Inflammatory profile of oxidized phospholipids

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Summary
Lipid oxidation products and in particular oxidized phospholipids (OxPL) are increasingly recognized as inducers of chronic inflammation characteristic of atherosclerosis. OxPL stimulate production of chemokines and adhesion of monocytes to endothelial cells. However, accumulating data suggest that, in addition to the proatherogenic and proinflammatory effects, OxPL can either stimulate or inhibit inflammation. In this review, the inflammatory properties of OxPL are discussed together with the underlying receptor, signalling and transcriptional mechanisms.

Keywords
Endothelial cells, phospholipids, inflammation

Introduction
Atherosclerosis is a chronic inflammatory disease characterized by accumulation of monocytes and T cells, but not neutrophils in the subendothelial space of the arterial wall. A substantial body of data implicates lipid oxidation products as agonists initiating and perpetuating inflammation in atherosclerotic vessels. Phospholipids containing polyunsaturated fatty acids represent one of the major targets for oxidation. Oxidized phospholipids (OxPL) are known to accumulate in atherosclerotic vessels (1–3). In addition, a number of in-vitro and in-vivo studies demonstrated that OxPL induce inflammatory reactions similar to those observed in atherosclerotic lesions. Therefore, OxPL are increasingly recognized as potential culprits inducing vascular wall inflammation (4). On the other hand, emerging evidence demonstrates that OxPL also can inhibit certain types of inflammatory reactions (5). This review discusses recent insights into the receptor, signalling and transcriptional mechanisms underlying pro- and antiinflammatory effects of OxPL.

Generation and structure of OxPL
Increased levels of phospholipid oxidation products have been detected in different organs and pathological states, including atherosclerotic vessels (1–3), inflamed lung (6, 7), non-alcoholic liver disease (8), plasma of patients with coronary artery disease (9), as well as in apoptotic cells (10, 11), virus-infected cells (12) and cells stimulated with inflammatory agonists (13). OxPL are generated by oxidation of polyunsaturated fatty acid residues, which are usually present in the phospholipids at the sn-2 position. Oxidation of phospholipids is initiated either enzymatically by lipoxigenases, or by reactive oxygen species, and propagates via the classical mechanism of lipid peroxidation chain reaction. In this respect OxPL differ from other mediators generated by oxidation of polyunsaturated fatty acids such as prostaglandins and leukotrienes, which are formed exclusively by enzymatic reactions. Therefore, production of OxPL cannot be regulated by adjusting the amount or activity of enzymes, thus increasing probability of the uncontrolled generation of OxPL during oxidative stress. Oxidation of a single molecular phospholipid species generates a number of OxPL with either full-length or fragmented oxidized residues. Figure 1 illustrates structures of several OxPL generated from a single precursor phospholipid – 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC). In addition to phosphatidylethanolamine, other classes of OxPL containing different polar groups and fatty acids can activate endothelial cells (EC) as well (2). This high structural variation may explain why OxPL demonstrate a remarkable variety of biological activities described below.

Inflammatory and procoagulant effects of OxPL
OxPL were initially characterized as an active principle of minimally modified LDL (MM-LDL), responsible for its ability to stimulate EC to bind leukocytes (14). It was shown that OxPL obtained by oxidation of pure synthetic phospholipid mimicked this effect (1, 14). A characteristic feature of MM-LDL and OxPL as inflammatory agonists is their ability to activate binding of
monocytes but not neutrophils (1, 15). This property of OxPL may play a role for the selective accumulation of mononuclear cells in atherosclerotic lesions. Some mechanisms of monocyte adhesion to OxPL-stimulated EC have been elucidated. In contrast to lipopolysaccharide (LPS), tumor necrosis factor α (TNFα), or interleukin 1 (IL-1), MM-LDL does not upregulate expression of ICAM-1, VCAM-1 and E-selectin on EC (16), but promotes surface deposition of CS-1-containing variant of fibronectin (CS-1 FN) serving as a ligand for the α4β1 (VLA-4) integrin expressed on the surface of monocytes (17). Similarly to MM-LDL, OxPL selectively stimulate adhesion of monocytes by a CS-1 FN-dependent mechanism (18). In addition, there is evidence for the role of P-selectin for the rolling and subsequent firm adhesion of monocytes induced by OxPL in murine carotid arteries (19). P-selectin is important for mononuclear cell attachment to the early atherosclerotic lesions (20). Furthermore, it has been shown that both inactivation of CS-1 FN using a synthetic peptidomimetic (21), as well as targeted deletion of P-selectin (22) slow down the progression of atherosclerosis. Thus, OxPL upregulate on the EC two types of adhesion molecules known to bind monocytes and play a role in atherogenesis.

