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Detection and Biological Activities of Carboxyethylpyrrole Ethanolamine Phospholipids (CEP-EPs)

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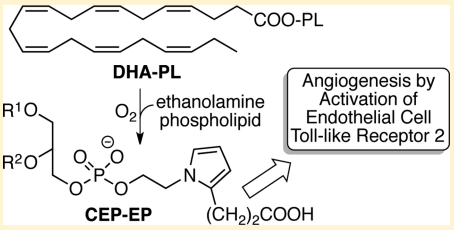
S *Supporting Information*

ABSTRACT: Oxidation of docosahexaenoate phospholipids produces 4-hydroxy-7-oxo-hept-5-eonyl phospholipids (HOHA-PLs) that react with protein lysyl ε-amino residues to generate 2-ω-carboxyethylpyrrole (CEP) derivatives, endogenous factors that induce angiogenesis in the retina and tumors. It seemed likely, but remained unproven, that HOHA-PLs react with ethanolamine phospholipids (EPs) *in vivo* to generate CEP-EPs. We now show that CEP-EPs are present in human blood at 4.6-fold higher levels in age-related macular degeneration plasma than in normal plasma. We also show that CEP-EPs are pro-angiogenic, inducing tube formation by human umbilical vein endothelial cells by activating Toll-like receptor 2. CEP-EP levels may be a useful biomarker for clinical assessment of AMD risk and CEP-associated tumor progression and a tool for monitoring the efficacy of therapeutic interventions.

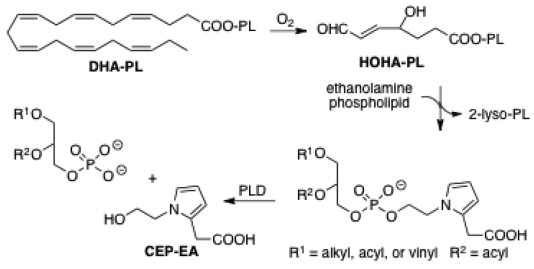


INTRODUCTION

2-ω-Carboxyethylpyrrole (CEP)-protein derivatives are formed through post-translational modification of the ε-amino groups of protein lysyl residues by 4-hydroxy-7-oxo-hept-5-eonyl phospholipids (HOHA-PLs), which are uniquely generated from oxidation of docosahexaenoate-containing phospholipids (DHA-PLs, Scheme 1).[1,2] Using anti-CEP antibodies raised against CEP-protein,[3] CEP immunoreactivity was first detected *in vivo* in photoreceptor rod outer segments that are DHA-rich tissues and in retinal pigmented epithelium that endocytose oxidatively damaged rod outer segment tips. CEPs are found in human Bruch's membrane/retinal pigmented epithelium/choroid tissues and extracellular deposits termed drusen, which are hallmarks of age-related macular degeneration (AMD).[4,5] That CEP immunoreactivity is associated with proteins was demonstrated by western blots of a protein extract, and it was noted that ocular tissues from AMD donors contain significantly higher levels of CEPs than that in normal healthy donors. CEP-protein and -peptide derivatives are novel factors that induce angiogenesis into the retina by the sprouting of new capillaries from the vasculature behind the retina, i.e., choroidal neovascularization.[6] Angiogenesis induced by endogenous CEP also promotes wound healing and tumor growth through a vascular endothelial growth factor (VEGF)-independent mechanism involving activation of Toll-like receptor 2.[7]

It seemed likely that the reaction of HOHA-PLs *in vivo* with ethanolamine phospholipids (EPs) in cell membranes would generate CEP-EPs. EPs are found *in vivo* as complex mixtures of ethanolamine phosphoglyderides that include 1,2-diacyl (phosphatidylethanolamines, PEs) and 1-alkyl-2-acyl and 1-alkenyl-2-acyl (plasmenylethanolamines) derivatives (Scheme 1). In humans, they account for 27−52% of total phospholipids in brain, spinal cord, heart, lung, liver, testis, kidney, spleen, erythrocytes, and platelets.[8,9] The primary amino group in EPs is prone to covalent modification by various electrophilic aldehydes *in vivo*, such as glyoxal, glucose, 4-hydroxynonenal

Scheme 1. Generation of CEP-EPs through Oxidative Cleavage of DHA-PLs to HOHA-PLs That React with Ethanolamine Phospholipids To Deliver CEP-EPs[a]



[a]Hydrolysis catalyzed by phospholipase D delivers CEP-EA.

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(HNE), acrolein, malonaldehyde, and isolevuglandins.[10−16] These aldehyde-PE adducts have important biological activities. For example, isolevugladin-PE is cytotoxic to HEK-293 cells, and Amadori-glycated PE has a pro-angiogenic effect on human umbilical vein endothelial cells (HUVECs).[17,18]

To test the hypothesis that CEP-EPs are produced *in vivo* and possess similar biological activities as those of CEP-proteins, we prepared pure samples of a CEP-PE by an unambiguous chemical synthesis. We applied liquid chromatography-tandem mass spectrometry (LC-MS/MS) to specifically determine the presence of CEP-EPs in human plasma. Elevated levels of CEP immunoreactivity in blood have been associated with AMD.[19,20] Previously, we developed immunoassays to measure levels of CEPs and anti-CEP autoantibodies that are both elevated in AMD plasma.[3] Because variability in level of anti-CEP autoantibodies in AMD blood complicates the immunoassay of CEP, we anticipate that CEP-EPs could be a superior blood-borne biomarker for CEP production *in vivo*, e.g., for monitoring the efficacy of therapeutic measures.[21] We also showed that CEP-PE is pro-angiogenic by demonstrating its ability to induce tube formation by endothelial cells. That this biological activity involves activation of the Toll-like receptor 2 (TLR2) was established by showing that a TLR2 inhibitor or knockdown of TLR2 expression blocks CEP-PE-induced tube formation by HUVECs.

