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

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Abstract **Lipid oxidation is implicated in a wide range of pathophysiogical disorders, and leads to reactive compounds such as fatty aldehydes, of which the most well known is 4-hydroxy-2*E*-nonenal (4-HNE) issued from 15-hydroperoxy-eicosatetraenoic acid (15-HpETE), an arachidonic acid (AA) product. In addition to 15-HpETE, 12(S)-HpETE is synthesized by 12-lipoxygenation of platelet AA. We first show that 12-HpETE can be degraded in vitro into 4-hydroxydodeca-(2*E*,6*Z*)-dienal (4-HDDE), a specific aldehyde homologous to 4-HNE. Moreover, 4-HDDE can be detected in human plasma. Second, we compare the ability of 4-HNE, 4-HDDE, and 4-hydroxy-2*E*-hexenal (4-HHE) from n-3 fatty acids to covalently modify different ethanolamine phospholipids (PEs) chosen for their biological relevance, namely AA- (20:4n-6) or docosahexaenoic acid- (22:6n-3) containing diacyl-glycerophosphoethanolamine (diacyl-GPE) and alkenylacyl-glycerophosphoethanolamine (alkenylacyl-GPE) molecular species. The most hydrophobic aldehyde used, 4-HDDE, generates more adducts with the PE subclasses than does 4-HNE, which itself appears more reactive than 4-HHE. Moreover, the aldehydes show higher reactivity toward alkenylacyl-GPE compared with diacyl-GPE, because the docosahexaenoyl-containing species are more reactive than those containing arachidonoyl. 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, alkenylacyl-GPEs may efficiently scavenge fatty aldehydes.**—Bacot, S., N. Bernoud-Hubac, N. Baddas, B. Chantegrel, C. Deshayes, A. Doutheau, M. Lagarde, and M. Guichardant. **Covalent binding of hydroxy-alkenals 4-HDDE, 4-HHE, and 4-HNE to ethanolamine phospholipid subclasses. *J. Lipid Res.* 2003.** 44: **917–926.**

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Ethanolamine phospholipids (PEs) in biological membranes consist of two main subclasses: diacyl-glycerophosphoethanolamine (diacyl-GPE) and alkenylacyl-glycerophosphoethanolamine (alkenylacyl-GPE). 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 *sn*-1 position of the glycerol backbone. The *sn*-2 position is acylated primarily by PUFAs such as arachidonic acid (AA) (20:4n-6) and docosahexaenoic acid (DHA) (22:6n-3). Plasmalogens represent 15–20% of total phospholipids (1–3) and more than 50% of PEs (4). Cell antioxidant defenses such as glutathione peroxidases (GPx), superoxide dismutase, or vitamins E and C prevent and/or scavenge highly 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, of which 4-hydroxy-2*E*-nonenal (4-HNE) has been widely investigated. 4-HNE is produced from the most abundant n-6 PUFA-derived hydroperoxides, namely 15-hydroperoxyeicosatetraenoic acid(15-HpETE) and 13-hydroperoxy-linoleic acid (12, 13). Although the breakdown of 15-HpETE has been well de-

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Abbreviations: AA, arachidonic acid (20:4n-6); alkenylacyl-GPE, alkenylacyl-glycerophosphoethanolamine; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; diacyl-GPE, diacyl-glycerophosphoethanolamine; DIBAL, diisobutylaluminium hydride; DHA, docosahexaenoic acid (22:6n-3); EI, electron ionization; FAME, fatty acid methyl ester; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; (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; GPx, glutathione peroxidase; 4-HDDE, 4-hydroxydodeca-(2*E*,6*Z*)-dienal; 4-HHE, 4-hydroxy-2*E*-hexenal; 4-HNE, 4-hydroxy-2*E*-nonenal; HpETE, hydroperoxyeicosatetraenoic acid; NICI, negative ion chemical ionization; PE, ethanolamine phospholipid; PFB, pentafluorobenzyl; ROS, reactive oxygen species; RP-HPLC, reverse-phase HPLC; TMS, trimethylsilyl.

