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Bioactive 4-Oxoheptanedioic Monoamide Derivatives of Proteins and Ethanolaminephospholipids: Products of Docosaheptaenoate Oxidation

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Abstract

Oxidative stress causes lipid-derived oxidative modification of biomolecules that has been implicated in many pathological states. Phospholipids containing polyunsaturated fatty acids are major targets of free radical-initiated oxidation. Phospholipids that incorporate docosaheptaenoate (DHA) are highly enriched in important neural structures including the brain and retina, where DHA comprises 40% and 60% of total fatty acids, respectively. Oxidative fragmentation of 2-docosaheptaenoyl-1-palmityl-sn-glycerophosphocholine generates esters of 4-hydroxy-7-oxohept-5-enoic acid (HOHA) and 4-keto-7-oxohept-5-enoic acid (KOHA) with 2-lysophosphatidylcholine, HOHA-PC and KOHA-PC. Covalent HOHA adducts that incorporate the primary amino groups of proteins and ethanolamine phospholipids in carboxyethylpyrrole (CEP) derivatives were detected immunologically with anti-CEP antibodies in human tumors, retina and blood. Now, we generated an anti-OHdiA antibody to test the hypothesis that KOHA adducts, which incorporate the primary amino groups of proteins or ethanolamine phospholipids in 4-oxo-heptanedioic (OHdiA) monoamide derivatives, are present in vivo. However, whereas the anti-CEP antibody is highly specific and does not cross-react with the OHdiA monoamide epitope, the anti-OHdiA monoamide antibody cross-reacted with CEP epitopes making it of little value as an analytical tool for OHdiA monoamides, but suggesting the possibility that OHdiA monoamides

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SUPPORTING INFORMATION

Synthesis of 4-oxoheptanedioic monoamide derivative of bovine serum albumin (OHdiA-BSA); synthesis of 4-oxoheptanedioic monoamide derivative of keyhole limpet hemocyanin (OHdiA-KLH); Table S1. Optimized parameters for the mass spectrometer; Table S2. MRM transition ion pairs and optimized collision energy for the analytes; Figure S1. Serum antibody titre (1:10,000 dilution) in New Zealand white rabbit immunized with OHdiA-KLH; Figure S2. Purification curve. B, binding buffer; E, eluting buffer; Figure S3. Inhibition curve for binding of the anti-CEP polyclonal antibody to CEP-BSA by OHdiA-HSA, CEP-HSA, CPP-HSA and CHP-HSA; Figure S4. Hydrolysis of OHdiA-ethanolamine amide (**6**) to 4-oxoheptanedioic acid by 6 N HCl. (Left panel) ¹H NMR of authentic 4-oxoheptanedioic acid. (Right panel) ¹H NMR of hydrolysis product from OHdiA-ethanolamine amide **6**; Figure S5. CID spectrum of the m/z 173.2 ion from authentic 4-oxoheptanedioic acid (**1**); Figure S6. CID spectrum of m/z 535.0 of authentic OHdiA-diPFB (**3**); Figure S7. HPLC/ESI/MS/MS analysis of OHdiA derived from reaction of DHA-PC with HSA after (A) 0 h incubation and (B) 48 h incubation followed by hydrolysis in the presence of 6 N HCl at 100 °C; Figure S8. HPLC/ESI/MS/MS analysis of OHdiA in the reaction mixture of DHA-PC with HSA prior to oxidation (0 h incubation) and (B) after 48 h oxidation followed by hydrolysis at 100°C with 6 N HCl and esterification with pentafluorobenzyl bromide; Figure S9. HPLC/ESI/MS/MS analysis of OHdiA-diPFB generated after acid hydrolysis followed by pentafluorobenzyl esterification of the reaction product mixtures from copper-catalyzed oxidation of (A) DHA-PC for 48 h or (B) DHA-PC with DPPE for 0 h or (C) DHA-PC with DPPE for 48 h. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Graphical Abstract

Oxidative stress causes lipid-derived oxidative modification of biomolecules that has been implicated in many pathological states including inflammation, atherosclerosis, neurodegenerative diseases, and cancer.^{11, 12} Besides serving as endogenous receptor ligands, γ -oxygenated- α,β -unsaturated aldehyde lipid peroxidation products form covalent adducts with proteins. We discovered that 4-HNE forms adducts that incorporate the ϵ -amino group of protein lysyl residues in 2-pentylpyrrole (PP) derivatives.¹³ Similar

chemistry converts the γ -hydroxy- α,β -unsaturated aldehydes HODA-PC, HOOA-PC and HOHA-PC into pyrrole derivatives of the primary amino groups of protein lysyl residues or ethanolamine phospholipids.^{10, 14} Among these, 2-(ω -carboxyethyl)pyrrole (CEP) derivatives are uniquely derived (Scheme 2) from oxidative cleavage of phospholipids that incorporate docosahexaenoic acid (DHA), a PUFA that is highly enriched in important neural structures including the brain and retina, where it comprises 40% and 60% of total fatty acids, respectively.^{15, 16} We detected CEP in vivo immunologically with an anti-CEP antibody in human tumors, retina and blood.^{17–19}

Modification of protein lysyl residues by γ -keto- α,β -unsaturated aldehydes such as 4-ONE and KODA produces γ -ketoamide derivatives of primary amines.^{20–22} We postulated that KOHA-PC modifies biomolecules to give 4-oxo-heptanedioic monoamide (OHdiA) derivatives of the primary amino groups of proteins and ethanolamine phospholipids (Scheme 2). To test this hypothesis, we generated an anti-OHdiA antibody. Whereas our anti-CEP antibody was highly specific, the anti-OHdiA monoamide antibody cross-reacted with CEP epitopes making it of little value as an analytical tool for OHdiA monoamides, but suggesting the possibility that OHdiA monoamides would exhibit receptor-mediated biological activity similar to that of CEP. An LC-MS/MS method was developed that allows quantification of OHdiA derivatives in biological samples. We now find that KOHA-PC forms OHdiA monoamide adducts of proteins and ethanolamine phospholipids and that OHdiA-protein levels are significantly higher than OHdiA-ethanolamine phospholipid levels in blood from healthy human subjects, 0.45 μ M and 0.18 μ M respectively ($n = 3$, $p = 0.027$). We also found that OHdiA monoamide epitopes are angiogenic, causing TLR2-dependent adhesion and tube formation by human umbilical vein endothelial cells (HUVECs).

EXPERIMENTAL PROCEDURES

Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), 1-tridecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (lyso-PC) and 1-palmityl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (DHA-PC) were purchased from Avanti Polar Lipids (Alabaster, AL). 2-(4-Oxo-7-oxohept-6-enoyl)-1-palmitoyl-*sn*-glycero-3-phosphatidylcholine (KOHA-PC) was prepared as described previously.¹⁰ Human serum albumin (HSA, Cat. A3782) was purchased from Sigma (Milwaukee, WI). Goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody was procured from Santa Cruz Biotechnology (Santa Cruz, CA). Methyl 6-amino-2-((2-acetylamino)acetyl)amino)hexanoate (Ac-Gly-Lys-OMe) was purchased from Bachem (Torrance, CA). CEP-HSA, CPP-HSA, CHP-HSA and CEP-dipeptide were prepared as described previously.²³ 2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate (ABTS), sodium deuterioxide (30 % in D₂O, 99%) and deuterium oxide (99.9%) and all other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO).

General Methods

Proton magnetic resonance (¹H NMR) spectra and carbon magnetic resonance (¹³C NMR) spectra were recorded on a Varian Inova AS400 spectrometer operating at 400 MHz and 100 MHz, respectively. Proton chemical shifts are reported as parts per million (ppm) on the δ

scale relative to CDCl_3 (δ 7.26), CD_3OD (δ 3.31) or D_2O (δ 4.79). ^1H NMR spectral data are tabulated in terms of multiplicity of proton absorption (s, singlet; d, doublet; dd, doublet of doublet; t, triplet; q, quartet; m, multiplet; br, broad), coupling constants (Hz), number of protons. Carbon chemical shifts are reported relative to CDCl_3 (δ 77.0) or CD_3OD (δ 49.0). Flash chromatography was performed with ACS grade solvents from Fisher Scientific (Hanover Park, IL). R_f values are quoted for TLC plates of thickness 0.25 mm from Whatman (Florham Park, NJ). The plates were visualized with iodine, dinitrophenylhydrazine or phosphomolybdic acid reagents. For all reactions performed in an inert atmosphere, argon was used unless otherwise specified.