EXPERIMENTAL PROCEDURES

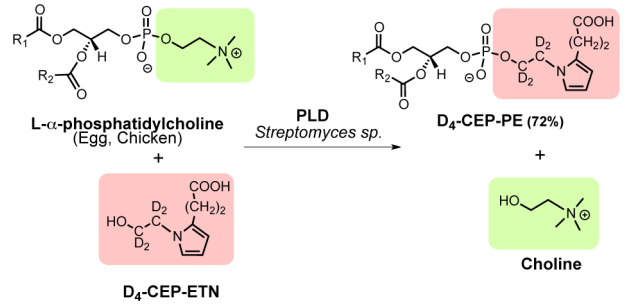
Materials. 1,2-Dipalmitoyl-*sn*-glycero-3-phospho-ethanolamine (DPPE) and egg PC (L-α-phosphatidylcholine) were purchased from Avanti Polar Lipids (Alabaster, AL). Recombinant mouse TLR2-Fc protein was purchased from R&D Systems (Minneapolis, MN). Phospholipase D (PLD) from *Streptomyces* sp. and *Streptomyces chromofuscus* were obtained from Enzo Life Sciences (Farmingdale, NY). Horseradish peroxidase-labeled goat anti-human IgG Fc antibody was purchased from Millipore (Billerica, MA). ABTS solution substrate for horseradish peroxidase was from Invitrogen (Grand Island, NY). OxPAPC was from InvivoGen (San Diego, CA). The fluorenemethyl ester of 3,6-dioxohexanoic acid (DOHA-Fm), CEP-modified human serum albumin (CEP-HSA) and chicken egg ovalbumin (CEP-CEO), and polyclonal rabbit anti-CEP-KLH antibody were prepared as described previously.[1] Mouse anti-human TLR2 LEAF antibody (CD282) was from BioLegend (San Diego, CA). Calcein AM and Accutase were from BD Biosciences (San Jose, CA). Goat anti-mouse IgG-AlexaFluor 488 was from Invitrogen (Carlsbad, CA). All other chemicals were from Sigma-Aldrich and were analytical grade.

Synthesis of CEP-DPPE. The CEP derivative of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (CEP-DPPE) was prepared in a similar method as that described by Lu et al.[22] In brief, to a solution of DPPE (45 mg, 0.065 mmol) in 500 μL of $CHCl_3$ was subsequently added triethylamine (25 μL, 0.247 mmol) and a solution of DOHA-Fm (22 mg, 0.065 mmol) in 500 μL of $CHCl_3$. The resulting mixture was stirred overnight at rt under argon. After the reaction was complete, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (20 μL, 0.13 mmol) was added to the mixture, which was stirred for another 3 h. Then, the solution was washed with 2 mL of pH 5.5 sodium phosphate buffer. The organic phase was washed with brine and dried over magnesium sulfate. Solvent was removed by rotary evaporation under reduced pressure, and the residue was purified by silica gel flash chromatography ($CHCl_3$/MeOH = 10:1, R_f = 0.25) to give pure CEP-DPPE (37.6 mg, 0.046 mmol, 71%). ^{1}H NMR for CEP-DPPE (CD_3OD/$CDCl_3$ = 1:1) δ 7.00 (m, 1H), 6.39 (dd, 1H), 6.24 (m, 1H), 5.55 (m, 1H), 4.87 (m, 4H), 4.73 (dd, *J* = 12.1, 3.3 Hz, 1H), 4.50 (m, 4H), 4.18 (s, 2H), 3.27 (t, *J* = 7.2 Hz, 2H), 2.99 (t, *J* = 7.2 Hz, 2H), 2.68 (t, *J* = 7.5 Hz, 4H), 1.98 (m, 4H), 1.78−1.53 (48H), 1.26 (t, *J* = 6.9 Hz, 6H). ESI-MS: *m*/*z* calcd for $C_{44}H_{79}NO_{10}P$ $[M - H]^-$, 812.54; found, 812.53.

Synthesis of CEP-Ethanolamines (CEP-ETN and d_4-CEP-ETN). To a solution of DOHA-Fm (33.6 mg, 0.1 mmol) in 2 mL of CH_2Cl_2 was slowly added ethanolamine or ethanol-1,1,2,2-d_4-amine (99 atom % D, C/D/N Isotopes Inc., Quebec, Canada) (0.12 mmol) in 1 mL of methanol. The mixture was stirred at room temperature for 5 h under argon. Then, 20 μL of DBU was added, and the mixture was stirred for another 6 h. After removal of solvent, 5 mL of ddH_2O was added. The pH of the mixture was adjusted to 3 by addition of 0.1 N HCl, and the solution was extracted three times with 5 mL of chloroform. The combined organic extracts were dried over anhydrous sodium sulfate for 3 h and evaporated to dryness under reduced pressure. The residue was purified by flash chromatography on a silica gel column ($CHCl_3$/MeOH = 20:1, TLC R_f = 0.18) to give pure CEP-ETN (11.3 mg, 62%) or d_4-CEP-ETN (12.5 mg, 67%). CEP-ETN: ^{1}H NMR (400 MHz, CD_3OD) δ 6.61 (dd, *J* = 2.7, 1.8 Hz, 1H), 5.99−5.89 (m, 1H), 5.83−5.77 (m, 1H), 3.94 (t, *J* = 5.9 Hz, 2H), 3.72 (t, *J* = 5.9 Hz, 2H), 2.90−2.80 (m, 2H), 2.60 (dd, *J* = 8.4, 6.9 Hz, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ 175.73, 131.36, 120.45, 106.58, 104.99, 62.01, 48.34, 33.32, 21.34. d_4-CEP-ETN: ^{1}H NMR (400 MHz, CD_3OD) δ 6.61 (dd, *J* = 2.8, 1.8 Hz, 1H), 5.98−5.89 (m, 1H), 5.80 (ddt, *J* = 3.5, 1.7, 0.8 Hz, 1H), 2.85 (t, *J* = 7.6 Hz, 2H), 2.67−2.54 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ 175.65, 131.33, 120.38, 106.58, 104.98, 33.27, 21.31.

LC-MS/MS Assay for CEP-PE in Human Plasma. Synthesis of Internal Standard d_4-CEP-PE. The internal standard, d_4-CEP-PE, was synthesized through a PLD (*Streptomyces* sp.) catalyzed transphosphatidylation reaction (Scheme 2).[23]

Scheme 2. Chemical Synthesis of Internal Standard d_4-CEP-PE from Egg PC and d_4-CEP-ETN through Transphosphatidylation Mediated by PLD (*Streptomyces* sp.)



