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Biological and pathophysiological roles of end-products of DHA oxidation

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Abstract

Background—Polyunsaturated fatty acids (PUFA) are known to be present and/or enriched in vegetable and fish oils. Among fatty acids, n-3 PUFA are generally considered to be protective in inflammation-related diseases. The guidelines for substituting saturated fatty acids for PUFAs have been highly publicized for decades by numerous health organizations. Recently, however, the beneficial properties of n-3 PUFA are questioned by detailed analyses of multiple randomized controlled clinical trials. The reported heterogeneity of results is likely due not only to differential effects of PUFAs on various pathological processes in humans, but also to the wide spectrum of PUFA's derived products generated *in vivo*.

Scope of review—The goal of this review is to discuss the studies focused on well-defined end-products of PUFAs oxidation, their generation, presence in various pathological and physiological conditions, their biological activities and known receptors. Carboxyethylpyrrole (CEP), a DHA-derived oxidized product, is especially emphasized due to recent data demonstrating its pathophysiological significance in many inflammation-associated diseases, including atherosclerosis, hyperlipidemia, thrombosis, macular degeneration, and tumor progression.

Major conclusions—CEP is a product of radical-based oxidation of PUFA that forms adducts with proteins and lipids in blood and tissues, generating new powerful ligands for TLRs and scavenger receptors. The interaction of CEP with these receptors affects inflammatory response, angiogenesis, and wound healing.

General Significance—The detailed understanding of CEP-mediated cellular responses may provide a basis for the development of novel therapeutic strategies and dietary recommendations.

Keywords

Polyunsaturated fatty acids; carboxyethylpyrrole; docosahexaenoic acid; inflammation; toll-like receptors; CD36

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Polyunsaturated fatty acids (PUFA) are fatty acids with more than one double bond in their backbone structure. The subgroups of PUFA are classified based on the location of the first double bond from the methylated end. N-3 PUFAs (exemplified by docosahexaenoic DHA and Eicosapentaenoic (EPA)), enriched in fish oils and n-6 (exemplified by arachidonic, and linoleic acids) and present in most vegetable oils, are considered to be the most essential component for human health. The guideline for substituting saturated fatty acids for PUFAs has been highly publicized for decades by numerous health organizations.

Despite clear benefits of this substitution, n-6 PUFA may still contribute to the development of inflammation. However, n-3 PUFA was previously considered to be anti-inflammatory or at least neutral [1]. As n-3 and n-6 fatty acids compete for the same substrates in the human body, it has been postulated that the consumption of n-3 polyunsaturated fatty acids in place of saturated ones is beneficial to overall health, and the cardiovascular system in particular [2]. Indeed, selected studies have shown that consumption of n-3 PUFA instead of saturated fats may lower the risk and reduce complications of cardiovascular disease [3]. There are other studies suggesting a correlative rather than causative relationship between plasma levels of PUFA and the risk of certain cardiovascular complications [4].

However, more recent detailed analyses of past clinical trials have revealed quite different results. For example, a 2012 analysis of 20 different studies (from 3635 citations) revealed no statistically significant benefits of n-3 PUFAs when considering all-cause mortality, cardiac death, and myocardial infarction [5]. Analysis of a double blinded Sydney Diet Heart Study revealed that substitution of dietary saturated fats with PUFAs resulted in higher mortality rates in men from cardiovascular disease and other causes (17.6% vs 11.8%), [6]. Even more fascinating is a 2016 NIH-led analysis of previously unpublished results of a double blind, randomized, controlled, and the most rigorously executed trial, known as the Minnesota Coronary Experiment, which revealed no benefits of dietary PUFAs. While the replacement of saturated fats with unsaturated ones resulted in the lowering of serum cholesterol, there were no mortality benefits whatsoever, including mortality from cardiovascular disease, which was based on blind autopsy reports [7]. In fact, in many groups, especially in women over the age of 65, mortality in PUFAs group was higher than in the control group. There was no effect of a PUFAs diet on coronary atherosclerosis or myocardial infarcts at autopsy.

These new rigorously performed statistical analyses provide a more stable data set; a solid platform from which to completely re-evaluate the well-accepted paradigm that preferential PUFA consumption is beneficial to cardiovascular health. It has become obvious that the overall evidence no longer supports the PUFAs benefits hypothesis. For other diseases associated with inflammation, the situation appears to be similar. Meta-analysis of results from clinical trials that focused on type 2 diabetes revealed a rather heterogeneous response to n-3 PUFA and as a result, no significant benefit of PUFA consumption [8]. Even the antioxidant ability of n-3 fatty acids has been questioned, especially in high doses where these fats have been suggested to act as prooxidants [9,10]. The reported “paradoxes” and observed heterogeneity of responses are likely due not only to differential effects of PUFAs on various pathological processes in humans, but also to the wide spectrum of PUFA derived products generated *in vivo*, whose effects might be distinct from PUFAs themselves. Indeed,

the current model linking potential harmful effects of PUFAs to cellular events underlying cardiovascular disease is centered on free radical-based oxidation of PUFAs and the presence of their oxidized products in atherosclerotic lesions [11-13].

