Nonenzymatic modifications of proteins and lipids are ubiquitous and often greatly exaggerated in various metabolic conditions, such as in hyperglycemia, or in the presence of inflammation, for example, because of enhanced lipid peroxidation. Such modifications often alter the normal structure/function or confer novel and unwanted properties to the modified proteins or lipids. For example, nonenzymatic glycation of lysine residues of proteins, which is greatly exaggerated in hyperglycemia, can lead to the formation of glucoislyselline adducts that can alter function of proteins, such as the binding properties of albumin and the metabolic properties of low-density lipoprotein (LDL). Over time, these adducts can undergo even more complex chemical rearrangements to form a host of newly formed chemical structures, collectively termed advanced glycation end products, which can confer yet further novel properties, for example, proinflammatory properties mediated by various innate receptors, such as scavenger receptor CD36 and RAGE (receptor for advanced glycation end products).  

In a similar manner, reactive oxygen species, generated during inflammation as a result of both enzymatic and nonenzymatic reactions, lead to oxidative modifications of a wide range of substrates. Among these, polyunsaturated fatty acids present in phospholipids in extracellular lipoproteins, such as LDL, or in the cellular membranes of cells, are highly susceptible to such attack. One such widely studied example occurs during the formation of oxidized LDL (OxLDL), which leads to formation of oxidized cholesterol esters and oxidized phospholipids (OxPLs). For example, if the sn2 polyunsaturated fatty acid in a phosphocholine (PC) containing phospholipid undergoes free radical attack, generating an oxidized phospholipid (OxPL), a variety of highly reactive oxidative degradation split products can be formed, such as 4-hydroxyxenonenal and malondialdehyde, which both can then further modify proteins or amino-containing phospholipids. Both malondialdehyde and 4-hydroxyxenonenal adducts with apoB-100 of LDL can be demonstrated in OxLDL generated in vitro and in apoB-100 isolated from animal and human atherosclerotic plaques. In turn, the residual OxPL backbone, containing an sn1 saturated fatty acid and in this example, the PC headgroup, now contains a partially degraded sn2 fatty acid, often with reactive groups, such as aldehydes, which can itself undergo complex rearrangement chemistries. Alternatively, the entire OxPL can itself form covalent adducts with lysine residues of proteins via its reactive sn2 groups, leading to the formation of OxPL-protein adducts. Thus, oxidation of LDL in vitro can lead to the formation of approximately 80 moles of OxPL covalently linked to apoB-100 aside from the OxPL found in the lipid phase. These OxPLs and OxPL-apoB adducts form epitopes on OxLDL that are recognized by scavenger receptors CD36 and SR-B1, and thus likely play an important role in mediating foam-cell formation and atherosclerosis.

These OxPL have been shown to have a myriad of proinflammatory effects on both endothelial cells and macrophages and to be important mediators of adverse effects in multiple settings. Such oxidative modifications of both extracellular lipids, as in lipoproteins, and cellular membranes likely occur at some basal rate even in normal situations, but are greatly accelerated in nearly all inflammatory settings, both sterile and pathogen induced. Furthermore, during programmed cell death, the oxidative events that accompany apoptosis also lead to the formation of similar OxPLs, which are also displayed on the cell surface, and shown to be capable of both mediating many of the same proinflammatory events as has the isolated OxPLs, as well as to serve as “eat me” signals for macrophage phagocytosis.

We have termed these oxidation-specific epitopes (OSE) to emphasize that the same oxidative modification, irrespective of the protein to which they are bound, can mediate such proinflammatory effects and can serve as ligands for macrophage scavenger receptors. Thus, OSE can be regarded as a general class of danger-associated molecular patterns (DAMPs). In turn, there is a concerted innate immune response to maintain homeostasis to DAMPs, mediated by innate pattern recognition receptors (PRRs), such as scavenger receptors, natural antibodies, and other soluble innate proteins. In the example discussed of PC-containing OxPL, the PC headgroup of OxPL, but not of unoxidized phospholipids, is recognized by scavenger receptors CD36 and SR-B1, by the IgM natural antibody E06, and by CRP. In a similar manner, malondialdehyde-modified proteins are recognized by scavenger receptor SR-A, by the innate IgM NAb E014, and by CRP. In a similar manner, malondialdehyde-modified proteins are recognized by scavenger receptor SR-A, by the innate IgM NAb E014, and by the innate protein Complement Factor H. Remarkably, it seems that many if not most OSE that are DAMPs also have molecular identity or mimicry with similar pathogen-associated molecular patterns (PAMPs) present on infectious pathogens. Thus, the PC present on the cell wall of many pathogens, such as Streptococcus pneumoniae (but not as part of a phospholipid) is also recognized by CD36, E06 and CRP. This suggests that a motif common to both a pathogen (PAMP) and
OSE (DAMP), such as PC, will have exerted selective pressure for the conservation of a set of innate PRRs to maintain homeostasis against the common PAMP/DAMP. In turn, this leads to the prediction that in a similar manner, there will be a set of innate PRRs that will have been selected to recognize other important pathological motifs, such as CEP, an OSE that is the subject of the article by Kim et al in the current issue of Circulation Research.

