Inflammation related gene expression by lipid oxidation derived products in the progression of atherosclerosis

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Abstract

Vascular areas of atherosclerotic development persist in a state of inflammation, and any further inflammatory stimulus in the subintimal area becomes a pro-atherogenic response; this alters the behavior of the artery wall cells and recruits further inflammatory cells. In association with the inflammatory response, oxidative events are also involved in the development of atherosclerotic plaques. It is now unanimously recognized that lipid oxidation-derived products are key players in the initiation and progression of atherosclerotic lesions.

Oxidized lipids, derived from oxidatively modified low-density lipoproteins (LDL) which accumulate in the intima, strongly modulate inflammation-related gene expression, through involvement of various signaling pathways. In addition, considerable evidence supports a pro-atherogenic role of a large group of potent bioactive lipids called eicosanoids, which derive from oxidation of arachidonic acid, a component of membrane phospholipids. Of note, LDL lipid oxidation products might regulate eicosanoid production, modulating the enzymatic degradation of arachidonic acid by clycooxygenases and lipoxygenases; these enzymes might also directly contribute to LDL oxidation.

This review provides a comprehensive overview of current knowledge on signal transduction pathways and inflammatory gene expression, modulated by lipid oxidation derived products, in the progression of atherosclerosis.

Keywords: atherosclerosis; oxidized LDL; cell signaling; inflammation; oxysterols; aldehydes; core-aldehydes; oxidized phospholipids; prostanoids; leukotrienes.

Contents

Introduction: modulation of inflammatory signaling by oxidized lipid products

It is generally accepted that vascular areas of atherosclerotic progression are in a state of persistent inflammation [1,2]. As a consequence, any further inflammatory stimulus in the subintimal area automatically becomes a pro-atherogenic stimulus, altering the behavior of the intrinsic cells of the artery wall, and recruiting further inflammatory cells that interact to promote lesion formation and complications. Of note, leukocytes are present even in the very earliest fatty streak lesions [3-8]. The association of hypercholesterolemia with atherosclerosis is also well established [9,10]. Given that numerous oxidative events are associated with the development of atherosclerotic plaques [11], it is now accepted that oxidized low-density lipoproteins (oxLDLs), which accumulate in the intima, play a major role in the initiation and promotion of fatty streaks and fibrotic plaques [12,13]. In addition, considerable evidence supports a pro-atherogenic role of oxLDLs. The biological effects of oxLDLs are mediated through signaling pathways, especially involving receptors, protein kinases, and activation of transcription factors, which in turn stimulate the expression of genes involved in oxidative stress and the inflammatory response during generation of the atherosclerotic plaque [14,15]. Indeed, oxidative stress and inflammation go hand-in-hand, because oxidative stress induces the production of inflammatory cytokines, and the cytokines in turn induce free radical production.

The main lipid oxidation products present in oxLDLs, and which may be responsible for inflammatory processes, are oxysterols and cholesteryl ester oxidation products, lipid-derived free aldehydes and oxidized phospholipids. Another quantitatively important component of oxLDLs, alongside the oxidized lipid products proper, are the lysophospholipids, hydrolytic derivatives of oxidized phospholipids. Moreover, among the oxidized lipid products implicated in atherogenesis, there is a large group of potent bioactive lipids, known as prostanoids, isoprostanoids, and leukotrienes, which derive from the oxidation of arachidonic acid, a component of membrane phospholipids. All these compounds have been directly linked to the induction and propagation of

monocytic subendothelial accumulation, and to other inflammatory reactions associated with chronic vascular inflammation. Macrophages in the atheroma may also have a pro-inflammatory array of functions, characteristic of M1 macrophages, which produce high levels of effectors such as cytokines, chemokines, growth factors, adhesion molecules, and other inflammatory molecules.

Conversely, accumulating data suggest that oxidized lipids may also have anti-inflammatory potential, and could, in certain cases, act as inhibitors of the nuclear factor-κB (NF-κB)-dependent pro-inflammatory cascade [16].

This review aims to comprehensively summarize current knowledge on the signal transduction pathways, and on inflammatory gene expression modulated by lipid oxidation derived products, in the progression of atherosclerosis.

Oxysterols

Oxysterols are 27-carbon atom molecules resulting from non-enzymatic or enzymatic oxidation of cholesterol, which may either originate in the blood, cells and tissues, or may derive from the diet. Several reviews have comprehensively discussed the sources of major biological oxysterols and the routes for their formation [17-24]. In general, biological oxysterols can be divided into two main groups: 1) those oxygenated in the sterol ring, mainly at position 7 (e.g. 7α/β-hydroxycholesterol, 7α/β-hydroperoxycholesterol, and 7-ketocholesterol), which have non-enzymatic origin; 2) those oxygenated on the side-chain (e.g. 24S-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol) which usually have enzymatic origin. Some oxysterols, however, can be produced by either non-enzymatic or enzymatic routes: these include 25-hydroxycholesterol and 7α-hydroxycholesterol.

Oxysterols are a large and diverse group of compounds with a multiplicity of biological activities; they have been found at increased levels in the plasma of patients with cardiovascular

diseases, and within atherosclerotic lesions. Depending on the nature and location of the oxygen substitution, oxysterols have distinct effects on the biophysical properties of cell membranes, with which they interact faster than does cholesterol, due to higher polarity and poorer membrane packing. Besides the impact of oxysterols on the biophysical properties of membranes, endogenous cellular oxysterols are thought to drive important functions, by interacting with receptor proteins [23,25,26]. In this connection, oxysterols not only participate in basic metabolic processes, but are also involved in signaling pathways leading to disease development, through induction of inflammation, apoptosis, and fibrosis.

Being multifunctional molecules, oxysterols appear to exert a number of pro-inflammatory effects during the progression of atherosclerosis; however, it is extremely difficult to study all routes involved, and the molecular mechanisms whereby oxysterols induce inflammation at the transcriptional level are not yet fully elucidated. Oxysterol-dependent gene expression is probably regulated by various different transcriptional regulators. In this connection, activation of liver X receptors (LXR) can only be induced by a small number of pro-inflammatory oxysterols, acting as natural ligands [27], while it has been demonstrated that NF-κB or activator protein-1 (AP-1) nuclear binding can be induced by a biologically representative oxysterol mixture [28,29]. Similarly, the oxysterol-induced metabolic pathways at the translational and post-translational levels are still unclear, although mitogen-activated protein kinases (MAPKs) are implicated [28-31]. MAPKs include extracellular signaling-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK, which are involved in several cellular functions, ranging from proliferation to differentiation and apoptosis, and which thus play an important role in the pathogenesis of cardiac and vascular disease [32].

Oxysterol-mediated up-regulation of chemokines, inflammatory cytokines and metalloproteinases

Oxysterols have been shown to induce the expression of various key inflammatory molecules. They stimulate not only the adhesion of leukocytes to the arterial endothelium, but also their transmigration to sub-intimal spaces, especially by up-regulating chemotactic cytokines such as monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8).

Up-regulation of MCP-1 has been reported in U937 promonocytic cells stimulated by a biologically relevant oxysterol mixture, through the ERK and NF-κB pathways [28]. Macrophages isolated from human atherosclerotic plaques produce IL-8, and oxysterols appear to be involved in production of this cytokine [33]; IL-8 is up-regulated in human monocytes/macrophages by various oxysterols, including 25-hydroxycholesterol (25-OH), 24-hydroxycholesterol (24-OH), 7β-hydroxycholestetrol (7β-OH), 5α,6α-epoxycholesterol (α-EPOX), 7-ketocholesterol (7-K), and cholestan-3β,5α,6β-triol (TRIOL) [34-37]. Using calcium channel blockers, it has been shown that both 7β-OH-induced and 25-OH-induced IL-8 secretion by THP-1 cells involves calcium-dependent activation of c-fos (AP-1) via the ERK1/2 signaling pathway [37].

The oxysterols 25-OH, 7β-OH and 7-K have been found to stimulate IL-8 expression in human macrophages, independently of Toll-like receptor (TLR) signaling, although this pathway has been shown to contribute to the inflammatory events occurring during atherosclerosis progression [36]. IL-8 production is also induced in Caco2 cells by treatment with 25-OH followed by IL-1β stimulation [38]. The chemotactic cytokine IL-8 might be pro-atherogenic, acting not only by recruiting leukocytes, but also by inhibiting expression of the tissue inhibitor of metalloproteinase-1 (TIMP-1). This event causes an imbalance in the metalloproteinases (MMPs)/TIMPs activity ratio, with a consequent excessive degradation of extracellular matrix components, followed by destabilization and eventual rupture of the atherosclerotic plaque [39]. In this connection, in human promonocytic U937 cells it is reported that an oxysterol mixture of composition similar to that detectable in advanced human carotid plaques induces expression and synthesis of MMP-9, without affecting its endogenous inhibitors TIMP-1 and TIMP-2. Using

antioxidants, or specific inhibitors, or siRNAs, it has been demonstrated that the oxysterol mixture induces MMP-9 expression through: i) over-production of reactive oxygen species (ROS), likely by NADPH oxidase and mitochondria, ii) up-regulation of MAPKs signaling pathways via protein kinase C (PKC), iii) up-regulation of AP-1 and NF-κB DNA binding [29].