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scribed, that of 12-HpETE, the 12-lipoxygenase product of AA, remains partially unknown. The putative 12-HpETE-derived aldehyde, 4-hydroxydodeca-(2*E*,6*Z*)-dienal (4-HDDE) was characterized in the present study and measured in human plasma. Also, 4-hydroxy-2*E*-hexenal (4-HHE) is described as a major degradation product of n-3 PUFA peroxidation, such as DHA (14). 4-HNE is produced in many degenerative diseases, such as atherosclerosis (15), diabetes (16), inflammation (17–19), and neurodegenerative diseases (20, 21) such as Alzheimer's disease (22–24) and Parkinson's (25) disease. It is highly reactive and covalently binds 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 of which was 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 PEs 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). [^{3}H]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-(2*E*,6*Z*)-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 (4*Z*)-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 yield pure methyl 4-hydroxydodeca-(2*E*,6*Z*)-dienoate (0.8 g, yield 71%).

Synthesis of 4-HDDE. To a stirred solution of methyl 4-hydroxydodeca-(2*E*,6*Z*)-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 from −75°C to −70°C, and the mixture was stirred for 1 h. A 1 M 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 yield 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; and 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 citric acid-trisodium citrate-dextrose was centrifuged at 150 *g* for 10 min. The supernatant platelet-rich plasma was acidified to pH 6.4 with citric acid and centrifuged at 900 *g* for 10 min, and the pellets were resuspended into Tyrode HEPES buffer, pH 7.35.

Preparation of 12-HpETE

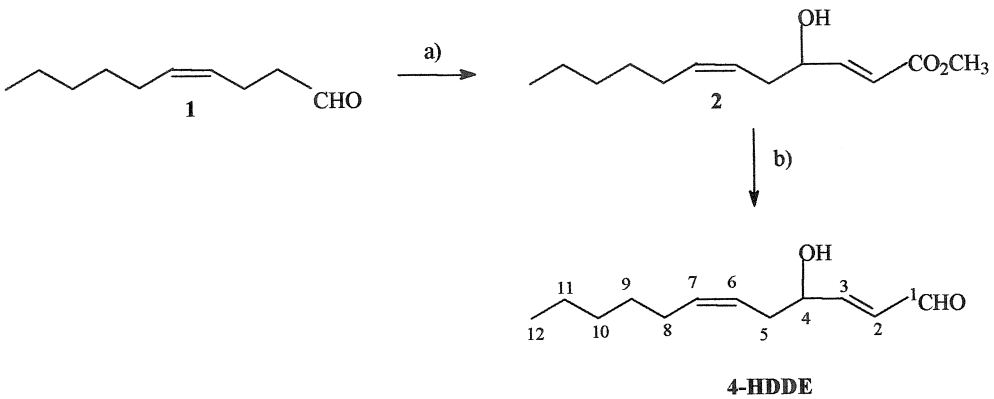
Platelet suspension was preincubated under gentle stirring with 200 μM diamide to lower reduced glutathione (41) and then slow down GPx, and with 200 μM acetylsalicylic acid as a cyclooxygenase inhibitor (42), for 5 min at 37°C. Platelets were then incubated with 300 μM AA labeled with 37 kBq of [5,6,8,9,11,12,14,15-^{3}H]AA (7.77 TBq/mmol) for 5 min in the presence of oxygen. Platelet suspension was then acidified to pH 3 with 3 M HCl and treated three times with 3 vol 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-diethylether-acetic acid (60:40:1; v/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 h at room temperature with 5 ml of 0.1 M HCl containing 0.5 M ascorbate and 0.02 M $FeSO_4$ 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, 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



Scheme 1. Chemical synthesis of 4-HDDE.

(50 mM in 0.1 M PIPES buffer, pH 6.5) for 30 min at room temperature, according to the procedure described by Van Kuijk et al. (44). After acidification with 100 μl of 98% H_2SO_4, pentafluorobenzyloxime 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 trimethylsilylether after an overnight treatment with *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) at room temperature. The pentafluorobenzyl oxime, trimethylsilylether-

