Impact of oxidized low density lipoprotein on vascular cells

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Abstract

Oxidized LDL (OxLDL) is a proatherogenic lipoprotein, accumulating in the vascular wall and contributing to the pathogenesis of vascular dysfunction early in the development of atherosclerosis. Enhanced serum levels of OxLDL, as well as antibodies against its epitopes, are predictive for endothelial dysfunction and coronary heart disease. While enhanced oxidative stress is one factor triggering formation of OxLDL, OxLDL itself has been identified as a potent stimulus for vascular oxygen radical formation, causing a vicious circle. OxLDL-induced O2− formation, largely through activation of NADPH oxidase, but also through uncoupling of endothelial NO-synthase and through direct O2− release, leads to endothelial dysfunction. Furthermore, OxLDL-induced O2− formation has a strong impact on tissue remodeling, resulting in either cell growth – proliferation or hyperplasia – or apoptotic cell death. The effect of OxLDL on cell cycle regulation is mediated by activation of the small GTPase RhoA and consequent regulation of p27KIP1, a key enzyme of the cell cycle. In addition, OxLDL-induced activation of RhoA sensitizes the contractile apparatus of the vessel wall, enhancing the contractile tonus and favoring vasospasm. Thus, through a variety of mechanisms, OxLDL importantly contributes to vascular dysfunction and remodeling.

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Keywords: Oxidative stress; NAD(P)H-oxidase; NO-synthase; Atherosclerosis; Lipoprotein; Proliferation; GTPase; RhoA

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1. Introduction

The progression of atherosclerosis and the stability of an atherosclerotic plaque determine cardiovascular disease outcome. The development of atherosclerosis depends on a fragile balance between proinflammatory stimuli on one side, and anti-inflammatory and anti-oxidative defense mechanisms on the other side [1,2]. Tissue remodeling in atherosclerosis is characterized by increased cellular turnover [3,4], with the parallel existence of cell proliferation and cell death, the latter being either of apoptotic or of necrotic nature [5,6]. Vascular proliferation and inflammation are closely linked [7], and excessive proliferation of vascular cells plays an important
role in the pathobiology of vascular occlusive disease. Enhanced oxidative stress is considered to play a causal role in this setting [2], contributing to the inflammatory cascade in the vessel wall [8] and influencing cell cycle decisions [9].

A typical feature of atherosclerosis is the accumulation of oxidatively modified low density lipoproteins (OxLDL) within plaques [10,11], and these lipoproteins are considered to contribute to the inflammatory state of atherosclerosis and to play a key role in its pathogenesis [1,12,13].

During recent years, great emphasis has been placed on analyzing the mechanisms how OxLDL could influence various steps of endothelial and vascular dysfunction during the development of atherosclerosis in vitro. Most of these studies were performed using human LDL, prepared from fresh human plasma oxidized in vitro in the presence of metal ions such as Cu2+ [14]. It should be stated that various other forms of LDL modification exist with relevance for the progression of atherosclerosis, such as enzymatically degraded LDL [15], or minimally oxidized LDL [16]. However, discussion of these other forms of LDL modification is beyond the scope of this brief review. Here we aim to summarize recent experimental data: (1) on the impact of Cu2+ oxidized OxLDL on generating oxidative stress in the vascular system; (2) on the relevance of oxidative stress for cell cycle decisions, resulting in cell growth or cell death; (3) on additional mechanisms of OxLDL-induced cell cycle regulation; (4) on new mechanisms of OxLDL-induced vascular dysfunction.