High Performance liquid chromatography/mass spectrometry

LC-ESI/MS/MS analysis of OHdiA adducts was performed on a Quattro Ultima triple-quadrupole mass spectrometer (Micromass, Wythenshawe, UK) equipped with a Waters Alliance 2690 HPLC system with an auto-injector (Waters, Milford, MA). The chromatographic separation was achieved using on a Luna C18 column (150 2.0 mm i.d. 5 μm , Phenomenex). The source and desolvation temperature was maintained at 120 $^\circ\text{C}$ and 250 $^\circ\text{C}$, respectively. The drying gas (N_2) and cone flow gas was kept at ca. 650 L/h and 65 L/h, respectively. The multiplier was set at an absolute value of 600.

Detection and quantification of 4-oxoheptanedioic acid by LC-MS/MS analysis was performed in the negative ion mode and the total run time was 30 min. The mobile phase consisted of solvent A (HPLC grade water containing 2 mM ammonium acetate) and solvent B (HPLC graded methanol containing 2 mM ammonium acetate). HPLC gradient steps were as follows: 0–8 min, isocratic at 5% solvent B; 8–10 min, linear gradient from 5 to 100% solvent B; 10–20 min, isocratic at 100% solvent B; 21–30 min, isocratic at 5% solvent B. The flow rate employed was 200 $\mu\text{L}/\text{min}$.

Detection and quantification of OHdiA-diPFB by LC-MS/MS was performed in the positive ion mode and the total run time was 35 min. The mobile phase consisted of solvent A (HPLC grade water containing 0.1% formic acid) and solvent B (HPLC graded methanol containing 0.1% formic acid). The HPLC gradient steps were set as follows: 0–2 min, isocratic at 75% solvent B; 2–15 min, linear gradient from 75 to 100% solvent B; 15–24 min, isocratic at 100% solvent B; 25–35 min, isocratic at 75% solvent B. The flow rate employed was 200 $\mu\text{L}/\text{min}$.

Optimized parameters for 4-oxoheptanedioic acid and OHdiA-diPFB were determined with authentic samples. MS scans at m/z 50–900 were obtained for standard compounds. For multiple reaction monitoring experiments, argon was used as collision gas at a pressure of 5 psi. The optimum collision energy and other parameters were determined for each individual analyte (Tables S1 and S2).

Bis(perfluorophenyl) 4-oxoheptanedioate (OHdiA-diPFB)

4-Oxoheptanedioic acid (18.4 mg, 0.1 mmol) was suspended in the 1.5 mL of anhydrous acetonitrile containing pentafluorobenzyle bromide (39.2 mg, 0.15 mmol) and *N,N*-diisopropylethylamine (DIPEA, 100 μL , 0.57 mmol). The reaction mixture was incubated at room temperature under Ar protection for 5.5 h. Then solvent was evaporated under a stream

of dry N₂. Water (1 mL) was added and the resulting mixture was extracted with ethyl acetate (3 × 2 mL). The combined organic extracts were dried with NaSO₄, filtered and the solvents were evaporated under reduced pressure and the diester was purified by flash chromatography on a silica gel column (hexane:ethyl acetate 4:1, TLC: R_f= 0.27) to give OHdiA-diPFB (48.2 mg, 0.09 mmol, 88%). ¹H NMR (400 MHz, CDCl₃) δ 5.18 (d, *J* = 1.4 Hz, 4H), 2.77 (t, *J* = 6.5 Hz, 4H), 2.61 (t, *J* = 6.5 Hz, 4H). ESI-MS: *m/z* calcd for C₂₁H₁₃F₁₀O₅ [M+H]⁺, 535.06, found 535.00; *m/z* calcd for C₂₁H₁₂F₁₀O₅Na [M+Na]⁺, 557.04, found 556.87.

4-Oxoheptanedioic-3,3,5,5-d₄ acid (4)

4-Oxoheptanedioic acid (522 mg, 3 mmol) was incubated overnight in 5 mL of mixture of D₂O and NaOD (764 mg of 40 % NaOD in D₂O, 7.6 mmol) at room temperature under Ar. The solvents were then evaporated under reduced pressure to remove the HOD product. Then another 5 mL of D₂O were added and 360 mg of 40% NaOD in D₂O was used to adjust the pH to 12. The mixture was allowed to react for another 6.5 h. The NMR spectrum showed that the wash out of α-hydrogens was complete. Then the pH of the reaction mixture was adjusted 2 by addition of concentrated deuterium chloride. The resulting mixture was extracted with ethyl acetate (3 × 10 mL). The combined organic extracts were dried with MgSO₄, filtered and the solvents were evaporated under reduced pressure to give pure 4-oxoheptanedioic-3,3,5,5-d₄ acid (499 mg, 2.8 mmol, 93%). ¹H NMR (400 MHz, D₂O) δ 2.26 (s, 4H). ESI-MS: *m/z* calcd for C₇H₅D₄O₅ [M-H]⁻, 177.07, found 177.13.

Bis((perfluorophenyl)methyl) 4-oxoheptanedioate-3,3,5,5-d₄ (OHdiA-diPFB-D₄, 5)

Pure **4** (38.3 mg, 0.2 mmol) was suspended in 2.0 mL of anhydrous acetonitrile containing pentafluorobenzyl bromide (78.4 mg, 0.3 mmol) and N,N-diisopropylethylamine (DIPEA, 200 μL, 1.14 mmol). The reaction mixture was incubated for 8 h at room temperature under Ar. Then the solvents were evaporated under reduced pressure. Water (2 mL) was added and the resulting mixture was extracted with ethyl acetate (3 × 4 mL). The combined organic extracts were dried with anhydrous NaSO₄, filtered and the solvents were evaporated under reduced pressure and the diester was purified by flash chromatography on a silica gel column (hexane/ethyl acetate 4:1, TLC: R_f= 0.27) to give OHdiA-diPFB-D₄ (97.8 mg, 0.18 mmol, 91%). ¹H NMR (400 MHz, CDCl₃) δ 5.18 (s, 4H), 2.59 (s, 4H). ESI-MS: *m/z* calcd for C₂₁H₉D₄F₁₀O₅ [M+H]⁺, 539.09, found 539.24.

7-((2-Hydroxyethyl)amino)-4,7-dioxoheptanoic acid (OHdiA-ethanolamine)

4-Oxoheptanedioic acid (0.8814 g, 5 mmol) was heated in acetyl chloride (10 mL) overnight at 60 °C under Ar. The solvents were then evaporated under reduced pressure to give an anhydride, 1,5-dioxocane-2,8-dione, (750 mg, 4.8 mmol, 96%) that was used without further purification for the synthesis of OHdiA-ethanolamine (*vide infra*). ¹H NMR (400 MHz, CDCl₃) δ 2.87 (ddd, *J* = 17.9, 11.3, 9.1 Hz, 2H), 2.68–2.53 (m, 4H), 2.40 (ddd, *J* = 13.8, 11.3, 9.5 Hz, 2H).

A solution of tert-butyldimethylchlorosilane (TBDMSCl, 3.15 g, 20.9 mmol) in dichloromethane (10 mL) was added dropwise to a stirred solution of ethanolamine (1.2 mL, 20.7 mmol) and imidazole (2.78 g, 40.8 mmol) in dichloromethane (20 mL) at room

temperature, and the resulting mixture was stirred overnight at room temperature. Water (20 mL) was then added, and the phases were separated. The aqueous phase was extracted with dichloromethane (3 × 20 mL), and the combined organic phases were dried with MgSO₄, filtered and the solvents were evaporated under reduced pressure to give the 2-(tert-butyltrimethylsiloxy)ethanamine (2.54 g, 14.5 mmol, 70%) that was used without further purification for the synthesis of OHdiA-ethanolamine. ¹H NMR (400 MHz, CDCl₃) δ 3.67 – 3.49 (m, 2H), 2.75 (t, *J* = 5.2 Hz, 2H), 0.82 – 0.80 (m, 9H), –0.02 (dd, *J* = 4.4, 2.6 Hz, 6H).

