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The role of oxysterols in vascular aging

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Abstract The aging endothelium progressively loses its remarkable and crucial ability to maintain homeostasis of the vasculature, as it acquires a proinflammatory phenotype. Cellular and structural changes gradually accumulate in the blood vessels, and markedly in artery walls. Most changes in aged arteries are comparable to those occurring during the atherogenic process, the latter being more marked: prooxidant and proinflammatory molecules, mainly deriving from or triggered by oxidized low density lipoproteins (oxLDLs), are undoubtedly a major driving force of this process. Oxysterols, quantitatively relevant components of oxLDLs, are likely candidate molecules in the pathogenesis of vascular aging, because of their marked prooxidant, proinflammatory and proapoptotic properties. An increasing bulk of experimental data point to the contribution of a variety of oxysterols of pathophysiological interest, also in the age-related genesis of endothelium dysfunction, intimal thickening due to lipid accumulation, and smooth muscle cell migration and arterial stiffness due to increasing collagen deposition and calcification. This review provides an updated analysis of the molecular mechanisms whereby oxysterols accumulating in the wall of aging blood vessels may “activate” endothelial and monocytic cells, through expression of an inflammatory phenotype, and “convince” smooth muscle cells to proliferate, migrate and, above all, to act as fibroblast-like cells.

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Keywords oxysterols, vascular aging, endothelial dysfunction, atherosclerosis

Abbreviations α-EPOX, 5α,6α-epoxide; β-EPOX, 5β,6β-epoxide; 25-OH, 25-hydroxycholesterol; 27-OH, 27-hydroxycholesterol; 7α-OH, 7α-hydroxycholesterol; 7β-OH, 7β-hydroxycholesterol; 7-K, 7-ketocholesterol; 24S-OH, 24S-hydroxycholesterol; AngII, angiotensin II; CVDs, cardiovascular diseases; ECs, endothelial cells; ECM, extracellular matrix; EDR, endothelium-dependent relaxation; EGFR, epidermal growth factor receptor; eNOS, endothelial nitric oxide
Introductory remarks

Aging is a gradual and irreversible process caused by environmental, genetic, and epigenetic factors and lifestyle conditions, which together cause progressive alterations in the homeostatic mechanisms of an organism. Although the improvement of lifestyle and hygienic conditions and the great advances in medicine have lengthened life expectancy, aging is considered to be an independent risk factor for age-related diseases in the developed countries.

The vascular endothelium plays a crucial role in maintaining the body’s health, adapting its functions and structures to different insults. In particular, the endothelium makes a major contribution to blood homeostasis, appropriate organ perfusion, immune response activation, and regulation of peripheral resistances (Busse & Fleming, 2006; Donato et al., 2015). The compensative mechanisms of the endothelium are progressively impaired during aging, causing peculiar changes in vascular cell morphology and vessel structure.

A growing bulk of evidence suggests that oxysterols, cholesterol oxidation products contained in oxidized low density lipoproteins (oxLDLs), significantly contribute to the vascular remodeling that occurs in atherosclerosis, since they are involved in various key steps of this age-related disease. Oxysterols are also involved in endothelial dysfunction: they favor platelet aggregation and impair arterial relaxation by reducing nitric oxide (NO) bioavailability; moreover, they promote migration and proliferation of vascular smooth muscle cells (VSMCs), causing intimal thickening, and collagen deposition and calcification with consequent arterial stiffness. Oxysterols might also play a crucial role in vascular aging thanks to their ability to induce inflammation, oxidative stress, and apoptosis (Fig.1).
Vascular aging

Aged blood vessels are characterized by arterial stiffness due to increased intimal-to-media thickness, reduced elasticity consequent on increased collagen accumulation and decreased elastin content, and vascular calcification (Wang & Bennett, 2012). Augmented proliferation and migration of medial VSMCs and increased numbers of inflammatory cells have been observed in the arteries of aged rats in vivo, together with an elevated ratio between matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) (Li et al., 1999; Monk & George, 2014). Moreover, collagen levels in the arteries of aged rodents are reported to be higher than in young animals, while the content of elastin remains unchanged, favoring arterial stiffness (Harvey et al. 2015). During aging, VSMCs shift their contractile phenotype into a fibroblast-like one, thus becoming able to secrete extracellular matrix (ECM) components, and to migrate and proliferate from the tunica media to the intima of the artery (Monk & George, 2014).

Endothelial dysfunction is one of the first signs of age-related alteration of the vasculature, and may appear well before the clinical manifestation of cardiovascular diseases (Donato et al., 2015). A healthy endothelium retains antithrombotic, vasodilatory, antiinflammatory, and antioxidant properties, which are tightly regulated to respond to the organism’s needs. During aging, these features gradually deteriorate, and the endothelium acquires a proinflammatory, proaggregating, and prooxidant phenotype. One hallmark of endothelium dysfunction is the progressively impaired vasodilatory response to blood flow and vasodilating compounds, that is altered endothelium-dependent relaxation (EDR) (Deanfield et al. 2007). The reduced vasodilatation is mainly due to a diminished bioavailability of NO, which is the most important vasodilating factor synthesized by endothelial NO synthase (eNOS) having modulatory effects on several autocrine and paracrine mediators (Michel & Vanhoutte, 2010). Moreover, NO exerts vasoprotective, cardioprotective, and antiatherogenic effects: it prevents vasospasm of the coronary arteries, inhibits the release of vasoconstrictor endothelin-1 (ET-1), and decreases endothelium permeability thus contrasting LDL infiltration; it also reduces VSMC proliferation and migration, as well as inflammation and endothelial cell (EC) apoptosis (Gradinaru et al. 2015).

