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Carboxyethylpyrroles: From Hypothesis to the Discovery of Biologically Active Natural Products

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Abstract

Our research on the roles of lipid oxidation in human disease is guided by chemical intuition. For example, we postulated that 2-(ω -carboxyethyl)pyrrole (CEP) derivatives of primary amines would be produced through covalent adduction of a γ -hydroxyalkenal generated, in turn, through oxidative fragmentation of docosahexaenoates. Our studies confirmed the natural occurrence of this chemistry, and the biological activities of these natural products and their extensive involvements in human physiology (wound healing) and pathology (age-related macular degeneration, autism, atherosclerosis, sickle cell disease and tumor growth) continue to emerge. This perspective recounts these discoveries and proposes new frontiers where further developments are likely. Perhaps more significantly, it depicts an effective chemistry-based approach to the discovery of novel biochemistry.

Graphical abstract

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Conflict of Interest

The mouse model for dry AMD described in this perspective is protected for commercialization by OHR Pharmaceuticals. R. G. Salomon is a coinventor. The CEP-immunization model patent is "Non-human model of autoimmune disease," number 20090155243.

INTRODUCTION

The classical approach to identifying natural products begins with detection and isolation, often by monitoring a biological activity, followed by confirming identity through total chemical synthesis. This approach is minimally useful when the natural product is a complex mixture of post-translationally and promiscuously modified proteins. We have relied on an alternative approach, hypothesis of products that are likely to be generated by lipid oxidation, followed by synthesis, and then detection in vivo guided by the availability of authentic standards, and last, characterization of biological activities.¹ Previously, 4-hydroxy-2-nonenal (HNE) had been shown to react with primary amines to produce 2-pentylpyrrole (PP) derivatives (Scheme 1).² To test the hypothesis that PP derivatives are produced in the biological milieu, we generated antibodies that recognize PPs, and detected the time-dependent appearance of the PP epitope in protein exposed to HNE.³ PP immunoreactivity was found in brain tissue from individuals with Alzheimer's disease, not only in neurofibrillary tangles, but also in neurons lacking neurofibrillary tangles,⁴ and in Rosenthal fibers, the histological hallmark of Alexander's disease,⁵ but not in age-matched control brain, suggesting that the accumulation of post-translationally modified proteins contributes to the pathogenesis of Alexander's and Alzheimer's diseases. PP immunoreactivity was also detected in atherosclerotic plaques.⁶

Free radical-induced cogeneration of 2-(ω -carboxyheptyl)pyrroles with pentylpyrroles

We confirmed that nonenzymatic, free radical-induced oxidation of low-density lipoprotein (LDL) produces PP epitopes.⁶ The levels of PP immunoreactivity detected in human plasma

were found to be significantly elevated in renal failure and atherosclerosis patients when compared to those in healthy volunteers.⁶ The initial intermediate of free-radical induced oxidation of linoleate, a pentadienyl radical, is expected to produce both 9- and 13-hydroperoxide intermediates. Fragmentation of the 9-hydroperoxide delivers HNE. We anticipated that fragmentation of a 13-hydroperoxide would generate a 9-hydroxy-12-oxodocohept-10-enoate, e.g., HODA-PL, and that protein adduction would then produce 2-(ω -carboxyheptyl)pyrrole (CHP) derivatives (Scheme 1). To test this hypothesis, we raised antibodies against a CHP-modified protein, and used them to confirm that free radical-induced oxidation of LDL also produces CHP epitopes.⁷ Since ester hydrolysis with KOH markedly elevated levels of immunoreactive epitopes detected in oxidized LDL, the CHP is presumably generated by reactions of oxidized cholesteryl esters, triglycerides, and phospholipids with LDL protein, and only some of these oxidized esters are hydrolyzed, e.g., by phospholipase activity associated with LDL. CHP immunoreactivity was detected in human plasma, and levels were significantly elevated in blood from renal failure and atherosclerosis patients compared with healthy volunteers.⁷