1,5-Dioxocane-2,8-dione (234 mg, 1.5 mmol) was combined with 2-(tert-butyltrimethylsiloxy)ethanamine (179 mg, 1 mmol) in 15.0 mL of dichloromethane and kept at room temperature under Ar for 24 h. The solvents were then evaporated under reduced pressure and the residue was purified by flash chromatography on a silica gel column (CH₂Cl₂ : MeOH = 25:1, TLC: *R*_f = 0.18) to give 7-((2-(tert-butyltrimethylsilyl)oxy)ethyl)amino)-4,7-dioxoheptanoic acid (165 mg, 0.5 mmol, 50%). ¹H NMR (400 MHz, CDCl₃) δ 6.36 – 6.13 (m, 1H), 3.71 – 3.57 (m, 2H), 3.30 (p, *J* = 5.0 Hz, 1H), 2.85 – 2.65 (m, 1H), 2.63 – 2.53 (m, 1H), 2.44 (dd, *J* = 9.7, 6.5 Hz, 1H), 0.97 – 0.77 (m, 2H), 0.19 – –0.15 (m, 1H). ESI-MS: *m/z* calcd for C₁₅H₂₈NO₅Si [M–H][–], 330.17, found 330.33.

7-((2-(Tert-butyltrimethylsilyl)oxy)ethyl)amino)-4,7-dioxoheptanoic acid (33 mg, 0.1 mmol) was incubated in 10 mL of mixture of AcOH, H₂O and THF (3:1:1, V:V:V) for 3.5 h at 50 °C under Ar. The solvents were then evaporated under reduced pressure and the residue was purified by flash chromatography on a silica gel column (CH₂Cl₂:MeOH 5:1, TLC: *R*_f = 0.2) to give pure 7-((2-hydroxyethyl)amino)-4,7-dioxoheptanoic acid (OHdiA-ethanolamine, 10 mg, 46%). ¹H NMR (400 MHz, CD₃OD) δ 3.58 (dd, *J* = 7.1, 4.6 Hz, 2H), 3.27 (dd, *J* = 8.0, 3.6 Hz, 2H), 2.83 – 2.72 (m, 4H), 2.54 (t, *J* = 6.5 Hz, 2H), 2.47 (t, *J* = 6.8 Hz, 2H). ESI-MS: *m/z* calcd for C₉H₁₄NO₅[–] [M–H][–], 216.09, found 216.67; *m/z* calcd for C₉H₁₅NO₅Na [M+Na]⁺, 240.08, found 240.80.

7-((9H-Fluoren-9-yl)methoxy)-4,7-dioxoheptanoic acid

1,5-Dioxocane-2,8-dione (156 mg, 1 mmol) and (9H-fluoren-9-yl)-methanol (FmOH, 196 mg, 1 mmol) were dissolved in chloroform (5 mL). After stirring at approximately 110 °C overnight, the mixture was evaporated, and the residue was distilled, bp 124 °C/0.2 torr, to give the pure product acid 2.3 (257 mg, 73 %). ¹H NMR (CDCl₃, 400 MHz), δ 9.78 (s, 1H), 7.77 (d, *J* = 6.7 Hz, 2H), 7.60 (d, *J* = 7.7 Hz, 2H), 7.42 (dd, *J* = 6.7, 7.7 Hz, 2H), 7.30 (dd, *J* = 6.7, 7.7 Hz, 2H), 4.39 (d, *J* = 7.4 Hz, 2H), 4.20 (t, *J* = 7.4 Hz, 1H), 2.61–2.79 (8H). HRMS (FAB): *m/z* calcd for C₂₁H₂₀O₅Na (MNa⁺), 375.1203; found, 375.1204.

4-Oxoheptanedioic monoamide protein derivatives: human serum albumin (OHdiA-HSA)

N-Hydroxysulfosuccinimide (sulfo-NHS, 198 mg, 1.7 mmol) and 1-ethyl-3-[3-(dimethylaminopropyl)carbodiimide (EDC, 93 mg, 0.6 mmol) were added to 5 mL of a DMF/0.1 M MES (pH 6.0) (1:1, v/v) solution containing 7-[(9H-fluoren-9-yl)methoxy]-4,7-dioxoheptanoic acid (12 mg, 0.06 mmol), was stirred at room temperature for 15 minutes. Then 200 μL of saturated sodium bicarbonate solution was added to adjust the pH to above 7.0. Then 60 mg of HSA in 3.5 mL PBS buffer solution was added to the mixture. The

solution was stirred under argon for 8 hours and then 200 μ L DBU was added and stirring continued overnight. This was followed by two successive 6 h dialyses (Mr cutoff 14000) against 500 mL 40 % DMF in 0.1 M MES (pH 6.0) and two additional 6 h dialyses against 500 mL 10 mM PBS (pH 7.4) at 4 °C. The final protein concentration (3.60 mg/mL) was determined by the Pierce bicinchoninic acid (BCA) protein assay and the OHdiA concentration (720 μ M) was determined by trinitrobenzene sulfonic acid (TNBS) assay. First, protein solution (20–200 μ g/mL) was dissolved in 0.1 M sodium bicarbonate buffer (pH 8.5) and 5% TNBS solution was diluted 500-fold in 0.1 M sodium bicarbonate buffer (pH 8.5). Diluted TNBS solution (0.5 mL) was added to the protein solution (1 mL) and mixed well. Absorbance of the mixture at 335 nm was measured. Concentration of primary amines was determined by calculation from the extinction coefficient or by comparison to amino acid standards. 4-Oxoheptanedioic monoamide protein derivatives of bovine serum albumin (**OHdiA-BSA**) and keyhole limpet hemocyanin (**OHdiA-KLH**) were prepared similarly (see Supplemental Information).

7-(5-(2-Acetamidocaetamido)-6-methoxy-6-oxohexylamino-4,7-dioxoheptanoic acid (OHdiA-dipeptide)

Methyl 6-amino-2-((2-acetylamino)acetyl)amino hexanoate (Ac-Gly-Lys-OMe, 130 mg, 0.5 mmol) and anhydride **7** (78 mg, 0.5 mmol) was refluxed in acetic acid (4 mL) overnight. The solvent was removed by rotary evaporation, and the crude compound was purified by silica gel chromatography (10% methanol in chloroform) to give 162 mg (78%) of pure OHdiA-dipeptide, TLC (10% methanol in chloroform): R_f = 0.24. T. ^1H NMR (CDCl_3 , 400 MHz), δ 7.77 (d, J = 6.7 Hz, 2H), 7.60 (d, J = 7.7 Hz, 2H), 7.42 (dd, J = 6.7, 7.7 Hz, 2H), 7.30 (dd, J = 6.7, 7.7 Hz, 2H), 4.39 (d, J = 7.4 Hz, 2H), 4.20 (t, J = 7.4 Hz, 1H), 2.60–2.79 (8H). ^{13}C NMR (CDCl_3 , 100 MHz) δ 175.98, 175.86, 174.47, 172.68, 171.05, 169.38, 102.68, 52.69, 52.28, 43.18, 39.41, 33.72, 31.42, 29.61, 28.73, 28.65, 23.15, 22.83. HRMS (FAB): m/z calcd for $\text{C}_{18}\text{H}_{29}\text{N}_3\text{O}_8\text{Na}$ (MNa^+), 438.1741; found, 438.1742.