Several studies support the implication of vascular oxidative stress in diminishing endothelium relaxation and NO production (Moncada et al. 1991; Cohen et al. 1997; Taddei et al. 2001; Puca et al. 2013). The bioavailability of NO is reduced by oxidative stress because of the reaction with superoxide anion (O$_2^-$) (Harrison, 1997; Hamilton et al. 2001) and because of a reduced eNOS activity (Gradinaru et al. 2015). Moreover, NO interaction with O$_2^-$ generates
peroxynitrite (ONOO$^-$), a highly reactive radical molecule able to damage proteins, lipids and DNA (Bosch-Marce et al. 2007).

Oxidative stress is known to be a major driving force in aging and in the development of cardiovascular diseases (CVDs). Reactive oxygen species (ROS) are produced by enzyme systems, such as NOX (reduced nicotinamide adenine dinucleotide phosphate oxidases: NADPH oxidases), xanthine oxidase, cytochrome P$_{450}$, as well as by mitochondria through the electron transfer chain. Several studies have demonstrated the crucial role of NOX enzymes in aging (Krause, 2007; Brandes et al. 2014), and stressed the association between up-regulation of NOX isoforms and endothelial dysfunction in CVDs (Wind et al. 2010; Montezano et al. 2011; Montezano & Touyz, 2012). Another relevant source of ROS is the mitochondrial dysfunction induced by biological aging, which leads to altered electron flow in the respiratory complexes and increases oxygen consumption (Kokoszka et al. 2001). A vicious circle thus comes about, since ROS production in turn causes mitochondrial DNA and respiratory chain damage. The increased cellular ROS steady-state due to altered NOX and mitochondria during aging is further supported by the quite frequent dysregulation of antioxidant defense, for example through decreased levels of reduced glutathione (GSH), decreased superoxide dismutase and nuclear factor erythroid 2 p45- related factor 2 (Nrf2) transcription factor activities (Puca et al. 2013).

It has been demonstrated that oxidative stress is one of the main triggers of cell apoptosis, via both mitochondria-dependent and -independent pathways (Sinha et al. 2013). Apoptosis, or programmed cell death, is a crucial mechanism necessary to regulate cell turnover and homeostasis; this process depends on the balance between proapoptotic and antiapoptotic genes. Inflammation, oxidative stress, genomic damage, and other stimuli impair this complex equilibrium, making different cell types more sensitive to apoptosis. Among these stimuli, aging is responsible for mitochondrial DNA damage (Szkłarczyk et al. 2014), impairment of human retinal vessels (Catita et al. 2015) and alterations of cardiac function (Quarles et al. 2015), reflecting an increase in the rate of cell apoptosis.

Cell loss by programmed death, and alteration of cell repair mechanisms, are amplified by cellular senescence, a key marker of vascular aging. Senescence is described as the loss of replicative potential, and occurs at the end of proliferative cell lifespan, or prematurely under the influence of stress factors, such as hypoxia, shearing, mechanical injury, and oxidative stress. Senescent ECs are detached from the basement membrane, with consequent enhanced permeability, infiltration of inflammatory molecules and cells, and thrombotic events (Lakatta, 2013). Moreover, premature VSMC senescence has been observed in old arterial wall, probably associated with an increase of NOX4 activity and angiotensin II (AngII) signaling (Yang et al. 2007). The AngII
pathway is implicated in arterial remodeling during the aging process: its age-related perturbation, as well as that of aldosterone/mineralcorticoid receptor and ET-1/ET-1 receptor A systems, represents a chronic proinflammatory stimulus for ECs and VMSCs. These pathways trigger and sustain inflammation by releasing several molecules, such as various MMPs, the chemokine monocyte chemoattractant protein 1 (MCP-1), transforming growth factor-β1 (TGF-β1), NOX and ROS (Wang et al. 2014). Chronic inflammation is certainly the pivotal force in the development of the above structural and functional changes occurring in the arterial wall during aging.

Of note, the molecular, cellular, and structural changes occurring in aged vessels, leading to endothelial dysfunction, vascular inflammation, remodeling, and increased arterial stiffness, are comparable to those occurring in the process of atherogenesis (Harvey et al. 2015). For this solid reason, aging is considered to be a non-modifiable and important risk factor, associated with the incidence and prevalence of CVDs; importantly, traditional risk factors of atherosclerosis, such as hypercholesterolemia, obesity, and smoking, promote the pathogenesis of atherosclerosis by accelerating the vascular aging process (Wang & Bennett, 2012).

**Atherosclerosis, oxLDLs, and oxysterols**

Atherosclerosis is a multifactorial, degenerative disease which affects large- and medium-sized arteries, and is characterized by chronic inflammation of the vascular wall. This inflammation entails continuous monocyte recruitment and migration from the blood into the subendothelial space; it is favored by the up-regulation of endothelial adhesion molecules, chemokines, cytokines, and growth factors. Once in the sub-intimal space, monocytes differentiate into macrophages and take up oxLDLs through the scavenger receptors CD36 and SR-A. Unlike LDL receptors, scavenger receptors are not regulated by a negative feedback loop, so that macrophages avidly accumulate oxidized lipids, becoming foam cells, and meanwhile release a large variety of proinflammatory cytokines. Perpetuation of this process promotes a chronic inflammatory state, and progression of the atherosclerotic lesion.

