Covalent binding of hydroxy-alkenals (4-HDDE, 4-HHE and 4-HNE) to ethanolamine phospholipid subclasses

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Running title: Aldehyde reactivity with ethanolamine phospholipids

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Abbreviations:

PE, ethanolamine phospholipids; diacyl-GPE, diacyl-glycerophosphoethanolamine; alkenylacyl-GPE or plasmalogen PE, alkenylacyl-glycerophosphoethanolamine; 20:4n-6 or AA, arachidonic acid; 22:6n-3, docosahexaenoic acid; GPx, glutathione peroxidase; ROS, reactive oxygen species; 4-HNE, 4-hydroxynonenal; 15-HpETE, 15-hydroperoxyeicosatetraenoic acid; 12-HpETE, 12-hydroperoxyeicosatetraenoic acid; 13-HpODE, 13-hydroperoxyoctadecadienoic acid; 4-HDDE, 4-hydroxydodecadienal; 4-HHE, 4-hydroxyhexenal; DIBAL, diisobutylaluminium hydride; ACD, citric acid-trisodium citrate-dextrose; PRP, platelet-rich plasma; PUFAs, polyunsaturated fatty acids; TLC, thin-layer chromatography; RP-HPLC, reverse phase high pressure liquid chromatography; DMA, dimethylacetals; FAMEs, fatty acid methyl esters; (18:0/20:4-GPE), 1-stearoyl,2-arachidonoyl-GPE; (18:0/22:6-GPE), 1-stearoyl,2-docosahexaenoyl-GPE; (18:0p/20:4-GPE), 1-O-stearyl-1′-enyl,2-arachidonoyl-GPE; (18:0p/22:6-GPE) 1-O-stearyl-1′-enyl,2-docosahexaenoyl-GPE; TMS, trimethylsilyl; PFBHA, O-2,3,4,5,6-pentafluorobenzyl hydroxylamine hydrochloride; BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; PFB Br, 2,3,4,5,6-pentafluorobenzyl bromide; GC-MS, gas chromatography-mass spectrometry; GC, gas-chromatography; NMR, nuclear magnetic resonance; EI, electron ionization; NICI, negative ion chemical ionization; FID, flame ionization detector.
Abstract

Lipid oxidation is implicated in a wide range of pathophysiological disorders, which leads to reactive compounds such as fatty aldehydes, the most well-known being 4-hydroxynonenal (4-HNE) issued from 15-hydroperoxycicosatetraenoic acid (15-HpETE), an arachidonic acid product. In addition to 15-HpETE, 12(S)-HpETE is synthesized by 12-lipoxygenation of platelet arachidonic acid. We first show that 12-HpETE can be degraded *in vitro* into 4-hydroxydodecadienal (4-HDDE), a specific aldehyde homologous to 4-HNE. Moreover, 4-HDDE can be detected in human plasma. Secondly, we compared the ability of 4-HNE, 4-HDDE and 4-hydroxyhexenal (4-HHE) from n-3 fatty acids to covalently modify different ethanolamine phospholipids (PE) chosen for their biological relevance, namely arachidonoyl- (20:4n-6) or docosahexaenoyl- (22:6n-3) containing diacylglycerophosphoethanolamine (diacyl-GPE) and alkenylacyl-GPE (plasmalogen PE) molecular species. 4-HDDE, the most hydrophobic aldehyde used, generates more adducts with the PE subclasses than 4-HNE, which itself appears more reactive than 4-HHE. Moreover, the aldehydes show higher reactivity toward plasmalogen PE compared to diacyl-GPE, the docosahexaenoyl-containing species being more reactive than the arachidonoyl-containing ones. We conclude that the different PE species are differently targeted by fatty aldehydes, the higher their hydrophobicity, the higher the amount of adducts made. In addition to their antioxidant potential, plasmalogen PE may efficiently scavenge fatty aldehydes.
Ethanolamine phospholipids (PE) in biological membranes consist in two main subclasses: diacyl-glycerophosphoethanolamine (diacyl-GPE) and alkenylacyl-GPE (plasmalogen PE). The brain is particularly rich in plasmalogens, a unique class of glycerophospholipids exhibiting antioxidant properties due to the presence of a vinyl ether moiety at the \textit{sn-1} position of the glycerol backbone. The \textit{sn-2} position is mainly acylated by polyunsaturated fatty acids (PUFAs) such as arachidonic (AA) (20:4n-6) and docosahexaenoic (22:6n-3) acids. Plasmalogens represent 15-20\% of total phospholipids (1-3) and more than 50\% of PE (4). Cell antioxidant defenses such as glutathione peroxidases (GPx), superoxide dismutase or vitamins E, C... prevent and/or scavenge high reactive oxygen species (ROS) (5-8). Uncontrolled formation of ROS leads to oxidative stress, responsible for lipid peroxidation which generates lipid hydroperoxides. The latter compounds are normally reduced into monohydroxylated fatty acids by GPx (9), but oxidative stress conditions, in which GPx activities may be decreased such as in aging and diabetes (10,11), favor the accumulation of hydroperoxides. The breakdown of these compounds generates a variety of products such as aldehydes, among which 4-hydroxy-2E-nonenal (4-HNE) has been widely investigated. 4-HNE is produced from the most abundant n-6 PUFA-derived hydroperoxides, namely 15-hydroperoxy-arachidonic acid (15-HpETE) and 13-hydroperoxy-linoleic acid (13-HpODE) (12,13). Whereas the breakdown of 15-HpETE has been well described, that of 12-HpETE, the 12-lipoxygenase product of arachidonic acid, remains partially unknown. The putative 12-HpETE-derived aldehyde, namely 4-hydroxydodeca-(2E,6Z)-dienal (4-HDDE), was characterized in the present study and measured in human plasma. Also, 4-hydroxy-2E-hexenal (4-HHE) is described as a major degradation product of n-3 PUFA-peroxidation such as 22:6n-3 (14). 4-HNE is produced in many degenerative diseases, like atherosclerosis (15), diabetes (16), inflammation (17-19), and neurodegenerative diseases (20,21), for example Alzheimer (22-24) and Parkinson (25) diseases. It is highly reactive and binds covalently the
NH$_2$ group of lysine residues (26-29) to form Michael and Schiff base adducts. It may also react with thiol groups (30-32) and produce Michael adducts in this case. Subsequently, 4-HNE has been described to alter many proteins such as aldose reductase (33), glucose dehydrogenase (34), and low density lipoproteins (26-29). Covalent modifications with 4-HNE have also been described for nucleotides (35,36) and aminophospholipids (37). The main resulting compounds found with PE were Michael and Schiff base adducts, the latter being partially cyclized. Other covalent modifications may occur with carbonyl-containing molecules. As a matter of fact, PE can make adducts with glucose (38) and protein lysine residues with tyrosine-derived aldehydes (39).