The goal of this review is to discuss the studies focused on well-defined end-products of PUFA oxidation and their generation, presence in various pathological and physiological conditions, and biological activities and receptors.

2. PUFA oxidation products OxPC_{CD36} and pyrroles

The presence of more than one double bond in the backbone structure makes PUFAs highly susceptible to oxidation and fragmentation. PUFAs are oxidized either by enzymes, such as lipoxygenases or cyclooxygenases, or by non-enzymatic oxidation mediated by free radicals or photooxidation, propagating via the classical mechanism of lipid peroxidation chain reaction. There are multiple products that can be formed as a result of this process. For example, enzymatic oxidation of PUFA containing phospholipids can lead to the formation of hydroperoxides such as resolvins. [14] or prostaglandins, such as 15 deoxy- Δ 12, 14 prostaglandin I₂ (PGI₂) and 9 α ,15S-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic acid (PGD₂); further rearrangement of prostaglandin H₂ results in the formation of levuglandins [15].

Peroxidation of PUFA-containing phospholipids can lead to the formation of 1-palmitoyl-2-oxovaleroyl-sn-glycero-3-phosphorylcholine (POVPC), 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphorylcholine (GPC) or 1-palmitoyl-2-(5,6-epoxyisoprostane E₂)-sn-glycero-3-phosphorylcholine (PEIPC) [16]. A high degree of structural variation explains a wide spectrum of biological activities of PUFA's derived products.

Particular interest in this review represents the family of oxidized phospholipids possessing CD36 binding activity. This family was first described by Podrez and coauthors [17,18], and is comprised of phospholipid- γ -hydroxyalkenals and their more oxidized derivatives, collectively called oxPC_{CD36}. This novel family of oxidized phospholipids participates in macrophage CD36-mediated recognition of oxidized lipoproteins and foam cell formation *in vivo*. (Fig.1). Moreover, oxPC_{CD36} is a precursor of ω -carboxyalkylpyrrole protein adducts, the existence of which was predicted and then confirmed by Robert G. Salomon's group at CWRU. This particular group made major contribution to our current understanding of lipid oxidation and metabolism as well as the generation and properties of lipid-derived protein adducts [19-23]. It was postulated that hydroxy- ω -oxoalkenoic acids generated due to hydrolysis of phospholipid γ -hydroxyalkenals react with the primary amino group of proteins resulting in the formation of ω -carboxyalkylpyrrole modification of proteins. Initial studies were focused on PUFAs, which are abundant in low density lipoprotein — linoleate and arachidonate. Oxidation of linoleic or arachidonic acids produces γ -hydroxyalkenals, such as 9-hydroxy-12-oxododec-10-enoic acid (HODA) or 5-hydroxy-8-oxooct-6-enoic acid (HOOA), respectively. In the presence of protein, they then generate 2-(ω -carboxyheptyl)pyrrole (CHP) or 2-(ω -carboxypropyl)pyrrole (CPP) adducts, respectively [24] (Fig. 2). The antibodies produced against CHP and CPP synthesized *in vitro* recognized antigens in oxidized low density lipoprotein, in plasma and in atherosclerotic lesions of

patients, confirming the presence of these adducts *in vivo* and their association with oxidative stress [24,25].

It has been suggested that oxidation of other PUFAs can generate similar carboxyalkylpyrrole adducts. Of particular interest is oxidation of DHA, as DHA represents a unique example of PUFA in mammalian species. DHA is found at a very high concentration in several tissues, such as the retinal rod outer segment [26,27], certain cells in the brain [28], synaptosomes [29], and sperm [30], among others. DHA levels can approach 50 mol% of the total phospholipid acyl chains in these membranes, but interestingly, the high DHA level in these tissues is not further increased by diet. In contrast, the level of DHA in many other tissues are found below 5 mol% of the total phospholipid acyl chains, but can be enhanced up to 10-fold through dietary supplementation. Available DHA is rapidly incorporated in a variety of cells, primarily into phospholipids of the plasma membrane [31] that make DHA the important source of PUFA in various tissues. DHA has been shown as the most oxidizable fatty acid in humans due to high content of double bonds [32].

Therefore, the following models were suggested for oxidative cleavage of DHA phospholipids: PLA₂-catalyzed hydrolysis of DHA generates 4-hydroxy-7-oxo-hept-5-eonate (HOHA), which, in turn, produces 2- ω -Carboxyethylpyrrole (CEP)-protein derivatives through condensation with the primary amino groups of protein lysyl residues (Fig. 2 and Fig.3). Alternatively, oxidative cleavage of DHA-PC generates DHA-derived phospholipid γ -hydroxyalkenal, such as 2-(4-hydroxy-7-oxohept-5-enoyl)-1-palmitoyl-*sn*-glycero-3-phosphatidylcholine (HOHA-PC), which reacts with proteins followed by hydrolysis of an intermediate phospholipid adduct and generates CEP-modification **through** primary amino groups **of protein lysyl residues**. Notably, oxidation of several PUFAs can lead to the formation of CPP or CHP, while CEP adduct exclusively originates from oxidative modification of DHA and cannot arise from any other common polyunsaturated fatty acid [22].