Free radical-induced oxidation of DHA-PC in the presence of HSA

Small unilamellar vesicles (SUVs) comprised of DHA-PC (2 mg/mL) were prepared by extrusion (11 times) through a 0.1 μ m polycarbonate filter using an Avanti Mini-Extruder (Avanti Polar Lipids, Inc., Alabaster, AL) in argon sparged phosphate buffered saline (PBS, 10 mM, pH 7.4). Liposome solution (1 mL) containing DHA-PC (2 mg/mL) was mixed with HSA (16.5 mg) in PBS (4 mL) to give final concentrations of DHA-PC (0.4 mg/mL) and HSA (3.3 mg/mL). Autoxidation was initiated by the addition of 1 M CuSO_4 (5.0 μ L) and 5 M H_2O_2 (10 μ L). The autoxidation proceeded at 37 °C. Aliquots (1200 μ L/each) were withdrawn and autoxidation was terminated by the addition of 200 mM BHT (6.0 μ L) and 0.1 mM EDTA (2.4 μ L) after various reaction times (0 h, 20 h, 30 h and 48 h). Then, all aliquots were dialyzed against 10 mM PBS, pH 7.4 (3 $\times$ 2 L) containing Na_2EDTA (1 mg/mL) for 48 h at room temperature. After dialysis, each aliquot was divided into six portions (200 μ L/each). Each aliquot (200 μ L) at various reaction times was hydrolyzed in 2000 μ L of 6 N HCl at 100 °C using a Fisher Scientific™ isotemp™ block heater (Model 2050FS) under Ar protection for 6 h. After adjusting the pH to 1–2 with 10 N NaOH, the mixture was extracted with ethyl acetate (3 $\times$ 4 mL). Volatiles were removed from the combined organic extracts under reduced pressure. The dried sample was redissolved in 800

μL of methanol and was divided equally into two vials. The solvent was then removed under a stream of dry N_2 . Then half of the dried sample was kept at -80°C under argon until LC-MS/MS analysis. The other half of the dried sample was used for PFB derivatization followed by extraction with ethyl acetate and ddH_2O as described above. The PFB derivatized product was dried and kept at -80°C under argon until LC-MS/MS analysis. For LC-MS/MS analysis, the dried samples were redissolved in MeOH ($60\ \mu\text{L}$). After vortexing and centrifuging for 10 min, $20.0\ \mu\text{L}$ of this solution was used for LC-MS/MS.

Free radical-induced oxidation of DHA-PC in the presence of DPPE

Small unilamellar vesicles (SUVs) comprised of DHA-PC ($1\ \text{mg/mL}$) and DPPE ($0.09\ \text{mg/mL}$) were prepared by extrusion (11 times) through a $0.1\ \mu\text{m}$ polycarbonate filter using an Avanti Mini-Extruder (Avanti Polar Lipids, Inc., Alabaster, AL) in argon sparged pH 7.4 $10\ \text{mM}$ PBS. Autoxidation was initiated by exposure to $1\ \text{mM}$ CuSO_4 and $10\ \text{mM}$ H_2O_2 . The autoxidation proceeded at 37°C . Aliquots ($600\ \mu\text{L}/\text{each}$) were withdrawn and the autoxidation was terminated by the addition of $100\ \text{mM}$ BHT ($6.0\ \mu\text{L}$) and $0.2\ \text{mM}$ EDTA ($0.6\ \mu\text{L}$) after various reaction times (0 h, 2 h, 5 h, 10 h, 22 h, 34 h and 48 h). Each aliquot was divided into six portions ($100\ \mu\text{L}/\text{each}$). Then, $100\ \mu\text{L}$ of each aliquot was hydrolyzed using $6\ \text{N}$ HCl at 100°C using a Fisher Scientific™ isotemp™ block heater (Model 2050FS) under Ar protection for 6 h. After adjusting the pH to 2–3 with $10\ \text{N}$ NaOH, the mixture was extracted with ethyl acetate ($3 \times 3\ \text{mL}$). The combined organic phases were dried under reduced pressure. Then half of the dried sample was kept at -80°C under argon before LC-MS/MS analysis. The other half of the dried sample was used for PFB derivatization followed by extraction with ethyl acetate and ddH_2O as described above. The PFB derivatization product was dried and kept at -80°C under argon until LC-MS/MS analysis. For LC-MS/MS analysis, the dried samples were redissolved in $50\ \mu\text{L}$ of MeOH. After vortexing and centrifuging for 10 min, $15.0\ \mu\text{L}$ of this solution was used for LC-MS/MS.

Extraction of human plasma lipids

Blood was collected into 7-ml vacutubes containing EDTA ($10.5\ \text{mg}$). Cells were removed by centrifugation at $2500\ \text{rpm}$ ($1300 \times g$) for 10 min, and the plasma was transferred to plastic vials containing butylated hydroxytoluene (BHT; $1\ \text{mg/mL}$), leupeptin ($35\ \mu\text{M}$), pepstatin ($5\ \mu\text{M}$), and aprotinin ($0.1\ \text{trypsin inhibitor unit/mL}$). Plasma was then quenched-frozen in liquid nitrogen and stored at -80°C until used. To separate protein from phospholipids, plasma samples were extracted by a modified Bligh & Dyer method adapted for small-scale extraction with precautions to prevent *in vitro* oxidation. In brief, $200\ \mu\text{L}$ of human plasma from healthy volunteers was mixed with $750\ \mu\text{L}$ of chloroform/methanol (1:2, v/v) containing $1\ \text{mM}$ BHT and the resulting mixture was vortexed vigorously for 1 min. Then $250\ \mu\text{L}$ of chloroform was added and the resulting mixture was vortexed vigorously for 1 min. Then $250\ \mu\text{L}$ of 1.5% aqueous sodium chloride solution was added and the resulting mixture was vortexed vigorously for 1 min. The resulting mixture was then centrifuged for 10 min at $14,000\ \text{rpm}$ to give a three-phase system (aqueous top, protein disk and organic bottom). The lower organic layer containing phospholipids was carefully transferred to a new tube, making sure to avoid the interface or upper layer. After transferring the upper aqueous layer to another separate tube, the protein disk at the interphase between the aqueous and organic phase was left. The solvents were then evaporated from the fractions

under a stream of dry nitrogen at room temperature. The dry protein and lipid fractions were hydrolyzed using 6 N HCl at 100 °C as described above. After adjusting the pH of reaction mixture to 2–3 with 10 N NaOH, the resulting mixture was extracted with ethyl acetate three times. The organic phases were combined and dried under a stream of dry nitrogen. Half of the dried sample was stored under argon at –80 °C until being analyzed using LC-MS/MS and half was used for PFB derivatization. After PFB derivatization, ethyl acetate and ddH₂O was used to extract the PFB derivative from the reaction product mixture. The organic phase was combined and dried under a stream of nitrogen. The dried sample was stored under argon at –80 °C until being analyzed using LC-MS/MS. For LC-MS/MS analysis, the dried sample was redissolved in 50 µL of MeOH containing OHdiA-diPFB-D₄ (5.0 µg/mL) as quantitation standard. After vortexing and centrifuging for 10 min, 15.0 µL of the solution was injected for LC-MS/MS analysis.

Cell adhesion assay

A 96-well plate was coated with fibronectin (1µg/mL) for 1 h at 37 °C followed by blocking with 1% BSA for 1 h. Then HUVECs were added to the fibronectin coated 96-well plate (10⁴ cells per well) and incubated for 1 h in the presence of various concentrations of OHdiA-BSA, BSA or VEGF as indicated. The cells were then washed with Dulbecco's modified Eagle's (DMEM)/F12 medium and fixed with 4% paraformaldehyde (PFA) in PBS. Then hematoxylin was added to stain the cells for 30 min. Then the cells were washed with PBS and photographs were taken.

Tube formation assay

HUVECs were seeded onto matrigel-coated 48-well plates (BD Bioscience). Cells were incubated with various concentrations of OHdiA-BSA, BSA or VEGF as indicated at 37 °C for 8 h. Tube formation was observed using a phase contrast inverted microscope, and photographs were taken of each well. The data were quantified by measuring the length of tubes with ImagePro software.

Statistical analysis

Statistical analyses were performed by using Student's *t* test. *P* value <0.05 is considered as statistically significant. All data are presented as mean ± SD for all studies. Probability values were based on the paired *t* test: NS – not significant, * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001.