γ -Hydroxyalkenal phospholipids

To test the hypothesis that oxidation of LDL produces γ -hydroxyalkenal phospholipids, e.g., HODA-PL (Scheme 1), we prepared authentic samples of oxidatively truncated phosphatidylcholines by unambiguous total chemical syntheses.^{8,9} Not only did this allow confirmation of their presence in oxidized LDL, but it also allowed characterization of a family of more highly oxidized derivatives.¹⁰ Thus, we postulated their formation, prepared authentic samples by unambiguous chemical syntheses, and then established their presence by LC-MS/MS comparison with components of the LDL oxidation reaction product mixture.¹¹ The pure samples of these oxidatively truncated phospholipids that were only available by unambiguous chemical syntheses were absolutely essential for characterization of their biological activities. For example, 5-hydroxy-8-oxohept-6-enoyl phosphatidylcholine (HOOA-PC) dose-dependently activates human aortic endothelial cells (ECs) to bind monocytes, and causes a dose-dependent two- to threefold increase in levels of monocyte chemotactic protein-1 and interleukin-8 – chemokines that are important in monocyte entry into chronic lesions.⁹ HOOA-PC also inhibits LPS-induced expression of E-Selectin, a major adhesion molecule that mediates neutrophil endothelial interactions.⁹ γ -Hydroxyalkenal phospholipids and their more oxidized derivatives are also ligands for the scavenger receptor CD36 that trigger endocytosis of oxidized LDL by macrophage cells.¹⁰ They also promote the physiologically important CD36 mediated phagocytosis of oxidatively damaged rod photoreceptor cell tips by retinal pigmented epithelial cells.¹² On platelets they promote aggregation, accounting for the prothrombotic phenotype that is linked with hyperlipidemia and oxidant stress.¹³ They also inhibit scavenger receptor B1-mediated selective uptake of cholesteryl esters in hepatocytes, and thus, may have an inhibitory effect on reverse cholesterol transport.¹⁴

DISCUSSION

2-(ω -Carboxyethyl)pyrroles¹⁵

By analogy with the chemistry that we had established for oxidative fragmentation of linoleyl and arachidonyl phospholipids, we postulated that oxidation of docosahexaenoyl phospholipids would produce 4-hydroxy-7-oxohept-5-enoate (HOHA) phospholipids, and that their reaction with primary amino groups of protein lysyl residues would lead to the production of 2-(ω -carboxyethyl)pyrrole (CEP) derivatives (Scheme 1). Immunological evidence for the presence of CEPs in vivo was obtained with anti-CEP antibodies raised against a CEP-modified protein. Because CEP is uniquely derived from docosahexaenoate (DHA) and because DHA is especially abundant in the brain and retina, we first looked for CEP in retina. Immunocytochemistry localized CEP to photoreceptor rod outer segments and retinal pigment epithelium in mouse retina and demonstrated more intense CEP immunoreactivity in photoreceptors from a human age-related macular degeneration (AMD) donor compared with healthy human retina.¹⁶ CEP immunoreactivity was found to be associated with drusen, extracellular deposits that accumulate below the retinal pigment epithelium and are risk factors for developing AMD.^{17, 18}

In a pilot clinical study, the mean level of anti-CEP immunoreactivity in AMD human plasma (n = 19 donors) was 1.5-fold higher (p = 0.004) than in age-matched controls (n = 19 donors). Sera from AMD patients demonstrated mean titers of anti-CEP autoantibody 2.3-fold higher than controls (p = 0.02). Of individuals (n = 13) exhibiting both antigen and autoantibody levels above the mean for non-AMD controls, 92% had AMD. These results suggested that together CEP immunoreactivity and autoantibody titer have diagnostic utility in predicting AMD susceptibility. A larger clinical investigation, with 916 AMD and 488 control donors, confirmed the correlation of elevated blood CEP and CEP autoantibodies with AMD.¹⁹ Mean CEP adduct and autoantibody levels are elevated in AMD plasma by approximately 60 and approximately 30%, respectively, and these markers can discriminate between AMD and control plasma donors with approximately 76% accuracy.²⁰

A surprising twist in the road between HOHA-PC and CEP

To facilitate investigations of the chemistry and biology of γ -hydroxyalkenal phospholipids, we devised an efficient total synthesis that generates the sensitive functional array of the target phospholipids under mild conditions.²¹ Unexpectedly, HOHA-PC spontaneously deacylated under physiological conditions ($t_{1/2}$ = 30 min at 37 °C and pH 7.4). The reaction proceeds through an intramolecular transesterification that is especially favorable for HOHA-PC because it generates a 5-membered lactone, HOHA-lactone (Scheme 2).²² This proclivity toward spontaneous deacylation would interfere with isolation of HOHA-PC from biological samples. The availability of pure HOHA-PC through unambiguous chemical synthesis enabled the discovery of its extraordinary instability. HOOA-PC undergoes a much slower deacylation that generates a 6-membered lactone, and HODA-PC shows no proclivity toward spontaneous deacylation.