L-α-Phosphatidylcholine (egg PC) (9.1 mg, 0.012 mmol) was mixed with 300 μL of ethyl acetate. Then, d_4-CEP-ETN (5.8 mg, 0.031 mmol) in 200 μL of 0.2 M sodium acetate buffer (pH 5.6) containing 80 mM $CaCl_2$ and 80 units of PLD (*Streptomyces* sp.) was added. The biphasic reaction was vigorously stirred at 37 °C for 3 h and then terminated by extraction with three portions of 500 μL of ethyl acetate. The organic layers were combined, and solvent was evaporated under reduced pressure. The residue was purified by flash silica gel chromatography. Unreacted d_4-CEP-ethanolamine was eluted first with $CHCl_3$/MeOH (20:1, v/v). Then, pure d_4-CEP-PE (9.3 mg, 72.1%) was eluted with $CHCl_3$/MeOH (10:1, v/v. R_f = 0.2). ESI-MS analysis confirmed the generation of d_4-CEP-PE (Supporting Information Figure S1). The average molecular weight (MW) of d_4-CEP-PE was calculated to be 853.1 according to the average MW of egg PC (770.1) plus a mass increment of 83 for CEP modification.

Human Plasma Sample Preparation. Human blood plasma samples from donors with AMD and age-matched donors with no disease (controls) were provided by the Cole Eye Institute of the Cleveland Clinic. Total lipids were isolated from human blood plasma (from blood collected in the presence of EDTA) as follows: 100 μL of plasma was mixed with 200 μL of acetone in the presence of 0.5 mM butylated hydroxytoluene (BHT) to precipitate proteins. After centrifugation for 20 min at 10 000 rpm, the supernatant was transferred to a clean vial and dried under a stream of nitrogen gas. The samples were stored under nitrogen at −80 °C until analysis.

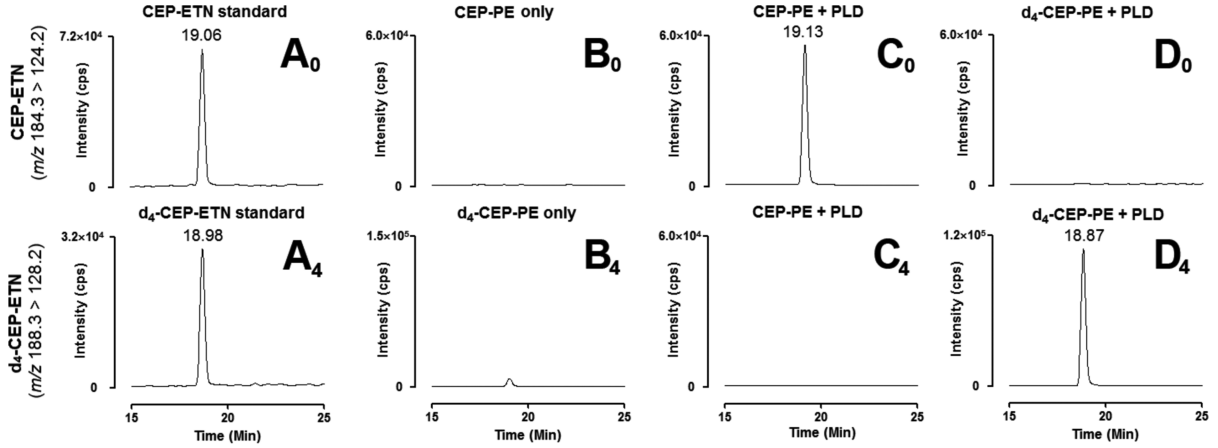


Figure 1. LC-MS/MS analysis showing that PLD (*Streptomyces chromofuscus*) can effectively hydrolyze CEP-PE and d_4-CEP-PE to CEP-ETN and d_4-CEP-ETN, respectively. CEP-PE (panels A_0−D_0) or d_4-CEP-PE (panels A_4−D_4) standards were treated either without (panels A_0, B_0, A_4, B_4) or with (panels C_0, D_0, C_4, D_4) PLD for 2 h. Then, CEP-ETN and d_4-CEP-ETN were analyzed by MRM transitions m/z 184.3 → 124.2 or 188.3 → 128.2, respectively.