Further, in human monocytic cells, 7β-OH and 25-OH, but also to a lesser extent 7-K, are potent *in vitro* inducers of MCP-1, IL-8, IL-1β, tumor necrosis factor-α (TNF-α), macrophage inflammatory protein-1β (MIP-1β), as well as of other inflammatory molecules [31]. The same study demonstrated that IL-8 secretion was associated with activation of the ERK1/2 signaling pathway [31]. The oxysterols 7-K and 25-OH have also been observed to enhance IL-1β, IL-6, IL-8 and TNF-α mRNA and secretion levels, in a dose-dependent manner, although to different extents. These effects were associated with increased ROS production, through enhanced expression of NADPH oxidase; net posphorylation of MAPKs and NF-κB activation also occurred [40].

Up-regulation of IL-1β is another important event, because this cytokine increases the surface expression of endothelial adhesion molecules, by facilitating inflammatory cells' attachment to the artery endothelium. Expression and synthesis of IL-1β were found to be stimulated by 25-OH in human macrophages, through involvement of LXR, as well as, but less strongly, by 27-hydroxycholesterol (27-OH). 25-OH was also capable of potentiating lipopolysaccharide (LPS)-induced IL-1β secretion [41]. IL-1β secretion was also markedly induced by 7β-OH, 7-K and 7α-hydroxycholesterol (7α-OH) in human promonocytic cells U937 and U4 [35,42] and in human umbilical vein endothelial cells (HUVECs) [43].

Increased levels of IL-6 have also been found in human macrophages stimulated with 25-OH or 27-OH [41]. IL-6 secretion was increased by 7-K treatment in human aorta smooth muscle cells (SMCs) through MAPK activation, in particular by p38 MAPK, but also via JNK pathways [44]. Furthermore, IL-6 regulates the expression of other inflammatory cytokines, such as IL-1 and TNF-α [45].

Expression of TNF-α is stimulated by 25-OH in macrophages, only when the cells were co-treated with 9-cis-retinoic acid, through heterodimerisation of the LXR [46]. Production of the pro-inflammatory cytokines TNF-α and IL-1 is also induced by 25-OH in adherent human peripheral blood mononuclear leukocytes, through phosphorylation of p38 MAPK [47]. Further, synthesis of TNF-α has been reported in human peripheral monocytes and in monocytic THP-1 cells treated with 22-hydroxycholesterol (22-OH) [46]. Another effect of 7-K and 25-OH, which may enhance the inflammatory response, is inhibition of secretion of IL-10, a key anti-inflammatory cytokine, in SMCs [48]. Treatment of vascular SMCs with 7-K enhances synthesis of the vascular endothelial growth factor (VEGF) [49].

Up-regulation of transforming growth factor β1 (TGFβ1), the main pro-fibrogenic cytokine contributing to the progression of inflammation and fibrosclerosis within the damaged arterial wall, has also been shown to be exerted by a biologically representative oxysterol mixture, in J774.A1 murine macrophagic cell line [50]. In particular, production of TGFβ1 plays a pivotal role in SMC chemoattraction and differentiation into myofibroblast-like cells. 25-OH has also been shown to increase the expression of basic fibroblast growth factor (bFGF), a strong mitogenic and fibrogenic cytokine, in SMC [51].

Induction of the endothelial inflammatory phenotype

Oxysterols can contribute to increased expression of several adhesion molecules, which play key roles in endothelial adhesion and transmigration of leukocytes. Oxysterols, such as 7-K, 7α-OH and 7β-OH, increase the level of adhesion molecules involved in the recruitment of immunocompetent cells, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin, in HUVECs [43,52,53]. Moreover, incubation with 7-K markedly induces ROS-dependent secretion of VCAM-1 in human aortic endothelial cells

(ECs) and U937 cells [54]. It has also been shown that 25-OH can augment eicosanoid release from cultured ECs and increase endothelial-leukocyte interaction by up-regulating VCAM [55].

It is known that endothelial dysfunction plays a central role in atherosclerosis, and 7-K has been found to increase expression of the actin-binding protein profilin-1 in aortic ECs. Up-regulation of the pro-atherogenic protein profilin-1 appears to occur through activation of a transduction molecule known as signal transducer and activator of transcription 3 (STAT3), which requires Janus kinase 2 (JAK2) and tyrosine 394 phosphorylation of oxysterol-binding protein-1 (OSBP-1) [56]. Again, oxysterols can be inserted into the cell membrane, where they modify the functions of membrane-bound proteins: 7-K has been found to induce endothelial dysfunction by disrupting ion transport by Na/K-ATPase, and to perturb the membrane structure in human ECs. For these reasons, this oxysterol-induced effect may contribute to the rigidity of plasma membranes [57].

Monocyte differentiation and foam cell formation

Oxysterols contribute to sustaining inflammation, by favoring monocyte differentiation into macrophages. It has been shown that a biologically compatible mixture of oxysterols up-regulates the expression and synthesis of the scavenger receptor CD36, with consequent uptake of oxLDLs, involving PKC-δ, ERK, and the peroxisome proliferator-activated receptor γ (PPARγ) pathways [58]. Indeed, the monocyte differentiation into macrophages observed in oxLDL-treated THP-1 cells has been attributed to its major oxysterols, 7β-OH and 7-K [59]. Foam cell formation, a key process during the development of atherosclerosis, which leads to vascular inflammation, is also sustained by TLR4: deficiency of TLR4 significantly reduces macrophage lipid accumulation in vascular lesions [60]. Furthermore, the common Asp299Gly TLR4 polymorphism, which causes loss of function, is associated with lower plasma levels of pro-inflammatory cytokines, adhesion molecules and acute-phase proteins, and a decreased atherosclerosis risk [61].

The accumulation of lipids in the macrophages present at the subendothelial level might depend on some oxysterol profile present in the atheromasic lesion: the main cytotoxic oxysterols, i.e. 7-K, 7β-OH, and 5β,6β-epoxycholesterol (β-EPOX), are potent inducers of endolysosomal phospholipidosis, because they trigger the formation of multilamellar cytoplasmic structures, called myelin figures, cointaining high levels of phospholipids [62-64]. Phospholipid accumulation has been shown to be connected with oxidative stress and apoptosis induced by oxysterols, and could contribute to *in vivo* vascular injury [65]. Moreover, 7-K-induced phospholipidosis involves the phosphatidylinositol 3 kinase (PI3K)/Akt signaling pathway, and is partially reversed by vitamin E in U937 monocytes [66]. Of note, endolysosomal lipid storage induces strong pro-inflammatory and pro-atherogenic responses in macrophages [67].

Induction of other inflammatory mediators: phospholypase A_2 and cyclooxigenase-2

Treatment of rat aortic SMCs with 25-OH or 22R-OH causes accumulation of group IIA secretory phospholipase A_2 (PLA_2) and increases enzyme activity, with involvement of the LXR pathway [68]. Oxysterols are also able to activate the cytosolic PLA_2: in macrophages, activation of cytosolic PLA_2 by 25-OH increases arachidonic acid release [69] and is involved in the initiation of the apoptosis pathways triggered by 7-K [70]. In agreement with these observations, eicosanoid production has been found to be stimulated in bovine coronary artery ECs treated with 25-OH [62]. In contrast, in ECs, 7-K inhibits phosphorylation of cytosolic PLA_2 and arachidonic acid release, by altering a Ca^{2+}-independent upstream step of the PI3K and ERK1/2 cascade [72].

The oxysterol TRIOL enhances cyclooxygenase-2 (COX-2) expression and synthesis, leading to prostaglandin E_2 (PGE_2) production in HUVECs. This effect requires the involvement of PI3K/Akt and endothelial nitric oxide synthase (NOS) and the activation of p38 MAPK and NF-κB [73].