It is especially noteworthy that this nonenzymatic deacylation produces 2-lysophosphatidylcholine (lysoPC). Elevated levels of lysoPC, present in oxidatively

damaged low-density lipoprotein, are implicated in cardiovascular complications associated with numerous pathological conditions.^{23–31} LysoPC is generated by free radical-catalyzed oxidation of polyunsaturated PCs to oxidatively truncated PCs that are especially susceptible to hydrolysis by platelet-activating factor acetylhydrolase, a phospholipase A2 (PLA2) that exists in plasma largely in association with LDL.³² Drugs that aim to prevent the generation of lysoPC by inhibiting this PLA2-catalyzed hydrolysis are in advanced clinical trials.³³ Our discovery that the spontaneous deacylation of HOHA-PC occurs readily under physiological conditions suggests a limitation for the efficacy of antiphospholipase drugs because they cannot block this nonenzymatic pathway to lysoPC.

HOHA-lactone is a major precursor of CEP

In model studies, the reaction of HOHA-PC with a dipeptide, Ac-Gly-LysOMe, produced a pyrrole adduct with the CEP esterified to lysoPC³⁴ as well as unesterified CEP-dipeptide.³⁵ Most importantly, the majority of CEP-dipeptide generated in the reaction of HOHA-PC with Ac-Gly-Lys-OMe is produced through spontaneous deacylation followed by reaction of the resulting HOHA-lactone with Ac-Gly-Lys-OMe, and not by hydrolysis of CEP esterified to lysoPC.³⁵ In other words, the nonenzymatic deacylation of HOHA-PC to produce HOHA-lactone and its reactions with the ϵ -amino group of protein lysyl residues to form CEPs occur more readily than the reaction of HOHA-PC to generate CEP derivatives esterified to lysoPC.

HOHA-lactone diffuses into RPE cells that metabolize it and secrete the metabolites

The likely biological significance of this chemistry includes the fact that HOHA-lactone (CLogP: -1.02) is expected to be comparable to cortisone (CLogP: -0.93) in the ability to diffuse across cell membranes. In contrast, HOHA esterified to phospholipids is membrane bound. Diffusion of HOHA-lactone can result in the generation of CEPs in locations remote from the site of phospholipid oxidation in vivo. HOHA-lactone also reacts with the primary amino group of ethanolamine phospholipids to produce the corresponding CEP derivatives.³⁵ Exposure of rats to bright light generates elevated levels of CEP in their retinas and blood.³⁶ It seems reasonable to postulate that light exposure produces HOHA-lactone in the retina and that this product of photooxidative injury in the eye diffuses into the blood of these animals where it forms CEP derivatives of blood proteins and ethanolamine phospholipids. Diffusion into the blood of HOHA-lactone generated in photoreceptor disk membranes in the eye may contribute to the elevated levels of CEP present in blood from AMD patients noted above.

HOHA-lactone is biologically active

It may contribute to the advanced “wet” form of AMD by causing the secretion of vascular endothelial growth factor (VEGF) that fosters sprouting of choroidal capillaries through the retinal pigmented endothelium (RPE) into the neural retina. Low concentrations ($0.1\text{--}1\text{ }\mu\text{M}$) of HOHA-lactone promote the secretion of vascular endothelial growth factor by ARPE-19 cells.³⁵ Metabolism of HOHA-lactone by RPE cells can also protect the retina from its pathological activities and those of the derived CEP (vide infra). HOHA-lactone readily diffuses into and is metabolized by RPE cells.³⁷ A reduced glutathione (GSH) Michael adduct of HOHA-lactone is the most prominent metabolite that appears inside of ARPE-19

cells within seconds after exposure to HOHA-lactone, and is then exported from the cytosol to the extracellular medium.³⁷ This metabolism can provide protection against the pathological involvements of HOHA-lactone and CEP. However, it also causes depletion of intracellular GSH that is needed to combat oxidative stress, making the cells vulnerable to further oxidative damage.

