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## Role of Oxidized Lipids in Atherosclerosis

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### 1. Introduction

The role of oxidized lipids in cardiovascular diseases (CVD) has been investigated over the last three decades extensively. A number of studies have been carried out on the mechanisms, and pathways leading to the arterial atherosclerosis. These studies originated from the oxidation hypothesis of the atherosclerosis which was originally proposed more than 25 years ago (Steinberg et al., 1989), and since then experiments were performed by many investigators to further examine and explore the contribution of oxidation and oxidized lipids to cardiovascular diseases. Oxidized fatty acids in the ester and free forms, their decomposition products, cholesterol and its oxidized products, proteins with oxidized amino acid residues and cross-links, and polypeptides with varying extents of covalent modification with lipid oxidation products, and many others substances derived from oxidation have been the subject of detailed studies by many investigators. These products originated *in vivo* from oxidized lipoproteins and lipid membranes were linked to initiation and propagation of atherosclerosis (Zhang & Salomon, 2005; Mitra et al., 2011; Hulsmans et al., 2010). The effect of dietary oxidized fat as a contributor to the oxidative stress was also investigated by several groups including our group (Catapano et al., 2000; Drüeke et al., 2001; Garelnabi et al., 2008; Mitra et al., 2011). While there is a consensus in understanding of initial oxidative steps in the generation of early fatty streak lesions as well as the role of products of peroxidized lipid decomposition such as aldehydes in atherosclerosis, the role of further oxidation into neutral carboxylic acids is still obscure. In this chapter we will review the background of the oxidation theory of lipoproteins and the current state of the knowledge. We will review and summarize data leading to the current understanding of the role of oxidized lipids in atherosclerosis and some pathways involved in this process. We will also discuss recent studies that elucidate factors leading to oxidative stress including chemical, physical and biological factors. In addition, we will explain the current knowledge of the use of antioxidants; and explain their benefits if any to inhibit oxidation of LDL. This part will discuss in brief some selected clinical data.

## 1.1 Atherosclerosis

Atherosclerosis is the principal contributor to the pathogenesis of myocardial and cerebral infarction, gangrene, and loss of function in the extremities. The process, which under normal circumstances is a protective response to insults against the endothelium and smooth muscle cells of arterial walls, consists of the formation of fibrofatty and fibrous lesions, and is preceded and accompanied by inflammation. The advanced lesions of atherosclerosis become pathologic, and may cause occlusion of the affected artery, result from an excessive inflammatory-fibroproliferative response to numerous different forms of insult (Ross, 1986).

The earliest recognizable lesion of atherosclerosis is the so-called 'fatty streak', an aggregation of lipid-rich macrophages and T lymphocytes within the innermost layer of the arterial wall, the intima. The ubiquity of the atherosclerotic process is attested by the finding of fatty streaks in the coronary arteries of half of the autopsy specimens from children aged 10 to 14 years (WHO, 1985). Animal observations have shown that fatty streaks precede the development of intermediate lesions, which are composed of layers of macrophages and smooth muscle cells and, in turn, develop into the more advanced, complex, occlusive lesions called fibrous plaques (Fig 1). The fibrous plaques increase in size and, by projecting into the arterial lumen, may impede the flow of blood. They are covered by a dense cap of connective tissue with embedded smooth muscle cells that usually overlie a core of lipid and necrotic debris (Garelnabi, 2010).

Most of the sudden deaths from myocardial infarcts are due to ruptures or fissures, particularly in the margins of the fibrous cap where there are more macrophages, resulting in hemorrhage into the plaque, thrombosis, and occlusion of the artery (Ross, 1993). As the process continues, migrating cells reach further beneath the arterial surface, where the monocytes become macrophages, accumulate lipid, become foam cells, and together with the accompanying lymphocytes, become the fatty streak. These often form at sites of pre-existing collections of intimal smooth muscle. Thereafter, continued cell influx and proliferation lead to the more advanced lesions, distinguished by their fibrous character, and ultimately to the fibrous plaque (Ross, 1993).

Studies on animals with artificially induced hypercholesterolemia have confirmed that three processes are involved in the formation of atherosclerotic lesions : (1) The proliferation of smooth muscle cells, macrophages, and possibly lymphocytes; (2) the formation of a connective tissue matrix by smooth muscle cells comprised of elastic fiber proteins, collagen, and proteoglycans; and (3) the accumulation of lipid and mostly free esterified cholesterol in the surrounding matrix and the associate cells (Daley et al., 1994).

There are numerous signals, biochemical in nature, which underlie smooth muscle proliferation. Platelet derived growth factor (PDGF), the first postulated growth factor in atherogenesis is produced by many of the cells involved in the process (i.e., platelets, macrophages, endothelial cells and smooth muscle cells). Activated macrophages can also synthesize fibroblast growth factor (FGF), endothelial derived growth factor (EDGF), and transforming growth factor beta ( $\beta$ -TGF). The combination of these growth factors has been shown to be extremely potent in stimulating the migration and proliferation of fibroblasts and smooth muscle cells, as well as the formation of connective tissue element.

When platelets interact with or adhere to sub-endothelial connective tissue, they are stimulated to release their granule contents. Endothelial cells normally prevent platelet adherence because of the non-thrombogenic character of their surface and their capacity to form antithrombotic substances (e.g., prostacyclin and heparin). When endothelium is injured, platelets are promoted to adhere to its surface and thus, the release of platelet constituents, although it is not clear that platelet adherence to modified endothelium is a common event (Ross, 1986). Several investigators have demonstrated that if platelets are absent from the site of endothelial injury, or if are prevented from the injury sites pharmacologically as in experimental models, then the intimal proliferative lesions that usually accompany such injury will not occur (Friedman et al., 1977; Haker et al., 1983). Oxidized low density lipoproteins (OxLDLs) have been shown to play a key role in the pathogenesis of atherosclerosis, since they are present in atherosclerotic lesions. Indeed, oxidized LDLs inhibit endothelium-dependent relaxation of the rabbit aorta in response to acetylcholine, as well as of porcine coronary artery in response to serotonin and platelets (Tanner et al., 1990).