However, despite their marked pro-inflammatory effects, in certain experimental conditions, oxysterols have also been reported to exert anti-inflammatory effects. For instance, LPS-induced TNF-α secretion was significantly decreased when human macrophages were incubated with 7β-OH, 25-OH and 27-OH before LPS challenge [74]. Moreover, exposure of human macrophages to chylomicron remnant-like particles loaded with 7β-OH, but not similar particles loaded with 7-K, significantly reduced IL-6 and TNF-α secretion [75]. It is also reported that 22R-OH and 7-K inhibit protein expression of both inducible NOS and COX-2 in LPS-stimulated primary rat microglial cells [76].

LXR-dependent effects of oxysterols

LXR has been implicated in many biological processes, ranging from regulating lipid metabolism to inflammation and immunity [77-79]. Of note, LXR is involved in the pathogenesis of cardiovascular diseases, and LXR agonists prevent development of atherosclerosis, by modulating metabolic and inflammatory gene expression in rodent models. Indeed, activation of LXR leads to maintenance of cholesterol homeostasis through transcriptional activation, and to suppression of the inflammatory response through transrepression. Among the oxysterol family, 20S-hydroxycholesterol (20S-OH), 22R-OH, 24S-OH, 24S,25-epoxycholesterol (24S,25-EPOX), α-EPOX, 25-OH, and 27-OH are endogenous ligands of LXR, but 7-K and 7β-OH are not [27,80-82]. However, among these LXR ligands, only 27-OH appears to be present in significant amounts in cholesterol-loaded human macrophages, cells that play a key role in atherogenesis [83].

Activation of both LXR isotypes (α and β) in cultured macrophages represses the expression of inflammatory mediators, such as inducible NOS, COX-2, IL-6 and IL-1β, MCP-1 and MCP-3, and MMP-9, in response to inflammatory stimuli, and, at the same time, it regulates the expression of genes involved in lipid homeostasis. In this connection, LXR appears to play a major role in

translating lipid signaling into inflammatory responses [77,78,84]. Activation of LXR also inhibits inflammatory gene expression in the aortas of atherosclerotic mice [85]. However, it has recently been reported that agonists of the oxysterol LXR stimulate inflammatory pathways in ECs, leading to the up-regulation of adhesion molecules (e.g. ICAM, VCAM), chemokines (e.g. IL-8, IL-1α), transcription factors (e.g. AP-1), and enzymes (e.g. COX-2), and to the down-regulation of endothelial NOS, through LXR-independent mechanisms [86].

Importantly, cross-talk between LXR and TLR signaling has been demonstrated in macrophages, as well as in aortic tissue. This cross-talk is mediated by the transcriptional factor IRF3, a specific effector of TLR3 and TLR4 that inhibits the transcriptional activity of LXR on its target promoters. Thus, activation of TLR3 and TLR4 can block the induction of LXR target genes by antagonizing LXR [77]. Conversely, activation of LXR by oxysterols or by synthetic agonists in macrophages inhibits TLR-inducible inflammatory genes, such as inducible NOS, IL-1β, MCP-1, by interfering with NF-κB signaling [78,87]. In this regard, the repression pathways mediated by LXR involve the nuclear receptor co-repressor. Nuclear receptor co-repressor-deficient macrophages exhibit derepression of a subset of genes that are normally activated by NF-κB and AP-1 in response to pro-inflammatory signals [88,89].

Cholesteryl ester oxidation products

In the lipid moiety of LDLs, cholesterol is present both in the unesterified and in the esterified form, i.e. bound or not to fatty acids. The atherogenic properties of oxLDLs are also conferred by oxidized cholesteryl esters, in which oxidation involves either cholesterol itself or monounsaturated fatty acids and polyunsaturated fatty acids (PUFAs), producing several products, including cholesteryl ester hydroperoxides [90] and core-aldehydes [91]. Of note, at least in *in vitro* models, at more advanced stages of LDL oxidation, decomposition of the initially formed cholesteryl ester hydroperoxides may lead to the formation of oxysterols [92,93].

Induced formation of foam cells

Cholesteryl ester hydroperoxides are responsible for many of the biological activities of minimally oxLDLs, and they may be relevant to inflammatory activation of macrophages in atherosclerotic lesions [94]. Moreover, in differentiated human macrophages, it has been observed that oxidized derivatives of cholesteryl esters contribute to the formation of foam cells, by inducing scavenger receptor CD36 expression with involvement of PPARα [95].

Although accumulation of cholesteryl oleate in the plasma has been reported to be a predictor of atherosclerosis in animal models, whereas cholesteryl esters of *n*-3 and *n*-6 PUFAs may provide athero-protection by reducing that accumulation [96], oxidized derivatives of cholesteryl ester containing *n*-6 PUFAs have been found in oxLDLs and in atherosclerotic lesions. In particular, high levels of 9-oxononanoyl cholesterol (9-ONC) and 5-oxovaleroyl cholesterol (5-OVC), respectively derived from linoleic acid and from arachidonic acid, have been found in oxLDLs and in atherosclerotic lesions [97-99]. These so-called core-aldehydes have been shown to stimulate adhesion molecule production by HUVECs, favoring blood monocytes and U937 cell adhesion [100]. Furthermore, these products can react covalently with amino groups of apoprotein B100, with consequent uptake of the modified oxLDLs by activated macrophages through scavenger receptors and foam cell formation [101].

Pro-fibrogenic effect

9-ONC has also been found to stimulate expression and synthesis of TGFβ1, in both J774.A1 murine macrophages and human U937 promonocytic cells, contributing to inflammation and fibrosclerosis in atherosclerotic plaques [102,103]. An increased membrane presentation of TGFβ type II receptor has also been observed. The increased levels of the cytokine and of its

specific receptors in 9-ONC-treated cells appear to be mediated by the stimulation of ERK1/2 induced by the oxidized lipid. The core-aldehyde 9-ONC might thus sustain further vascular remodeling due to atherosclerosis, not simply by stimulating synthesis of the pro-fibrogenic cytokine TGFβ1 in vascular cells, but also and chiefly by enhancing the TGFβ1 autocrine loop [103].

Lipid peroxidation-derived free aldehydes

Lipids containing PUFAs are susceptible to free radical-initiated oxidation, and can participate in chain reactions that increase damage to biomolecules. The chain process of lipid peroxidation includes simultaneously generated lipid hydroperoxides and aldehydes of various chain lengths [104-108].

The enzymatic and non-enzymatic peroxidation of *n*-3 and *n*-6 PUFAs generates several reactive aldehyde species, which have been shown to exert regulatory roles as well as detrimental effects in various cell types and organs. These aldehydic end-products of lipid peroxidation are known to be among the molecules responsible for the pro-atherogenic effect of oxLDL.

Peroxidation of *n*-3 PUFAs, such as linolenic, eicosapentaenoic, and docosahexaenoic acids, generates the molecule 4-hydroxy-2-hexenal (HHE). Peroxidation of *n*-6 PUFAs, such as arachidonic and linoleic acids, as well as of their 15- and 13-lipoxygenase (15-LO, 13-LO) metabolites (namely 15-hydroperoxyeicosatetraenoic acid and 13-hydroperoxyoctadecadienoic acid) yields an aldehyde with remarkable biochemical effects, namely 4-hydroxy-2-nonenal (HNE). Another reactive peroxidation product is 4-hydroxy-2,6-dodecadienal (HDDE), which is derived from 12-hydroperoxyeicosatetraenoic acid, the 12-lipoxygenase metabolite of arachidonic acid. These aldehydes, and in particular HNE, have multiple pathophysiological effects, due to their high chemical reactivity and lipophilicity, and to their formation of covalent adducts with macromolecules (proteins, peptides, lipids, and nucleic acids). Indeed, they react with the thiol and

amino groups, especially of the amino acids cysteine, histidine and lysine. The progressive accumulation of these adducts alters normal cell functions, and may lead to cell death [104,109-111]. Nonetheless, at low and noncytotoxic concentrations, these molecules can act as second messengers in signal transduction in physiological and/or pathophysiological conditions [111-115].

The most representative unsaturated hydroxyalkenal in tissues and cells is HNE [116-118]. This aldehyde has been investigated in depth thanks to its contribution to the pathogenesis of major chronic human diseases, including atherosclerosis, and this molecule has been reported to possess both signaling and cytotoxic effects [112,116,119,120].

HNE-induced endothelial dysfunction and foam cell formation

HNE can directly impair the barrier function of the endothelium by increasing its permeability [115]: it may induce changes in cellular thiol redox status, thus perturbing cell signaling pathways and leading to endothelial barrier dysfunction. Following induction of ROS, HNE may affect EC permeability, modulating cell-cell adhesion by suppressing focal adhesion kinase (FAK) phosphorylation, which in turn affects focal adhesion, adherence, and tight junction proteins, as well as integrins, which are natural FAK receptors [121]. Modification of cytoskeletal proteins, including actin and microtubules, by HNE and by other unsaturated aldehydes, plays an important role in regulating cell-cell contacts and endothelial barrier function [121]. HNE may stimulate the adhesion of macrophages to the vascular endothelium in the early stages of atherosclerosis, as well as that of lymphocytes, pro-inflammatory immune cells [122]. This aldehyde may potentiate inflammation and immune responses by inducing COX-2 and in consequence prostaglandin production [123].

