Evidence for in situ ethanolamine phospholipid adducts with hydroxy-alkenals

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Abstract Hydroxy-alkenals, such as 4-hydroxy-2(\(E\))-nonenal (4-HNE; from \(n\)-6 fatty acids), are degradation products of fatty acid hydroperoxides, including those generated by free radical attack of membrane polyunsaturated fatty acyl moieties. The cytotoxic effects of hydroxy-alkenals are well known and are mainly attributable to their interaction with different molecules to form covalent adducts. Indeed, ethanolamine phospholipids (PEs) can be covalently modified in a cellular system by hydroxy-alkenals, such as 4-HNE, 4-hydroxy-2(\(E\))-hexenal (4-HHE; from \(n\)-3 fatty acids), and 4-hydroxy-dodecadienal (4-HDDE; from the 12-lipoxygenase product of arachidonic acid), to form mainly Michael adducts. In this study, we describe the formation of PE Michael adducts in human blood platelets in response to oxidative stress and in retinas of streptozotocin-induced diabetic rats. We have successfully characterized and evaluated, for the first time, PEs coupled with 4-HHE, 4-HNE, and 4-HDDE by gas chromatography-mass spectrometry measurement of their ethanolamine moieties. We also report that aggregation of isolated human blood platelets enriched with PE-4-hydroxy-alkenal Michael adducts was altered. These data suggest that these adducts could be used as specific markers of membrane disorders occurring in pathophysiological states with associated oxidative stress and might affect cell function.—Bacot, S., N. Bernoud-Hubac, B. Chantegrel, C. Deshayes, A. Doutheau, G. Ponsin, M. Lagarde, and M. Guichardant. Evidence for in situ ethanolamine phospholipid adducts with hydroxy-alkenals. J. Lipid Res. 2007. 48: 816–825.

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Oxidative stress is involved in many pathophysiological states, such as aging, atherosclerosis, diabetes, and neurodegenerative disorders. Reactive oxygen species initiate deleterious effects on different biological components, especially polyunsaturated fatty acids, which lead to lipid hydroperoxide formation. Those hydroperoxides are normally reduced to their corresponding alcohols by glutathione peroxidases (1). However, glutathione peroxidase activities have been shown to be decreased in aging (2) and diabetes (3, 4), resulting in a transient accumulation of lipid hydroperoxides that favors their degradation into several compounds, including hydroxy-alkenals. The most well known of these are 4-hydroxy-2(\(E\))-nonenal (4-HNE) (5) and 4-hydroxy-2(\(E\))-hexenal (4-HHE) (6), which derive from \(n\)-6 and \(n\)-3 fatty acid peroxidation, respectively. We have shown previously the occurrence of 4-hydroxy-2(\(E\),6(\(Z\))-dodecadienal (4-HDDE), mainly issued from the 12-lipoxygenase product of arachidonic acid (AA; \(20:4n-6\)), 12-hydroperoxyeicosatetraenoic acid (7). Those three hydroxy-alkenals are highly reactive because of a double bond conjugated with the carbonyl group. 4-HNE has been shown to make covalent adducts with amine moieties of amino acid residues such as lysine or histidine (8–11) and of nucleotides (12, 13). It may also react with thiol groups such as in glutathione (5) to form Michael adducts. We have found that 4-HNE may also react with amino phospholipids, particularly ethanolamine phospholipids (PEs) (7, 14, 15), to form Michael and Schiff base adducts, the latter being partially cyclized. The reactivity of 4-HHE, 4-HNE, and 4-HDDE toward PE depends on their hydrophobicity. Indeed, 4-HDDE is more active in making covalent adducts than 4-HNE, which is also more reactive than 4-HHE (7). Our hypothesis is that the formation of PE-alkenal Michael adducts could be involved in diseases associated with oxidative injury.

The first aim of this study was to investigate the occurrence of such Michael adducts formed between PEs and the above-mentioned aldehydes in oxidative stress conditions, induced either in human blood platelets in vitro or
in retinas of streptozotocin-induced diabetic rats in vivo. For this purpose, we used a sensitive GC-MS negative ion chemical ionization (NICI) method to measure the adducts, after cleavage of the phosphodiester bonds and derivatization of the ethanolamine-alkenal moiety. For the first time, we have successfully measured PE-4-HHE, PE-4-HNE, and/or PE-4-HDDE Michael adducts in the two biological systems described above. We also have explored the biological effect of PE-4-HNE Michael adduct on human blood platelet aggregation.