## 2. Oxidation of LDL

The major constituents of plaques are lipid-laden foam cells are formed and their remains. Foam cells form when macrophages or other cells uptake an excessive amount of LDL, and die. An oxidative hypothesis of atherosclerosis was proposed in 1989 and suggested modification of LDL as a primary reason of foam cell formation and development of atherosclerosis (Steinberget al, 1989; Parthasarathy et al., 2010). A massive amount of confirming data was collected since then. It is well accepted now that oxidative processes and oxidized lipids play pivotal role in initiation and progression of the disease.

LDL is a microparticle consisting of one ApoB protein molecule and a mixture of triacylglycerol, cholesterol and its esters, phospholipids, and vitamin E. Oxidation of LDL is a gradual process starting with oxidation of vitamin E and polyunsaturated fatty acids. Peroxides, the primary oxidation products, undergo further transformations with generation of aldehydes among other products. Aldehydes modify amino acid residues of ApoB, primarily lysine, resulting in malondialdehyde modified ApoB (MDA-ApoB) and 4-hydroxy-2-nonenal modified ApoB (4-HNE-ApoB). Biological effect of oxidized LDL varies greatly depending on the grade of oxidation. There are several terms for oxidized LDL that indicate the level of oxidation, such as MM-LDL (minimally modified LDL), fully oxidized LDL, and MDA-LDL (malondialdehyde-modified LDL). It is difficult to determine the level of oxidation in many cases. The term OxLDL (oxidized LDL) is used for any oxidized LDL regardless of the extent of oxidation.

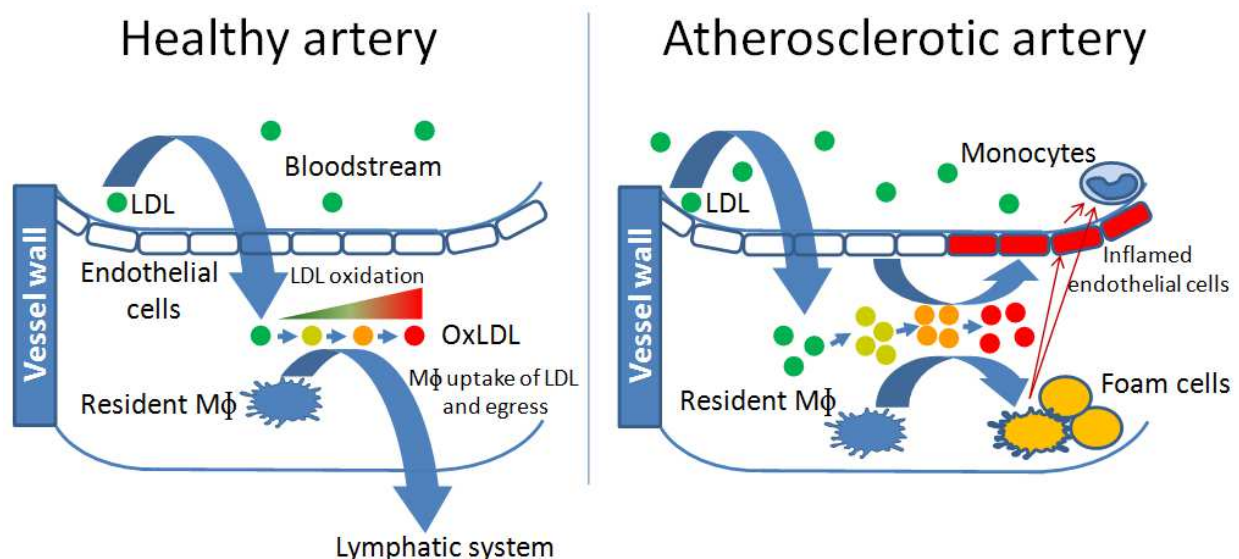
Development of atherosclerotic lesion starts with accumulation of OxLDL in intima, the innermost part of vessel, consisting of single layer of endothelial cells that rest on basement membrane. Intimal basement membrane separates endothelial cells and smooth muscle cells in arterial blood vessels. It consists of extracellular matrix, mostly collagen and proteoglycans, with sparse immune cells and smooth muscle cells (SMC) in it.

There is detectable level of OxLDL in circulating blood, and OxLDL is observed in vascular wall. Immunoglobulin M (IgM) is essential for noninflammatory clearance of OxLDL by macrophages. IgM co-localizes with CD68-positive macrophages in lesions. Double

knockout *Ldlr*<sup>-/-</sup> and soluble *IgM*<sup>-/-</sup> mice develop lesions seven time bigger than *Ldlr*<sup>-/-</sup> control. C1qa is a complement participating in IgM-mediated clearance. There is a pronounced increase in the size of aortic root lesion in double knockout *Ldlr*<sup>-/-</sup>, *C1qa*<sup>-/-</sup> mouse as compared to *Ldlr*<sup>-/-</sup> mouse/- (Lewis et al., 2009).

Immunization of atherosclerosis-prone *Ldlr*<sup>-/-</sup> mice with MDA-LDL or native LDL before feeding with cholesterol-rich atherogenic diet resulted in smaller lesion areas without significant reduction of plasma cholesterol (Freigang et al., 1998). Both type of immunization generated antibodies that recognize a wide pattern of modified and oxidized LDL likely because of some oxidation of LDL during immunization. Binding of OxLDL with antibodies demonstrated antiatherogenic effect, whether it limits the influx of OxLDL into artery wall or helps to clear retained OxLDL. Similar results were obtained in rabbit (Ameli et al., 1996).

While immunization with MDA-LDL prior or at initial stages of atherosclerosis suppresses growth of lesions in mouse and rabbit, there is a controversy in whether higher titer of antibodies to OxLDL in blood correlates with higher or lower grade of atherosclerosis (Palinski et al., 1995; Tsimikas et al., 2007, reviewed in Shoenfeld et al., 2004).



**Fig. 1. OxLDL effects and fate in healthy and atherosclerotic artery wall.** LDL (green circles) enter vessel wall and become gradually oxidized (depicted by changing circle color from green to red). In healthy artery tissue lymphocytes, primarily macrophages (Mφ), uptake OxLDL, and egress the vessel to lymphatic system. The removal of OxLDL is impaired in atherosclerotic artery. Macrophages get overloaded with OxLDL and die generating foam cells. Overloaded macrophages release inflammatory signals that affect endothelial cells and patrolling leukocytes on the vessel surface (depicted with red arrows). Endothelial cells respond to accumulating OxLDL by inflammation as well.