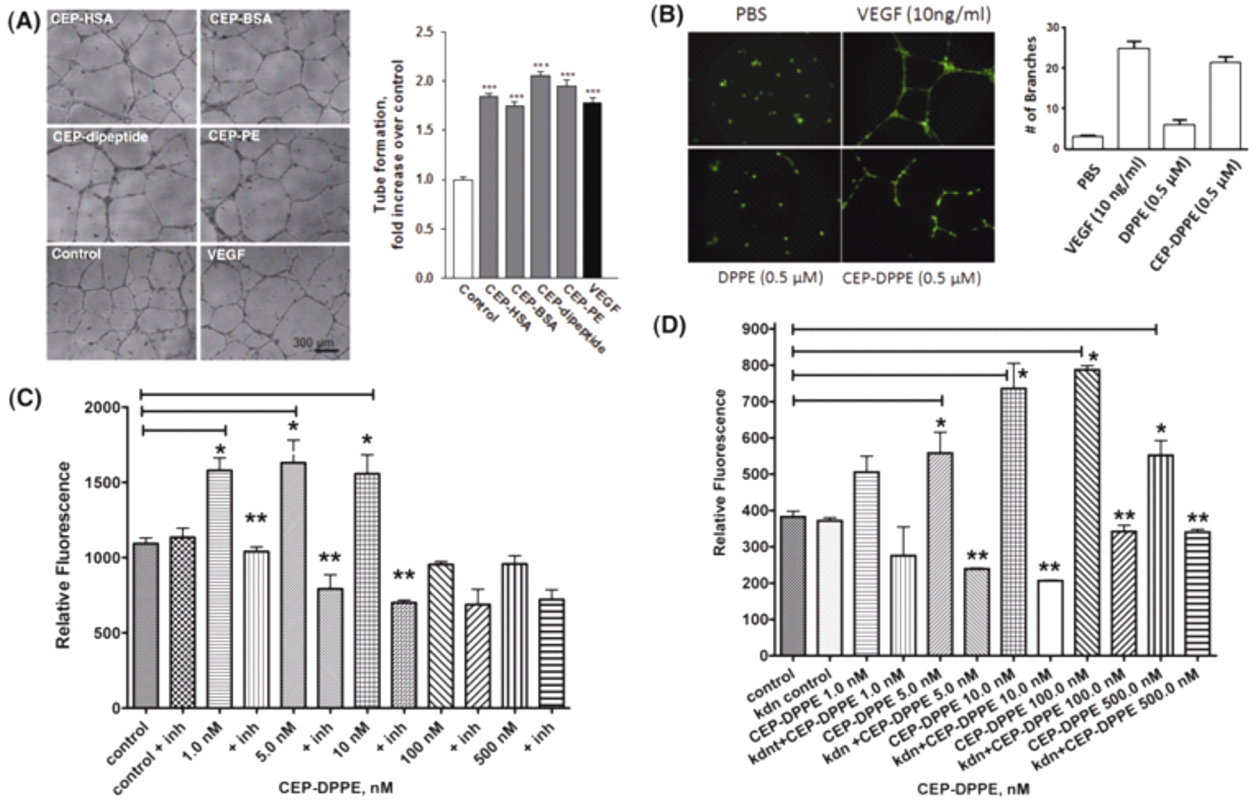


Figure 2. Effects of CEP-DPPE on tube formation by HUVECs. (A) Assay used PBS as negative control. HUVEC cells cocultured with Matrigel in the absence (control) or presence of test samples: 60 ng/mL VEGF, 1.3 μM CEP-dipeptide, 0.5 μM CEP-DPPE, and 0.5 μg/mL of CEP in CEP-BSA and CEP-MSA protein derivatives for 8 h. Right: quantification of tube formation assay reported as mean ± SD (n = 3); (B) Assay used DPPE as negative control. Left: representative micrographs of cells with various treatments as indicated. Right: quantification of tube formation assay reported as mean ± SD (n = 3); (C) Effect of oxPAPC (30.0 μg/mL), a TLR2/TLR4 inhibitor, on the tube-formation ability of HUVEC cells in the absence and presence of various concentrations of CEP-DPPE. Results shown are the mean of duplicate cultures ± SD and are representative of two similar experiments. *, $p < 0.001$ versus control (ANOVA); **, $p < 0.02$ (t-test) HUVEC CEP-DPPE treatment versus the respective concentration of CEP-DPPE treatment in the presence of oxPAPC inhibitor; (D) Effect of TLR2 knockdown (kdn) on the tube formation ability of HUVECs in the absence and presence of various concentrations of CEP-DPPE. Results shown are the mean of duplicate cultures ± SD and are representative of two similar experiments. *, $p < 0.005$ versus control (ANOVA); **, $p < 0.03$ (t-test) HUVEC CEP-DPPE treatment versus the respective concentration of CEP-DPPE treatment in the presence of oxPAPC inhibitor.

Lipid Extraction and PLD Hydrolysis. The above samples were extracted by a modified Bligh and Dyer method to isolate phospholipids from nonlipid matrix. In brief, the residue was resuspended in 200 μL of PBS, followed by addition of 750 μL of chloroform/methanol (1:2, v/v) containing 1 mM BHT and 20 ng of internal standard (d_4-CEP-PE, 0.023 nmol), followed by addition of

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250 μL chloroform and 250 μL aqueous sodium chloride solution (1.5%). The resulting mixture was vortexed vigorously and then centrifuged for 10 min at 3000 rpm. The lower organic layer was collected and dried under a stream of nitrogen. The residue was stored under argon at −80 °C until analysis.

The isolated lipids were hydrolyzed with PLD (*S. chromofuscus*) by a modification of the method described previously for hydrolysis of isolevuglandin-modified PEs. Lipids were resuspended in 50 μL of methanol. Then, 450 μL of HBSS buffer (Thermo Scientific, Waltham, MA) supplemented with 5 mM $CaCl_2$ and 0.1 mM EDTA were added. The mixture was incubated at 37 °C for 30 min and then was sonicated for 10 min. The cloudy lipid mixture was passed through a 0.1 μm polycarbonate filter (20 times) for extrusion using an Avanti mini-extruder set (Avanti Polar Lipids, Inc., Alabaster, AL) to generate a homogeneous unilammelar lipid vesicle solution. Then, 280 units of PLD (*S. chromofuscus*) were added to each sample. The mixture was incubated under argon with gentle shaking at 37 °C overnight. The next day the samples were evaporated to dryness under a stream of nitrogen for 2 h. The residue was then reconstituted with 100 μL of methanol under argon and stored at −80 °C before LC-MS/MS analysis. Twenty microliters of the above solutions were injected into the LC-MS/MS for each analysis.

LC-MS/MS Analysis. Mass spectrometric analyses of CEP-ETN and d_4-CEP-ETN were performed on a Quattro Ultima triple-quadrupole mass spectrometer (Micromass, Wythenshawe, UK) equipped with a Waters Alliance 2690 HPLC system (pump and autosampler) (Waters, Milford, MA). Chromatographic separation was achieved using a 150 × 2.0 mm i.d. Prodigy ODS 5 μm column (Phenomenex). Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in methanol. 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% B; 22−30 min, isocratic at 100% B; 30−31 min, linear gradient from 100 to 5% B; 31−40 min, isocratic at 5% B. The flow rate employed was 200 μL/min. The analytes were measured mass spectrometrically in the positive ion mode with the source temperature at 120 °C, the desolvation temperature at 250 °C, the drying gas N_2 at 450 L/h, a cone gas flow rate of 70 L/h, and multiplier at 600, collision gas at 5 psi, and collision energy at 30 eV. Multiple reaction monitoring (MRM) of the transitions m/z 184.3 → 124.2 and 188.3 → 128.2 was used to analyze CEP-ETN and d_4-CEP-ETN, respectively (Figure 1A_0,A_4). No interference from the isobaric phosphocholine (exact mass: 183.07) was detected (Supporting Information Figure S2).

HUVEC Tube Formation Assays. For comparing the ability of various CEP derivatives to promote tube formation (Figure 2A), HUVECs were seeded on Matrigel-coated 48-well plates (BD Bioscience). DMEM-F12 medium (Media Lab, Cleveland Clinic, Cleveland, OH) was supplemented with various CEP derivatives as indicated and incubated with cultured HUVECs at 37 °C for 8 h. Tube formation by HUVECs was observed using a phase-contrast inverted microscope. Two independent assays were performed by two operators: one used PBS as control and the other used DPPE vesicles as control. The data were quantified either by measuring the length of tubes or by the number of branches.

