Free Radical Biology and Medicine 208 (2023) 178–185

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

When nitrosative stress hits the endoplasmic reticulum: Possible implications in oxLDL/oxysterols-induced endothelial dysfunction

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ARTICLE INFO

Keywords:
Endothelial dysfunction
Ox-LDLs
Oxysterols
Nitric oxide
Nitrosative stress
s-Nitrosylation
ER stress

ABSTRACT

Oxidized LDL (oxLDL) and oxysterols are known to play a crucial role in endothelial dysfunction (ED) by inducing endoplasmic reticulum stress (ERS), inflammation, and apoptosis. However, the precise molecular mechanisms underlying these pathophysiological processes remain incompletely understood. Emerging evidence strongly implicates excessive nitric oxide (NO) production in the progression of various pathological conditions. The accumulation of reactive nitrogen species (RNS) leading to nitrosative stress (NSS) and aberrant protein S-nitrosylation contribute to NO toxicity. Studies have highlighted the involvement of NSS and S-nitrosylation in perturbing ER signaling through the modification of ER sensors and resident isomerases in neurons. This review focuses on the existing evidence that strongly associates NO with ERS and the possible implications in the context of ED induced by oxLDL and oxysterols. The potential effects of perturbed NO synthesis on signaling effectors linking NSS with ERS in endothelial cells are discussed to provide a conceptual framework for further investigations and the development of novel therapeutic strategies targeting ED.

1. Introduction

The endothelium, consisting of a single layer of endothelial cells, functions as a homeostatic organ that maintains the structure and tone of the vascular system by producing substances that either dilate or constrict blood vessels. Vasodilating factors such as nitric oxide (NO) and prostacyclin promote vessel relaxation, while vasoconstricting factors like endothelin-1 and thromboxane An induce constriction. An imbalance between these factors leads to endothelial dysfunction (ED) [1]. Among these molecules, NO is particularly important since it acts as a potent signaling effector in vascular regulation; indeed, an alteration in the production of NO can cause ED. The beneficial effects of NO occur within a specific intracellular concentration range of 1–10 nM [2]; higher levels of NO (>1 μM) lead to nitrosative stress (NSS) [3], which can cause cell dysfunction if not resolved.

Reactive nitrogen species (RNS) are formed when there is an abnormal increase in the production of NO, either through the inducible (iNOS) or the uncoupled endothelial (eNOS) nitric oxide synthase. These reactive species readily react with various macromolecules, including proteins. S-nitrosylation is a reversible covalent chemical reaction that involves the addition of NO to critical cysteine thiol (-SH) groups on target proteins. Protein S-nitrosylation can modulate enzymatic activity, localization, and/or structure [4]. Aberrant S-nitrosylation of proteins is a feature observed in various pathological conditions associated with NSS. The detrimental effects caused by RNS are linked to numerous disorders, including atherosclerosis, hypertension, and diabetes, among others [5]. However, the precise role of NSS in the pathophysiology of cardiovascular diseases remains incompletely explored.

Some studies have revealed that NSS induces endoplasmic reticulum stress (ERS) by means of S-nitrosylation affecting ER sensors and ER resident isomerases, particularly in neurodegenerative diseases. On the other hand, a growing body of evidence has established a strong association between excessive accumulation of oxidized low-density lipoprotein (oxLDL) and oxysterols with atherosclerosis. Specifically, it has been demonstrated that oxLDL/oxysterols have the ability to induce ERS and activate the unfolded protein response (UPR) in endothelial cells [6].

The purpose of this review is to provide a comprehensive overview of the current understanding regarding the involvement of NSS and S-nitrosylation in ERS, as well as to explore the potential implications of this relationship in the development of ED induced by oxLDL/oxysterols.

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https://doi.org/10.1016/j.freeradbiomed.2023.08.008
Received 12 June 2023; Received in revised form 14 July 2023; Accepted 3 August 2023
Available online 5 August 2023

Abbreviazioni

ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
ED	endothelial dysfunction
eIF2α	eukaryotic translation initiation factor 2α
ER	endoplasmic reticulum
ERAD	ER-associated degradation
ERO-1	ER oxidoreductin 1
ERS	endoplasmic reticulum stress
eNOS	endothelial nitric oxide synthase
GNAI2	guanine nucleotide-binding protein G(i) subunit alpha-2
GRP78	chaperone-binding immunoglobulin protein/78 kDa
HMGB1	high mobility group box 1 protein
iNOS	inducible nitric-oxide synthase
IRE1	inositol-requiring kinase 1
LOX-1	lectin-like oxidized low-density lipoprotein receptor-1
NAC	N-acetyl-cysteine
NO	nitric oxide
NSS	nitrosative stress
Nox	NADPH oxidase
oxLDL	oxidized low-density lipoprotein
PDI	protein disulfide isomerase
PERK	pancreatic ER kinase
PTMs	posttranslational modifications
RNS	reactive nitrogen species
ROS	reactive oxygen species
SERCA	sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase
SNO	S-nitrosothiol
UBE2D1	ubiquitin-conjugating enzyme
UPR	unfolded protein response
XBP1	x-box-binding protein 1