MATERIALS AND METHODS

Materials

Diamide, mercaptosuccinic acid, aspirin, AA, α-ω-1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphoethanolamine [(16:0/18:2)-PE], 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine [(18:1/18:1)-PE], bovine brain phosphatidylethanolamine, 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, 1,2-ω-1-palmitoyl,2-arachidonoyl-sn-glycero-3-phosphoethanolamine [(16:0/20:4)-PE], boron trifluoride in methanol, sodium borohydride, 1-(trifluoroacetyl)-2,3,4,5,6-pentafluorobenzyl hydroxylamine, N-acetyl-α-thioglycoside, and 1,3-dimethyl-2-imidazole (TFAI), O2,3,4,5,6-pentfluoro benzyl hydroxylamine hydrochloride, N-α-bis(3-methylsilyl) trifluoroacetamidate, and streptozotocin (S0130) were purchased from Sigma-Fluka-Aldrich Chemical Co. (St. Quentin Fallavier, France). The silica HPLC column (4.6 × 250 mm, 3 μm) was from Macherey-Nagel AG (Hoerdt, France). 4-HHE, 4-HNE, 4-HDDE, and their corresponding terminal CD3 analogs (deuterated HHE, deuterated HNE, and deuterated HDE, respectively) used as internal standards were chemically synthesized.

Chemical synthesis of PE-alkenal Michael adducts and their CD3 analogs

Two equivalents of 4-HHE, 4-HNE, or 4-HDDE were incubated with one equivalent of 1-palmitoyl,2-linoleoyl-sn-glycero-3-phosphoethanolamine or bovine brain phosphatidylethanolamine as described previously by Bacot et al. (7) to produce standard PE-alkenal Michael adducts. The corresponding deuterated internal standards labeled with deuterium at the methyl terminus (CD3) of the hydroxy-alkenals were synthesized by the same procedure.

Platelet isolation, induction of oxidative stress, and lipid extraction

Blood platelets were isolated from a blood bank according to Lagarde et al. (16) with the following modifications. Briefly, blood taken onto anticoagulant citric acid, trisodium citrate, and dextrose (ACD) was centrifuged at 150 g for 10 min. The supernatant platelet-rich plasma (PRP) was incubated with 200 μM aspirin for 30 min at room temperature to minimize platelet activation. Under these conditions, the cyclooxygenase pathway was inhibited and AA metabolism was shifted to the 12-lipoxygenase pathway. After acidification to pH 6.4 with 0.15 M citric acid and centrifugation at 900 g for 10 min, platelet pellets were resuspended into a Tyrod-HEPES buffer solution (in mmol/l: 137 NaCl, 2.7 KCl, 0.41 NaH2PO4, 11.9 NaHCO3, 1 MgCl2, 5.5 glucose, and 5 HEPES, pH 7.35). Platelets were treated in the presence or absence (controls) of 200 μM diamide for 24 h at 37°C and in the presence or absence (controls) of 2 mM aspirin plus 2 mM mercaptosuccinic acid for 30 min, followed by 20 μM AA for 2 h at 37°C and by 100 μl of red cell lysate for 22 h at 37°C. Such a high concentration of aspirin (10-fold higher than that required for cyclooxygenase inhibition) is known to inhibit the peroxidase activity associated with the lipoxygenase pathway (17). Mercaptosuccinic acid is a specific inhibitor of seleno-enzymes, including glutathione peroxidase (18). Platelet lipids were extracted twice with a solvent mixture of chloroform-ethanol (2:1, v/v) containing the deuterated PE-4-HNE Michael adducts as a deuterated internal standard.

Animals and induction of type 1 diabetes

Ten male Wistar rats (Charles River Laboratories, l’Abresle, France), each 6 weeks old, weighing ~200 g, received a single intravenous injection of streptozotocin (60 mg/kg in 10 mM citrate buffer, pH 4.5). Ten rats received an equivalent injection of citrate buffer to serve as control animals. All rats were allowed free access to water and food before euthanasia. Streptozotocin induced type 1 diabetes in rats by the destruction of pancreatic β-cells of the Langerhans islets. Five months after streptozotocin treatment, both control and diabetic rats were euthanized and the eyes were enucleated. Rat retinas were prepared, and total lipids were extracted twice with a solvent mixture of chloroform-ethanol (2:1, v/v) and stored at −80°C until analysis.

Separation of phospholipids

Nonphosphorus lipids and phospholipids, from both blood platelets and retinas, were separated on 6 ml Silica-Sep-Pak cartridges (500 mg of silica) according to Juareda and Rocquelin (19). Briefly, lipid extracts were dissolved in chloroform and loaded onto silica cartridges. Neutral lipids were washed through with chloroform, and total phospholipids were subsequently eluted with methanol. Alcoholic fractions were evaporated to dryness, and residues were dissolved in a mixture of chloroform-ethanol (2:1, v/v).