For testing the effect of oxPAPC, a TLR2 inhibitor,[24] (Figure 2C) or TLR2 knockdown (Figure 2D) on the ability of CEP-PE to promote tube formation, growth factor-reduced (GFR) basement membrane extract (Cultrex BME, Trevigen, Gaithersburg, MD; 175 μL/well) was added to the wells of an ice-cold 48-well plate and then incubated at 37 °C for 1 h to allow the gel to solidify. Overnight-starved, trypsin-detached, and extensively washed HUVECs (2.5×10^4 cells/well; Promocell, GmbH, Heidelberg, Germany) in a basal growth medium 2 (GM2; Promocell, GmbH) were seeded onto the BME-coated wells. For testing the effect of a TLR2 inhibitor (Figure 2C), HUVECs, untreated or treated with 30.0 μg/mL of oxPAPC (InvivoGen; San Diego, CA) for 1 h prior to the experiment, were challenged with a basal medium alone (GM2) or a basal medium (GM2) containing 1−500 nM CEP-DPPE for at least 16−18 h in 5% CO_2/95% air at 37 °C. The following day, the cells were stained with Calcein AM solution (BD Biosciences; San Jose, CA, 1:100 dilution of a stock 1 mg/mL solution in DMSO in PBS) by adding 50 μL of working solution to each well.[25] After incubating HUVECs for 1 h in 5% CO_2/95% air at 37 °C, images (Supporting Information Figure S3) of the HUVECs (untreated, treated with CEP-DPPE, and treated with CEP-DPPE and 30.0 μg/mL of oxPAPC) were obtained using a Leica DMI 6000 B inverted microscope (5× magnification, FITC filter, Leica Microsystems, Wetzlar, Germany) equipped with a Retiga EXI camera (Q-imaging, Vancouver, British Columbia). Image analysis was performed using Metamorph Imaging Software (Molecular Devices, Downington, PA) in angiogenesis tube formation mode. For testing the effect of TLR2 knockdown (Figure 2D), HUVECs and HUVECs with a transient TLR2 gene knockdown (24 h, see below) were starved for 12 h in GM2 basal medium, treated with Accutase (BD Biosciences; San Jose, CA) as suggested by the manufacturer, and harvested from the plates using a rubber policeman. They were further thoroughly washed three times with GM2 basal medium, counted, and seeded onto BME-coated wells. HUVECs and HUVECs with transient TLR2 knockdown were challenged with basal medium (GM2) alone or GM2 cell culture media containing 1−500 nM CEP-DPPE for 12 h in 5% CO_2/95% air at 37 °C. The cells were then stained with Calcein AM solution, and images (Supporting Information Figure S4) were obtained and processed as described above.

Transient TLR2 Knockdown. HUVECs were seeded in six-well plates at a density of 0.27×10^6 cells/well in antibiotic-free normal GM2 growth medium to achieve 60−70% confluence on day 2. For transfection of each well, 6−12 μL of TLR2 siRNA duplex (Qiagen, Valencia, CA; 0.18−0.36 μg) was diluted in 100 μL of HBSS and was labeled A. Then, 6−12 μL transfection reagent (HiPerFect; Qiagen, Valencia, CA) was diluted in 100 μL of HBSS and labeled B. Solutions A and B were mixed together and incubated at room temperature for 45 min. The cell monolayer was rinsed in basal GM2 medium, and the siRNA transfection mix was added dropwise onto the monolayer in GM2 containing 2% FBS and incubated for 3 h in 5% CO_2/95% air at 37 °C. Fresh GM2 medium containing 10% FBS was added on the monolayer without removing the transfection mixture and was incubated for an additional 12 h in 5% CO_2/95% air at 37 °C.

Flow Cytometry. Monolayers of normal HUVECs and HUVECs with a transient TLR2 gene knockdown were detached from the plates by Accutase cell detachment solution (BD Biosciences; San Jose, CA). The cells were then washed with HBSS containing 1% FCS (PBS-FCS) and then were incubated with 2.0 μg of unconjugated primary mouse anti-human TLR2 LEAF antibody (CD282, BioLegend, San Diego, CA) in 0.2 mL of PBS−FCS for 45 min at 4 °C as described.[26] After washing three times with PBS−FCS, the cells were incubated with the secondary goat anti-mouse IgG-AlexaFluor 488 (Invitrogen, Carlsbad, CA) for 45 min at 4 °C. Normal and transiently transfected HUVECs were then washed with PBS−FCS and then were analyzed on a BD LSR II flow cytometer (BD Biosciences; San Jose, CA). The FACS data on HUVECs with transient TLR2 gene knockdown showed that TLR2 was completely absent in 20−30% of the cells.

Competitive Binding of CEP-PE vs CEP-HSA to Recombinant Mouse TLR2-Fc Protein. Small unilamellar vesicles of CEP-DPPE (125 μM) in phosphate buffered saline (PBS) were prepared by extruding (20 times) the hydrated phospholipids through a 0.1 μm polycarbonate filter using an Avanti mini-extruder (Avanti Polar Lipids, Inc., Alabaster, AL). The resulting solution was diluted to various concentrations by addition of PBS. To a 96-well ELISA plate was added 100 μL of 5 μM CEP in CEP-HSA protein in PBS solution into each well, and the plate was incubated at 37 °C for 1 h. PBS (100 μL) was used as control. Then, the wells were washed with PBS, and the nonbinding sites on wells were blocked with 350 μL of 4% bovine serum albumin (BSA) in PBS buffer for 1 h at 37 °C. After blocking was completed, a mixture of recombinant anti-mouse TLR2-Fc protein at a fixed concentration (1 μg/mL, in 1% BSA-PBS) with an equal volume of CEP-PE vesicles at concentrations from 0 to 125 μM was added to each well, and the plate was allowed to incubate for 1 h at 37 °C. DPPE vesicle solutions at 0, 50, 75, and 120 μM, prepared by sonication, were used as parallel samples. After washing the plate with PBS contining 0.1% Tween-20 (PBST) buffer, 100 μL of 1:2000 diluted goat anti-human Fc antibody in PBST buffer was added to each

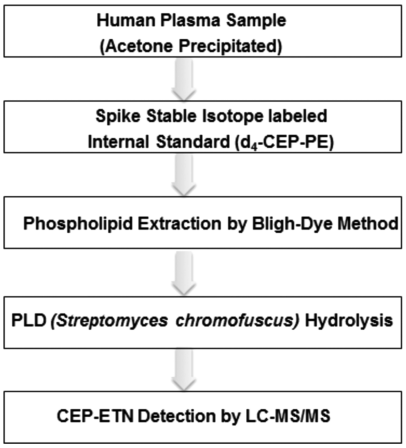
well. The plate was then incubated at room temperature for 1 h followed by washing with PBST buffer and was then developed by adding 100 μL of ABTS solution to each well. After an additional 30 min incubation, the absorbance of each well was read at 405 nm with a microplate reader (Bio-Rad, Hercules, CA).