1.1. The endoplasmic reticulum and the unfolded protein response

The endoplasmic reticulum (ER) is an organelle responsible for the folding of proteins entering the secretory pathway or that are inserted into membranes. The integrity of the secretory proteome is maintained by biological pathways within the ER, known as the ER quality control system [7]. Indeed, protein folding in the ER is not always efficient, thus cells must continually assess the pool of folding proteins and remove the unfolded or misfolded ones. These aberrant protein forms are degraded through two pathways: ER-associated degradation (ERAD) or autophagy [8].

When a significant proportion of ER-synthesized proteins fail to fold correctly, the ER triggers a response known as the unfolded protein response (UPR). Imbalances in ER quality control systems caused by genetic, environmental, or age-related factors can lead to a condition called ER stress (ERS) [9]. The UPR primarily aims at reducing the accumulation of misfolded proteins in the ER and restoring proper ER quality control and secretory proteostasis in response to ERS. This response is initiated by three transmembrane transducers: inositol-requiring kinase 1 (IRE1), pancreatic ER kinase (PERK), and activating transcription factor 6 (ATF6). Under normal conditions, these transducers remain inactive due to their association with the chaperone-binding immunoglobulin protein/78 kDa (Bip or GRP78).

Upon dissociation from Bip, IRE1/α dimerizes and undergoes autotransphosphorylation of its kinase domain, becoming active. Activated IRE1/α cleaves 26 nucleotides from X-box-binding protein 1 (XBP1) mRNA, enabling the translation of a functional transcription factor called XBP1s. Phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) occurs when Bip dissociates from PERK, leading to a reduction in global protein synthesis and alleviation of ER load. This process also promotes preferential translation of the activating transcription factor 4 (ATF4). ATF6 activation involves its packaging into COPII vesicles and transfer to the cis-Golgi compartment, where it undergoes intramembrane proteolysis by site 1 and 2 proteases (S1P and S2P) to generate a transcriptionally active fragment called pATF6α. XBP1, ATF4, and pATF6α collectively activate the transcription of specific UPR genes involved in various pathways such as autophagy, apoptosis, redox control, protein folding and transport, and lipid metabolism [10].

1.2. OxLDL/oxysterols-induced ER stress in endothelial dysfunction

OxLDL and oxysterols have emerged as significant contributors to ED in atherosclerotic vessels. OxLDL exhibit various forms with different degrees of oxidation and mixtures of bioactive components [11]. Within the bioactive lipids present in oxLDL, oxysterols play a role in all stages of atherosclerosis development [12]. Oxysterols are formed through the oxidation of cholesterol, incorporating carbonyl, ketoxyl, hydroxyl, or epoxy groups into the sterol ring and/or side chain [13,14]. They can be found in animal-derived food due to food preparation, storage, and processing, while in biological systems, they are generated by enzymatic processes catalyzed by cytochrome P450 enzymes or non-enzymatic reactions involving the oxidation of sterol rings.

OxLDL and oxysterols have dramatic effects on vascular and endothelial cell homeostasis. In the early stage of atherosclerosis, oxysterols contribute to endothelial nitric-oxide synthase (eNOS) dysfunction, reactive oxygen species (ROS) production, and alteration of barrier permeability. In the late stage of atherosclerosis, an increased amount of oxysterols was shown to promote endothelial cell apoptosis and degradation of the extracellular matrix, leading to instability of the plaque [15,16]. Moreover, oxLDL and oxysterols induce an inflammatory response in the vascular wall which is characterized by chemokine secretion (CCL2, CCL5), expression of adhesion molecules (ICAM-1, VCAM, E-selectina) and smooth muscle cells migration, thus contributing to atherosclerosis development [17–19]. An increasing body of evidence supports the association between excessive accumulation of these compounds and ERS in the pathogenicity of cardiovascular disease [6,20]. Negre-Salvayre and co-workers [21] first described the effects of oxLDL and oxysterols on ERS and UPR in endothelial cells. Specifically, the authors provided evidence that oxLDL can induce different effects on ERS depending on its concentration. Lower concentrations of oxLDL stimulate the expression of ER-resident chaperone proteins through the UPR, while toxic concentrations lead to prolonged ERS characterized by CHOP overexpression, IRE1α phosphorylation, and JNK activation, promoting apoptosis (Fig. 1). These findings align with the results obtained by our group, demonstrating that a cholesterol autoxidation product called 3β-hydroxy-5β-hydroxy-B-norcholestane-6βcarboxalde hyde (SEC-B), identified in atherosclerotic plaques, significantly expands the ER in HUVEC cells [8]. At lower doses, cells attempt to cope with this stress by activating autophagy and the ubiquitin proteasome system in an effort to restore ER function. However, at higher doses, cell apoptosis occurs through a pathway involving early phosphorylation of eIF2α and NF-kB activation, indicating a failure of the adaptive program and activation of the apoptotic program. OxLDL-mediated apoptosis has also been observed in endothelial cells, primarily through the PERK/eIF2α/CHOP signaling pathway of ERS [22]. Additionally, Dong et al. [23] demonstrated that both oxLDL and glycated-LDL trigger ERS by oxidizing and subsequently inhibiting sarcoplasmic/endoplasmic reticulum Ca2+ ATPase (SERCA) in bovine aortic endothelial cells (BAECs). ERS and the UPR have been associated with inflammation in various human pathologies, including autoimmune, neurodegenerative, and metabolic disorders. It has been shown that different UPR inducers stimulate the production of low levels of pro-inflammatory cytokines, a