In an extensive, elegant, and seminal set of articles, Byzova, Podrez, Salomon, Hollyfield, and colleagues have demonstrated that CEP (2-(ω-carboxyethyl)pyrrole), an adduct between an oxidative fragment of docosahexaenoic acid, (E)-4-hydroxy-7-oxohept-5-enoic acid (HOHA), and lysines of proteins (or aminophospholipids), is just such an important and ubiquitous OSE. In addition to a detailed characterization of its chemistry, they have convincingly shown that CEP adducts, on a variety of different proteins, have multiple proinflammatory and proangiogenic properties. The CEP adducts on autologous proteins are also immunogenic, giving rise to highly specific CEP antibodies that recognize CEP on a variety of proteins, but do not bind to closely related lipid peroxidation-derived protein epitopes, such as ethylpyrrole, which differs in only lacking a carboxy group. Furthermore, the presence of CEP has been shown by both immunochromatography and LC-MS/MS (liquid chromatography-tandem mass spectrometry) to accumulate in a variety of different oxidative and proinflammatory settings, including inflammatory tumors, age-related macular degeneration, and atherosclerosis in both experimental models and humans. In the current studies, they extend these studies to demonstrate that CEP levels accumulate progressively in melanomas in mice over time as the tumor progresses, and that plasma and arterial levels of CEP are substantially elevated in Western diet–fed ApoE−/− mice.

In aggregate, these studies convincingly demonstrate that CEP is generated in vivo, is biologically active, and progressively accumulates under inflammatory or aging processes. The enhanced oxidative events that accompany inflammation and aging logically lead to the conclusion that CEP generation will be induced in these settings, but accumulation only occurs if the rate of CEP formation is in excess of the rate of CEP clearance and degradation. As is true for most extracellular modifications, while considerable information has been generated concerning the characterization and formation of these products, such as OSE, surprisingly little is known of the mechanisms responsible for their clearance and degradation. However, it might be predicted from the paradigm offered above that innate PRRs present on macrophages would be centrally involved in maintaining homeostasis to these highly prevalent OSE, and importantly, be chiefly responsible for mediating their clearance and degradation.

In the current article by Kim et al, the authors present important new studies that now begin to address the mechanisms by which CEP is cleared from tissues. Using a variety of elegant in vitro and in vivo techniques, they convincingly demonstrate that macrophages use the PRRs CD36 and TLR2, individually or cooperatively, to bind to and mediate tissue removal of CEP-decorated proteins. Using chimeric CD36-Fc, TLR2-Fc, and CD14-Fc constructs and surface plasmon resonance techniques, they demonstrate direct binding of CEP to both CD36 and TLR2 but not CD14. Because TLR2 is known to heterodimerize with TLR1 or TLR6, they also demonstrate direct binding to these 2 TLRs as well, although the binding to TLR6 was considerably higher, suggesting that TLR2/TLR6 pairing is more likely responsible for the CEP binding.

To determine the relevance of these in vitro binding studies to CD36 and TLR2 chimeric proteins, they generated Cd36−/− and Tlr2−/− double knockout mice and directly examined the binding of CEP to macrophages from these mice. Whereas CEP binding to Cd36−/− macrophages was reduced by 75% and to Tlr2−/− macrophages by 60%, binding to macrophages from double knockout mice was reduced by 90%. In parallel studies, the amount of CEP removed from culture over 24 hours by macrophages from wild type, Cd36−/−, Tlr2−/−, and double knockout mice was 70%, 40%, 55%, and 20% respectively. The relevance of these data in vivo was demonstrated by the qualitative observation that immunochromatographically detected CEP clearance from tissues was impaired in Cd36−/− mice and that this led to accelerated angiogenesis.

These data convincingly demonstrate that macrophage CD36 and TLR2 bind to CEP in a saturable manner and either independently or cooperatively account for ≈80% of the macrophage uptake of CEP-modified proteins in cell culture. These timely studies thus begin to address the all-important question of how OSE are cleared in vivo, and the information gained should have broad applicability to the wide range of OSE formed. Like all seminal studies, they raise many new questions and suggest further areas that need to be explored. Although it may be true that CD36 and TLR2 account for ≈80% of the uptake of CEP in cultured macrophages, how does this relate to clearance of CEP in vivo, and in a setting where formation of CEP may be greatly accelerated and tissue concentrations may be greatly increased. For example, the authors show that in Western diet–fed ApoE−/− mice, the plasma levels of CEP rose >8-fold, reflecting the enhanced generalized inflammation and oxidation in this setting. No doubt, absolute CEP levels in relevant tissues where these adducts are generated, such as in the artery, were even more greatly increased. Because of their higher affinity for CEP, CD36, and TLR2 may be the major receptors mediating uptake during a given time in a tissue culture dish with fixed levels of CEP. Nevertheless, in an in vivo setting with a great excess of CEP, the residual 20% of uptake accounted for by lower affinity or even nonsaturable linear uptake pathways may become quantitatively important and account for a steadily increasing proportion of overall uptake as CEP levels rise. The situation may be analogous to that with the contrasting roles of CD36 and SR-A in mediating macrophage uptake of OxLDL in cell culture and in vivo. In culture, CD36/SR-A clearly accounted for ≈80% of uptake, predicting that uptake of OxLDL would be greatly diminished in double knockout mice and thus should lead to decreased atherosclerosis. Yet considerable angst has been generated by conflicting studies of atherosclerosis in hypercholesterolemic Cd36−/− and Sra−/− double knockout mice in the ApoE−/− and Ldlr−/− background. In this setting of excess OxLDL, the uptake of OxLDL via lower affinity non-CD36 and non-SR-A alternative scavenger receptor pathways can nevertheless become quantitatively just as important when integrated over long periods of time, leading to nearly similar or even the same
extent of atherosclerosis. By analogy, understanding the non-CD36 and non-TLR2 pathways responsible for uptake of CEP is likely to be of similar importance. In addition, the authors demonstrated that OxLDL competed with CEP for the same site on CD36, implying that in the setting of excess OxLDL formation in the artery wall, as occurs with Western diet–fed mice, both CEP and OxLDL would compete with each other for macrophage uptake. In a similar manner, because CD36 mediates efferocytosis of apoptotic cells via the same site that binds OxLDL, it is possible that CEP also prevents this process, contributing to tissue necrosis, as has been postulated for OxLDL.