In addition, HNE covalently modifies LDLs by binding to lysine and histidine residues, leading to adduct formation [124]; this modification could make them available to scavenger receptors on macrophages, thus resulting in macrophage activation and foam cell formation, a

crucial event in the vascular inflammation that occurs in atherosclerotic lesions. HNE also promotes foam cell formation, by increasing synthesis of class A scavenger receptors at the transcriptional level, as well as increasing synthesis of the scavenger receptor CD36 [125]. HNE also induces induction of CD36 in vascular SMCs by enhancing nuclear accumulation of nuclear factor E2 related factor 2 (Nrf2), a second important transcription factor, alongside PPARγ, in the induction of this scavenger receptor [126]. In this connection, it is also reported that nuclear translocation of Nrf2 requires activation of PKC [127]. After its translocation into the nucleus induced by HNE, Nrf2 transactivates the antioxidant responsive element (ARE). The HNE-induced Nrf2-ARE transcription pathway can up-regulate molecules such as thioredoxin (Trx), Trx reductase, and heme oxygenase-1 (HO-1), all of which are antioxidant proteins involved in the mechanisms of adapted cytoprotection against oxidative stress [128,129].

Pro-inflammatory effects of HNE

Again in regard to inflammation, HNE has been shown to increase MCP-1 release by J774.A1 macrophages, by activating PKC-βI and -βII. The aldehyde has a dual effect on these enzymes: their activity is increased if cells are incubated with relatively low concentrations of HNE, but progressively decreases if concentrations of the aldehyde are higher [130]. Strong up-regulation of the inflammatory and fibrogenic cytokine TGFβ1 in promonocytic and macrophagic cells challenged with pathophysiological concentrations of HNE has also been demonstrated [131-133].

It has been postulated that HNE-induced activation of NF-κB mediates its pro-inflammatory and oxidative effects [134,135]. However, it is also reported that HNE may inactivate NF-κB, by preventing proteolysis of the inhibitor of NF-κB (IκB) [136,137]. The dual effect of HNE on this redox transcriptional factor appears to depend on the type of cells used. For example, in THP-1 monocytes HNE inhibited NF-κB activation induced by LPS and the subsequent TNF-α release, by

inhibiting phosphorylation and proteasome degradation of IκB [136]. Further, in human aortic ECs, down-regulation of NF-κB activity by HNE resulted in inhibition of the expression of various adhesion molecules induced by inflammatory stimuli [138]. In agreement with these findings, activation of NF-κB induced by exposure of THP-1 cells to *Chlamydia pneumonia* was significantly reduced by HNE addition [139]. Addition of HNE to macrophages did not modify the constitutive extent of NF-κB nuclear translocation [140]. In contrast, net stimulation of NF-κB by HNE was demonstrated in vascular SMCs [141].

The different ways in which HNE can modulate the NF-κB-signaling pathway may have a significant impact on atherosclerosis development. For instance, down-regulated expression of NF-κB-dependent genes, as a consequence of HNE's inhibition of this nuclear factor, may interfere with the immune response and thus indirectly amplify and sustain inflammation and degenerative processes.

Conversely, marked activation of AP-1 nuclear binding by HNE has been reported. Activation of AP-1 has been demonstrated in murine and human macrophages [140], and in aortic SMCs [142]. HNE appears to up-regulate AP-1 DNA binding, mainly through activation of PKC-δ and, subsequently, through PKC-mediated activation of JNK [143].

Modulation of growth factor receptors by HNE

HNE interacts with epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), which are transmembrane glycoproteins involved in various biological processes, as well as in the development of human diseases. In cultured human ECs, HNE was found to directly react with these membrane proteins, inducing formation of HNE-EGFR or HNE-PDGFR adducts [144]. However, HNE showed a dual effect on PDGFR-β, which regulates SMC migration and proliferation in the vascular wall. Short-term incubation of SMCs with low

concentrations of HNE induced autophosphorylation of PDGFR-β and activation of a signaling cascade involving the PI3K/Akt pathway and stimulating SMC proliferation [145]. Long-term incubation and high concentrations of HNE increased formation of HNE-PDGFR-β adducts and progressively inhibited PDGFR-β phosphorylation and SMC proliferation. The latter event may contribute to defective SMC proliferation, and decrease the stability of a vulnerable plaque [146].

Exposure to this aldehyde also resulted in increased proliferation of SMCs through MAPK activation. In rat aortic SMCs treated with HNE, strong but transient activation of ERK1/2 occurred, with induction of *c-fos* and *c-jun* protein synthesis, and increased AP-1 DNA binding activity; in addition, HNE induced PDGF-AA protein synthesis in rat aortic SMCs [142]. Again using SMCs, it was than shown that this aldehyde may activate not only ERK1/2 but also JNK and p38 MAPK [147]. Moreover, prolonged treatment of mouse aortic SMCs with a low concentration of HNE increased cell growth when young SMCs were used, but showed cytotoxic effects in aged SMCs. In young cells, the aldehyde induced strong activation of ERK and enhanced cyclin D1 expression [148]. Activation of PDGFR-β and ERK1/2 by HNE in human coronary SMCs also appeared to be the key mechanism in the production of MMP-1, which plays a role in SMC migration into the intima and in plaque instability [149].

HNE treatment of murine macrophages has been observed to enhance 5-LO, following its regulation at the transcriptional level by EGFR, through activation of the stimulating protein-1 (SP-1)/p38 and NF-κB/ERK pathways [135]. Since HNE and 5-LO co-exist in macrophages in atherosclerotic lesions, it has been suggested that they may cooperate in modulating SMC migration, proliferation and atherosclerotic plaque instability. Indeed, the incubation of J774.A1 macrophagic cells with HNE induces production of 5-LO derivatives. The association of these products increases MMP-9 secretion via activation of ERK and p38 pathways [150]. The same research group also demonstrated a similar synergic action between HNE and 5-LO derivatives in vascular SMCs, in which marked induction of MMP-2 occurred, again following activation of the

ERK and p38 pathways [134,151]. Moreover, HNE has also been shown to stimulate MMP-2 production in the same type of cells, via mitochondrial ROS-mediated activation of the Akt/NF-κB signaling pathways [134].

Pro-inflammatory effects of unsaturated aldehydes other than HNE

Notably, the finding that HNE and HDDE may act as endogenous ligands of PPARδ in vascular ECs is particularly important in view of the remarkable metabolic and regulatory functions that this nuclear receptor mediates. For instance, selective PPARδ ligands possess anti-inflammatory properties [152]; other reports have shown that PPARδ also increases cholesterol export and represses inflammatory gene expression in macrophages [153], as well as augmenting fatty acid oxidation [154,155].

HHE, an end-product of *n*-3 PUFA peroxidation, is quite abundant but, due to its lower lipophilicity and lower reactivity, it is considered less physiologically active and less damaging than HNE or HDDE [104]. Further, HHE has properties in common with HNE, but there are important differences, particularly with regard to adduction targets and detoxification pathways [156]. It has been suggested that HHE may activate NF-κB in vascular ECs, with a consequent increase in inducible NOS gene expression, which in turn leads to cell dysfunction due to excessive generation of nitric oxide radicals [157]. This aldehyde also appears to be responsible for EC apoptosis [158].

Another reactive unsaturated aldehyde is acrolein, which is generated from threonine by neutrophil myeloperoxidase at the site of inflammation, and which has been identified as both a product and an initiator of lipid peroxidation [159,160]. Elevated acrolein levels have been found in various diseases, including atherosclerosis, and it contributes to ROS generation, which in turn may lead to inflammation and cell dysfunction [161]. It has been demonstrated that acrolein induces COX-2 expression and PGE_2 production in HUVECs, through the activation of PKC, p38 MAPK,

and cAMP response element-binding protein (CREB). Moreover, this aldehyde inactivates Trx reductase, a primary antioxidant enzyme [161]. In this connection, acrolein, like HNE, reacts with the thiol groups of Trx1, a protein that regulates antioxidant functions in ECs. Modification of Trx1 by either aldehydes may potentiate monocyte adhesion to ECs during the early events of atherosclerosis [122]. Further, exposure to acrolein induces differentiated THP-1 macrophages to secrete MMP-9 via a mechanism that involves xanthine oxidase activation and increased ROS generation [162].