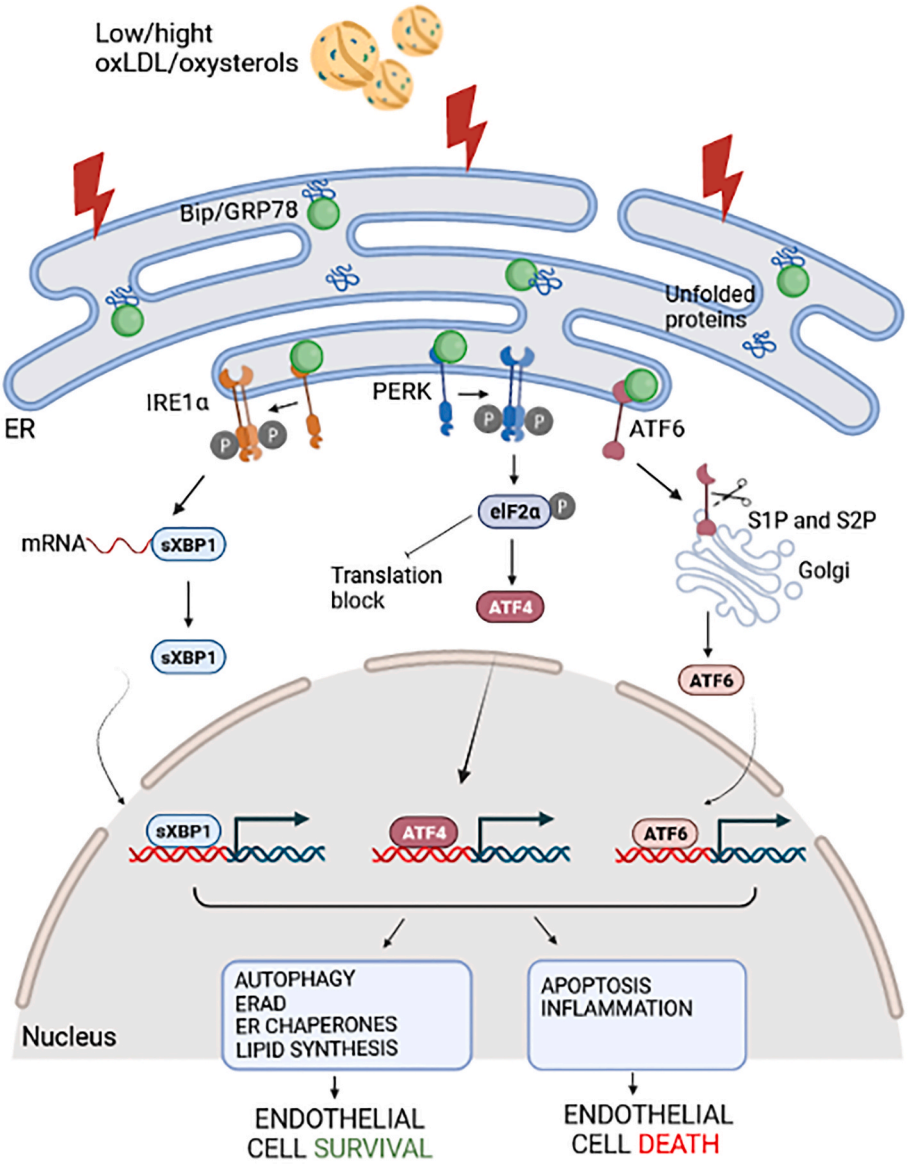

Fig. 1. ERS and the UPR activation induced by oxLDL/oxysterols in endothelial cells. After exposure to oxLDL/oxysterols all the three branches of the UPR are activated in endothelial cells. The amount of oxidized lipids determines the severity of the stress and thus the ability of the cell to cope or not with the stress. Among the expressed genes are those involved in: lipid metabolism which serve to expand ER membranes and accommodate higher amounts of misfolded proteins; ER associated degradation (ERAD) and autophagy which serve to degrade unfolded proteins; inflammation and apoptosis which induce cell damage and commit cells to apoptosis. UPR, unfolded protein response; ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; eIF2α, eukaryotic translation initiation factor 2α; GRP78, chaperone-binding immunoglobulin protein/78 kDa; PERK, pancreatic ER kinase; XBP1, x-box-binding protein 1.