The first part of the present study was to examine whether 12-HpETE could be degraded into its putative aldehyde 4-HDDE as from 15-HpETE to 4-HNE, and to provide evidence that such a compound is formed in vivo. The second part of the study was to compare the ability of 4-HHE, 4-HNE and 4-HDDE, which have different hydrophobicity, to covalently modify four molecular species of PE. These species were arachidonoyl- and docosahexaenoyl- containing PE in both diacyl and plasmalogen subclasses from rat brain, chosen because of their biological relevance.
EXPERIMENTAL PROCEDURES

Materials

All chemicals and reagents were analytical grade and purchased from Sigma-Fluka-Aldrich Chemical Co. (St. Quentin Fallavier, France). Analytical grade organic solvents and silica TLC plates were from Merck (Nogent/Marne, France). HPLC columns were from Waters (St Quentin en Yvelines, France). (3H)-AA was from N.E.N. Dupont de Nemours (Les Ulis, France).

Isolation and characterization of 4-HDDE

Chemical synthesis of 4-HDDE

HDDE was prepared from methyl 4-hydroxydodeca-(2E,6Z)-dienoate.

- Synthesis of methyl 4-hydroxydodeca-(2E,6Z)-dienoate

To a solution of methyl 4-chlorophenylsulfinylacetate (1.15 g, 4.94 mmol) and piperidine (0.6 ml, 6.0 mmol) in acetonitrile (10 ml) was added dropwise a solution of (4Z)-decenal (0.95 g, 6.2 mmol) in acetonitrile (2.5 ml). The mixture was stirred overnight at room temperature. The solvent was evaporated under reduced pressure and the residue was flash-chromatographed on silica gel with dichloromethane/ether (19:1, v/v) as eluent to afford pure methyl 4-hydroxydodeca-(2E,6Z)-dienoate (0.8 g, yield 71%).

- Synthesis of 4-HDDE

To a stirred solution of methyl 4-hydroxydodeca-(2E,6Z)-dienoate (202 mg, 0.89 mmol) in anhydrous dichloromethane (10 ml) at −90°C under nitrogen was added dropwise a solution of 1.8 ml of 1M diisobutylaluminium hydride (DIBAL) in anhydrous dichloromethane diluted with 10 ml of anhydrous dichloromethane. The temperature was allowed to rise to −75/-70 °C and the mixture was stirred for 1 h. A 1M hydrochloric acid aqueous solution (10 ml) was then added at −70°C and the mixture allowed to warm to room
temperature. The organic layer was separated and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure and the residue was flash-chromatographed on silica gel with pentane/ether (1:1, v/v) as eluent to afford pure 4-HDDE (77 mg, yield 44%).

*Nuclear Magnetic Resonance of 4-HDDE*

Nuclear magnetic resonance spectra were recorded in CDCl$_3$ with a Bruker AC 200 (200/50 MHz) spectrometer. Chemical shifts are given in ppm and are referenced to CHCl$_3$ resonances (7.26 and 77.0 ppm). Splitting pattern abbreviations are s, singlet; d, doublet; dd, doublet of doublet; ddd, doublet of doublet of doublet; t, triplet; m, multiplet.

