CHOLESTERYL ESTER ACYL OXIDATION AND REMODELING IN MURINE MACROPHAGES: FORMATION OF OXIDIZED PHOSPHATIDYLCHOLINE

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SUPPLEMENTAL MATERIAL

Experimental Procedures

Materials- [1-^{14}C]-linoleate was purchased from Perkin Elmer (Waltham, MA). [1-^{14}C]-13-HpODE was synthesized by incubating [1-^{14}C]-linoleate with soybean 15-lipoxygenase (250 pmols) (Cayman Chemcical) in 100 μL borate buffer (0.1 M, pH 9) at ambient temperature for ~1 h. Reactions were terminated with 1 volume ice cold MeOH, and the product ([1-^{14}C]-13-HpODE) was extracted twice using 500 μL 75:25 isooctane:ethyl acetate (v/v).

Ex-vivo Macrophage Incubations- Peritoneal macrophages were collected as described in the main body of the text. To the adherent cells of HEPES buffered HBSS (0.5 mL) containing fatty acid-free bovine serum albumin (0.5% w/w) was added. To the medium [1-^{14}C]-13-HpODE or [1-^{14}C]-linoleate (1.5 μM; 0.05 μCi) was added in and adhered cells were incubated for 20 h. After incubation the medium was removed, the cells were washed with 500 μL of medium, and adherent cells were then scraped twice with 500 μL of 90% MeOH in water containing $SnCl_2$ (2mM) before lipid were extracted and separated by TLC.

Chromatography- The same chiral chromatography used for intact CEs was also employed for the separation of 12-HETE and 15-HETE methyl esters. Free HETEs and HODEs were separated using RP-HPLC-MS/MS (RP-HPLC-MS/MS) as previously reported (29).

TLC separations were carried out on pre-washed and activated Gel G TLC plates (Alltech, Deerfield, IL), which were developed in two steps. The first mobile phase, 50:25:8:4 (v/v/v/v) DCM:MeOH:acetic aicd:H_2O, was allowed to migrate approximately 5 cm from the origin, the plate was then dried briefly before the second mobile phase, 80:20:1 (v/v/v) hexane:ethyl acetate:acetic acid, was allowed to migrate the entire length of the plate. Spots were visualized by spraying with phosphomolybdic acid (20 weight % in EtOH) (Sigma Aldrich, Milwaukee, WI), followed by development on a hot plate. Spots were identified by comparing their R_f values with those of standards. Radioactive lipids were detected using a System 200 TLC image scanner (Bioscan, Wasgington DC).

Saponification and Methylation- CE(HETE) was isolated by NP-HPLC-MS/MS and saponified exactly as previously described (9). Free HETE was methylated by reaction with diazomethane in diethyl ether for 5 min at room temperature. The solution was then dried completely, resuspended in initial HPLC conditions (35:65 (v/v) methyl *tert*-butyl ether:isooctane) for chiral analysis by HPLC-MS/MS

Mass Spectrometry- Online RP-LCMS/MS of fatty acids, eicosanoids and other oxylipins was performed using negative ion MRM as previously reported (29), however the current analyses were carried out using the AB Sciex 5500 instrument. Also, additional MRM transitions were included to analyze the numerous HETE and HODE isomers; these mass transitions were based on previously published eicosanoid ESI-MS/MS studies (31). Some experiments included a continuous enhanced product ion scan (EPI scan) of m/z 319.3 to obtain online product ion spectra of unknown HETE analytes.

Results

Characterization of CE(12(S)-HETE)- The most abundant oxCE following macrophage incubation with human LP was a single CE(HETE) isomer, likely CE(12-HETE) or CE(15-HETE), based on its NP-LCMS/MS retention time (Figure 2A). To further characterize this CE(HETE) the peak was isolated by NP-HPLC-MS/MS. An aliquot of this material was re-chromatographed on a chiral column and it eluted as a single enantiomer indicting enzymatic oxygenation (Figure 2A inset *i*). The remainder of the isolated CE(HETE) was saponified and an aliquot of the free acid was subjected to RP-HPLC-MS/MS. The unknown HETE produced a peak corresponding to the MRM transition (*m/z* 319 to 179) and retention time of authentic 12-HETE, and did not produce any response for the MRM transition for 15-HETE (*m/z* 319 to 219)(Supplemental Figure 3A,B). When the unknown HETE was subjected to CID the resulting product ion spectra exactly match that of authentic 12-HETE (Supplemental Figure 3C,D). By methylating the remaining hydrolyzed material

