Chemical Research in Toxicology

Article

pubs.acs.org/crt

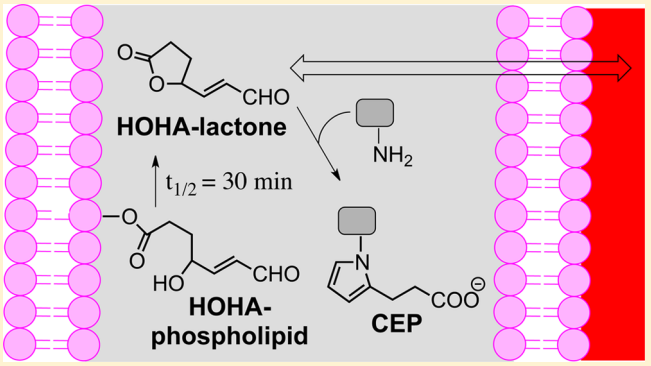
4-Hydroxy-7-oxo-5-heptenoic Acid (HOHA) Lactone is a Biologically Active Precursor for the Generation of 2-(ω-Carboxyethyl)pyrrole (CEP) Derivatives of Proteins and Ethanolamine Phospholipids

Hua Wang, Mikhail Linetsky, Junhong Guo, Jaewoo Choi, Li Hong, Amanda S. Chamberlain, Scott J. Howell, Andrew M. Howes, and Robert G. Salomon*

Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106, United States

Supporting Information

ABSTRACT: 2-(ω-Carboxyethyl)pyrrole (CEP) derivatives of proteins were previously shown to have significant pathological and physiological relevance to age-related macular degeneration, cancer and wound healing. Previously, we showed that CEPs are generated in the reaction of ε-amino groups of protein lysyl residues with 1-palmityl-2-(4-hydroxy-7-oxo-5-heptenoyl)-*sn*-glycero-3-phosphatidylcholine (HOHA-PC), a lipid oxidation product uniquely generated by oxidative truncation of docosahexanenate-containing phosphatidylcholine. More recently, we found that HOHA-PC rapidly releases HOHA-lactone and 2-lyso-PC ($t_{1/2}$ = 30 min at 37 °C) by nonenzymatic transesterification/deacylation. Now we report that HOHA-lactone reacts with Ac-Gly-Lys-OMe or human serum albumin to form CEP derivatives in vitro. Incubation of human red blood cell ghosts with HOHA-lactone generates CEP derivatives of membrane proteins and ethanolamine phospholipids. Quantitative analysis of the products generated in the reaction HOHA-PC with Ac-Gly-Lys-OMe showed that HOHA-PC mainly forms CEP-dipeptide that is not esterified to 2-lysophosphatidycholine. Thus, the HOHA-lactone pathway predominates over the direct reaction of HOHA-PC to produce the CEP-PC-dipeptide derivative. Myeloperoxidase/H_2O_2/NO_2^- promoted in vitro oxidation of either 1-palmityl-2-docosahexaneoyl-*sn*-glycero-3-phosphatidylcholine (DHA-PC) or docosahexaenoic acid (DHA) generates HOHA-lactone in yields of 0.45% and 0.78%, respectively. Lipid oxidation in human red blood cell ghosts also releases HOHA-lactone. Oxidative injury of ARPE-19 human retinal pigmented epithelial cells by exposure to H_2O_2 generated CEP derivatives. Treatment of ARPE-19 cells with HOHA-lactone generated CEP-modified proteins. Low (submicromolar), but not high, concentrations of HOHA-lactone promote increased vascular endothelial growth factor (VEGF) secretion by ARPE-19 cells. Therefore, HOHA-lactone not only serves as an intermediate for the generation of CEPs but also is a biologically active oxidative truncation product from docosahexaenoate lipids.



INTRODUCTION

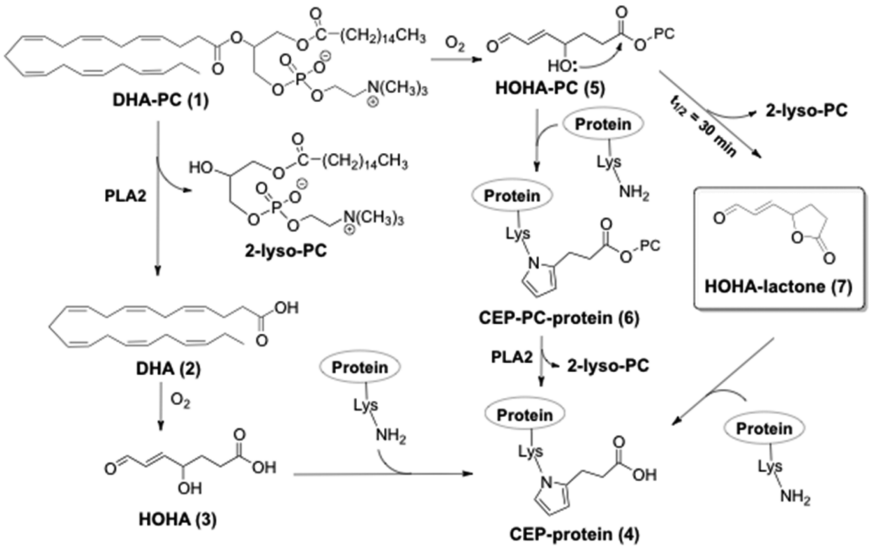
Our basic research on the chemical biology of lipid oxidation led to the discovery of 2-(ω-carboxyethyl)pyrrole (CEP)-modifications of proteins and ethanolamine phospholipids.[1] Using anti-CEP antibodies raised against CEP-protein,[2] CEP immunoreactivity was first detected in vivo in photoreceptor rod outer segments and in retinal pigmented epithelium. CEP derivatives were found in plasma[2] from individuals with age-related macular degeneration (AMD) and in drusen from AMD donor eye tissues,[3] in brain tissue from autistic children,[4] and in human melanoma skin tissue and healing wounds.[5] CEP-protein, CEP-peptide, and CEP-ethanolamine phospholipids promote vascular angiogenesis and platelet activation through Toll-like receptor-2[5] and Toll-like receptor-9[6] dependent signaling pathways, respectively. In addition, immunization of mice with CEP-modified mouse serum albumin induces AMD-like lesions in their retinas.[7] Such important physiological and pathological activities tightly link CEPs to human diseases and oxidative stress.

The generation of CEP-protein derivatives **4** (Scheme 1) results from an oxidative cleavage of docosahexaenoic acid (DHA)-containing phospholipids, such as 1-palmityl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (DHA-PC, **1**). Oxidative cleavage might occur after PLA2-catalyzed hydrolysis to DHA (**2**) to generate 4-hydroxy-7-oxo-hept-5-eonate (HOHA, **3**) that produces CEP-protein derivatives **4** through condensation with the primary amino groups of protein lysyl residues. Release of free fatty acid from membrane phospholipids under catalysis of phospholipase (PL) A2 occurs during oxidative stress.[8] Alternatively, oxidative cleavage of DHA-PC (**1**) might occur first to deliver 1-palmityl-2-(4-

Received: January 1, 2015

DOI: 10.1021/acs.chemrestox.5b00001
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

Scheme 1



hydroxy-7-oxo-5-heptenoyl)-*sn*-glycero-3-phosphatidylcholine (HOHA-PC, **5**). Condensation of this oxidatively truncated phospholipid with the primary amino groups of protein lysyl residues could be followed by PLA2-catalyzed hydrolysis of an intermediate CEP-PC-protein derivative **6** to deliver **4**.[9,10] In vitro experiments demonstrated that CEP-PC derivatives could be generated during autoxidation of DHA-PC in the presence of protein.

Another plausible pathway for the generation of CEPs was suggested by our finding that HOHA-PC (**5**) undergoes spontaneous deacylation with a half-life of 30 min under physiological conditions, that is, 37 °C and pH 7.4.[11] Intramolecular transesterification of HOHA-PC (**5**) generates 2-lyso-PC and a five-member ring lactone aldehyde, HOHA-lactone (**7**, Scheme 1). It seemed reasonable to expect that HOHA-lactone would react with primary amino groups in biomolecules to generate CEPs. We now report that HOHA-lactone readily forms CEP derivatives by reaction with Ac-Gly-Lys-OMe, proteins and ethanolamine phospholipids. We also find that HOHA-lactone is generated through autoxidation not only of DHA-PC but also of unesterified DHA. Thus, the equilibrium between lactone **7** and γ-hydroxy acid **3** strongly favors the lactone even in an aqueous environment. Evidence is also presented showing that deacylation of HOHA-PC (**5**) can predominate over its reaction with primary amines, and therefore the production of CEPs from HOHA-PC in vivo can occur entirely nonenzymatically, for example, without the need for phospholipase activity that is required to convert CEP-PC-protein (**6**) into CEP-protein (**4**).