GC analysis of retina phospholipids

Total phospholipids, and especially PE from control and diabetic rats, were quantified by measuring their fatty acid content. They were transesterified with 10% boron trifluoride in methanol as described (7). Diheptadecanoyl-glycerophosphoethanolamine was used as an internal standard before transesterification.

HPLC isolation of Michael adducts

PE-alkenal Michael adducts, chemically synthesized or biologically formed during oxidative stress, were isolated by HPLC as described previously (14) and modified as follows. Lipid extracts were applied to a 4.6 × 250 mm packed silica Nucleosil 100 column (particle size, 3 μm), and phospholipid adducts were eluted with a gradient of solvents consisting of hexane-isopropanol-water (60:80:2, v/v) (solvent A) and hexane-isopropanol-water (60:80:16, v/v) (solvent B). Solvent A was pumped from 0 to 5 min at 100% and was then progressively replaced by 5, 20, and 100% solvent B at 20, 30, and 40 min, respectively. Solvent B was held for 15 min, and then the system was returned to the initial conditions within 15 min. A flow rate of 1 ml/min was used, and detection was achieved by monitoring UV absorbance at 203 nm. PE-hydroxy-alkenal Michael adducts were collected between 18 and 25 min.

Derivationization of alkenals produced by oxidative stress in blood platelets

The free fatty hydroxy-alkenals 4-HHE, 4-HNE, and 4-HDDE, released during oxidation, were analyzed and quantified by GC-MS according to the procedure described by Van Kuijk et al. (20) and modified as follows. Deuterated 4-HNE (15 ng) used as an internal standard was added to a platelet suspension (500 μl) subjected or not subjected to oxidative stress. Samples were treated with 1 ml of O2,3,4,5,6-pentfluoro benzyl hydroxylamine hydrochloride (50 mM in 0.1 M PIPES buffer, pH 6.5) for 30 min.
at room temperature. After acidification with 60 μl of concentrated H₂SO₄, pentafluorobenzoxime derivatives were extracted with 2.5 ml of methanol and 5 ml of hexane. The hydroxyl group was then converted into trimethylsilyl ether after an overnight treatment with 100 μl of N,O-bis(trimethylsilyl)trifluoroacetamide at room temperature. The pentafluorobenzoxime trimethylsilyl ether derivatives of 4-HHE (OPFB-TMS-4-HHE), 4-HNE (OPFB-TMS-4-HNE), and 4-HDDE (OPFB-TMS-4-HDDE) were analyzed by GC-MS as described previously (7).

**GC-MS derivatization and analysis of PE-alkenal Michael adducts**

PE-alkenal Michael adducts, isolated by HPLC, were treated as described for Fig. 1. Briefly, the adducts were reduced by sodium borohydride (NaBH₄) in anhydrous ethanol (1 ml) for 2 h at room temperature to transform the aldehyde group into alcohol. The resulting adducts were then treated with 10% KOH in methanol solution for 3 h at 100°C to release the ethanolamine coupled with the initial aldehyde, which were further derivatized by 100 μl of TFAI for 30 min at 60°C and extracted with toluene-water (1:4, v/v) before NICI GC-MS analysis.

GC-MS was carried out on a Hewlett-Packard quadrupole mass spectrometer interfaced with a Hewlett-Packard gas chromatograph (Les Ulis, France). The gas chromatograph was equipped with an HP-5MS fused-silica capillary column (60 m × 0.25 mm inner diameter, 0.25 μm film thickness; Agilent Technologies). The oven temperature gradient program used was as follows: 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 pulse splitless injector with a head pressure of 7.9 p.s.i. The interface, injector, and ion source temperatures were set at 280, 260, and 150°C, respectively. Electron energy was set at 70 eV. Helium and methane were used as carrier and reagent gases, respectively. NICI mode was used, and the mass spectra were acquired from 100 to 800 Da. PE-Michael adducts were quantified in the selected ion monitoring (SIM) mode using the main specific characteristic fragments of derivatized ethanolamine-alkenal adducts at m/z 561 and 541 for PE-4-HHE, 603 and 583 for PE-4-HNE, 643 and 623 for PE-4-HDDE, and 564 and 544, 606 and 586, and 646 and 626 for their deuterated counterparts used as internal standards, respectively.

As a general control for the detection of all three adducts, a blank with pure 1-palmitoyl,2-arachidonoyl-phosphoethanolamine was treated as described for Fig. 1. This blank did not show any detectable adducts (results not shown). This indicates that the adducts are not generated during the handling steps.

