

Modulation of nitric oxide synthases by oxyLDL: role in vascular inflammation and atherosclerosis development

Micaela Gliozzi¹, Miriam Scicchitano¹, Francesca Bosco¹, Vincenzo Musolino¹, Cristina Carresi¹, Federica Scarano¹, Jessica Maiuolo¹, Saverio Nucera¹, Alessia Maretta¹, Sara Paone¹, Rocco Mollace², Stefano Ruga¹, Maria Caterina Zito¹, Roberta Macrì¹, Francesca Oppedisano¹, Ernesto Palma¹, Carolina Muscoli^{1,3} and Vincenzo Mollace^{1,3*}

¹Institute of Research for Food Safety & Health IRC-FSH, University Magna Graecia, Catanzaro, Italy.

²Department of Medicine, Chair of Cardiology, University of Rome Tor Vergata,

³IRCCS San Raffaele Pisana, Roma, Italy.

*Corresponding author: Prof. Vincenzo Mollace, Institute of Research for Food Safety & Health IRC-FSH, University Magna Graecia, Catanzaro, Italy. Tel.0039-09613694128, Fax 0039-09613695737.e-mail: mollace@libero.it

Abstract

The release of nanomolar concentrations of nitric oxide (NO) by endothelial cells (EC), via activation of constitutive NO synthase (eNOS), represents the pre-requisite for the vaso-protective role of vascular endothelium. On the other hand, exaggerated release of NO as a consequence of activation of inducible NO synthase (iNOS), leads to endothelial dysfunction and, at the late stages, to the development of atherothrombosis. Oxidized LDLs (OxyLDL) represent the major candidate to trigger biomolecular processes accompanying endothelial dysfunction and vascular inflammation leading to atherosclerosis development though the pathophysiological mechanism still remains to be elucidated.

Here, we summarize recent evidence suggesting that oxyLDL produce significant impairment in the balance in the eNOS/iNOS machinery, downregulating eNOS via HMGB1-TLR4-Caveolin-1 pathway. On the other hand, a sustained activation of the scavenger receptor LOX-1 leads to NFkB activation which, in turn, increases iNOS, leading to EC oxidative stress. Finally, these events are associated to reduced protective autophagic response and accelerated apoptotic EC death which activates atherosclerotic development.

Taken together, these informations shed new light into the pathophysiological mechanisms of oxy-LDL-related impairment of EC functionality and open new perspective in atherothrombosis prevention.

Key words: oxidized LDL, eNOS, iNOS, endothelial dysfunction.

1. Introduction

Endothelial cells (EC) are the major component of the innermost layer of mammal blood vessels thereby representing a natural barrier which functionally serves to maintain separate the bloodstream from extra-vascular tissues. As a consequence, the integrity of vascular endothelium represents a key pre-requisite for regulating regional blood flow and many other mechanisms modulating vascular responses [1,2] including the inhibition of atherothrombotic disease development.

Among pathophysiological factors which have been identified to generate endothelial dysfunction, overproduction of oxidized low-density lipoproteins (oxyLDL) appears to play a crucial role in the development of atherosclerosis. Indeed, evidence has been accumulated demonstrating that oxyLDL produce pathophysiological effects, characterized by release of proinflammatory cytokines, overexpression of cell adhesion molecules, monocyte chemoattractant protein-1, smooth muscle cells (SMC) growth factors, and impairment of endothelium-dependent vasorelaxation [3,4]. In addition, EC dysfunction due to overproduction of oxyLDL leads to imbalanced activation of constitutive (eNOS) thereby highlighting inducible (iNOS) isoform of nitric oxide (NO) synthase which, in turn, enhances inflammatory processes within vascular wall and contributes to atherosclerosis progression [5,6,7].

The present review will summarize some recent advances in the bio-molecular mechanisms involved in the imbalanced modulation of NO biosynthesis and release, leading to EC dysfunction. In addition, the effect of oxyLDL-dependent impairment of NO-related pathway on proinflammatory mechanisms in the development of atherosclerosis will be focused.