A causative role in atherosclerosis has now been established for oxLDLs; these micelles are involved in the initiation, formation, progression, and destabilization of the fibrotic plaque, as they induce inflammation, apoptosis, VSMC proliferation and migration, and endothelial activation (Lusis, 2000). It thus appears possible that oxLDLs might also significantly contribute to vascular aging. Although the implication of oxidized lipoproteins in atherogenesis and aging is undeniable, the different roles of the various components present in oxLDLs has not yet been fully elucidated.
Several reactive molecules derive from oxidation of the LDL lipid fraction, including peroxides, hydroxides, aldehydes, oxidized phospholipids and cholesterol oxidation products (Parthasarathy et al. 2010; Leonarduzzi et al. 2012). The latter class of compounds, quantitatively relevant in oxLDLs, has drawn the attention of various research groups; oxysterols, especially, are of interest as they are thought to be closely involved in the pathogenesis of atherosclerosis. Oxysterols are 27-atom carbon compounds that originate from cholesterol oxidation by both enzymatic and non-enzymatic mechanisms and present one or more carbonyl, keto, hydroxyl or epoxide groups in the sterol ring and/or in the side chain (Fig. 2). They are present in both free and esterified form. Several foods of animal origin contain oxysterols, as the consequence of food preparation, storage, and processing; the amount of oxysterols thus produced in foods can reach or even exceed 10% of total cholesterol (Lordan et al. 2009) (Table 1). In biological systems, oxidation of the cholesterol side chain is an enzymatic process, generally catalyzed by enzymes of the cytochrome P<sub>450</sub> family, while oxidation of the sterol ring occurs through non-enzymatic reactions, with the exception of the formation of 7α-hydroxycholesterol (7α-OH), which is produced by the enzyme cholesterol 7α-hydroxylase. From the quantitative stand point, 27-hydroxycholesterol (27-OH), 7-ketocholesterol (7-K), 5α,6α-epoxide (α-EPOX), 5β,6β-epoxide (β-EPOX) and cholesterol-3β,5α,6β-triol (Triol) are the most abundant oxysterols in the plasma and atherosclerotic lesions (Lordan et al. 2009; Poli et al. 2009). Of note, oxysterols are involved in many physiological processes, such as cholesterol metabolism, and the synthesis of steroid hormones and vitamin D, and are present in cholesterol-rich domains of membranes (lipid rafts), where they contribute to modulating membrane fluidity and permeability, flux of ions, and the activity of membrane-bound enzymes. Conversely, an excessive accumulation of these compounds in tissues and organs has been related to the progression of major chronic disease processes. Indeed, certain oxysterols exercise strong prooxidant, proinflammatory, and proapoptotic effects at pathological concentrations detectable in the lesions typical of atherosclerosis, neurodegenerative diseases, inflammatory bowel diseases, age-related macular degeneration, and other pathological conditions characterized by altered cholesterol uptake and/or metabolism (Poli et al. 2013). In particular, there is no longer any doubt that oxysterols of pathophysiological relevance play a pivotal role in contributing to the various steps of atheroma formation, from endothelial dysfunction to plaque fibrosis and rupture, through macrophage infiltration and VSMC differentiation (Table 2). In this connection, an excessive amount of oxysterols has been detected in human atherosclerotic plaques (Table 3). It has been observed that the oxysterols commonly found in plasma from hypercholesterolemic patients are recovered in atherosclerotic plaques, and a strong direct correlation between the total oxysterols and the total amount of cholesterol have been observed in the same plaques. This finding further points
to cholesterol oxidation as an important or even crucial event in vascular remodeling due to atherosclerosis. Conversely, it has been observed that unoxidized cholesterol does not exert a pro-atherosclerotic effect, since it is less polar and reactive than oxysterols (Leonarduzzi et al. 2007; Poli et al. 2009; Leonarduzzi et al. 2012; Zarrouk et al. 2014).

Further, among the aldehydic end-products of polyunsaturated fatty acid peroxidation 4-hydroxy-2-nonenal (HNE) may contribute to the pathophysiology of vascular aging by forming adducts on cellular proteins, leading to a progressive protein dysfunction (Leonarduzzi et al. 2012). HNE increases endothelial permeability (Usatyuk & Natarajan 2012), modifies cytoskeletal proteins, including actin and microtubules, by regulating cell-cell contacts and endothelial barrier function (Usatyuk et al. 2006). HNE may also stimulate the adhesion of macrophages to the vascular endothelium during atherosclerosis (Go et al. 2007). In addition, HNE covalently modifies LDLs by binding to lysine and histidine residues, leading to adduct formation: this favors LDL uptake by macrophages, thus resulting in macrophage activation and foam cell formation, a crucial event in atherosclerosis lesion development (Annangudi et al. 2008).

Aldehyde-adducts have been detected in human aorta, and their levels were increased in aged aortas, however, it is not known whether aldeydes accumulate on ECM proteins, contributing to arterial stiffness. Although in these aged aortas the structure of elastin fiber is strongly altered, elastin was shown to be very poorly modified by HNE-adducts (Zarkovic et al. 2015).

**Prooxidant effect of oxysterols**

Vascular ROS mainly derive from the activity of multi-subunit enzyme NOXs present in ECs and smooth muscle cells (SMCs), which catalyze the reduction of oxygen to O$_2^-$ using NAD(P)H as electron donor. These enzymes comprise five subunits (p47phox, p67phox, p22phox, p40phox and gp91phox) which assembly into the membrane upon complex activation by different stimuli. Different NOX isoenzymes, namely NOX1, NOX4, and NOX5, are constitutively present in the vasculature, while NOX2, typically expressed in cells of the macrophage lineage, may be involved when ECs show an inflammatory phenotype. All these enzymes release O$_2^-$ which acts as signaling molecule in many pathophysiological processes, such as cell growth, migration, fibrosis, apoptosis, and inflammation. Excessive activation of these enzymes, by AngII, proinflammatory cytokines and oxLDLs, occurs in age-related diseases, including diabetes, hypertension, and atherosclerosis.