3. Carboxyalkylpyrrols synthesis and specific antibodies for their detection

To develop tools for testing the hypothesis of CEP generation, ω -carboxyethylpyrrole-modified proteins were created using Paal-Knorr reactions of γ -dicarbonyl compounds (DOHA) with ϵ -amino group of lysyl residues of proteins [23]. The specific synthesis of DOHA was performed as described in detail in [22] and then DOHA was used to prepare CEP-modified keyhole limpet hemocyanin (CEP-KLH), bovine serum albumin (CEP-BSA), human serum albumin (CEP-HSA), and glyceraldehyde-3-phosphate dehydrogenase (CEP-GPDH). Pyrrole modification of proteins was quantified using the Ehrlich reagent, 4-(dimethylamino)benzaldehyde. Protein CEP-adducts were digested with trypsin and analyzed using liquid chromatography tandem mass spectrometry that revealed several peptides with modification of ϵ -amino groups with CEP. CEP-modified proteins were used as antigen for the anti-CEP antibody production. Several monoclonal and polyclonal antibodies were generated [22].

Importantly, the generated antibodies against CPP, CHP and CEP have very low cross-reactivity. For example, rabbit polyclonal anti-CEP antibodies bind to the CEP-modified

human serum albumin (HSA) ≈ 1000 times stronger as compared to CPP-HSA. Anti-CPP antibodies interact with CPP-HSA 80 times stronger than with CEP-HAS. Binding of either anti-CEP or anti-CPP antibodies to CHP-HSA was below detection [22]. Of note, another protein modification, ethylpyrrole (EP), is generated through the alternative oxidative cleavage of DHA to give 4-hydroxyhex-2-enal followed by condensation of 4-hydroxyhex-2-enal with the ϵ -amino group of lysyl residues (Fig. 3). This modification contains only one structural difference — the lack of a carboxyl group, which makes it an excellent control for CEP functional studies [33].

4. Identification of CEP in pathologies *in vivo*

CEP formation *in vivo* was initially verified using mouse retinal tissue, as DHA-containing phospholipids are highly enriched within the photoreceptor rod's outer segment. Immunohistochemical analysis using anti-CEP antibodies identified a specific signal in retinas, which was diminished in the control sample preincubated with CEP-HAS [22]. Thus antibodies, which were generated for the hypothetical product of DHA oxidation (CEP protein adducts produced through chemical synthesis), recognized a specific antigen in tissue, thereby confirming the presence of CEP *in vivo*. More importantly, anti-CEP specific antibodies provided an opportunity for the analysis of CEP accumulation in different organs and tissues [22]. As phospholipid oxidation is dramatically increased in many pathological conditions associated with inflammation, it would be reasonable to anticipate the augmented deposition of CEP in such conditions.

Age-related macular degeneration (AMD) is the first and most studied pathology characterized by elevated levels of CEP. AMD is a prevalent cause of legal blindness in elderly populations; it is characterized by the breakdown of the macula, the small portion of the central retina responsible for high-acuity vision [34], and the accumulation of focal extracellular deposits on the Bruch's membrane below the retinal pigment epithelium in the macula, initiating the dysfunction of retinal pigment epithelium cells and resulting in photoreceptor cell death and loss of central vision. AMD has two forms – dry and wet. Dry AMD is characterized by drusen formation in visible yellow spots, while wet AMD is a more severe form of the disease involving choroidal neovascularization, an abnormal type of angiogenesis in subretinal tissues. Recent data has demonstrated that AMD progression is associated with the development of chronic inflammation and macrophage accumulation in the inter photoreceptor matrix in close proximity to retinal pigment epithelium cells [35].

In retinal tissue and plasma samples of AMD patients, elevated levels of CEP were identified as compared to healthy donor controls using anti-CEP antibodies in Western Blot analysis, immunohistochemistry, and ELISA [22]. In addition, the proteins from the drusen isolated from the patients with dry AMD were analyzed by 2-D Western Blot, and CEP immunoprecipitated proteins were identified using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry. Several CEP-modified proteins including albumin were identified, albumin being the most abundant CEP-modified protein present in serum of AMD patients in another study from this group [36]. The recent understanding of the mechanism for CEP formation suggests that protein concentration in the particular tissue

regulates the degree of protein modification by CEP. Namely, the most concentrated protein in a tissue/plasma will be the major source for CEP modifications.

The experimental results from the AMD model were adapted and served as a base for the development of several directions in the investigation of CEP role in pathologies as discussed below (Fig. 4).

The discovery of CEP's contribution to angiogenesis is a good example of AMD-initiated studies. The first observation of CEP's role in angiogenesis was demonstrated in an AMD related mouse model by Ebrahem, *et al.* [37]. This study showed that picomole levels of CEP-HSA and CEP-dipeptide stimulated neovascularization *in vivo*. These studies also implicated the VEGF-independent nature of angiogenesis induced by CEP. While the anti-CEP antibody completely inhibited blood vessel growth stimulated by CEP-HSA, the anti-VEGF antibody had only a partial effect [37].