2. Oxidative stress in atherosclerosis and cardiovascular disease

Formation of reactive oxygen species (ROS) is part of the unspecific defense system of an organism against, e.g., bacteria and other microbes. However, ROS may also affect cells of the host organism, in particular at sites of inflammation. Indeed, inflammation and oxidative stress seem to be closely linked. Atherosclerosis is considered to be a chronic inflammatory disease [1], and growing evidence indicates that chronic and acute overproduction of ROS under pathophysiological conditions is relevant for the development of cardiovascular diseases (CVD) [17]. Various sources for vascular ROS production have been identified: ROS can be produced from nicotinamide adenine dinucleotide (phosphate) oxidase [NAD(P)H-oxidase], xanthine oxidase, lipoxigenase, mitochondria, or the uncoupling nitric oxide synthase (NOS) [18–24]. Stimuli for enhanced vascular ROS formation are numerous, including catecholamines, physical stress, inflammation mediators such as C-reactive protein (CRP) and TNFα, advanced glycation end products (AGEs), thrombin, platelet-derived growth factors, peroxisome proliferator-activated receptor α agonists, angiostatin IL-10, androgenic lipoproteins, and various forms of modified LDLs (the list by far not being complete) [25]. Among these factors, we consider OxLDL-induced O2− formation to be of particular relevance for vascular remodeling and the stability of an atherosclerotic plaque, since it accumulates at the site of inflammation within atherosclerotic plaques, thus in direct vicinity to all types of cells which are involved in the pathogenesis of the disease; only recently, it was shown that OxLDL levels are elevated after percutaneous coronary intervention [26]. We therefore focus in the following section on OxLDL-induced oxidative stress.

3. Mechanisms of oxidative stress induced by OxLDL

Animal studies with cholesterol fed rabbits provided first indirect evidence for a role of LDL in the induction of oxidative stress. Aortas from hypercholesterolemic rabbits produced significantly more superoxide than control aortas [27,28]. Later, we and others could show that incubation of cultured human umbilical vein endothelial cells (HUVEC), isolated arteries, and other blood cells with oxidized LDL, or Lp(a) stimulated O2− formation [29–36]. Induction of free radical formation has also been demonstrated in mesangial cells after stimulation with OxLDL [37].

It is a general observation that oxidation of LDL strongly increases its capacity to stimulate O2− formation; native, non-oxidized lipoproteins elicit no or only weak effects on ROS production. This observation hints to metabolites of the lipid peroxidation process itself, and/or to the specific receptors for OxLDL, which are distinct from the ApoB100 receptor for native LDL. During the lipoprotein oxidation, various more or less stable products are formed, including lysophosphatidylcholine (LPC), aldehyde lipid peroxidation products, and fatty acids produced by phospholipase A2 [38–40]. Indeed, LPC, a by-product of cholesterol esterification, increases O2− formation in human endothelial cells [41] and in vascular smooth muscle cells via stimulation of protein kinase C [42]. We could identify LPC as a strong stimulator of the NAD(P)H-oxidase subunits p22phox and gp91phox [43,44], indicating that it is one component of OxLDL-induced oxidative stress. Superoxide can also be formed from all NOS isoforms under specific conditions, e.g., lack of essential cofactors [45]. In collaboration we could recently show that treatment of endothelial cells with OxLDL resulted in a decrease in the phosphorylation of Thr495, causing dissociation of the eNOS signalling complex and enhanced production of eNOS-derived O2− [23]. What translates stimulation of O2− formation by OxLDL to the cell? Several findings indicate an increase in ROS production upon receptor ligation [46–50]. E.g., activation of the lectin-like receptor LOX-1 by OxLDL results in increased OxLDL-induced O2− formation [51,52]. The importance of the LOX-1 receptor is underlined by data obtained in cell lines: Chinese hamster ovarian (CHO)-cells stably expressing bovine LOX-1 show increased ROS formation compared to native CHO-cells, further confirming its role in OxLDL-induced O2− formation [36].

Another mechanism, also at least partly involving the LOX-1 receptor, is the impact of OxLDL on asymmetric
dimethyl L-arginine (ADMA), an endogenous inhibitor of NOS and a novel cardiovascular risk factor [53,54]. OxLDL can acutely increase the concentration of ADMA in endothelial cells [55], and leads to an upregulation of the expression of protein arginine N-methyltransferases, the enzymes that generate ADMA. In endothelial cells, ADMA increases oxidative stress [56], and in activated macrophages it upregulates the expression of LOX-1 [57], thus causing a vicious circle.