**Incorporation of Michael adducts or PE in platelets**

PE-4-HNE and PE-4-HDDE Michael adducts were both chosen because of the well-known effect of 4-HNE on platelet aggregation and because a specific PE-4-HDDE increase was observed in stressed platelets. Briefly, PRP was treated with a low and non-aggregatory concentration of collagen (0.125 μg/ml) to facilitate the incorporation of exogenous phospholipids, PE (bovine brain), PE-4-HNE, or PE-4-HDDE Michael adducts. Incorporation was done as described by Ibrahim et al. (21) and modified as follows. Different concentrations of Michael adducts or PE alone (1, 10, 25, 50, 100, and 200 μM) were dissolved in a suitable

![Diagram of the procedure used to evaluate ethanolamine phospholipid (PE)-alkenal Michael adducts by GC-MS, as their ethanolamine-alkenal residue. Two equivalents of 4-hydroxy-2(\(E\))-hexenal (4-HHE), 4-hydroxy-2(\(E\))-nonenal (4-HNE), or 4-hydroxy-2(\(E\),6(\(Z\))-dodecadienal (4-HDDE) were incubated with one equivalent of 1-palmitoyl,2-linoleoyl-sn-glycero-3-phosphoethanolamine as described in Materials and Methods to produce standard ethanolamine phospholipid-alkenal Michael adducts. These adducts, isolated by HPLC, were reduced by sodium borohydride in anhydrous ethanol for 2 h at room temperature. The resulting adducts were then hydrolyzed with 10% KOH in methanol solution for 3 h at 100°C, derivatized by 100 μl of 1-(trifluoroacetyl)imidazole (TFAI) for 30 min at 60°C, and extracted before negative ion chemical ionization (NICI) GC-MS analysis. For 4-HHE, \(R = \text{C}_2\text{H}_5\); for 4-HNE, \(R = \text{C}_5\text{H}_{11}\); for 4-HDDE, \(R = \text{C}_8\text{H}_{15}\).](image)
concentration of ethanol (0.5%, v/v) and incubated with PRP for 2 h at 25°C with gentle shaking (an equivalent volume of ethanol was added into control PRP). After acidification and centrifugation, platelets were resuspended into a Tyrode-HEPES buffer. Platelet suspensions were left for 1 h at room temperature before experiments were started.

**Platelet aggregation tests**

Platelet suspensions enriched or not with Michael adducts or PE were preincubated for 1 min at 37°C. Aggregation was then induced by collagen (0.50 µg/ml) and measured by a turbidimetric method (22) using a Chronolog dual-channel aggregometer (Coulter, Margency, France).

**Statistical analysis**

Statistical analysis was performed using Student’s t-test.

**RESULTS**

Characterization of chemically synthesized PE-alkenal Michael adducts

Michael adducts resulting from (16:0/18:2)-PE treated with 4-HHE, 4-HNE, or 4-HDDE were isolated by HPLC. They were then reduced and hydrolyzed, and the resulting ethanola mine adducts were derivatized (Fig. 1) and finally analyzed by NICI GC-MS as described previously. The derivatized ethanola mine adducts were eluted at 17.34, 18.80, and 20.73 min, respectively. Their mass spectra (Fig. 2) showed that ions at m/z 561, 603, and 643 correspond to the molecular anion radicals [M]− of the ethanola mine moiety modified by 4-HHE, 4-HNE, and 4-HDDE, respectively. Minor ions resulting from the loss of fluorhydric acid [M-20] were found at m/z 541, 583, and 623, respectively. A rearrangement of the molecular anion radicals with loss of CF3-COOH ([M-114]−) was also detectable at m/z 447 for ethanola mine-4-HHE, 489 for ethanola mine-4-HNE, and 529 for ethanola mine-4-HDDE. The ion at m/z 227 is common to the three adducts and presumably derives from the [M-114]− ion by molecular rearrangement, whereas m/z 113 corresponds to the [CF3-COO]− ion. Typical SIM profiles of standard ethanola mine adducts derivatized by TFAI are shown in Fig. 3.

**Human blood platelets**

*Free hydroxy-alkenal measurements.* 4-HHE, 4-HNE, and 4-HDDE were detected by NICI GC-MS using deuterated 4-HHE as an internal standard (Table 1). 4-HNE was increased significantly in platelets treated with diamide compared with controls (1.61 ± 0.12 vs. 1.15 ± 0.08 ng/ml). We also observed a significant increase in 4-HHNE (0.74 ± 0.24 vs. 0.54 ± 0.20 mg/ml), whereas 4-HHDDE did not significantly increase 4-HHDDE. The treatment with combined high concentrations of aspirin and mercaptosuccinic acid inhibited the peroxidase activity of the 12-lipoxygenase pathway and led to a strong and significant increase of 4-HHDDE compared with controls (1.54 ± 0.80 vs. 0.01 ± 0.07 mg/ml). The 12-lipoxygenase pathway was specifically targeted, because only a slight and nonsignificant increase of both 4-HHE and 4-HNE was observed.