The further molecular mechanisms of CEP-induced vascular responses were established by West, *et al.* [38]. It was demonstrated that CEP deposition is not limited to retina, but it also affects various tissues as a result of inflammation or injury, for example, in wounds and in highly vascularized tumors with a strong inflammatory component. As CEP is an end-product of lipid oxidation, it is not surprising that it is continuously accumulated during long-term processes, *i.e.* in aging vasculature [38]. Another key finding in this study is the identification and characterization of TLR2 as a receptor for CEP and other pyrrole-containing adducts on endothelial cells. Importantly, TLR2 specifically recognizes CEP modifications, but not merely denatured proteins, since a structurally similar modification, ethylpyrrole (EP), does not bind to TLR2 [32]. CEP promoted angiogenesis in hind limb ischemia and wound healing models through TLR2 and MyD88 dependent signaling [38]. It is remarkable that in last five years, multiple manuscripts from various labs have validated and firmly established the role of TLRs and TLR2, in particular, in endothelial cell responses as well as in angiogenesis [33,39-47],

The causative role of CEP in pathologies was later confirmed using antibodies to neutralize endogenously generated CEP, which impaired wound healing and tissue revascularization as well as diminished tumor angiogenesis [38]. Therefore, it appears that the series of original AMD-related studies provided a base for the in-depth understanding of a general mechanism of CEP proangiogenic function in different physiological and pathophysiological conditions.

Another example of CEP contribution to pathophysiological processes is the accumulation of CEP-modified proteins in neurofilaments in the brains of patients with autism spectrum disorder [48]. The interest in this pathology was stimulated by the knowledge that DHA is the most abundant fatty acid in brain tissues and is known to be required for normal brain development [48]. Moreover, oxidative damage in tissues of autistic patients has been previously documented [49]. Recent analyses of autism pathogenesis emphasize the importance of inflammation, which, in turn, might lead to lipid peroxidation and lipid-protein adducts formation [50]. Immunohistochemical analysis using anti-CEP antibodies demonstrated CEP deposition in cortical brain tissues and by ELISA in blood plasma of patients [48]. Interestingly, the neurofilament heavy chain was a major target for CEP

modifications, which was confirmed by Western blotting and immunoprecipitation assays. Apparently, the inflammation and oxidative injury of proteins in the brain is associated with neurological abnormalities and might provide a molecular mechanism for the autism spectrum disorders.

A prothrombotic state and increased platelet reactivity are common pathophysiological responses during oxidative stress, inflammation, and infections. Panigiani, *et al.*, demonstrated that CEP-modified proteins promote platelet activation, granule secretion and aggregation *in vitro*, and thrombosis *in vivo* via TLR9-related pathway [51]. The molecular analysis revealed that CEP-TLR9 interaction activates MyD88 signaling pathway that utilized IRAK1 downstream and depends on AKT1 and AKT2, but not AKT3 in murine platelets. Therefore, CEP via interaction with platelet TLR9 connects oxidative stress, innate immunity, and thrombosis. Further studies from the same group detected the presence of CEP adducts of phosphatidylethanolamine in circulation of hyperlipidemic ApoE^{-/-} mice [52]. Phosphatidylethanolamine, the second most abundant phospholipid in eukaryotes, possesses a primary amino group that can be modified by the hydroxy-omega-oxoalkenoic acids with the formation of carboxyalkyl pyrroles such as CEP. Carboxyalkyl pyrroles of phosphatidylethanolamine promoted platelet hyper-reactivity and thrombosis in mice by engaging innate immune system in platelets and inducing SFK-Syk-PLC γ 2 activation leading to platelet integrin activation signaling pathway. Signaling was independent of the platelet scavenger receptors class B, and was mediated by TLR2/TLR1 complex and activation of TRAF6 [52].

Inflammation and oxidative stress critically contribute to the development of myocardial infarction and atherosclerosis. Carboxyalkyl pyrroles were found in heart sections of mice with myocardial infarction [53] and in human atherosclerotic lesions [25] in macrophages and extracellularly. Accumulation of carboxyalkyl pyrrole adducts in serum of ApoE^{-/-} mice fed western diet was reported recently [52]. Interestingly, the plasma level of CEP in ApoE^{-/-} mice on a standard chow diet was also elevated as compared to wild type animals, possibly reflecting the contribution of oxidative stress in dyslipidemic ApoE^{-/-} mice on normal chow. The Western diet dramatically increased CEP concentration in the plasma and this level remained continually high during the progression of atherosclerotic lesions. Immunofluorescence analysis of the aortic root demonstrated dramatic accumulation of CEP adducts in ApoE^{-/-} mice fed western diet, while in control mice no CEP immunoreactivity was detected [33,51]. CEP accumulation correlated with the macrophage presence in the aortic root [33] (Fig. 5).

Although CEP is continually accumulated in pathologies associated with chronic inflammation, exemplified by atherosclerosis, tumor progression, macular degeneration, and other diseases, the CEP level normalizes during the completion of wound healing, suggesting the existence of a specific clearance mechanism, recently elucidated by our laboratory [33]. It has been shown that macrophages bind, internalize, and metabolize CEP *in vitro* (Fig. 6). The role of macrophages in this process was further confirmed *in vivo*, as macrophage depletion resulted in increased accumulation of CEP in tissues. It was demonstrated that CEP clearance is mediated by anti-inflammatory M2 macrophages. Using

in vitro and *in vivo* approaches, it was established that two macrophage pattern recognition receptors, TLR2 and CD36, cooperate during CEP clearance [33].