Similarly to other inflammatory agonists, OxPL stimulate production of cyto- and chemokines. OxPL are known to upregulate expression of IL-6, IL-8, MCP-1, GROα, MIP-1α, MIP-1β and CXCL3 (13, 19, 23–27). Chemokines MCP-1, MIP-1α, and MIP-1β attract different groups of mononuclear inflammatory cells. In contrast, GROα, IL-8 and their mouse analog K Ca re chemokceptors for neutrophils but not monocytes. However, in addition to the chemoattractant properties, these chemokines stimulate firm arrest of monocytes on the endothelium (28). It has been suggested that two groups of cytokines act in concert: MCP-1 and other CCR2 receptor ligands predominantly stimulate monocyte migration, while IL-8 and other CXCR2 receptor ligands are more efficiently coupled to the monocyte-EC adhesion mechanisms (29). Thus, OxPL induce several chemokines known to be important for extravasation of mononuclear cells, which is in agreement with their suggested role as initiating factors in atherogenesis.

Activation of endothelium by inflammatory agonists leads to increased thrombogenicity mediated by increased expression of clotting tissue factor (TF) on the surface of EC. It has been shown that OxPL stimulate production of TF in cultured human EC (30). Thrombogenic shift in EC can be further enhanced by downregulation of an anticoagulant glycoprotein thrombomodulin (TM) resulting from OxPL-induced decrease of TM gene transcription (31). Moreover, OxPL reduce the anticoagulant activity of tissue factor pathway inhibitor (TFPI) directly interacting with the C-terminal basic region of the TFPI molecule (32). In summary, several lines of evidence suggest that OxPL promote a transition from an anticoagulant phenotype of healthy endothelium to the procoagulant state characteristic of atherosclerosis and inflammation. The thrombogenic switch induced by OxPL in endothelium may be further exacerbated by direct platelet-activating action of oxidatively fragmented 1-alkyl- and 1-acyl-phosphatidylcholines (33, 34).

In addition to the inflammatory and procoagulant activation of endothelium, OxPL also increase generation of reactive oxygen species. In bovine aortic EC, OxPL activated generation of superoxide anion radical by NADPH oxidase and upregulated Nox4 subunit of NADPH oxidase (35). Furthermore, endothelial nitric oxide synthase (eNOS) has been characterized as a source of superoxide anion radicals produced in OxPL-treated human aortic EC, thus suggesting that OxPL induce both activation and uncoupling of eNOS (36). These data show that OxPL increase oxidant stress, which may further impair function of endothelium in atherosclerotic vessels.

**Receptors for OxPL**

Available evidence suggests that OxPL interact with several signal-transducing receptors.

Peroxidation of phospholipids leads to accumulation of lysoforms as a result of both non-enzymatic deacylation and enzymatic hydrolysis catalysed to a large extent by lipoprotein-associated phospholipase A2 (also known as PAF acetylhydrolase), which has high substrate selectivity toward polar phospholipids, including the oxidized forms (37). Some lysophospholipids bind and activate G protein-coupled receptors (GPCR). In particular,
lysoosphatidylcholine and lysophosphatidic acid stimulate activity of G2A and LPA1 – LPA4 receptors, respectively (38, 39). Thus, phospholipid peroxidation may stimulate generation of these lysophospholipids known to accumulate in oxidized LDL (OxLDL) and atherosclerotic lesions (40, 41).