**GC-MS evaluation of PE-alkenal Michael adducts isolated from human blood platelets treated by diamide.** PE-alkenal Michael adducts were extracted, purified by HPLC, and treated as described for Fig. 1 to analyze the derivatized ethanolamine adducts by NICI GC-MS using SIM detection mode, as described previously. Derivatized ethanolamine-4-HNE residue was detected, as attested by the signal of ions at m/z 603 (molecular anion radical) and 583 (loss of fluorhydric acid) (Fig. 4A) and by the retention time at 18.80 min, as already observed for the ethanolamine-4-HNE standard derivatized according to the same procedure (Fig. 3B). In addition, the common ions at m/z 227 and 113 were observed (results not shown) at the same retention time, and the relative abundance of the different ions was also close to that observed for the standard, confirming again the presence of the derivatized ethanolamine-4-HNE residue. Under these conditions, neither derivatized ethanolamine-4-HDDE nor derivatized ethanolamine-4-HHE residues from PE-4-HHE and PE-4-HDDE adducts, respectively, were detectable.

**GC-MS evaluation of PE-alkenal Michael adducts isolated from human blood platelets treated with the combination of aspirin and mercaptosuccinic acid.** The two inhibitors induced a more drastic oxidative stress than diamide and led to significant formation of 4-HHDDE, as shown in Table 1. In this experiment, the SIM profile (Fig. 4B) of two characteristic fragments of ethanolamine-4-HDDE residues at m/z 643 (molecular anion radical) and 623 (loss of fluorhydric acid) eluted at the same retention time (20.77 min) as that observed for the corresponding ethanolamine standard derivatized (20.73 min) (Fig. 3C) ascertained the formation of PE-4-HDDE Michael adducts. Moreover, signals of both ions at m/z 227 and 113 were also detected at the same retention time (results not shown). The derivatized ethanolamine-4-HNE residue was also detected in the same extract (18.80 min), with a chromatogram similar to the NICI GC-MS profile already observed after the diamide treatment (Fig. 4A). Traces of molecular ion at m/z 541 were detectable at 17.39 min, suggesting that PE-4-HHNE Michael adducts could also be present in small amounts (results not shown). PE-4-HHE and PE-4-HDDE Michael adducts were evaluated through their corresponding derivatized ethanolamine-alkenal residue using the ion signal at m/z 606 corresponding to the initial deuterated PE-4-HHNE used as an internal standard and added to the platelet suspension before lipid extraction. The amount of PE-alkenal Michael adducts, expressed as a percentage of the total PE initially present in platelets, can be evaluated to 2% and 1% of total platelet PE content for PE-4-HHE and PE-4-HDDE Michael adducts, respectively.

**Retinas of streptozotocin-induced diabetic rats**

*Effect of diabetes on phospholipid AA and docosahexaenoic acid.* The fatty acid proportions in total phospholipids and PE from both control (10 rats) and diabetic (10 rats) rat retinas were measured by GC. All rats received the same diet. Only the most relevant ones, AA and docosahexaenoic acid (DHA; 22:6n-3), were compared in this study.
The proportion of AA was 3- to 4-fold lower than that of DHA in total phospholipids as well as in PEs. The percentage of DHA in total phospholipids of diabetic rat retinas was decreased (~10%) compared with that of controls, 28.3% versus 31.4%. A similar decrease (~8%) of DHA present in PEs of diabetic rat retinas was also observed (38.4% vs. 41.7%). In contrast, the percentage of AA in both lipid compartments was not different between diabetic and control rats.

**Fig. 2.** Typical NICI mass spectra of standards corresponding to ethanolamine covalently bound with 4-HHE, 4-HNE, or 4-HDDE and derivatized by TFAL. Standard Michael adducts resulting from 1-palmitoyl,2-linoleoyl-sn-glycero-3-phosphoethanolamine treated with 4-HHE, 4-HNE, or 4-HDDE were isolated by HPLC as described in Materials and Methods. They were then reduced and hydrolyzed, and the resulting ethanolamine adducts were finally derivatized and analyzed by NICI GC-MS as described for Fig. 1. A: Derivatized ethanolamine-4-HHE adducts. B: Derivatized ethanolamine-4-HNE adducts. C: Derivatized ethanolamine-4-HDDE adducts.