However, OxLDL can cause oxidative stress through an additional mechanism, independent of intracellular enzymes or receptor ligation. The generation of OxLDL is a self-preserving and lipid radical producing chain reaction [58]. Lipid peroxide radicals are generated when a single oxygen molecule is mounted at the active site of radical generation [59]. These lipid peroxide radicals can release superoxide anion and its protonated version, the hydroperoxyl radical (O$_2^\cdot$ and HO$_2^\cdot$, respectively). The former locally mediates oxidative stress, while the latter (that makes up approximately 0.3% of the number of superoxide anion radical molecules [60]) is known to be able to transfer the oxidative chain reaction from one unsaturated fatty acid-containing micelle to another [61]. The release of superoxide anion radicals is believed to be driven by a chemical elimination reaction giving rise to a new conjugated double bond in the affected (poly-)unsaturated fatty acid. Along this line, we (unpublished data) and others [62] have seen that unsaturated fatty acids being components of LDL and phospholipids, respectively, are also able to release superoxide anion radicals. As discussed nicely by Jerek and Hlavata [63], ROS are a unique stress factor for polyunsaturated fatty acids (PUFA). PUFA doubtlessly carry the risk to become part of the self-maintaining generation of lipid peroxides, regardless of their location. Any unsaturated fatty acid can be affected, whenever the resulting lipid radical can be stabilized by conjugated double bonds. Whether dietetic consumption of unsaturated fatty acids has any impact on OxLDL-induced release of reactive oxygen species in vivo is presently unknown.

Taken together, OxLDL contributes to oxidative stress in the vasculature through multiple pathways, as illustrated in Fig. 1.

### 3.1. Oxidative stress and cell proliferation/cell death

Oxidative stress has multitude effects on progression of the cell cycle, depending upon the amount and type of reactive oxygen species, the type of cells and the duration of exposure of the cells to ROS. Hence, oxidative stress can result in diverse and even oppositional effects such as cell growth, as well as cell death [64–69]. Exposure of non-proliferating cells to low doses of ROS usually results in an activation of mitogenic signal transduction pathways, leading to cellular proliferation. Growth factors such as angiotensin II and VEGF get activated by ROS and lead to cellular proliferation of endothelial cells and other cell types [69,70]. Additionally, growth factors such as VEGF can also stimu-

![Fig. 1. Overview of effects of OxLDL on vascular cells. OxLDL stimulates O$_2^\cdot$ formation in endothelial cells and smooth muscle cells through stimulation of NOX, eNOS, and also enzyme-independently from LPR via a lipid radical producing chain reaction. Superoxide radicals cause endothelial dysfunction. Oxidative stress also influences the cell cycle. Cell cycle arrest occurs upon sustained activation of growth factors [72]. Sustained activation of growth signal transduction pathways by ROS, as has been demonstrated for the MAPK pathway, can also result in an arrested cell cycle in the G1 phase leading to cellular hypertrophy [73]. High amounts of ROS not only alter signal transduction pathways, but also affect cellular processes such as ubiquitination essential for cyclin functions, proteasomal degradation, and protein and lipid oxidation. Under such conditions, cells can arrest in all phases of the cell cycle, and subsequently undergo apoptosis, or, in severe cases, even necrotic cell death [74,75]. Oxygen radicals were also identified as messengers for the expression of chemokines such as MCP-1 [76]. Attracted macrophages are then a major source of TNF-α release, activating transcription factors, cytokines, growth factors, cell surface receptors, cell adhesion molecules, and other mediators of inflammatory processes resulting in apoptotic and necrotic cell death [73].](image)
amount and the duration of exposure of the cells to OxLDL [35,43,77]. Thus, OxLDL-induced $O_2^-$ formation has a dual effect on the cell cycle, inducing cell growth as well as cell death [78]. Proliferation and apoptosis are well known end-points of cell cycle progression, while cellular hypertrophy (increase in cell size) results from an arrest in the G1 phase of the cell cycle [79]. The cell cycle is closely regulated by the activity of certain proteins, the cyclins and their partners, the cyclin dependent kinases (CDKs) [80]. One important representative of these cell cycle regulators is CDK-2 [81–85]. Activation of CDK-2 is considered to be essential for the progression of the cell cycle from the G1 to the G2 phase [80], and its activity is controlled by a cyclin-dependent kinase inhibitor called p27Kip1 [86,87]. The CDK inhibitor p27Kip1 plays different roles in cell cycle progression. Besides inhibiting CDK-2 activity, it facilitates the formation of the active cyclin D/CDK complex that triggers the transition from G1 to S phase of the cell cycle [88–90]. Therefore, the CDK-2 inhibitor p27Kip1 acts as an important regulator for cellular signals controlling cell growth and cell death. It is generally believed that p27Kip1 levels have to be decreased in a cell in order to pass through the cell cycle. Recent studies provide evidence that OxLDL regulates endothelial cell proliferation and hypertrophy via regulation of p27Kip1 expression [91].