2. Mechanisms of NOS regulation

The generation of NO represents the product of oxidation of the terminal guanidino nitrogen of L-arginine catalyzed by NOS through a highly regulated process. The three major isoforms of NOS have been distinguished on the basis of several parameters; in particular, amino acid sequences

(only 50–60% identity), tissue and cellular localization, and different regulation mechanisms have been considered for the classification. The calcium/calmodulin dependent NOS isoforms are expressed constitutively (cNOS) in endothelial cells (eNOS) and brain tissue (astrocytes and neurons in particular; nNOS), respectively [8,9,10]. The third NOS isoform is represented by the cytokine-inducible and calcium/calmodulin-independent form named (iNOS) [8,9,11,12].

The distinct properties of each NOS isoform, such as the features of activation and the tissue localization, define both physiological or pathophysiological effects[13]. For example, the release of NO from cNOS occurs transiently, at nanomolar concentrations and cNOS-derived NO regulates organ blood flow distribution in the cardiovascular and renal system. In particular, at vascular level, it inhibits platelet aggregation, platelet and leukocyte adhesion and smooth muscle cell proliferation, whereas it promotes diuresis and natriuresis within the kidney [8,9,14]. In addition, it is involved in neurotransmission [15,16]. These effects are mainly due to the binding of NO to Fe^{2+} in the heme prosthetic group of soluble guanylate cyclase, responsible for the conversion of GTP to cyclic GMP [8,9].

On the other hand, iNOS is overexpressed in response to different inflammatory stimuli, such as endogenous cytokines and bacterial lipopolysaccharide endotoxin (LPS) and causes a delayed but persistent synthesis of a large amount of NO [8,9,12].

The cross-modulation of eNOS and iNOS actions in cardiovascular system represents a crucial event, aimed to ensure the right equilibrium between the constitutive, anti-atherogenic release of pulsed NO at nanomolar concentrations and the suppression of its inducible release at micromolar concentrations [17]. Thus, the maintenance of this balance reduces the potential dangerous effect of NO overproduction, which, after the reaction with superoxide anions, can trigger peroxidative processes [18].

It has been demonstrated that in different tissues expressing both cNOS and iNOS, as well as in cells expressing only inducible iNOS, both NO and NO donors can influence the production of either isoforms of these enzymes [19,5]. In particular, under basal conditions, cNOS-derived NO

causes the inhibition of iNOS expression, suppressing NF- κ B signalling [20,21]. On the other hand, extracellular stimuli, such as bacterial endotoxin and endogenous inflammatory cytokine, are able to induce NF- κ B, thus triggering iNOS expression [22,23].

The NO-related inhibition of NF- κ B is characterized by different mechanisms, which are not affecting the activation and translocation of NF- κ B and are characterized by the interference of the interaction between NO and DNA. In particular, NO is able to modify the binding process of NF- κ B to its promoter response element [24,25], and it has been shown to also inhibit NF- κ B/DNA binding through S-nitrosylation of the Cys 62 residue of p50 subunit [26].

In addition, NO interacts with NF- κ B by stabilizing its endogenous inhibitor I κ B [1]. Indeed, both NO and NO donors are able to prevent I κ B degradation and its dissociation from NF- κ B and to increase I κ B mRNA expression without modulating mRNA expression of NF- κ B (p65 and p50 subunits) [27]. As a consequence, after activation of iNOS by extracellular inducers of NF- κ B, the early inhibitory effect of NO is replaced by its overproduction, which represent the main cause of peroxynitrite formation [28]. Peroxynitrite, in turn, probably via nitration of IKK, favours the shift of NF- κ B in its active state, thus permitting iNOS activation [1].