**GC-MS evaluation of PE-alkenal Michael adducts isolated from rat retinas.** Oxidative stress associated with diabetic retinopathy was also used as a model to measure PE-alkenal. The derivatized ethanolamine-4-HHE residue from PE-4-HHE Michael adducts was found and characterized by NICI GC-MS (Fig. 4C) in comparison with its chemically synthesized standard (Fig. 3A). Indeed, prominent signals corresponding to the molecular ion radical at m/z 561 and the ion at m/z 541 (loss of fluorhydric acid) were detected.
at the same retention time (17.39 min) as the synthesized standard (17.34 min). In addition, signals corresponding to the common ions at m/z 227 and 113 (results not shown) were found. Results presented in Fig. 5 are expressed as percentages of total PE initially present in retinas. A 5.3-fold increase of PE-4-HHE Michael adducts was observed in diabetic rat retinas compared with controls of total PE. An increase of PE-4-HNE Michael adducts (3.2-fold) could also be assessed in diabetic rat retinas. Finally, undetectable in control retinas, PE-4-HDDE was detected at a very low percentage in diabetic retinas.

Effect of PE-4-HNE on the platelet aggregation response. Preliminary data showed that normal platelets incubated in the presence or absence of either PE-4-HNE or PE-4-HDDE Michael adducts or PE (see Materials and Meth-

### Table 1. Free hydroxyl-alkenal levels in human platelets after oxidative stress

| Hydroxyl-alkenal                          | Diamide Treatment | Aspirin + Mercaptosuccinic Acid + AA Treatment |
|-------------------------------------------|-------------------|-----------------------------------------------|
|                                           | Controls          | Treated Platelets | Controls          | Treated Platelets |
| 4-Hydroxy-2(E)-hexenal                    | 0.54 ± 0.20       | 0.74 ± 0.24<sup>a</sup>                        | 1.18 ± 0.63       | 2.11 ± 1.08       |
| 4-Hydroxy-2(E)-nonenal                    | 1.15 ± 0.08       | 1.61 ± 0.12<sup>a</sup>                        | 1.01 ± 0.60       | 1.26 ± 0.66       |
| 4-Hydroxy-2(E),6(Z)-dodecadienal          | 0.10 ± 0.05       | 0.25 ± 0.10                                      | 0.01 ± 0.07       | 1.54 ± 0.80<sup>b</sup> |

AA, arachidonic acid. Platelets were incubated in the absence (controls) or presence of 200 μM diamide in the first treatment. Platelets were incubated in the absence (controls) or presence of 2 mM aspirin, 2 mM mercaptosuccinic acid, and 20 μM AA in the second treatment. These reactions took place for 24 h at 37°C. Results are expressed in ng/ml platelet suspension and represent means ± SEM of six platelet suspensions.

<sup>a</sup> P < 0.05.
<sup>b</sup> P < 0.001.

Fig. 3. Typical NICI GC-MS using the selected ion monitoring (SIM) profiles of the derivatized ethanolamine-alkenal residues issued from standards corresponding to ethanolamine covalently bound with 4-HHE, 4-HNE, or 4-HDDE and derivatized by TFAI. A: Derivatized ethanolamine-4-HHE adducts. B: Derivatized ethanolamine-4-HNE adducts. C: Derivatized ethanolamine-4-HDDE adducts.
ods) can incorporate ~1% of total exogenous Michael adducts initially added to PRP (results not shown). Different concentrations of PE-4-HNE or PE-4-HDDE Michael adducts incorporated separately into platelets as described in Materials and Methods were tested for platelet aggregation induced by collagen and compared with similar amounts of PE incorporated at the same concentration. Two parameters were investigated to determine PE adduct effects on platelet aggregation: the lag time between the collagen addition and the beginning of aggregation, and the maximum aggregation percentage triggered by collagen. The results (Fig. 6) are expressed relative to the aggregation response after PE incorporation as a control; a ratio of <1 indicates an inhibitory effect of PE adducts and vice versa. It is worth noting that PE did not significantly alter platelet aggregation compared with the vehicle. At ≥ 50 μM PE-4-HDDE, the aggregation was abolished, whereas 200 μM PE-4-HNE did not abolish aggregation. This indicates that PE-4-HDDE is more powerful than PE-4-HNE for that inhibition. Interestingly, low concentrations of PE-4-HNE (10 and 25 μM) potentiated aggregation, which is confirmed by the absence of a lag phase in response to collagen (Fig. 6, dotted line). This finding suggests that low amounts of PE-hydroxy-alkenal adducts might potentiate platelet aggregation.