EXPERIMENTAL PROCEDURES

Materials. Human serum albumin (HSA, fraction V), DHA, and phospholipase D *Streptomyces chromofuscus* (PLD) were purchased from Sigma (St. Louis, MO). The Ac-Gly-Lys-OMe dipeptide was purchased from Bachem (Torrance, CA). DHA-PC and LysoPC (13:0) (1-tridecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). CEP-dipeptide (**7**) and CEP-lysine,[12] HOHA-PC and HOHA-lactone,[13] mouse monoclonal anti-CEP antibody,[14] CEP-ethanolamine (**14**), and d_4-CEP-ethanolamine[1] were prepared as described previously. CEP-PC-dipeptide (**11**) was prepared through a Paar–Knorr pyrrole synthesis as described in the Supporting Information. 2,2′-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) was from Thermo Scientific (Rockford, IL). Dulbecco's modified Eagle's medium, high glucose (DMEM/HG), fetal bovine serum, penicillin–streptomycin, and L-glutamine were obtained from Gibco (Life Technologies, Grand Island, NY). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Invitrogen. ARPE-19 cells were obtained from American Type Culture Collection (CRL-2302; ATCC, Rockville, MD). A human VEGF-A ELISA kit was purchased from Piercenet (Thermo Fisher Scientific, Rockford, IL).

Reaction of Ac-Gly-Lys-OMe with HOHA-PC or HOHA-Lactone. Ac-Gly-Lys-OMe (2 mM) was incubated with either HOHA-PC (0.2 mM) or HOHA-lactone (1 mM) in a binary solution mixture of phosphate buffered saline (PBS) and methanol (9:1, v/v). Reaction vials were blanketed with argon, and were incubated at 37 °C. For the reaction with HOHA-PC, 50 μL aliquots of the reaction product mixture were withdrawn after various reaction times. Methanol (200 μL), internal standards CEP-lysine (50 ng) and lysoPC (13:0) (50 ng) were added. The above solution (10 μL) was injected into the LC-MS/MS for analysis. CEP-dipeptide and CEP-PC-dipeptide concentrations were determined using calibration curves that were obtained by using pure CEP-dipeptide and CEP-PC-dipeptide standards at various known concentrations spiked with 50 ng of CEP-lysine and lysoPC (13:0). For the reaction of Ac-Gly-Lys-OMe with HOHA-lactone, 20 μL aliquots of the reaction mixture were withdrawn after various reaction times. Methanol (200 μL) and 50 ng of CEP-lysine internal standard were added and the resulting solution (10 μL) was injected into HPLC-ESI/MS/MS for analysis.

Reaction of Human Serum Albumin with HOHA-Lactone. A sterile solution of human serum albumin (HSA, 1 mg/mL) was incubated with 0.5 mM HOHA-lactone in 1 mL PBS for up to 3 days at 37 °C. Aliquots of the reaction mixture were withdrawn at various times and then quenched by four dialyses against 1 L of 1× PBS. The final protein concentration after dialysis was determined by BCA protein assay (Piercenet, Rockford, IL). An aliquot (10 μL) of each modified HSA-protein was loaded onto an SDS PAGE gel and then analyzed by either Western blot with monoclonal mouse anti-CEP antibody or by Coomassie blue staining. For Western blot analysis, proteins were transferred to a nitrocellulose membrane (0.45 μ). The membrane was subsequently blocked with 5% BSA-TBST. The CEP modifications on proteins were detected with 0.2 μg/mL monoclonal anti-CEP antibody and 1:10 000 diluted HRP conjugated goat antimouse antibody. The membrane was developed with SuperSignal West Pico Chemiluminescent substrate solution (Thermo Scientific, Rockford, IL) and was then exposed to X-ray film.

Incubation of Human Erythrocyte Ghosts with HOHA-Lactone. Human erythrocyte ghosts in PBS (1.15 mg/mL protein) were prepared by hemolysis of packed human red blood cells in

DOI: 10.1021/acs.chemrestox.5b00001
Chem. Res. Toxicol. XXXX, XXX, XXX–XXX

isotonic solution as described previously.[15] Then, 1 mL of erythrocyte ghosts were incubated with 1 mM HOHA-lactone at 37 °C. Untreated erythrocyte ghosts that were incubated at 37 °C for 3 days were used as control. Aliquots (200 μL) withdrawn after 1 day and 3 days, as well as a control sample (200 μL) were extracted with chloroform/methanol by the Bligh and Dyer method to separate proteins and phospholipids.[16] The lower layer fractions containing phospholipids were dried under a nitrogen gas stream, and then redissolved in diethyl ether (1 mL). Then 500 Units of PLD in MES buffer (250 μL, pH 5.5) supplemented with 15 mM of $CaCl_2$ were added. The biphasic solution was shaken vigorously overnight at 37 °C. Then diethyl ether was evaporated under a stream of nitrogen. The residue was mixed with 1 mL of methanol and then centrifuged at 10 000 × *g* for 10 min. The supernatant was dried under nitrogen and was redissolved in 100 μL of methanol. Deuterium labeled CEP-ethanolamine (d_4-CEP-ETN, 50 ng) was spiked into the solution as internal standard. Twenty microliters of solution was injected into the LC-MS for analysis.

Protein disks obtained during the Bligh and Dyer extraction were resuspended in 200 μL of PBS, mixed with 200 μL of 2× Laemelli SDS sample buffer, and then heated at 95 °C for 5 min. An aliquot (30 μL) of the protein solution (approximately 15 μg of protein) was separated by SDS PAGE gel electrophoresis followed by transfer of the proteins to a PVDF membrane. CEP modified proteins were then analyzed by Western blot as described above.

Myeloperoxdase/H_2O_2/NO_2^- Induced Autoxidation of DHA-PC Vesicles, DHA, and Human Erythrocyte Ghosts. DHA-PC vesicles in PBS (1 mL of 1.24 mM solution) were prepared by passing hydrated DHA-PC through a 0.1 μm polycarbonate filter 20 times using an Avanti Mini-Extruder Set (Avanti Polar Lipids, Inc., Alabaster, AL). DHA (2 mM) was mixed with 1 mL of PBS, and then the mixture was sonicated for 5 min to obtain a homogeneous solution. Human erythrocyte ghosts in PBS (1.15 mg/mL in protein concentration) were used without further treatment for MPO/H_2O_2/NO_2^- system-induced oxidation. DHA-PC vesicles, DHA or human erythrocyte ghosts in PBS solution (1.0 mL) were incubated in air at 37 °C in the presence of 30 nM myeloperoxidase (MPO), 100 ng/mL glucose oxidase, 100 μg/mL glucose and 500 μM $NaNO_2$. Aliquots (50 μL) of the reaction mixture were withdrawn after various reaction times and were extracted three times with 200 μL of ethyl acetate. Organic layers were pooled and evaporated to dryness under a stream of nitrogen. The residues were stored under −80 °C until analysis.

Pentafluorobenzyl Oxime Derivatization of HOHA-Lactone (HOHA-Lactone-PFBHA). Reaction mixtures from MPO/H_2O_2/NO_2^- promoted lipid oxidation were derivatized as described previously[17] with pentafluorobenzyl hydroxylamine (PFBHA) for LC-MS analysis. In brief, the residue obtained from 50 μL of oxidation reaction mixture was treated for 1 h with 0.05 M pentafluorobenzyl hydroxylamine hydrochloride aqueous solution (pH was adjusted to 7.0 by 0.1 N NaOH). Then 500 μL methanol and 2.5 mL hexane were added. After adding 6 drops of concentrated sulfuric acid, the mixture was immediately vortex vigorously and centrifuged for 5 min at 3,000 × g. The upper organic layer was collected and evaporated to dryness under a stream of nitrogen. The residue was redissolved in methanol (100 μL). An aliquot of this solution (10 μL) was injected into an LC-MS for analysis. Authentic HOHA-lactone-PFB oxime was prepared in the same way by incubating a sample of pure HOHA-lactone with PFB-HA hydrochloride in methanol.

High Performance Liquid Chromatography/Mass Spectrometry. Chromatographic separation was achieved with a Waters Alliance 2690 HPLC system (Waters, Milford, MA) equipped with a Luna C18(2) column (2.0 mm i.d. × 150 mm length, 5 μm, Phenomenex). Mobile phase A consisted of HPLC grade water containing 0.1% formic acid. Mobile phase B was HPLC grade methanol containing 0.1% formic acid. For analysis of Ac-Gly-Lys-OMe after treatment with HOHA-PC or HOHA-lactone, the total run time was 36 min. HPLC gradient steps were as follows: 0−15 min, linear gradient from 2 to 100% solvent B; 15−25 min, isocratic at 100% solvent B; 25−26 min, linear gradient from 100 to 2% solvent B; 26−36 min, isocratic at 2% solvent B. The flow rate was 200 μL/min. For analysis of PLD-digested RBC ghost phospholipids, the total run time was 40 min. The HPLC gradient steps were set as follows: 0−5 min, isocratic at 5% solvent B; 5−22 min, linear gradient from 5% to 100% solvent B; 22−30 min, isocratic at 100% solvent B; 30−31 min, linear gradient from 100% to 5% solvent B; 31−40 min, isocratic at 5% solvent B. The flow rate was 200 μL/min. For analysis of HOHA-lactone-PFB oxime, the total run was 25 min. HPLC gradient steps were set as follows: 0−7 min, linear gradient from 25% to 100% solvent B; 7−15 min, isocratic at 100% solvent B; 15−16 min, linear gradient from 100% to 25% solvent B; 16−25 min, isocratic at 25% solvent B. The flow rate was 200 μL/min.