3. The effect of oxyLDL in the modulation of NOS isoforms

Clear evidence suggests that oxyLDL produce early endothelial dysfunction. Indeed, the occurrence of high concentrations of circulating oxyLDL, as found in hyperlipemic patients as well as in subjects undergoing metabolic syndrome, have been associated to an altered reactive vasodilatation which represents the early stage of endothelial dysfunction [29]. The mechanism underlying oxyLDL-related endothelial dysfunction and inflammation of vascular tissues is still unclear. However, the relationship among the internalization of lipoproteins, the modulation of EC mediators, such as NO, and the activation of transcription factors, such as NF- κ B, appears to have a relevant role in the development of atherosclerosis, though the mechanism is to be better clarified. In particular, it has been hypothesized that oxyLDL may suppress constitutive NO release via direct

or indirect inhibition of eNOS, thereby leading to exaggerated activation of iNOS and subsequent toxic effect due to the reaction of NO with superoxide anions, generating peroxynitrite, which is responsible to EC damage [30]. In this context, a crucial role is played by the activity of the caveolae/NO coupled system.

Caveolae, 50-100 nm vesicular invaginations of the cell plasma membrane, represents the site of the important mechanisms occurring at the plasma membrane, such as vesicular trafficking and signal transduction [31]. The scaffolding protein Caveolin-1 is the main structural and signalling component of caveolae in different cells, including EC, which can interact with several molecules, thereby promoting important signalling functions [32].

eNOS is mostly targeted to caveolae in the plasma membrane of EC through interaction with caveolin-1 [32]. In particular, the binding of caveolin-1 to eNOS is a negative regulator of eNOS activity, and hypercholesterolemia-induced decrease of NO production is probably due to enhanced interaction of caveolin-1 with eNOS [33,34], suggesting its involvement in endothelial dysfunction. The regulatory role of caveolae in endocytosis and transcytosis processes in EC is mainly influenced by its carrier function aimed to mediate the uptake and transcytosis of oxyLDL. This is confirmed by the action of two inhibitors, filipin and nocodazole, which counteracted oxyLDL uptake and transcytosis attenuating the crosstalk between oxyLDL and caveolae across the endothelial cell membrane [35].

OxyLDL have been also shown to upregulate caveolin-1 in a time-dependent manner, thus promoting the translocation of NF- κ B and regulating the transcription of iNOS and LOX-1 [35]. In this scenario, caveolin-1 might promote oxyLDL uptake by EC and NF- κ B might be involved in this pathway.

The activity of Caveolin-1 is regulated by TRL4. In particular, evidence exists that Caveolin-1 Tyr14 phosphorylation is a crucial step in TLR4 signalling and, consequently, it mediates the inflammatory response in EC [36]. In particular, TLR4-mediated recruitment of the adaptor protein MyD88 induces the phosphorylation and the degradation of I κ B, favouring an early activation and

translocation of NF- κ B into the nucleus and, finally, cell death.

It has been hypothesized that high mobility group box 1 protein (HMGB1) may play a crucial role in the OxyLDL-induced development of atherosclerosis [37]. Furthermore, on the basis of the studies on HMGB1, it has been suggested a potential role for oxy-LDL-mediated TLR4/Caveolin-1 expression in EC, which represents a negative modulator of eNOS activity and induces high permeability in EC layer [38].

Summarizing, oxyLDL impair the balance between constitutive eNOS and inflammatory inducible iNOS in EC. This occurs via direct modulation of caveolin-1 (probably via HMGB-1 and subsequent TLR4 signalling), which promotes the translocation of NF- κ B into the nucleus. This inhibits protective mechanisms such as eNOS function and protective autophagy leading, at the end stage, to EC apoptosis and subsequent endothelial dysfunction (Figure 1).

4. Role of LOX-1 scavenger receptor in iNOS activation

Evidence exists that vascular inflammation is mediated by several mechanisms depending on the overexpression of the lectin-like oxidized LDL (LOX-1), a scavenger receptor which selectively internalizes oxyLDL in EC [39]. LOX-1 is highly modulated by stimuli which have been shown to be involved in the development and progression of atherosclerosis, such as cytokines, mechanical forces and angiotensin II action, oxidative stress and directly by the occurrence of LDL oxidation [6,39,40].