RESULTS

Production and characterization of an anti-OHdiA rabbit polyclonal antibody

To develop an immunoassay for OHdiA derivatives, a polyclonal rabbit antibody (anti-OHdiA) was raised against OHdiA-keyhole limpet hemocyanin (KLH). Following immunization of a New Zealand white rabbit, the anti-OHdiA antibody titer increased and then plateaued after 80 days (Figure S1). The anti-OHdiA polyclonal antibody was purified on a protein A/G column. Briefly, rabbit serum was diluted with binding buffer (PBS, 10 mM, pH 7.4) and then loaded onto an equilibrated protein A/G column packed with immunopure protein A/G plus-agarose. To remove non-immunoglobulin G (non-IgG)

proteins, pH 7.4 binding buffer was first passed through the column (Figure S2). Then the bound IgG was eluted with elution buffer (100 mM glycine-HCl containing 150 mM NaCl, pH 2.5). The purified polyclonal antibody solution was neutralized immediately and then dialyzed against 10 mM PBS (pH 7.4) at 4 °C for 38 h. The resulting solutions of anti-OHdiA polyclonal antibody contained 0.528 mg/mL purified IgGs, as determined by Pierce bicinchoninic acid (BCA) protein assay.

Cross-reactivity of the anti-OHdiA monoamide polyclonal antibody

The structural specificity of the anti-OHdiA monoamide polyclonal antibody was assessed by competitive inhibition of antibody binding to OHdiA-BSA by various haptens was examined through enzyme-linked immunosorbent assay (ELISA) studies. For these studies, OHdiA-BSA was used as the coating agent and OHdiA-human serum albumin (HSA) was used as a standard. The concentration at 50% inhibition (IC_{50}) for OHdiA-HSA was defined as 100% cross-reactivity. The ELISAs were done as described previously for similar studies with CEP-KLH antibodies.¹⁷ As shown in Figure 1, CEP-HSA, which contains a carboxyethyl group appended to the 2-position of a pyrrole (Scheme 3), showed a significant inhibition of OHdiA monoamide antibody binding to OHdiA-BSA. The IC_{50} values for OHdiA-HSA and CEP-HSA using OHdiA-BSA as coating agent were 0.084 μ M and 0.291 μ M, respectively. Thus, the OHdiA monoamide antibody exhibits 29% cross reactivity with CEP-HSA. Since CEP-HSA showed significant cross-reactivity with the anti-OHdiA polyclonal antibody, it was of interest to compare this with the cross-reactivity of the anti-CEP polyclonal antibody with OHdiA-protein. For this purpose, CEP-BSA was used as coating agent and CEP-HSA as a standard for binding anti-CEP polyclonal antibody. The concentration at 50% inhibition (IC_{50}) for CEP-HSA was defined as 100% cross-reactivity. The IC_{50} value for CEP-HSA using CEP-BSA as coating agent was 0.1555 μ M (Figure S3).

Release of OHdiA from monoamide derivatives of biological amines

The low structural selectivity of anti-OHdiA monoamide antibodies limits their utility for specific immunological detection of OHdiA derivatives in biological samples. Therefore, an alternative analytical method was developed. As shown in Scheme 3, hydrolysis of OHdiA monoamide derivatives was expected to release OHdiA that was characterized by LC-tandem mass spectrometry (LC-MS/MS) comparison with authentic samples of both the diacid OHdiA and a bispentafluorobenzyl ester (OHdiA-diPFB) generated by treatment of the hydrolysis reaction product mixture with pentafluorobenzyl bromide (PFB-Br). The overall workflow for analysis of OHdiA derivatives is shown in Scheme 4.

Syntheses of OHdiA-diPFB and OHdiA-diPFB-D₄

To facilitate characterization of OHdiA derived from biological samples by LC-ESI-MS/MS, authentic OHdiA-diPFB (**3**) was prepared by chemical synthesis (Scheme 5). To allow quantification by isotope dilution, a tetradeutero isotopomer 3,3,5,5-*d*₄-4-oxoheptanedioic acid (**4**) was prepared by a base catalyzed deuterium exchange of keto diacid **1**. The carboxyl groups in **4** were also derivatized with PFB-Br (**2**) to provide 3,3,5,5-*d*₄-OHdiA-diPFB (**5**).

Validation of acid-catalyzed hydrolysis for OHdiA adducts

The efficacy of HCl-mediated amide hydrolysis was tested with the ethanolamine monoamide **6** of OHdiA that was prepared as outlined in Scheme 6. OHdiA-ethanolamine amide **6** in 6 N HCl was heated at 100 °C for 5 hours. As shown in Figure S4, the hydrolysis of OHdiA-ethanolamine amide **6** went to completion delivering 4-oxoheptanedioic acid, as confirmed by ¹H NMR identity with an authentic sample.

Reaction of KOHA-PC with Ac-Gly-Lys-OMe followed by acid hydrolysis forms OHdiA

We tested the hypothesis that KOHA-PC reacts with the ε-amino group of a peptide lysyl residue to form an OHdiA adduct. A 2.5-fold excess (100 ng/μL) of the ketoaldehyde KOHA-PC (**10**) was incubated with the dipeptide Ac-Gly-Lys-OMe (**11**, 40 ng/μL) in methanol at room temperature for 48 hours. To detect the formation of OHdiA-PC-dipeptide (**12**), after the removal of solvents, the crude reaction product mixture was hydrolyzed with 6 N HCl at 100 °C for 5.0 h and the product mixture was analyzed by reverse phase HPLC-ESI/MS in the negative ion mode to detect the formation of **1** by comparison with a sample of authentic **1** (Scheme 7). In a control experiment, the absence of a significant peak in the SIC at *m/z* 173.2 (Figure 2B) corresponding to the peak at 2.42 min for the authentic sample of **1** (Figure 2A) established that KOHA-PC itself does not generate OHdiA upon acid hydrolysis in the absence of pretreatment with dipeptide **11**. In contrast, a significant peak at 2.40 min corresponding to **1** was detected in the product mixture from reaction of KOHA-PC with dipeptide **11** followed by acid hydrolysis of the putative amide intermediate **12** (Figure 2C). Thus, KOHA-PC reacts with the dipeptide to produce OHdiA-PC-dipeptide (**12**) that is hydrolyzed to form **1**.

To confirm the identity of the putative product **1** from incubation of KOHA-PC with dipeptide followed by hydrolysis, it was further analyzed by reverse phase HPLC/ESI/MS/MS using multiple reaction monitoring (MRM) in the negative ion mode. A MRM method was developed based on the collision induced dissociation (CID) spectrum of [M-H]⁻ of the parent ion for authentic 4-oxoheptanedioic acid (**1**) (Figure S5). Four representative daughter ions, *m/z* 85, 111, 129.2, and 137.1, were chosen as MRM transitions for fragmentations of the parent ion *m/z* 173.2 for analysis of 4-oxoheptanedioic acid. The proposed structure of each daughter ion is shown in the inset. As shown in Figure 3, treatment of KOHA-PC with dipeptide Ac-Gly-Lys-OMe for 48 h followed by acid-catalyzed hydrolysis generates a significant peak in the negative-ion LC-MS/MS chromatogram at 2.11 min. An identical peak appears in the chromatogram from authentic 4-oxoheptanedioic acid (**1**).

To further confirm the production of 4-oxoheptanedioic acid in the acid-catalyzed hydrolysis of the product mixture from the reaction of KOHA-PC with the dipeptide, the product mixture was treated with pentafluorobenzyl bromide (PFB-Br) to derivatize the two carboxylic acid functional groups for analysis by reverse phase HPLC/ESI/MS/MS using multiple reaction monitoring (MRM) in the positive ion mode. A MRM method was developed based on the CID spectrum of [M+H]⁺ the parent ion for authentic OHdiA-diPFB (**3**) (Figure S6). Three representative daughter ions, *m/z* 111.1, 181.2, and 337.0, were

chosen as MRM transitions for fragmentations of the parent ion m/z 535 for OHdiA-diPFB analysis. Possible structures of each daughter ion are shown in the inset.