and co-injecting the methyl ester onto a chiral column with authentic d_8-12(*S*)-HETE methyl ester the absolute stereochemistry was confirmed *S* (Supplemental Figure 3E). Analysis of authentic (±)12-HETE methyl ester ensured adequate separation and confirmed that the slight retention time shift between the deuterated material and the unlabeled 12-HETE methyl ester was due to isotopic effects and not structural differences (Supplemental Figure 3F).

Incorporation of [1-^{14}C]-linoleate and [1-^{14}C]-13-HpODE by Murine Peritoneal Macrophages- To investigate reacylation of CE-derived fatty acyl components, specifically linoleate and it oxidation products, 1[^{14}C]-linoleate and 1[^{14}C]-13-HpODE was incubated with macrophages for 20 h. Following incubation adherent cells were isolated, scintillation counting revealed the radioactivity in the adherent cells following 1[^{14}C]-linoleate incubation was approximately five times the amount in cells following 1[^{14}C]-13-HpODE incubation. Total lipids were extracted from the remainder of the cells and separated by TLC. Radiolabeled cellular lipids, determined by radioactive TLC image scanner, were virtually identical between the two substrates (Supplemental Figure 5). In both cases, essentially all the radioactivity was incorporated into PLs, and showed no incorporation into neutral lipids. The vast majority of the radiolabeled PL migrated with a PC standard.

Supplemental Figure Legends

Supplemental Figure 1. Loss of regio- and stereospecificity among the CE(HODE) isomer distribution of murine peritoneal macrophages over time during incubation with either CE(d_4-18:2)-containing synthetic lipid vesicles (A) or human lipoproteins (B). The second and forth peaks in the series, labeled with asterisks, are the all-trans forms, CE(13-EE-HODE) and CE(9-EE-HODE) respectively. Insets show chiral separations after CE(13-HODE) or CE(d_4-13-HODE) isomers were collected and re-chromatographed.

Supplemental Figure 2. Representative NP-HPLC-MS/MS chromatograms of CE(d_4-HODE) isomers after 20 h incubations of peritoneal macrophages with synthetic lipid vesicles containing CE(d_4-18:2) and various anti-oxidants at 3 mole percent (A). Insets show chiral analysis of isolated CE(d_4-13-(ZE)-HODE). (B) The bars indicate average peak area ratios of CE(d_4-13-(ZE)-HODE)/CE(d4-9-(EZ)-HODE) (filled bars) or CE(13(*S*)-(ZE)-HODE)/CE(13(*R*)-(ZE)-HODE) (open bars), error bars indicate mean±SEM (*n*=3). The dashed line indicates a ratio of one, i.e. complete loss of regio- or stereospecificity.

Supplemental Figure 3. Characterization of the unknown CE(HETE) isomer. RP-LCMS/MS analysis and product ion spectra of authentic 12-HETE and 15-HETE (A,B) or hydrolysis products of the unknown CE(HETE) (C,D). (C) Chiral separation of methylated CE(HETE) hydrolysis products co-injected with authentic d_8-12(*S*)-HETE methyl ester. (D) Chiral separation of authentic (±)12-HETE methyl ester co-injected with authentic d_8-12(*S*)-HETE methyl ester.

Supplemental Figure 4. Representative RP-HPLC-MS/MS MRM chromatograms of free d_4-13-HODE and 13-HODE (A) or d_4-9-HODE and 9-HODE (B) following 20 h incubations of peritoneal macrophages with synthetic lipid vesicles containing CE(d4-18:2) (*n*=3). Lipids were extracted

from cells and incubation medium before analysis. Certain aliquots were co-injected with authentic 9-hydroxy-9,10,12,13-d_4-HODE and 13-hydroxy-9,10,12,13-d_4-HODE which could be independently detected for the non-deuterated HODEs and the 17,17,18,18-d_4-HODE isomers derived from CE(d_4-18:2). These experiments confirmed the identifications of the peaks, especially important in the 9-HODE analyses where a later eluting compound formed spurious peak (at 15.5 min) in the chromatogram.