Recent data suggest that the detrimental effect of oxyLDL is mediated by an overexpression of the scavenger receptor LOX-1 in EC, which also appears to play a pivotal role in attenuating protective autophagy against apoptosis. Indeed, silencing LOX-1 receptor via ShRNA showed to restore autophagy and protect against oxyLDL-induced apoptotic cell death, thus suggesting the essential role of LOX-1 in mediating oxyLDL-dependent impairment of autophagy (Figure 1) [41].

Similar results have recently been found also in animal models of atherosclerotic disorders in

carotid arteries; indeed, it has been demonstrated that oxidized LDL uptake through LOX-1 contributes to induce endothelial dysfunction observed in the early stages of this pathology [38,42].

In particular, an increased production of reactive oxygen species (ROS), such as superoxide anions (O_2^-), occurs directly via LOX-1-induced NADPH oxidase activation [43,44]. In this context, ROS overproduction and the subsequent LOX-1 activation seems to impair PI-3-kinase/Akt pathway, causing an early attenuation of constitutive eNOS activity through inhibition of its phosphorylation/activation [44,45].

The central role of eNOS dysfunction in the onset of atherosclerosis has been further confirmed by *in vitro* discoveries showing that in EC, oxyLDL elicited a time-dependent decrease in serine 1179 phosphorylation of eNOS, causing its inactivation and, at the same time, the impairment of physiological NO-mediated suppression of iNOS gene expression [46,21]. As a consequence, NO production by iNOS causes the generation of high levels of peroxynitrite, which has been correlated with EC death via apoptosis, as demonstrated by the enhancement of caspase-3 expression [47,48].

The involvement of oxidative stress triggered by the interaction oxyLDL/LOX-1 was confirmed by detecting the effect of N-acetylcysteine (NAC), a thiol-containing radical scavenger and glutathione precursor, able to counteract EC mortality induced by the oxidative damage [49]. Moreover, a similar effect was observed after recovering physiological NO levels, an effect achieved by treating BAEC with the NO donor S-nitroso-N-acetylpenicillamine (SNAP) [41].

This direct correlation between LOX-1 activation and free radical-induced apoptosis of EC has been also proven by using pterostilbene, a naturally occurring analogue of antioxidant resveratrol, which has been shown to inhibit oxyLDL-induced apoptosis of human EC through the down-regulation of LOX-1 expression and the suppression of intracellular oxidative stress [50].

5. The role of LOX-1/iNOS activation in the crosstalk between apoptosis and autophagy of EC.

In the few last years, it has been hypothesized a more complex mechanism responsible for EC death via apoptosis. In particular, Lu et al showed that LOX-1 activation by L5, an electronegative component of LDL abundant in dyslipidemic but not in normolipidemic human plasma, selectively inhibited Bcl-xL and anti-apoptotic Bcl-2 expression and Akt and eNOS phosphorylation; moreover, LOX-1 activation induced an enhancement of proapoptotic Bax and Bad expression. Finally, L5 had been shown to cause the activation of caspase-3 and mitochondrial release of cytochrome c, thus further favouring apoptosis [47,51,52].

Bcl-2 protein family is divided into two different subgroups based on the presence of their Bcl-2 homology (BH) domain(s): anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, and pro-apoptotic proteins, such as Bad and Bax. Bcl-2 family proteins regulate autophagy, which is also considered as a cellular defensive mechanism able to eliminate ROS induced damaged proteins [53,54].

Beclin 1 is an important effector of autophagy, belonging to Bcl-2 family proteins which binds to a hydrophobic groove in Bcl-2/Bcl-xL similarly to pro-apoptotic proteins of Bcl-2 family. The formation of this complex, in turn, impairs autophagy and its inhibitory effect can be suppressed by the dissociation of Beclin 1 mediated by pro-apoptotic proteins [55,56]. This suggests that apoptosis and autophagy may be co-regulated in the same directions; as a consequence, in the presence of stimuli (i.e. LOX-1 activation by L5 or oxyLDL) able to down-regulate Bcl-2/Bcl-xL and to induce pro-apoptotic proteins [57,58], it is conceivable that the switch between autophagy and apoptosis is regulated through alternative mechanisms.