Currently, the general consensus is that oxidation of LDL occurs mostly within vascular wall. Both native LDL and OxLDL are able to pass through endothelial layer passively through interendothelial junctions, or by endothelial transcytosis, an active transport process ( von Eckardstein & Rohrer, 2009). LDL and OxLDL are retained in intima through interaction of the LDL protein ApoB-100 and proteoglycans. LDL undergoes oxidation in

intima and becomes absorbed by macrophages through scavenger receptors. There are many scavenger receptors that vary in the substrate specificity, expression in different tissues, and biological roles. Some of them play essential role in atherosclerosis (Table 1). Excessive loading of macrophages by OxLDL convert them to dysfunctional "foam" cells. OxLDL itself or products of spontaneous or enzyme-assisted decomposition act as pro-inflammatory, chemotactic, growth-promoting factors (Fig 1).

### 2.1 Induction of oxidative stress by OxLDL

OxLDL are cytotoxic for all spectra of atherosclerosis-related cells: T-cells (Alcouffe et al., 1999), macrophages, endothelial cells, smooth muscle cells. OxLDL cytotoxicity in human fibroblasts is mediated through OxLDL-derived lipid peroxides and hydroperoxides, but not superoxide (Coffey et al., 1995).

High load of OxLDL induces two separate lethal processes in macrophages. The first process is activation of caspases-3 in Fas-independent manner. Other caspases, caspase-6, caspase-8, caspase-9, are likely involved as well. It ultimately leads to apoptosis with characteristic DNA fragmentation. The second process is OxLDL-induced plasma membrane lysis (necrosis) mediated by reactive oxygen species (ROS). Both processes occur concurrently, however lysis of plasma membrane is likely the actual reason for macrophages death.

Caspase activation might contribute to macrophage death, however some experiments demonstrate that the extent of the activation is not enough for OxLDL cytotoxicity, since a higher level of caspase-3 activity through activation of Fas is not lethal for macrophages. At the same time inhibitors of caspase-3 do not suppress macrophage lysis by OxLDL, while peroxy radical scavengers Trolox, and N,N'-diphenyl-1,4-phenylene diamine (DPPD) inhibit cytotoxicity of OxLDL. Generation of peroxy radical as primary reactive oxygen species (ROS) in OxLDL-activated macrophages was confirmed with several specific ROS-sensitive fluorescent dyes. So, OxLDL cytotoxicity is mediated by peroxy radicals, but not superoxide. ROS-mediated lysis and caspase activation are independent processes since inhibitors of caspase-3 do not suppress macrophage lysis by OxLDL, and Trolox does not inhibit caspase activation when it inhibits OxLDL-induced macrophage lysis (Asmis & Begley, 2003).

In response to OxLDL, macrophages start to generate intracellularly an increased amount of ROS. Excessive load with OxLDL and ROS generation leads to necrosis of foam cells. There are several NADPH oxidases expressed in macrophages. Nox2 (Gp91phox), a heme-containing subunit of NADPH oxidase, is the major source of ROS during phagocytosis. Nox2 likely does not contribute to atherosclerosis, since Nox2 knockout mouse does not slow development of lesions (Kirk et al., 2000).

Nox4 is another NADPH oxidase. Protein expression of Nox4 and its binding partner p22phox in macrophages is increased by OxLDL but not by native LDL through MEK1/2 pathway. Inhibition of MEK1/2 or siRNA knockdown of Nox4 suppresses ROS production and macrophage death assessed by membrane integrity (Lee et al., 2010).

### 2.2 NF- $\kappa$ B response to OxLDL and atherosclerosis

NF- $\kappa$ B is a family of transcription factors and their precursors sharing Rel homology domain. They function as homo or heterodimers, such as RelA/p50. In resting cells, NF- $\kappa$ B

dimer is associated with I $\kappa$ B, an inhibitory subunit of NF- $\kappa$ B. There are several members in I $\kappa$ B family. The canonical pathway of NF- $\kappa$ B activation is I $\kappa$ B phosphorylation by activated I $\kappa$ B kinase complex consisting of IKK $\alpha$  and IKK $\beta$  subunits and regulatory protein NEMO. Phosphorylated I $\kappa$ B becomes ubiquitinated and undergoes degradation. Degradation of inhibitory subunit releases NF- $\kappa$ B dimer, which translocates from cytoplasm to nucleus and initiates transcription of target genes. Various signals activate IKK complex including tumor necrosis factor (TNF) and interleukin-1 (IL-1). In an alternative pathway, activated NF- $\kappa$ B inducing kinase (NIK) phosphorylates precursor protein p100 that results in ubiquitination and proteasomal processing of a precursor protein p100 into mature p52 subunit. The subunit binds with RelB, and RelB/p52 dimer is an active transcription factor. B-cell-activating factor and other stimuli can activate NIK and thus initiate the alternative pathway. Factors such as lipopolysaccharide (LPS), CD40 ligand can activate both pathways, canonical and alternative. However, there is no data yet on regulation of NF- $\kappa$ B via alternative pathway in smooth muscle cells, macrophages, and endothelial cells (de Winther et al., 2005). OxLDL initiates inflammatory response in endothelial cells and leukocytes. Inflamed cells induce factors that attract leukocytes. Activation of NF- $\kappa$ B is one of the pathways that are involved in atherosclerosis. Activation of this pathway is observed in lesions in endothelial cells, macrophages and SMC (Brand et al., 1996).

OxLDL exerts dual effect on NF- $\kappa$ B activation in monocytes and macrophages. It activates NF- $\kappa$ B in short term, and suppresses it in long term (Brand et al., 1997; Eligini et al., 2002). Activation of NF- $\kappa$ B by OxLDL in atherosclerotic endothelial cells is more stable. An essential mechanism of NF- $\kappa$ B activation is mediated through scavenger receptor LOX-1 (lectin-like oxidized low-density lipoprotein receptor 1). Binding of OxLDL to LOX-1 induces superoxide and hydrogen peroxide generation, and NF- $\kappa$ B activation through activation of p38 MAP kinase, PI3K, ERK1/2 pathway (Cominacini et al., 2000; Tanigawa et al., 2006). Knockdown of LOX-1 gene suppresses endothelial cell injury measured as LDH release, abates expression of MCP-1 and decreases monocyte adhesion to endothelial cells (Li & Mehta, 2000). Knockout of LOX-1 in *Ldlr*<sup>-/-</sup> mouse suppresses activation of p38 MAPK, decreases NF- $\kappa$ B p65 protein level, and inhibits development of atherosclerosis (Mehta et al., 2007).