A small proportion of phosphatidylcholine present in LDL contains sn-1 residues attached to the glycerol backbone by the ether (alkyl) bonds. Upon oxidative fragmentation of sn-2 residue these alkyl-PC acquire structural similarity to platelet activating factor (PAF) and become agonists for PAF receptors (42). The major PAF-like lipid in OxLDL is 1-0-hexadecyl-2-(butanoyl or butenoyl)-sn-glycero-3-phosphocholine (43). Thus, some effects of OxPL may be mediated by PAF receptors, especially on platelets and leukocytes. However, our unpublished observations show that certain types of cultured EC readily respond to OxPL by up-regulation of inflammatory genes, but are insensitive to PAF or PAF antagonists, thus suggesting the involvement of receptor mechanisms other than the PAF receptor.

Prostaglandin receptors have been recently implicated into OxPL-induced inflammation. OxPAPC and its component lipid PEIPC are able to stimulate prostaglandin E2 and D2 receptors (EP2 and DP, respectively) and to compete with receptor binding of radiolabeled prostaglandin E2 (44). An EP2 receptor agonist, butaprost, stimulated the ability of EC to bind monocytes, which is consistent with the role of EP2 receptors in mediating inflammatory effects of OxPL (44).

In addition to the GPCR mentioned above, OxPL activate other classes of receptors as well. Some data point to peroxisome proliferator-activated receptors (PPAR) as targets for OxPL. Alkyl-OxPL, such as azeloyl phosphatidylcholine, were identified as ligands for PPARγ (45). OxPAPC and its components POVPC and PGPC were shown to activate PPAR response element-driven reporter construct in transfected EC (23). Furthermore, it has been shown that OxPL are the major fraction of OxLDL responsible for activation of PPARγ and that the mechanism of activation involves phospholipase A2-dependent release of hydroxylated fatty acids directly stimulating PPAR (46). There is additional evidence for the involvement of the receptors of innate immunity. MM-LDL have been shown to stimulate secretion of chemokine MIP-2 (but not of several other cytokines) in a Toll-like receptor 4 (TLR4)-dependent manner (47). Furthermore, Walton et al. demonstrated that OxPL activate TLR4 acting in concert with a GPI-anchored protein different from the common TLR4 co-receptor CD14 (48). In addition, OxPL have been shown to interact with pattern-recognition receptors C-reactive protein and scavenger receptors CD36 and SR-BI, thus implicating OxPL into the recognition of apoptotic cells and formation of foam cells (3, 49–52).

Some effects of OxPL probably are not mediated by signal-transducing receptors in the classical meaning of this term. Modulation of cellular cholesterol deposits has been suggested as a non-receptor mechanism of OxPL sensing by cells. It has been shown that OxPAPC induces depletion and redistribution of cellular cholesterol stores finally leading to the activation of a transcription factor SREBP, a well-recognized sensor for cellular cholesterol contents. In turn, SREBP activates IL-8 production (53). Furthermore, it has been found that OxPAPC stimulates in human aortic EC expression of genes known to be upregulated in response to accumulation of unfolded proteins in the endoplasmic reticulum (ER) (54). It was shown that siRNA-mediated knock-down of transcription factors ATF4 and XBP1, which are key effectors of the unfolded protein response induced by ER stress, strongly downregulated basal and OxPAPC-simulated expression of IL-8, IL-6, CXCL3 and MCP-1 genes in human aortic EC (27). In addition to cholesterol redistribution and ER stress, some biological effects of OxPL may result from the direct chemical reactions of the oxidation-generated esterified aldehydes with lysine, histidine and cysteine residues, thus leading to the modification of protein function (55).

In summary, available data suggest that OxPL activate cells both by receptor-mediated and receptor-independent mechanisms. There is evidence for the involvement of several receptor types, belonging to different protein families, including GPCR, LPS-receptors and nuclear receptors. It is not yet clear to which extent this pharmacological promiscuity is due to high structural heterogeneity of OxPL. Further studies are required in order to clarify the relationships between individual compounds, their receptors and biological effects.