Fig. 4. NICI GC-MS using the SIM profiles of the derivatized ethanolamine coupled with alkenals in two different biological systems. A: Profiles of the derivatized ethanolamine-4-HNE residues issued from PE-4-HNE Michael adducts in platelets. A limited oxidative stress was induced in platelet suspension by adding 200 μM diamide for 24 h at 37°C. B: Profiles of the derivatized ethanolamine-4-HDDE residues issued from PE-4-HDDE Michael adducts in platelets. Oxidative stress was induced in platelet suspension by adding 2 mM aspirin plus 2 mM mercaptoacetic acid for 30 min, followed by 20 μM arachidonic acid for 2 h at 37°C in the presence of 100 μl of red cell lysate for 22 h at 37°C. C: Profiles of the derivatized ethanolamine-4-HHE residues issued from PE-4-HHE Michael adducts in rat retinas. Five months after streptozotocin treatment (single intravenous injection, 60 mg/kg in 10 mM citrate buffer, pH 4.5) or equivalent injection of citrate buffer, both diabetic and control rats were euthanized and the eyes were enucleated.

Fig. 5. PE-alkenal Michael adducts formed in the retinas of streptozotocin-induced diabetic rats. Values are expressed as percentages of total PE. Values obtained from diabetic rat retinas are compared with those of controls. Data represent means of two determinations.
Fig. 6. Dose response of PE-4-HNE Michael adducts on platelet aggregation induced by collagen. Platelet-rich plasma (PRP) was treated with a nonaggregatory concentration of collagen (0.1 μg/ml) to facilitate the incorporation of both PE and PE-4-HNE Michael adducts at different concentrations: 1, 10, 25, 50, and 200 μM. The incubation was performed for 2 h at 25°C with gentle shaking. An equivalent volume of ethanol (vehicle) was added to control PRP. After acidification to pH 6.4 and centrifugation, platelets were resuspended into a Tyrode-HEPES buffer. Platelet suspensions were left for 1 h at room temperature before measuring their ability to aggregate in the presence of collagen (0.50 μg/ml). Measurements were done in duplicate. Diamonds, percentage aggregation (PE-4-HNE Michael adducts)/percentage aggregation (PE); circles, lag time (PE-4-HNE Michael adducts)/lag time (PE); triangles, percentage aggregation (PE-4-HDDE Michael adducts)/percentage aggregation (PE).

DISCUSSION

Hydroxy-alkenals issued from lipid peroxidation are responsible for most oxidative damage associated with aging and with pathophysiologic states such as atherosclerosis and diabetes. Among them, 4-HNE and 4-HHE are formed from the breakdown of fatty acid hydroperoxides issued from the peroxidation of n-6 (mainly AA and linoleic acid) and n-3 (mainly linolenic acid and DHA) PUFAs, respectively. These hydroxy-alkenals are harmful because they form covalent adducts with biomolecules such as proteins (23, 24), DNA (12, 13), and amino phospholipids (7, 14).

Modification of the amino groups of PE by 4-HNE generates Michael adducts and minor Schiff base adducts, the latter being partly cyclized as a pyrrole derivative (14). We recently characterized another hydroxy-alkenal, 4-HDDE, which derives from AA peroxidation catalyzed by 12-lipoxygenase, and we showed that 4-HDDE was more reactive than 4-HNE and 4-HHE toward PE as a function of their hydrophobicity (7). In this study, we clearly show that Michael adducts can be produced within cells in response to oxidative stress. Indeed, the occurrence of PE-4-HHE, PE-4-HNE, and/or PE-4-HDDE Michael adducts was measured in human blood platelets in response to oxidative stress as well as in retinas of streptozotocin-induced diabetic rats.

First, platelets were treated with diamide, which generates a limited oxidative stress, as assessed by a significant but modest increase of both 4-HHE and 4-HNE. Diamide, a well-known thiol-oxidizing agent, has been reported to decrease the level of glutathione (25), a cofactor of glutathione peroxidase. The inhibition of glutathione peroxidase activity appears limiting, and such an inhibition leads to transient accumulation of hydroperoxides, which favors their degradation into aldehydes. Under this moderate oxidative stress (8.7 ng of free hydroxy-alkenals/10^9 platelets), only PE-4-HNE Michael adducts were measurable; neither PE-4-HHE nor PE-4-HDDE Michael adducts were detectable. Those results agree with the fact that both the remaining free 4-HHE and 4-HDDE concentrations were relatively low in these conditions. In contrast, treatment that combined high concentrations of aspirin (2 mM), able to inhibit both the cyclooxygenase and glutathione peroxidase activities (17), and mercaptosuccinic acid (2 mM), which is a more specific inhibitor of glutathione peroxidases (18) than diamide, generated 4-HDDE more specifically. The global oxidative stress assessed by the hydroxy-alkenals formed in the latter case was almost 24-fold higher (16.3 ng/10^9 platelets) than that obtained with diamide treatment (8.7 ng/10^9 platelets) but remained comparable to those reported in plasma from Alzheimer disease patients (26).

4-HDDE formation was also facilitated by the use of exogenous AA converted directly into 12-hydroperoxycisatetraenoic acid and further to 4-HDDE. Under those conditions, we succeeded in characterizing PE-4-HDDE as well as PE-4-HNE. Levels of PE-4-HNE and PE-4-HDDE Michael adducts were evaluated by NICI using deuterated PE-4-HNE as an internal standard. More PE-4-HNE than PE-4-HDDE was formed (2% vs. 1% of the total amount of platelet PE), despite 4-HDDE being more reactive toward PE than 4-HNE in an acellular system (7). This could be explained by the fact that the amount of 4-HDDE surrounding the membrane is likely limited because of its production by 12-lipoxygenase located in the cytosol. In contrast, 4-HNE is formed mainly by the autoxidation of AA and linoleic acid in membrane phospholipids, which allows their peroxide-derived alkenal to react with PE in the surrounding area. This also agrees with the higher amount of remaining free 4-HDDE compared with 4-HNE. The relatively low formation of PE-4-HHE adducts in platelets treated with the combination of mercaptosuccinic acid and aspirin might be attributable to the rapid diffusion of 4-HHE in the cytosol, because of its high polarity compared with 4-HNE and 4-HDDE and also because its polyunsaturated precursors, n-3 fatty acids, are much lower in platelet membranes than n-6 fatty acids.

In contrast, we have found PE-4-HHE Michael adducts, resulting from n-3 fatty acid peroxidation, in retinas that are especially rich in DHA (27). Indeed, our results indicate that in diabetic rat retinas, DHA represented 28.3% and 38.4% of total phospholipids and PEs, respectively, whereas AA was 4-fold lower. Moreover, we observed that the mol% of DHA was decreased (by almost 10%) in diabetic rats compared with controls in these two analyzed lipid compartments. This observation, which likely results from a loss attributable to peroxidation, reflects the relationship with oxidative stress and diabetes and is in agreement with reports indicating that diabetic complications are especially located in retinas (28). In those conditions, DHA peroxidation would lead to
the formation of 4-HHE, which could react with PEs to form covalent adducts. Indeed, under these conditions of diabetic retinopathy, we successfully showed the occurrence of PE-4-HHE Michael adducts. In diabetic rat retinas, ~0.1% of PE-4-HHE Michael adduct were found as a percentage of total PE. The amount of PE-4-HHE adducts was ~5-fold higher than in control retinas. From the same retina samples, we also measured PE-4-HNE and PE-4-HDDE adducts. In control retinas, only traces of PE-4-HNE adducts were found, although 3-fold enhanced in diabetic retinas, whereas PE-4-HDDE adducts were not detected in control retinas and were found in trace amounts in diabetic retinas.

The formation of PE-alkenal adducts may be important in pathologies involving oxidative stress to modulate the cell signaling-dependent phospholipases. We have already shown (15) that such adducts are poor substrates for secreted phospholipase A₂, and they are barely hydrolyzed by cabbage phospholipase D (29). Michael adducts could also alter membrane fluidity and the activity of anchored proteins in the adduct-containing membranes as well as the accessibility of ligands and other functional components. Although we cannot elaborate on the biological relevance of the adducts we have characterized in oxidatively stressed biological samples at present, we may suggest some alteration of platelet function. Indeed, ~1% of the total exogenous Michael adducts initially added to PRP appears to produce a bimodal effect on platelet aggregation induced by collagen, an agonist that requires the liberation of endogenous AA from phospholipids to trigger the response. Interestingly, we observed that low concentrations of PE-4-HNE Michael adducts (10 and 25 μM) added to PRP abolished the lag time to collagen-induced aggregation. This hyperactivity was already observed at 1 μM, with a lag time that was 30% shorter than that measured for PE at the same concentration. Similar hyperactivity assessed by a reduced lag time was also observed with 50 μM adducts. In contrast, 200 μM PE-4-HNE Michael adducts incubated with PRP inhibited platelet aggregation by ~70%. Such a bimodal effect has already been described with 4-HNE itself (30) and strengthens the role of such adducts in modulating platelet function. In contrast, PE-4-HDDE Michael adducts, which result from the platelet 12-lipoxygenase activation, appear to be more potent inhibitors of platelet aggregation. Further investigation should reveal the mechanism of such alterations.

In conclusion, we report for the first time the occurrence of PE-alkenal Michael adducts in biological membranes, which could be used as specific markers in pathophysiologic states associated with oxidative stress. The formation of such adducts appears well related to the amount of free hydroxyalkenals detected. Moreover, we suggest that the formation of such adducts may affect biological functions.

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