The importance of NF- $\kappa$ B in endothelial cells in progression of atherosclerosis is demonstrated in *ApoE*<sup>-/-</sup> mouse. NF- $\kappa$ B pathway was disrupted by ablation of NEMO/IKK $\gamma$  or expression of dominant-negative I $\kappa$ B $\alpha$  in endothelial cells. In both cases the lesions developed slower than in control *ApoE*<sup>-/-</sup> mouse (Gareus et al., 2008).

Inflammation is central process in development of atherosclerosis. Presentation of P-, E-, L-selectins by endothelial cells initiates vascular recruitment of circulating monocytes through selectin ligands that are expressed on surface of leukocytes, such as PSGL-1 (Yang et al., 1999; Sperandio et al., 2003). Inhibition of leukocyte recruitment slows development of atherosclerosis. Indeed, P-selectin knockout mice have smaller lesions than control animals (Dong et al., 2000). NF- $\kappa$ B regulates expression of P-selectin and other inflammation-related genes including E-selectin, ICAM-1, VCAM-1, and MCP-1 (Cominacini et al., 1997).

MCP-1 is another cytokine essential for development of atherosclerosis: *Ldlr*<sup>-/-</sup> *Mcp1*<sup>-/-</sup> mouse has smaller lesions compare to *Ldlr*<sup>-/-</sup> (Gu et al., 1998). VCAM-1 on endothelial cells

participates in tight adhesion of monocytes. VCAM-1 knockout is lethal for mouse; however a study of a transgenic mouse with suppressed expression of VCAM-1(D4D) demonstrated reduced lesion development (Cybulsky et al., 2001).

While NF- $\kappa$ B pathway responds to OxLDL, activation of NF- $\kappa$ B stimulates expression of Lox-1 and OxLDL uptake. A study of transgenic ApoE-/-, SIRT1+/- mouse with decreased SIRT1 function revealed that NF- $\kappa$ B inhibition decreases expression of Lox-1 and Ox-LDL uptake. SIRT1, a NAD-dependent class III deacetylases, is known to inhibit NF- $\kappa$ B activity by deacetylating RelA/p65. Indeed transgenic ApoE-/-, SIRT1+/- mouse has decreased SIRT1 activity an increased level of Lox-1 in aorta, and develops atherosclerosis faster compared to ApoE-/-, SIRT1+/+ mouse. Experiments with bone marrow transplantation revealed that pro-atherogenic effect of decreased SIRT1 function is mostly associated with leukocytes. ApoE-/-, SIRT1+/- peritoneal thioglycolate-elicited macrophages uptake showed increased uptake of OxLDL (Stein et al., 2010).

### 3. Lipid peroxidation: NO Implication

It is believed that lipid peroxidation is involved in the oxidative modification of low density lipoprotein (LDL) and the formation of the potent oxidant peroxynitrite (ONOO-) (Roger et al., 1994). Despite intensive research into this key step, the identity of the radical is still a mystery, especially for the in vivo situation. It may result from preformed or lipoxygenase-derived lipid hydroperoxides or hydrogen peroxide, which decompose in the presence of metal ions to lipid alkoxy radicals and lipid peroxy radicals and to hydroxyl radical, respectively. Once formed, the carbon-centred PUFA radical reacts very quickly with molecular oxygen yielding a lipid peroxy radical which in turn abstracts a hydrogen atom from an adjacent PUFA, yielding a lipid hydroperoxide and a new PUFA radical. It is the latter reaction that carries the lipid peroxidation chain. If no chain termination took place, a single initiating event could convert all LDL. The precise length of the chain, i.e., the number of PUFAs oxidized per one initiating radical depends on many factors especially on the antioxidants. The antioxidants of LDL compete with chain propagation by very efficiently scavenging lipid peroxy radicals.

Lipid peroxidation can be measured in a laboratory setting by a variety of methods. Oxidized lipid extracts is measurable in spectrophotometer technique. Recent methods of analysis includes the free oxygen radicals monitor (FORM) system (Garelnabi et al, 2008), Electron Spin Resonance Spin Trapping Techniques (ESRT), and several other traditional techniques. Peroxidation of fatty acids containing three or more double bonds will produce malondialdehyde (MDA). Malondialdehyde produced by peroxidation can cause cross-linking and polymerization of membrane components (Nielsen, 1981). This can alter intrinsic membrane properties such as deformability, ion transport, enzyme activity, and the aggregation state of cell surface determinants. Because MDA is diffusible, it will also react with nitrogenous bases of DNA (Bruce & James 1982). Increased formation of MDA has been associated with arachidonic acid metabolism and platelet aggregation (Marie, 1979; Macfarlane et al., 1977; Garelnabi et al. 2008; Garelnabi et al. 2010). Experimental studies have shown that free radicals promote platelet aggregation and thrombosis and chain breaking antioxidants, such as vitamin E, inhibit or delay arterial thrombogenesis (Ikeda et al., 1994; Jourdan et al., 1995).