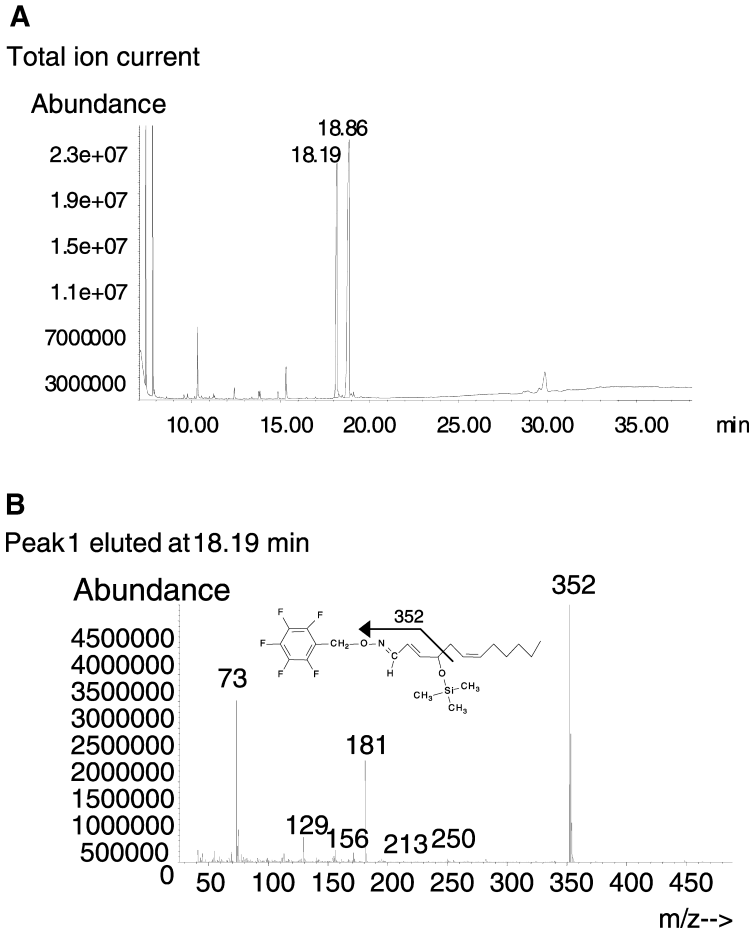


Fig. 1. Electron ionization (EI) mass spectrum of chemically synthesized 4-hydroxydodeca-(2*E*,6*Z*)-dienal (4-HDDE) derivatized as *O*-pentafluorobenzyl oxime, trimethylsilylether. Analytical conditions were as described in Experimental Procedures. A: Total ion current chromatogram. B: EI mass spectrum of the isomer eluted at 18.19 min.