Analyses following treatment with PFB-Br are shown in Figure 4. After reaction with PFB-Br, a major peak with a retention time at 11.2 min that is identical to that for a sample prepared from the pure diacid **1** (Figure 4A) is present in the positive-ion MRM chromatogram of the product mixture from KOHA-PC after reaction with the dipeptide followed by acid hydrolysis and pentafluorobenzoylation. (Figure 4B), demonstrating the generation of the OHdiA monoamide derivative.

Generation of OHdiA derivatives by in vitro oxidation of DHA-PC in the presence of human serum albumin (HSA)

Previously, it was demonstrated that free radical-induced oxidative cleavage of DHA-PC promoted by myeloperoxidase or copper ions generates a large number of reactive electrophiles such as HOHA-PC and KOHA-PC.¹⁰ It was established that condensation of HOHA-PC with the ϵ -amino group of a lysine containing dipeptide or protein could form CEP derivatives.^{10, 17} In vitro experiments also demonstrated that CEP derivatives could be generated during autoxidation of DHA-PC in the presence of protein.¹⁷ Since the studies described above showed that KOHA-PC could modify Ac-Gly-Lys-OMe dipeptide to generate OHdiA monoamide derivatives, it seemed likely that OHdiA monoamide derivatives would be also generated during autoxidation of DHA-PC in the presence of protein. Therefore, we next investigated the formation of OHdiA monoamide derivatives during in vitro free radical initiated oxidation of DHA-PC in the presence of HSA by the HPLC-ESI/MS/MS analysis using MRM developed as described above. As shown in Figure S7A, no significant peaks corresponding to OHdiA were detected in the reaction mixture before (0 h) oxidation of DHA-PC in presence of HSA followed by acid-catalyzed hydrolysis. After 48 h oxidation followed by acid-catalyzed hydrolysis, a significant peak was observed in the negative-ion LC-MS/MS chromatogram at 2.12 min as also observed for an authentic 4-oxoheptanedioic acid standard (Figure S7B). These results demonstrated that an OHdiA monoamide derivative is produced during autoxidation of DHA-PC in the presence of protein.

To further characterize the putative OHdiA formed in the oxidation product mixture from DHA-PC in the presence of HSA after acid-catalyzed hydrolysis, it was subjected to pentafluorobenzyl esterification followed by analysis by the reverse phase HPLC-ESI/MS/MS using MRM in the positive ion mode as described above. Analyses following treatment with PFB-Br are shown in Figure S8. DHA-PC in the presence of HSA prior to oxidation (0 h) produced barely detectable levels of OHdiA while oxidation for 48 h resulted in a high level of OHdiA. These results further confirmed the generation of OHdiA monoamide derivatives during autoxidation of DHA-PC in the presence of protein.

The time course for formation of OHdiA monoamide derivatives during autoxidation of DHA-PC in the presence of HSA was determined. The copper-promoted oxidation of DHA-PC vesicles with HSA in PBS was performed up to 48 h at 37 °C under air and aliquots were withdrawn after various reaction times and quenched by the addition of butylated hydroxytoluene (BHT) and ethylenediaminetetraacetic acid (EDTA). The reaction mixture

was then dialyzed against PBS containing EDTA for 48 h. The reaction mixture was then combined with 6 N HCl in a vial and heated for 5 h at 100 °C. The concentration of OHdiA was quantified by LC-MS/MS using a calibration curve established with authentic standard 4-oxoheptanedioic acid in methanol (Figure 5A). The product evolution profile showed that the OHdiA monoamide derivative level increased steadily in the first 30 h and more slowly afterward during autooxidation of DHA-PC in the presence of HSA (Figure 5B).

Generation of OHdiA by in vitro oxidation of DHA-PC in the presence of DPPE followed by hydrolysis with 6 N HCl at 100 °C

Although lys ε-amino groups in proteins are a major target of lipid peroxidation-derived aldehydes, such as α,β-unsaturated aldehydes, and various adducts have been identified, the primary amino groups of phosphatidylethanolamines (PEs) are also targets for covalent modification by lipid peroxidation products such as 4-HNE, acrolein and isolevuglandins.^{25–27} Therefore, it was expected that PEs would be modified by KOHA-PC generated from oxidation of DHA-PC to produce OHdiA monoamide derivatives. To test this hypothesis, the copper-catalyzed oxidation of DHA-PC was performed in the presence of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) followed by hydrolysis with HCl at 100 °C and analysis of the reaction product mixture by the LC-MS/MS method described above for detecting OHdiA. As shown in Figure 6, the copper-promoted oxidation of DHA-PC in the presence of DPPE for 48 h followed by acid-catalyzed hydrolysis, produced high levels of OHdiA as demonstrated by the detection of significant peaks with the same retention time as the authentic standard 4-oxoheptanedioic acid in the corresponding MRM transitions (Figure 6C) while a barely detectable level of OHdiA was observed prior to oxidation of DHA-PC in the presence of DPPE followed by acid-catalyzed hydrolysis (Figure 6B). In addition, the copper-promoted oxidation of DHA-PC in the absence of DPPE for 48 h followed by acid hydrolysis produced very low levels of OHdiA (Figure 6A). Collectively, these results indicated that covalent binding of KOHA-PC, generated from free radical-induced oxidation of DHA-PC *in vitro*, reacts with the primary amino group of PEs to produce OHdiA monoamide derivatives.

Further evidence for the formation of OHdiA derivatives by copper-promoted oxidation of DHA-PC in the presence of DPPE was secured by LC-MS/MS analysis after treatment of the product mixture with PFB-Br. As shown in Figure S9, strong peaks with the same retention time as authentic standard OHdiA-diPFB appeared in the MRM chromatograms of the product mixture generated from DHA-PC oxidation in the presence of DPPE (C) for 48 h, but not (B) 0 h or (A) for 48 h in the absence of DPPE as detected in peaks for three MRM transitions with a retention time that is identical to that of an authentic OHdiA-diPFB standard.

The time course for the formation of OHdiA derivatives during oxidation of DHA-PC in the presence of DPPE was also investigated and the concentration of endogenous OHdiA was quantified by LC-MS/MS using a calibration curve established with the commercially available authentic standard 4-oxoheptanedioic acid in methanol (Figure 7A). The product evolution profile for oxidation of DHA-PC in the presence of DPPE is shown in Figure 7B. The OHdiA monoamide level rose rapidly in the first 10 h and then more slowly afterward.

Detection of OHdiA derivatives in human plasma

We anticipated that OHdiA derivatives are formed *in vivo*. Therefore, we examined whether they could be detected in human blood. Analysis of human plasma after acid hydrolysis followed by PFB esterification provided evidence for the presence of OHdiA derivatives *in vivo*. Representative LC-MS/MS chromatograms of OHdiA detected in human plasma are shown in Figure 8B. The MRM transitions corresponding to OHdiA-diPFB coeluted at a retention time identical with that of the pure synthetic OHdiA-diPFB standard (Figure 8A).

Comparison of OHdiA modification levels in human plasma PE and protein

The levels of the putative OHdiA-PE and OHdiA-protein adducts in human plasma samples were compared. Before acid hydrolysis, blood plasma was extracted with chloroform/methanol by the Bligh and Dyer method to separate proteins and phospholipids.²⁸ To minimize interference owing to mass detector fluctuations and thereby increase accuracy, an isotope-labeled internal standard OHdiA-diPFB-d₄ was introduced prior to LC-MS analysis. The integrated peak area of OHdiA-diPFB in the MRM transition m/z 535>181.2 from different runs was normalized by comparing with that of the internal standard OHdiA-diPFB-d₄ in the MRM transition m/z 539>181. As shown in Figure 9, both OHdiA-PE adducts and protein adducts were detected in human plasma samples ($n = 3$) and the ratio of OHdiA-PE to protein adducts was 0.35, indicating that OHdiA-protein adducts are approximately three times as abundant as PE adducts in human plasma. Previously, it was shown that modification of proteins by LPO-derived aldehydes such as 4-HNE and isolevuglandin (also referred to by the misnomer “isoketal”) inhibits their proteasomal degradation.^{29, 30} Therefore, it is likely that OHdiA modification of proteins would similarly impair their clearance, potentially leading to their accumulation, and their observed predominance compared to OHdiA derivatives of ethanolamine phospholipids.