Oxysterols appear to up-regulate the activity of these NOX isoenzymes, at least that of constitutive NOX1, NOX4 and induced NOX2. With regard to NOX1, a dietary representative
mixture of oxysterols has been reported to up-regulate this enzyme in a differentiated epithelial colonic cell line (CaCo2) leading to the overexpression and synthesis of various proinflammatory molecules, more efficiently than unoxidized cholesterol (Biasi et al. 2009; Mascia et al. 2010); in the same cell model, NOX1 up-regulation induced by 7α-OH, 7β-hydroxycholesterol (7β-OH) and α-EPOX was observed to trigger apoptosis (Biasi et al. 2013). The NOX4 content is increased in atherosclerotic plaques, and it has been demonstrated that 7-K promotes endoplasmic reticulum (ER) stress and apoptosis by up-regulating NOX4 in human aortic SMCs (Pedruzzi et al. 2004). ER stress occurs when stress signals, such as oxidative stress, cause the accumulation of misfolded or unfolded proteins in the organelle. Under normal conditions, the unfolded protein response is then activated to restore ER homeostasis; if this response is not sufficient to contrast the stress stimuli, ER triggers apoptosis (Chaudhari et al. 2014). As regards NOX2, in U937 promonocytic cells, an oxysterol mixture mimicking that present in atherosclerotic plaques induced the early generation of ROS, the effect being at least partly dependent on activation of this enzyme (Gargiulo et al. 2011).

More recently, in the same cell model 27-OH was shown to markedly up-regulate NOX2, but also induced the derangement of the mitochondrial membrane potential, thus amplifying ROS production (Vurusaner et al. 2014). In aged ECs, it appears reasonable to expect reduced mitochondrial function; oxysterols might then further interfere with these aged organelles, and either favor the onset of, or sustain, an oxidative imbalance of EC redox state.

**Oxysterols and inflammation**

Atherosclerosis, which resembles the accelerated aging of certain tracts of the arterial system, is characterized by chronic inflammation. This process begins as a protective mechanism, and then becomes detrimental when it is activated in an uncontrolled and continuous manner in the arterial wall. Inflammation is a crucial trigger of the initial stage of atherosclerosis, and supports its pathogenesis at all stages, including the rupture of unstable plaques. In the atherosclerotic process, oxLDLs are the typical trigger of the expression of adhesion molecules and chemokines on ECs, with consequent recruitment of circulating monocytes; the inflammation-dependent increase in the permeability of the endothelial layer allows inflammatory cells to transmigrate into the subendothelial space, where they differentiate into macrophages and start secreting chemokines and cytokines to attract other inflammatory cells and activate vascular cells, i.e. ECs and VSMCs. Indeed, inflammation stimulates the shift of VSMC from the contractile phenotype to the fibroblast-like phenotype, enabling the cells to proliferate and migrate from the tunica media into the intima,
thus determining a net and irreversible alteration of the structure of the arterial wall. All these events lead to endothelium dysfunction and arterial stiffness. Inflammation also participates in vascular remodeling due to aging, by progressively disrupting the ECM, both by stimulating fibrogenesis (the inflammatory cytokine TGF-β1 is strongly profibrogenic) and by markedly up-regulating MMPs. In this connection, oxysterols have been shown to induce expression and synthesis of TGF-β1 in U937 human promonocytic cells (Leonarduzzi et al. 2001) and in Raw264.7 murine macrophages (Ferré et al. 2009). Both of these studies used a biologically compatible mixture of oxysterols, in which 7-K was preponderant. Conversely, no effect on TGF-β1 expression and synthesis was found with unoxidized cholesterol in U937 cells (Leonarduzzi et al. 2001).

Irregular and uncontrolled ECM degradation would clearly contribute to the age-related disruption of vascular wall homeostasis. MMPs are the main enzymes responsible for this process, together with their specific inhibitors TIMPs; their expression may be modulated by inflammation. In this connection, oxysterols are among the active molecules that contribute to disrupting arterial ECM homeostasis: MMP-2 and -9 have been found to increase in VSMCs by 7-K and α-EPOX through activation of extracellular signal-regulated kinase (ERK) (Liao et al. 2010), a signaling kinase involved in nuclear factor-κB (NF-κB) activation, and thus in overexpression of inflammatory genes. Interestingly, 7-K can orientate human macrophages from peripheral blood monocytes towards a proinflammatory phenotype, by inducing many proinflammatory mediators, including MMP-9 (Buttari et al. 2013). In addition, one the most represented oxysterols in human blood, i.e. 27-OH, has been shown to markedly up-regulate MMP-9 expression and synthesis in human promonocytic U937 cells, by activating Toll-like receptor 4 (TLR4) (Gargiulo et al. 2015) and/or by inducing CD14, a coreceptor of TLR4 (Kim et al. 2015). As far as TIMPs are concerned, 27-OH is reported not to modulate their levels in promonocytic cells (Gargiulo et al. 2011).

Of great interest, arteries from oxysterol-fed ApoE−/− mice showed accelerated plaque destabilization and rupture, associated with increased monocyte infiltration, and MCP-1 and MMP activity. All these effects were prevented, in the same animal model, when oxysterol intestinal absorption was inhibited by administering ezetimibe; this points to a role of oxysterols in plaque vulnerability in high-risk patients (Sato et al. 2012). Moreover, MCP-1 as well as β1-integrin were found to be up-regulated in U937 by an oxysterol mixture mimicking that detectable in the plasma of hypercholesterolemic individuals (Leonarduzzi et al. 2005; Gargiulo et al. 2012). The same oxysterol mixture was also found to induce foam cell formation through up-regulation of scavenger receptor CD36 (Leonarduzzi et al. 2008, 2010). Unlike the oxysterol mixture, unoxidized cholesterol didn’t modulate MCP-1 and β1-integrin expression and synthesis as well as CD36 (Leonarduzzi et al. 2005, 2008, 2010; Gargiulo et al. 2012).
Quite a number of oxysterols appear able to trigger and sustain an inflammatory process within the vasculature, through overexpression of a variety of inflammatory cytokines, chemokines, and adhesion molecules. 7β-OH, and 7-K induce interleukin-1β (IL-1β) expression in human vascular ECs, as well as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule (VCAM-1) (Lemaire et al. 1998). Moreover, 27-OH was found to up-regulate IL-1β, IL-8, TNF-α, and MMP-9 in U937 cells (Gargiulo et al. 2015). In addition, 7β-OH, 7-K, and 25-hydroxycholesterol (25-OH) display potent induction of MCP-1, macrophage inflammatory protein-1β (MIP-1β), tumor necrosis factor-α (TNF-α), and IL-8 in human macrophagic cell lines (Prunet et al. 2006). IL-8 appears to be up-regulated by 7β-OH and 25-OH in THP-1 cells via the ERK/activator protein 1 (AP-1) pathway (Lemaire-Ewing et al. 2009) and by 27-OH in U937 cells via the TLR4/NF-κB pathway (Gargiulo et al. 2015). Again in connection with the oxysterol-stimulated expression of adhesion molecules, 25-OH has been shown to enhance VCAM-1 expression in human aortic ECs, as well as augmenting monocyte adhesion to ECs (Naito et al. 2005).