process referred to as "sterile inflammation." Cytokine synthesis occurs through the modulation of NF-kB and MAPK-signaling cascades by UPR pathways, as well as through the direct recruitment of UPR transcription factors to cytokine promoter regions [24]. More recently, IRE1α and PERK have been found to induce the NLRP3 inflammasome [25]. Consistent with these findings, Hang demonstrated that oxLDL reduces cholesterol efflux from endothelial cells, accompanied by the activation of NLRP3 inflammasome signaling, apoptosis signal-regulating kinase 1 (ASK1) and elevated levels of ERS [26].

1.3. NO and nitrosative stress in endothelial dysfunction

NO is a short-lived free radical that plays a role in numerous signaling pathways involved in various physiological and pathological processes. Extensive scientific research has established NO as the endothelium-derived relaxing factor, critical in the mechanism of vasodilation. However, studies have also demonstrated that NO, apart from its vasodilatory function, has significant implications in oxidative stress and inflammation [27,28]. NO is a small and highly diffusible molecule produced by a family of enzymes called nitric oxide synthases (NOS). These enzymes convert L-arginine to L-citrulline using molecular oxygen and NADPH [27]. The NOS family consists of three members: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). Furthermore, NO can enter cells either through cell-surface protein disulfide isomerase (PDI) reactions or by diffusion through the cell membrane [29].

The production of NO by eNOS requires various co-factors, including tetrahydrobiopterin (BH4), flavin adenine dinucleotide, flavin mononucleotide, calmodulin, and iron protoporphyrin IX (heme) [30]. Activation of eNOS can occur in a calcium-dependent or calcium-independent manner. An increase in intracellular calcium leads to its binding to calmodulin (CaM), resulting in the activation of the calmodulin-binding domain of eNOS and subsequent NO production. Alternatively, in response to hemodynamic shear stress and hormones, phosphorylation of specific sites on eNOS, namely Ser635 and Ser1179, independent of calcium concentration, mediates enzyme activation [30].

Once released, NO can act in an autocrine manner on endothelial cells or in a paracrine manner on adjacent cells, such as other endothelial cells, vascular smooth muscle cells (VSMCs), and platelets

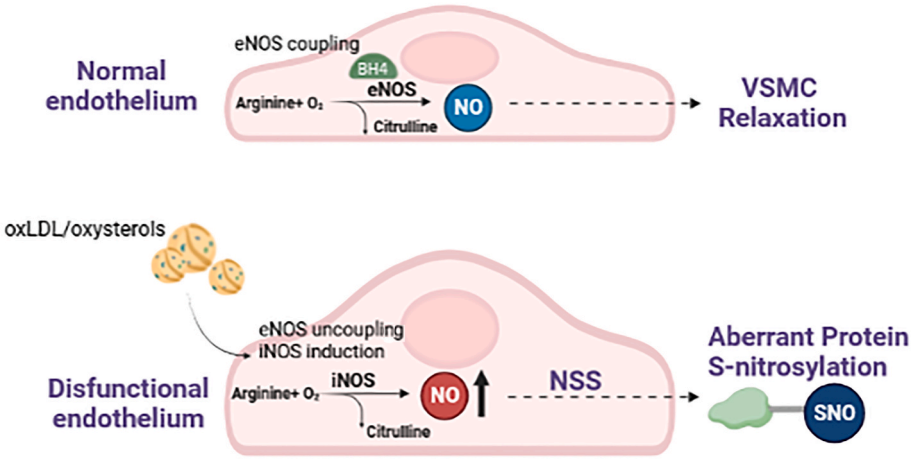

Fig. 2. Role of NO in oxLDL/oxysterols-mediated endothelial dysfunction. An overview of nitric oxide (NO) signaling in both normal and dysfunctional endothelium. Healthy endothelium maintains physiological vascular smooth muscle cells (VSMC) function through the beneficial effects of eNOS coupling. OxLDL and oxysterols can promote eNOS uncoupling and/or stimulate iNOS expression to produce an excessive amount of nitric oxide (NO) leading to nitrosative stress (NSS) and an aberrant protein S-nitrosylation (-SNO).

(Fig. 2). NO signaling can be classified into classical and non-classical schemes. In the classical scheme, NO activates soluble guanylate cyclase (sGC), initiating various cellular signaling pathways. sGC selectively and efficiently binds NO, leading to the conversion of GTP into cGMP. This, in turn, activates multiple substrates, including protein kinase G (PKG) [31].