CEP promotes global retinal atrophy

The appearance of anti-CEP autoantibodies in blood from patients with AMD suggested that an immune response might contribute to the pathogenesis of the disease. To create an animal model, mice were immunized with CEP-modified³⁸ mouse serum albumin (CEP-MSA). These mice develop antibodies to this hapten, fix complement component-3 in Bruch's membrane, accumulate drusen below the RPE during aging, and develop AMD-like lesions in the RPE that resemble the geographic atrophy, the blinding end-stage condition characteristic of "dry" AMD.³⁹ A close relationship was observed between the CEP specific antibody titer and the severity of outer retina pathology. Presumably, the immunized mice are sensitized to the generation of CEP in the outer retina, where DHA is abundant, oxygen tension is high and photoinduced generation of radical species provide a permissive environment for oxidative damage. Histology of the CEP-MSA eyes revealed deposition of C3d, vesiculation and swelling of individual or multiple adjacent RPE cells as well as cell lysis, pyknosis and the presence of monocytes in the interphotoreceptor matrix. An antibody-mediated response to CEP-MSA is evidenced, inter alia, by the deposition of C3d on Bruch's membrane below the RPE. C3d is a degradation product of C3b, a key complement protein required for the generation of the C3 and C5 convertases in the classical, lectin and alternate pathways.⁴⁰ This process requires an intact immune system, because CEP-MSA-immunized Rag-deficient mice, which are missing mature T cells and B cells,⁴¹ showed none of the changes observed in CEP-MSA-immunized normal mice.

There was a time-dependent increase in the number of macrophages within the interphotoreceptor matrix (IPM), between the retinal pigment epithelium and photoreceptor outer segments in immunized mice relative to young age-matched controls prior to overt retinal degeneration.⁴² Intracellular staining showing the production of tumor necrosis factor- α and interleukin (IL)-12 but not IL-8, established that the macrophages in immunized mice are polarized toward the proinflammatory M1 and not the anti-inflammatory M2 phenotype. This conclusion was substantiated by mRNA quantification on IPM-infiltrating macrophages isolated by laser capture. M1 marker genes (IL-6, TNF- α , and IL-1 β) were observed only in CEP-immunized mice, whereas IL-10 expression was not detected. The observation of elevated M1/M2 ratios in human AMD eyes⁴³ in contrast with a predominance of M2 macrophages in normal human aging eyes supports the disease relevance of CEP-induced M1 polarization in the CEP-immunized mouse model. CEP-immunized mice also exhibited increased expression of Ccl2, a monocyte chemoattractant that has been implicated in AMD, suggesting that the Ccl2/Ccr2 axis may play a role in CEP-induced pathology.⁴² Macrophages were not present in the IPM and no retinal lesions were observed in CEP-immunized Ccr2-deficient mice, suggesting a deleterious role for these cells in this mouse model of "dry" AMD.

CEP eye injections in mice induced acute pro-inflammatory gene expression in the retina.⁴⁴ CEP acts directly and indirectly to influence M1 macrophage polarization. Interferon (IFN)- γ and IL-17-producing CEP-specific T cells were identified ex vivo after CEP immunization. These T-cells induced M1 macrophage polarization in vitro. CEP-mediated retinal pathology also occurs in mice lacking mature B cells, indicating that AMD-like pathology in the CEP-immunized model is antibody-independent and T cell-mediated. Analysis of mice with defects in several T cell differentiation pathways suggests that Th1 (IFN- γ producing) cells are important for development of disease. Thus, M1 macrophages and antigen-specific T cells activated by oxidative damage-induced CEP derivatives work together at the early onset stage of dry AMD. These discoveries led to the hypothesis that pharmacological inhibition of T cell activation can prevent CEP-mediated retinal pathology. Treatment CEP-immunized mice with a combination drug therapy aimed at suppressing T cell responses. Cyclosporine A and Rapamycin caused downregulation of anti-CEP titers and prevented CEP-induced retinal pathology.⁴⁴