The analytes were detected with a Quattro Ultima triple-quadrupole mass spectrometer (Micromass, Wythenshawe, U.K.) operated in the positive ion mode. The source temperature was maintained at 120 °C, the desolvation temperature was kept at 250 °C, the drying gas (N_2) was maintained at ∼450 L/h, the cone gas flow was kept at ∼70 L/h, and the multiplier was set at an absolute value of 600. Optimized parameters for detecting each analyte were determined using authentic samples. MS scans from 50 to 1000 were obtained for standard compounds. For multiple reaction monitoring (MRM) 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. (Supporting Information Table S1)

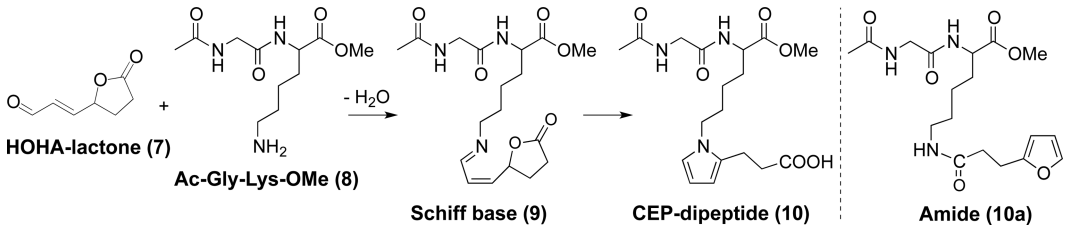
^{1}H NMR Analysis of HOHA-Lactone Incubated in Aqueous Buffer under Physiomimetic Conditions. To evaluate the stability of HOHA-lactone under physiological conditions, 2.5 mg of pure HOHA-lactone was dissolved in 725 μL of D_2O-reconstituted phosphate buffered saline solution (pH 7.4), HOHA-lactone solution was then transferred into a NMR tube and incubated in an oven set at 37 °C. ^{1}H NMR data was collected on Varian Inova AS400 spectrometer operating 400 MHz after 0, 12, and 24 h of incubation.

Cell Culture. Human retinal pigmented epithelial cells (ARPE-19) were cultured in complete DMEM/F12 medium supplemented with 10% FBS, and allowed to attach to culture plates in a humidified CO_2 incubator at 37 °C and 5% CO_2 overnight. All studies were conducted by using ARPE-19 cells of passages 20−40 following 12 h quiescence in DMEM/F12 serum-free medium. All the experiments were repeated two to three times with n = 8 in each experiment for cell viability studies and VEGF secretion, and n = 3 for measurements of metabolites in cell lysates and extracellular medium by either LC-MS or GC-MS. For metabolism studies, 1 × 10^6 of ARPE-19 cells/plate were grown in a 60 mm culture dish and washed with PBS, then 2 mL of PBS containing HOHA-lactone were added and incubated at 37 °C for up to 2 h. For cell viability assay and VEGF assay, 25 000 cells/well were grown in 96 well plates with 200 μL culture medium per well before analysis.

Cell Viability Assay (MTT Assay). ARPE19 cells (25 000 cells/per well) were seeded in a 96-well flat bottom plate in 200 μL of complete DMEM/HG medium supplemented with 10% FBS and allowed to attach to culture plates in a humidified CO_2 incubator at 37 °C and 5% CO_2. The following day, cells were washed with basal DMEM/HG medium and starved in 180 μL of basal DMEM/HG medium for 4−5 h. To these cells (8 replicate wells were used for each concentration), 20 μL of stock solutions of HOHA-lactone in PBS were added to create 0 to 50 μM final concentrations of HOHA-lactone followed by 2-h incubation in a humidified CO_2 incubator at 37 °C and 5% CO_2. The plates were then centrifuged at 1000*g* for 5 min and the medium was aspirated from each well and the cells were washed with DMEM/HG medium three times and then incubated for 4 h at 37 °C with 20 μL of filter-sterilized 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in PBS) plus 180 μL of DMEM/HG medium. The plates were then centrifuged at 1000*g* for 5 min again and the medium was aspirated from each well. Dimethyl sulfoxide (DMSO) was added to each well (200 μL) and the water-insoluble intracellular formazan crystals were dissolved by carefully pipetting the content of the wells. The optical density (OD) of formazan solutions were measured by using a plate reader (Model M3, Molecular Device) set at λ = 540 nm and a reference wavelength set λ = 620 nm.

DOI: 10.1021/acs.chemrestox.5b00001
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

Scheme 2



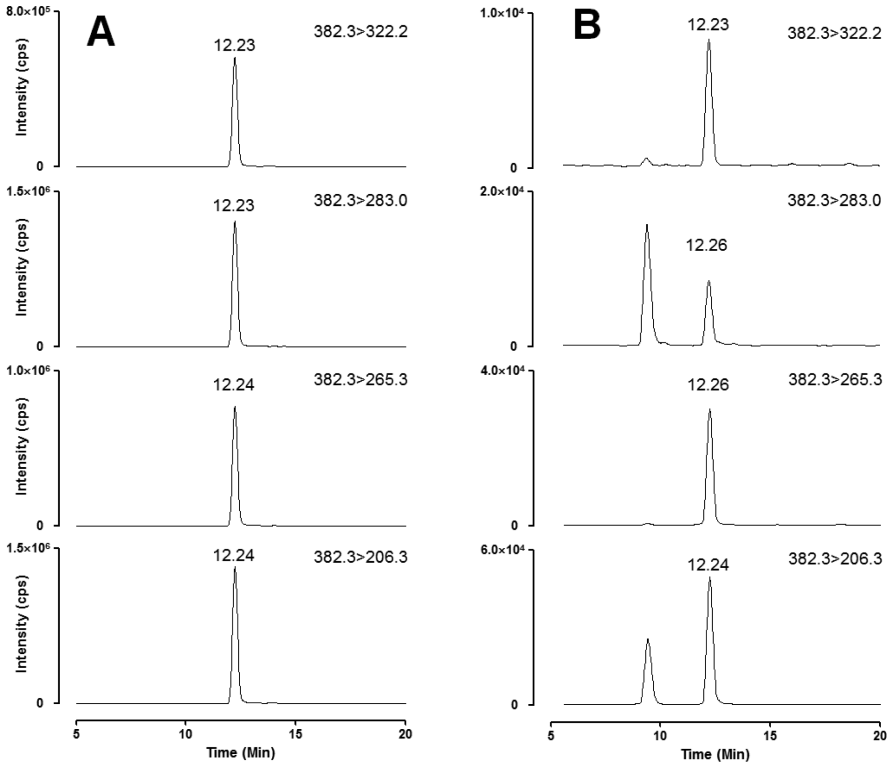


Figure 1. HPLC/ESI/MS/MS analysis of CEP-dipeptide production in the reaction of HOHA-lactone with Ac-Gly-Lys-OMe dipeptide. (A) Authentic CEP-dipeptide standard. (B) Reaction product mixture after 24 h incubation at 37 °C.

Measurement of Secreted VEGF-A from ARPE-19 Cells. After they were starved for 12 h, ARPE-19 cells grown in 96-well plates (25 000 cells/well) were incubated with HOHA-lactone (0−25 μM) dissolved in 200 μL of serum-free DMEM/HG medium. After 12 h, supernatants were collected to estimate secreted VEGF using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturers protocol (Human VEGF-A ELISA kit, Thermo Scientific, Rockford, IL). Briefly, 100 μL of supernatant samples, recombinant VEGF standards, and blank samples were added to an ELISA plate, which was precoated overnight with the coating antibody (antihuman VEGF-A antibody) and blocked 1 h with 4% BSA in PBS. After incubation for 1 h at room temperature, the plates were aspirated and washed three times with 300 μL per well of PBS with 0.05% Tween-20 (wash buffer). Then 1:100 diluted antihuman VEGF-A detection antibody in 4% BSA in PBS was added to each well and incubated for 1 h at room temperature. Then the plate was washed three times with wash buffer described above, and 100 μL of 1:400 diluted Streptavidin-HRP reagent was added to each well. After incubation at room temperature for 30 min, the plate was washed three times with wash buffer. Substrate solution (100 μL) was added to each well and incubated for 20 min until the reaction was stopped with 0.16 M sulfuric acid. All the absorbances were measured using a microplate reader with a test wavelength of 450 nm and a reference wavelength set at 550 nm.

3-(Furan-2-yl)propanoyl amide 10a. *N*-hydroxysulfosuccinimide (80 mg, 0.37 mmol) and 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide (88 mg, 0.46 mmol) were added into 6.0 mL of anhydrous dimethylformamide solution containing 2-furanpropanoic acid (60.0 mg, 0.43 mmol). After stirring for 3.0 h at room temperature under Ar protection, the reaction was filtered and 2.0 mL of DMF was used to wash the filter three times. The filtrate was added to a solution of methyl 6-amino-2-((2-acetylamino)acetyl)-amino) hexanoate(Ac-Gly-Lys-OMe.acetate, 78 mg, 0.30 mmol) with *N*,*N*-diisopropylethylamine (100.0 μL, 0.76 mmol) in the 3.0 mL anhydrous DMF at room temperature The resulting mixture was stirred for another 2.0 h. Then ddH_2O was added to the reaction mixture and 5 N HCl was used to adjust the pH to 2−3. The mixture was extracted with ethyl acetate three times. The organic phase was collected and dried with anhydrous sodium sulfate. After the removal of solvent by rotarary evaporation, the crude product was purified by silica gel chromatography to give target compound **10a** (80.0 mg, 70%, R_f = 0.30, 6.3% methanol in chloroform). ^{1}H NMR (400 M Hz, CD_3OD): 7.99 (broad, 1 H), 7.35 (dd, J = 0.8, 1.6 Hz, 1 H), 6.28 (dd, J = 2.0, 2.8 Hz, 1H), 6.04 (dd, J = 0.8, 3.2 Hz, 1 H), 4.41 (dd, J = 5.2, 9.2 Hz, 1 H), 3.90 (dd, J = 16.4, 28.8 Hz, 2 H), 3.71 (s, 3 H), 3.16 (dd, J = 6.4, 12.4 Hz, 2 H), 2.92 (t, J = 7.6 Hz, 2 H), 2.49 (t, J = 7.6 Hz, 2 H), 2.00 (s, 3 H), 1.82 (m, 1 H), 1.69 (m, 1 H), 1.47 (m, 2 H), 1.34 (m, 2 H). ^{13}C NMR (101 MHz, CD_3OD): δ 173.55, 172.79, 172.53, 170.45, 154.53, 141.19, 109.98, 105.09, 52.38, 51.53, 42.10, 38.90, 34.36, 30.93, 28.62, 23.95, 22.84, 21.24. ESI-MS: m/z calcd for $C_{18}H_{26}N_3O_6$ $[M - H]^-$, 380.18; found 380.73; calcd for $C_{18}H_{28}N_3O_6$ $[M + H]^+$382.20, found 382.60.