Signalling and transcriptional mechanisms induced by OxPL

As discussed above, OxPL activate several receptor mechanisms, and it is to be expected that such stimulation leads to the activation of a variety of signalling pathways. Indeed, current evidence shows that OxPL activate several signalling and transcriptional pathways, including the cAMP pathway (18). Activation of monocyte adhesion by OxPL is mediated by increasing cAMP levels leading to the stimulation of R-Ras and PI3K, finally resulting in the activation of α5β1 integrin responsible for surface deposition of CS-1 fibronectin, which in turn binds monocytes (56). Dibutylryl-cAMP is able to activate β1-integrin and to stimulate adhesion of monocytes to EC (44). A candidate receptor mediating cAMP elevation in OxPAPC-treated cells is prostaglandin receptor EP2 (44).

In addition to raising cAMP levels, treatment of EC with OxPL induces rapid and reversible elevation of cytosolic Ca2+, which is important for OxPL-induced expression of TF (30). Elevated Ca2+ activates calcineurin phosphatase and its downstream transcription factor NFAT, which upon stimulation of EC with OxPL translocates from the cytoplasm to the nucleus and stimulates transcription of TF (30). The importance of the Ca2+/NFAT-pathway is demonstrated by the ability of cyclosporin A, known to prevent activation of NFAT, to inhibit OxPL-induced expression of TF (30).

Apart from NFAT, OxPL have been shown to stimulate several key transcription factors such as EGR-1, STAT3, SREBP and CREB, known as effectors of a variety of inflammatory and non-inflammatory stimuli. It has been shown that EGR-1 is important for OxPL-induced up-regulation of TF (30) and that STAT3 and SREBP play a role in IL-8 induction (53, 57), while CREB is necessary for the OxPL-induced elevation of heme oxygenase-1 (HO-1) (58).

Some genes are induced both by OxPL and classical inflammatory mediators such as LPS, TNFα or IL-1. Induction of the same genes by OxPL and inflammatory mediators is sometimes achieved through different transcription mechanisms. For
example, in has been shown that while TNFα induces IL-8 transcription mainly through the NFKB site in the promoter, the response to OxPL is mediated by a different promoter region (59). Further studies revealed candidate transcription factors mediating induction of IL-8 by OxPL, including STAT3 (57), PPARγ (23) and SREBP (53). Another example is induction of TF. In contrast to classical inflammatory agonists acting through the NFKB pathway, OxPL stimulate transcription of TF via transcription factors EGR-1 and NFAT and do not activate NFKB (30). Further studies are required in order to establish whether the activation of transcriptional pathways alternative to those induced by mediators of acute inflammation is a characteristic property of OxPL.

In summary, OxPL induce a specific pattern of inflammatory events (Fig. 2) apparently reflecting specific signalling mechanisms. In particular, signalling events activated in EC by OxPL are very different from those induced by mediators of acute inflammation such as LPS, TNFα and IL-1. Effects of these agonists are to a large extent mediated by the activation of transcription factor NFKB. In contrast, OxPL do not activate NFKB in cultured EC, do not upregulate cell adhesion molecules induced during the acute inflammation, such as E-selectin, ICAM-1 and VCAM-1, and do not stimulate binding of granulocytes to the endothelium.

**Antiinflammatory and tissue-protective effects**

OxPL initially were described as proinflammatory agonists likely responsible for the initiation of atherogenic inflammation. However, more recent data suggest that in addition to the proatherogenic and proinflammatory effects, OxPL can stimulate antiinflammatory and tissue-protective mechanisms. The best-documented example of the antiinflammatory action of OxPL is their ability to inhibit effects of bacterial lipopolysaccharide (LPS). OxPL inhibit action of LPS on different cell types *in vitro*, including EC and monocytes/macrophages (5, 18, 60, 61). Furthermore, under the *in vivo* conditions, OxPL exert protective effects against acute LPS-induced sepsis and rescue mice from lethal doses of endotoxin (5, 61). Therefore it is likely that OxPL generated at sites of acute inflammation as a by-product of leukocyte oxidative burst can serve as a negative feedback shutting down excessive inflammation. One mechanism underlying the antiendotoxin effect is based on the inhibition of LPS-binding protein (LBP) and CD14 (5), which are crucially important for the activation of the major LPS receptor, TLR4. Our unpublished data show that OxPL directly bind to LBP and CD14, form stable complexes with these proteins and thus prevent further binding of LPS and its presentation to the TLR4. Thus, OxPL act as LPS-receptor antagonists, rather than LPS scavengers. In addition, it has been shown that OxPL inhibit LPS-induced membrane redistribution of TLR4, thus probably uncoupling it from the intracellular signalling machinery (60). A recent study (62) presented evidence that alterations in membrane caveolar fraction leading to TLR4 desensitisation may be induced by ceramide known to be produced in OxPL-activated EC and vascular smooth muscle cells by sphingomyelinases (62, 63). Apart from the TLR4, OxPL have been shown to inhibit activation of TLR9 by CpG-oligodeoxynucleotides (61) and TLR2 by 19-kDa lipo-