As another critique, it should be appreciated that the fate of the internalized CEP has not been defined in these studies. A detailed and quantitative analysis of this aspect needs to be accomplished in a similar manner as has been done for uptake of LDL and OxLDL by macrophages. The in vivo studies strongly suggest that tissue accumulation of CEP was modulated by macrophage CD36 content, but we do not know if this was simply because of uptake and emigration of the macrophages from the tissue or to actual degradation of the CEP intracellularly. As the authors note, fast turnover of CEP is not likely to occur as the pyrrole moiety of CEP is stabilized by aromaticity. Does macrophage uptake of CEP-modified proteins lead to extracellular CEP clearance, but result in intracellular degradation of the protein with retention of the CEP, for example, in lysosomes associated with CD68 as shown immunohistochemically in tissues? Could this be another example of a Trojan Horse paradigm, as occurs with uptake of excessive amounts of OxLDL, resulting in tissue clearance of oxidized lipids but macrophage accumulation of oxidized lipids and cholesterol ester accumulation, leading to foam-cell formation along with ultimate malfunction of the macrophage and apoptosis or necrosis. These skilled investigators, whose work is characterized by state-of-the-art biochemical techniques, are ideally suited to address the fate of the ingested CEP and if indeed it can be degraded within macrophages, and if not, what is its ultimate fate after macrophage uptake. These data have important implications not only for CEP but also likely as a paradigm for the large number of OSE that are continuously generated.

Yet another interesting and important question is the relationship of binding of CEP to CD36 and TLR2 and its copartners in mediating cellular signaling. Indeed, consistent with prior work,18,19 Kim et al16 demonstrate that CEP mediates activation of proinflammatory signaling, for example, of TNF-α. Does CEP need to be internalized to initiate this signaling and are these same set of PRRs involved? Previous studies have suggested that CD36 pairs with TLR2/TLR6 to mediate OxLDL- or OxPL-induced apoptosis20 or to pair with TLR4 and TLR6 to mediate OxLDL stimulation of sterile inflammation.21 Of direct relevance to this study, it was recently reported that CEP-modified proteins specifically augmented macrophage inflammatory signaling mediated by TLR2, but paired with TLR1 and not TLR6, although the role of CD36 was not examined.18 These data all indicate considerably complexity in the manner by which CEP is recognized and internalized and how it affects cellular signaling pathways.

In summary, the authors have contributed an original and substantial body of work that CEP is a ubiquitous and important OSE that likely accumulates whenever oxidative and inflammatory events are increased. This work is valuable not only as CEP seems to mediate considerable pathological events but also as an important model OSE, and thus, understanding the immune mechanisms that respond to maintain homeostasis has broad implications for the many OSE formed. According to the paradigm presented above, it would be predicted that natural selection has led to the conservation of multiple innate PRRs to provide homeostasis for CEP, as has occurred for PC-containing OxPL. In this article, Kim et al16 provide compelling evidence that both CD36 and TLR2 represent macrophage scavenger receptors as one component of this innate response. The authors have previously demonstrated that CEP is immunogenic and that there are CEP-specific autoantibodies of IgG isotype likely of adaptive immunity, although it is not clear if these include IgM or IgA isotype natural antibodies of innate origin, which we predict are likely present. We might further speculate that there is also a soluble innate protein that will bind CEP with high affinity, in analogy to the binding of PC-containing OxPL by CRP, and malondialdehyde-modified proteins by Complement Factor H. In turn, there may be similar molecular structures or molecular mimics of CEP on pathogens, which further contributed to the maintenance of the various innate PRRs that recognize CEP. Were this to be the case, we would need to consider the implications of immune responses cross reactive between CEP present on proteins as a DAMP and a CEP mimic on pathogens as a PAMP.

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CEP Is an Important and Ubiquitous Oxidation Specific Epitope Recognized by Innate Pattern Recognition Receptors

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