DOI: 10.1021/acs.chemrestox.5b00001
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

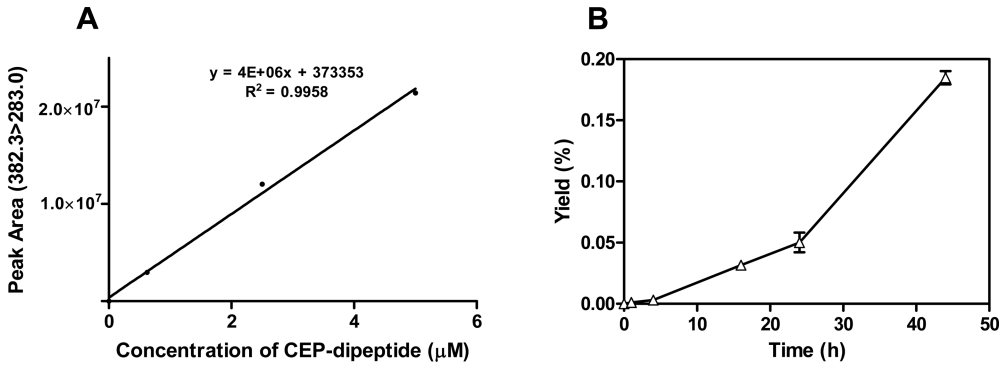
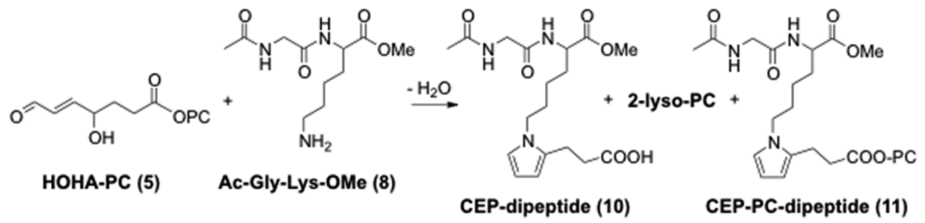


Figure 2. (A) Calibration curve for CEP-dipeptide MRM transition 382.3 → 283.0 and (B) evolution profile of CEP-dipeptide generation in the reaction of HOHA-lactone with Ac-Gly-Lys-OMe.

Scheme 3



LC-MS Comparison of Furan-Dipeptide 10a and CEP-Dipeptide 10. ESI mass spectrometry was performed on a Thermo Finnigan LCQ Deca instrument in the positive ion mode using nitrogen as the sheath and auxiliary gas. The Surveyor LC system was equipped with a Luna C18 (2) column (2.0 mm i.d. × 150 mm length, 5 μm, Phenomenex). Mobile-phase A consisted of HPLC grade water containing 0.1% formic acid. Mobile phase B was HPLC grade methanol containing 0.1% formic acid. The total run time was 36 min. HPLC gradient steps were as follows: 0−15 min, linear gradient from 2% to 100% solvent B; 15−25 min, isocratic at 100% solvent B; 25−26 min, linear gradient from 100% to 2% solvent B; 26−36 min, isocratic at 2% solvent B. The flow rate was 200 μL/min. The heated capillary temperature was 300 °C, the source voltage was 4.5 kV, and the capillary voltage was 31.00 V. The data were processed with Qual browser in Xcalibur software.

RESULTS

HOHA-Lactone (7) Reacts with Ac-Gly-Lys-OMe (8) to Form CEP-Dipeptide 9. Incubation of HOHA-lactone (7) with the dipeptide Ac-Gly-Lys-OMe (**8**) in pH 7.4 phosphate buffered saline (PBS) at 37 °C generated CEP-dipeptide **10** (Scheme 2) that was analyzed 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 collision induced dissociation spectrum for authentic CEP-dipeptide **10** (Supporting Information Figure S1). Four representative daughter ions, *m*/*z* 206.3, 265.3, 283.0, and 322.2, were chosen as MRM transitions for fragmentations of the parent ion *m*/*z* 382.3 for CEP-dipeptide analysis. HOHA-lactone treated dipeptide after 24 h has a major peak in LC-MS/MS spectrum at 12.24 min in all four MRM channels, as does the authentic CEP-dipeptide (Figure 1A, B). The peak that elutes at 9.37 min in the transitions 382.3 → 283.0 and 382.3 → 206.3 might correspond to the isobaric Schiff base adduct (**9**) or 3-(furan-2-yl)propanoyl amide (**10a**) (Figure 1C). An authentic sample of **10a** was readily distinguished from CEP-dipeptide (**10**) by LC retention time (Supporting Information Figure S2). The concentration of CEP-dipeptide generated from the reaction of HOHA-lactone (7) with dipeptide (**8**) was calculated from the peak area of the transition 382.3 → 283.0 by comparison with a calibration curve generated from authentic CEP-dipeptide standard (Figure 2A). The profile for the evolution of CEP-dipeptide shows a time dependent increase (Figure 2B).

CEP-Dipeptide (10), Instead of CEP-PC-Dipeptide (11), is the Major CEP Derivative Generated in the Reaction between HOHA-PC (2) and Ac-Gly-Lys-OMe (8). Previously, we found that reaction of HOHA-PC (**5**) with Ac-Gly-Lys-OMe (**8**) produced CEP-PC-dipeptide (**11**, Scheme 3) in 0.7% yield after 30 h incubation.[9] Since we now know that HOHA-PC (**5**) produces to HOHA-lactone (7) under the reaction conditions, it seemed likely that CEP-dipeptide (**10**) would be generated through the intermediacy of **7** in addition to CEP-PC-dipeptide (**11**). Using HPLC/ESI/MS/MS analysis, we now find that both **10** and **11** are indeed cogenerated in the reaction of **5** with **8** after 24 h incubation at 37 °C (Figure 3). CEP-dipeptide (**10**) and CEP-PC-dipeptide (**11**) in reaction mixture were simultaneously measured by LC-MS/MS analysis using MRM. Quantification of concentrations was accomplished using CEP-lysine and 2-lyso-PC (13:0) as internal standards, the peak area ratio of the CEP-dipeptide MRM transition 382.3 → 206.3 to the CEP-lysine transition 269.2 → 84, and the CEP-PC-dipeptide MRM transition 859.6 → 184 to the 2-lyso-PC (13:0) transition 454 → 184. Calibration curves of each analyte were generated using authentic standards (Figure 4A). CEP-dipeptide was initially generated at the same rate as CEP-PC-dipeptide. But after the first 2 days, CEP-dipeptide generation was faster than CEP-PC-dipeptide (Figure 4B). The concentration of CEP-dipeptide continued to increase and it became the major CEP product after 3 days incubation. After 5 days incubation, the concentration of CEP-dipeptide was 2.36 ± 0.23 μM, nearly 3 times that of CEP-PC-dipeptide, 0.71 ± 0.10 μM.