In summary, generation and accumulation of CEP and other carboxyalkyl pyrroles during atherosclerosis, hyperlipidemia, aging, macular degeneration, tumorigenesis, and wound healing demonstrate their importance for the pathogenesis of a wide spectrum of diseases. While the role of TLRs in CEP-induced processes have been shown, many key questions, including the contribution of co-receptors and the molecular mechanisms underlying differential responses to CEP in various cell types, remain unanswered. Also, the translational value of CEP accumulation in the progression of the above mentioned diseases needs to be further elucidated. Considering all of these CEP-related diseases are characterized by the presence of a strong inflammatory component, it is tempting to speculate that CEP, generated by ROS during inflammation, is able to promote inflammation further, creating a positive feedback loop. Therefore, the contribution of CEP to inflammatory processes is of a particular interest and becomes the focus for investigation in many laboratories.

5. Role of CEP in pro-inflammatory cytokines induction and macrophage polarization

The induction of pro-inflammatory cytokines in macrophages in response to CEP represents the most significant piece of evidence that CEP is involved in augmentation of inflammatory responses. Most of the results discussed in this section were obtained using mouse models of macular degeneration. The experimental mouse model of AMD was developed [54] by immunization of mice with CEP-modified mouse serum albumin. Immunized mice produced anti-CEP autoantibodies, and exhibited complement deposition in the Bruch's membrane, the damage of retinal pigment epithelium, and drusen formation in the macula and other pathologies, which are characteristics of dry AMD [55]. This model represents inducible rather than genetically modified AMD with the symptoms similar to that in human patients. Using this model, the authors demonstrated that macrophages accumulate in the retina of CEP-immunized mice [55]. Importantly, macrophage accumulation was diminished in CCR2-deficient mice, the knockout, which leads to defective recruitment of monocytes/macrophages during inflammation. Moreover, it was demonstrated that retinas of these mice contained an increased concentration of TNF α and IL-12. Analysis of infiltrating macrophages revealed the upregulation of mRNA for TNF α , IL-1 β and IL-6, all well-described markers for M1 macrophages, while the markers for M2 macrophages, IL-10 and Arg1, were not affected by CEP immunization [55]. More recently, these authors demonstrated that CEP protein adducts induce M1 polarization of bone marrow derived macrophages (BMDM) [56]. Several M1 markers genes, including iNOS, IL-1 β , TNF α , and IL-12, were upregulated after CEP-MSA stimulation in macrophages. CEP-induced M1 polarization was further confirmed on the protein level [56]. In concert with these findings, we demonstrated that treatment of macrophages with CEP-BSA led to upregulation of TNF α , both at mRNA and protein levels [33]. Importantly, the specificity of CEP-induced responses was verified using sham-MSA [56] or sham-BSA [33], the albumin that was treated the same way as CEP-albumin during chemical synthesis. No upregulation of pro-

inflammatory cytokines in macrophages was observed after the stimulation with sham modified albumin, demonstrating the specific requirement for CEP.

In agreement with these studies, Doyle and co-authors also detected the effect of CEP on the cytokine production [57]. Interestingly, the authors found that treatment of blood leukocytes and BMDM with CEP-HSA has no effect on expression of IL-1 β . However, they demonstrated that preincubation of macrophages with ATP or complement C1Q led to significant upregulation of IL-1 β production in response to CEP, indicating a synergism between these agonists. These authors independently evaluated the importance of TLR2 in CEP-mediated cytokine expression and demonstrated that BMDM isolated from TLR2-deficient mice were unable to upregulate IL-1 β , thereby emphasizing the critical role of TLR2. This comprehensive analysis allowed for the conclusion that CEP signaling may require an additional activator for the induction of pro-inflammatory cytokines [57]. A similar synergistic mechanism was more recently suggested by Saeed and co-authors [58]. The authors found that CEP synergistically act with a low dose of TLR-2 agonists to induce inflammatory cytokines, such as TNF α and IL-12. This is not surprising, as CEP is an endogenously generated ligand for TLR2 and, therefore, it is anticipated to be reasonably well tolerated. An excessively robust response of inflammatory cells to CEP alone may lead to severe consequences.

Taken together, these studies demonstrate that CEP accumulation stimulates monocyte/macrophage recruitment, production of several pro-inflammatory cytokines and, probably, leads to M1 macrophage polarization (Table 1). The synergism between the endogenously generated “natural” TLR ligand, CEP, and exogenous bacterial ligands is intriguing and requires additional studies to clarify the underlying molecular mechanism. Future studies would help to understand the mechanism of CEP-mediated macrophage migration/accumulation at the sites of inflammation, since TNF α , IL-1 β , and IL-6 secretion alone is not likely to induce substantial monocyte/macrophage recruitment. Nevertheless, published studies provide compelling evidence that CEP modifications generated by PUFA oxidation create pro-inflammatory environment, which, in turn, may contribute to a number of pathologies.