Scavenger receptor	Expression	LDL-related substrates	Other substrates	Effect of knockout in mouse
Class A: SR-AI, SR-AII	Tissue macrophages, arterial endothelial cells, smooth muscle cells	Acetylated LDL, lower affinity for OxLDL; recognize modified ApoB	Apoptotic cells, beta-amyloid peptide, anionic phospholipids, advanced glycation end-products, Gram-negative and Gram-positive pathogen-related molecules	Controversial results on atherosclerosis development in knockout of both SR-AI and SR-AII genes ( <i>Msr</i> <sup>-/-</sup> ) in <i>Apo</i> <sup>-/-</sup> or <i>Ldlr</i> <sup>-/-</sup> mice
Class B: SR-B1 (and another minor splice variant of the same gene SR-B2)	Liver, macrophages; adrenal glands, ovaries, and testes - reverse cholesterol transport	OxLDL	Native LDL, HDL, apoptotic cells, beta-amyloid, anionic phospholipids, advanced glycation end-products, amyloid	<i>Srb1</i> knockout in <i>Apo</i> <sup>e</sup> <sup>-/-</sup> or <i>Ldlr</i> <sup>-/-</sup> mouse promotes atherosclerosis
Class B: CD36	Macrophages, dendritic cells, endothelial cells	Moderately oxidized LDL, POV-PC (1-palmytoyl-2-(5-oxovaleryl)-sn-glycero-3-phosphocholine); does not bind acetylated LDL or extensively oxidized LDL	Native LDL, HDL, apoptotic cells, beta-amyloid, anionic phospholipids, advanced glycation end-products, thrombospondin-1, collagen, fatty acids, protozoan and bacterial peptides and lipopeptides	Knockout of <i>Cd36</i> in <i>Apo</i> <sup>e</sup> <sup>-/-</sup> mouse partly protects from atherosclerosis
Class E: LOX-1	Endothelial cells, macrophages, SMC	OxLDL		<i>Lox1</i> knockout inhibits atherosclerosis in <i>Ldlr</i> <sup>-/-</sup> mouse (Mehta et al., 2007)

Less studied scavenger receptors such as MARCO, SRCL (Class A), CD68 (Class D), SREC-1 (Class F), SR-PSOX/CXCL16 (Class G) are not included in the table. The table is based on review (Moore & Freeman, 2006)

Table 1. Scavenger receptors involved in atherosclerosis

The autoxidation of polyunsaturated lipids is an irreversible destructive process; and in tissues it may be associated with accelerated cell aging and premature cell death. Because such biological autoxidation is essentially slow process, the quantitative measurement of susceptibility to oxidation requires standard experimental stress conduction (Dildar et al., 1998).

#### 4. Cellular defenses against ROS

The biochemical defenses that protect organism from the ROS include both small molecules (low molecular weight compounds such as antioxidants and free radical scavengers) and complex enzyme systems. These defenses serve to lower concentrations of free radical species such as superoxide ( $O_2^{\bullet-}$ ), nitric oxide ( $\bullet NO$ ) hydroxyl radical ( $\bullet OH$ ), lipid peroxy radicals ( $L-OO^{\bullet}$ ), and strong oxidants and precursors of free radicals such as hydrogen peroxide ( $H_2O_2$ ) and peroxynitrite ( $ONOO^-$ ). If ROS generation exceeds defense capacity of the cell, ROS will cause excessive damage to cell components. ROS scavengers have also been used to characterize the production, nature, and toxicity of free radical species in *in vitro* and *in vivo* systems.

##### 4.1 Lipid soluble scavengers

A variety of molecules that preferentially partition into membranes function by reducing lipophilic free radical species to less toxic forms. Vitamin E (a series of isomers of tocopherol) will reduce superoxide ( $O_2^{\bullet-}$ ), hydroxyl radical ( $\bullet OH$ ), singlet oxygen ( $^1O_2$ ), lipid peroxy radicals, and other radical species. Ascorbate is proposed to have similar properties and may serve to maintain tocopherols in the reduced active form. Ascorbate serves as a water-soluble reductant and radical scavenger (Bruce & James 1982). The ascorbate-glutathione pathway represent an avenue through which ascorbate consumed in  $H_2O_2$  reduction get recycled at the expense of NADPH. In the first step of this pathway,  $H_2O_2$  is reduced to water by ascorbate peroxidase (APX) using ascorbate as the electron donor. The oxidized ascorbate (monodehydroascorbate) is regenerated by monodehydroascorbate; a radical and if not rapidly reduced it disproportionates into ascorbate and dehydroascorbate. Dehydroascorbate is reduced to ascorbate by dehydroascorbate reductase at the expense of GSH, yielding oxidized glutathione GSSG which is reduced by glutathione reductase (GR) using NADPH as electron donor (Fig 2), (Blokhina and Fagerstedt KV, 2010; Palma et. al, 2009; Halliwell, 2009). Enzymatic ROS scavengers: Catalase and peroxidases lower the steady state concentration of  $H_2O_2$  which is a precursor of potent radical species. Thus, the cytotoxic potential of  $H_2O_2$  is in large part a function of intracellular catalase and peroxidase activities that scavenge  $H_2O_2$ , and concentration of free ions of transition metals that promote generation of  $\bullet OH$  from  $H_2O_2$ . Three glutathione peroxidase (GPx; EC1.11.1.9) isozymes are known, cellular GPx, extracellular GPx, and phospholipid hydroperoxide GPx, and each contains a selenocysteine in its catalytic center. Cellular GPx; the most characterized form, can react with hydrogen peroxide and organic peroxides but not lipid hydroperoxide (Michio et al., 1995). Platelet GPx has been shown to influence the platelet arachidonic acid metabolism by stimulating lipoxygenase and inhibiting cyclooxygenase, since oxidative stress enhances the arachidonic acid metabolism and thereby creates greater demands on the regulatory systems (Malmgren et al., 1990). Phospholipid hydroperoxide

glutathione peroxidase (PHGPx) is an intracellular antioxidant selenoenzyme which interacts directly with peroxidized phospholipids and cholesterol and cholesteryl esters (Imai and Nakagawa 2003). Selenium (Se) is an essential micronutrient for animals and humans that exerts its biological functions through selenoproteins. These proteins contain Se in the form of selenocysteine (Sec), Phospholipid hydroperoxide glutathione peroxidase (PHGPx or GPx4, E.C. 1.11.1.12) is characterized by the presence of selenocysteine at the active site, and belongs to the important family of glutathione peroxidases (GPx). Since the discovery of PHGPx, a number of studies have demonstrated that this seleno-enzyme is essential to organisms. However on the other hand glutathione-S-transferase possessing glutathione peroxidase activity toward lipid peroxides, but not having selenocysteine in its active site (Ursini et al. 1982; Yagi et al. 1996)

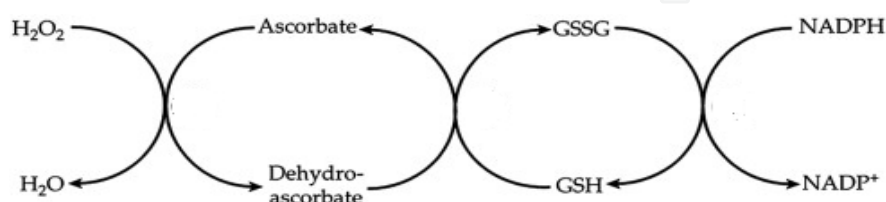


Fig. 2. The glutathione-ascorbate cycle.