After 4 days, the CEP-PC-dipeptide concentration reached a maximum of 0.94 ± 0.11 μM and then its concentration started

DOI: 10.1021/acs.chemrestox.5b00001
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

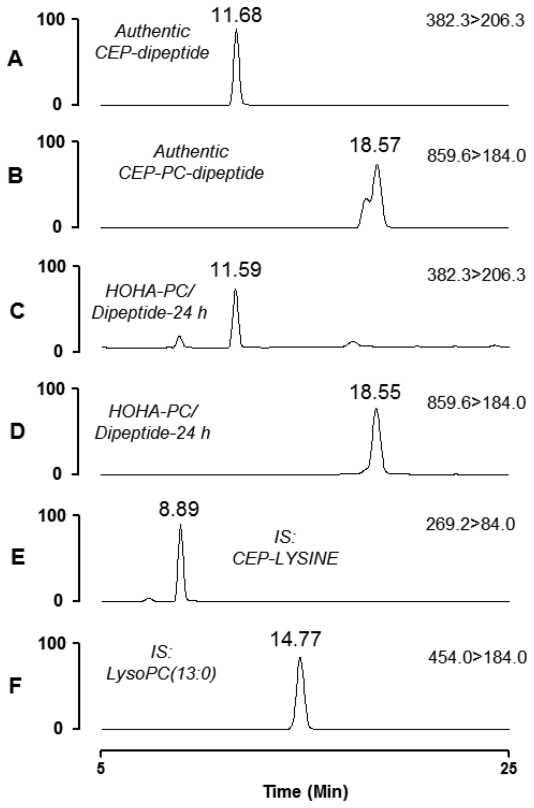


Figure 3. HPLC/ESI/MS/MS analysis of CEP-dipeptide and CEP-PC-dipeptide adducts. All chromatograms were monitored by LC-MS in the positive ion mode with MRM of appropriate mass transitions as noted. (A) Authentic CEP-dipeptide. (B) Authentic CEP-PC-dipeptide. (C) CEP-dipeptide produced from the reaction mixture of HOHA-PC and Ac-Gly-Lys-OMe dipeptide for 24 h incubation. (D) CEP-PC-dipeptide produced from the reaction mixture of HOHA-PC and Ac-Gly-Lys-OMe dipeptide for 24 h incubation. (E) Internal standard, CEP-lysine. (F) Internal standard, lysoPC (13:0).

to decrease owing presumably to nonenzymatic hydrolysis. Nevertheless, the majority of nonesterified CEP-dipeptide was apparently generated through the HOHA-lactone pathway. To explore the possibility that nonenzymatic hydrolysis of CEP-PC-dipeptide accounts for the majority of CEP-dipeptide generation, we determined the yield of CEP-dipeptide generated upon incubation of 20 μM CEP-PC-dipeptide (Figure 4B). CEP-PC-dipeptide only generated CEP-dipeptide in 17.6% yield after incubation for 5 days. Thus, the concentration of CEP-dipeptide produced by spontaneous hydrolysis of CEP-PC-dipeptide observed at 5 days is expected to be at most 0.35 μM based on the maximum concentration of CEP-PC-dipeptide produced in the reaction of the HOHA-PC with dipeptide **8**. This only accounts for 15% of the total CEP-dipeptide detected in the latter reaction mixture. This confirms that *the majority of CEP-dipeptide generated in the reaction of HOHA-PC with Ac-Gly-Lys-OMe is produced through the HOHA-lactone pathway. This suggests that HOHA-lactone is an important precursor of CEPs during oxidative damage in vivo.*

HOHA-Lactone Forms CEP Derivatives by Reaction with Proteins and Amino Phospholipids. The reactivity of HOHA-lactone toward biomolecules such as protein and ethanolamine phospholipids was tested by treating human serum albumin (HSA) and human red blood cell ghosts (RBC ghosts) with HOHA-lactone. Reaction of HSA in PBS with HOHA-lactone at 37 °C followed by removal of excess HOHA-lactone by dialysis generated CEP-protein. Thus, enzyme-linked immunosorbent assay (ELISA) of HSA protein after incubation with HOHA-lactone for 3 days exhibited significant CEP immunoreactivity to mouse monoclonal anti-CEP antibody, while control HSA showed no CEP immunoreactivity (Figure 5A).

Human erythrocyte ghosts were incubated with 1 mM HOHA-lactone in PBS for 0, 24, and 72 h and then were extracted with chloroform and methanol to isolate proteins and phospholipids. Immunoblot analysis of the protein fraction showed that HOHA-lactone formed CEP protein adducts in human erythrocyte ghosts in a time-dependent manner (Figure 5B). The phospholipid fraction was dried, and then hydrolyzed by phospholipase D (*Streptomyces chromofuscus*). The presence of CEP modified ethanolamine phospholipids (CEP-EA-PL, **13**, Scheme 4) was then determined by isotope dilution analysis of the resulting CEP-ethanolamine (CEP-EA, **14**) by HPLC/ESI/MS/MS. MRM was performed in the positive ion mode monitoring the transition 184 → 124 for CEP-EA and 188 → 128 for d_4-CEP-EA standard, according to daughter ion scan of CEP-EA and d_4-CEP-EA, respectively (Supporting Information Figure S2). LC-MS/MS showed that treatment of the RBC ghosts with HOHA-lactone for 24 and 72 h resulted in a significant peak that elutes at 15.47 min in the CEP-EA channel, at the same retention time as the internal standard d_4-CEP-EA, while the untreated RBC ghosts had no peak at this retention time (Figure 5C).

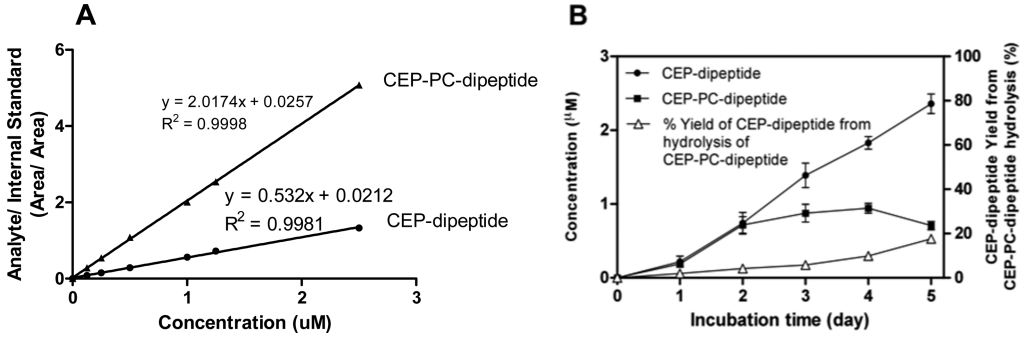


Figure 4. (A) Calibration curve for CEP-dipeptide and CEP-PC-dipeptide generated by peak area ratio of each analyte to internal standard. (Peak area ratio of MRM transition 382.3 → 206.3 to 269.2 →84.0 for CEP-dipeptide, 859.6 → 184.0 to 454.0 → 184.0 for CEP-PC-dipeptide) (B) Evolution profile of CEP adducts generation in the reaction mixture of HOHA-PC and Ac-Gly-Lys-OMe dipeptide and the yield of CEP-dipeptide generated from spontaneous hydrolysis of 20 μM CEP-PC-dipeptide in aqueous solution.

DOI: 10.1021/acs.chemrestox.5b00001
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

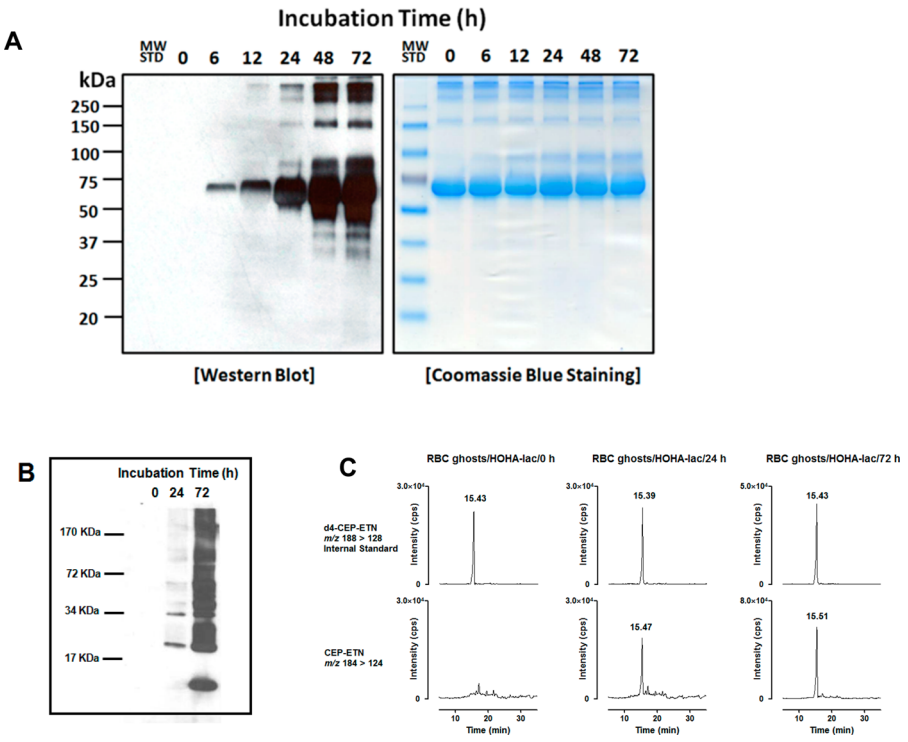
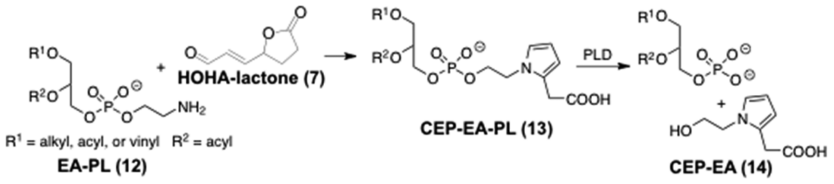
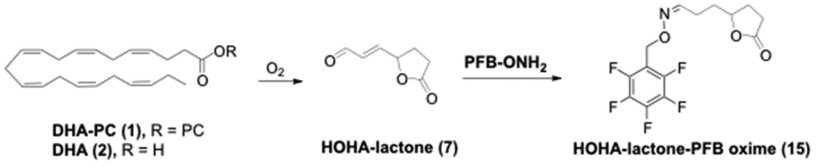


Figure 5. HOHA-lactone modifies biomolecules to form CEP adducts. (A) Ten μg per well of HOHA-lactone (0.5 mM) treated HSA protein (1 mg/mL) from 0 to 72 h incubation was either analyzed by Western blot (Left) using monoclonal mouse anti-CEP IgG antibody or Coomassie blue staining (Right). (B) Western blot analysis of the protein fraction isolated from HOHA-lactone treated human RBC ghosts (incubation for 0, 24, and 72 h) by monoclonal anti-CEP mouse IgG antibody. (C) LC-MS/MS analysis of CEP-ethanolamine adducts in PLD digested phospholipids isolated from HOHA-lactone treated human RBC ghosts. A fixed amount of deuterium labeled CEP-ethanolamine (d_4-CEP-ETN) was added as internal standard.