CEP promotes activation of the NLRP3 inflammasome

CEP-HSA primes the ATP-induced secretion of active inflammatory cytokines IL-1 β and IL-18 through a Toll-like receptor (TLR)-2 dependent activation of the macrophage NACHT, LRR and PYD domains-containing protein (NLRP)3 inflammasome (Figure 1).⁴⁵ Cleaved caspase-1 and NLRP3 were found in activated macrophages in the retinas of mice immunized with CEP-MSA, modeling a dry-AMD-like pathology. Activation of the NLRP3 inflammasome is expected to contribute to a vicious cycle of inflammation⁴⁶ that fosters the generation of reactive oxygen and nitrogen species⁴⁷ that promote lipid oxidation and the production of more CEP.

CEP promotes VEGF-independent laser-induced choroidal neovascularization

Laser-induced choroidal neovascularization (CNV), a model of the advanced stage AMD is referred to as “wet” AMD because new capillaries that sprout from the choroidal vasculature through the RPE into the neural retina are leaky. To test the hypothesis that CEP protein adducts stimulate angiogenesis and possibly contribute to CNV in AMD, the angiogenicity of CEP-modified human serum albumin (CEP-HSA) or CEP-modified acetyl-Gly-Lys-O-methyl ester (CEP-dipeptide) were tested in a rat corneal micropocket assay.⁴⁸ Low picomole amounts of CEP-HSA or CEP-dipeptide stimulate CNV. Mouse monoclonal anti-CEP antibody neutralized the limbal vessel growth stimulated by CEP-HSA, whereas anti-VEGF antibody only partially neutralized vessel growth. Subretinal injections of CEP-MSA exacerbated laser-induced CNV in mice.⁴⁸ In vitro treatments of human retinal pigment epithelial cells with CEP-dipeptide or CEP-HSA did not induce increased secretion of vascular endothelial growth factor (VEGF) that is a well-known inducer of CNV. These results demonstrate that CEP-induced angiogenesis utilizes VEGF-independent pathways and suggest that anti-CEP therapeutic modalities might be of value in limiting CNV in AMD.

As noted above, HOHA-lactone promotes secretion of VEGF. Ironically, CEP-dependent stimulation of angiogenesis is opposed by the ability of CEP to indirectly inhibit VEGF secretion. A protective role for CEP-induced NLRP3 activation and secretion of IL-18 in the

progression of CNV through inhibition of VEGF synthesis was postulated because VEGF-dependent laser-induced CNV is exacerbated in *Nlrp3*($-/-$) but not *Il1r1*($-/-$) mice, directly implicating IL-18 in the regulation of CNV development. Treatment of ARPE-19 cells and a mouse brain microvascular endothelial cell line (bEnd.3) with recombinant IL-18 significantly decreased the amount of VEGF secreted by both ARPE-19 and bEnd.3 cells. These findings implicate a role for IL-18 in the regulation of VEGF expression and could explain the exacerbated CNV in *Nlrp3* $-/-$ and *IL18* $-/-$ mice.

CEP-promoted angiogenesis in tumor vascularization and wound healing is TLR2-dependent

The involvement of TLR2 in biological responses to CEP was first demonstrated in studies on tumor vascularization.⁴⁹ CEP accumulates at high levels in ageing tissues in mice and in highly vascularized tumours in both murine and human melanoma. Recognition of CEP by TLR2, but not TLR4 or scavenger receptors on ECs was demonstrated exploiting receptor knockout and blocking antibodies. The TLR2-dependent angiogenic response to CEP is independent of VEGF. CEP also promotes angiogenesis in hind limb ischaemia and wound healing models through MyD88-dependent TLR2 signalling.⁴⁹ Neutralization of endogenous CEP with an anti-CEP antibody impaired wound healing and tissue revascularization and diminished tumour angiogenesis and growth. Both TLR2 and MyD88 are required for CEP-induced stimulation of Rac1 and endothelial migration. These findings established a new function of TLR2 as a sensor of oxidation-associated molecular patterns, providing a key link connecting inflammation, oxidative stress, innate immunity and angiogenesis.