6. CEP is recognized by scavenger receptors and TLRs

Although the studies on CEP precursors [17,18] hint that similar products are likely to be recognized by a class of molecular pattern recognition receptors, which includes scavenger receptors and TLRs, the first characterized CEP receptor is a member of TLR family, TLR2 [38]. A number of studies implicate TLRs in recognition of the endogenous ligands, particularly so-called “altered-self” ligands [59,60]. CEP modifications provide an excellent example of such “altered-self” ligands. As discussed above, CEP-mediated endothelial responses during angiogenesis and wound healing require TLR2 [38].

The role of TLRs and TLR2 in vascularization was confirmed by a number of recent studies using a variety of experimental approaches and models. A recent thorough study by Aplin, *et al.* [41] from the laboratory that developed the *in vitro* aortic ring angiogenesis assay firmly established the presence of specific TLRs on various cell types involved in angiogenesis, and

then demonstrated a clear angiogenic response to TLR ligands. Numerous studies show TLRs are involved in angiogenic responses to bacterial [41,46,61-63] as well as endogenous ligands [64,65]. Among those, results of Sachdev, *et al.* [66], showing the proangiogenic function of TLR2 are especially noteworthy. In many instances the proangiogenic effect was mediated by TLRs on endothelial cells as demonstrated by TLR2 knockdown [39], knockout or using specific TLR2 ligands [41,61,63,66,67]. Impaired vascularization in TLR2 null mice was reported in several models, including hind limb ischemia models [62,63,66]. Reduced rate of wound vascularization and healing in both, TLR4 and TLR2 null mice was also independently demonstrated [68]. TLR2 was proposed as a hallmark of angiogenesis in certain pathologies [69]. Thus, the collective body of evidence from different groups (with an exception of a rather controversial report by Gounarides, *et al.* [70], which contradicted a number of previously published studies) establishes TLRs as important contributors to endothelial functions and angiogenesis.

The studies using CEP as a TLR2 ligand [33,38,58,71] emphasize that activity of CEP-modified proteins depends on the number of modifications per a molecule of protein carrier. Preparations with low CEP content exhibited lower activity toward TLRs. Therefore, it is important for subsequent studies to pay special attention to the quality of CEP preparations and the stoichiometry of CEP-modifications [23].

As TLR2 is well expressed on the surface of macrophages, it is also a perfect candidate for CEP-induced effects in inflammation. Indeed, Doyle and co-authors using TLR2 deficient bone marrow derived macrophages demonstrated TLR2-dependent induction of expression of IL-1 β and IL-6 by CEP-BSA [57]. Kim, *et al.* demonstrated that CEP clearance by macrophages (phagocytes) is reduced in TLR2-deficiency [33]. These data together clearly establish the importance of TLR2 for a number of macrophage functions. Furthermore, using surface plasmon resonance and solid-phase binding assay, it was shown that TLR2 binds in a concentration dependent manner to CEP-BSA immobilized via amino-coupling to sensor chip [33]. A similar result was obtained in the reverse system, where varying concentrations of CEP-BSA were flowed over immobilized TLR2 [33]. Independently, Wang, *et al.* [71] demonstrated the role of TLR2 in functional responses to CEP-lipid modifications using TLR2 overexpressing cells.

TLR2-mediated functional responses require either TLR2/TLR1 or TLR2/TLR6 heterodimeric complexes on the cell surface. Saeed, *et al.* [58] aimed to identify a critical co-receptor on inflammatory cells for CEP-induced upregulation of cytokines. The comparison of TLR1 agonists (Pam3CSK4 and lipomannan) with TLR6 agonists (Pam2CSK4 and lipoteichoic acid) revealed that CEP synergizes with TLR1/TLR2 ligands, but not with TLR1/TLR6 ligands. Of note, TLR1/TLR2 ligand Pam3CSK4 induced robust sprouting of endothelial cells from aortic rings and tubulogenesis, thereby further supporting the role of TLR1/TLR2 in CEP-induced effects on macrophages and endothelium [38]. Intriguingly, it appears that the co-receptor requirement for CEP clearance, which is mainly an anti-inflammatory function, is distinct from its proangiogenic and proinflammatory activity and depends on TLR2/TLR6 complex [33]. This suggests that co-receptors of TLR2 define the nature of downstream effects initiated by CEP. To test whether purified TLR1 and TLR6 can directly bind CEP, surface plasmon resonance technique was employed. Surprisingly,

both toll-like receptors interacted with CEP, but the binding to TLR6 was substantially stronger. Similar results were obtained using standard solid-phase binding assay [33]. Of note, CEP did not interact with CD14, a well-known partner of TLR2 on many cells [33].

On platelets, the involvement of TLRs to CEP-induced responses seems to be rather unique and depends on the carrier of CEP modifications. Panigrani, *et al.* demonstrated the key role of platelet TLR9 as a receptor for CEP-protein-modifications [51]. The direct interaction between TLR9 and CEP-BSA was documented using surface plasmon resonance. In addition, TLR9 from human and mouse platelet lysates was co-immunoprecipitated with CEP-BSA [51]. In contrast to CEP-protein modifications, CEP-phosphatidylethanolamine derivative binds to TLR2 on platelets and induces platelet activation via MyD88/TIRAP [52]. Intravital thrombosis studies demonstrated that CEP-phosphatidylethanolamine derivatives accelerate thrombosis in TLR2-dependent manner and TLR2 substantially contributes to accelerated thrombosis in mice in the settings of hyperlipidemia.

