental composition and typical fragmentation pattern in the mass spectra. The limit of detection for each PCOOH were 0.1 pmol at signal-to-noise ratio above 6. The relative concentration of PCOOH was expressed as a ratio of its peak intensity to corresponding molecular species of phosphatidylcholine. Sum of the relative concentration of all detected species of PCOOH is designated as total PCOOH.

Results

We identified 12 molecular species of PCOOH species in TRL and/or plasma using the criterion consisting of 1) elementary composition and high-resolution *m/z* with mass accuracy within 5 ppm, 2) RT in LC, and 3) fragmentation pattern in MS2 and MS3.

A typical LC/MS extracted-ion chromatogram (EIC) and the corresponding chromatograms of PC34:2-OOH obtained from the standard, VLDL and IDL, and their MS2 and MS3 spectra are provided in Supplementary Figure 1. The major fragment ions of PCOOH observed in MS2 spectra were resulted by the loss of H_2O from hydroperoxide group, fatty acids (FA) from sn-1 carbon and FAOOH from sn-2 carbon (Supplementary Figure 2). In contrast the fragment ions of phosphatidylcholine in MS2 spectra were formed by the natural loss of the phosphatidylcholine head-group and FA. All characteristic ions of phosphatidylcholine and PCOOH in VLDL and IDL, corresponding RT and fragment ions can be found in the Supplementary Table 1. In VLDL, 12 species of PCOOH were identified, among which, 11 species were also detected in IDL, and 10 species in plasma. PC36:4-OOH was most often detected in VLDL, IDL, and plasma. PC34:2-OOH, PC36:2-OOH, and PC36:4-OOH were detected in all plasma samples. PC34:2-OOH, PC36:2-OOH, PC36:4-OOH, PC38:4-OOH, PC38:6-OOH, and PC40:6-OOH were major PCOOH species detected in TRL (Supplementary Table 2).

Oxidized VLDL and IDL showed the increase in electrophoretic mobility, indicating the loss of positive charge at the amino groups of apolipoprotein B100 due to the Schiff-base formation with the aldehyde groups from oxidized acyl chains (Figure 1(a) and 1(b). Accordingly, TBARS for VLDL and IDL increased progressively and peaked at 4 h during oxidation. The ratio of total PCOOH to phosphatidylcholine progressively increased with

the duration of oxidation in both VLDL (Figure 1(a')) and IDL (Figure 1(b')). Based on the ratio of PCOOH to its corresponding phosphatidylcholine, the phosphatidylcholine with polyunsaturated FA particularly, PC34:3, PC36:3, PC 36:5, and PC38:5 were comparatively more susceptible to oxidation (Supplementary Figure 3).

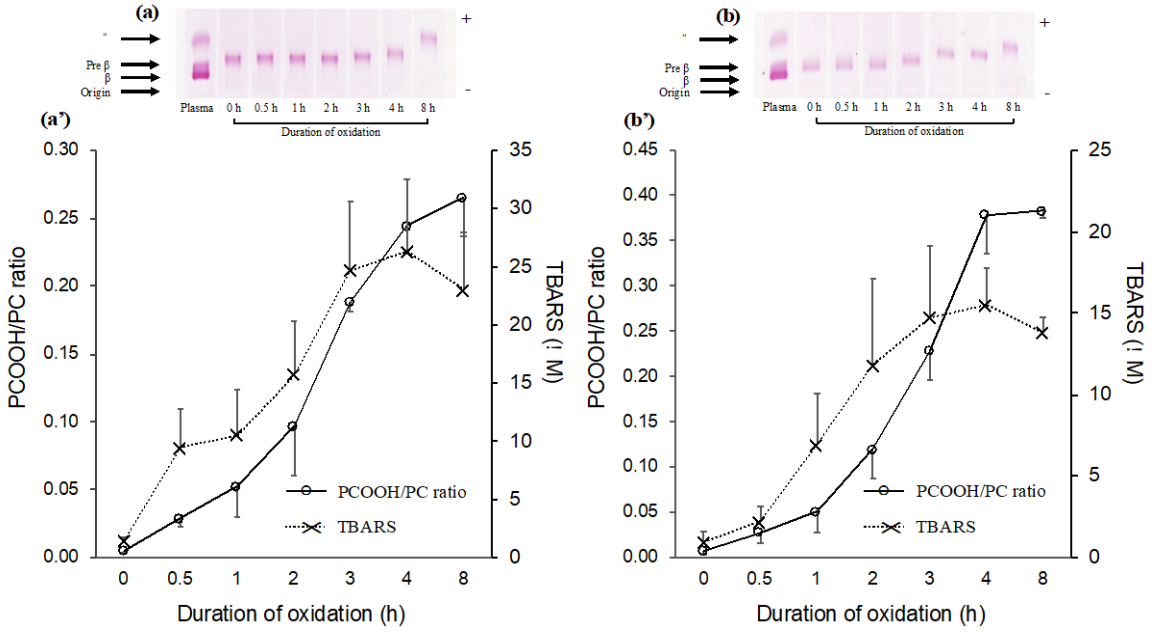

Figure 1. Agarose gel electrophoresis of copper-oxidized VLDL (a) and IDL (b). Lipoproteins (3 μg lipid) was electrophoresed in 50 mmol barbital buffer (pH 8.6) for 30 min at 100 V followed by staining with fat red 7B. Time course of generation of phosphatidylcholine hydroperoxides (PCOOH) in term of its ratio to phosphatidylcholine (PC) and TBARS during oxidation of VLDL (a') and IDL (b').

Discussion

This is the first study to identify molecular species of PCOOH in TRL in its native form. The use of a high-sensitivity and high-resolution Orbitrap mass spectrometer, coupled with a reversed-phase liquid chromatograph and synthetic PCOOH standards, enabled us to perform a high-quality detection and identification of PCOOH molecular species in TRL. PC34:2-OOH, PC36:2-OOH, PC36:4-OOH, PC38:4-OOH, PC38:6-OOH, and PC40:6-OOH were frequently identified in native TRL. The relative contents of PCOOH significantly increased with the increase in the degree of artificial oxidation (Figure 1). The time course of PCOOH generation in TRL is similar to that of LDL and HDL published previously.[5] As like oxidized LDL, the majority of PCOOH species show lag phase up to 1 h followed by rapid propagation phase (Supplementary Figure 3).

Although the generation mechanism of PCOOH in TRL is not determined, the oxidation of phosphatidylcholine in TRL might occur due to the interaction with ROS in the circulation as phosphatidylcholine resides in the outer layer of the lipoproteins, and are susceptible to oxidative damage. Moreover, we have identified that both VLDL and IDL contain hydroperoxides of triglycerides and choletserylester.[3,4] These FA are liberated from the TRL by the action of lipoprotein lipase during its metabolism. It is known that the metabolism of TRL is accompanied by the release of a significant amount of potentially toxic oxidized FA.[6] Therefore, the capillaries endothelial cells are constantly exposed to hydroperoxides as TRL deliver triacylglycerol to tissues including cardiac muscle. Perhaps this phenomenon may result in endothelial dysfunction and inflammation which are the crucial features in early atherogenesis. Furthermore, various molecular species of oxidized phospholipid molecules have been detected in both oxLDL and human atherosclerotic

plaque. Therefore, it is of significance to measure PCOOH in TRL for the better understanding of the pathogenicity of these lipoproteins.

The present study revealed the molecular species of PCOOH, which in turn become the target of future quantitative study. Our study was primarily limited to identify PCOOH in the native lipoproteins. Further works are needed to find the association of PCOOH in the atherosclerotic process. Nevertheless, our demonstration of the existence of PCOOH would provide a new insight to understand the pathogenicity of TRL.

Conclusion

This study demonstrated the presence of 12 molecular species of PCOOH in native TRL. The existence of PCOOH in TRL provides a molecular basis of for the atherogenicity of TRL, and its measurement might contribute to early prediction and risk assessment of CHD.

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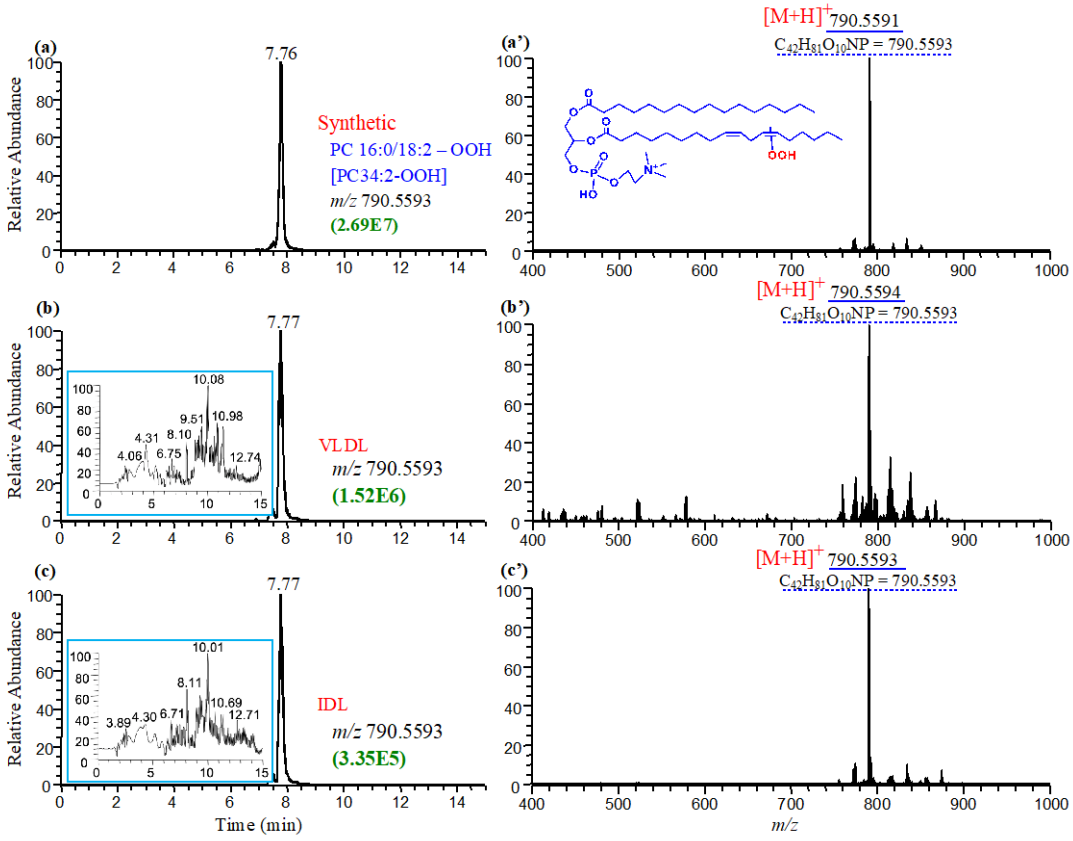
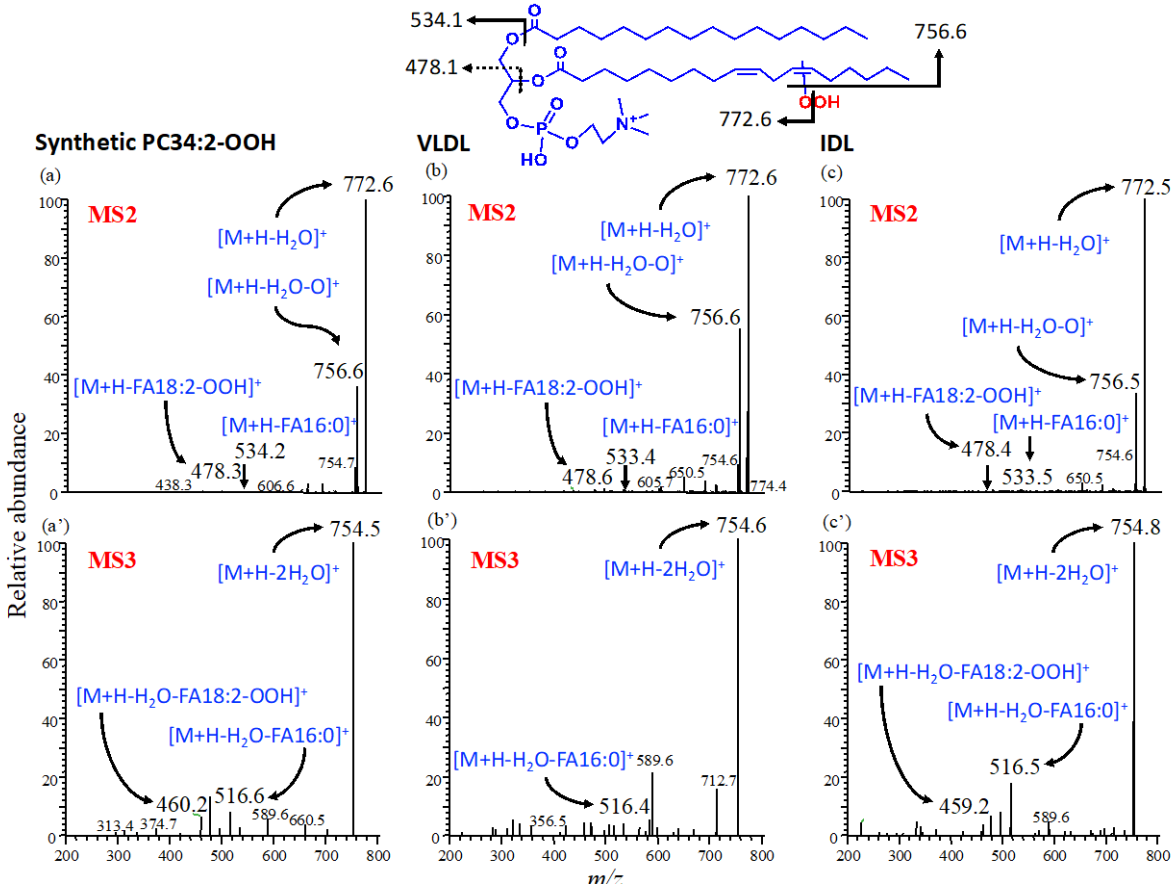
Supplementary Materials:



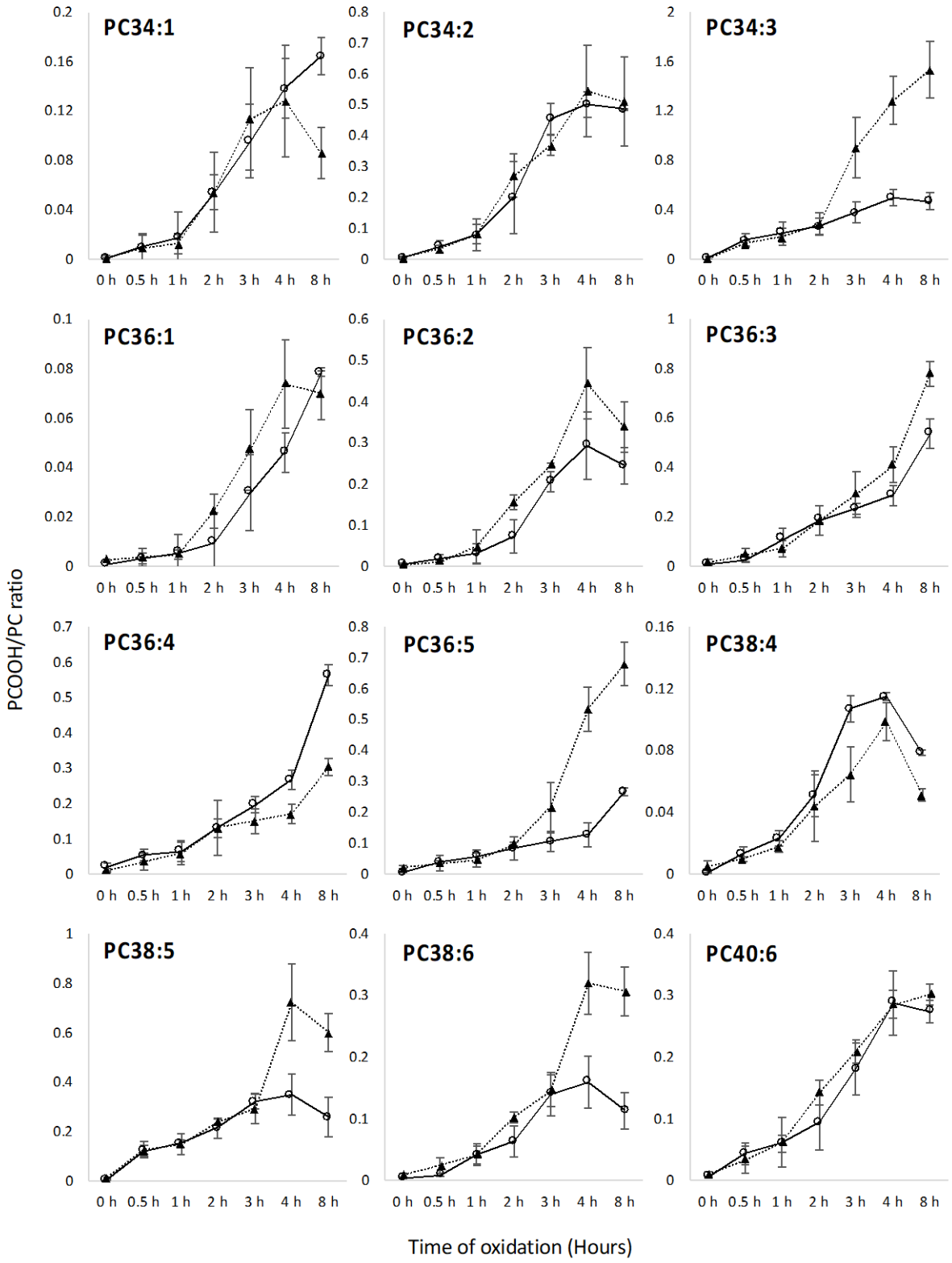
Supplementary Figure 1. LC/LTQ Orbitrap profiles of PC34:2-OOH in positive-ion mode: (a) extracted ion (*m/z* 790.5593) chromatogram of synthetic PC34:2-OOH (40 pmol); (a') mass spectrum of the peak at RT 7.76 min in (a); (b) total ion chromatogram (inset) and extracted ion (*m/z* 790.5593) chromatogram of VLDL; (b') mass spectrum of the peak at RT 7.77 min in (b); (c) total ion chromatogram (inset) and extracted ion (*m/z* 790.5593) chromatogram of IDL; (c') mass spectrum of the peak at RT 7.77 min in (c).

The synthetic PC16:0/18:2-OOH (PC34:2-OOH) eluted at 7.76 min and its corresponding mass spectrum contained the $[M+H]^+$ at *m/z* 790.5591 (elemental composition $C_{42}H_{81}O_{10}NP$, theoretical mass 790.5593). The EIC for VLDL and IDL showed a single peak at RT of 7.77. The base peak observed at *m/z* 790.5594 in (b') and *m/z* 790.5593 in (c') were corresponding to the $[M+H]^+$ with the same elemental composition and exact mass as the corresponding ion for the synthetic standard. Thus, the EIC peaks of (b) and (c) were identified as PC34:2-OOH.



Supplementary Figure 2. MS2, MS3 spectra and structure with fragmentation pattern of PC34:2-OOH at *m/z* 790.5593 in synthetic standard, VLDL, and IDL

The MS2 and MS3 spectra of the standard shows a base peak at *m/z* 772.6 and *m/z* 754.5 corresponding to $[M+H-H_2O]^+$ and $[M+H-2H_2O]^+$, respectively. Ion peaks corresponding to $[M+H-H_2O-O]^+$, $[M+H-FA16:0]^+$, $[M+H-FA18:2-OOH]^+$ were also detected. Identical fragmentation patterns in MS2 and MS3 spectra were observed for the ion at *m/z* 790.5593 detected in the VLDL and IDL.



Supplementary Figure 3. Change in the ratio of PCOOH to corresponding PC during oxidation of VLDL (○, solid line) and IDL (▲, dashed line).

Supplementary Table 1. Mass spectrometric properties of PC and PCOOH in VLDL and IDL obtained by LC/LTQ Orbitrap in positive-ion mode

PC/PCOOH Molecular species	Element composition	Theoretical [M+H]+	VLDL				IDL			
			Retention time	Experimental [M+H]+	Mass accuracy	Product ions	Retention time	Experimental [M+H]+	Mass accuracy	Product ions
		(m/z)	(Min)	*(m/z)*	(ppm)	*(m/z)*	(Min)	*(m/z)*	(ppm)	*(m/z)*
PC34:1	$C_{42}H_{82}O_8NP$	760.5851	9.82	760.5865	1.84	504.6, 496.5, 478.5, 576.9	9.85	760.5869	2.39	504.4, 496.5, 478.6, 522.5
PC34:2	$C_{42}H_{80}O_8NP$	758.5694	9.32	758.5701	0.93	502.6, 496.5, 478.6	9.40	758.5705	1.48	502.5, 496.5, 478.5
PC34:3	$C_{42}H_{78}O_8NP$	756.5538	9.04	756.5539	0.07	500.6, 496.5, 518.4, 573.6	9.07	756.5543	0.62	500.5, 496.4, 479.5, 573.6
PC36:1	$C_{44}H_{86}O_8NP$	788.6164	10.69	788.6178	1.79	504.4, 524.5, 604.9	10.69	788.6182	2.34	503.6, 523.5, 604.4
PC36:2	$C_{44}H_{84}O_8NP$	786.6007	10.07	786.6015	1.00	524.4, 502.6, 506.7, 726.4	10.07	786.6019	1.55	502.5, 726.7
PC36:3	$C_{44}H_{82}O_8NP$	784.5851	9.53	784.5887	4.63	524.4, 601.5	9.59	784.5850	-0.11	524.5, 601.9
PC36:4	$C_{44}H_{80}O_8NP$	782.5694	9.20	782.5713	2.42	722.6, 526.5, 496.3, 478.5	9.26	782.5717	2.97	722.6, 496.4, 526.5, 598.7
PC36:5	$C_{44}H_{78}O_8NP$	780.5538	8.94	780.5575	4.74	524.5, 721.6	8.97	780.5538	0.02	524.3, 597.5, 721.7
PC38:4	$C_{46}H_{84}O_8NP$	810.6007	9.98	810.6033	3.23	751.6, 627.7, 524.4, 506.5	10.01	810.6038	3.78	751.6, 627.6, 524.5, 506.6
PC38:5	$C_{46}H_{82}O_8NP$	808.5851	9.58	808.5890	4.81	542.6, 624.9	9.59	808.5851	-0.01	542.5, 625.2
PC38:6	$C_{46}H_{80}O_8NP$	806.5694	9.15	806.5692	-0.27	478.6, 568.5, 747.7, 623.7	9.20	806.5696	0.28	623.6, 496.3, 478.5
PC40:6	$C_{48}H_{84}O_8NP$	834.6007	9.87	834.6039	3.87	506.5, 524.1, 568.6	9.92	834.6044	4.42	524.3, 506.4, 569.1, 775.6
PC34:1-OOH	$C_{42}H_{83}O_{10}NP$	792.5749	7.95	792.5769	2.46	773.6, 757.6, 478.4	7.96	792.5773	3.01	773.6
PC34:2-OOH	$C_{42}H_{81}O_{10}NP$	790.5593	7.77	790.5621	3.57	772.6 , 756.6	7.77	790.5626	4.13	772.5, 756.5
PC34:3-OOH	$C_{42}H_{79}O_{10}NP$	788.5436	7.58	788.5467	3.94	770.5, 754.6, 532.4, 478.7	7.62	788.5471	4.49	770.6, 754.7, 532.4, 478.4
PC36:1-OOH	$C_{44}H_{87}O_{10}NP$	820.6062	8.42	820.6067	0.62	802.8	8.42	820.6072	1.17	802.7
PC36:2-OOH	$C_{44}H_{85}O_{10}NP$	818.5906	8.18	818.5904	-0.26	800.7, 784.6	8.19	818.5908	0.29	800.6, 784.6
PC36:3-OOH	$C_{44}H_{83}O_{10}NP$	816.5749	7.93	816.5771	2.67	798.6, 782.6		ND	-	
PC36:4-OOH	$C_{44}H_{81}O_{10}NP$	814.5593	7.86	814.5624	3.82	796.6, 795.7, 558.7, 478.5	7.88	814.5629	4.37	796.5, 780.5
PC36:5-OOH	$C_{44}H_{79}O_{10}NP$	812.5436	7.69	812.5464	3.49	794.6	7.69	812.5469	4.05	-
PC38:4-OOH	$C_{46}H_{85}O_{10}NP$	842.5906	8.29	842.5934	3.37	824.6, 808.7, 506.5	8.30	842.5939	3.92	824.7, 808.8, 506.6
PC38:5-OOH	$C_{46}H_{83}O_{10}NP$	840.5749	7.95	840.5784	4.21	822.6	7.96	840.5789	4.76	822.7
PC38:6-OOH	$C_{46}H_{81}O_{10}NP$	838.5593	7.86	838.5615	2.60	820.6, 804.6, 478.3		ND	-	
PC40:6-OOH	$C_{48}H_{85}O_{10}NP$	866.5906	8.26	866.5918	1.39	849.0		ND	-	

ND: Not detected.

Mass tolerance within ± 5.0 ppm.

Supplementary Table 2. Distribution of PC-OOH species in VLDL, IDL and Plasma

PCOOH Species	VLDL (n=6)	IDL (n=6)	Plasma (n=6)
PC34:1-OOH	4	5	5
PC34:2-OOH	5	5	6
PC34:3-OOH	2	3	1
PC36:1-OOH	2	3	4
PC36:2-OOH	5	5	6
PC36:3-OOH	4	ND	5
PC36:4-OOH	6	5	6
PC36:5-OOH	3	1	ND
PC38:4-OOH	5	5	4
PC38:5-OOH	3	2	5
PC38:6-OOH	6	5	2
PC40:6-OOH	4	2	ND

ND: Not detected