Oxidized phospholipids

Oxidized phospholipids (oxPLs) derive from oxidative modification of LDLs, but also from the membranes of cells undergoing apoptosis. Enzymatic or non-enzymatic oxidation of fatty acids linked to the *sn*-1 and *sn*-2 positions of glycerophospholipids leads to many different reaction products, depending on chain length and degree of unsaturation [111,163]. These products are implicated as modulators of inflammation, and increased levels of oxPLs are involved in the pathogenesis of various diseases, including atherosclerosis [164-166]. However, oxPLs play a dual role in modulating inflammation, since they may exhibit both pro-inflammatory and anti-inflammatory effects [16,165,167,168].

Primary role in stimulating adhesion of monocytes to the endothelial barrier

One major phospholipid present in minimally modified LDLs is 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (PAPC), which generates a heterogeneous group of oxygenated full-length products, or compounds with truncated oxidized residues present at the *sn*-2 position (oxPAPC); these accumulate in atherosclerotic lesions [169,170]. The atherogenic potential of oxPLs, in particular of oxPAPC, has been demonstrated in cell culture studies, in terms of

enhanced binding of monocytes, but not of neutrophils, to oxPLs-stimulated ECs [170] and of concomitant induction of MCP-1 and IL-8 [171]: monocyte/EC adhesion is a hallmark of chronic inflammation. Regarding the mechanisms mediating adhesion of monocytes to ECs, oxPLs were not found to up-regulate ICAM, VCAM or E-selectin, but rather to induce MCP-1, connecting segment-1 and P-selectin. In this connection, oxPAPC have been shown to stimulate adhesion of monocytic cells through activation of β1-integrin, which subsequently binds to a splice variant of fibronectin, containing the sequence known as connecting segment-1 [172]. The activation of β1-integrin by oxPLs appears to be mediated by a cAMP-dependent R-Ras/PI3K pathway [173]. Of note, it has also been observed that oxPAPC induces monocyte adhesion to ECs by activating the PGE_2 receptor subtype 2, leading to increased cAMP levels and activation of PKA [174]. Another mechanism involves P-selectin, which is known to bind both monocytes and neutrophils to ECs. OxPLs up-regulates P-selectin in human aortic ECs [175] and in murine carotid arteries [176]. Up-regulation of P-selectin protein and P-selectin-dependent adhesion of leukocytes are also reported in $ApoE^{-/-}$ mouse aortic segments [177]. Moreover, it has been suggested that oxPLs could stimulate formation of platelet-monocyte aggregates, an event that may play a role in the pathogenesis of vascular diseases, by enhancing P-selectin expression [178].

Increasing evidence also indicates that phosphatidylcholine (PC) hydroperoxide plays a role in atherosclerosis. In particular, it has been reported that treating THP-1 monocytic cells with PC hydroperoxide stimulates their adhesion to immobilized vascular EC adhesion molecules. In addition, THP-1 cell adhesion to ICAM-1, an effect exerted by this peroxide, was found to be dose-dependent [179] and to require activation of GTPases of the Rho subfamily named Rac (Ras-related C3 botulinum toxin substrate) [180].

Up-regulation of chemokines, inflammatory cytokines and growth factors

OxPLs, in particular oxPAPC, contribute to the recruitment of inflammatory cells in the atherosclerotic lesion, inducing not only adhesion molecules but also growth factors and cytokines in vascular ECs [181]. Moreover, induction by oxPLs of chemotactic activity in monocytes requires activation of MAPKs [182-184]. The induction of chemokines is regulated by genetic and/or epigenetic factors: in human aortic ECs derived from multiple heart transplant donors, the level of inflammatory gene induction by oxPLs differed. In particular, production of IL-8, but also of IL-6 and MCP-1, was increased in those cells through regulation of the unfolded protein response [185,186]. Further, immunohistochemical analysis of human atherosclerotic lesions indicated activation of the unfolded protein response in areas containing oxPLs [186]. Importantly, it has also been reported that up-regulation of IL-8 and MCP-1 expression induced by oxPLs is independent of the classical NF-κB pathway, but that it involves activation of c-Src kinase. OxPLs bind to the TLR4 complex and lead to rapid and transient activation of c-Src kinase, which in turn induces phosphorylation of the transcription factor STAT3 [187]. Subsequently, STAT3 dimerizes, translocates to the nucleus and induces transcription of IL-8. In ECs, oxPAPC induces the synthesis of chemotactic factors, such as IL-8, involving c-Src kinase-dependent activation of JAK2, which leads to enhanced levels of STAT3 activity in inflammatory areas of atherosclerotic lesions [188]. Moreover, oxPAPC may modulate cytokine expression by binding to human macrophages via the platelet activating factor (PAF) receptor. Occupation of the PAF receptor by oxPLs stimulates intracellular calcium signaling, which modifies the transcription levels of highly pro-inflammatory cytokines, including IL-8 [189]. Another mechanism has also been proposed: oxPAPC might increase IL-8 and MCP-1 expression in ECs, involving activation of PPARα [190,191].

The compound 1-palmitoyl-2-(5-oxovaleryl)-*sn*-glycero-phosphocholine (POVPC), an oxidatively fragmented component of oxPAPC, stimulates expression of IL-8, IL-1β, and TNF-α in human monocyte-derived macrophages [189]. POVPC has also been found to activate cytosolic PLA_2 via the MAPK pathway in HUVECs, resulting in the release of free arachidonic acid [192],

which is used by LO to produce 12-hydroxyeicosatetraenoic acid that, in turn, contributes to stimulating monocyte binding to human aortic ECs [193].

In addition, hydroxy and oxo alkenal phospholipids, another form of oxPLs, may locate at the surface of oxLDLs and act as ligands of the scavenger receptor CD36 on macrophages, with consequent uptake of oxLDLs and foam cell formation [194].

In monocytes, oxPLs have also been found to stimulate VEGF that, in addition to its angiogenic growth factor activity, is another factor involved in leukocytes' migration [195]. In addition, up-regulation of VEGF induces expression of VCAM-1 and platelet endothelial cell adhesion molecule (PECAM-1) on the endothelium, and stimulates monocyte adhesion, leading to the formation of more inflamed atherosclerotic lesions in $ApoE^{-/-}$mice [196]. Again, in human aortic ECs, VEGF receptor 2 is reported to regulate ERK1/2-mediated activation of the transcription factor sterol regulatory element-binding protein 1 (SREBP-1), and the subsequent transcription of tissue factor, LDL receptor, and IL-8 [197]. Endothelial NOS has been reported to play a role in the activation of SREBP by oxPAPC [198].

OxPAPC may contribute to oxidative stress by inducing ROS generation, which in turn stimulates inflammatory gene expression. VEGF receptor 2 was found to mediate oxPAPC-induced recruitment of Rac1 to the NADPH oxidase-4 complex, resulting in strong ROS generation by ECs [199]. Another pathway used by oxPAPC to generate ROS includes uncoupled endothelial NOS and increased mitochondrial metabolism [200]. In parallel with an increased production of ROS, oxPAPC may stimulate the transcription of oxidative stress response genes, such as OKL38, a key regulator of important inflammatory and anti-inflammatory molecules [201]. Expression of OKL38 has been observed to be mediated by Nrf2 [202].

Anti-inflammatory effects

Despite their likely implication in inflammatory reactions, oxPLs are also thought to exert anti-inflammatory effects in leukocytes. For example, oxPLs, acting via PGE_2 receptor subtype 2, inhibit basal production of TNF-α and enhance production of the anti-inflammatory cytokine IL-10 in macrophages [203]. Inhibition of IL-12 synthesis, with a concomitant increase in IL-10 levels, has also been observed in primary human monocytes stimulated by TLR2 and TLR1 ligands [204]. Furthermore, oxPLs may exert anti-inflammatory effects, by inducing drug metabolism phase II genes, which mediate protection from oxidative stress. Low concentrations of oxPLs do not damage cells, but induce antioxidant enzymes, such as HO-1, known for its anti-inflammatory activity [205]. Induction of HO-1 expression by oxPAPC in HUVECs involves the phosphorylation of CREB, which is also dependent on MAPK pathways [206]. COX-2 is another enzyme involved in oxPL-mediated anti-inflammatory signaling. The expression of COX-2 appears to be modulated by oxPLs, in a cAMP-response-element-binding-protein-dependent and PPARγ-dependent manner [207]. OxPLs may also increase NO production, with possible anti-inflammatory effects, including down-regulation of adhesion molecules and chemokines, as well as inhibition of leukocyte migration [198].