Superoxide dismutases (SOD; EC 1.15.1.1) are metalloproteins that catalyze dismutation of superoxide anion radical to H<sub>2</sub>O<sub>2</sub>. Several types of SOD have been discovered. Mn-SOD (MW 85,000) has been found in mitochondria matrices and CuZn-SOD (MW 33,000) is contained in cellular cytosol. However, Mn-SOD and CuZn-SOD have been found also in extracellular fluids (Wesiger & Fridovich, 1973; Marklund et al., 1982). The superoxide radical has been reported as being produced from stimulated platelets (Levine et al., 1981) but its biological value in platelet function is not clearly understood (Violi et al., 1985). A decrease in cytosolic SOD the main defense against superoxide, could lead to increased cellular peroxides. Role of diet in the activity of Cu,Zn-SOD in platelets was studied and found to be influenced by the availability of Cu in diet (Catherine et al., 1993). Furthermore insufficiency in dietary copper was found to increase platelet thromboxane production, which in turn significantly correlated with endogenous lipid hydroperoxides. Evidence obtained from *in vitro* experiments indicates that superoxide dismutase may also inhibit platelet aggregation. That is, SOD given as adjuvant therapy with thrombolysis may both blunt free radicals mediated reperfusion injury and limit the incidence of spontaneous reocclusion after restoration of blood flow (Karin & Robert, 1993). Superoxide dismutase may protect endogenous •NO from inactivation by scavenging superoxide anion. *In vitro* the inhibitory action of •NO on platelet aggregation as well as their adhesion to endothelium induced by thrombin is potentiated by SOD consistent with its preventing inactivation of endothelium-derived •NO (Meng et al., 1995).

Nitric oxide derived reactive nitrogen species (RNS) such as nitrogen dioxide (•NO<sub>2</sub>) and peroxynitrite (ONOO<sup>-</sup>) are indicated in the mediation of oxidative damage. Nitric oxide reacts very rapidly with oxygen radicals. Thus •NO reacting with O<sub>2</sub><sup>•-</sup> generates peroxynitrite (IUPAC-recommended name is oxoperoxonitrate O=N-O-O<sup>-</sup>). The peroxynitrite anion (ONOO<sup>-</sup>) is relatively stable but its acid form (ONOOH) decays to nitrite with a half life of at most 1 sec at physiological pH and temperature (Ducrocq et al., 1999). Peroxynitrite mediates several of the cytotoxic effects of •NO such as the destruction

of FeS centres in enzymes. Persistent blockade of cytochrome c oxidase by  $\bullet\text{NO}$  may lead to the release of free calcium ions ( $\text{Ca}^{2+}$ ) from the mitochondrial matrix into the cell cytosol. Nitric oxide also reacts with lipophilic peroxy radicals, important propagating species in biological chain reaction of lipid peroxidation, to generate alkyl peroxy nitrates (LOONO). These appear far more stable than  $\text{ONOO}^-$ . If LOONO derivatives can be metabolised without the release of toxic free radicals then the reaction of  $\bullet\text{NO}$  with peroxy radicals is potentially beneficial because it allows  $\bullet\text{NO}$  to stop lipid peroxidation.  $\bullet\text{NO}$  inhibits platelet and phagocyte adhesion to the endothelium. However, in atherosclerotic lesions excess production of  $\text{O}_2\bullet^-$  may cause loss of the modulatory action of  $\bullet\text{NO}$  and at the same time yield  $\text{ONOO}^-$  which is pro-aggregatory and so could commit platelets in this environment to thrombus formation (Roger et al., 1994).

Protective mechanism: Several antioxidants can scavenge  $\text{ONOO}^-$ , a molecule responsible for irreversibly oxidation of thiols to higher oxidation states, but nitrosothiols can also form, and later may act as  $\bullet\text{NO}$  donors. Indeed, when isolated vascular tissues are exposed to  $\text{ONOO}^-$  vasorelaxation occurs by a mechanism characteristic of release of  $\bullet\text{NO}$  from a carrier molecule such as nitrosothiol (Liu et al., 1994). Repeated exposure to  $\text{ONOO}^-$  results in a progressive decrease in the efficiency of the vasorelaxing effect.

#### 4.2 Benefits of antioxidants against lipid peroxidation

There are a vast number of studies on the role of anti-oxidants particularly in the area of atherosclerosis and CVD. These studies are controversial, and do not provide clear evidences on the benefits of antioxidants for prevention or treatment of the diseases. Supplementation of antioxidant vitamins such as  $\alpha$ -tocopherol, ascorbic acid and  $\beta$ -carotene used alone or in combination had long been considered to be cardio protective. However, controlled clinical trials using antioxidant vitamin supplements to prevent CVD have yielded conflicting results (Raghavamenon et al., 2009). While some secondary prevention interventions have been shown with  $\alpha$ -tocopherol supplementation alone or in combination with ascorbic acid is reported to reduce CVD risk, other studies have shown no effect of  $\alpha$ -tocopherol supplementation in both primary and secondary prevention.

Vitamin E ( $\alpha$ -tocopherol) is found in plant oils (Honarbakshsh & Schachter, 2009). This vitamin is extensively studied as a possible antioxidant agent against oxidation-induced cardiovascular diseases. Administration of 1000 IU/day  $\alpha$ -tocopherol has been shown to reduce LDL oxidation (Princen et al., 1992). A human study shown that  $\alpha$ -tocopherol supplementation of 150 IU/day to 1200 IU/day increases it level in plasma and in LDL in concentration-dependent manner. *In vitro* oxidation of LDL was partly inhibited in LDL with higher tocopherol content (Dieber-Rotheneder et al., 1991).  $\alpha$ -Tocopherol is reported to reduce plasma OxLDL levels at 25 IU/day in both men and women, and the effect rises with increased supplementation until 800 IU/day (Princen et al., 1995). Tocopherol accumulation in monocytes decreases stress-induced adhesion of monocytes to endothelial cells (Islam et al., 1998; Devraj et al., 1996; Faruqi et al., 1994; Zapolska-Downar et al., 2000), which in turn inhibit the formation of atherosclerotic lesions. Overall, a number of *in vitro* studies demonstrate anti-atherogenic effect of vitamin E by decreasing the production of ROS, lipid oxidation, monocyte endothelial cell adhesion and cytokines secretion. However clinical studies have not revealed anti-atherogenic effect in human (Yusuf et al., 2000).