**Oxysterols and apoptosis**

It is known that cell death and survival are important events in arterial wall degeneration and atherosclerosis progression. Macrophage apoptosis and defective clearance of these cells contribute to the formation and enlargement of the lipid-rich necrotic core, a structural change that favors the further development of atherosclerotic lesions. In this connection, apoptosis of VSMCs eventually favors plaque instability, since the thickness of the atheroma’s fibrotic cap essentially depends on proliferation and activity of these cells: an increased number of apoptotic VSMCs has, indeed, been found in symptomatic plaques (Khatib & Vaya, 2014).

Apoptosis is genetically-programmed cell death that has been conserved throughout evolution, and which is used to maintain normal cell numbers in the organism, and during growth. It is a coordinated and active process characterized by specific morphological changes: chromatin condensation and nuclear fragmentation, membrane blebbing and apoptotic body formation.

Apoptosis is triggered both through an extrinsic or death-receptor-mediated pathway, and through an intrinsic or mitochondrial pathway. Both pathways appear to be activated by a variety of biologically-relevant oxysterols that exert strong proapoptotic effects. Some findings support the involvement of death receptors in oxysterol-induced apoptosis. 27-OH and 22-hydroxycholesterol induce TNF-α in macrophagic cells (Landis et al. 2002; Kim et al. 2013; Gargiulo et al. 2015) and
the up-regulation of TNF receptors has been observed in human aortic SMCs after challenge with 7-K (Lee et al. 2005).

With regard to the intrinsic pathway of apoptosis, a large number of reports corroborate its activation by oxysterols. To give some examples, 25-OH and 7-K have been shown to regulate mitochondrial proapoptotic Bax translocation and ROS release in VSMCs via activation of soluble adenylyl cyclase/protein kinase A (Appukuttan et al. 2013). Again in VSMCs, Triol was found to markedly impair mitochondrial membrane potential (Tang et al. 2005); 25-OH caused cytochrome c release and activation of caspase-3 and -9 in CHO-K1 (Yang & Sinensky, 2000) while 7β-OH activated caspase-3 and -9 in U937 cells (Ryan et al. 2004).

An important point in oxysterol-induced apoptosis is the effect of the compounds on cell cycle regulation: oxysterols can lead to p53 activation, a key protein involved in DNA surveillance, promotion of cell cycle arrest, repair, and eventually apoptosis. The role of p53 in cell death associated with atherosclerosis progression is confirmed by the finding that p53 expression is correlated with necrotic core formation in human carotid atherosclerotic plaques (Yuan et al. 2010). Challenging macrophagic cells (THP-1 and J774 cell lines) with either 7β-OH or 7-K has been observed to promote p53 phosphorylation, and its translocation into the nucleus, with consequent triggering of a series of cell reactions, namely lysosomal membrane permeabilization, cathepsin release, and eventually mitochondrial membrane damage (Li W et al. 2011). The activation of p53 by these two oxysterols is thought to be dependent upon an early induction of ROS and a transient up-regulation of early growth response protein, a proinflammatory protein also present in human atheroma (Miah et al. 2013). Besides up-regulation of p53 function, another common mechanism that interferes with cell cycle completion is the inhibition of certain cyclin-dependent kinases, with consequent accumulation of cells in the G1, S, and G2/M phases of the cycle. In this connection, treatment of THP-1 monocyte-like cells with 7β-OH or with 25-OH blocked them in the G2/M phase, through an oxysterol-dependent inhibition of cyclin B1 and its dependent kinase cdc2. The cells then underwent apoptosis via reduction of the antiapoptotic protein Bcl-2 and activation of caspase-9 and -3 (Lim et al. 2003).

Further evidence of primary interest with regard to proapoptotic effects of some oxysterols is that the ability to induce apoptosis appears consistently to be correlated with an increased intracellular ROS steady-state (Cheng et al. 2005; Sottero et al. 2009; Miah et al. 2013). The key role exerted by ROS in oxysterol-mediated apoptosis is indirectly validated by its inhibition upon cell pre-treatment with antioxidants. For instance, when promonocytic U937 cells were challenged with 7-K, it provoked oxidation of GSH, a versatile molecule that also controls transmembrane mitochondrial potential; the consequence was an increased rate of cell apoptosis. However, when
oxysterol-treated cells were supplemented with GSH or N-acetylcysteine, the number of apoptotic cells was drastically diminished (Lizard et al. 1998). Supplementation of U937 cells with α-tocopherol counteracted 7-K-provoked lysosomal degradation, caspase activation, and phospholipidosis (Vejux et al. 2009).