Modulation of vascular smooth muscle cell phenotype

Increasing experimental evidence suggests that oxPLs may play a role in phenotypic modulation of vascular SMCs. It has been found that oxPAPC and POVPC switch the phenotype of SMCs to an inflammatory state. Suppressed transcription of the SMC differentiation marker genes induces expression of pro-inflammatory genes, enhances the rate of cellular proliferation, and increases synthesis of extracellular matrix proteins; further, oxPLs can also suppress expression of smooth muscle actin and myosin heavy chain, and increase expression of MCP-1 and MCP-3 [208]. OxPL derivatives POVPC and 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphorylcholine (PGPC) have been shown, both *in vivo* and *in vitro*, to induce vascular SMC proliferation, by reducing

expression levels of connexin 43, another important factor in the pathogenesis of atherosclerosis [209]. In addition, POVPC may alter extracellular matrix production and stimulate vascular SMC migration [210]; it has also been shown that oxPLs stimulate expression of the metalloproteinase known as disintegrin, and that of metalloproteinase with thrombospondin motifs-1 (ADAMTS-1) [195]. These observations suggest a role of oxPLs in plaque destabilization.

Effects on endothelial cells

OxPLs, which accumulate in atherosclerotic lesions, may also exert intraplaque angiogenetic effects, by stimulating production of VEGF, IL-8, and COX-2-derived prostaglandins that act in concert to induce the angiogenic switch in ECs [195]. A later study found that oxPLs induced up-regulation of VEGF by activating transcription factor 4 (ATF4)-dependent transcription [186]. Moreover, it has recently been reported that oxPLs regulate expression of ATF4 and VEGF in ECs, through a Nrf2-dependent mechanism [211].

OxPLs may have a dual effect on barrier function. Some *sn*-2 fragmented oxPLs, such as POVPC and PGPC, increase endothelial permeability even at low concentrations [212,213]. In contrast, both oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoserine (oxPAPS) and oxPAPC exhibit potent barrier-protective effects by attenuating the agonist-induced EC permeability triggered by transient activation of the Rho pathway [214]. Indeed, treatment of the endothelium with oxPAPC suppresses Rho-dependent induction of EC permeability, as stimulated by inflammatory molecules or other agonists, such as IL-6, thrombin, and LPS [212,214]. It has also been observed that, during protection of the endothelial barrier, oxPLs cause specific rearrangements of the cytoskeleton, also enlarging the adherens junctions and their colocalization with focal adhesion protein complexes [215].

Very recently, to elucidate the major pathways involved in oxPAPC action, a systems analysis of endothelial cell gene expression after exposure to these products was performed

[201,216]. This network analysis provided novel hypotheses about molecular interactions, as well as candidate molecular regulators of inflammation. In particular, it was found that oxPLs activate specific signaling pathways, and regulate a large number of genes involved in homeostasis of the cytoskeleton, junctional components, and tyrosine kinases, all cell elements that may contribute to the phenotypic and molecular changes observed in ECs treated with oxPAPC.

Other oxPLs derivatives: lysophospholipids

Hydrolysis of oxPLs by secretory PLA_2, or by lipoprotein associated-PLA_2, yields bioactive lipids that include oxidized fatty acids and lysophospholipids, such as lyso-phosphatidylcholine (lyso-PC) [217]. Lyso-PC, the precursor of lysophosphatidic acid, is found in atherosclerotic lesions and may in principle exert pro- or anti-atherogenic effects, depending on the arterial cell type and inflammation status. However, lyso-PC has been consistently shown to be associated with endothelial dysfunction in early coronary atherosclerosis in humans, thus supporting a role of these phospholipases in the pathogenesis of vascular inflammation and atheromasic lesions [218].

Various signaling effects relevant to atherosclerosis progression are reported to be exerted by lyso-PC. This lysophospholipid has been shown to: i) increase intracellular Ca^{2+}, mainly by activating the PAF receptor, in macrophagic cells [219]; ii) modulate the MAPK pathway in macrophages [220]; iii) induce pro-inflammatory cytokines in circulating mononuclear cells, by activating the PAF receptor [221]; iv) activate G-protein-coupled receptor A, a molecule involved in immune cell migration and apoptosis, both in T lymphocytes and in macrophagic cells [222,223]. Further, lyso-PC has also been found to stimulate the release of pro-inflammatory cytokines, such as IL-8 and IL-6, and of growth factors, such as bFGF, in human coronary artery SMCs [224]. Even nanomolar concentrations of lyso-PC are able to stimulate cytokine expression and monocyte recruitment [225]. In addition, monocyte migration driven by lysophospholipids appears to

implicate a certain protein kinase D (PKD)-dependent signaling pathway [226] and to require non-selective cation channel activation, independent of G-protein and phospholipase C [227].

Arachidonic acid derivatives in atherosclerosis

Among the oxidized lipid products implicated in atherogenesis, there is a large group of potent bioactive lipids called eicosanoids (prostanoids, isprostanes, and leukotrienes) which derive from the oxidation of arachidonic acid, a component of membrane phospholipids released by PLA_2. Prostanoids and leukotrienes are formed enzymatically from arachidonic acid, by COX and LO, and the various sub-classes are generated by distinct enzymatic pathways. Isoprostanes are a class of prostaglandin-like products formed via free-radical-mediated oxidation of arachidonic acid, esterified in membrane phospholipids. All these lipid mediators are consistently observed in atherosclerotic lesions where, together with the oxLDL-derived oxidized lipids, they contribute to the inflammatory responses and to plaque progression [228-232].

Of note, LDL lipid oxidation products could regulate eicosanoid production, by modulating the enzymatic degradation of arachidonic acid via cyclooxygenases and lipoxygenases. In particular, oxLDL has been shown to downregulate inducible COX-2 in human macrophages exposed to LPS. This finding may support the hypothesis that transformation of macrophages into foam cells results in the attenuation of the inflammatory response, thus contributing to the progression of atherogenesis [233]. Inhibition of COX-2 by oxLDL might also promote thrombotic events, by disturbing the balance between platelet thromboxanes (TX) and PG [234]. In contrast, the aldehyde HNE is reported to induce gene expression of COX-2 [235]. It has also been hypothesized that 12-LO and 15-LO might directly contribute to LDL oxidation [236].

Prostanoids

Prostanoids, consisting of PG and TX, are produced from arachidonic acid by the sequential actions of PLA_2, COX-1 or COX-2, and the respective prostanoid synthases. They exert a variety of biological activities in the atheromasic lesion, by binding to specific G-protein-coupled receptors, known as the thromboxane receptor (TP) and the prostaglandin receptors (e.g. EP, IP), that are expressed in the target cells [237,238]. These receptors are highly expressed in ECs and SMCs, as well as in platelets and monocytes/macrophages. Of note, a significant increase in TP receptor expression and activation induces the expression of adhesion molecules, such as ICAM-1 and VCAM-1, in ECs [239,240], as well as inducing IL-1β-dependent VCAM-1 expression in vascular SMCs [241] which facilitate the migration of monocytes to the vessel wall.

Prostanoids are known to promote the initiation and progression of atherosclerosis, not only via platelet activation, but also through leukocyte-endothelial adhesion and vasoconstriction. Of note, PGI_2 and TXA_2, the major prostanoids in cardiovascular diseases, have opposing actions: PGI_2 induces vascular relaxation, and potently inhibits platelet activation and vascular SMC proliferation, whereas TXA_2 is a potent vasoconstrictor, a strong platelet activator, and it also stimulates vascular SMC proliferation. Because of their opposed actions, the balance between these two bioactive lipids is known to be a critical factor causing a tendency to thrombosis. Moreover, it has been suggested that PGI_2 suppresses the development of vascular remodeling, due to its inhibitory actions on vascular SMC proliferation and on the platelet function [242] but also due to its regulatory role over endothelial progenitor cells, which are known to be the cells involved in reendothelialization, a process which limites vascular remodeling [243]. PGI_2 is also reported to play a role in the pro-angiogenic function of endothelial progenitor cells, consisting in proliferation of these cells and tube and capillary formation [244]. In contrast, platelets activated by TXA_2 serve as a source of growth factors, pro-inflammatory cytokines, and chemokines [245]. TXA_2 and its TP receptor are also critical to atherogenesis, promoting leukocyte recruitment and adhesion through enhancement of the transmembrane chemokine fractalkine (CX3CL1) [246].

The prostanoid 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ_2) has also been shown to inhibit the cytokine-induced expression of several adhesion molecules, including E-selectin, ICAM-1 and VCAM-1. For example, 15d-PGJ2 inhibits E-selectin expression via PPARγ-dependent transcriptional induction of ATF3 [247]. Furthermore, 15d-PGJ_2 has a dual action on ICAM-1 in ECs: it directly induces ICAM-1 expression through an increase of AP-1 binding to its promoter, but it simultaneously blocks TNF-α-induced ICAM-1 expression through inhibition of NF-κB DNA binding. Both these actions are PPARγ-dependent [248]. TNF-α-induced VCAM-1 expression is also blocked by activation of the transmembrane PGI receptor, known as IP [249].