OHdiA-modified proteins induce angiogenesis *in vitro*

The biological activity of an OHdiA-modified protein was investigated using two different *in vitro* models of angiogenesis with human umbilical vein endothelial cells (HUVECs). Both OHdiA-BSA and vascular endothelial growth factor (VEGF) exhibit proangiogenic effects in adhesion and tube formation assays (Figure 10). In both adhesion and tube formation experiments, although 1 $\mu\text{g/mL}$ of OHdiA had no significant effect on angiogenesis compared to control BSA without OHdiA modification, 10 $\mu\text{g/mL}$ of OHdiA-BSA greatly promoted angiogenesis in HUVECs. As for OHdiA monoamides, CEP derivatives are generated through the peroxidation of DHA and are proangiogenic.¹⁹ The proangiogenic effects of OHdiA monoamide derivatives and CEP derivatives were compared using a tube formation assay. The tube length increased with increasing concentrations of both the CEP and OHdiA derivatives of the dipeptide Ac-Gly-Lys-OMe (**11**) and then plateaued (Figure 11). In the concentration range tested, the OHdiA-dipeptide showed comparable but significantly lower proangiogenic effects than the CEP-dipeptide.

Induction of angiogenesis by OHdiA-modified proteins is toll-like receptor (TLR)2-dependent

Having established that OHdiA-BSA has proangiogenic effects on HUVECs, the mechanism of OHdiA-BSA-induced angiogenesis was explored. Because it recognizes a broad range of protein and lipid ligands including CEP, we suspected that the angiogenic activity of OHdiA derivatives is TLR2-dependent. TLR2 and TLR4 are expressed on the endothelium³¹ and implicated in angiogenesis.^{18, 19, 32} Tube formation assays with HUVECs treated with OHdiA-BSA, VEGF or control BSA in the presence of anti-TLR2, anti-TLR4 and anti-isotype control (IgG2a) antibodies are shown in Figure 12. Anti-TLR2, but not antibodies against TLR4, inhibited OHdiA-BSA-induced, but not VEGF-induced, tube formation. Thus, OHdiA-driven angiogenesis is similar to that promoted by CEP which is also TLR2-dependent and VEGF-independent.

DISCUSSION

Polyclonal rabbit anti-OHdiA monoamide antibodies cross-react with CEP epitopes

In a previous study,¹⁷ a polyclonal rabbit anti-CEP antibody showed high structural selectivity for not binding with CPP-HSA or CHP-HSA in competitive binding inhibition assays with CPP-HSA and CHP-HSA even though the CEP epitope differs from the CPP epitope by only a single CH₂ group (Scheme 8). Especially noteworthy is the fact that in the present study, OHdiA-HSA also did not show significant inhibition of anti-CEP binding to CEP-HSA. Thus, the remarkable selectivity of rabbit polyclonal anti-CEP antibodies for CEP versus CPP or CHP epitopes is now reconfirmed and extended to include a high level of discrimination for CEP versus OHdiA monoamide derivatives.

Perhaps the OHdiA monoamide antibodies cross-react with CEP because they recognize the cyclic OHdiA-BSA pyrrolidone tautomer that has a carboxyethyl group appended to the pyrrolidone 2 position (Scheme 8). Under equilibrating conditions, γ -keto amides tautomerize completely to the cyclic carbinol amide, i.e., pyrrolidone, form in aqueous solution.²⁴ The antigen-binding site of the OHdiA monoamide antibody might accommodate the CEP epitope owing to their similar structures, i.e., ω -carboxyethyl group appended to the 2-position of a 5-membered nitrogen heterocycle. In contrast, the carboxypropylpyrrole (CPP)-HSA and carboxyheptylpyrrole (CHP)-HSA epitopes, which have larger carboxylic acid side chains (three and seven CH₂ groups, respectively) than CEP-HSA and would not be expected to fit the OHdiA monoamide binding site, exhibit no cross-reactivity with the OHdiA monoamide. As a consequence of the nonplanarity of the 2-(ω -carboxyethyl)pyrrolidone tautomer of the OHdiA monoamide epitope, its binding pocket is expected to be slightly larger than that required for the planer CEP pyrrole ring of CEP-HSA. In contrast, the bulkier OHdiA monoamide epitope does not fit the antigen binding site of the anti-CEP antibody. This cross-reactivity suggested the possibility that OHdiA monoamides would exhibit receptor-mediated biological activity similar to that of CEP.

LC-MS/MS analysis of OHdiA monoamides provides a measure of docosahexaenoate phospholipid oxidation

Previously, we measured levels of CEP-modified ethanolamine phospholipids in human blood by LC-MS/MS. The present study demonstrated that KOHA-PC, an oxidatively-truncated docosahexaenoate phospholipid, reacts with proteins and ethanolamine phospholipids to generate OHdiA monoamide derivatives. This chemistry is expected to generate an extraordinarily complex mixture of modified biomolecules *in vivo*. This complexity makes the detection of individual OHdiA modified biomolecules challenging. On the other hand, we also demonstrated that biomolecule-derived monoamides, as well as the simple monoamide of ethanolamine, could all be hydrolyzed to provide an identical DHA-derived diacid, OHdiA. Similar treatment of KOHA-PC in the absence of protein, peptide or ethanolamine catalysts, does not produce OHdiA. Thus, acid hydrolysis followed by LC-MS/MS analysis, with or without conversion of the diacid OHdiA into a pentafluorobenzyl diester, provides a sensitive and specific assay for total OHdiA monoamide derivatives in biological samples. Quantitation of these assays is readily achieved using deuterated isotopomers as standards. OHdiA monoamide derivatives were detected in human blood, and levels of total protein OHdiA monoamides were significantly higher than levels of total phospholipid OHdiA monoamides, perhaps reflecting accumulation of the protein derivatives because clearance by proteasomal processing is impaired by covalent modification of protein lysyl residues.

OHdiA monoamides promote angiogenesis in a TLR2-dependent manner

OHdiA monoamide derivatives promote angiogenesis of endothelial cells in a TLR2-dependent manner with slightly lower efficacy than CEP derivatives. The similar receptor-mediated biological activity of CEP and OHdiA monoamide derivatives and binding with rabbit polyclonal anti-OHdiA antibodies suggests structurally similar epitopes, and further suggests that recognition of OHdiA amides may involve the 2-(ω -carboxyethyl)pyrrolidone tautomer that incorporates the lysyl ϵ -amino group in a 5-membered nitrogen heterocycle as is the case for CEP derivatives.

CONCLUSIONS

By analogy with the chemistry of ONE, we postulated that KOHA-PC, an oxidatively truncated docosahexaenoate phospholipid that incorporates identical 4-oxo-2-alkenal functionality, modifies biomolecules to give 4-ketoamide derivatives. The remarkably high specificity of our rabbit polyclonal anti-CEP antibody reported previously has now been expanded to include the observation of no cross-reactivity with OHdiA-HSA. In contrast, we now generated a rabbit polyclonal anti-OHdiA monoamide antibody that exhibit significant cross-reactivity with CEP-HSA. Anti-OHdiA monoamide binds with both CEP and OHdiA monoamide epitopes making it of no value for the specific analysis of OHdiA monoamides in biological samples. OHdiA was detected by LC-MS/MS in the reaction mixture produced by incubation of KOHA-PC in the presence of the dipeptide Ac-Gly-Lys-OMe followed by HCl-catalyzed hydrolysis of putative OHdiA monoamide derivatives. In contrast, similar treatment of KOHA-PC in the absence of the dipeptide did not produce OHdiA. In addition, the formation of OHdiA was detected by LC-MS/MS of the reaction product mixture from

autoxidation of DHA-PC in the presence of HSA or DPPE followed by HCl-catalyzed hydrolysis, indicating that KOHA-PC not only modifies the primary ϵ -amino group of protein lysyl residues but also those of the ethanolamine head group of phosphatidylethanolamines. OHdiA monoamides were detected in human blood. Both adhesion and tube formation assays demonstrated that OHdiA-BSA is angiogenic toward HUVECs. OHdiA-driven angiogenesis is TLR2-dependent similar to that promoted by CEP but different from VEGF-promoted angiogenesis. These observations provide additional evidence for the importance of lipid oxidation in promoting angiogenesis. The unselective binding of anti-OHdiA monoamide antibodies with both OHdiA monoamides and CEPs may be useful for antiangiogenic therapeutic applications as decoy receptors that inhibit TLR2-dependent pathological effects of CEP and OHdiA monoamide epitopes. Such applications might complement antiangiogenic anti-VEGF therapy that is clinically important for inhibiting tumor growth and the choroidal neovascularization associated with age-related macular degeneration, especially for AMD and cancers resistant to or after failure of anti-VEGF therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