Moreover, some oxysterols, for example 7-K, 7β-OH, and 24S-hydroxycholesterol (24S-OH), are reported to trigger apoptosis and, at the same time, autophagy; activation of the autophagic process was demonstrated by the presence of cytoplasmic phagosomes, and by increased levels of the specific marker of autophagy, the microtubule-associated protein light chain 3 (LC3)-II derived from LC3-I cleavage (Nury et al. 2015).

Oxysterols thus appear able to induce a complex type of cellular death mechanism, which includes oxidative stress, apoptosis, and autophagy; this has very recently been termed “oxiapoptophagy”. Of interest, α-tocopherol and docosahexaenoic acid effectively contrasted the various aspects associated to oxiapoptophagy; this was observed in murine oligodendrocytes (Nury et al. 2015).

Oxysterols and endothelial dysfunction

Alteration of the endothelium is an early sign of vascular aging and atherosclerosis development, leading over time to altered endothelial relaxation and vascular stiffness. Quiescent ECs become senescent when exposed to vascular risk factors; for example, oxLDLs promote the senescence of human umbilical vein ECs (HUVECs), by decreasing sirtuin 1 and up-regulating both the ROS steady-state level and the inflammatory reaction (Tian & Li, 2014), likely through hyperactivation of NOX (Zarzuelo et al. 2013). On becoming senescent, ECs gradually lose their replicative potential, so that endothelium repair is markedly or even dramatically impaired. The latter event, together with intima stiffness, leads to the loss of endothelial integrity, which in the long run and in localized areas of medium and large arteries may also initiate the atherosclerotic process (Huynh et al. 2011).

Oxysterols may contribute to endothelial senescence by making ECs more susceptible to stress stimuli and to CVDs pathogenesis: oxysterols may alter the endothelium layer in at least two ways: i) in circulating oxLDLs, these compounds are localized on the surface of the micelles and may directly affect endothelial structure and function, thus favoring the deposition of LDLs and inflammatory cells in the subendothelial space; ii) once they have accumulated in the subintimal
spaces, and thus reached relatively high concentrations, oxysterols promote EC detachment from basement membrane, enhance inflammatory reactions, and favor thrombotic events.

In the initial stage of atherosclerosis, oxysterols are involved in the impairment of several endothelial functions, such as endothelial-eNOS-dependent NO generation, ROS production, and cell permeability. Among the various lipid oxidation products present in oxLDLs, 7-K, 7α-OH and 27-OH are reported to be the most effective at endothelial stiffening, suggesting these compounds play an important role in EDR and endothelium dysfunction. Of note, unoxidized cholesterol has no effect on endothelial stiffness. Interestingly, enriching the cells with cholesterol before oxysterol treatment protect ECs against the detrimental effect of oxysterols (Shentu et al. 2012). It has been hypothesized that oxysterols alter the endothelium by inserting themselves into the lipid bilayer and decreasing lipid packing of the ordered domains rich in cholesterol, as was experimentally observed for 7-K (Sleer et al. 2001; Shentu et al. 2010), and that, conversely, increasing the levels of membrane cholesterol may be protective (Shentu et al. 2012). The insertion of oxysterols into biomembranes might also induce endothelial dysfunction by affecting the activity of membrane-bound enzymes, such Na/K-ATPase, as was found to occur in cells treated with Triol and 7-K (Ramasamy et al. 1992; Duran et al. 2010). The different effects of different oxysterols on endothelial stiffness may be due to their differing ability to modify lipid membrane content and structure, depending on their chemical structure and properties. The incubation of aortic rings with 7-K, 7α-OH, and 7β-OH confirmed the ability of these oxysterols to affect EDR; of note, arterial relaxation was inhibited more markedly when oxysterols were used in a mixture, suggesting possible synergistic reactions in vivo. This effect on rabbit aortic rings was due to diminished NO availability following direct interaction of oxysterols with NO, and not to eNOS inhibition (Wong et al. 2011). Conversely, eNOS inhibition was observed in histamine-stimulated HUVECs, in which 7-K or 7β-OH significantly decreased NO release, while cholesterol, β-EPOX, 19-hydroxycholesterol did not (Deckert et al. 1998). It is known that a decline in NO bioavailability may also be due to NO accelerated degradation by ROS (Harrison, 1997). Since oxysterols are well-known NOX activators and ROS inducers, they may increase the production of O2•−, which rapidly reacts with NO, thus decreasing its bioavailability (Bosch-Marce et al. 2007).

Again with regard to EDR, pretreatment of rabbit aortic segments with native LDLs did not affect endothelial relaxation, while oxLDLs significantly did so; remarkably, among the major components derived from LDL oxidation, e.g. lysophosphatidylcholine and the lipoperoxides, oxysterols most strongly impaired EDR (Deckert et al. 1997; Wong et al. 2011). These results strengthen the case for the oxidation of cholesterol being a crucial reaction in rendering LDLs proatherogenic molecules, and also point to cholesterol oxidation derivatives as key actors in
endothelium dysfunction related to hypercholesterolemia. Another mechanism whereby oxysterols, such as 25-OH, 7-K, α-EPOX and Triol, could alter endothelial integrity is by inhibiting prostaglandin (PG) I₂ synthesis and release by HUVECs, in consequence enhancing platelet adhesion to ECs. Unoxidized cholesterol, conversely, had no effect on PGI₂ production or platelet adhesion (Peng et al. 1993).

A recently characterized oxysterol, 5,6-secocholesterol, stemming from a non-free-radical-mediated autoxidation process, was demonstrated to affect endothelial integrity by inducing p53-dependent apoptosis and by strongly inhibiting EDR in in vivo and in vitro models (Luchetti et al. 2015). Further, the ability of 7-oxysterols, which derive from cholesterol oxidation at C₇, including 7β-OH and 7-K, to induce endothelial dysfunction was suggested by research showing that these oxysterols induced EC apoptosis by accumulating in lysosomes at an early stage, followed by lysosomal activation, oxidative stress, and mitochondrial pathway of programmed death. In particular, 7β-OH and 7-K induced cell release of lysosomal cathepsin and von Willebrand factor, thus further contributing to endothelial damage. Again, cell treatment with cholesterol did not show any toxic effect (Li et al. 2011).

Among the various factors involved in the regulation of endothelial function is AngII, which stems from the renin-angiotensin system (RAS). This peptide has a vasoconstrictor effect, and stimulates the release of aldosterone from the adrenal glands, regulating reabsorption of water and sodium by kidneys. This system is thus crucial to the regulation of vascular tone and systemic blood-pressure levels, and its dysregulation is associated to vascular damage in hypertension and atherosclerosis (Schiffrin & Touyz, 2004). The classical source of renin are the kidneys, but it has recently been reported that angiotensin peptides may be formed in the brain, adrenal glands, reproductive tissue, pituitary glands, gastrointestinal tract, hematopoietic tissues, heart and blood vessels (Ferrario et al. 2014). The effects of AngII on vascular cells is mediated by binding to its G-protein coupled receptor AT₁R and receptor AT₂R, and by the activation of several signaling pathways. AngII may contribute to cell proliferation, apoptosis, and inflammation by activating the mitogen-activated protein kinases ERK, p38 and c-Jun N-terminal kinases (JNK), Janus kinase/signal transducer and activator of transcription (JAK/STAT); further, AngII may promote arterial remodeling by activating tyrosine kinase receptors, such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor, and insulin-like 1 growth factor receptor (Montezano et al. 2014).

Increasing evidence supports the synergistic effect of hyperlipidemia and RAS in endothelial dysfunction during atherogenesis. Indeed, oxLDLs can induce expression of angiotensin converting enzyme and AT₁R in primary human HUVECs (Catar et al. 2007); this interaction was confirmed in
ApoE<sup>-/-</sup> mice fed a high-cholesterol diet, in which the combined administration of the statin rosvustatin and the AT<sub>1</sub>R inhibitor candesartan significantly counterbalanced the up-regulation of lectin-like oxLDL receptor-I and p38 activation, as provoked by the dietary regimen adopted (Chen <i>et al.</i> 2006). Again, the combination of pravastatin and irbesartan in patients with stable coronary artery disease improved endothelial function (Morawietz <i>et al.</i> 2006). Blocking of AT<sub>1</sub>R abolished oxLDLs-induced EDR alteration in murine aortic vascular ring (Yamamoto <i>et al.</i> 2015) and foam cell formation in human macrophages (Osada-Oka <i>et al.</i> 2012).

The role of oxysterols as important mediators of the oxLDL effect on RAS has been little investigated to date. It has been demonstrated that 27-OH and 24S-OH up-regulate RAS, in particular angiotensin converting enzyme activity, and angiotensinogen levels, in mouse brain and in primary neurons and astrocytes (Mateos <i>et al.</i> 2011). Since AngII activates signaling pathways similar to that promoted by oxysterols, and since this peptide is involved in key events in atherosclerosis, it might be interesting to investigate the relationship between these molecules and their likely synergistic interaction in greater depth.

**Oxysterols and VSMC phenotypic changes**

VSMCs are crucial to plaque formation, progression, and stability, and the changes observed in these cells during aging reflect that occurring during atherogenesis. As described above, VSMCs migrate and proliferate, causing intimal thickening, and they contribute to arterial stiffness by increasing collagen deposition and calcification. These cells undergo a phenotypic change and start synthesizing ECM components (a process called SMC activation); in addition, SMCs migrate into the intimal layer of the arterial wall and they determine the fibrous cap formation.

With regard to the ability of compounds in the oxysterol family to activate VSMCs, it has been shown that low doses of 7-K or of α-EPOX (2.5 μM) induce proliferation and migration of rat aortic SMCs, by activating the EGFR/phosphoinositide 3-kinase (PI3K)/Akt pathway and up-regulating MMP-2 and MMP-9 activities; cell treatment with the same concentration of cholesterol had no significant effect on SMC proliferation (Liao <i>et al.</i> 2010). The activation of survival pathways by oxLDLs has been confirmed in a study that demonstrated that induced MMP-2 via PI3K/Akt pathway in primary cultures of rat aortic VSMCs (Li HX <i>et al.</i> 2012). Conversely, 7-K and α-EPOX at high concentration (>25 μM) markedly decreased the number of viable cells by activating apoptotic death (Liao <i>et al.</i> 2010); this dual effect of the two oxysterols fits quite well with the view that they may first initiate impairment of arterial wall structures by favoring
activation of VSMCs, and subsequently, following excessive accumulation, they could contribute to a dramatic derangement of the entire vascular wall, the latter stage being especially evident in the vascular areas affected by the atherosclerotic process. It has been hypothesized that oxysterols might activate VSMCs by a different mechanism: 25-OH induced severe morphological changes in SMCs and ECs of rabbit pulmonary artery by up-regulating PGG/H synthase-2 activity thus enhancing eicosanoid production, mainly PGE\textsubscript{2} (Wohlfeil & Campbell, 1999); PGE\textsubscript{2} increases endothelial permeability VSMC proliferation and migration, and vascular tone (Gomez et al. 2013). The enzyme PGG/H synthase-2 is expressed after vascular cellular activation, often in response to inflammatory stimuli; thus, 25-OH could induce it directly or by overexpressing proinflammatory cytokines.

**The contribution of oxysterols to vascular calcification**

Vascular calcification is a dynamic process similar to osteogenesis, which is often associated with arterial stiffness and atherogenic degeneration. It principally depends on the VSMCs’ acquiring an osteoblastic phenotype, whereupon they begin producing and secreting hydroxyapatite crystals. This specific cell shift may, for instance, be promoted by oxidative stress, in turn induced by high phosphatemia, advanced glycation end-products, bone morphogenetic proteins, and inflammatory cytokines (McCarty & DiNicolantonio, 2014).