Non-classical signaling refers to the occurrence of posttranslational modifications (PTMs) induced by NO, specifically S-nitrosylation, S-glutathionylation, and tyrosine nitration [32]. S-nitrosylation is a reversible covalent chemical reaction in which an NO moiety is added to a critical cysteine thiol (-SH) group or thiolate anion –S on a target protein, thereby regulating its function [33]. This reaction forms an S-nitrosothiol (-SNO), and the S-nitrosylated protein is thus referred to as a SNO-protein. Physiological levels of S-nitrosylation can mediate neuroprotective effects, similar to phosphorylation mechanisms [33]. Numerous proteins in endothelial cells have been found to undergo nitrosylation [34], although the functional consequences of this post-translational modification are not yet fully understood. Studies have demonstrated that eNOS promotes S-nitrosylation of pyruvate kinase M2, reducing its activity and increasing the generation of reducing equivalents (NADPH and GSH), thus protecting endothelial cells from oxidative stress [35]. Similarly, S-nitrosylation of caveolin-1 (Cav-1) leads to its degradation, thus establishing a reciprocal regulation between Cav-1 and eNOS [36].

Under non-physiological conditions such as induction of iNOS by inflammatory mediators or uncoupling of eNOS, abnormal amounts of NO and/or ROS are produced within cells [5,37]. eNOS uncoupling disrupts enzyme dimerization and its ability to transfer electrons to L-arginine, resulting in the production of O^{2-} instead of NO. NO can react with O^{2-} to form various RNS, with the predominant species being peroxynitrite ion ($ONOO^{-}$), which is highly cytotoxic due to its strong oxidizing and nitrating properties. Dysfunction of eNOS and reactivity of NO with other chemical species/groups lead to decreased NO levels and impaired NO signaling, while the byproducts of NO induce oxidative damage and protein modifications. The molecular mechanisms underlying RNS-induced damage, particularly in endothelial cells, remain poorly investigated. However, it has been established that S-nitrosylation in combination with oxidative stress is necessary to induce ED [5,38,39]. In bovine aortic endothelial cells, S-nitrosating agents caused metabolic stress and cell death by affecting mitochondrial function, but only in combination with redox-cycling agents [40]. S-nitrosylation/denitrosylation serves as an important regulatory mechanism in endothelial cells, contributing to the maintenance of endothelial cell integrity and functionality. Ravi et al. demonstrated a direct link between protein S-nitrosylation and ED, showing that exogenous NO treatment in BAECs reduced eNOS activity by impairing eNOS dimer formation through S-nitrosylation of cysteine residues at the dimer interface [41]. Various pathological conditions are associated with altered protein S-nitrosylation, either increased or decreased, affecting the activity and stability of different target molecules. Hyperglycemia and hyperlipidemia decrease the number of S-nitrosylated proteins. Among these proteins, both IKKβ and transcription factor p65 have been identified as targets of reduced nitrosylation associated with inflammation and ED [42]. The authors also demonstrated that the treatment with bFGF increased S-nitrosylated IKKβ and p65 alleviating ED and angiogenic defects in diabetes. In vivo, homocysteine reduces protein nitrosylation in rat aortic endothelial cells. Mechanistically, homocysteine reduces eNOS phosphorylation and NO levels and induces the expression of vascular cell adhesion molecules by limiting p65 nitrosylation in HUVEC [43]. On the contrary, Yin and coworkers reported that hyperhomocysteinemia or homocysteine thiolactone, a major metabolite of homocysteine, induces NSS and GCH1 S-nitrosylation at cysteine 141 via inducible NO synthase (iNOS) in wildtype mice [44]. The authors demonstrated that S-nitrosylated GCH1 protein inhibits its activity leading to endothelial senescence and cerebrovascular stiffness. Furthermore, stimuli such as shear stress and exposure to the adipocytokine resistin have been shown to increase protein nitrosylation [45].

Taken together, these observations suggest that regulation of protein SNO and iNOS expression have a key role in ED and in vascular diseases. In perspective, pharmacological compounds that are able to affect SNO and iNOS should be investigated.

1.4. Role of NO in oxLDL/oxysterols-mediated endothelial dysfunction

OxLDL and oxysterols can contribute to ED by dysregulation of NO/iNOS levels. These molecular events are associated with impaired eNOS activity, reduced NO bioavailability, increased ROS production, and activation of inflammatory pathways. iNOS expression, typically absent in normal vessels, is induced in both inflammatory and endothelial cells in early and advanced atherosclerotic plaques [46]. It has been demonstrated that oxysterols promote ED through eNOS uncoupling, resulting in increased production of superoxide anions (O^{2-}) and reduced NO levels [47,48]. OxLDL can directly generate ROS via LOX-1-induced NADPH oxidase activation, inhibit Akt-mediated eNOS phosphorylation, and upregulate Cav-1 expression, leading to down-regulation of eNOS activity and decreased NO availability [49].