Low concentrations of OxLDL-induced cell cycle progression in endothelial cells, paralleled by downregulation of the p27Kip1 expression [91]. Ongoing stimulation of endothelial cells with higher concentrations of OxLDL caused a delayed increase of p27Kip1 expression, presumably arresting cell cycle progression by inhibition of CDK-2, facilitating cellular hypertrophy [91].

One important pathway regulating p27Kip1 activity is the RhoA/Rho-kinase pathway. Recently it was demonstrated that proliferation of vascular smooth muscle cells results from activation of the small GTPase RhoA, down-regulating the expression of p27Kip1 [92]. Furthermore, decreased p27Kip1 expression, due to activation of RhoA, is believed to be responsible for cellular proliferation in thrombin stimulated aortic smooth muscle cells [92]. RhoA activation depends on its conversion from the cytosolic GDP- to the membrane associated GTP-bound state. As further outlined in the next section, we have previously shown in smooth muscle cells and endothelial cells that OxLDL stimulates RhoA [91,93] (Fig. 2). We found that activation of RhoA is an essential process for the signaling of endothelial cell proliferation in response to OxLDL stimulation, since inhibition of RhoA activation by transient transfection with dominant inhibitory RhoA N19 prevented downregulation of p27Kip1.

Fig. 2. Direct stimulation of RhoA by OxLDL (reproduced from JASN 2003 [93], with permission). Translocation of RhoA from the cytosolic to the membrane fraction was taken as parameter of its stimulation. Immunocytochemistry with a RhoA-specific antibody in cultured bovine aortic smooth muscle cells using confocal laser scan microscopy for direct visualization of RhoA translocation. OxLDL 5 μg/ml (or its buffer as control) was added to SMC for 1, 5 or 30 min. OxLDL rapidly induced translocation of RhoA into the membrane, as can be depicted by the intensive red staining, the effect being maximal at 5 min.
and almost completely abolished the proliferative response of HUVEC to OxLDL stimulation [91]. We confirmed the role of the RhoA/Rho-kinase signaling pathway in OxLDL-induced endothelial cell proliferation by demonstrating a reduced proliferative response of OxLDL in endothelial cells treated with the Rho-kinase inhibitor Y27632. Rho-kinases are downstream effectors of RhoA [94–96], known to play an important role in cell cycle regulation by influencing cytokinesis, centrosome positioning, and G1 to S phase progression, depending on the cell type [97,98].