Therefore, several characteristics of CEP modifications, such as the structure of pyrrole-containing modifications (CEP vs. EP, Fig. 3), the stoichiometry of these modifications and the nature of the CEP carrier (protein vs. lipid) might define the involvement of specific receptors and, possibly, downstream effects of CEP.

We have recently found that besides TLRs, CD36 serves as an additional co-receptor on the surface of macrophages that assists in CEP recognition during the process of CEP clearance by macrophages [33]. Several lines of evidence demonstrated CD36/CEP interaction: 1) Cell lines transfected with CD36 demonstrated increased binding to CEP-BSA in FACS assay; 2) CD36-deficient macrophages have reduced CEP-BSA binding abilities and internalization; 3) CD36 directly binds CEP-BSA in solid phase binding assay and surface plasmon resonance assay. In addition, CEP-CD36 interaction was inhibited by an established CD36 ligand – an oxidized low density lipoprotein. This result suggests that CEP may share the ligand binding site on CD36 with oxidized LDL. This hypothesis is further supported by the demonstration that a specific peptide derived from an extracellular domain of human CD36 that is critical for oxLDL binding [72], inhibited CEP-CD36 interaction in a dose-dependent manner [33]. The specificity of CEP binding was established by several approaches using sham modified BSA and structurally similar protein modification, EP-BSA. The only structural difference between CEP and EP is the lack of carboxyl group, which eliminates a negative charge of CEP modification (Fig. 3). EP-BSA (and sham modified BSA) do not bind to macrophages in multiple assays, including FACS analysis [33] and the surface plasmon resonance assay using isolated TLR2 and CD36 (Yakubenko, Byzova, manuscript in preparation). This indicates that the negative charge of the carboxyl group of CEP is absolutely critical for the interaction with the receptor. The abundance of polyanionic components represents a common mechanism of ligand recognition by various receptors; therefore, one can expect identifications of new receptors for CEP.

Thus, CEP seems to be recognized by several molecular pattern recognition receptors on the cell surface, and the carboxyl group of CEP is indispensable for this recognition. The involvement of different receptors and co-receptors may be due to the presence of carboxyl group in CEP, resulting in the generation of proteins with substantial negative charge on the

surface, which, in turn, may mimic the negative charge of a bacterial cell wall, known to serve as the primary target for the toll-like and scavenger receptors. The information regarding known CEP receptors is summarized in Fig. 4.

Conclusions

Oxidation of DHA leads to the generation of end-products exemplified by CEP modifications of proteins and lipids. Numerous studies from multiple laboratories have demonstrated the presence of CEP modifications in blood and tissues in a variety of pathological conditions including, but not limited to, hyperlipidemia, atherosclerosis, macular degeneration, and tumor progression. ROS-mediated modification of proteins and lipids with CEP seems to represent a process of endogenous ligand generation (*self-alteration*), which engages and activates Toll-like and scavenger receptors. The process of CEP recognition by its respective receptors is highly specific and dependent on the presence of carboxyl group, the stoichiometry of CEP to carrier and the nature of modified substrate (protein vs. lipid). None of the published results using well-characterized CEP with appropriate controls, such as sham-modifications and EP-modifications, can be merely explained by protein denaturation.

Studies from several laboratories have established a causative relationship between CEP and angiogenesis in pathologies, exemplified by injury response, AMD, and cancer. Other studies have established a causative role for CEP modifications in platelet activation and hyperlipidemia-associated thrombosis. All of these results are further supported by multiple and independent lines of evidence demonstrating the similar function of CEP receptors, especially TLR2, in “sterile” pathologies, namely proangiogenic response and atherothrombosis. Another set of studies establish that CEP interacts with macrophages leading to the release of pro-inflammatory mediators. Likewise, CEP presence may switch the balance toward the formation of proinflammatory M1 macrophages, thereby further augmenting inflammatory milieu.

Despite this rather impressive range of pathological activities, CEP-modifications contribute to timely and successful wound healing. Therefore, it would be unwise to propose that CEP modifications serve as uniformly pathological agents. The amount of CEP in tissues can be controlled by a specific receptor-mediated removal by macrophages, a mechanism preventing excessive accumulation and potentially negative effects of these modifications. It appears that it is not CEP generation *per se*, but the imbalance between its generation and removal is a contributing factor to inflammation-associated pathologies. The normalization of CEP removal by macrophages may be a powerful anti-inflammatory strategy.

The mechanism of CEP generation *in vivo* still requires clarification. Therefore, analysis of CEP levels in plasma of patients consuming DHA supplements may provide critical evidence regarding the main source of CEP. These experiments will link DHA uptake and CEP generation and address the possible pro-inflammatory effects of DHA supplementation.