Scheme 4



Scheme 5



Oxidation DHA-Containing Phospholipidipids or DHA Generates HOHA-Lactone. DHA-containing phospholipids (DHA-PC vesicle), free docosahexaenoic acid (DHA), and human red blood cell ghosts were subjected to myeloperoxidase/H_2O_2/NO_2^- system-induced lipid oxidation. Measurement of HOHA-lactone in those lipid peroxidation products was achieved by detection of the derived pentafluorobenzyl oximes by LC-ESI-MS/MS (Scheme 5). MS scan spectrum of authentic HOHA-lactone-PFB oxime (**11**) in positive ion mode showed a strong peak at m/z 336, which corresponds to protonated HOHA-lactone-PFB oxime. Collision induced dissociation spectrum of m/z 336 with 25 eV collision energy showed three major daughter ions, m/z 181, 139, and 95 (Supporting Information Figures S3 and S4).

A selected reaction monitoring (SRM) of the transition 336 → 139 was used to analyze HOHA-lactone-PFB oxime. A typical LC-MS/MS chromatogram of authentic standard shows that HOHA-lactone-PFB oxime eluated at 12.34 min. DHA-PC vesicle, free DHA acid and RBC ghosts were oxidized by the MPO/H_2O_2/NO_2^- system, and then the lipid oxidation products were isolated by ethyl acetate extraction and derivatized with pentafluorobenzyl hydroxyamine. LC-MS/MS analysis showed that peaks that eluted at the same retention time as authentic HOHA-lactone-PFB oxime were present in the products from oxidation of DHA-PC vesicles, DHA or RBC ghosts, but not in those nonoxidized samples (Figure 6). The yield of HOHA-lactone from DHA-PC oxidation and DHA were determined as 0.45% and 0.78% after 24 h incubation, by

DOI: 10.1021/acs.chemrestox.5b00001
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

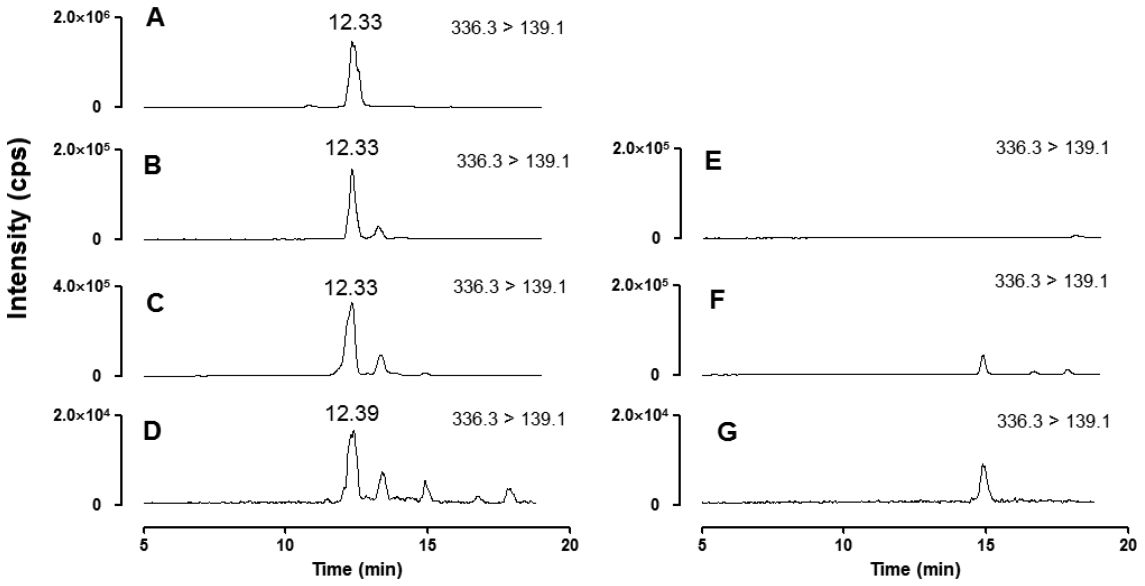


Figure 6. HOHA-lactone is generated during MPO//H_2O_2/NO_2^- system induced lipid oxidation. HOHA-lactone was measured by LC-MS/MS as pentafluorobenzyl oxime derivative. Left: LC chromatograms of (A) authentic HOHA-lactone-PFB oxime, (B) oxidized DHA-PC vesicle, (C) oxidized free DHA acid, (D) oxidized human RBC ghosts. Right: LC chromatograms of (E) nonoxidized DHA-PC vesicle, (F) nonoxidized free DHA acid, (G) nonoxidized human RBC ghosts.

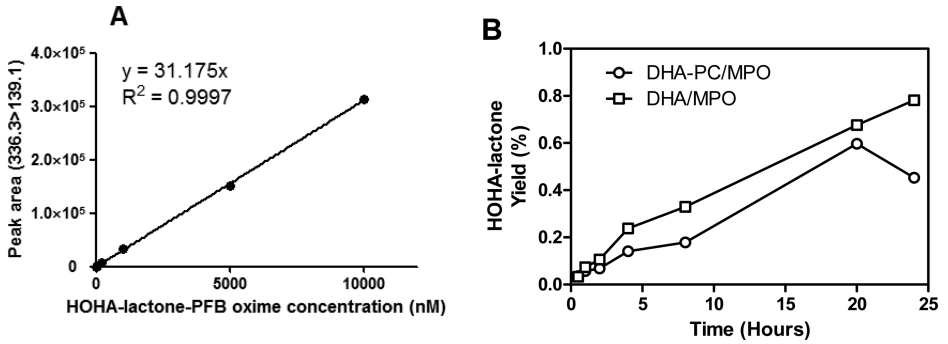


Figure 7. (A) Calibration curve of HOHA-lactone-PFB oxime determined by LC-MS/MS with SRM method 336.3 → 139.1. (B) Evolution profile of HOHA-lactone generation in MPO//H_2O_2/NO_2^- system induced oxidized DHA-PC vesicle (open circle) and free DHA acid (open square).

quantifying the peak area of HOHA-lactone-PFB oxime at 12.34 min in the 336 → 139 transition and comparing to the calibration curve generated with authentic HOHA-lactone-PFB oxime (Figure 7).

HOHA-Lactone Is Stable under Physiological Conditions of pH and Temperature. To confirm the stability of HOHA-lactone relative to the free acid HOHA under physiological conditions, pure HOHA-lactone (2.5 mg) was dissolved in 725 μL of phosphate buffered saline solution (pH 7.4) in D_2O and transferred into a NMR tube. ^{1}H NMR data was collected immediately and then the tube was placed in an incubator set at 37 °C. Another two ^{1}H NMR analyses were recorded after incubation for 12 and 24 h at 37 °C. Comparison of the NMR data at 0, 12, and 24 h showed no significant differences (Figure 8). Moreover, peaks with chemical shift range from 5 to 10 ppm correspond to four hydrogen atoms: an aldehydic hydrogen H_a at 9.33 ppm; two vinyl hydrogens, H_b and H_c, at 6.18 and 6.94 ppm and H_d, the allylic ring hydrogen α to the ester oxygen that exhibits a unique chemical shift at 5.25 ppm. The peaks of these four hydrogens were integrated, and the integration values were compared at different time points. The ratio of aldehyde proton to other three protons suggests that there is no significant structure change of HOHA-lactone.

HOHA-Lactone Induces ARPE-19 Cell Growth and Vascular Endothelial Growth Factor (VEGF) Secretion. Oxidative injury of ARPE-19 human retinal pigmented epithelial cells by exposure to H_2O_2 generated CEP derivatives (Supporting Information Figure S5). Treatment of ARPE-19 cells with HOHA-lactone generated CEP-modified proteins (Supporting Information Figure S6). A recent study showed that low levels of 4-hydroxynonenal (4-HNE), a structural analog of HOHA-lactone, cause secretion of VEGF from RPE cells.[18,19] To test whether HOHA-lactone could induce cell signaling and stimulate the secretion of VEGF from RPE cells in a similar pattern as 4-HNE, we treated ARPE-19 cells with various concentrations of HOHA-lactone. The cytotoxicity of HOHA-lactone to ARPE-19 cells was assessed by MTT assay. And VEGF-A levels in the extracellular medium were examined using a human VEGF-A ELISA kit (Thermo Scientific, Rockford, IL). Cell viability results showed that HOHA-lactone significantly stimulated cell proliferation at relatively low concentrations (<0.5 μM). However, it significantly decreased cell viability at higher concentration levels (>20 μM, Figure 9A).