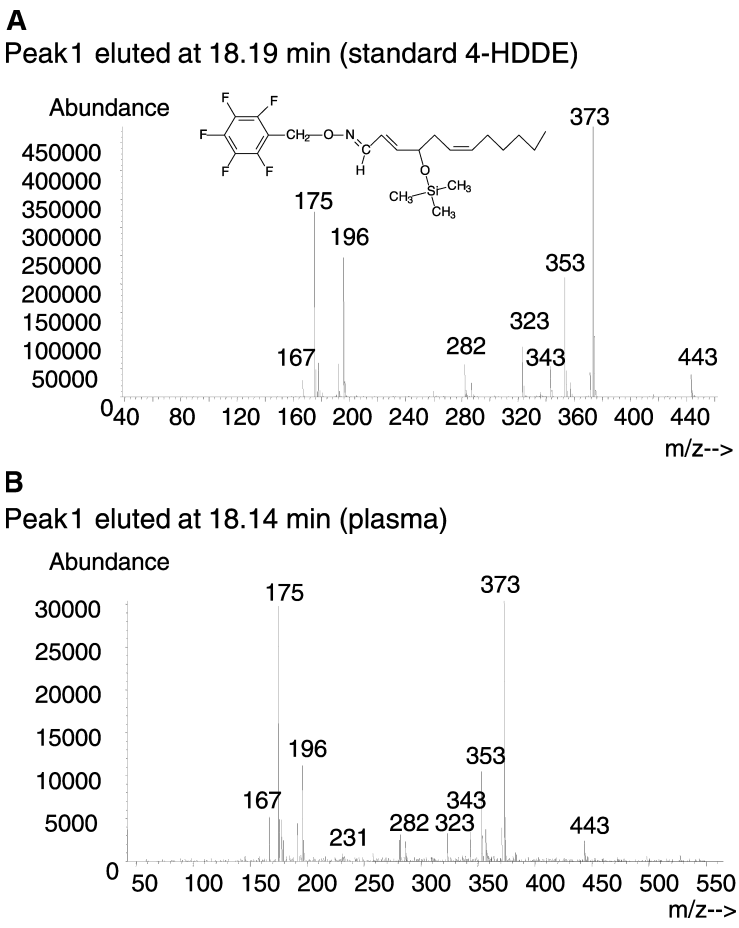


Fig. 2. Negative-ion chemical ionization (NICI) mass spectrum of 4-HDDE derivatized as *O*-pentafluorobenzyl oxime, trimethylsilylether. Analytical conditions were as described in Experimental Procedures. A: NICI mass spectrum of the first isomer of standard 4-HDDE (eluted at 18.19 min). B: Mass spectrum of the same isomer of 4-HDDE originating from human plasma (eluted at 18.14 min).

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.

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 an HP-1MS fused-silica capillary column (30 m × 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°C, 260°C, 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 1,400 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). Nonphosphorus lipids and phospholipids were separated according to Juaneda and Rocquelin (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/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).

Reverse-phase 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 and Murphy (46) and modified as follows. PE was loaded onto a 3.9 × 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/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/v). Eluent A was pumped from 0 to 10 min at 100%, then was replaced by a linearly increasing percentage of solvent B to reach 100% at 155 min. Eluent B was held for 15 min, and then the system returned to the initial conditions within 5 min.

