Chapter 3

Role of Oxidized LDL in Atherosclerosis

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Additional information is available at the end of the chapter

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1. Introduction

Nowadays, Atherosclerosis is the most important source of morbidity and mortality in the world, and is detected by the accumulation of lipids deposits (mainly cholesterol) in macrophages located not only in large but also in medium sized arteries. Currently, the association between atherosclerosis and heightened oxidative stress is widely accepted. Nevertheless, despite numerous efforts the role of oxidative stress in the progression of Atherosclerosis is still not clear.

Oxidation is a biochemical process of loss of electrons, which is essential for life due to its involvement in the production of cellular energy. However, when oxidation is excessive causing cellular damage is when Oxidative Stress appears. This process is complex; therefore, it cannot be measured or defined by a single parameter. For this reason, currently the interest lies on developing antioxidant therapies and diets enriched with antioxidants that prevent or at least decrease cellular damage and atheromatous plaque formation originated by the excess of oxidative stress.

Aim. The aim of this review is to analyze the state of the art on oxidized LDL role within the pathogenesis of atherosclerosis.

This chapter will be developed according to the following titles,

1. Oxidative stress and atherosclerosis.

2. LDL Oxidation

3. OxLDL in atheromatous plaque formation

4. Study Models

5. OXIDATIVE STRESS AND ATHEROSCLEROSIS

The word “Atherosclerosis” comes from the ancient Greeks where "sclerosis” means hardening and “athere” is gruel or accumulation of lipid. The physiopathological process is characterized by the aggregation of cholesterol, infiltration of macrophages and the proliferation of smooth muscle cells (SMCs) as accumulation of connective tissues and thrombus creation. In early stages of the disease, the growth of the lesion starts in the sub endothelial space and its progress may even cause total cessation in blood flow with intermittent periods of quiescence. The accumulation of lipids and other organic molecules lead to a proliferation of certain cell types within the arterial wall that gradually impinge the vessel lumen and block up the blood flow in large and medium sized arteries. Furthermore, this disease tends to be more common in white than black men [1]. The magnitude of this problem is deep, because atherosclerosis claims more lives than all types of combined cancer and economic costs are considerably high[2]. Currently, the idea that atherosclerosis constitutes a state of high levels of oxidative stress is widely accepted and this phenomenon is associated with lipid and protein oxidation in the vascular wall[3, 4].

Despite the countless efforts made to explain the role of oxidative stress in progression of atherosclerosis, its predictive role is still not clear. Goldstein and Brown discovered the LDL incorporation process in peripheral cells as fibroblasts, macrophages and others -which meant for them the Nobel Prize- has been the basis for a series of subsequent discoveries, from 1979 up to now, which have intended to explain the development of the atherosclerosis’ process [5-8]. The hypothesis of oxidative modification in atherosclerosis, reviewed by Steinberg and others in several opportunities argues that the oxidation of low-density lipoprotein (LDL) is an early stage of the disease and that oxidized LDL (OxLDL) would contribute to atherogenesis [9-12].

Until 1991, the strength of the scientific evidence regarding the role of the oxidation of LDL in the phenomenon of atherosclerosis was such that the National Heart, Lung and Blood Institute recommended the initiation of clinical trials [8, 13-17]. In relationship to this hypothesis, based on *in vitro* assays, the evidence showed the following relevant aspects: 1) the LDL oxidation is the first event in the foam cell formations [18, 19] the LDL lipids in human arterial lesions are extensively oxidized and 3) the presence of Ox LDL is evident *in vivo*[20]. On the other hand, the existence of several structurally unrelated compounds such as probucol and vit E that inhibit atherosclerosis in animals, prevent the initiation of the disease due to a reduction of the oxidization of LDL [21]. In relationship to probucol, It seems to be a more effective protection against lesion formation on an early-stage of the disease than the statin-mediated lipid-lowering effects [22].

The events involving the process of atherosclerosis begin with LDL oxidation in the vascular wall. This happens due to the production of reactive oxygen species (ROS) and nitrogen species (NOS) by endothelial cells, therefore, oxidative modifications would be crucial in the clinical aspect of coronary artery disease such as endothelial dysfunction and plaque disruption [23].

Although it is known by scientific evidence that LDL oxidation plays a central role in the pathophysiology of atherosclerosis, up to now there is no convincing proofs related to the protective effect of antioxidant therapy as a way to prevent the damage caused by that process on vital macromolecules such as lipids, proteins and DNA.This may be due to the discrepancies

between human and animal studies that use antioxidant therapies either to try or to limit the atherosclerotic process and cardiovascular events. It is not clear if oxidative stress is cause or consequence of the atherogenic process. In this sense, it has been proposed that inflammation could be considered as a primary process and oxidative stress as a secondary event of atherosclerosis [24].