*Platelet isolation*

Blood platelets were isolated from human volunteers (local blood bank) according to Lagarde et al. (40). Briefly, blood taken onto anticoagulant (ACD) was centrifuged at 150 g for 10 min. The supernatant platelet-rich plasma (PRP) was acidified to pH 6.4 with citric acid, centrifuged at 900 g for 10 min, and the pellets were resuspended into Tyrode HEPES buffer pH 7.35.

*Preparation of 12-HpETE*

Platelet suspension was preincubated under gentle stirring with 200 μM diamide, to lower reduced glutathione (41) and then slow down glutathione peroxidase, and with 200 μM acetylsalicylic acid as a cyclooxygenase inhibitor (42), for 5 min at 37°C. Platelets were then incubated with 300 μM AA labeled with 37 kBq of (5,6,8,9,11,12,14,15-$^3$H)-AA (7.77 TBq/mmol) for 5 min in the presence of oxygen. Platelet suspension was then acidified to pH 3 with HCl 3 M and treated three times with 3 volumes of diethyl ether to extract AA derivatives. The organic phase was evaporated to dryness under vacuum. The 12-HpETE formation was checked with an aliquot of the lipid extract separated by TLC using the solvent mixture hexane/diethyl ether/acetic acid (60:40:1, v/v). Labeled compounds were detected by a Berthold TLC linear analyser.
Preparation of the 12-HpETE-derived aldehyde

The dry lipid extract, which contained the radioactive 12-HpETE, was treated for 22 hours at room temperature with 5 ml of 0.1 M HCl containing 0.5 M ascorbate and 0.02 M FeSO₄ according to the procedure previously used by Lang et al. (43) to synthesize 4-hydroxynonenal from 15-HpETE. The incubate was treated with 45 ml of the mixture chloroform/ethanol (2:1, v/v). The organic phase was removed, dried under vacuum and the residue purified by TLC using the solvent mixture pentane/diethylether (50:50, v/v) as eluent. Standard 4-HDDE (30 µg), used as a reference, was spotted on the same plate, and the radioactive band corresponding to the standard was scraped off and the aldehyde was extracted three times with chloroform/ethanol (2:1, v/v).

Aldehyde derivatization and purification

The dry purified aldehyde or crude plasma was treated with 200 µl of O-2,3,4,5,6-pentafluorobenzyl hydroxylamine hydrochloride (PFBHA) (50 mM in 0.1 M PIPES buffer pH 6.5) for 30 min at room temperature, according to the procedure described by Van Kuij et al. (44). After acidification with 100 µl of 98 % H₂SO₄, pentafluorobenzyl oxime derivatives were extracted with 500 µl methanol and 2 ml hexane, and then purified by TLC using pentane/diethylether (70:30, v/v) as eluent. The radioactive band, corresponding to standard pentafluorobenzyl oxime derivative of 4-HDDE, was scraped off and extracted three times with chloroform/ethanol (2:1, v/v). The solvent was removed under nitrogen and the hydroxyl group was converted into trimethylsilyl ether after an overnight treatment with N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) at room temperature. The pentafluorobenzyl oxime, trimethylsilyl-4-HDDE derivative (O-PFB-TMS-4-HDDE) was then analyzed by gas chromatography - mass spectrometry (GC-MS).

Synthesis of 4-HNE and 4-HHE

These two aldehydes have been synthesized similarly to 4-HDDE.
Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS was carried out on a Hewlett Packard quadripole mass spectrometer interfaced with a Hewlett Packard gas chromatograph (Les Ullis-France). The gas chromatograph was equipped with a HP-1MS fused-silica capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness) (Hewlett Packard) which was held at 57 °C. The following oven temperature program was used: 2 min at 57 °C, then increased to 180 °C at 20 °C/min, followed by an increase to 280 °C at 4 °C/min. Samples were injected with a splitless injector with a head pressure of 7.9 psi. The interface, injector and ion source were kept at 280, 260 and 130 °C, respectively. Electron energy was set at 70 eV. Helium and methane were used as carrier and reagent gases, respectively. Mass spectra were acquired from 50 to 800 Da using both the electron ionization (EI) and the negative ion chemical ionization (NICI) modes. The electron multiplier voltage was usually set at 1400 V.

Aldehyde reactivity toward PE subclasses

Lipid extraction and separation

Rat brain lipids were extracted twice with a solvent mixture of chloroform/ethanol (2:1, v/v). Non phosphorus lipids and phospholipids were separated according to Juaneda et al. (45). Briefly, the lipid residue was dissolved in chloroform and the mixture was loaded onto a Silica-Sep-Pak cartridge (solid phase extraction) equilibrated with chloroform. Neutral lipids were washed through with chloroform and total phospholipids were subsequently eluted with methanol. The alcoholic fraction was taken to dryness by rotary evaporation and 2 ml of chloroform/ethanol (2:1, v/v) was added to each residue. Aliquots (500 µl) were taken for further TLC analysis.
TLC of phospholipids

The different phospholipid classes were then separated by TLC using the solvent mixture chloroform/methanol/aqueous methylamine solution (14%) (60:20:5, v/v) as eluent. PE was detected by spraying the silica gel plate with 0.2% dichlofluorescein in ethanol. Silica gel was scraped off and PE was extracted by a mixture of chloroform/ethanol (2:1, v/v).