CEP-MSA activates innate immune signaling in murine bone-marrow derived macrophages by specifically synergizing with low-dose TLR2-agonists, but not agonists for other TLRs, to induce the production of inflammatory cytokines.⁵⁰ Moreover, CEP selectively augments TLR2/TLR1-signaling instead of TLR2/TLR6-signaling.

CEP-modified ethanolamine phospholipids

Besides the ϵ -amino group of protein lysyl residues, oxidation of DHA lipids also leads to the modification of primary amino groups in ethanolamine phospholipids (EPs). LC-MS/MS analysis revealed the presence of CEP-EPs in human blood, and levels are 4.6-fold higher in AMD plasma than in normal plasma.⁵¹ Using an LC-MS/MS method that simultaneously measures PP-, CEP-, 2-(ω -carboxypropyl)pyrrole (CPP)- and CHP-modified EPs, elevated levels of all of these products of nonenzymatic lipid oxidation were found in blood from individuals with sickle cell disease (SCD).⁵² As for the corresponding CEP-modified proteins, CEP-EPs are pro-angiogenic, inducing tube formation by human umbilical vein ECs in a TLR2-dependent manner.⁵¹

CEP and CPP promote platelet aggregation in a TLR9- and TLR2-dependent manner

CEP- and CPP-modified proteins promote platelet activation, granule secretion, and aggregation of human and murine platelets in vitro and thrombosis in vivo via the TLR9/MyD88 pathway. This showed, for the first time, that TLR9 is a functional platelet receptor that links oxidative stress, innate immunity, and thrombosis. CEP- and CPP-PEs are present

in the plasma of hyperlipidemic ApoE(−/−) mice. In contrast with the analogous protein modifications, they bind directly to TLR2 and induce platelet integrin $\alpha\text{IIb}\beta 3$ activation and P-selectin expression in a TLR2-dependent manner.⁵³ They also accelerate murine intravital thrombosis in TLR2-dependent manner. Thus, these end products of lipid peroxidation, which accumulate in the circulation in hyperlipidemia, induce platelet activation by promoting crosstalk between innate immunity and integrin activation signaling pathways.

Pathophysiological conditions associated with oxidative stress, such as acute or chronic infections, dyslipidemia and diabetes, are frequently associated with the prothrombotic state. CEP- and CPP-modified proteins and ethanolamine phospholipids in SCD blood can play an essential role in vaso-occlusive events. Elevated levels of these products of nonenzymatic lipid oxidation are likely to contribute to a permanent condition of hypercoagulability causing thrombosis and thereby a critical pathophysiologic feature of SCD. These endogenous TLR ligands may provide new mechanism-based targets for developing therapeutic measures to combat the prothrombotic state associated with SCD pathology. The observation that individuals with elevations in various measures of platelet reactivity are at an increased prospective risk for coronary events and death supports the clinical importance of increased platelet reactivity. Therapies that block the CEP/PPP-dependent signaling cascades leading to platelet activation can complement the antiplatelet and anticoagulation treatments previously under investigation.

Clearance of CEP is receptor-mediated

Besides the pro-angiogenic effect of CEP, it has a pro-inflammatory role, making its clearance critical.^{42, 44, 45} In contrast with ageing tissues in mice and in highly vascularized tumors where CEP accumulates at high levels, CEP is only transiently present during wound healing, reaching a maximum 3d after injury before returning to original levels when the wound is healed.⁴⁹ Exposure of macrophages to CEP-BSA, caused upregulation of the pro-inflammatory cytokine, TNF- α , at a protein as well as at an mRNA level. Anti-inflammatory M2-skewed macrophages were found to be much more efficient at CEP binding and scavenging than inflammatory M1-skewed macrophages.⁵⁴ Depletion of macrophages leads to increased CEP accumulation in vivo. Although knockout of either CD36 or TLR2 results in diminished CEP clearance, the lack of both receptors almost completely abrogates the ability of macrophages to scavenge CEP-modified protein. Thus, CEP binding and clearance by macrophages is dependent on both CD36 and TLR2. Macrophages bound, scavenged, and metabolized CEP-BSA but not structurally similar ethylpyrrole derivatives, demonstrating the high specificity of the process and the importance of the CEP carboxyl for receptor recognition.⁵⁴ The M1 polarization of macrophages in AMD eyes presumably contributes to the pathological accumulation of CEP.