RESULTS

PLD from *S. chromofuscus* Catalyzes Hydrolysis of CEP-PE To Generate CEP-ETN. The complexity of lipid side chains in naturally occurring EPs presents a challenging problem for CEP-EP detection by LC-MS because CEP derivatives are distributed into dozens of different diacyl, 1-alkyl-2-acyl, and 1-alkenyl-2-acyl ethanolamine phosphoglycerides. However, the analysis can be simplified by enzymatic hydrolysis of the phospholipids. Phospholipase D (PLD) from *S. chromofuscus* was used previously to efficiently hydrolyze isolevuglandin-EPs to form phosphatidic acid and isolevuglandin-ethanolamine (isoLG-ETN).[18] Analogously, we exploited the ability of this PLD to release CEP-ethanolamine (CEP-ETN) from the polar head of CEP-EP derivatives (Scheme 1). We anticipated that this method would eliminate the diversity inherent in naturally occurring EPs by converting all of the different CEP-EPs into a single molecule, CEP-ETN.

To verify CEP-PEs are substrates of PLD, CEP-DPPE and d_4-CEP-PE standards were treated with PLD from *S. chromofuscus*. Then, the generation of CEP-ETN and d_4-CEP-ETN was monitored by LC-MS/MS. LC-MS/MS data in Figure 1 showed that prominent levels of CEP-ETN and d_4-CEP-ETN peaks were observed in the hydrolyzed CEP-PE or d_4-CEP-PE samples, suggesting that these CEP-PEs are readily hydrolyzed by PLD (*S. chromofuscus*) to release CEP-modified ethanolamine headgroups (Figure 1C$_0$,D$_4$).

Scheme 3. Schematic Workflow of Analysis of CEP-PE Adduct by LC-MS/MS with PLD-Mediated Hydrolysis



LC-MS/MS Demonstration that CEP-EPs Are Present in Human Plasma. The workflow developed for detection of CEP-EPs in human plasma is shown in Scheme 3. In brief, lipid extracts from 200 μL of AMD and normal human plasma samples were hydrolyzed by 280 units of PLD (*S. chromofuscus*). d_4-CEP-PE (20 ng, 0.023 nmol) was added to each sample as internal standard before PLD hydrolysis. Then the CEP-ETN levels in the hydrolysis reaction product mixtures were determined by LC-MS/MS.

To minimize possible interference from variations in phospholipolysis efficiency and interference from the complex biological matrices that may vary among samples, a phospholipid internal standard was implemented for the LC-MS/MS analysis. A d_4-CEP-PE standard was prepared from L-α-phosphatidylcholine (egg PC) by enzyme-mediated trans-phosphatidylation. The d_4-CEP-PE standard obtained was a mixture containing deuterated CEP-PEs with various fatty acyl side chains, which strongly mimics the natural multiplicity of CEP-PE fatty acyl side chains in biological matrices. Figure 1B$_4$,D$_0$,D$_4$ shows the LC-MS/MS chromatograms from 20 ng of d_4-CEP-PE standard treated either with or without PLD hydrolysis. Figure 1D$_0$ suggests that hydrolyzed d_4-CEP-PE shows no interference from the MRM transition m/z 184.3 → 124.2 found in the chromatograph of CEP-ETN. In Figure 1B$_4$, although a trace amount of d_4-CEP-ETN was present in the d_4-CEP-PE, its molar percentage was less than 7%, or 1.6% in mass percentage (data not shown). A correction for this trace impurity could be applied during data analysis (*vide infra*).

A calibration curve for CEP-ETN quantification was established with samples containing pure CEP-ETN in HBSS buffer in the concentration range from 2 to 30 nM. A fixed amount of internal standard, d_4-CEP-ETN (0.023 nmol), was added to 200 μL of each calibrator standard. The peak area ratio of CEP-ETN to d_4-CEP-ETN from the LC-MS/MS analysis was plotted against calibrator concentrations to generate the equation as shown in Figure 3A. For human plasma samples, the CEP-PE concentration was calculated as follows

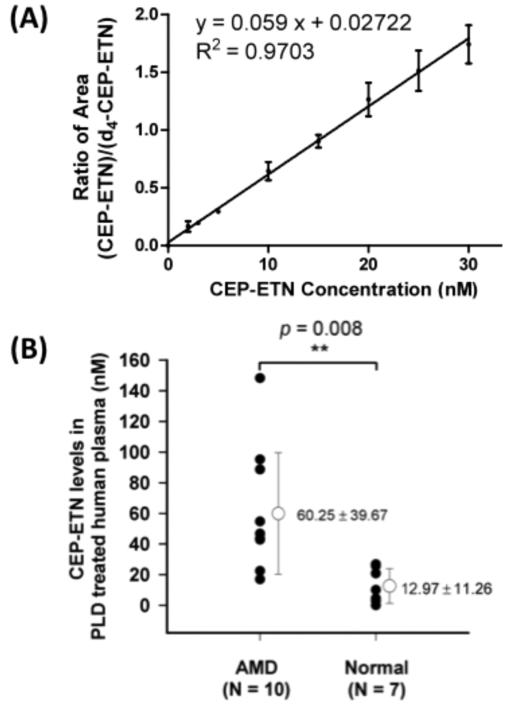


Figure 3. (A) Calibration curve of CEP-ETN. Data points represent mean ± SD of three independent experiments; (B) LC-MS/MS results indicate that CEP-ETN levels were significantly elevated in human plasma samples from AMD patients (n = 10) vs normal plasma samples (n = 7). Results are presented as mean ± SD.

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$$[\text{CEP-PE}] = [\text{peak area ratio of (CEP-ETN)} / (d_4\text{-CEP-ETN}) \times 1.074 - 0.0272] / 0.059\ (\text{nM})$$

where 1.074 is the factor to correct for interference caused by the trace d_4-CEP-ETN present in the d_4-CEP-PE standard.