protein from *M. tuberculosis* (60). In summary, available data show that OxPL prevent initiation of inflammatory reactions by several receptors of the innate immune system.

In addition to the LPS antagonism, OxPL activate intracellular signalling mechanisms, some of which may exert antiinflammatory effects in the context of acute inflammation. In particular, cAMP has been shown to interfere with inflammatory reactions in various cell types. Among other antiinflammatory effects, cAMP inhibits E-selectin expression (64), suppresses p38 MAP kinase (65), inhibits the transactivation potential of p65 (66) and upregulates suppressor of cytokine signalling (SOCS) proteins (67). Treatment of human monocyte-derived macrophages by OxPAPC, dibutyryl-cAMP or PGE2 receptor agonist butaprost dramatically decreases expression of TNFα, while at the same time increasing expression of antiinflammatory IL-10 (44). In addition, OxPL have been shown to activate endothelial nitric oxide synthase (36). Nitric oxide demonstrates a number of antiinflammatory activities including downregulation of adhesion molecules and suppression of chemokine production resulting in inhibition of leukocyte extravasation (68). Furthermore, OxPL-activated transcription factors CREB, EGR-1 and PPARs are known to interfere with the activation of NFKB-dependent transcription (69–71). To summarize, OxPL induce a number of signalling and transcriptional pathways that have been shown previously to inhibit certain facets of inflammation. Further studies are necessary to demonstrate which of these signalling antiinflammatory mechanisms are activated by OxPL *in vivo*.

Another potentially beneficial mechanism activated by OxPL during acute inflammation is protection of lung EC barrier function, which is impaired during acute lung injury and sepsis, often leading to a severe complication of pulmonary edema. These conditions are accompanied by increased oxidative stress and generation of OxPL in lung. However, in this case OxPL may act in a protective way. It has been shown that OxPL produce a sustained increase in transendothelial electrical resistance of human pulmonary EC and restore barrier disruption induced by edemagenic agonist thrombin (72). The EC barrier-protective ef-

![Figure 2: Major mechanisms of proinflammatory action of OxPL](image-url)
effect of OxPL is mediated by the activation of small GTPases Rac and Cdc42 acting in concert to induce unique cytoskeleton rearrangements such as peripheral actin rim formation (72). The abilities of OxPL to antagonize LPS effects and to protect endothelial barrier may supplement each other. It has been shown that intravenously injected OxPAPC but not unoxidized PAPC protects rats from lung inflammation and injury induced by intratracheal application of LPS (73). Thus, OxPL have a potential to preserve function of lung during life-threatening systemic inflammation. This subject is discussed in more detail in a recent review (74).

An additional antiinflammatory mechanism activated by OxPL is induction of HO-1, a gene with well-documented cytoprotective and antiinflammatory properties (75, 76). HO-1 catalyzes the rate-limiting reaction in heme degradation, producing carbon monoxide as one of the products. Carbon monoxide inhibits synthesis of inflammatory cytokines, stimulates generation of antiinflammatory cytokines (77) and inhibits apoptosis of EC (78). OxPL induce HO-1 in vitro and in vivo (26, 79). Induction of HO-1 by OxPL in EC is mediated by CREB-dependent stimulation of HO-1 gene transcription. Activation of CREB in OxPL-treated cells results from the combined action of several signalling mechanisms activated by OxPL, including protein kinases A and C, as well as MAP kinases p38 and ERK (58).