DOI: 10.1021/acs.chemrestox.5b00001
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

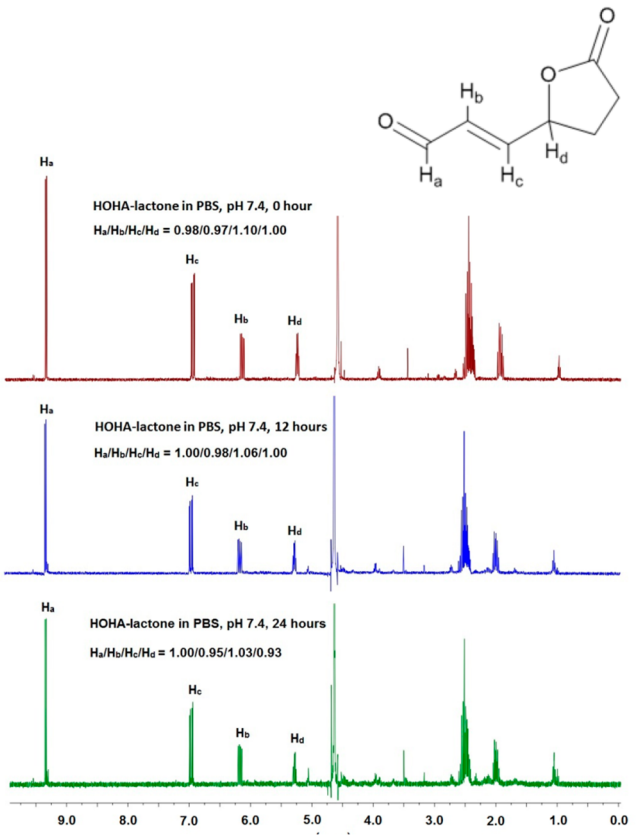


Figure 8. ^{1}H NMR spectrum analysis of 2.5 mg of HOHA-lactone in 725 μL of PBS-D_2O solution, pH 7.4, after incubated in 37 °C for 0, 12, and 24 h. The proton peaks that correspond to hydrogen atoms H_a, H_b, H_c and H_d on HOHA-lactone was integrated and then the ratio of these four H atoms were determined.

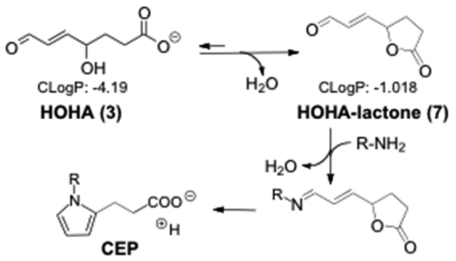
We also found that HOHA-lactone induces VEGF secretion from ARPE-19 cells in a dose dependent manner. At submicromolar concentrations of HOHA-lactone, 0.1−1.0 μM, ARPE-19 cells secreted significantly increased levels of VEGF into the extracellular medium (Figure 9B). But at nontoxic higher levels, from 2.5 to 10 μM, HOHA-lactone did not induce increased secretion of VEGF. At high levels of HOHA-lactone, that is, 25 μM, it significantly inhibited the secretion of VEGF.

DISCUSSION

In the present study, we demonstrated that the oxidation of both DHA containing phospholipids and nonesterified DHA produce HOHA-lactone, and that HOHA-lactone converts primary amino groups of biomolecules into CEP derivatives. Human red blood cells are usually exposed to high oxygen tension, which results in a high level of oxidative stress,[20] and they possess a significant amount of membrane-associated docosahexaenoic acid.[21] We showed that peroxidation of red blood cell membrane lipids can produce HOHA-lactone, providing a biologic model in which HOHA-lactone is generated and released from membrane phospholipids during oxidative damage.

We also found that the spontaneous (nonenzymatic) deacylation of phospholipids to produce HOHA-lactone and its reactions to form CEPs occurs more readily than the reaction of HOHA-containing phospholipids to generate CEP esterified to the hydroxyl of a lyso phospholipid. These findings are likely to be biologically significant for several reasons. Whereas HOHA esterified to phospholipids is membrane bound and unesterified HOHA is anionic, lipophobic (CLogP = −4.19) and unable to penetrate cell membranes, HOHA-lactone is orders of magnitude less lipophobic (CLogP = −1.02) and is expected to be comparable to cortisone (CLogP = −0.93) in the ability to diffuse across cell membranes (Scheme 6). It is also likely to diffuse through confluent cell

Scheme 6



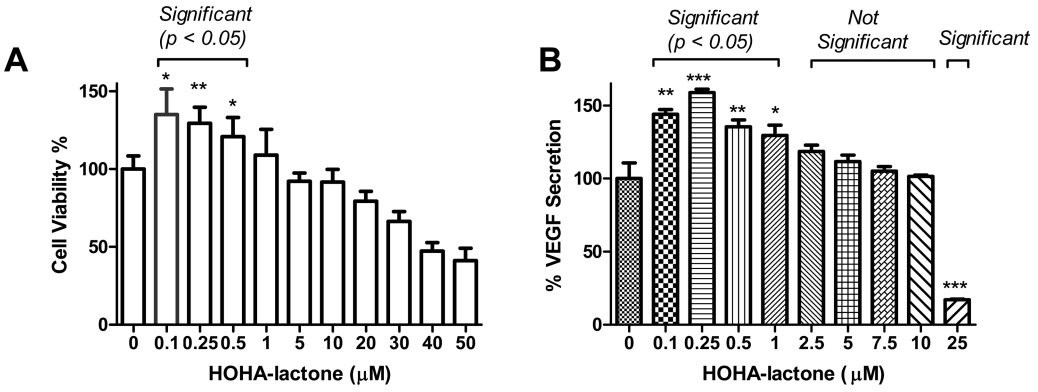


Figure 9. (A) Cytotoxicity of HOHA-lactone to ARPE-19 cells (25 000 cells/well). Cells were treated with 0−50 μM HOHA-lactone for 2 h and then assayed for cytotoxicity by MTT assay. Cell survival values (%) were presented as means ± SD of 8 independent experiments. (B) Effect of HOHA-lactone on VEGF secretion by ARPE-19 cells. ARPE-19 cells grown in a 96-well plate (25,000 cells per well) were treated with serum-free culture media containing varying concentrations (0−25 μM) of HOHA-lactone. After 12 h of incubation, supernatants were collected and secreted VEGF was measured using a human VEGF-A ELISA kit. The data represent the mean ± SD (n = 8).

DOI: 10.1021/acs.chemrestox.5b00001
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

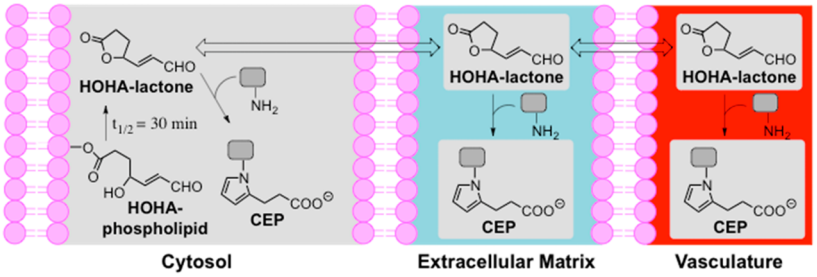


Figure 10. Diffusion of HOHA-lactone through cell membranes might provide a route for the generation CEPs at locations that are remote, for example, in the blood, from their photooxidative generation from docosahexaenoyl phospholipids that are especially abundant in retinal photoreceptor disk membranes.

monolayers resulting in the generation of CEPs in locations remote from the site of phospholipid oxidation in vivo (Figure 10).

CEPs are especially abundant in the retina and blood from individuals with age-related macular degeneration (AMD). Diffusion of HOHA-lactone generated in photoreceptor disk membranes in the eye into the blood may contribute to the elevated levels of CEP present in blood from AMD patients. Our previous studies found that exposure of rats to bright light generates elevated levels of CEP in their retinas ***and blood***.[22] It seems reasonable to postulate that light exposure produces HOHA-lactone in the retina and that this product of photooxidative injury in the eye migrates into the blood of these animals where it forms CEP derivatives of blood proteins.

In the present study, we demonstrated that exposure of ARPE-19 cells to HOHA-lactone increases secretion of VEGF from these cells in a concentration dependent manner. Similar to the previously reported effect of 4-HNE, the secreted VEGF can exert a paracrine effect on the surrounding endothelial cells, resulting in the proliferation of endothelial cells in the retina, and contributing to their migration and tube formation that ultimately promotes the pathologic processes of retinal neovascularization.

CONCLUSIONS

Owing to an extraordinary proclivity toward lactone formation, HOHA – both as the free acid and esterified within phospholipids—is readily converted under physiological conditions into HOHA-lactone. This product of nonenzymatic, free radical-induced, lipid oxidation is expected to readily diffuse from the site of oxidative injury and serve as an important precursor of CEPs. This may help to explain why CEPs in the blood are markers of oxidative injury associated with pathologies, such as age-related macular degeneration. Thus, although AMD involves oxidative injury in the retina, CEP levels are elevated in the blood of AMD patients. CEPs are also lipid-derived biologically active derivatives of proteins and ethanolamine phospholipids that contribute to disease progression, for example, AMD and cancer. In view of the importance of HOHA-lactone in the generation of CEPs, this molecule may provide a new target for the design of therapeutic interventions to block its contribution to CEP formation and its pathological consequences. Besides serving as an important precursor of CEPs, HOHA-lactone itself may possess biological activities that contribute to the choroidal neovascularization that characterizes "wet" AMD.