Regulation of the cell cycle by OxLDL is also achieved by modulators other than RhoA/Rho-kinase and p27 Kip1. Activation of the EGFR/PI-3K/Akt pathway is involved in promoting cell survival and resistance against the toxic effect of OxLDL [99]. The sphingomyelin/ceramide/sphingosine-1-phosphate pathway participates in OxLDL-induced SMC proliferation, activating ERK1/2 and DNA synthesis via release of activated matrix-metalloproteinase-2 (MMP-2) [100]. Apoptosis, induced by high concentrations of OxLDL, is initiated by down-regulation of antiapoptotic proteins and followed by activation of the caspase cascade. Thereby, OxLDL decreases the expression of the antiapoptotic proteins Bcl-2 and c-IAP-1 (inhibitory apoptotic protein)-1, through a LOX-1 receptor mediated pathway [101]. Down-regulation of Bcl-2 and c-IAP-1 is followed by a significant release of cytochrome c and Smac from mitochondria to the cytoplasmic compartment, activating caspase-9 and caspase-3, promoting apoptotic cell death [101].

An additional mechanism how OxLDL may modulate the cell cycle is the inhibition of import of cell cycle proteins into the cell nucleus [102].

5. OxLDL-induced vasoconstriction

Enhanced vascular tone has frequently been observed in hypercholesterolemic monkeys [103], dogs [104], miniature swine [105], and rabbits [106,107], and has usually been attributed to attenuation of endothelium-dependent dilations. Indeed, OxLDL has been identified as an important trigger for endothelial dysfunction for many years, and OxLDL-induced oxidative stress is an important mechanism leading to endothelial dysfunction: through generation of O2−, OxLDL induces the expression of the antiapoptotic proteins Bcl-2 and c-IAP-1 (inhibitory apoptotic protein)-1, through a LOX-1 receptor mediated pathway [101]. Down-regulation of Bcl-2 and c-IAP-1 is followed by a significant release of cytochrome c and Smac from mitochondria to the cytoplasmic compartment, activating caspase-9 and caspase-3, promoting apoptotic cell death [101].

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However, enhanced vascular tone can also be caused by smooth muscle cell activation, independently of endothelial (dys-)function. On a molecular basis, it has been suggested that increase of intracellular Ca2+ in smooth muscle cells stimulated by LDLs may contribute to enhanced vascular contractility [111]. E.g., we could demonstrate that OxLDL potentiates agonist-induced constrictor responses, and that this effect could partly be diminished by Ca2+-antagonists [112]. However, contraction of smooth muscle cells is not only regulated by the cytosolic calcium concentration via Ca2+-dependent myosin light chain phosphorylation, but also by changes in the activity of the smooth muscle myosin phosphatase that is regulated by the RhoA-Rho-kinase pathway [113]. We therefore investigated whether OxLDL interferes with the RhoA-Rho-kinase pathway, and found strong evidence that OxLDL, as well as its constituent LPC – stimulates the constrictor apparatus in isolated resistance arteries [114] as well as in large arteries [93] via stimulation of RhoA.

Fig. 2 depicts direct stimulation of RhoA, as measured by detection of its translocation to the cell membrane after stimulation of smooth muscle cells with OxLDL. Since upregulation of RhoA and Rho-kinase seems to play a key role for vasospasm in large conduit arteries [115,116], and since Rho-kinase inhibitors prevent agonist-induced vasospasm in human internal mammary artery [117], we suggest that stimulation of RhoA by OxLDL may contribute to vasospasm in atherosclerotic arteries, as illustrated in the scheme in Fig. 2.

6. Summary

Plasma levels of OxLDL, as well as antibodies against its epitopes, are predictive for endothelial dysfunction and coronary heart disease. OxLDL has been identified as a potent stimulus for vascular oxygen radical formation. OxLDL-induced O2− formation leads to endothelial dysfunction and results in either cell growth or apoptotic cell death, thereby eliciting a strong impact on tissue remodeling. Activation of the small GTPase RhoA by OxLDL and consequent regulation of p21Rho [119], a key enzyme of the cell cycle, play an important role in this context. In addition, OxLDL-induced activation of RhoA sensitizes the contractile apparatus of the vessel wall. Thus, through a variety of mechanisms, OxLDL importantly contributes to vascular dysfunction and remodeling.

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