Recently, it was demonstrated that OxPL can stimulate angiogenic reactions. OxPL activated migration and sprouting of EC in cell culture and promoted neovascularization in Matrigel plugs in vivo (80). VEGF was identified as one of the autocrine mediators of these effects (80). In addition to the EC, OxPL stimulated production of VEGF by human monocytes and monocyte-derived macrophages, mouse and human fibroblasts and keratinocytes, lung epithelial cells and several tumor cell lines of epithelial origin (80). VEGF is known to protect target cells from apoptosis, to stimulate proliferation, and to induce growth of capillaries. These data suggest that OxPL accumulating as a result of tissue damage and inflammatory response may stimulate repair of injuries and regeneration of tissue.

In summary, OxPL has a potential to antagonize inflammatory reactions and stimulate tissue repair acting through several mechanisms, including inhibition of inflammatory receptor activation, induction of intracellular antiinflammatory signalling, enhancement of endothelial barrier function and upregulation of cytoprotective and angiogenic genes.

Conclusion

The inflammatory profile of OxPL combines both pro- and anti-inflammatory features (Figs. 2 and 3). It is well documented that OxPL exert proinflammatory effects on different cell types including endothelium, where they induce a shift from antithrombotic and antiinflammatory state to the procoagulant and inflammatory phenotype of EC. Furthermore, OxPL upregulate monocyte-specific chemokines and stimulate EC to bind monocytes, thus initiating monocytic inflammation. We do not have a simple explanation why OxPL induce an inflammatory response. One possibility is that OxPL are structurally similar to some bacterial components. It has been shown that oxidation of phosphatidylcholine induces conformational changes in the phospholipolysaccharide (PC) group in such a way that it mimicks the PC moiety of microbial capsule polysaccharide (81). However, phospholipids containing other types of head groups than PC are proinflammatory as well (2). Seong and Matzinger hypothesized that exposed hydrophobic fragments, which they called ‘hypos’, represent a structural motive common for both pathogen-associated molecular patterns and tissue-derived endogenous alarm signals (82). OxPL may belong to the structural class of ‘hypsos’ since their molecules contain an abnormal combination of hydrophobic unoxidized residues and more polar oxygenated residues, which likely leads to destabilization of membrane bilayers and lipoproteins, thus resulting in exposure of hydrophobic portions of OxPL and surrounding lipids and proteins. Generation of endogenous OxPL is increased when tissues contact damaging stimuli initiating the defensive reaction. Therefore, it is likely that OxPL are recognized by known and as yet unidentified receptor mechanisms as a danger signal of the ‘modified-self’ type, thus switching on mononuclear inflammation.

Although OxPL stimulate a number of classical inflammatory mechanisms, they are not capable of activating many signalling and adhesion events characteristic of acute inflammation, such as activation of the NFκB pathway, expression of ICAM-1 and E-selectin or adhesion of granulocytes. The effects of OxPL are more alike to chronic inflammation than acute, or to the resolution phase of acute inflammation. Furthermore, OxPL inhibit acute inflammation induced by bacterial products and most probably other inflammatory agonists. Therefore, in the course of acute inflammation OxPL may represent the ‘fire alarm’ signal indicating that oxidative burst, which is a protective but potentially harmful reaction, has reached the level at which it becomes deleterious and has to be turned off.

In summary, depending on the biological situation, OxPL can either stimulate or inhibit inflammation. OxPL initiate inflammation when they contact normal tissues but inhibit full-blown inflammation induced by strong inflammatory mediators. The inflammatory profile of OxPL is reminiscent of two faces of Janus looking in opposite directions. This ancient God symbolized change and transitions between conditions or visions. We are currently observing a transition between a traditional view of OxPL as ‘bad’ proinflammatory agonists into a broader vision of
these lipids as compounds demonstrating both deleterious and beneficial properties in the variable context of acute and chronic inflammation.

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