In the progression of atherosclerotic lesions, vascular SMCs migrate to the subendothelial space, where they proliferate and secrete extracellular matrix proteins. This process is partly mediated by endothelial production of PDGF, and 15d-PGJ_2 has been reported to inhibit PDGF synthesis in ECs, by decreasing the expression of the SP-1 transcription factor [250]. Of note, it has also been demonstrated that overexpression of COX-2 and of microsomal PGE synthase 1 play key roles in plaque instability, through enhanced MMP generation and activity [251]. In this connection, the prostaglandin PGE_2 might contribute to plaque destabilization, by inducing MMP-2 and MMP-9 activity in the macrophages present in the shoulder of atherosclerotic plaques [252,253]. In addition, PGE_2 could trigger neovascularization in the lesion, which enhances plaque growth and instability, by acting directly on ECs and inducing VEGF secretion through activation of ERK2 and JNK1 [254]. 15d-PGJ_2 might also have pro-angiogenic activity, by up-regulating VEGF expression with the involvement of PPARγ [255]. PGE_2 might also exert pro- or anti-inflammatory effects, depending on its receptor subtypes (EP), the cell type, and the activation context. For example, it has been shown that EP3, a receptor for PGE, mediates PGE_2-exacerbated atherothrombosis [256] while the EP4 receptor inhibits production of chemokines by macrophages [257]. However, it is also reported that the EP4 receptor is involved in PGE_2-dependent MMP overexpression in human atherosclerotic plaques [258].

Isoprostanes

Several lines of evidence suggest that isoprostane generation may reflect oxidative stress and inflammation in experimental and human atherosclerosis [230,259]. Moreover, isoprostanes are useful biomarkers of cardiovascular diseases [260]. Some F_2-isoprostanes, in particular 8-iso-$PGF_{2\alpha}$, have been demonstrated to have biological activities that may contribute to the progression of vascular damage [259].

Concerning the pro-inflammatory effects of F_2-isoprostanes, it has been observed that these arachidonic acid derivatives promote the formation of TXA_2, platelet activation, mitogenesis of vascular SMCs, proliferation of fibroblasts and ECs, and that they increase endothelin 1 expression in aortic ECs. All their biological activities involve activation of the TP receptor [261,262]. In particular, during promotion of platelet activation and adhesion, 8-iso-$PGF_{2\alpha}$, induced activation of tyrosine kinase and of p38 MAPK [263] whereas in SMCs F_2-isoprostanes activated PKC, as well as Rho/Rho kinase and tyrosine kinases. In addition, activation of ERK1/2 by 8-iso-$PGF_{2\alpha}$ has been implicated in SMC proliferation while activation of p38 MAPK by the same isoprostane has been shown in the pro-adhesive phenotype of ECs [264,265]. Of note, 8-iso-PGF_2 has been observed to induce monocyte adhesion to ECs, although it was unable to induce the expression of E-selectin or VCAM-1: it induced monocyte adhesion via TP receptor and PKA/MAPK pathway activation [265]. Moreover, the isoprostane 8-iso-PG2α may induce formation of foam cells, by inducing scavenger receptor A type 1 expression, and it may stimulate oxLDL to induce MMP-9 and TIMP-1 gene expression in THP-1 cells [266].

Leukotrienes

Leukotrienes (LTs) are generated from arachidonic acid by the 5-, 12-, and 15-LO pathways [232]. Several studies have suggested the existence of a potential link between the LT signaling cascade and the progression of atherosclerosis. LTs exert pro-inflammatory effects, by interacting with specific LT receptors expressed in inflammatoty cells within vascular wall [267].

Among LTs, LTB_4 produces increased leukocyte adhesion to the endothelium, followed by transendothelial migration. This inflammatory effect is mediated through the LTB_4-specific G-protein-coupled receptors, the BLT_1 and BLT_2 receptors [268]. In HUVECs, LTD_4 has been found to induce endothelial P-selectin expression through $CysLT_2$ receptor activation [269] and levels of various chemokines and of IL-8 were enhanced after stimulation of HUVECs with LTD_4 [270,271].

It has also been found that, within human atherosclerotic lesions, SMCs express receptors for LTB_4, mediating migration and proliferation of vascular SMCs [272]. Migration and proliferation of vascular SMCs appear to be modulated by β3-integrin-associated signaling pathway activation, including FAK phosphorylation, mobilization of the actin cytoskeleton, association of FAK to PI3K, ERK phosphorylation, and NF-κB activation [273]. Regarding the NF-κB transcriptional factor, in cultured monocytic cells it has been found that LTB4 phosphorylates MAPKs and stimulates NF-κB activity, through the BLT_1 and BLT_2 membrane receptors. Activation of the transcriptional factor contributes to NF-κB-dependent inflammatory responses [274].

It is also reported that 15-hydroxyeicosatetraenoic acid, generated by 15-lypoxygenase 2, plays a role in the regulation of vascular SMC migration and neointima formation, involving CREB-mediated IL-6 expression [275]. Another metabolite of 12- and 15-LO, 12-hydroxyeicosatetraenoic acid, can contribute to vascular SMC migration, by inducing MCP-1 and IL-6 expression, through Src tyrosine kinase signaling activation [276]. Further, 12-hydroxyeicosatetraenoic acid might activate PKC-α, which forms a complex with RhoA; this

complex is required for activation of NF-κB and the subsequent stimulation of ICAM expression, which events favor monocyte adhesion to the endothelium [277].

Furthermore, LTB_4 has been shown to increase the intima/media thickness and expression of MMP-2 and MMP-9 in human arteries [278]. Again, production of LTB_4 and signaling through the BLT_1 receptor have recently been shown to be essential for MMP-2 secretion in vascular SMCs treated with HNE, leading to atherosclerostic plaque instability [279]. In this connection, the colocalization of 5-LO/LT generation with MMP in human carotid atherosclerotic lesions is reported [280].

Conclusions

All of the principal events in the multistep process of atherosclerosis, from initial EC damage, to atheroma development and progression, until its possible rupture, are now clearly recognized, and some insight into the relevant molecular mechanisms and cellular interplay has been achieved.

LDL lipid oxidation products, such as oxysterols, oxidized phospholipids, free and core-aldehydes, as well as lyso-PC, have all been shown to accumulate in atherosclerotic lesions, pointing to these lipids as important factors, not only in initiating but also in promoting the inflammatory process underlying atherosclerosis. Especially in the last decade, significant progress has been made in elucidating the role of these lipid oxidation products in cell signaling and gene transcription, related to (i) activation of ECs and SMCs, (ii) foam cell formation, extracellular matrix deposition and arterial wall remodeling, and (iii) modulation of the chronic inflammatory reactions that occur during development of the atherosclerotic lesion.

Figure 1 schematically depicts the putative involvement of oxidized lipids in the various steps of atherosclerosis. Current knowledge of oxLDL-mediated cell signaling definitely points to oxPLs as the pivotal molecules among LDL lipid oxidation products promoting endothelial cell

dysfunction. Its derivative compound lyso-PC also appears to be implicated in this early step of atherogenesis. Because of their ability to up-regulate MMP, oxPLs might also contribute to the progression of atherosclerotic lesions, especially those becoming vulnerable and unstable. However, a major role in the inflammation-driven formation of atheroma appears likely to be ascribable to oxysterols and to certain aldehydic end-products of PUFA oxidation, in particular to HNE. Both types of compounds have been shown to be crucially involved in foam cell formation, through net overexpression of the CD36 scavenger receptor; they are also very probably involved in up-regulating macrophage-SMC cross-talk, mainly through overexpression of the TGFβ profibrogenic cytokine. In this connection, the demonstrated ability of the cholesteryl linoleate oxidation product 9-ONC to increase steady-state levels of both TGFβ and TGFβ receptors, in cells of the macrophage lineage, also appears interesting. Here, too, oxysterols, and less obviously free aldehydic products like HNE, are strong candidates for a key role in the process leading to the instability and rupture of the atherosclerotic plaque, because of their marked pro-apoptotic effect, and above all because of their demonstrated ability to up-regulate expression and activity of macrophage MMP-9, while constitutive levels of its specific inhibitors TIMP-1 and TIMP-2 remain unchanged.