ASSOCIATED CONTENT

Supporting Information

Synthesis of CEP-PC-dipeptide (**11**),1-palmityl-2-(4,7-dioxoheptanoyl)-*sn*-glycero-3-phosphatidylcholine and 1-palmityl-2-(Ac-Gly-Lys-OMe-CEP)-*sn*-glycerophosphatidylcholine; Table S1, optimized mass spectrometer parameters, as well as the MRM transitions and collision energies for each individual analyte; Figure S1, ESI-MS scan of authentic CEP-dipeptide **9** and ESI-MS/MS scan of *m*/*z* 382.5 at collision energy 30 eV and the proposed daughter ion structures; Figure S2, LC-MS chromatogram for authentic furan-dipeptide **10a** and CEP-dipeptide **10**, as well as their mixture; Figure S3, ESI-MS spectrum of authentic CEP-EA, **14**, and d_4-CEP-EA, as well as their daughter ions in the positive ion mode; Figure S4, ESI-MS scan of HOHA-lactone-PFB oxime in the positive ion mode and ESI-MS/MS scan of *m*/*z* 336 at collision energy 25 eV and the proposed daughter ion structures; post-translational modification of ARPE-19 cells by HOHA-lactone; PAGE and Western blot analysis of proteins in ARPE cell lysates; detection CEP in ARPE-19 cells incubated with H_2O_2; Figure S5, modification of ARPE-19 cells by HOHA-lactone; Figure S6, ARPE-19 cells treated with 5 mM H_2O_2 and then immunostained with mouse monoclonal anti-CEP antibody; Figure S7, ^{1}H NMR spectrum of 3-(furan-2-yl)propanoyl amide **10a**; Figure S8, ^{13}C NMR spectrum of 3-(Furan-2-yl)propanoyl amide **10a**. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: rgs@case.edu. Phone: 216-368-2592. Fax: 216-368-3006.

Funding

This work was supported by NIH Grants EY011373, EY016813, and GM021249.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

ABTS, 2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt; AMD, age-related macular degeneration; CEP, 2-(ω-carboxyethyl)pyrrole; DHA, docosahexaenoic acid; DHA-PC, 1-palmityl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine; ECL, enhanced chemiluminescence; HOHA, 4-hydroxy-7-oxo-hept-5-eonate; HOHA-PC, 1-palmityl-2-(4-hydroxy-7-oxo-5-heptenoyl)-*sn*-glycero-3-phosphatidylcholine; HRP, horse radish peroxidase; MES, 2-(*N*-morpholino)-

DOI: 10.1021/acs.chemrestox.5b00001
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

ethanesulfonic acid; PLA2, phospholipase A2; PLD, phospholipase D.

REFERENCES

(1) Wang, H., Guo, J., West, X. Z., Bid, H. K., Lu, L., Hong, L., Jang, G. F., Zhang, L., Crabb, J. W., Linetsky, M., and Salomon, R. G. (2014) Detection and biological activities of carboxyethylpyrrole ethanolamine phospholipids (CEP-EPs). *Chem. Res. Toxicol. 27*, 2015−2022.

(2) Gu, X., Meer, S. G., Miyagi, M., Rayborn, M. E., Hollyfield, J. G., Crabb, J. W., and Salomon, R. G. (2003) Carboxyethylpyrrole protein adducts and autoantibodies, biomarkers for age-related macular degeneration. *J. Biol. Chem. 278*, 42027−42035.

(3) Crabb, J. W., Miyagi, M., Gu, X., Shadrach, K., West, K. A., Sakaguchi, H., Kamei, M., Hasan, A., Yan, L., Rayborn, M. E., Salomon, R. G., and Hollyfield, J. G. (2002) Drusen proteome analysis: an approach to the etiology of age-related macular degeneration. *Proc. Natl. Acad. Sci. U.S.A. 99*, 14682−14687.

(4) Evans, T. A., Siedlak, S. L., Lu, L., Fu, X., Wang, Z., McGinnis, W. R., Fakhoury, E., Castellani, R. J., Hazen, S. L., Walsh, W. J., Lewis, A. T., Salomon, R. G., Smith, M. A., Perry, G., and Zhu, X. (2008) The autistic phenotype exhibits a remarkably localized modification of brain protein by products of free radical-induced lipid oxidation. *Am. J. Biotechnol. Biochem. 4*, 61−72.

(5) West, X. Z., Malinin, N. L., Merkulova, A. A., Tischenko, M., Kerr, B. A., Borden, E. C., Podrez, E. A., Salomon, R. G., and Byzova, T. V. (2010) Oxidative stress induces angiogenesis by activating TLR2 with novel endogenous ligands. *Nature 467*, 972−976.

(6) Panigrahi, S., Ma, Y., Hong, L., Gao, D., West, X. Z., Salomon, R. G., Byzova, T. V., and Podrez, E. A. (2013) Engagement of platelet toll-like receptor 9 by novel endogenous ligands promotes platelet hyperreactivity and thrombosis. *Circ. Res. 112*, 103−112.

(7) Hollyfield, J. G., Bonilha, V. L., Rayborn, M. E., Yang, X., Shadrach, K. G., Lu, L., Ufret, R. L., Salomon, R. G., and Perez, V. L. (2008) Oxidative damage-induced inflammation initiates age-related macular degeneration. *Nat. Med. (N. Y., NY, U. S.) 14*, 194−198.

(8) Balsinde, J., Winstead, M. V., and Dennis, E. A. (2002) Phospholipase A(2) regulation of arachidonic acid mobilization. *FEBS Lett. 531*, 2−6.

(9) Gu, X., Sun, M., Gugiu, B., Hazen, S., Crabb, J. W., and Salomon, R. G. (2003) Oxidatively truncated docosahexaenoate phospholipids: Total synthesis, generation, and Peptide adduction chemistry. *J. Org. Chem. 68*, 3749−3761.

(10) Salomon, R. G., Hong, L., and Hollyfield, J. G. (2011) Discovery of carboxyethylpyrroles (CEPs): critical insights into AMD, autism, cancer, and wound healing from basic research on the chemistry of oxidized phospholipids. *Chem. Res. Toxicol. 24*, 1803−1816.

(11) Choi, J., Zhang, W., Gu, X., Chen, X., Hong, L., Laird, J. M., and Salomon, R. G. (2011) Lysophosphatidylcholine is generated by spontaneous deacylation of oxidized phospholipids. *Chem. Res. Toxicol. 24*, 111−118.

(12) Lu, L., Gu, X., Hong, L., Laird, J., Jaffe, K., Choi, J., Crabb, J., and Salomon, R. G. (2009) Synthesis and structural characterization of carboxyethylpyrrole-modified proteins: Mediators of age-related macular degeneration. *Bioorg. Med. Chem. 17*, 7548−7561.

(13) Choi, J., Laird, J. M., and Salomon, R. G. (2011) An efficient synthesis of gamma-hydroxy-alpha,beta-unsaturated aldehydic esters of 2-lysophosphatidylcholine. *Bioorg. Med. Chem. 19*, 580−587.

(14) Ebrahem, Q., Renganathan, K., Sears, J., Vasanji, A., Gu, X., Lu, L., Salomon, R. G., Crabb, J. W., and Anand-Apte, B. (2006) Carboxyethylpyrrole oxidative protein modifications stimulate neovascularization: Implications for age-related macular degeneration. *Proc. Natl. Acad. Sci. U.S.A. 103*, 13480−13484.

(15) Steck, T. L., and Kant, J. A. (1974) Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membranes. *Meth. Enzymol. 31*, 172−180.

(16) Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol. 37*, 911−917.

(17) Spies-Martin, D., Sommerburg, O., Langhans, C. D., and Leichsenring, M. (2002) Measurement of 4-hydroxynonenal in small volume blood plasma samples: modification of a gas chromatographic-mass spectrometric method for clinical settings. *J. Chromatog. B, Anal. Technol. Biomed. Life Sci. 774*, 231−239.

(18) Vatsyayan, R., Lelsani, P. C., Chaudhary, P., Kumar, S., Awasthi, S., and Awasthi, Y. C. (2012) The expression and function of vascular endothelial growth factor in retinal pigment epithelial (RPE) cells is regulated by 4-hydroxynonenal (HNE) and glutathione S-transferaseA4−4. *Biochem. Biophys. Res. Commun. 417*, 346−351.

(19) Ayalasomayajula, S. P., and Kompella, U. B. (2002) Induction of vascular endothelial growth factor by 4-hydroxynonenal and its prevention by glutathione precursors in retinal pigment epithelial cells. *Eur. J. Pharmacol. 449*, 213−220.

(20) Fruehauf, J. P., and Meyskens, F. L., Jr. (2007) Reactive oxygen species: a breath of life or death? *Clin. Cancer Res. 13*, 789−794.

(21) Hoffman, D. R., and Birch, D. G. (1995) Docosahexaenoic acid in red blood cells of patients with X-linked pigmentosa. *Invest. Ophthalmol. Vis. Sci. 36*, 1009−1018.

(22) Renganathan, K., Gu, J., Rayborn, M. E., Crabb, J. S., Salomon, R. G., Collier, R. J., Kapin, M. A., Romano, C., Hollyfield, J. G., and Crabb, J. W. (2013) CEP biomarkers as potential tools for monitoring therapeutics. *PloS One 8*, No. e76325.

DOI: 10.1021/acs.chemrestox.5b00001
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX