Review article

Endothelial cells, endoplasmic reticulum stress and oxysterols

F. Luchetti\textsuperscript{a,}\textsuperscript{*}, R. Crinelli\textsuperscript{b}, E. Cesari\textsuperscript{a}, B. Canonico\textsuperscript{a}, L. Guidi\textsuperscript{a}, C. Zerbinati\textsuperscript{b}, G. Di Sario\textsuperscript{a}, L. Zamai\textsuperscript{a}, M. Magnani\textsuperscript{a}, S. Papa\textsuperscript{a}, L. Iuliano\textsuperscript{b}

\textsuperscript{a} Departments of Biomolecular Sciences, University of Urbino Carlo Bo, Urbino, Italy
\textsuperscript{b} Department of Medico-Surgical Sciences and Biotechnologies Vascular Biology, Atherothrombosis & Mass Spectrometry, Sapienza University of Rome, Latina, Italy

A R T I C L E  I N F O

Keywords:
Endothelial cell
Oxysterols
Endoplasmic reticulum stress
Unfolded protein response
Autophagy

A B S T R A C T

Oxysterols are bioactive lipids that act as regulators of lipid metabolism, inflammation, cell viability and are involved in several diseases, including atherosclerosis. Mounting evidence linked the atherosclerosis to endoplasmic reticulum dysfunction; in fact, the endothelium regulates the vascular system with roles in processes such as hemostasis, cell cholesterol, hormone trafficking, signal transduction and inflammation. Several papers shed light the ability of oxysterols to induce apoptosis in different cell lines including endothelial cells. Apoptotic endothelial cell and endothelial denudation may constitute a critical step in the transition to plaque erosion and vessel thrombosis, so preventing the endothelial damaged has garnered considerable attention as a novel means of treating atherosclerosis. Endoplasmic reticulum (ER) is the site where the proteins are synthesized and folded and is necessary for most cellular activity; perturbations of ER homeostasis leads to a condition known as endoplasmic reticulum stress. This condition evokes the unfolded protein response (UPR) an adaptive pathway that aims to restore ER homeostasis. Mounting evidence suggests that chronic activation of UPR leads to cell dysfunction and death and recently has been implicated in pathogenesis of endothelial dysfunction. Autophagy is an essential catabolic mechanism that delivers misfolded proteins and damaged organelles to the lysosome for degradation, maintaining basal levels of autophagic activity it is critical for cell survival. Several evidence suggests that persistent ER stress often results in stimulation of autophagic activities, likely as a compensatory mechanism to relieve ER stress and consequently cell death. In this review, we summarize evidence for the effect of oxysterols on endothelial cells, especially focusing on oxysterols-mediated induction of endoplasmic reticulum stress.

1. Introduction

The endothelial cell (EC) lining of vessels walls is a critical regulatory component of cardiovascular homeostasis that can be seen as an organ supporting important functions. On one hand, it influences vascular tone and inhibits coagulation and platelet activation allowing the blood to flow in the vessel conduits. Among others, endothelial cells produce nitric oxide and prostacyclin that modulate function of cells both in the intravascular compartment, i.e platelets, and in the vessel wall, i.e smooth muscle cells. On the other hand, the endothelial lining is the gate to the interstitial space for nutrients and cells of the immune system. Alteration in the EC barrier function has important implications in precipitating the loss of vascular homeostasis. The term endothelial dysfunction is used to refer to diverse changes in endothelial cell metabolism that are a prerequisite to triggering the mechanisms of atherosclerosis, which is the underlying cause of the myocardial infarction, stroke, unstable angina, and sudden cardiac death \cite{1}. EC dysfunction occurs in conditions of high blood cholesterol and glucose levels, in insulin resistance, and in conditions of up-regulated oxidative stress.

It is well established that atherosclerotic lesions develop in a non-random fashion typically around areas where blood vessels branch or curve. Physical computational models have determined that these susceptible regions have low time-average shear stress, a high oscillatory shear index, and a steep temporal and spatial gradient in shear stress. In contrast, unbranched arteries that are exposed to uniform laminar shear stress largely do not develop lesions \cite{2}.

Fluid dynamics in atherosclerosis-prone areas translates into impaired barrier function and higher rates of turnover and senescence compared with cells present in atherosclerosis-resistant regions.

Among the potential drivers of EC dysfunction, recent evidence highlighted the role of endoplasmic reticulum stress (ERS). Atherosclerotic plaques express markers of chronic ERS \cite{3} that...
precipitate apoptosis and eventually promote athero-thrombosis mechanisms. It is plausible that ERS may promote atherosclerosis by initially causing cell dysfunction and later inducing endothelial apoptosis [4].

The early detectable changes of atherosclerosis focus on endothelial dysfunction that favors the transfer and modification of circulating lipoproteins into the sub-endothelial space. In particular, free radicals and the oxidative modification of low-density lipoprotein (LDL) are mediators for inflammatory cells recruitment and foam cell formation. Oxidized-LDLs (Ox-LDL) induce different biological effects in cultured vascular cells depending on several factors, including the nature of the oxidized lipids [5].

LDLs are the main carriers of cholesterol in the circulation and are very prone to oxidative insult. Oxidation of the lipid components transported by LDL includes cholesterol that is transformed into a number of oxysterols, biologically active products that are emerging candidates in several disease settings [6].

Given the central role of endoplasmic reticulum (ER) pathway in cellular metabolism and function, and the bioactivity of oxysterols in the context of atherosclerosis, this review focuses on the potential links between of oxysterols on the ER pathway.

2. Endoplasmic reticulum physiology

The ER plays a central role in lipid and protein synthesis, and governs several signaling pathways by controlling Ca^{2+} movements. ER is composed by a series of continuous membranes organized into sub-domains that include the rough-, smooth- and transitional-ER, and the nuclear envelope. The rough ER, which is mainly lamellar, is associated with polyribosomes for protein synthesis and Ca^{2+} signaling. The smooth ER is primarily composed of tubular structures providing the site of lipid biosynthesis, has a main role in Ca^{2+} signaling, and is referred as the chief point of contact with other organelles [7].

The ER is the main cellular biosynthesis compartment of a variety of lipids - including phospholipids, cholesterol and ceramides – subsequently transported to others organelles and cellular membranes via vesicles of the secretory pathway [8]. The ER is involved in monitoring lipid membrane composition, helping to activate appropriate responses to preserve lipid homeostasis. Newly synthesized proteins are transferred into the ER to sustain specific modification, including folding, glycosylation and disulphide bond formation [9]. The ER quality control system prevents protein aggregation by promoting correct folding or selective degradation of the improperly folded polypeptide [10]. The varied ER functions are tightly connected. For example, an accumulation of misfolded proteins can alter Ca^{2+} homeostasis and, vice versa, a change in the luminal content of Ca^{2+} has a major effect on the process of protein synthesis [11]. Up to 30% of all proteins in the eukaryotic cells are targeted to the secretory pathway. Proteins destined for secretion are translocated across or inserted into the ER membrane, whereupon they fold and assemble to their native state before being transported to the Golgi apparatus. Proteins that fail to fold correctly are translocated back across the ER membrane to the cytosol where they become substrates for a cytosolic degradation machinery, the proteasome, in a process known as ER-associated degradation (ERAD) [12,13].

The ER is capable of both signal reception and signal transmission. The input signals include Ca^{2+}, inositol 1,4,5-trisphosphate, sphingosine-1-phosphate, reactive oxygen species (ROS), and steroids. In response to these input signals, the ER generates a variety of output signals such as Ca^{2+} transients, activators of store-operated channels, stress signals, arachidonic acid metabolites, and various transcription factors (NF-kB, CHOP, ATF6, and SREBP1s). Structural differences in the organelle morphology correlate with differences in ER function [7]. As mentioned above the ER serves as a nexus for the folding and maturation of proteins that transit to secretory pathways. Protein folding is both regulated and sensed by ER resident chaperones, such as GRP78/BiP and GRP94 [14–17]. GRP78 was first discovery as a 78,000 Da protein whose synthesis was enhanced in cultured cells grown in medium deprived of glucose. Subsequently, GRP78 was determined to be an ER resident protein whose synthesis could be stimulated by a variety of environmental and physiological stress conditions able to perturb ER function and homeostasis. GRP78, commonly referred to as BiP, the immunoglobulin heavy chain-binding protein, is a well established marker of ER stress (ERS) [18]. GRP94, like GRP78, participates in protein folding, interacts with other components of the ER protein folding machinery, governs calcium storage, and assists the targeting of misfolded proteins to ERAD [19,20]. Compared to GRP78, the client proteins of GRP94 are more selective and have critical roles in immunity, growth signaling, and cell adhesion.

3. ER stress

In the last decade, several studies showed that lipid oxidation products, including oxysterols, contribute to trigger ERS and eventually play a key role in the progression of atherosclerosis [4,21,22]. In particular, activation of different ERS markers has been demonstrated to occur in endothelial cells exposed to oxysterols and ox-LDL. Proteins are particularly vulnerable to oxidative stress. It has been estimated that under oxidative stress 69% of proteins are oxidized compared to 31% that accounts for lipids and DNA [23]. Free radicals-derived protein modification can result in either gain- or loss-of-function due to the protein misfolding or unfolding [24–26]. Given that proteins are the machinery that virtually performs all major cellular functions, it is not surprising that cells invest a significant energetic effort to retain a fully functional proteome. Cells placed under proteotoxic stress activate specific and integrated cellular pathways involved in maintaining the integrity of the proteome. This is referred to as the proteostasis network that is composed of several protein quality control machineries, which under conditions of proteotoxic stress aims at rescuing or degrading unfolded, misfolded or non-native polypeptides. The first function is usually accomplished by molecular chaperones, while the ubiquitin proteolytic system is mainly responsible for protein degradation.

Protein misfolding in the ER is sensed by the unfolded protein response (UPR), a signaling network able to re-establish homeostasis of the protein folding function. UPR induces the synthesis of folding catalysis to increase folding activity and attenuates the global translation to reduce folding load. On the other hand, ERAD eliminates misfolded proteins from the ER. Removal of abnormal protein products by ERAD occurs after their selective retro-translocation into the cytosol where they are degraded by the ubiquitin proteasome system (UPS) [12,13].

The UPS, which is highly coordinated with autophagy to maintain cellular homeostasis [27], specifically targets proteins for proteasomal degradation by adding the small polypeptide ubiquitin (Ub) at various lysine residues. The UPS pathway ensures high levels of specificity in labeling proteins for degradation through three classes of enzymes - E1 Ub-activating, E2 Ub-conjugating, and E3 Ub-ligase. ER substrates are ubiquitinated on the cytosolic side of the ER by ER-transmembrane E3 Ub-ligases. In the context of ERAD, Ub tagging serves for proteasome recognition, and as a signal for the retro-translocation and regulation of the ERAD machinery components. In addition, ubiquitinated proteins can be selectively degraded by autophagy. Suppression of the UPS pathway by siRNA is offset by an increase in autophagy. However, inhibition of autophagy results in inhibited degradation of UPS substrate.

4. UPR in the regulation of ER stress

In higher eukaryotes ER is constantly monitored by at least three ER trans-membrane sensors, each initiating a set of distinct but intersecting signaling pathways oriented in maintaining ER homeostasis. The trans-membrane sensors acting as transducers of ER stress signaling include the serine/threonine kinase and endoribonuclease IRE1; PERK serine/threonine kinase (also referred as PEB); and the basic leucine-zipper transcription factor ATF6. The first pathway is the IRE1 pathway, which
regulates the transcriptional induction of genes encoding ERAD components. IRE1 is a type I trans-membrane protein residing on the ER membrane [28-31], whose cytosolic portion contains kinase and RNAse domains [32-34]. IRE1 is an inactive monomer in normal growth conditions, but becomes an active oligomer and forms clusters on the ER membrane in response to ER stress [35,36]. IRE1 oligomers autophosphorylate each other to activate the RNase domain. IRE1 cleaves the pre-mRNA of XBP1 at two levels, and an unidentified RNA-ligase binds the two exons of the XBP1 mRNA, resulting in splicing of XBP1 mRNA and the excision of a small intron [37-39]. Because the length of the intron is 26 nt, splicing of XBP1 mRNA by IRE1 causes a frame shift. Thus, the pre-mRNA and mature mRNA of XBP1 encode different proteins, pXB1P1(U) and pXB1P1(S), respectively. pXB1P1(S) is an active transcription factor and contains both the DNA-binding domain and the transcriptional activation domain. pXB1P1(S) forms a heterodimer with pATF6(N) and binds to the enhancer element called the unfolded protein response element, resulting in the transcriptional activation of ERAD genes such as HRD1, EDEM and Derlin [40,41]. pXB1P1(S) is a very unstable protein that is degraded by the proteasome, unless bound to UBC9 [42]. The second pathway is based on PERK, a sensor molecule residing on the ER membrane [43,44]. The molecular structure of PERK is similar to that of IRE1, but the cytosolic domain of the PERK contains only the kinase domain. In the absence of ER stress, PERK is an inactive monomer, whereas PERK becomes an active oligomer upon ER stress, like IRE1. Activated PERK phosphorylates the α subunit of eukaryotic transcriptional initiation factor (eIF2α), resulting in the inactivation of eIF2α and translational attenuation, which prevents further accumulation of unfolded proteins in the ER. ATF6, consisting of the closely related ATF6α and ATF6β in mammals, is constitutively synthesized as a type II trans-membrane protein in the ER, designed as pATF6 α/β. Upon ERS, pATF6 α/β relocates from ER to the Golgi apparatus to be cleaved by site-1 and site-2 proteases. The resulting cytoplasmic fragment liberated from the membrane - designated pATF6 α/β (N) - enters the nucleus to activate transcription of its target genes [45,46], such as GRP78/Bip, CHOP and XBP1. In this manner, ATF6 indirectly regulates autophagy and apoptosis via XBP-1 and CHOP [47].

5. ER stress and autophagy

Oxidative stress and ERS are relevant factors in the mechanisms that induce autophagy. Oxidative stress also triggers ERS that in turn induces autophagy. A certain degree of autophagy brings to removal of ubiquitinated unfolded/misfolded proteins and as a consequence reduces ER stress. However, excessive activation of autophagy after persevering ER stress can aggravate cell injury eventually leading to apoptosis and cell self-digestion. Autophagy is a tightly regulated intracellular bulk degradation/recycling system that has fundamental roles in cellular homeostasis. Autophagy is a major catabolic process that delivers proteins, cytoplasmic components, and organelles to lysosomes for degradation and recycling. A well-orchestrated program including over 30 AuTophaGy-related (ATG) genes controls autophagy, which can be activated by nutrient starvation and subsequent inhibition of mechanistic target of rapamycin (mTOR) signaling [48], or by the UPR in response to accumulation of aggregated misfolded proteins [49]. A long-standing question in the autophagy field is the origin of the autophagosome membrane. Apparently, autophagy has a direct bidirectional connection with the ER membrane. The double-layer lipid membrane of autophagosomes most likely originates from two main sources, the ER and mitochondria [50,51]. A peculiar form of ER stress-induced autophagy is referred to reticulophagy, which is essential to counterbalance ER expansion during UPR [52,53]. Recently, Ge and co-workers [54] suggested the ER-Golgi intermediate compartment (ERGIC) as the most efficient membrane substrate for LC3 lipidation. The authors demonstrated that the ERGIC plays an essential role in triggering LC3 lipidation and autophagosome biogenesis by recruiting the key early autophagic factor ATG14. The ERGIC is a recycling compartment characterized by a tubule-vesicular structure, which is located between ER and cis-Golgi compartment, subjected to a constant flux of membrane traffic. A subset of the ERGIC may become diverted to specialized events such as the formation of phagophore membranes in starved cells. Considering the location of the ERGIC, which is adjacent to the ER, a burst initiation of autophagosome biogenesis could quickly mobilize the ER for further membrane acquisition and expansion. Cellular stress induced by different stimuli can activate several processes aimed at either restoring cellular homeostasis or committing cell death. ERS response, which includes UPR and autophagy, represents a mechanism used by the cell in the attempt of restoring the cellular homeostasis. It is important to underline that the degree of initial insult, often regarded as the degree of ERS and UPR activation, can determine the balance between pro- and anti-survival signals, in which autophagy may serve to either promote or attenuate ERS and UPR signals. In the canonical ERAD system, ubiquitinated unfolded/misfolded proteins are degraded by the UPS and the autophagic pathway is viewed as a secondary response to ERS. However, recent evidence suggests that autophagy can be engaged both in the canonical and non-canonical ERAD system. B’Chir et al. [55] showed that the eIF2α/ATF4 pathway drives an autophagy gene transcriptional program in response to amino acid starvation or ERS. The eIF2α/β kinases GCN2 PERK, the transcription factors ATF4, and CHOP are also required to increase the transcription of a set of genes implicated in the formation, elongation, and function of autophagosome. Furthermore, Haberzettl et al. [56] showed that IRE1 is also implicated in the activation of the autophagy. Increased IRE1 activity and IRE1-dependent inflammation was observed in intestinal epithelial cells of ATG-knockout mice. This observation implies that deregulated autophagy may also trigger IRE1 activation and concomitant activation of the xBP1 arm of the UPR, suggesting a possible feedback mechanism in the control of UPR signaling.

6. Oxysterols: metabolism and signaling

LDL are the main carriers of cholesterol in the circulation and are very prone to oxidative insults. Oxidation of the lipid components transported by LDL includes cholesterol that is transformed into a number of biologically active oxysterols [6]. A mounting body of evidence highlights the role of oxysterols in health and disease.

Diverse oxysterols are cytotoxic, induce apoptosis to cultured cells [57], and are implicated in inflammatory diseases, atherosclerosis, neurodegenerative diseases and cancer [22,58-60]. Pioneering work by Lizard et al. demonstrated that oxysterols are able to activate apoptotic signaling pathways in endothelial cells [61,62], thus contributing to the development of atherosclerosis. Notably, among investigated oxysterols, 7beta-hydroxycholesterol and 7-ketocholesterol (7KC) were the most potent inducers of apoptosis [63].

In addition, oxysterols are emerging candidates for the control of cell function by acting as signaling molecules at cellular level [64]. For example, 7KC through its ability to activate PKC (Protein Kinase C) is able to block the release of NO from vascular endothelial cells thus affecting the relaxation of arterial wall [65,66]. Moreover, several lines of evidence suggest that oxysterols act as stressors that can lead to prolonged activation of the UPR [4,5]. 7KC, in analogy to that observed with LDL, has been shown to induce ER stress in human EC and mediate apoptosis [67]. Several authors showed that changes in the antioxidant status correlate to the induction of redox-sensitive transcription factors, which in turn trigger autophagy [68,69]. Lizard and co-workers [70] reported that 7KC induces a mixed type of cell death on 158N cells, associated to ROS overproduction, apoptosis and autophagy, suggesting the new designation of "oxiapoptophagy". Yuan et al. [71] reported that the induction of autophagy, in a human monocytic cell line, is associated to a significant reduction in 7KC-mediated cell death. On the other hand, Muller [72] reported that ox-LDL induce the unfolded protein response (UPR) and trigger ERS and autophagy in HMEC-1 cells. The authors suggested that autophagy could act as anti-
atherogenic mechanism by favouring the degradation of cellular components after ox-LDL insult. In agreement, Yuan et al. [71] reported that autophagy minimizes cellular lipid accumulation induced by oxysterols, preventing the formation of atherosclerotic plaques.

Oxysterols induce the activation of several transcription factors that appear to be redox modulated and are in connection with the ER. Among them the most important and extensively characterized are the LXRs. Activation of LXRs by oxysterols induces the expression of genes involved in cellular cholesterol trafficking, including Niemann Pick type C1 and 2 proteins, and cholesterol efflux, including ABCA1, ABCG1 and apolipoprotein E [73]. Moreover, LXRs maintain cholesterol homeostasis by suppressing LDL uptake through transcriptional induction of Idol (inducible degrader of the LDL receptor), an E3 ubiquitin ligase that triggers ubiquitination of the LDL receptor. In addition, LXR is reportedly to regulate ER stress by dynamically modulating the incorporation of polyunsaturated fatty acids into phospholipids through the induction of Lpcat3 [74].

On the other hand, LXRs regulate fatty-acid-related genes, such as FAS (fatty acid synthase), SCD (stearyl-CoA desaturase), FADS (fatty acid desaturase), ELOVL5 (elongation of long-chain fatty acids), and SREBP1c (sterol regulatory element-binding protein 1c). SREBPs control cholesterol synthesis by a regulated transport from the ER to the Golgi that is inhibited by oxysterols [75,76].

By modulating LXRs activity, oxysterols can negatively affect the signaling of Toll like receptors (TLRs). Joseph et al. [77] demonstrated that activation of LXRs by oxysterols in macrophages inhibits NF-κB signaling and the consequent expression of TLR-inducible inflammatory genes such as inducible nitric oxide synthase (iNOS), interleukin-1β, and monocyte chemoattractant protein-1 in response to bacterial infection or LPS stimulation. On the other hand, Gargiulo et al. [78] recently reported an enhanced NF-κB nuclear translocation promoted by the activation of TLRs in response to oxysterol treatment of promonocytic cells. In analogy, NF-κB activation in response to oxysterols has been shown in bovine aortic endothelial cells by Umetani et al. [79]. In this paper the pro-inflammatory effect exhibited by oxysterols was attributed, at least in part, to the strong activation and nuclear translocation of NF-κB promoted by the ER/c-Jun N-terminal kinase (JNK) pathway. Involvement of NF-κB in transducing proinflammatory signals was also indirectly demonstrated in HUVEC exposed to a mixture of oxysterols [80]. Thus, oxysterols appear to have proinflammatory activity in resting cells, while they function as immunosuppressants upon cell activation by inflammatory agents such as LPS.

According to recent reports [79,81] the pro-inflammatory effect exhibited by oxysterols is based, at least in part, on the strong activation and nuclear translocation of NF-κB, through the ER/c-Jun N-terminal kinase pathway.

Side-chain oxysterols are efficient ligands and/or activators of SREBPs, which are synthesized and located on the ER membrane in an inactive form. Nuclear translocation requires that the active N-terminal region of the HILH be cleaved. The SREBP cleavage-activating protein (SCAP) and Insig function as cholesterol and oxysterol sensors, respectively. When the cellular cholesterol levels are depleted, SCAP binds to and escorts SREBP in COPII vesicles to the Golgi apparatus, where site 1 and site 2 proteases cleave the SREBPs [82,83]. Upon restoration of cellular cholesterol, Insig, a key regulator of ER membrane proteins, traps and retains the SREBP–SCAP complex in the ER to inhibit SREBP cleavage in the Golgi. Interestingly, 25-hydroxycholesterol was found to potently inhibit proteolytic processing of SREBPs, presumably by binding to Insig proteins. Therefore, oxysterols appear to negatively regulate cholesterol cellular content by promoting efflux, by limiting uptake through LXRs, and by inhibiting de novo synthesis through SREBPs. In this regard, Shentu et al. [84] have shown that exposure to ox-LDL in vitro, and in dyslipidemia in vivo, significantly increases the stiffness of aortic endothelial cells, which in turn is associated with an increase in endothelial contractility, enhanced angiogenic potential, and sensitivity to shear stress. The authors hypothesized that ox-LDL induces endothelial dysfunction by inserting oxysterols into the plasma membrane, resulting in the disruption of cholesterol-rich membrane domains and causing endothelial stiffening [84]. However, it cannot be excluded that the effects may be mediated by the ability of oxysterols to affect cholesterol metabolism, although total content of membrane-associated cholesterol resulted apparently unaffected.

Another, possible target of oxysterols-mediated cell signaling is nuclear factor-erythroid 2 p45-related factor 2 (Nrf2). Nrf2 is a master transcriptional activator of protective genes. It activates transcription in response to electrophiles and reactive oxygen species. Under normal conditions, Nrf2 is constantly ubiquitinigated by Keap1 (Kelch-like ECH-associated protein 1)/Cul 3 (cullin RING E3 ligase complex) and degraded by the proteasome. Exposure to oxidative stress leads to Keap1 inactivation and Nrf2 stabilization. Nrf2 migrates into the nucleus where it binds to the ARE (Antioxidant Response Element) sequences in the promoter region of genes encoding key components of the glutathione-based and thioredoxin-based anti-oxidant systems [85]. Although Nrf2 activation in response to oxysterol-induced ROS production has been reported in promonocytic cells [86], elevated expression of Nrf2 has been observed in HUVECs only after exposure to ox-LDL, but a direct evidence of oxysterol involvement in activating Nrf2 in endothelial cells is still missing [87]. Nrf2 is thought to protect cells against oxidative stress, and has displays anti-inflammatory and angiogenic functions in endothelial cells [88].

Oxysterols are also recognized by the OSBP and OSBP-related protein (ORP) family, which have been postulated as intracellular lipid sensors or transporters, and carry targeting determinants for ER [89]. Finally, a very rapid increase in cytoplasmic Ca2+ is observed in response to oxysterols in a time frame that precedes the increases in levels of reactive oxygen species, or changes in gene expression [90].

7. ER stress and oxysterols

In the last two decades, the physiology and pathophysiology of the ER has become a very active area of research due to the evidence that malfunction of the ER stress response caused by aging, genetic mutations, or environmental factors can result in various diseases [91]. The involvement of ER stress in atherosclerosis initiation and progression has been well established [92,93]. In addition, pro-atherogenic effects of ERS have been reported almost in every cell types present in atherosclerotic lesions and in endothelial cells. To this regard Zeng et al. [94] have demonstrated how the transient activation of XBPl splicing is related to EC proliferation, while sustained activation induced EC apoptosis, cell loss from vessel walls, and atherosclerotic lesion development.

Both physical factors (disturbed blood flow) and pro-atherogenic molecules such as ox-LDL have been proposed to contribute to ER stress in endothelial cells [95–98]. In human microvascular endothelial cells activation of the ER stress sensors IRE1α, PERK and ATF6 and subsequent activation of eIF2α, XB1, CHOP and the chaperons Grp78, Grp94 and ORP150 have been found associated to ox-LDL treatment [67,72]. Significantly, ox-LDL also triggered the activation of autophagic processes [72]. The evidence that ER stress takes part in the apoptotic effect of ox-LDL through the IRE1α/c-Jun N-terminal kinase pathway was demonstrated by the protective effect exerted by specific small interfering RNAs and c-Jun N-terminal kinase inhibitors [67]. Similarly, induction of the unfolded protein response was observed in bovine aortic endothelial cells (BAECs) exposed to ox-LDL, as demonstrated by phosphorylation of PERK and increased expression of GRP78 [99], and in HUVEC [21]. In the latter case ox-LDL induced cell apoptosis through activation of the ER stress sensors IRE1 and PERK, and nuclear translocation of ATF6, in agreement with the results obtained by Muller et al. [72] and Tao et al. [100]. Consistently, downstream pathways resulted activated as evidenced by the occurrence of eIF2α phosphorylation, increased expression of JNK, XB1 and...
chaperone GRP78, and up-regulation of the proapoptotic proteins CHOP and Bcl-2 [21].

Although the link between ox-LDL and ER stress has been well established by several reports, investigation of the activity/contribution of the different oxidized components is still in its emphasis. As far as oxysterols are concerned, several reports showed their ability to induce sustained ER stress in different cell types and in different pathological contexts. However, different oxysterols may elicit different cellular effects in a dose and cell-specific manner, highlighting the need of further investigation. To the best of our knowledge, only in one case, a comparative analysis of the effects of ox-LDL and 7KC in endothelial cells has been conducted [67]. The authors concluded that 7KC, used at concentrations relevant to those generated during LDL oxidation, mimicked the effect of ox-LDL by triggering the phosphorylation of Ire1α and eIF2α.

The molecular mechanism(s) used by oxysterols to induce ER stress are largely unknown. By modulating cholesterol/lipid metabolism and trafficking or by directly being inserted in ER membranes, oxysterols could alter lipid packing/composition, which is expected to be detrimental to endothelial function (Fig. 1). Interestingly, perturbation of the lipid composition could impair the folding of ER proteins, thereby activating the UPR [101]. On the other hand, oxysterols may activate the UPR by directly causing accumulation of unfolded proteins. Dong et al., showed that ox-LDL oxidize sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) causing its partial inactivation [102]. It is well known that inhibitors of SERCA, such as thapsigargin induce a decrease in ER calcium levels. When calcium levels are lowered in the ER, the calcium-dependent ER chaperones, lose their activity, leading to the accumulation of unfolded proteins [103].
8. Concluding remarks

ER plays a central role in cell metabolism as it governs protein and lipid synthesis, and controls Ca²⁺ movements. In response to oxidative insult ER activate a series of responses aimed at preserving cell function or drive signals towards apoptosis and autophagy. ER stress has been implicated in EC dysfunction and atherothrombosis. Ox-LDL, which are found in ox-LDL and in almost all biological fluids, have been implicated in several pathophysiological processes and are potential candidates in the mechanisms of ER stress. However, detailed studies focusing on relevant physiological concentrations and comparing the dose-dependent effect of the diverse oxysterols in inducing ER stress is still lacking and deserve further investigation.

References

[1] A.J. Lusis, Atherosclerosis, Nature 407 (2000) 233–241.
[2] I. Tabas, G. Garcia-Cardenas, G.K. Owens, Recent insights into the cellular biology of atherosclerosis, J. Cell Biol. 209 (2015) 13–22.
[3] M. Civelek, E. Manduchi, R.J. Riley, C.J. Stocekert Jr., P.F. Davies, Chronic endoplasmic reticulum stress activates unfolded protein response in arterial endothelium in regions of susceptibility to atherosclerosis, Circ. Res. 105 (2009) 453–461.
[4] I. Tabas, The role of endoplasmic reticulum stress in the progression of atherosclerosis, Circ. Res. 107 (2010) 839–850.
[5] A. Negre-Salvayre, N. Auge, C. Camane, T. Bacchetti, G. Ferretti, R. Salvayre, Dual signaling evoked by oxidized LDLs in vascular cells, Free Radic. Biol. Med. 106 (2017) 118–133.
[6] C. Zerbinati, L. Iuliano, Cholesterol and related steroids autoxidation, Free Radic. Biol. Med. (2017).
[7] S.H. Park, C. Blackstone, Further assembly required: construction and dynamics of the endoplasmic reticulum network, EMBO Rep. 11 (2010) 515–521.
[8] S. van Meer, D.R. Voelker, G.W. Feigenson, Membrane lipids: where they are and how they behave, Nat. Rev. Mol. Cell Biol. 9 (2008) 112–124.
[9] S.W. Fewell, K.J. Travers, J.S. Weissman, J.L. Brodsky, The action of molecular chaperones in the early secretory pathway, Annu. Rev. Genet. 35 (2001) 149–191.
[10] L. Eligaard, A. Helenius, Quality control in the endoplasmic reticulum, Nat. Rev. Mol. Cell Biol. 4 (2003) 181–191.
[11] M.J. Berridge, The endoplasmic reticulum: a multifunctional signaling organelle, Cell Calcium 32 (2002) 235–249.
[12] B. Meusser, C. Hirsch, E. Jarosch, T. Sommer, ERAD: the long road to destruction, Nat. Cell Biol. 7 (2005) 271.
[13] A.J. Lusis, The action of molecular chaperones in the early secretory pathway, Annu. Rev. Genet. 35 (2001) 149–191.
[14] A.H. Lee, N.N. Iwakoshi, L.H. Glimcher, XBP-1 regulates a subset of endoplasmic reticulum stress genes in response to ER stress, Cell 107 (2001) 881–891.
[15] A. Bertolotti, Y. Zhang, L. Hendershot, H. Zhang, A. Bertolotti, H. Zeng, D. Ron, Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase, Nature 397 (1999) 271–274.
[16] M. Imai, H. Yanagita, T. Okada, K. Mori, Bip mRNA is induced by ATP-γS, and spliced by IRE1 in response to ER stress to produce a highly active transcription factor, Cell 107 (2001) 881–891.
[17] H. Yoshida, T. Matsu, A. Yamamoto, T. Okada, K. Mori, Bip mRNA is induced by ADP-β-S and spliced by IRE1 in response to ER stress to produce a highly active transcription factor, Cell 107 (2001) 881–891.
[18] S. Deegan, S. Saveljeva, A.M. Gorman, A. Samali, Stress-induced self-cannibalism: cell death at the core of protein catabolism and proteostasis network deregulation in cell stress, Cell 173 (2018) 842–853.
[19] X.Z. Wang, P.H. Harding, Y. Zhang, E.M. Jolicoeur, M. Kudora, D. Ron, Cloning of mammalian Ire1 reveals diversity in the ER stress responses, EMBO J. 17 (1998) 5081–5090.
[20] T. Iwawaki, A. Hosoda, T. Okuda, Y. Kamigori, C. Nomura-Furukawatari, Y. Kimata, et al., Translational control by the ER transmembrane kinase/ribonuclease Ire1 under ER stress, Nat. Cell Biol. 3 (2001) 158–164.
[21] S. Cox, P. Walter, A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response, Cell 87 (1996) 391–404.
[22] C. Sidiras, J.S. Cox, P. Walter, RNA ligase is required for regulated mRNA splicing in the unfolded protein response, Cell 87 (1996) 405–413.