RP-HPLC separation of molecular species of PE

PE subclasses were then fractionated by reverse phase HPLC (RP-HPLC) using an Agilent Technologies instrument model 1100 according to the procedure described by Khaselev et al. (46) and modified as follows. PE was loaded onto a 3.9 x 300 mm NovaPak column packed with C18 silica (particle size 4 µm). A flow rate of 1 ml/min was used and the detection was achieved by monitoring UV absorbance of the effluent at 205 nm. The mobile phase consisted of a linear gradient elution with two eluents A and B. Eluent A was a mixture of methanol/water/acetonitrile (100:14:2.5, v/v) containing 1 mM ammonium acetate adjusted to pH 7.4, and eluent B contained the same solvents as A but in different proportions (70:4:2.5, v/v), respectively. Eluent A was pumped from 0 to 10 min at 100%. Then eluent A was replaced by linearly increasing percentage of solvent B to reach 100% at 155 min. B was held for 15 min and then the system returned to the initial conditions within 5 min.

Only four fractions were collected and taken to dryness under nitrogen. They were 1-stearoyl,2-arachidonoyl-GPE (18:0/20:4-GPE), 1-stearoyl,2-docosahexaenoyl-GPE (18:0/22:6-GPE), 1-O-stearyl-"-enyl,2-arachidonoyl-GPE (18:0p/20:4-GPE), and 1-O-stearyl-"-enyl,2-docosahexaenoyl-GPE (18:0p/22:6-GPE). Their purity was checked by measuring their fatty acyl content by gas chromatography (GC).

Quantification of phospholipid fractions

Each fraction was treated separately with 500 µl of toluene/methanol (2:3, v/v) and 500 µl of 14% boron trifluoride in methanol. After 90 min at 100°C the tubes were cooled to
0°C and 1.5 ml K$_2$CO$_3$ in 10 % water was added. The resulting fatty acid methyl esters (FAMEs) from diacyl-GPE, and FAME and dimethylacetals (DMA) from plasmalogen PE were extracted by 2 ml of isoctane and analyzed by gas chromatography (GC) with a DELSI instrument model DI 200 equipped with a fused silica capillary SP-2380 column (60 m X 0.22 mm). Helium was used as the carrier gas at 1 ml/min. Temperatures of the Ross injector and the flame ionization detector (FID) were set at 230°C and 250°C, respectively. Diheptadecanoyl-GPE was used as internal standard and added to each fraction before derivatization.

Synthesis of PE/aldehydes adducts

One equivalent of each molecular species of PE collected by HPLC was incubated under nitrogen with two equivalents of either 4-HHE, 4-HNE or 4-HDDE in a biphasic system containing 200 µl buffer pH 8 (0.75 M NaCl and 1 mM HEPES) and 800 µl of diethylether. The aldehyde was replaced by ethanol in the control. The incubation was performed for 2 h at room temperature under continuous vigorous stirring. The resulting adducts and the unreacted PE were then extracted with a mixture of chloroform:ethanol (2:1, v/v) and separated by TLC using the solvent mixture chloroform/methanol/aqueous methylamine solution (14%) (60:20:5, v/v) as eluent. Michael and Schiff base adducts were extracted altogether from the silica, and were quantified by measuring their fatty acyl content by GC as previously described in “Experimental Procedures”.
RESULTS

Chemical synthesis and characterization of 4-HDDE

For this study, in which conventional mass spectrometry was used, racemic mixtures of 4-HDDE were elaborated in 2 steps from commercially available (4Z)-decenal 1 through the key intermediate ester 2, according to Scheme 1.

\[
\begin{align*}
1 & \quad \text{CHO} \\
& \quad \text{a)} \\
& \quad \text{b)} \\
2 & \quad \text{OH} \\
& \quad \text{CO}_2\text{CH}_3
\end{align*}
\]

a) 4-ClC\textsubscript{6}H\textsubscript{4}SOCH\textsubscript{2}CO\textsubscript{2}CH\textsubscript{3}, piperidine, CH\textsubscript{3}CN, r.t.

b) DIBAL, CH\textsubscript{2}Cl\textsubscript{2}, -90\degree C to -70\degree C.

Scheme 1

Reaction of 1 with methyl 4-chlorophenylsulfinylacetate (SPAC reaction (47,48)) afforded methyl 4-hydroxydodeca-(2\textit{E},6\textit{Z})-dienoate 2 (49) (yield: 71\%). 4-HDDE was easily obtained by reduction of ester 2 with DIBAL (50) (yield: 44\%).