In this context, Cav-1 upregulation promotes the translocation of NF-κB and regulates the transcription of iNOS and LOX-1. High mobility group box 1 protein (HMGB1) has also been identified as a negative modulator of eNOS activity and induces high permeability in the endothelial cell layer [50]. The activation of LOX-1 by oxLDL trigger an inflammatory response, characterized by the activation of adhesion

molecules (VCAM-1 and ICAM-1), chemotactic factors, and excessive ROS formation [51]. OxLDL and oxysterols induce the expression of inflammatory cytokines, including IL-6, in endothelial cells, further supporting the role of IL-6 in upregulating LOX-1 and iNOS expression [18,52,53]. In vivo studies using rats fed with food containing 5α, 6α-epoxycholesterol demonstrated that oxycholesterols increase NSS and inflammatory cytokine production [54]. Some studies reported that mildly oxidized LDL has been shown to decrease protein S-nitrosylation in endothelial cells. The authors propose that this reduction may be due to an active denitrosylation process rather than a decline in NO levels resulting from the inhibition of eNOS expression/activity or oxidative stress [55]. Conversely, other studies have reported that oxLDL down-regulates eNOS expression while upregulating iNOS, leading to increased NO production and protein S-nitrosylation (Fig. 2). Wang et al. demonstrated that oxLDL induces S-nitrosylation of Cys94 and Cys99 of eNOS through iNOS induction. Despite the belief that S-nitrosylation inhibits eNOS activity, the authors showed that this modification enhances the interaction between eNOS and β-catenin, resulting in β-catenin activation and ED [56]. More recently, it has been demonstrated that oxLDL increase iNOS protein expression, while phosphorylated eNOS, total eNOS, S-nitrosoglutathione reductase (GSNOR), and Trx remain unchanged in HUVEC. This leads to S-nitrosylation of guanine nucleotide-binding protein G(i) subunit alpha-2 (GNAI2). iNOS-induced S-nitrosylation of GNAI2 enhances endothelial inflammation by inactivating Hippo-YAP signaling [57]. Besides, the elevation of SNO-Hsp90 was observed in atherosclerotic human and rodent aortas as well as in oxLDL-treated ECs. Inhibition of iNOS or transfection with Hsp90 cysteine 521 (Cys521) mutation plasmid decreased the level of SNO-Hsp90 in oxLDL-cultured ECs alleviating endothelial adhesion, inflammation and oxidative stress via inhibited NF-κB signaling [58].

All together these evidences highlight that oxLDL/oxysterols can disrupt NO vascular homeostasis by interfering with multiple pathways involved in controlling NOS activity.

1.5. The relationship between NSS, protein S-nitrosylation and ERS

As previously discussed, NO plays a significant role in compromising various cellular functions, particularly by affecting protein folding and activity through cysteine nitrosylation. One specific area where NO can have an impact is in activating ERS pathways. The ER's redox homeostasis is highly sensitive to disturbances as it maintains an oxidizing environment necessary for oxidative protein folding. Essential to this process is the catalysis of disulfide bond formation by numerous ER oxidoreductases, including protein disulfide isomerase (PDI), a multifunctional oxidoreductase that facilitates disulfide bond formation, isomerization, and reduction [59]. Other ER-resident enzymes, such as ER Oxidoreductin 1 (ERO1), also contribute to the oxidative folding process by assisting PDI. ERO1, an ER-resident thiol oxidoreductase, facilitates disulfide bond formation in nascent polypeptide substrates through electron transfer via PDI, with oxygen serving as the final electron acceptor. The crosstalk between NO and PDI can have several consequences. Firstly, PDI can nitrosylate substrates, resulting in the formation of S-nitrosylated proteins, or denitrosylate substrates, removing NO from S-nitrosylated proteins. Furthermore, the reaction of NO with PDI can inhibit PDI's activity [60] (Fig. 3). In an influential study, Uehara et al. [61] demonstrated that NO-mediated S-nitrosylation of PDI inhibits PDI's function, leading to dysregulated protein folding within the ER, prolonged activation of the UPR, and neuronal cell death.

Disulfide isomerases have been shown to modulate UPR signaling by controlling the oligomerization of ER sensors, which involves the formation of intermolecular disulfide bonds. For instance, PDIA6 reduces these disulfide bonds, thereby preventing oligomerization and promoting the inactivation of IRE and PERK [62]. Conversely, PDIA5 promotes ATF6 activation by reducing disulfide bridges in its luminal domain, facilitating monomer formation and packaging of ATF6 into COPII vesicles for transfer to the Golgi compartment [63]. The impact of nitrosylation on these mechanisms remains unknown and warrants