CONCLUSIONS AND FUTURE PROSPECTS

Besides AMD, oxidative stress is key in the pathogenesis of numerous diseases including atherosclerosis, diabetes, and Alzheimer's disease. Although CEP is the product of nonenzymatic lipid oxidation and nonenzymatic reaction of HOHA-lactone with primary amino groups in biomolecules, biological responses to CEP are receptor-mediated. Those

biological activities can be important for the resolution of physiological inflammation and contribute to a vicious cycle of pathological inflammation. It is also important to note that those biological activities may contribute to the failure of therapeutic measures. For example, antiangiogenic therapy of “wet” AMD or glioblastoma multiforme (GBM) with bevacizumab, a drug that inhibits angiogenesis promoted by VEGF, exhibits a disturbing tendency to fail. “Resistance” develops to anti-VEGF therapy that sometimes limits its efficacy to a few months. For example, for GBM, the median survival is only 15 months because essentially all patients develop recurrent or progressive disease after initial therapy.⁵⁵ Bevacizumab monotherapy has proven effective for recurrent GBM, but failed to provide a survival advantage when added to standard therapy for newly diagnosed GBM.^{56, 57} It extended progression-free survival and improved patient quality of life in various clinical trials,⁵⁸ but outcomes after bevacizumab failure⁵⁹ for recurrent GBM are poor.⁶⁰ Bevacizumab therapy causes a phenotypic change in GBM cells including increased invasiveness.⁶¹ Bevacizumab “resistant” GBM cells exhibit increased expression of genes associated with inflammation and myeloid chemotaxis leading to changes in the tumor microenvironment such as an increase in levels of tumor associated macrophages (TAM).⁶¹

It is tempting to speculate that the production and biological activities of CEP contribute to the therapeutic failure of bevacizumab for which we propose multiple CEP-dependent mechanisms that promote tumor growth by their putative effects of CEP on glioma stem cells (GSCs) and tumor stromal cells (Figure 2). Hypoxia promotes expression of VEGF, HIF-1, TLR2, iNOS and inflammation producing CEP that activates the NLRP3 inflammasome causing more inflammation. Chronic inflammation is a crucial event for tumor progression.^{46, 62} Bevacizumab therapy of a GBM xenograft in mice caused a dramatic increase in the level of HIF-2 α , a hypoxia marker.⁶³ Hypoxia-induced factor-1 (HIF-1) binds to the TLR2 gene promoter causing upregulation of TLR2 expression,⁶⁴ and it upregulates inducible nitric oxide synthetase (iNOS) and VEGF expression (Figure 2).⁶⁵

Inducible nitric oxide synthetase iNOS promotes lipid oxidation and the generation of more CEP. Since CEP promotes NLRP3 inflammasome activation in macrophages⁴⁵, and since bevacizumab therapy causes hypoxia that promotes inflammation and the consequent lipid oxidation that generates CEP, we now postulate that anti-CEP therapy (Figure 2) can inhibit the NLRP3 inflammasome-promoted generation of CEP in GBM tumors and, thereby, short circuit this vicious cycle. Inhibition of inflammasome activation by anti-CEP would also block inflammasome mediated generation of IL-1 β , a strong inducer of pro-angiogenesis and pro-invasion factors such as VEGF and matrix metalloproteases (MMPs), in TAM and GBM cells.⁶⁶

High-grade gliomas are heterogeneous neoplasms that contain stromal cells. Besides ECs, and carcinoma-associated fibroblasts (CAFs), resident glioma-associated microglial cells (GAMs) and invaded tumor-associated microglial cells account for up to 30% of the entire glioma mass.^{67, 68} Activation of TLR2 and, subsequently, p38 mitogen-activated protein kinase signaling pathways leads to induction of membrane type 1 (MT1)-MMP⁶⁹ and release of MMP9⁷⁰ from GAMs which both promote tumor progression by degrading the extracellular matrix. Activation of TLR2 promotes tumor invasion by upregulating MMP2

and MMP9 in GSCs.⁷¹ We propose that CEP promotes some of all of these TLR2-dependent processes.