Vitamin C (ascorbic acid) is principally found in citrus fruits, broccoli, red pepper, and cauliflowers, etc. Ascorbate acts in combination with vitamin E and beta-carotene to protect them from excretion and recycle them for further use. It is also reported to inhibit OxLDL formation indirectly by protecting vitamin E and beta-carotene (Jialal & Grundy, 1991; Kagan et al., 1992). Apart from this vitamin C is reported to inhibit endothelial apoptosis initiated by inflammatory cytokines *in vitro*, and reduces circulating apoptotic microparticles in human (Rössig et al., 2001). Adhesion proteins such as ICAM-1 can be involved in atherosclerosis. Ascorbate supplementation of subjects with low baseline level of this vitamin suppresses mRNA and protein expression of ICAM-1 in monocytes (Rayment et al, 2003). While these and other studies suggest that vitamin C might have anti-atherogenic effect, there is no conclusive clinical evidence of such effect.

$\beta$ -Carotene is indicated in preventing oxidation of lipids which might decrease atherosclerotic lesions formation.  $\beta$ -Carotene is proposed to be efficient scavenger of singlet oxygen and it attenuates oxidative stress, however it does not directly inhibit lipid peroxidation (Briviba et al., 2004).

Polyphenols are another group of antioxidants which are abundant in vegetables and fruits and are found to reduce the risk of CVD (Naderi et al., 2003). They contain both hydrophilic and hydrophobic moieties (Woodman & Chan, 2004). Polyphenols are suggested to inhibit lipid peroxidation (Madrau et al, 2009). It has also been reported that flavonoids chelates copper and iron ions, rendering them inactive to participate in free radical generating reactions (Fernandez et al., 2002). Polyphenols are also known to inhibit enzymes responsible for generation of ROS such as NADPH oxidase, lipoxygenase, phospholipase A2, and xanthine oxidase (Rice-Evans et al., 1997). Indirectly inhibiting the formation of OxLDL, the benefits of flavonoids goes beyond the protection against LDL oxidation to protect the HDL-associated paraoxonase activity (Patel et al., 2007). The antiatherogenic effect of mulberry leaf extracts (MLE) and the polyphenolic extracts (MLPE), which contain polyphenols including quercetin (11.70%), naringenin (9.01%) and gallicocatechin gallate (10.02%) was studied by Yang et al. 2011. Both MLE and MLPE inhibited the oxidation and lipid peroxidation of LDL, while MLPE was shown to be more potent.

## 5. Clinical studies: OxLDL and antioxidants

A number of studies have demonstrated an association of circulating OxLDL with atherosclerosis disease (Itabe & Ueda, 2007; Hulthe & Fagerberg, 2002). The size of LDL particles might have an effect on LDL oxidation. Smaller LDL was associated with higher level of OxLDL. However the association was observed in diabetic subjects, but not in non-diabetic subjects (Scheffer et al., 2003).

OxLDL level normalized to LDL or ApoB protein levels was increased in diabetic subject with macrovascular diseases compared to diabetic subjects without such diseases. Increased OxLDL normalized level was associated with TT genotype of 108C/T polymorphism in PON1 promoter with lower level of expression of the gene (Tsuzura et al., 2004; Brinkley et al., 2009) have demonstrated for the first time that plasma OxLDL levels are related to arterial stiffness in elderly men and women; suggesting that the oxidative modification of LDL may be associated with changes in the elastic properties of blood vessels. Their findings suggest that

while antioxidant supplementation trials have been found to be largely ineffective in preventing cardiovascular outcomes, other interventions including aerobic exercise training and pharmacological treatment with lipid and blood pressure-lowering medications may have significant antioxidant effects that are related to reductions in CVD risk. Another study have shown that oxidized lipoprotein(a) is significantly correlated with blood glucose level among healthy young women, suggesting that lipoprotein(a) may be oxidized with increased glucose concentration even within the normal glucose level (Kotani et al., 2010).

There is some controversy on the role of antioxidants on development of atherosclerosis. A number of clinical studies have demonstrated an anti-atherosclerotic effect of antioxidants while a group of other studies do not see any appreciable benefit of the use of antioxidants. The following are examples of these studies that have suggested an inhibiting effect of antioxidants on lesion development. Gey & Puska (1989) have reported that vitamin E and A concentrations in the plasma were inversely proportional to cardiovascular risks. A study of 667 cases of atherosclerosis-induced coronary disease developed in originally healthy (not diagnosed with coronary heart disease, diabetes, or hypercholesterolemia) 39,910 US men have shown a protective effect of vitamin E but not vitamin C. Carotene appeared to be protective in non-smoking men, however increased the risk of coronary disease among smokers (Rimm et al., 1993). A protective effect of vitamin E was observed in similar study of 87,245 women developed 552 cases of major coronary disease in eight years (Stampfer et al., 1993).

However a large the Heart Outcomes Prevention Evaluation (HOPE) study did not show any anti-atherogenic effect of vitamin E (Yusuf et al., 2000). Subjects who were taking vitamin E and placebo developed atherosclerosis-related diseases such as myocardial infarction, stroke, unstable angina, congestive heart failure at the same rate. Potential explanation for the failure of antioxidants in clinical studies may include the type of dose, duration, time of introduction, i.e. stages of the disease at which the treatment/supplementation were introduced and the selection of an optimal doses of antioxidants. Also, most of the studies did not measure the oxidative stress markers in the plasma to take it into account (Parthasarathy et al., 2001).