NMR data and resonance attributions corresponding to 4-HDDE are as follows:

\(^1\text{H}-\text{NMR}: 9.59 (d, J = 7.8 \text{ Hz}, \text{H-1}); 6.85 (dd, J = 15.7, 4.3 \text{ Hz}, \text{H-3}); 6.35 (ddd, J = 15.7, 7.8, 1.4 \text{ Hz}, \text{H-2}); 5.73-5.60 (m, \text{H-6}); 5.45-5.31 (m, \text{H-7}); 4.60-4.40 (m, \text{H-4}); 2.43 (t, J = 7.0 \text{ Hz}, 2\text{H-5}); 2.08-2.00 (m, 2\text{H-8}); 1.45-1.20 (m, 2\text{H-9}, 2\text{H-10}, 2\text{H-11}); 0.89 (t, J = 7 \text{ Hz}, 3\text{H-12}).
$^{13}$C-NMR: 193.61 (C-1); 158.40 (C-3); 135.10 (C-7); 130.92 (C-6); 122.90 (C-2); 70.54 (C-4); 34.57 (C-5); 31.50 (C-10); 29.21 (C-9); 27.44 (C-8); 22.54 (C-11); 14.04 (C-12).

Chemically synthesized 4-HDDE was derivatized as previously described in “Experimental Procedures” and analyzed by GC-MS in the EI mode to confirm its chemical structure. Two syn and anti isomers were eluted at 18.19 and 18.86 min, respectively (Fig. 1A). Electron impact mass spectra of both syn and anti isomers show the same fragmentation pattern except for the relative abundance of the different ions; as an example the spectrum of the isomer eluted at 18.19 min is shown (Fig. 1B). The base peak at $m/z$ 352 corresponds to the loss of the alkyl chain ($C_8H_{15}$) from the molecular ion and two other important ions at $m/z$ 73 and 181 correspond to the TMS and PFB groups, respectively. Two minor characteristic ions were also detected: one at $m/z$ 156 corresponds to a loss of pentafluorobenzaldehyde from the fragment at $m/z$ 352 and the second at $m/z$ 129 derives from the $m/z$ 156 ion with a loss of cyanhydric acid.

In order to measure 4-HDDE with high sensitivity in biological samples, NICI was used and then performed with the chemical standard. The NICI mass spectra of both syn and anti isomers show the same fragmentation pattern except for the relative abundance of the different ions. The NICI spectrum of the isomer eluted at 18.19 min min is presented (Fig. 2A). Its spectrum shows characteristic ions at $m/z$ 373 (loss of TMSOH (ion (M-90)), $m/z$ 443 (loss of HF (M-20)), $m/z$ 353 (loss of both TMSOH and HF), $m/z$ 282 (loss of the PFB group (M-181)). The $m/z$ 196 ion refers to the pentafluorotropyloxy radical anion ($C_7F_5HO$). Ions at $m/z$ 343 as well as at $m/z$ 323 derive from the fragments 373 and 353, respectively, with a loss of formaldehyde.

Overall, $^1$H- and $^{13}$C-NMR, EI and NICI spectra confirm the expected 4-HDDE structures.
Aldehyde formation from 12-HpETE

Radioactive 12-HpETE was biosynthesized to study its conversion by iron and vitamin C treatment. About 13 % of AA was converted into 12-HpETE by platelets treated with aspirin and diamide as evaluated after TLC separation (not shown). We indeed observed that 12-HpETE-derived aldehyde was generated by treatment of 12-HpETE containing extracts with iron and vitamin C. The aldehyde was purified by TLC as described in “Experimental Procedure”. A radioactive band representing about 8 % of the initial 12-HpETE radioactivity migrated as the chemically synthesized 4-HDDE. The radioactive compound was extracted and its carbonyl group converted into O-PFB oxime before being separated by TLC again (see “Experimental Procedures”). The radioactive band corresponding to standard O-PFB-HDDE was extracted and derivatized with BSTFA as previously described to check its structure by GC-MS with the NICI mode.

Characterization and measurement of 4-HDDE in biological samples

Accounting for their relative volatility, aldehydes were first derivatized as O-PFB oxime before being extracted with hexane as previously described in “Experimental Procedures”. NICI mode was selected for its high sensitivity. Analyzing human plasma, two peaks were eluted at the same retention times as those observed for the derivatized standard 4-HDDE. The mass spectrum of the first isomer (Fig. 2B) indicates the same fragmentation pattern as that of standard 4-HDDE except for some contamination by minor unknown ions. Preliminary data indicate that 4-HDDE was much less abundant in human plasma from normal donors than 4-HNE (0.80 ± 0.06 ng/ml vs 529.7 ± 5.9 ng/ml, n=6, respectively), a value in the same order of magnitude as that of 4-HHE (0.43 ± 0.06 ng/ml, n=6).

RP-HPLC separation of brain PE molecular species

Figure 3 shows a representative HPLC profile of rat brain PE with a number of different molecular species. Among them four fractions were collected and identified by GC
as described in “Experimental Procedures”. Their purity estimated according to their fatty acyl content was found greater than 70 % (results not shown). They are: (18:0/20:4)-GPE, (18:0/22:6)-GPE, (18:0p/20:4)-GPE and (18:0p/22:6)-GPE.