ABTS	2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid diammonium salt
AMD	age-related macular degeneration
BSA	bovine serum albumin
CEP	2-(ω -carboxyethyl)pyrrole
CID	collision-induced dissociation
DHA	docosahexaenoic acid
DHA-PC	1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine
DPPE	1,2-dipalmitoyl- <i>sn</i> -glycero-3-phosphoethanolamine
ELISA	enzyme-linked immunosorbent assay
EP	ethanolamine phospholipid
ETN	ethanolamine

4-HNE	4-hydroxy-2-nonenal
HODA	9-hydroxy-12-oxododec-10-enoic acid
HOHA	4-hydroxy-7-oxo-hept-5-enoic acid
HOHA-PC	1-palmitoyl-2-(4-hydroxy-7-oxo-5-heptenoyl)- <i>sn</i> -glycero-3-phosphatidylcholine
HOOA	5-hydroxy-8-oxooct-6-enoic acid
HSA	human serum albumin
HUVECs	human umbilical vein endothelial cells
KLH	keyhole limpet hemocyanin
KODA	9-keto-12-oxododec-10-enoic acid
KOHA	4-keto-7-oxohept-5-enoic acid
KOOA	5-keto-8-oxooct-6-enoic acid
MRM	multiple reaction monitoring
OHdiA	4-oxo-heptanedioic acid
OHdiA-ethanolamine	7-((2-Hydroxyethyl)amino)-4,7-dioxoheptanoic acid
OHdiA-diPFB	bis(perfluorophenyl) 4-oxoheptanedioate
4-ONE	4-oxo-2-nonenal
oxLDL	oxidized low-density lipoprotein
PFB-Br	pentafluorobenzyl bromide
PP	2-pentylpyrrole
PUFA	polyunsaturated fatty acid
SICs	selected ion chromatograms
SUV	small unilamellar vesicle
TLR	Toll-like receptor

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Figure 1.

Inhibition curves for binding of the anti-OHdiA-KLH polyclonal antibody to OHdiA-BSA by OHdiA-HSA (closed circle), CEP-HSA (open triangle), CPP-HSA (closed square) and CHP-HSA (open diamond). In contrast, anti-CEP polyclonal rabbit antibody showed no significant inhibition of anti-CEP binding to CEP-HSA (Figure S3).

Figure 2.

Selected ion chromatograms (SICs) in the negative ion mode of m/z 173.2 from (A) pure authentic standard **1**, (B) the reaction product mixture from incubation of KOHA-PC for 48 h without dipeptide **11** followed by hydrolysis with 6 N HCl at 100 °C for 5 h, and (C) incubation of KOHA-PC with dipeptide **11** for 48 h followed by hydrolysis with 6 N HCl at 100 °C for 5 h.

Figure 3. HPLC/ESI/MS/MS analysis of OHdiA adduct production in the reaction of KOHA-PC with Ac-Gly-Lys-OMe dipeptide. (A) Authentic 4-oxoheptanedioic acid (**1**) standard. (B) Reaction product mixture after 48 h incubation at room temperature.

Figure 4. HPLC/ESI/MS/MS analysis after acid hydrolysis and pentafluorobenzyl esterification of the OHdiA monoamide produced by the reaction of KOHA-PC with Ac-Gly-Lys-OMe dipeptide. (A) Authentic OHdiA-diPFB standard. (B) Reaction product mixture from incubation of KOHA-PC with dipeptide for 48 h at room temperature followed by hydrolysis with 6 N HCl and pentafluorobenzyl esterification.

Figure 5. (A) Calibration curve for OHdiA using the MRM transition 173.2>111 and (B) evolution profile of OHdiA generation in the reaction of DHA-PC with HSA followed by acid-catalyzed hydrolysis. The data was obtained from three replicated experiments.

Figure 6. HPLC/ESI/MS/MS analysis of OHdiA produced by copper-catalyzed oxidation of DHA-PC for 48 h (A) without DPPE or (B) with DPPE after 0 h incubation or (C) with DPPE after 48 h incubation followed by hydrolysis with 6 N HCl at 100 °C for 5 h.

Figure 7.

(A) Calibration curve for OHdiA detected by the MRM transition 173.2>111 and (B) evolution profile of OHdiA generated by the copper-catalyzed oxidation of DHA-PC in the presence (filled square) or absence of DPPE (filled circle) followed by hydrolysis with 6 N HCl at 100 °C for 5 h. The data represents three replicated experiments.

Figure 8.

LC/ESI/MS/MS evidence for OHdiA derivatives in human plasma. OHdiA-diPFB was detected after acid hydrolysis followed by PFB esterification. (A) MRM for synthetic authentic standard OHdiA-diPFB. (B) MRM for PFB derivative of 4-oxoheptanedioic acid from human plasma after acid hydrolysis and PFB derivatization.

Figure 9. Levels of OHdiA-PE and OHdiA-protein derivatives in human plasma ($n = 3$, $p = 0.027$) measured by LC-MS/MS. Results are presented as mean $\pm$ SD. The peak area ratio of OHdiA-diPFB in the MRM transition m/z 535>181.2 to the internal standard OHdiA-diPFB- d_4 in the MRM transition m/z 539>181 from the LC-MS/MS analysis was used for the analyses.

Figure 10.

OHdiA monoamide derivatives induce angiogenesis in vitro in HUVECs in both (A) adhesion and (B) tube formation assays. Left: representative micrographs of HUVEC cells treated with OHdiA-BSA (1 or 10 μ g/mL), BSA (control), or VEGF (60 ng/mL). Right: quantification of adhesion and tube formation reported as means $\pm$ S.D. ($n = 3$). NS represent no significant difference, *** represents $p < 0.001$.

Figure 11. Comparison of the proangiogenic effect of CEP and OHdiA derivatives in HUVECs in a tube formation assay. Data reported as means $\pm$ S.D. (n = 3).

Figure 12.

HUVEC tube formation stimulated by OHdiA-BSA is TLR2-dependent. (A) Representative micrographs of HUVECs treated with OHdiA-BSA (10 μ g/mL), VEGF (60 ng/mL) or control BSA in the presence of anti-TLR2, anti-TLR4 or isotype control (IgG2a) antibodies as indicated. (B) Quantification of tube formation reported as means and showing $\pm$ S.D. (n = 4). NS represents no significant difference, *** represents $p < 0.001$.

Scheme 1.

γ -Oxygenated- α,β -unsaturated aldehyde lipid peroxidation products.

Scheme 2.

The formation of CEP and OHdiA adducts of primary amines.

R = protein or ethanolaminephospholipid

OHdiA monoamide

4-oxoheptanedioic acid

OHdiA-PFB

Scheme 3.

Strategy for the identification of OHdiA monoamide derivatives.

Scheme 4.

Workflow for analysis of OHdiA monoamides by LC-MS/MS.

Scheme 5.
Syntheses of OHdiA-diPFB and d_4 -OHdiA-diPFB.

Scheme 6.

Synthesis and hydrolysis of OHdiA-ethanolamine (6).

Scheme 7.

KOHA-PC reacts with Ac-Gly-Lys-OMe to form OHdiA-PC-dipeptide (**12**).

Scheme 8.

Carboxyalkylpyrroles, OHdiA monoamides, and the tautomeric 4-hydroxypyrolidin-2-ones.