In particular, it has been demonstrated that Beclin 1, being a direct caspase substrate, can lose its autophagy-inducing property after its cleavage; indeed, after the direct interaction of its C-terminal fragment with mitochondria, it causes the release of pro-apoptotic factors, thus promoting apoptosis [59].

In vitro experiments supported this theory. In particular, under starvation experimental settings

able to activate autophagic response, a reduced expression of oxyLDL-induced Beclin 1 was observed being this effect also correlated to an increased expression of iNOS and caspase-3. This suggests that the oxidative stimulus was not able to restore physiologic NO levels early, favouring free radical overproduction and caspase-induced Beclin-1 cleavage; the result of these mechanisms triggered by oxyLDL was represented by a suppression of autophagy associated with an enhanced apoptosis [41,60].

The balance between protective autophagy and apoptotic cell death by LOX-1 has also been further confirmed by the up-regulation of another marker of autophagy, LC3 II (microtubule associated protein 1A/1B-light chain 3 type II), an effect reverted after LOX-1 silencing, via LOX-1 shRNA [60,61].

6. Conclusion

Taken together, data reported in this review article suggest that oxyLDL lead to direct and indirect impairment of constitutive release of NO highlighting overexpression of inducible iNOS. This effect, combined with overproduction of ROS, leads to endothelial cell dysfunction and subsequent development of atherosclerosis. The activation of LOX-1 receptor and subsequent activation of NFkB represents key events in this complex dysregulation of NOS isoforms, contributing to attenuation of protective autophagic response and an accelerated EC apoptotic death which is the end stage of endothelial dysfunction. These informations allow to better define the pathophysiology of imbalanced regulation of NOS isoforms which occurs at early stages of atherosclerotic process and represent a perspective for selective therapeutic interventions in cardiovascular disease prevention.

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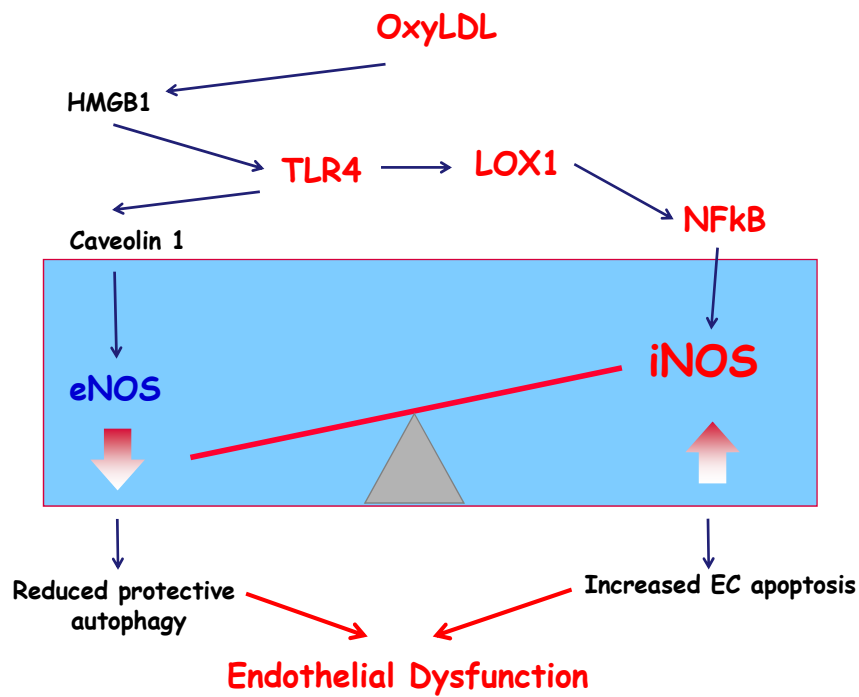


Figure 1

Proposed mechanism of oxy-LDL-related endothelial dysfunction via imbalanced regulation of eNOS/iNOS relationship. In particular, oxyLDL modulates HMGB1/TRL4/caveolin-1 signalling in eNOS/iNOS balance which impairs autophagic/apoptotic responses in vascular and non/vascular cells.

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