Metalloproteases, especially MMP2 and MMP9, are crucial for GBM invasion.⁷² After failure of anti-VEGF therapy,⁷³ GBM tumors become more invasive^{63, 74, 75} with increased expression of MMP2.⁷⁶ Previously, we showed that CEP promotes activation of TLR2 and TLR9.^{49, 77} We now postulate that CEP-promoted activation of TLR2, which leads to induction of MT1-MMP expression in microglia, which activates glioma-released MMP2 and thereby promotes glioma invasion and growth.⁶⁹ Activation of TLR2 also leads to release of MMP9⁷⁰ from GAMs as well as MMP3 from fibroblasts,⁷⁸ and CEP-promoted activation of TLR9, which leads to the release of MMP-13 from GBM cells,⁷⁹ foster tumor invasion by degrading the extracellular matrix (Figure 2). After short-term (4 day) treatment of a mouse GBM xenograft model with bevacizumab, intracerebral glioma was well demarcated with little expression of MMP9, but after 4 weeks of treatment, it was invasive into surrounding brain with up-regulation of MMP9 expression.⁸⁰ CEP can potentially promote any or all of these TLR2 or TLR9-dependent processes, and anti-CEP therapy can potentially inhibit them.

While GSH depletion can increase the lethality of radiotherapy, because it will impair the metabolism of HOHA-lactone, GSH depletion will also increase levels of HOHA-lactone and CEP that may promote recurrence of tumor growth. Finally, 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.

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ABBREVIATIONS

AMD	age-related macular degeneration
CAFs	carcinoma-associated fibroblasts
CEP	2-(ω -carboxyethyl)pyrrole
CEP-dipeptide	CEP-modified acetyl-Gly-Lys-O-methyl ester
CEP-HSA	CEP-modified human serum albumin
CEP-MSA	CEP-modified mouse serum albumin
CHP	2-(ω -carboxyheptyl)pyrrole
CNV	choroidal neovascularization
DHA	docosahexaenoate

DHA-PC	1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine
ECs	endothelial cells
EPs	ethanolamine phospholipids
GAMs	glioma-associated microglial cells
GBM	glioblastoma multiforme
GSCs	glioma stem cells
GSH	reduced glutathione
GST	glutathione S-transferase
HIF-1	hypoxia-induced factor-1
HNE	4-hydroxy-2-nonenal
HOHA	4-hydroxy-7-oxo-hept-5-enoic acid
HOHA-PC	1-palmitoyl-2-(4-hydroxy-7-oxo-5-heptenoyl)-sn-glycero-3-phosphatidylcholine
HOOA-PC	5-hydroxy-8-oxohept-6-enoyl phosphatidylcholine
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthetase
IPM	interphotoreceptor matrix
LDL	low-density lipoprotein
lysoPC	2-lysophosphatidylcholine
MMPs	matrix metalloproteases
NLRP	NACHT, LRR and PYD domains-containing protein
PLA2	phospholipase A2
PP	2-pentylpyrrole
RPE	retinal pigmented endothelium
SCD	sickle cell disease
TAM	tumor associated macrophages
TLR	Toll-like receptor
VEGF	vascular endothelial growth factor;

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Biography

Dr. Salomon did undergraduate studies at the University of Chicago, graduate and postdoctoral studies at the University of Wisconsin, and further postdoctoral studies at Indiana University. In 1973 he joined the faculty of the Department of Chemistry at Case Western Reserve University where he is now the Charles F. Mabery Professor of Research in Chemistry. His research on the chemistry and biology of lipid oxidation is focused on understanding the mechanisms of nonenzymatic lipid oxidation and its involvement in autism, age-related macular degeneration, Alzheimer's disease, atherosclerosis, cancer, glaucoma, renal and sickle cell diseases as well as wound healing.

Figure 1.
CEP induces activation of the NLRP3 inflammasome in bone marrow derived macrophages.

Scheme 1.

Oxidative fragmentation of linoleyl and docosahexaenoyl phospholipids produces γ -hydroxyalkenals that react with protein lysyl residues to deliver pyrrole derivatives.

Scheme 2.

Spontaneous deacylation of HOHA phospholipids, e.g., HOHA-PC, generates HOHA-lactone that reacts with proteins to generate CEP derivatives.