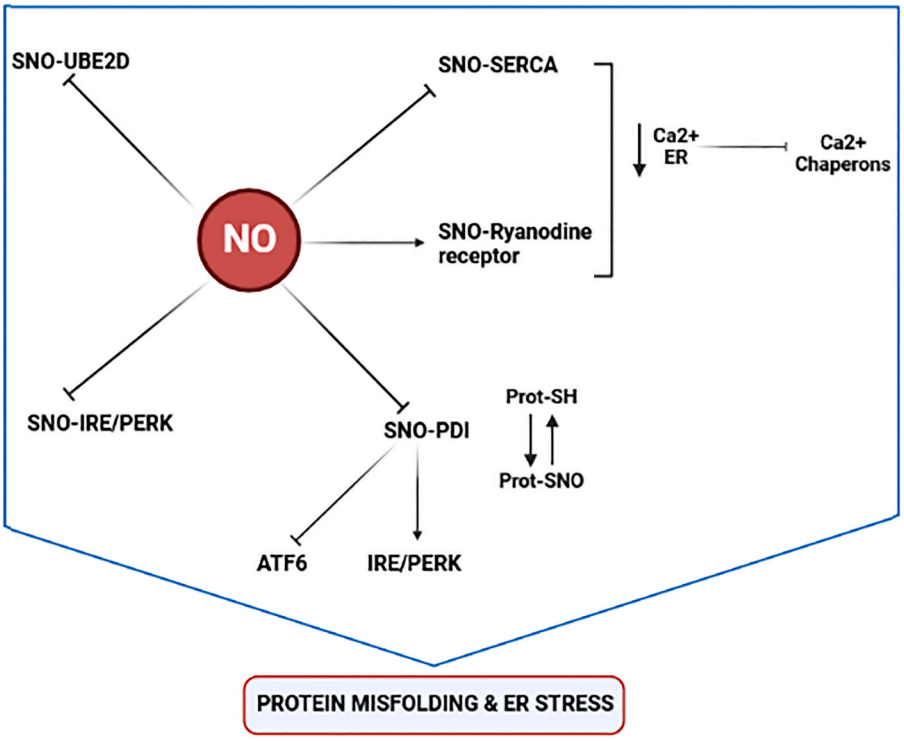

Fig. 3. The multiple effects of S-nitrosylation on ER proteins. Diagram outlining ER-associated pathways where protein S-nitrosylation (prot-SNO) may affect ER functioning leading to protein misfolding and ER stress. PDI, protein disulfide isomerase; UBE2D1, the ubiquitin-conjugating enzyme; ATF6, activating transcription factor 6; PERK, pancreatic ER kinase; IRE1, inositol-requiring kinase 1.

further investigation. Additionally, the S-nitrosylation of two cysteine residues in the Ca2+ sensor STIM has been shown to prevent its destabilization and oligomerization in response to low ER Ca^{2+} levels, enhancing the thermodynamic stability of its luminal domain [64]. These findings suggest that NO plays a role in controlling the oligomerization of sensor proteins.

Direct evidence of S-nitrosylation modification of ERS sensors IRE1α and PERK has been obtained by Nakato et al. [65]. When these ERS sensors are S-nitrosylated, downstream elements are affected. For example, S-nitrosylated IRE1 inhibits ribonuclease activity and attenuates XBP1 splicing. In contrast, S-nitrosylation of PERK activates its kinase activity and increases eIF2α phosphorylation.

Other proteins involved in ERS pathways can also undergo S-nitrosylation, influencing the UPR (Fig. 3). Maintaining calcium homeostasis is crucial for proper ER function since ER chaperones are calcium-dependent. NO protein modification has been reported to inhibit the calcium ATPase SERCA and activate ryanodine receptors, depleting ER calcium content [66,67]. More recently, NO has also been shown to modulate the ERAD pathway. Nitrosylation of the ubiquitin-conjugating enzyme UBE2D1 at its active cysteine site results in enzyme inactivation interfering with the ERAD pathway and leading to prolongation of ERS (Fig. 3) [68].

While dysregulated NO production is implicated in neuronal diseases associated with ER dysfunction, indicating a potential direct link between S-nitrosylation and ERS, this relationship needs further investigation in the specific context of the vascular system. Notably, NO/iNOS levels were found to be upregulated by ERS in endothelial cells exposed to microgravity [69], highlighting a crosstalk between NO and ERS. The work of Zhu et al. [70], demonstrate that oxLDL downregulates eNOS activity via lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) mediated ERS in BAECs, leading to eNOS dephosphorylation at Ser 1179. An analysis of the S-nitroproteome in the endothelial cell line EA. hy926 revealed that over 200 proteins exhibited abnormal S-nitrosylation following treatment with oxLDL [71]. The majority of these proteins are involved in splicing events and translational processes, indicating that changes in gene expression play a significant role in oxLDL-induced ED. Interestingly, Gene Ontology (GO) annotation for significantly regulated SNO-proteins by ox-LDL included both ER-associated proteins with increased nitrosylation and proteins involved in ERS with decreased nitrosylation. This preliminary evidence further supports the hypothesis of a strict connection between oxLDL/oxysterols-induced NSS and ERS in ED.