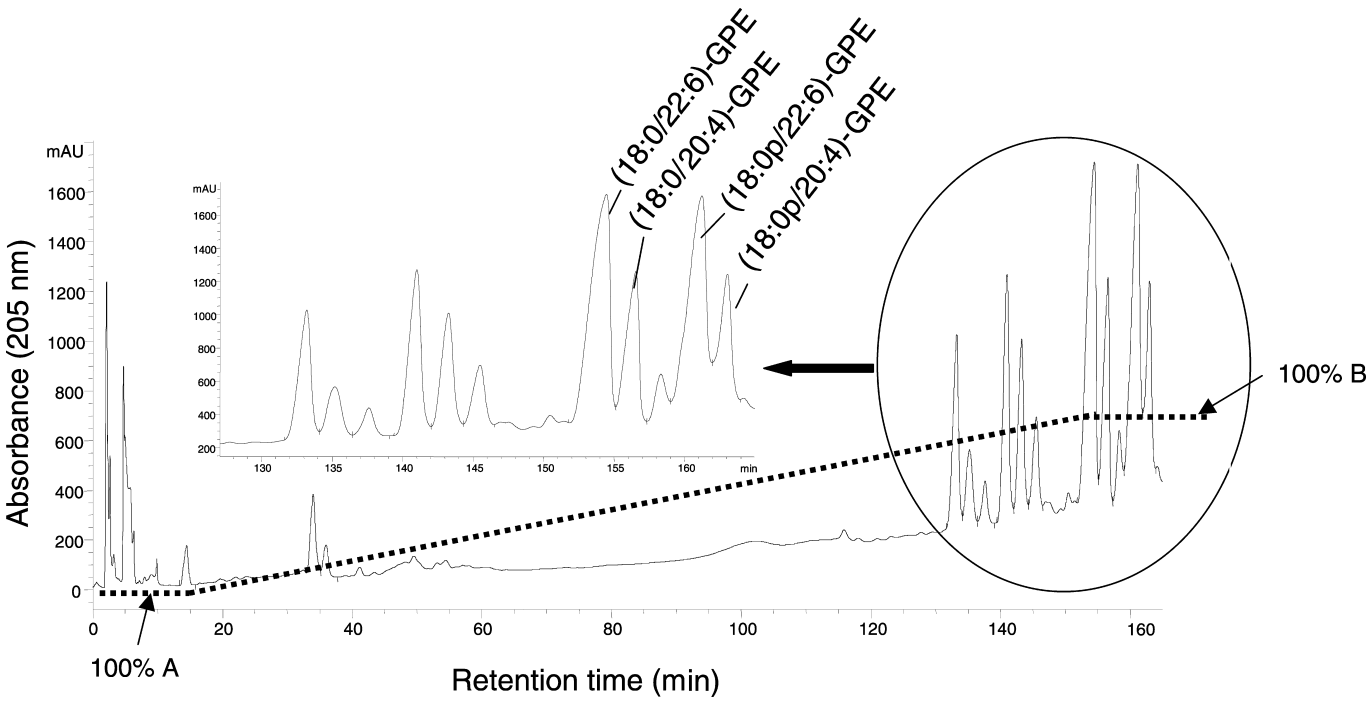


Fig. 3. Typical HPLC profile of ethanolamine phospholipids (PEs) extracted from rat brain. Phospholipids were extracted from rat brain and separated into phospholipid classes. PE isolated by TLC was further fractionated with a 3.9 × 300 mm NovaPak column packed with C18 silica (particle size, 4 μm). 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/v) containing 1 mM ammonium acetate, pH 7.4] to Eluent B [same eluent in different proportions (70:4:2.5, v/v/v)]. Dotted line, mobile phase in function of time.

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-1′-enyl,2-arachidonoyl-GPE (18:0p/20:4-GPE), and 1-*O*-stearyl-1′-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_2CO_3 in 10% water was added. The resulting fatty acid methyl esters (FAMEs) from diacyl-GPE, and FAME and dimethylacetals from alkenylacyl-GPE were extracted by 2 ml of isooctane and analyzed by GC with a DELSI instrument model DI 200 equipped with a fused silica capillary SP-2380 column (60 × 0.22 mm). Helium was used as the carrier gas at 1 ml/min. Temperatures of the Ross injector and the flame ionization detector 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/v) as eluent. Michael and Schiff base adducts were extracted all together 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 two steps from commercially available (4*Z*)-decenal (Ester 1) through the key intermediate ester (Ester 2), according to **Scheme 1**.

Reaction of Ester 1 with methyl 4-chlorophenylsulfinylacetate [SPAC reaction (47, 48)] yielded methyl 4-hydroxydodeca-(2*E*,6*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}H]NMR: 9.59 (d, J = 7.8 Hz, H-1); 6.85 (dd, J = 15.7, 4.3 Hz, H-3); 6.35 (ddd, J = 15.7, 7.8, 1.4 Hz, H-2); 5.73–5.60 (m, H-6); 5.45–5.31 (m, H-7); 4.60–4.40 (m, H-4); 2.43 (t, J = 7.0 Hz, 2H-5); 2.08–2.00 (m, 2H-8); 1.45–1.20 (m, 2H-9, 2H-10, 2H-11); 0.89 (t, J = 7 Hz, 3H-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).