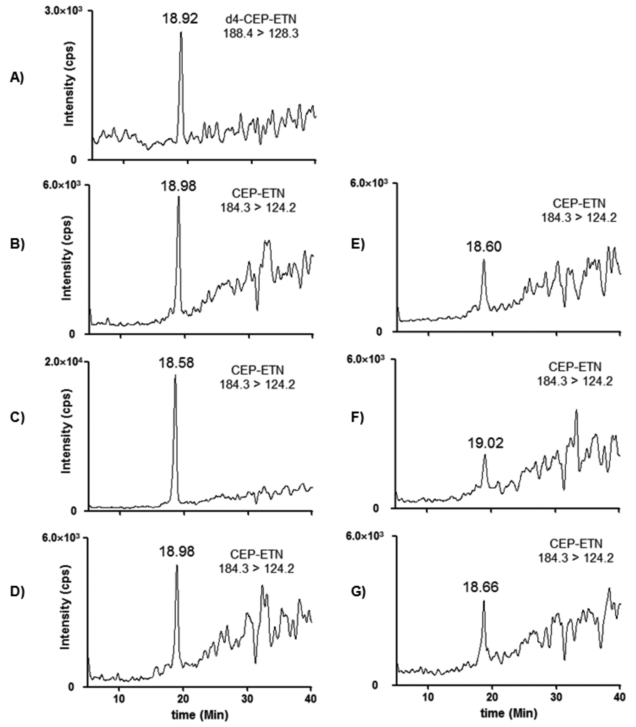

Figure 4. Representative LC-MS/MS chromatograms of (A) 20 ng of d_4-CEP-PE internal standard; (B–D) three representative LC-MS/MS chromatograms of CEP-ETN generated by PLD hydrolysis of phospholipids extracted from AMD patients' plasma samples; (E–G) three representative LC-MS/MS chromatograms of healthy control individual's plasma samples.

The LC-MS/MS data demonstrate that CEP-EP derivatives are present in human blood plasma. Representative LC-MS/MS chromatograms of CEP-ETN detected in PLD-hydrolyzed lipid extracts from human plasma samples are shown in Figure 4.

CEP-EP Levels Are Elevated in AMD Plasma. LC-MS/MS analysis of human plasma samples from donors with AMD (n = 10) and normal controls (n = 7) indicates that the CEP-EP derivative concentration in AMD samples is nearly 4.6-fold higher than its level in normal plasma samples (60 ± 40 vs 13 ± 11 pmol/mL; $p < 0.01$), as shown in Figure 3B. These results demonstrate that levels of CEP-EPs *in vivo* are strongly associated with age-related macular degeneration.

CEP-DPPE Induces Tube Formation of Cultured HUVECs. Using pure CEP-DPPE prepared by unambiguous chemical synthesis, we conducted *in vitro* tube formation assays to evaluate the pro-angiogenic effect of CEP-PE derivatives toward human umbilical vein endothelial cells (HUVECs). Two experiments, with three replicates each, were performed by two independent individuals: one used PBS buffer as the negative control and the other used DPPE as the negative control. Both experiments indicate that CEP-DPPE induces tube formation by HUVECs (Figures 2A,B), suggesting that CEP-DPPE promotes angiogenesis, similar to VEGF, CEP-protein, and CEP-peptide derivatives (Figure 2A).

Previously the mechanism of CEP-protein induced angiogenesis was shown to involve the TLR2 cell signaling pathway consequent to direct binding of CEP-protein to TLR2.[7] The CEP moiety was found to be responsible for the binding. Thus, it seemed reasonable to postulate that CEP-PE would also bind to TLR2 and induce tube formation through TLR2 signaling. To test this hypothesis, we measured the binding affinity of CEP-DPPE vesicles to a recombinant mouse TLR2-Fc fusion protein (mTLR2-Fc, R&D Systems, Minneapolis, MN) in a competitive binding assay. CEP-DPPE was shown to compete with CEP-HSA in binding with mTLR2-Fc protein, but DPPE does not inhibit the binding of CEP-HSA with TLR2 (Figure 5).

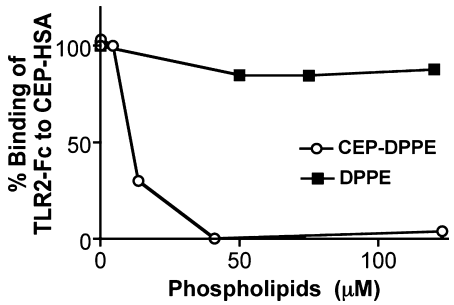

Figure 5. Binding assay in which CEP-DPPE, but not DPPE, competes with CEP-HSA to bind recombinant mouse TLR2-Fc fusion protein.

Direct evidence that CEP-PE induces angiogenesis through the activation of a TLR2 signaling pathway was then provided by the observation that oxPAPC, a TLR2/TLR4 inhibitor, inhibited ($p < 0.001$ or $p < 0.02$ versus control) the induction of HUVEC tube formation by CEP-DPPE (Figure 2C). In addition, knockdown of TLR2 (kdn) by TLR2 siRNA also abolished ($p < 0.005$ versus control or $p < 0.03$) the ability of CEP-DPPE to induce tube formation by HUVECs.

DISCUSSION

AMD is a progressive disease and a major cause of severe vision loss. Early identification of AMD risk could help to slow or prevent disease progression. Quantification of CEP-protein derivatives in human plasma by an enzyme-linked immunosorbent assay (ELISA) indicated that levels of CEP derivatives are significantly elevated in AMD plasma compared to that in normal controls.[1,19,20] However, the ELISA method cannot differentiate CEP-protein and CEP-PE derivatives, as we found that CEP-PE in vesicles also binds to anti-CEP antibody (Supporting Information Figure S5). Furthermore, the immunoassay is complicated by the presence of variable amounts of anti-CEP autoantibodies in AMD blood.[3]

We hypothesized that chronic inflammation in AMD patients would result in elevated plasma levels of oxidized lipid modified EPs. Previously, we showed that other modifications of PEs that are derived from oxidation of arachidonate phospholipids, i.e., isolevuglandin-PE derivatives, are present in human plasma and that their levels are significantly higher in blood from AMD donors than normal controls. In the present study, we detected and quantified CEP-EP derivatives by an LC-MS/MS method. Compared to our previous ELISA method, tandem mass spectrometric analysis is more specific because it can character-

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ize each analyte's molecular structural information, and the presence of immune complexes with autoantibodies does not interfere with the analysis.