Several oxysterols of pathophysiologic relevance have been shown to exert prooxidant effects, and also to stimulate phenotypic changes in VSMCs (see above). This makes them good candidate molecules for determining accumulation of calcium in the tunica media of arterial walls. In addition, the proapoptotic effect that they exert on vascular cells would provide suitable nucleating sites for the formation of hydroxyapatite crystals (Proudfoot et al. 2000).

An increasing bulk of data now points to the property of certain oxysterols of exerting potent osteogenic activity. The first evidence of this emerged from a study in which rat bone marrow stromal cells were treated with a 1:1 combination of 22(S)-hydroxycholesterol and 20(S)-hydroxycholesterol (Kha et al. 2004). The same oxysterol mixture was confirmed to induce osteogenic differentiation in a murine embryonic stem cell line, in which it enhanced mitochondrial activity and triggered the Hedgehog and Wnt/\(\beta\)-catenin signaling pathways (Kwon et al. 2015). Again, an oxysterol mixture enhanced calcium deposition and osteogenic protein expression, in particular alkaline phosphatase, osteopontin, and osteocalcin, in rat mesenchymal stem cells (Wöltje et al. 2015). Similar effects were exerted by 25-OH, and 7-K on bovine VSMCs (Watson et al. 2015).
1994; Saito et al. 2008) and Triol on rat VSMCs (Liu et al. 2004). At least with regard to Triol, experimental data point to the oxysterol’s prooxidant effect as being responsible for cell calcification, given that incubation with antioxidants (vitamin C + vitamin E) reversed that action (Liu et al. 2004); this explanation is also compelling for oxLDLs, since they are able to induce ROS and calcification by promoting inflammation, apoptosis, and the release of osteogenic factors (Farrokhi et al. 2015). A recent study provides indirect support to the hypothesis that oxysterols make a significant contribution to the abnormal calcium deposition in arterial wall; human coronary artery SMCs were challenged with oxLDLs, and induced net osteopontin synthesis and, through this protein, marked proliferation and migration of the cells; both these effects are primary events in arterial stiffness (Liu et al. 2014).

Conclusions

The accumulating experimental evidence in favor of oxysterols as candidate molecules involved in vascular aging mechanisms appears convincing, for a number of reasons. First, the biochemical effects exerted by a large variety of these oxysterols, in particular their proinflammatory properties, closely fit the type of events occurring in aging blood vessels. Second, oxysterols have a remarkable ability to induce vascular cells to modify their phenotypic features, and thus their function. Third, the relatively high concentrations that these compounds can reach in the vasculature, transported into the sub-intimal space by LDLs, is noteworthy; it also probably limits oxysterols’ contribution to vascular aging mainly to the case of the arteries and, specifically, to medium and large arteries, in which lipids do actually accumulate. However, a generalized effect on the vascular endothelium of oxysterols contained in circulating oxLDLs, favored by the fact of their being localized on the surface of these micelles, should not be excluded a priori (Fig. 3).

Acknowledgements

The authors thank the University of Torino for having enabled them to investigate in depth over the years the potential role of oxysterols in human pathophysiology.
References


Figure legends

Figure 1. Involvement of oxysterols in endothelial dysfunction
LDLs pass through the activated endothelium, where they are oxidized in the subendothelial space, leading to oxysterol formation. These compounds whereby induce and sustain inflammatory processes in the vascular wall, by upregulating adhesion molecules on the ECs surface and by augmenting the release of MCP-1, consequently amplifying recruitment of monocytes from the blood stream. In the intima layer, oxysterols promote monocyte differentiation into lipid-laden macrophages, called foam cells, which are the first sign of atherogenesis. Oxysterols alter endothelial permeability by inserting into EC membranes and modulating their fluidity and structure; further, they contribute to endothelial dysfunction by favoring platelet aggregation. In this connection, these oxysterols impair arterial relaxation by reducing NO bioavailability and promoting VSMC calcification, which leads to arterial stiffness. Finally, oxysterols are triggers of oxidative stress, one of the major processes implicated in the development of age-related disease.
ECs, endothelial cells; NO, nitric oxide; oxLDLs, oxidized low-density lipoproteins; VSMCs, vascular smooth muscle cells.

Figure 2. Chemical structures of the main oxysterols

Figure 3. Oxysterol-driven anticipation and/or amplification of main features of aging blood vessels

Table 1. Origin of the most relevant oxysterols

<table>
<thead>
<tr>
<th>ENDOGENOUS ORIGIN</th>
<th>NON-ENZYMATIC FORMATION</th>
<th>EXOGENOUS ORIGIN</th>
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<td>Cholesterol-3β,5α,6β-triol (Triol)</td>
<td>Cholesterol-3β,5α,6β-triol (Triol)</td>
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Table 2. Effects of oxysterols on atherogenesis and vascular aging

<table>
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<th>Effect</th>
<th>Experimental model</th>
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<td>CaCo-2</td>
<td>Biasi et al. 2009, 2013; Mascia et al. 2010</td>
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<td>CaCo-2</td>
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<td>MMP-9, IL-1β, IL-8, TNF-α↑</td>
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**Endothelial dysfunction/Cell phenotype changes**

**Vascular calcification**

**Table 3. Summary of recent studies quantifying cholesterol and oxysterols in human normal vessels and atherosclerotic plaques**

*(because of the style this table has been sent as separate file)*
Fig. 1
Fig. 2
Fig. 3

Oxysterols + Aging

Oxidative stress
Inflammation
Cell death/proliferation
Cellular senescence
Calcification

Endothelial dysfunction and arterial stiffness

Anticipated and enhanced vascular aging

Stimulation of atherosclerosis