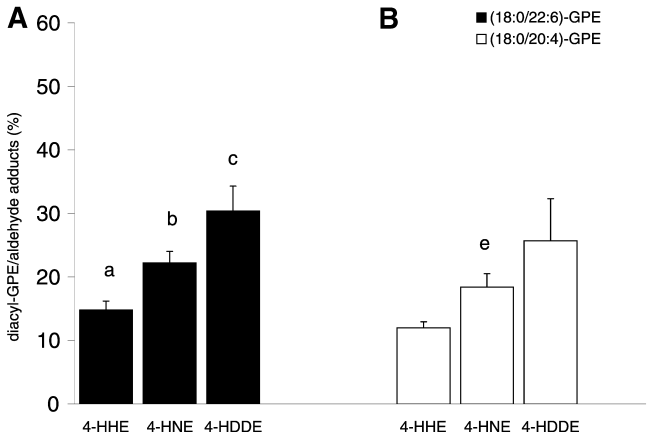


Fig. 4. Covalent modifications of (18:0/22:6-GPE) (A) and (18:0/20:4-GPE) (B) with 4-hydroxy-2*E*-hexenal (4-HHE), 4-hydroxy-2*E*-nonenal (4-HNE), and 4-HDDE. Diacyl-glycerophosphoethanolamines (diacyl-GPEs) isolated by HPLC from rat brain were incubated with the different aldehydes as described in Experimental Procedures. For each incubate, Michael and Schiff base adducts were extracted and quantified by gas chromatography (GC). Values represent the mean ± SEM of six (18:0/22:6-GPE) experiments and of four (18:0/20:4-GPE) experiments. [a] (18:0/22:6-GPE)-4-HHE adducts versus (18:0/22:6-GPE)-4-HDDE adducts, $P \leq 0.002$; [b] (18:0/22:6-GPE)-4-HNE adducts versus (18:0/22:6-GPE)-4-HHE adducts, $P \leq 0.001$; [c] (18:0/22:6-GPE)-4-HDDE adducts versus (18:0/22:6-GPE)-4-HNE adducts, $P \leq 0.02$; [e] (18:0/20:4-GPE)-4-HNE adducts versus (18:0/20:4-GPE)-4-HHE adducts, $P \leq 0.04$.

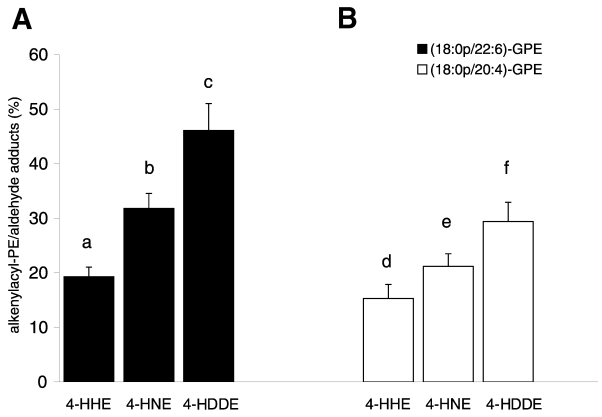


Fig. 5. Covalent modifications of (18:0p/22:6-GPE) (A) and (18:0p/20:4-GPE) (B) with 4-HHE, 4-HNE, and 4-HDDE. Alkenylacyl-glycerophosphoethanolamine isolated by HPLC from rat brain was incubated with the different aldehydes as stated in Experimental Procedures. For each incubate, Michael and Schiff base adducts were extracted and quantified by GC. Values represent the mean ± SEM of six (18:0p/22:6-GPE) experiments and of four (18:0p/20:4-GPE) experiments. [a] (18:0p/22:6-GPE)-4-HHE adducts versus (18:0p/22:6-GPE)-4-HDDE adducts, $P \leq 0.005$; [b] (18:0p/22:6-GPE)-4-HNE adducts versus (18:0p/22:6-GPE)-4-HHE adducts, $P \leq 0.01$; [c] (18:0p/22:6-GPE)-4-HDDE adducts versus (18:0p/22:6-GPE)-4-HNE adducts, $P \leq 0.01$; [d] (18:0p/20:4-GPE)-4-HHE adducts versus (18:0p/20:4-GPE)-4-HDDE adducts, $P \leq 0.003$; [e] (18:0p/20:4-GPE)-4-HNE adducts versus (18:0p/20:4-GPE)-4-HHE adducts, $P \leq 0.05$; [f] (18:0p/20:4-GPE)-4-HDDE adducts versus (18:0p/20:4-GPE)-4-HNE adducts, $P \leq 0.02$.

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 antiisomers were eluted at 18.19 and 18.86 min, respectively (**Fig. 1A**). Electron impact mass spectra of both syn- and antiisomers show the same fragmentation pattern, except for the relative abundance of the different ions; the spectrum of the isomer eluted at 18.19 min is shown as an example (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 trimethylsilyl (TMS) and pentafluorobenzyl (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 one 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 antiisomers 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 is shown in **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), and *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]NMR 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. Approximately 13% of AA was converted into 12-HpETE by platelets treated with aspirin and diamide, as evaluated after TLC separation (unpublished observations). 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 Procedures. 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

TABLE 1. Percentage of adducts formed with the different aldehydes

	Percent of PE Aldehyde Adducts	
	Diacyl-GPE	Alkenylacyl-GPE
4-HHE	13.7 ± 1.0	17.7 ± 1.5[a]
4-HNE	20.7 ± 1.3	27.5 ± 2.5[b]
4-HDDE	28.5 ± 3.4	39.4 ± 4.2[c]

PE, ethanolamine phospholipid; diacyl-GPE, diacyl-glycerophosphoethanolamine; alkenylacyl-GPE, alkenylacyl-glycerophosphoethanolamine; 4-HHE, 4-hydroxy-2*E*-hexenal, 4-HNE, 4-hydroxy-2*E*-nonenal; 4-HDDE, 4-hydroxydodeca-(2*E*,6*Z*)-dienal. Results are taken from Figs. 4 and 5. Values represent the mean ± SEM of 10 experiments and are compared as follows:

[a] Diacyl-GPE-4-HHE adducts versus alkenylacyl-GPE-4-HHE adducts $P \leq 0.02$.