Moreover, derivatives of arachidonic acid oxidation, besides affecting the initiation and progression of atherosclerotic lesions, play key roles in vascular tone modulation and vascular remodeling, mainly by regulating cell proliferation, platelet function and matrix deposition.

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Abbreviations: 5-OVC, 5-oxovaleroyl cholesterol; 7α-OH, 7α-hydroxycholesterol; 7β-OH, 7β-hydroxycholesterol; 7-K, 7-ketocholesterol; 9-ONC, 9-oxononanoyl cholesterol; 15d-PGJ_2, 15-deoxy-Δ12,14-prostaglandin J2; 20-OH, 20-hydroxycholesterol; 22-OH, 22-hydroxycholesterol; 24-OH, 24-hydroxycholesterol; 24S,25-EPOX, 24S,25-epoxycholesterol; 25-OH, 25-hydroxycholesterol; 27-OH, 27-hydroxycholesterol; α-EPOX, 5α,6α-epoxycholesterol;β-EPOX, 5β,6β-epoxycholesterol; AP-1, activator protein-1; ARE, antioxidant responsive element; ATF, activating transcription factor; bFGF, basic fibroblast growth factor; BLT, LTB receptor; COX, ciclooxygenase; CREB, cAMP response element-binding protein; ECs, endothelial cells; EGFR, epidermal growth factor receptor; EP, PGE receptor; ERK, extracellular signaling-regulated kinase; FAK, focal adhesion kinase; HDDE, 4-hydroxy-2,6-dodecadienal; HHE, 4-hydroxy-2-hexenal; HNE, 4-hydroxy-2-nonenal; HO-1, heme oxygenase-1; HUVECs, human umbilical vascular endothelial cells; ICAM, intercellular adhesion molecule; IκB, inhibitor of NF-κB; IL, interleukin; IP, PGI receptor; JAK, Janus kinase; JNK, c-Jun-N-terminal kinase; LDLs, low-density lipoprotein; LO, lipoxygenase; LPS, lipopolysaccharide; LT, leukotriene; LXR, liver X receptor; Lyso-PC, lyso-phosphatidylcholine; MAPKs, mitogen activated protein kinases; MCP-1, monocyte chemotactic protein-1; MIP-1β, macrophage inflammatory protein-1β; MMP, metalloproteinase; NF-κB, nuclear factor-κB; NOS, nitric oxide synthase; Nrf2, nuclear factor E2 related factor 2; ox LDLs, oxidized low-density lipoproteins; oxPLs, oxidized phospholipids; PAF, platelet activity factor; PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine; PAPS, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoserine; PDGFR, platelet-derived growth factor receptor; PG, prostaglandin; PGPC, 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphorylcholine; PI3K, phosphatidylinositol 3-kinase; PK, protein kinase; PLA_2, phospholipase A_2; POVPC, 1-palmitoyl-2-(5-oxovaleryl)-*sn*-glycero-phosphocholine; PPAR, peroxisome proliferator-activated receptor; PUFAs, polyunsaturated fatty acids; RAC, Ras-related C3 botulinum toxin substrate; ROS, reactive

oxygen species; SMCs, smooth muscle cells; SP-1, stimulatory protein-1; SREBP-1, sterol regulatory element-binding protein 1; STAT, signal transducer and activator of transcription; TGFβ1, transforming growth factor β1; TIMP, tissue inhibitor of metalloproteinase; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-α; TP, thrompoxane receptor; TRIOL, cholestan-3β,5α,6β-triol; Trx, thioredoxin; TX, thromboxane; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor.

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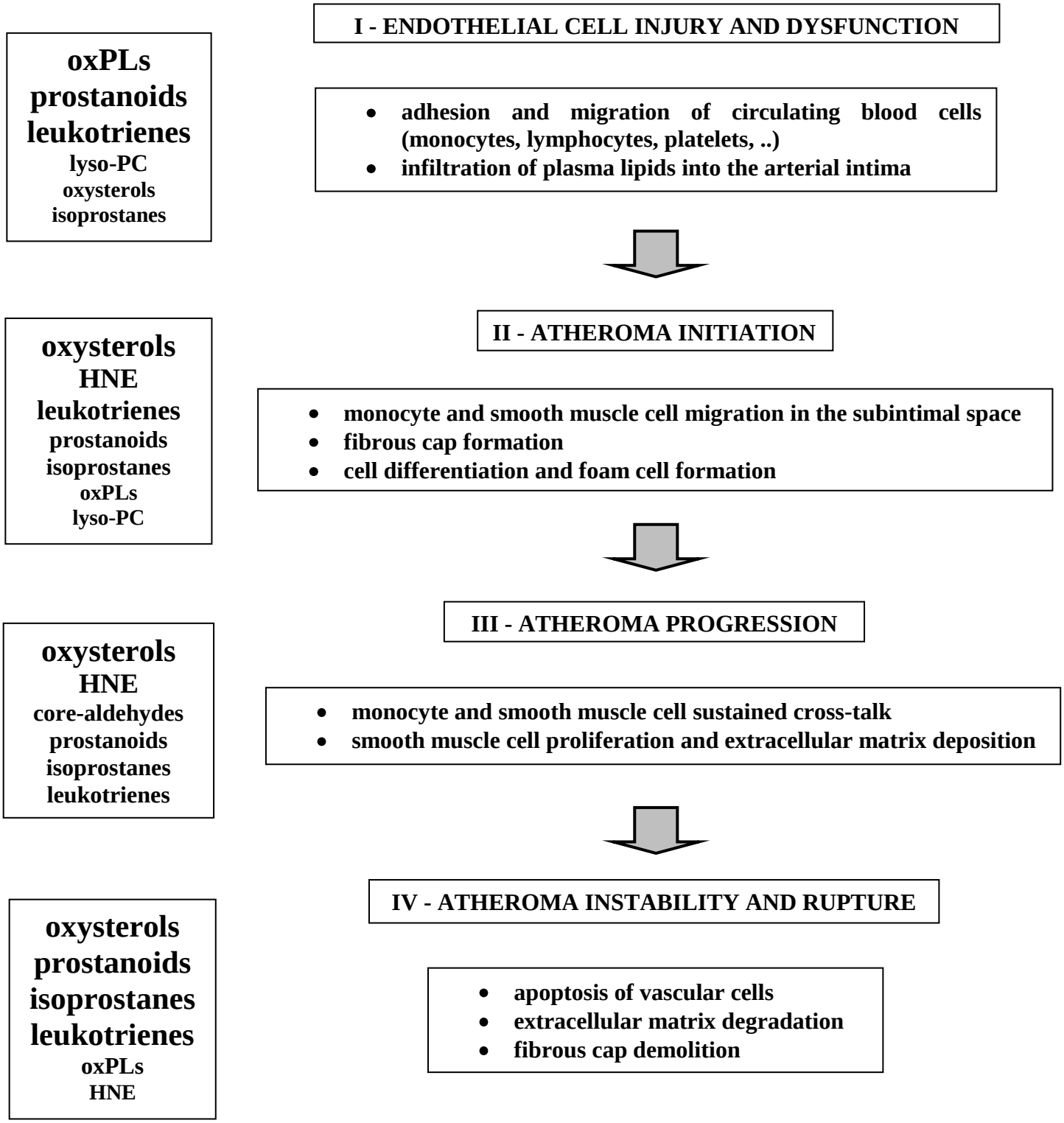
Figure caption

Figure 1. Inflammation-dependent progression of the atherosclerotic process: putative differential involvement of the various LDL lipid oxidation products and of arachidonic acid derivatives.

Abbreviations used: oxPLs, oxidized phospholipids; lyso-PC, lysophosphatidylcholine; HNE, 4-hydroxy-2-nonenal.


I - ENDOTHELIAL CELL INJURY AND DYSFUNCTION
oxPLs
prostanoids
leukotrienes
lyso-PC
oxysterols
isoprostanes
adhesion and migration of circulating blood cells (monocytes, lymphocytes, platelets, ..)
infiltration of plasma lipids into the arterial intima
II - ATHEROMA INITIATION
oxysterols
HNE
leukotrienes
prostanoids
isoprostanes
oxPLs
lyso-PC
monocyte and smooth muscle cell migration in the subintimal space
fibrous cap formation
cell differentiation and foam cell formation
III - ATHEROMA PROGRESSION
oxysterols
HNE
core-aldehydes
prostanoids
isoprostanes
leukotrienes
monocyte and smooth muscle cell sustained cross-talk
smooth muscle cell proliferation and extracellular matrix deposition
IV - ATHEROMA INSTABILITY AND RUPTURE
oxysterols
prostanoids
isoprostanes
leukotrienes
oxPLs
HNE
apoptosis of vascular cells
extracellular matrix degradation
fibrous cap demolition