The complexity of EP species mixtures present *in vivo* makes it difficult to analyze and quantify individual CEP-EPs by LC-MS/MS. Due to the varying fatty acyl, alkyl, and vinyl side chains in diacyl, alkyl acyl, or alkenyl acyl EPs, there are more than 30 different EP molecular species in cell membranes. The "dilution" of CEP into every type of EP molecule would result in the concentration of each different CEP-EP being low, making detection and quantification by mass spectrometric analysis difficult and less reliable. Our data show that, PLD from *S. chromofuscus* efficiently hydrolyzes the phosphodiester bond of CEP-EPs and releases a single molecule for analysis, CEP-ETN. Considering that the efficiency of the phospholipase activity may vary among different batches during sample preparation, we synthesized d_4-CEP-PE as an internal standard to minimize the impact of any variability and facilitate accurate determination of CEP-EP levels in human plasma samples. We used PLD from *Streptomyces* sp. to facilitate conversion of the headgroup of L-α-phosphatidylcholine (egg PC) to d_4-CEP-PE in high yield through transphosphatidylation. This method may be very useful for the synthesis of stable isotopically labeled internal standards for other biologically important modified PE derivatives, such as advanced glycated EP derivatives, i.e., carboxymethyl-EPs, carboxyethyl-EPs, and Amadori-EPs.

Our data show that, CEP-ETN was found in PLD *S. chromofuscus* hydrolyzed human plasma phospholipid extracts, which shows that CEP-EP is present in human blood plasma. In a pilot clinical study, we compared CEP-EP concentrations in plasma from AMD patients (n = 10) and normal controls (n = 7). The mean level of CEP-EP in AMD plasma is 4.6-fold higher than that in plasma from age-matched healthy controls (60 ± 40 vs 13 ± 11 pmol/mL; $p < 0.01$). These data exhibited higher differences between AMD and control than was found previously for CEP levels detected by an ELISA, i.e., only a 1.5-fold elevation in AMD plasma compared to that in plasma from age-matched healthy controls.[19] If this result is confirmed in a larger clinical study, then the new LC-MS/MS analysis of CEP-EP is likely to provide a superior biomarker for assessing risk and monitoring the efficacy of therapeutic measures for the diagnosis and treatment of age-related macular degeneration.

We are particularly interested in the biological activities that CEP-EPs may exhibit. Since CEP-protein derivatives were found to be novel factors that induce angiogenesis and were considered to be involved in many important biological events *in vivo* such as the choroidal neovascularization of "wet" AMD, wound healing, and tumor angiogenesis and growth, it is reasonable to expect that CEP-EP derivatives have similar biological activities. We now demonstrated that CEP-PE induces tube formation by HUVECs. For example, all of the CEP derivatives tested promoted the formation of longer tubes than that observed for the control (Figure 4A). Presumptive evidence that CEP-PE induces this effect through binding with TLR2 was provided by the observation that CEP-PE competes with CEP-protein for binding to recombinant mouse TLR2-Fc protein. Direct evidence that CEP-PE induces angiogenesis through the activation of a TLR2 signaling pathway was then provided by the observation that oxPAPC, a TLR2/TLR4 inhibitor, or knockdown of TLR2 with TLR2 siRNA abolished the ability of CEP-DPPE to induce tube formation by HUVECs.

A CAVEAT AND FUTURE PROSPECTS

In interpreting CEP-EP levels as a disease biomarker, it is important to recognize that although elevated CEP-EP levels correlate strongly with AMD they may also be generated in other chronic inflammatory environments, such as that present in tumors. Such environments promote free radical-induced lipid oxidation, leading to endogenous CEP, as was found in human melanoma.[7] It seems likely, but remains unproven, that CEP-EPs promote tumor angiogenesis and progression and that CEP-EP levels in plasma from cancer patients correlate with those consequences of CEP-EP generation in tumors. In view of the production of radical and reactive oxygen species during radiation therapy,[27] it is tempting to speculate that radiation-induced free radical generation will cause oxidation of docosahexaenoate phospholipids, especially in tissues such as brain and retina that have especially high levels of docosahexaenoate. The consequent production of CEP-protein and CEP-EPs may eventually lead to CEP-induced angiogenesis and tumor progression. The new LC-MS/MS assay for levels of blood CEP-EPs may prove to be useful for testing the hypothesis that elevated blood CEP-EP correlates with tumor progression after failure of radiation therapy or of anti-VEGF therapy, which blocks angiogenesis that is promoted by activation of the VEGF pathway but not that induced by CEP through the TLR2 pathway. In other words, elevated levels of CEP may contribute to the resistance that develops against these anticancer therapeutic modalities.

CONCLUSIONS

In summary, our studies indicate that a lipid oxidation product from docosahexaenoate-containing phospholipids modifies EPs *in vivo*, generating CEP-EPs. CEP-EPs are found in human plasma, and their levels are significantly elevated in plasma from donors with age-related macular degeneration. CEP-PE exhibits pro-angiogenic activity that is dependent on the TLR2 signaling pathway, as was found previously for CEP-protein and -peptide derivatives.

ASSOCIATED CONTENT

Supporting Information

Figure S1: Mass spectrometric analysis of authentic synthetic d_4-CEP-PE from transphosphatidylation of egg PC and d_4-CEP-ETN. Figure S2: LC-MS/MS analysis showing that the isobaric compound phosphocholine does not interfere the detection of CEP-ETN. Figure S3: Effect of oxPAPC (30.0 μg/mL) on tube formation by HUVECs in the absence and presence of various concentrations of CEP-DPPE. Figure S4: Effect of TLR2 knockdown on tube formation by HUVECs in the absence and presence of various concentrations of CEP-DPPE. Figure S5: (A) Structure of a CEP-PE derivative, e.g., CEP-DPPE; (B) CEP-PE inhibits the binding of CEP-protein to anti-CEP antibody with affinity similar to that of CEP-CEO. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

AMD, age-related macular degeneration; CEP, 2-ω-carboxyethylpyrrole; DHA-PL, docosahexaenoate containing phospholipid; EP, ethanolamine phospholipid; HUVEC, human umbilical vein endothelial cell; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLD, phospholipase D; TLR2, Toll-like receptor 2; VEGF, vascular endothelial growth factor

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