Supplemental Figure 5. Thin layer chromatography of cellular lipids extracted from peritoneal macrophages following 20 h incubation with [1-^{14}C]-18:2 or [1-^{14}C]-13-HpODE as detected by radioactive TLC image scanner. The dashed lines show the migration of radioactive components. The height of the line indicates the relative intensity of radioactivity at that distance from the origin, the traces where independently scaled. Arrows above the image indicate the migration of authentic lipid standards. PC: phosphocholine, PS: phosphoserine, PE: phosphoethanolamine, CL: cardiolipin, Chol: cholesterol, HpODE: 13-HpODE, 18:2: linoleate, TAG: triacylglyceride, CE: Cholesterol ester.

Supplemental Table 1. Phospholipid multiple reaction monitoring mass transitions.

		Q1 *m/z* [b]					Q3 *m/z* [d]	
		PC [c]	PE [c]	PG [c]	PI [c]	PS [c]	-	-
Acyl Components[a]		[M+OAc]$^-$	[M-H]$^-$	[M-H]$^-$	[M-H]$^-$	[M-H]$^-$	[M-H]$^-$	[M-H]$^-$
16:0	16:0	793	691	722	810	735	255	-
16:0	18:0/d418:2	821	719	750	838	763	255	283
16:0	18:1	819	717	748	836	761	255	281
16:0	18:2	817	715	746	834	759	255	279
16:0	20:4	841	739	770	858	783	255	303
18:0/d418:2	18:0/d418:2	849	747	778	866	791	283	-
18:0/d418:2	18:1	847	745	776	864	789	283	281
18:0/d418:2	18:2	845	743	774	862	787	283	279
18:0/d418:2	20:4	869	767	798	886	811	283	303
18:1	18:1	845	743	774	862	787	281	-
18:1	18:2	843	741	772	860	785	281	279
18:1	20:4	867	765	796	884	809	303	303
18:2	18:2	841	739	770	858	783	279	-
18:2	20:4	865	763	794	882	807	279	303
20:4	20:4	889	787	818	906	831	303	-
16:0	HODE	833	731	762	850	775	255	295
18:0/d418:2	HODE	861	759	790	878	803	283	295
18:1	HODE	859	757	788	876	801	281	295
18:2	HODE	857	755	786	874	799	279	295
20:4	HODE	881	779	810	898	823	303	295
16:0	oxoODE	831	729	760	848	773	255	293
18:0/d418:2	oxoODE	859	757	788	876	801	283	293
18:1	oxoODE	857	755	786	874	799	281	293
18:2	oxoODE	855	753	784	872	797	279	293
20:4	oxoODE	879	777	808	896	821	303	293
16:0	di-oxy-LA	849	747	778	866	791	255	311
18:0/d418:2	di-oxy-LA	877	775	806	894	819	283	311
18:1	di-oxy-LA	875	773	804	892	817	281	311
18:2	di-oxy-LA	873	771	802	890	815	279	311
20:4	di-oxy-LA	897	795	826	914	839	303	311
16:0	d4HODE	837	735	766	854	779	255	299
18:0/d418:2	d4HODE	865	763	794	882	807	283	299
18:1	d4HODE	863	761	792	880	805	281	299
18:2	d4HODE	861	759	790	878	803	279	299
20:4	d4HODE	885	783	814	902	827	303	299
16:0	d4oxoODE	835	733	764	852	777	255	297
18:0/d418:2	d4oxoODE	863	761	792	880	805	283	297
18:1	d4oxoODE	861	759	790	878	803	281	297
18:2	d4oxoODE	859	757	788	876	801	279	297
20:4	d4oxoODE	883	781	812	900	825	303	297
16:0	d4di-oxy-LA	853	751	782	870	795	255	315
18:0/d418:2	d4di-oxy-LA	881	779	810	898	823	283	315
18:1	d4di-oxy-LA	879	777	808	896	821	281	315
18:2	d4di-oxy-LA	877	775	806	894	819	279	315
20:4	d4di-oxy-LA	901	799	830	918	843	303	315

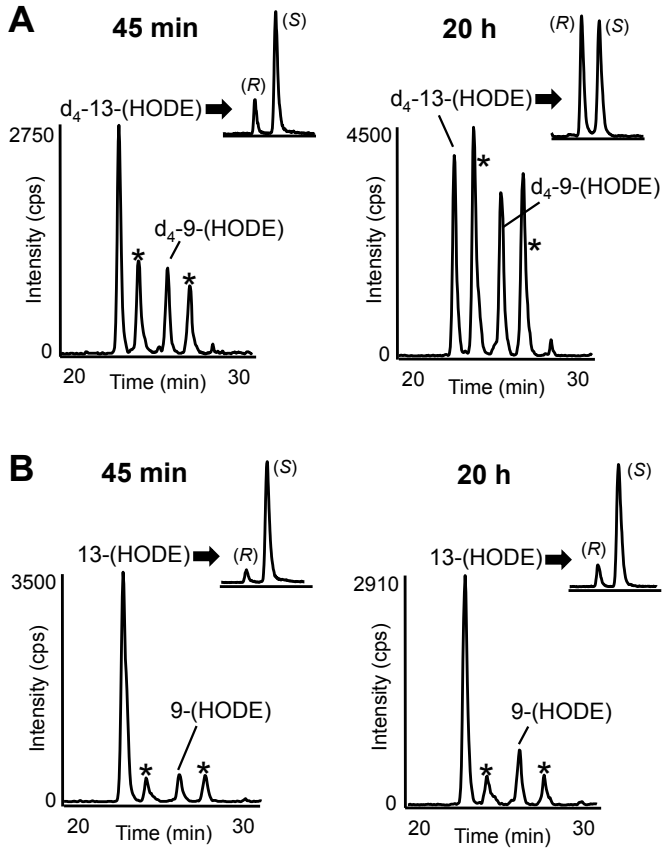
[a] Abbreviated names of esterified fatty acids; total acyl chain carbons:number of double bonds, "d4" denotes acyl chain containing 4 deuterium atoms, LA: linoleic acid

[b] Nominal m/z of parent ions; ion species are indicated in brackets

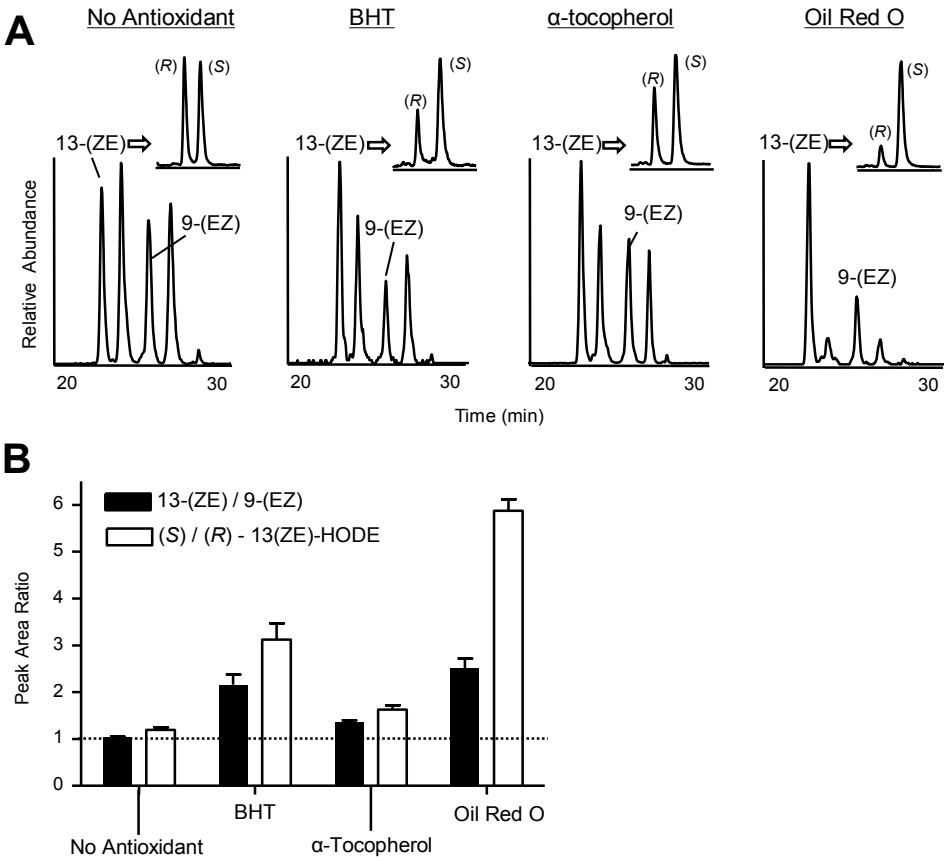
[c] PC: phosphocholine, PE: phosphoethanolamine, PG: phosphoglycerol, PI: phophoinositol, PS: phosphoserine

[d] Nominal m/z of predicted acyl carboxylate product ions; dashed indicate degenerate transitions

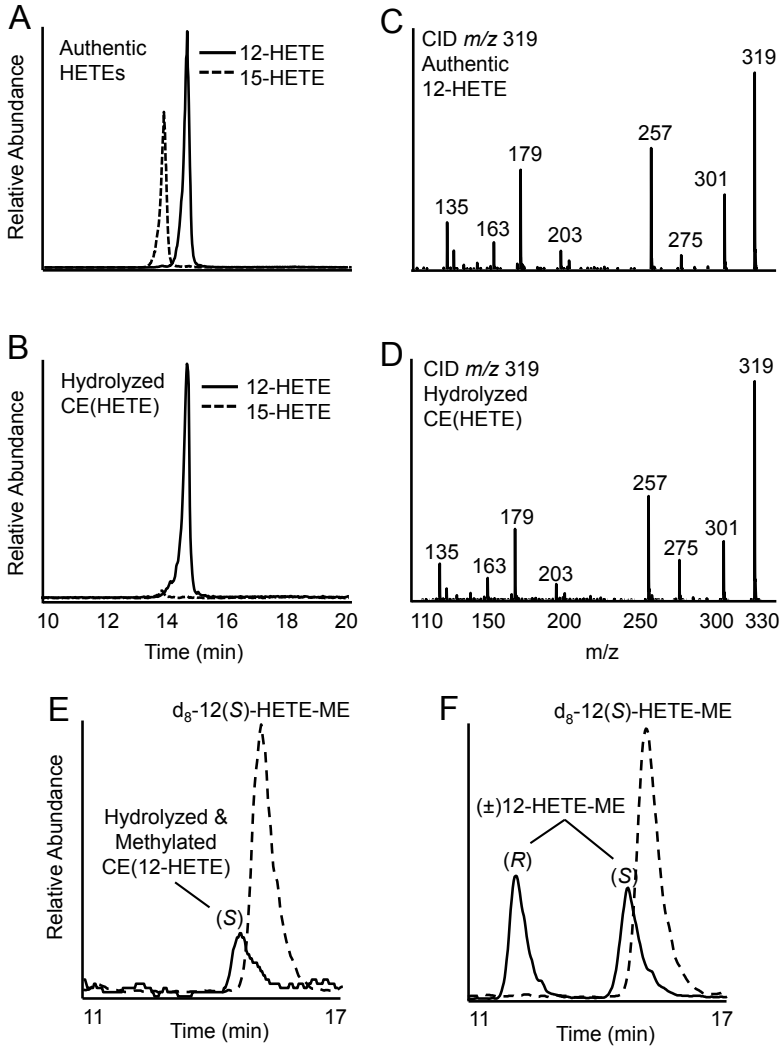
Supplemental Figure 1



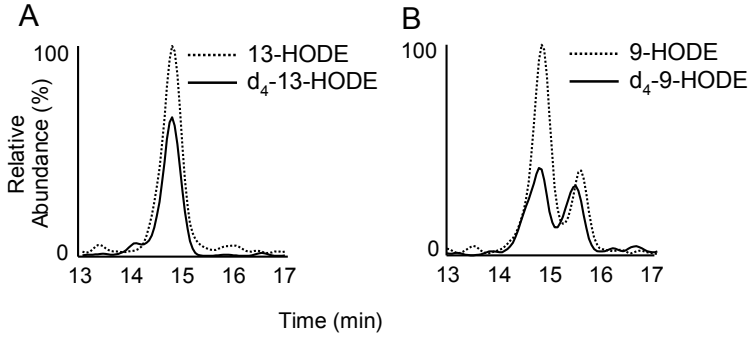
Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5