1.6. Drugs targeting nitrosative and ER stress: limitations and challenges

Given the role of ROS in both nitrosative and ER stress, natural and synthetic compounds with antioxidant properties as well as inhibitors of ROS generating enzymes are expected to mitigate both stress conditions. Converging preclinical and clinical evidence suggest that coenzyme Q10 (CoQ10) supplementation could provide therapeutic benefits for various neurodegenerative disorders, partly due to its effects on ERS. Moreover, CoQ10 has been shown to reduce pro-inflammatory cytokines and ROS levels, which can trigger the UPR and contribute to pathological states [72]. Similarly, Pal et al. [73,74] demonstrated that curcumin, masoprocol and EF-24, a curcumin analogue, rescue S-nitroso-PDI formation by scavenging nitrogen oxides (NOx) using dopaminergic SHSY-5Y cell lines treated with rotenone. The NSS in this experimental model was also investigated by Kabiraj and coworkers [75]. The authors provided evidence that Ferrostatin-1 inhibited the ROS/RNS generated by rotenone demonstrating a protective role on ER. Interestingly, statin treatment, commonly used in the setting of coronary heart disease prevention, protects against ERS and inhibits NADPH oxidase (Nox) activation in preclinical models [76–78]. Nox4, the major source of ROS in vascular endothelial cells, promotes ROS generation in response to ER stressors, leading a hyperoxidized state that affects protein folding prolonging UPR activation [79]. Nox-derived ROS in turn have been shown to induce eNOS uncoupling and mitochondrial dysfunction, resulting in sustained oxidative stress [80,81]. Therefore, Nox inhibitors hold potential for alleviating both nitrosative and ER stress. Clinical trials investigating Nox1/Nox4 inhibitors, such as GKT137831, have demonstrated good tolerability and attenuation of inflammatory markers, highlighting the effectiveness of Nox inhibition/modulation in treating various pathologies, including endothelial dysfunction [82]. In the future, the development of more selective inhibitors against the different Nox isoforms is expected to enhance their therapeutic potential and limit adverse effects. Among synthetic antioxidants, N-acetyl-cysteine (NAC), a cysteine pro-drug that replenishes intracellular glutathione, significantly attenuate atherosclerosis progression in animal models. However, it was unable to reverse pre-existing atherosclerotic lesions, suggesting that NAC may interfere with early events of the atherogenic process, such as endothelial dysfunction [83]. NAC is a well-known agent able to contrast both oxidative and nitrosative stress by reacting with and/or scavenging reactive oxygen and nitrogen species [84]. Furthermore, NAC treatment has been shown to ameliorate ERS under condition of oxidative stress and inflammation [85]. However, the timely administration of antioxidants and the challenge of achieving effective intracellular concentrations without disrupting the basal nitroso-redox balance remain major obstacles for their successful pharmacological application. To overcome these challenges, a deeper understanding of the initial molecular events in oxLDL/oxysterols-induced ED is crucial. This knowledge will help in designing more specific compounds, such as redox modulating agents that selectively target early responsive redox-sensitive proteins instead of broadly quenching intracellular ROS/RNS. Small molecules targeting redox-sensitive cysteines have shown promising effects in regulating protein structure and functions [86]. Additionally, several synthetic compounds generating NO or removing NO from S-nitrosothiols have been developed [87]. Notably, recent studies have demonstrated the potential of pharmacological approaches *in vitro* and *in vivo* model, including the use of NAC and melatonin, and selective inhibitor of iNOS, i.e. 1400 W, L-NIL, to prevent iNOS expression and protein S-nitrosylation in the context of vascular dysfunction [44,57]. However, the precise control of NO bioavailability in biological systems remain an intriguing but as yet unachievable goal. Further investigations and advancements in drug design are necessary to establish whether this research will progress to the translational phase.

2. Conclusions

In conclusion, NO may play a crucial role in regulating ERS and protein folding in pathological conditions. Multiple lines of evidence support the notion that NO can activate ERS pathways, disrupting ER redox homeostasis and compromising proper protein folding and activity through cysteine nitrosylation. Dysregulated NO production and S-nitrosylation have been implicated in ER dysfunction in neuronal diseases, although further investigation is needed to understand their specific impact on the vascular system.

The data available suggest a possible link between oxLDL/oxysterols induced ERS and NSS in ED. At the state of the art, this hypothesis is supported only by the proteomic analysis of Xu et al. indicating that the nitrosylation state of several proteins associated with the UPR and the ERS response changes after exposure of endothelial cells to oxLDL.

The intricate interplay between NO, ERS, and protein folding is crucial for unraveling the mechanisms underlying vascular pathologies and developing potential therapeutic strategies to restore ER homeostasis and endothelial function. Further research is needed to elucidate the precise molecular interactions, signaling pathways, and consequences of S-nitrosylation in the context of ERS and vascular diseases.

Acknowledgements

We would like to thank BioRender.com (https://biorender.com) for the creation of all figures.

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