Reactivities of 4-HHE, 4-HNE and 4-HDDE toward diacyl-GPE subclasses

The reactivity of 4-HHE, 4-HNE and 4-HDDE was determined by measuring their ability to covalently modify (18:0/20:4)- or (18:0/22:6)-GPE subclasses previously isolated by RP-HPLC. Michael and Schiff base adducts were measured altogether by GC as previously described. Results presented in Figure 4 show that the amount of (18:0/22:6)-GPE-4-HDDE adducts (measured as % of initial PE), was significantly higher than (18:0/22:6)-GPE-4-HNE adducts (30.4 ± 3.9 % vs. 22.2 ± 1.8 %, n=6), and than (18:0/22:6)-GPE-4-HHE adducts (14.8 ± 1.4 %, n=6) (Fig. 4A). Similar results were observed with (18:0/20:4)-GPE (Fig. 4B). (18:0/20:4)-GPE-4-HHE, (18:0/20:4)-GPE-4-HNE and (18:0/20:4)-GPE-4-HDDE adducts were 12.0 ± 1.8 %, 18.4 ± 2.1 % and 25.7 ± 6.6 %, n=4, of the initial PE, respectively. The aldehyde reactivity then seems dependent on their hydrophobicity, 4-HDDE being the most and 4-HHE the least hydrophobic aldehydes.

Reactivities of 4-HHE, 4-HNE and 4-HDDE toward plasmalogen PE subclasses

Similar investigations were performed with the corresponding plasmalogen PE subclasses (Fig. 5). Again 4-HHE, 4-HNE and 4-HDDE were incubated with (18:0p/22:6)-GPE (Fig. 5A) or (18:0p/20:4)-GPE (Fig. 5B). The amount of the resulting adducts (18:0p/22:6)-GPE-4-HHE, (18:0p/22:6)-GPE-4-HNE and (18:0p/22:6)-GPE-4-HDDE adducts represent 19.3 ± 1.8 %, 31.8 ± 2.7 % and 46.1 ± 4.9 % (n=6) of initial plasmalogen PE, respectively, and the amount of (18:0p/20:4)-GPE-4-HHE adducts, (18:0p/20:4)-GPE-4-HNE adducts and (18:0p/20:4)-GPE-4-HDDE adducts represent 15.3 ± 2.6 %, 21.2 ± 2.3 % and 29.4 ± 3.5 % (n=4), respectively. Again we observe that 4-HDDE, the most hydrophobic
aldehyde, was significantly more efficient to make covalent adducts with plasmalogen PE than 4-HNE and 4-HHE.

**Compared sensitivities of PE subclasses and molecular species toward aldehydes**

Values corresponding to experiments reported in Figures 4 and 5 were put together and compared according to the PE subclasses (plasmalogen PE vs diacyl-GPE) and to the nature of the fatty acid esterified at the sn 2 position. Table 1A indicates that, whatever the fatty acid esterified at the sn 2 position, aldehydes were more powerful to make adducts with the amino group of plasmalogen PE than with that of diacyl-GPE. The amount produced from the former was significantly higher than with the latter, whatever the aldehyde considered. The amino group of plasmalogen PE would then be more accessible than that of diacyl-GPE to make covalent adducts.

Also, the reactivity of different aldehydes toward PE subclasses shown in Figures 4 and 5 allows to evaluate the ability of these aldehydes to covalently modify PE according to the nature of the fatty acid esterified at the sn-2 position, disregarding the PE subclasses. The amount of the resulting adducts (Table 1B) was significantly (p < 0.02) more important with 22:6n-3-containing PE at the sn-2 position than with 20:4n-6-containing PE.
DISCUSSION

Lipid peroxidation leads to the formation of numerous fatty aldehydes, such as alkenals and 4-hydroxyalkenals (12,13). These aldehydes are obviously more stable than intermediate hydroperoxides and thus are likely to diffuse through cellular compartments. Among them, 4-HNE is one of the major products of membrane peroxidation, and exhibits many biological effects. It is formed by the breakdown of fatty acid hydroperoxides issued from the peroxidation of n-6 polyunsaturated fatty acids, including 15-HpETE from AA (51,52). However, 15-HpETE is not the only fatty acid hydroperoxide found in vivo. As a matter of fact, 12(S)-HpETE is substantially produced in cells expressing 12-lipoxygenase activity, especially blood platelets (53), in addition to racemic 12-HpETE generated by autoxidation of AA. Based on the 15-HpETE degradation, we undertook a series of experiments to determine the potential of 12-HpETE to form homologous aldehydes. For this purpose, the plausible compound 4-HDDE was successfully synthesized, and its chemical structure has been ascertained by NMR and GC-MS. Racemic 4-HDDE was prepared instead of pure enantiomers because our goal was to take into consideration 4-HDDE resulting from both autoxidation and 12-lipoxygenation of AA. This allowed a rapid two-step synthesis which is to be compared with the previously reported ten-step synthesis of enantiomerically pure 4-HDDE (54,55). In addition, the measurement of both enantiomers in the same GC-MS peak is considered as an advantage for the overall assessment of 4-HDDE issued from autoxidation and 12-lipoxygenation of AA.

Our results indicate that 12-HpETE prepared from human platelets, treated with a mixture of vitamin C and ferrous iron, is degraded into aldehyde with about the same yield (8%) as that observed for the degradation of 15-HpETE into 4-HNE in similar conditions. The structure of the aldehyde homologous to 4-HNE was determined by GC-MS after derivatization, and compared with that of derivatized standard 4-HDDE. NICI was used to
improve the detection sensitivity in biological samples. The mass spectra of both syn and anti isomers of the O-PFB derivatives issued from the 12-HpETE-derived aldehyde had the same GC retention time and the same NICI fragmentation pattern as those of chemically synthesized 4-HDDE. We may then conclude that 12-HpETE can be broken down by a chemical process which resembles that previously proposed by Pryor et al. (51) for the degradation of 15-HpETE into 4-HNE.

Interestingly, both 4-HNE and 4-HDDE were detected by GC-MS in human plasma, and preliminary results indicate that the amount of 4-HNE measured was about 500 fold higher than that of 4-HDDE. 4-HDDE may be produced in vivo from AA via 12-lipoxygenase, and/or reactive oxygen action. 12(S)-HpETE formation is enhanced when platelets are activated and when the glutathione peroxidase and/or glutathione reductase activities are depressed, which would eventually contribute to increased 4-HDDE production. In contrast, 4-HNE is assumed to be less specific as it derives from peroxidation of several n-6 polyunsaturated fatty acids, mainly AA and linoleic acid.

In the second part of the present study, we aimed to evaluate the reactivity of 4-HDDE with the primary amine of PE, as its less hydrophobic homolog 4-HNE has been shown to make covalent adducts with this phospholipid (37). Also, 4-HHE, issued from n-3 PUFA, was used as a less hydrophobic homolog. Different PE subclasses containing either 20:4n-6 or 22:6n-3 at the sn-2 position were investigated. The results in Figures 4A and 5A clearly show that 4-HDDE is significantly more reactive than 4-HNE, which is also significantly more reactive than 4-HHE, with 22:6n-3-containing PE subclasses. The same observation was done with the 20:4n-6-containing plasmalogen PE subclass (Figure 5B). The results with diacyl-GPE containing 20:4n-6 (Figure 4B) indicate that 4-HNE seems to make significantly more adducts than 4-HHE and that 4-HDDE tends to make more adducts than 4-HNE. Moreover, the comparison between diacyl-GPE and alkenylacyl-GPE toward the different aldehydes
indicates that ethanolamine plasmalogen fractions are more reactive than the diacyl ones. This can be due to a greater accessibility of the amino group as it has already been described by NMR and crystallography studies (56). Indeed, the reverse hexagonal phase \( H_{II} \) configuration and/or the conformation of plasmalogen PE enhance the ethanolamine exposure and facilitate its reaction with aldehydes. The higher reactivity of the plasmalogen PE might be biologically relevant because, in addition to their antioxidant properties linked to the presence of the vinyl ether moiety, plasmalogen PE may scavenge more efficiently aldehydes and then lower their toxicity.

Interestingly, the comparison between different PE molecular species containing either 20:4n-6 or 22:6n-3 esterified at the \( sn-2 \) position reveals that aldehydes make more adducts with 22:6n-3-containing PE (Table 1B). Indeed, with 6 cis double bounds, 22:6n-3 is likely to exhibit a stronger folding than 20:4n-6. Its conformation might then alter the location of the phospholipid in the membrane leaflet as it has been previously suggested for lipid bilayers (56), allowing a better exposure of the amino group to the aqueous phase. Our results obtained with a biphasic system are in agreement with a better access of the amino group to the aldehydes tested.

Preliminary results show that detectable amount of PE-derived covalent adducts could be found in platelets incubated with exogenous AA (not shown). If such covalent modifications of PE occur in vivo, that may alter the phospholipid membrane distribution and change its fluidity. Moreover, Michael PE-4-HNE adducts are poor substrate of various phospholipases (57). Such adducts issued from 4-HHE and/or 4-HDDE may modulate the phospholipase-dependent cell signaling as well.

In conclusion, 4-HDDE which is detectable in human plasma might be considered as a marker of 20:4n-6 oxidation by 12-lipoxygenase. The reactivity of the different fatty aldehydes to make adducts with PE is favored by their hydrophobicity and is more effective
with plasmalogen PE than with diacyl-GPE. This could be relevant to the control of the anti/prooxidant balance in the cell.
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Figure legends

Figure 1: Electron impact (EI) mass spectrum of chemically synthesized 4-hydroxy-
(2E,6Z)-dodecadienal (4-HDDE) derivatized as O-pentafluorobenzyl oxime,
trimethylsilyl ether. Analytical conditions were as described in "Experimental Procedures".
Figure 1A represents the total ion current chromatogram, and Figure 1B the EI mass spectrum
of the isomer eluted at 18.19 min.

Figure 2: Negative-Ion Chemical ionization (NICI) mass spectrum of 4-HDDE
derivatized as O-pentafluorobenzyl oxime, trimethylsilyl ether. Analytical conditions were
as described in "Experimental Procedures".
Figure 2A represents the NICI mass spectrum of the first isomer of standard 4-HDDE (eluted
at 18.19 min) and Figure 2B the mass spectrum of the same isomer of 4-HDDE originating
from human plasma (eluted at 18.14 min).