[b] Diacyl-GPE-4-HNE adducts versus alkenylacyl-GPE-4-HNE adducts, $P \leq 0.03$.

[c] Diacyl-GPE-4-HDDE adducts versus alkenylacyl-GPE-4-HDDE adducts, $P \leq 0.04$.

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 was 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 in 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 to be >70% (unpublished observations). 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-GPE) or (18:0/22:6-GPE) subclasses previously isolated by RP-HPLC. Michael and Schiff base adducts were measured all together by GC as previously described. Results presented in **Fig. 4** show that the amount of (18:0/22:6-GPE)-4-HDDE adducts (measured as percent 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 reactivity of the aldehydes, then, seems dependent on their hydrophobicity, with 4-HDDE the most and 4-HHE the least hydrophobic aldehydes.

TABLE 2. Percentage of adducts formed with the different PE subclasses

	Percent of Adducts
(18:0/22:6) + (18:0p/22:6)-GPE	27.4 ± 2.1[a]
(18:0/20:4) + (18:0p/20:4)-GPE	20.4 ± 1.8

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

[a] Values represent the mean ± SEM of experiments reported in Figs. 4 and 5; $P \leq 0.02$.

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

Similar investigations were performed with the corresponding alkenylacyl-GPE 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 amounts 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 represent 19.3 ± 1.8%, 31.8 ± 2.7%, and 46.1 ± 4.9% (n = 6) of initial alkenylacyl-GPE, respectively, and the amounts 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 in making covalent adducts with alkenylacyl-GPE than were 4-HNE and 4-HHE.

Compared sensitivities of PE subclasses and molecular species toward aldehydes

Values corresponding to experiments reported in Figs. 4 and 5 were combined and compared according to the PE subclasses (alkenylacyl-GPE vs. diacyl-GPE) and to the nature of the fatty acid esterified at the *sn*-2 position. **Table 1** indicates that whatever the fatty acid esterified at the *sn*-2 position, aldehydes were more powerful for making adducts with the amino group of alkenylacyl-GPE 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 alkenylacyl-GPE 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 Figs. 4 and 5, allows for evaluation of 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 2**) was significantly ($P < 0.02$) more important with DHA-containing PE at the *sn*-2 position than with AA-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 PUFAs, 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. On the basis of 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 was 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 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 and 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 under 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 antiisomers 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 and Porter (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 ~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 GPx 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 because it derives from peroxidation of several n-6 PUFAs, 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, because 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 AA or DHA at the *sn*-2 position were investigated. The results (Figs. 4A, 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 AA-containing alkenylacyl-GPE subclass (Fig. 5B). The results with diacyl-GPE containing AA (Fig. 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 alkenylacyl-GPE enhance the ethanolamine exposure and facilitate its reaction with aldehydes. The higher reactivity of the alkenylacyl-GPEs might be biologically relevant because, in addition to their antioxidant properties linked to the presence of the vinyl ether moiety, alkenylacyl-GPEs may scavenge aldehydes more efficiently and then lower their toxicity.

Interestingly, the comparison of different PE molecular species containing either AA or 22:6n-3 esterified at the *sn*-2 position reveals that aldehydes make more adducts with 22:6n-3-containing PE (Table 2). Indeed, with 6 *cis* double bonds, 22:6n-3 is likely to exhibit a stronger folding than AA. Its conformation might then alter the location of the phospholipid in the membrane leaflet, as 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 amounts of PE-derived covalent adducts could be found in platelets incubated with exogenous AA (unpublished observations). 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 substrates 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 AA 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 alkenylacyl-GPE than with diacyl-GPE. This could be relevant to the control of the anti/prooxidant balance in the cell.

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