Importantly, CEP (and similar pyrrolle-containing modifications), which exhibits a plethora of biological and pathological activities, represents only a fraction of numerous possible

metabolites of PUFAs. Thus, the complexity of responses, which may be affected by PUFA metabolites, is difficult to comprehend. Detailed and rigorous studies based on close collaboration between organic chemists, cell biologists, and translational researchers with a specific focus on well-defined processes may help put together the pieces of the PUFA puzzle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PUFA	Polyunsaturated fatty acids
CEP	2-(ω -Carboxyethyl)pyrrole
CPP	2-(ω -carboxypropyl)pyrrole
CHP	2-(ω -carboxyheptyl)pyrrole
DHA	docosahexaenoic acid
TLR	toll-like receptor
AMD	Age-related macular degeneration
ROS	reactive oxygen species
BMDM	bone marrow derived macrophages
EP	ethylpyrrole
BSA	bovine serum albumin
HSA	human serum albumin
MSA	mouse serum albumin
HODA	9-hydroxy-12-oxododec-10-enoic acid
HOOA	5-hydroxy-8-oxooct-6-enoic acid
HOHA	4-Hydroxy-7-oxo-5-heptenoic Acid
ELISA	enzyme-linked immunosorbent assay

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Highlights

- The products of PUFA oxidation exhibit a wide spectrum of biological activities.
- CEP, a product of PUFA oxidation, has activities underlying several pathologies.
- CEP is endogenous ligand for TLRs and CD36.
- CEP-receptor interaction promotes inflammatory cell response.

Fig. 1.
Binding site of CEP to CD36 corresponds to the common binding motif of CD36 ligands.

Fig. 2.

Generation of 2-(ω -carboxyalkyl)pyrroles. Oxidative fragmentation of polyunsaturated fatty acids generates a host of oxidation products, including the hydroxy- ω -oxoalkenoic acids HODA, HOOA, and HOHA, which give rise to a family of carboxyalkylpyrrole protein adducts CHP, CPP, and CEP. Adapted from [22].

Fig. 3. Hypothetical reaction mechanism for the generation of EP and CEP through oxidative fragmentation of a DHA phospholipid to γ -hydroxyalkenals HHE and HOHA followed by their reaction with ϵ -amino groups of protein lysyl residues.

Fig. 4. Pattern recognition receptors for CEP during the development of different pathophysiological conditions.

Fig. 5.

CEP accumulates in the atherosclerotic lesions and plasma of *ApoE*^{-/-} mice. **A**, Plasma levels of CEP from *ApoE*^{-/-} and WT mice after 5 weeks on a Western diet, measured by enzyme-linked immunosorbent assay (ELISA) (n=3). Fold increase over WT control is shown. **B**, Plasma levels of CEP from *ApoE*^{-/-} and WT mice, which were fed with a chow diet or Western diet for 16 weeks, were measured by ELISA. Fold increase over WT control on CD is shown. **C**, Fluorescence staining for CEP (green), CD68 (macrophages, red), and 4',6-diamidino-2-phenylindole (DAPI, nuclei, blue) of the aorta of *ApoE*^{-/-} mice fed with Western diet for 5 weeks. Adapted from [33].

Fig. 6. CEP scavenging by macrophages. Immunostaining for CEP, CD68, and DAPI on the primary resident mouse peritoneal macrophages is shown. Cells were incubated at 37°C for the times indicated; then washed, culture media were changed with fresh media without CEP-BSA, and incubated for additional 0, 30, and 90 minutes after 30 minutes for pulse treatment (**A**) or during the entire incubation (**B**) with 500 nmol/L of CEP-BSA. **C**, CEP was quantified by ELISA before and after incubation with macrophages. After 500 nmol/L CEP-BSA was added to the macrophages at 37°C, the supernatants were then collected at the times indicated, measured by ELISA, and normalized to the CEP only-containing culture media without macrophages. Percentage of remaining CEP is shown. Initial concentration of CEP (time point 0) was assigned a value of 100%. Adapted from [33].

Table 1

Secretion of pro-inflammatory cytokines from macrophages after CEP stimulation

Type of macrophages	Stimulation	Cytokines	Evaluated	Comments	Reference
Retinal macrophages [*]	CEP-MSA <i>in vivo</i> (AMD model)	TNF α IL-6 IL-1 β	mRNA	M1 phenotype was detected	(55)
BMDM ^{**}	CEP-MSA <i>in vitro</i>	TNF α IL-6 IL-1 β IL-12	mRNA protein	M1 phenotype was detected	(56)
PBMC ^{***} BMDM	Drusen isolated from AMD mice, CEP-HSA <i>in vitro</i>	IL-1 β IL-18	protein	Required co-stimulation with ATP or C1Q	(57)
BMDM	CEP-BSA <i>in vitro</i>	TNF α IL-6 IL-12	mRNA Protein	Required co-stimulation with TLR2 agonist ^{****}	(58)
Peritoneal macrophages ^{*****}	CEP-BSA <i>in vitro</i>	TNF α	mRNA protein		(33)

^{*} Interphotoreceptor matrix-infiltrating macrophages were isolated by laser capture

^{**} BMDM - bone marrow derived macrophages

^{***} PBMC - *peripheral blood mononuclear cell*

^{****} TLR2 agonist - cells were co-stimulated with Pam3CSK4

^{*****} Thioglycollate-induced and resident peritoneal macrophages were evaluated