Figure 3: Typical HPLC profile of ethanolamine phospholipids (PE) extracted from rat
brain. Phospholipids were extracted from rat brain and separated into phospholipid classes.
PE isolated by TLC was further fractionated with a 3.9 x 300 mm NovaPak column packed
with C18 silica (particle size 4 µm). A flow rate of 1 ml/min was used and the detection was
achieved by monitoring UV absorbance of the effluent at 205 nm. The mobile phase consisted
of a gradient from eluent A (methanol/water/acetonitrile (100:14:2.5, v/v) containing 1 mM
ammonium acetate pH 7.4) to eluent B (same eluent in different proportions (70:4:2.5, v/v)).
(       ) mobile phase in function of time.
Figure 4: Covalent modifications of \((18:0/22:6)\)-GPE (A) and \((18:0/20:4)\)-GPE (B) with 4-HHE, 4-HNE and 4-HDDE. Diacyl-GPE isolated by HPLC from rat brain were incubated with the different aldehydes as stated in "Experimental Procedures". For each incubate, Michael and Schiff base adducts were extracted and quantified by GC. Values represent the mean ± SEM of six experiments ((18:0/22:6)-GPE) and of four ((18:0/20:4)-GPE).

\( a \) \((18:0/22:6)\)-GPE-4-HHE adducts vs. \((18:0/22:6)\)-GPE-4-HDDE adducts \( p \leq 0.002 \)

\( b \) \((18:0/22:6)\)-GPE-4-HNE adducts vs. \((18:0/22:6)\)-GPE-4-HHE adducts \( p \leq 0.001 \)

\( c \) \((18:0/22:6)\)-GPE-4-HDDE adducts vs. \((18:0/22:6)\)-GPE-4-HNE adducts \( p \leq 0.02 \)

\( e \) \((18:0/20:4)\)-GPE-4-HNE adducts vs. \((18:0/20:4)\)-GPE-4-HHE adducts \( p \leq 0.04 \)

Figure 5: Covalent modifications of \((18:0p/22:6)\)-GPE (A) and \((18:0p/20:4)\)-GPE (B) with 4-HHE, 4-HNE and 4-HDDE. Plasmalogen PE isolated by HPLC from rat brain were incubated with the different aldehydes as stated in "Experimental Procedures". For each incubate, Michael and Schiff base adducts were extracted and quantified by GC. Values represent the mean ± SEM of six experiments ((18:0p/22:6)-GPE) and of four ((18:0p/20:4)-GPE).

\( a \) \((18:0p/22:6)\)-GPE-4-HHE adducts vs. \((18:0p/22:6)\)-GPE-4-HDDE adducts \( p \leq 0.005 \)

\( b \) \((18:0p/22:6)\)-GPE-4-HNE adducts vs. \((18:0p/22:6)\)-GPE-4-HHE adducts \( p \leq 0.01 \)

\( c \) \((18:0p/22:6)\)-GPE-4-HDDE adducts vs. \((18:0p/22:6)\)-GPE-4-HNE adducts \( p \leq 0.01 \)

\( d \) \((18:0p/20:4)\)-GPE-4-HHE adducts vs. \((18:0p/20:4)\)-GPE-4-HDDE adducts \( p \leq 0.003 \)

\( e \) \((18:0p/20:4)\)-GPE-4-HNE adducts vs. \((18:0p/20:4)\)-GPE-4-HHE adducts \( p \leq 0.05 \)

\( f \) \((18:0p/20:4)\)-GPE-4-HDDE adducts vs. \((18:0p/20:4)\)-GPE-4-HNE adducts \( p \leq 0.02 \)
Table 1: Percentage of adducts formed.

<table>
<thead>
<tr>
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<th>% of PE aldehyde adducts</th>
<th>% of adducts</th>
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<tbody>
<tr>
<td>4-HHE</td>
<td>13.7 ± 1.0</td>
<td>17.7 ± 1.5</td>
</tr>
<tr>
<td>4-HNE</td>
<td>20.7 ± 1.3</td>
<td>27.5 ± 2.5</td>
</tr>
<tr>
<td>4-HDDE</td>
<td>28.5 ± 3.4</td>
<td>39.4 ± 4.2</td>
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Results are taken from Fig 4 and Fig 5.

**Table 1A:** % modification of ethanolamine phospholipid subclasses. Values represent the mean ± SEM of ten experiments and are compared as follows:

* diacyl-GPE-4-HHE adducts vs. plasmalogen PE-4-HHE adducts p ≤0.02

** diacyl-GPE-4-HNE adducts vs. plasmalogen PE-4-HNE adducts p ≤0.03

*** diacyl-GPE-4-HDDE adducts vs. plasmalogen PE-4-HDDE adducts p ≤0.04

**Table 1B:** Relevance of the fatty acid esterified at the sn 2 position of PE on the adduct formation, whatever the aldehyde considered.

Values represent the mean ± SEM of experiments reported in Figures 4 and 5; ‡ p ≤0.02
eluted at 18.19 min

Figure 1
Figure 2
Figure 4
Figure 5