Research has provided strong evidence that LDL oxidation plays an important role in the pathogenesis of atherosclerosis and cardiovascular diseases. The involvement of lipid peroxidation in the propagation of the disease is well supported by clinical and scientific research using cell culture and animal models; these studies clearly point that modification of the LDL and the accompanied oxidative damage trigger an inflammation response that mediate the development of the atherosclerosis. One may assume that antioxidants should inhibit the oxidative damage and slow the inflammation processes that lead to CVD and associated with metabolic disorders. However despite of some positive findings, antioxidant compounds did not consistently prove to be potent protective agents against atherosclerosis. In animal atherosclerosis, which is studied in the short term, the emphasis is on establishing the lesions. Thus, antioxidants, such as  $\alpha$ -tocopherol, might affect predominantly the initial formation and progression of the lesion. In humans, particularly in those who already have clinically significant events, the early steps might have already occurred. In such cases,  $\alpha$ -tocopherol and similar antioxidants could affect the conversion of aldehydes into carboxylic

acids. The latter, are presumed to be nonatherogenic and are easily degraded *via* fatty-acid degradation pathways (Raghavamenon et al., 2009). Based on these arguments it may be necessary for the scientific community to revisit the topic and investigate in well structured studies the type, dose, duration of the antioxidants on a well defined population of subjects with various stages of CVD and its associated metabolic disorders such as diabetes, obesity and hyperlipidemia.

Anti-oxidants	Mechanism	References
$\beta$ -carotene	Scavenging ROS and excellent trapper of singlet oxygen, acts against LDL oxidation	Honarbakshsh & Schachter, 2009. Princen et al., 1992
Vitamin C	Scavenging ROS, reactivating other anti-oxidants such as Vitamin E; inhibit formation of OxLDL, Il-1 $\beta$ secretion or chemokines and monocyte – endothelial cell adhesion and $\beta$ -carotene which are anti-atherogenic	Dunstan et al., 2007 Honarbakshsh & Schachter, 2009. Jialal & Grundy, 1991. Kagan et al, 1992. Rössig et al, 2001. Gokce et al., 1999, Rayment et al., 2003. Heller et al., 1999
Vitamin E	Scavenging ROS, reported to inhibit formation of OxLDL, Il-1 $\beta$ secretion or chemokines and monocyte –endothelial cell adhesion	Dunstan et al., 2007 Honarbakshsh & Schachter, 2009. Princen et al., 1992. Islam et al., 1998. Devraj et al., 1996. Faruqi et al, 1994. Zapolska-Downar et al., 2000
Selenium	Cofactor for glutathione peroxidase. Has antioxidant capacity.	Michiels et al., 1994
Zinc	Cofactor for superoxide dismutase. Protects cells from oxidative damage.	Michiels et.al., 1994
Curcumin	Chelating of iron and copper ions, scavenging of ROS, inhibiting lipid peroxidation Protects anti-oxidant enzymes.	Wongcharoen & Phrommintikul, 2009
Quercetin	Scavenging of metals ions, and inhibition ROS. Activation of NF- $\kappa$ B, which is involved in development of atherosclerosis.	Cho et al., 2003
Resevetrol	Inhibits ROS production and lipid peroxidation	Ramprasath & Jones, 2010
Ergothioneine	Protects endothelial cells from oxidative damage by reactive nitrogen species.	Martin, 2010

The table describes the currently investigated antioxidants and their relation to markers of CVD.

Table 2. Role of Antioxidants in Cardiovascular Disease

Clinical study	Findings	No of patients	References
Department of Internal Medicine, Kochi Medical School, Kochi, Japan.	OxLDL increased in subjects with PON1 genotype that lead to decreased expression of PON1 protein	155	Tsuzura et al., 2004
AIR study	OxLDL role in atherosclerosis and inflammation	391	Hulthe & Fagerberg, 2002
CARDIA study	OxLDL indication metabolic syndrome and in abdominal obesity, hyperglycemia and hypertriglyceridemia	1889	Holvoet et al., 2008
Metabolic Laboratory, Department of Clinical Chemistry study, Netherlands	Smaller LDL are associated with higher level of OxLDL	116	Scheffer et al., 2003
HOPE Study	No effect of vitamin E on development of CVD	9541	Yusuf et al., 2000

Table summarizes some clinical studies measured OxLDL in plasma

Table 3. Clinical studies on OxLDL

## 6. Conclusions and perspectives

The low density lipoprotein oxidation hypothesis is pivotal to the explanation of the formation of fatty streak lesions. A wide range of atherogenic processes has been reported to be influenced by OxLDL and its components. The presence of OxLDL in lesions and plasma of patients with various forms of coronary artery diseases and other related metabolic disorder confirms the role of oxidized lipids in atherosclerosis. This conclusion led to numerous studies on the role of antioxidants in the prevention or treatment of atherosclerosis. However they did not yield uniformed outcome on the role of antioxidants in suppressing of the atherosclerotic process. Possible reasons might include discrepancies in experimental models, study designs, and schemes of treatment. Results shown in cell culture or animal models do not necessarily translate to similar results in human due to the major difference between the atherosclerosis development and stages in the animal models and human. Another factor that has not been tested yet is a possible inhibition of oxidation of OxLDL-released aldehydes by antioxidants. If oxidation of aldehydes is inhibited, they modify proteins and cause wide spectra of biological effects that exaggerate atherosclerotic processes. The future studies on the role of antioxidants in atherosclerosis should take in consideration these factors.

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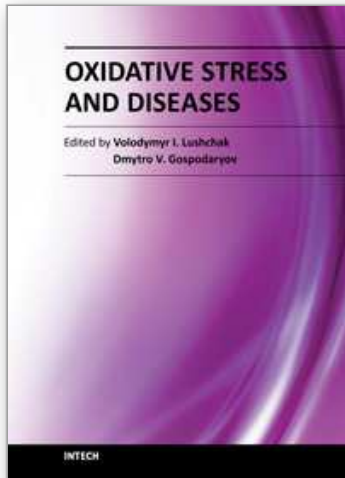
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## **Oxidative Stress and Diseases**

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The development of hypothesis of oxidative stress in the 1980s stimulated the interest of biological and biomedical sciences that extends to this day. The contributions in this book provide the reader with the knowledge accumulated to date on the involvement of reactive oxygen species in different pathologies in humans and animals. The chapters are organized into sections based on specific groups of pathologies such as cardiovascular diseases, diabetes, cancer, neuronal, hormonal, and systemic ones. A special section highlights potential of antioxidants to protect organisms against deleterious effects of reactive species. This book should appeal to many researchers, who should find its information useful for advancing their fields.

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