2. LDL oxidation

Oxidation is a biochemical process of loss of electrons, which is essential for life due to its involvement in the production of cellular energy. Oxidative stress appears when oxidation is excessive. This apparently simple process is actually complex in all biological levels, and cannot be measured or defined by a single parameter.

The oxidation process of lipids and proteins is the result of an excess of free radical and other oxidant species derived from oxygen, nitrogen and other chemical elements in the body. Chemically, the oxidative stress is associated with an increased production of oxidizing species or a significant decrease in the effectiveness of antioxidants defenses such as reduced glutathione, catalase, peroxidases and others. The cell proliferation and death are key processes in the progression of atherosclerosis and severe oxidative stress can cause cell death and even mild oxidation can trigger cellular stress and apoptosis, while more intense stress may cause necrosis [25].

There is a constant production of ROS and other oxidative species derived from the normal and xenobiotic metabolism, ionizing radiation and smoke snuff exposure, among others. Oxidative molecules can exert positive or negative effects over cells and tissues, depending on their concentration. ROS plays an important role in several physiological cell processes, such as signaling and regulation cascades, however excesses can induce chemical and structural modifications which has been proven that alter the function of cellular components, inhibit protein function, induce DNA damage, viral activation and lipid peroxidation which can promote cell death (Figure 1).

In addition, redox systems such as gluthation peroxidase, thioredoxine reductase and pyridine nucleotide redox status can change their physiological function when modified by ROS and others reactive species, affecting the normal cell signaling including apoptotic cell death [26].

Today there are clear proofs that LDL oxidation plays a significant role in atherogenesis. In fact, this has been demonstrated throughout time. So, between 1985 and 1989, 62 papers about OxLDL were published; between 1992 and January 1997, the number of publications related to OxLDL went up to 727, and up to day only considering PubMed entry, it is possible to find over 7000 publications associated with the key words Oxidized LDL. This growing interest is supported by the large amount of evidence which confirms that oxidative modification of LDL plays a pivotal role in atherosclerosis and hence, makes it an obvious target for therapeutic approaches [10, 27].

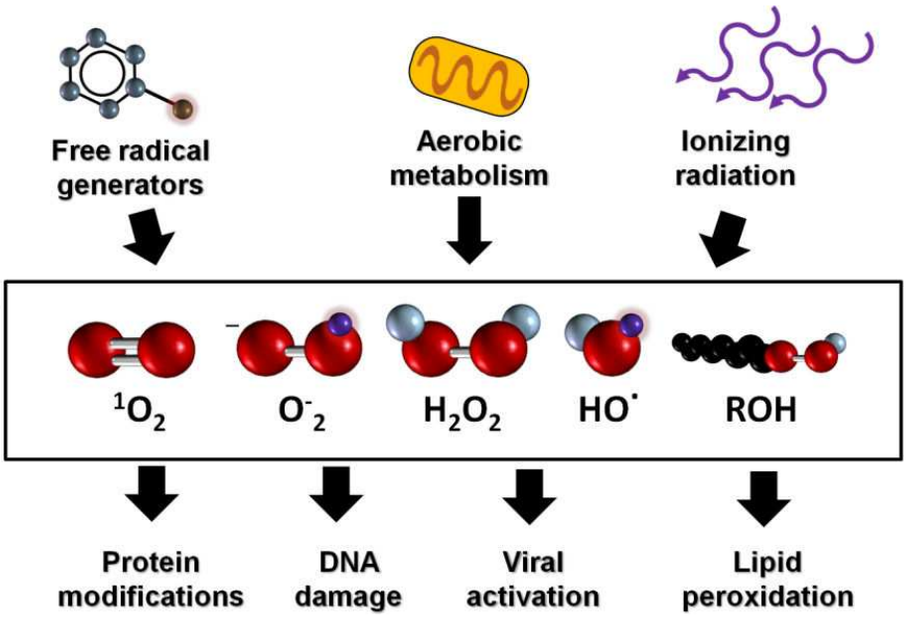

Figure 1. The figure shows some sources and consequences of oxidative stress.

In 2002, Friedman et al. showed that the oxidized lipids from OxLDL are biologically active. Specifically, polyunsaturated fatty acids (PUFA) either free or bound to an ester from phospholipid are converted into hydroperoxides, which break down to form highly reactive molecules, such as malondialdehyde and 4-hydroxynonenal among other metabolic products. These reactive aldehydes can then form Schiff-bases, covalent Michael-type adducts with lysine residues of apolipoprotein B in LDL molecules. Besides, the sn-2 oxidized fatty acid fragments which can remain attached via ester bridges may also contain terminal reactive aldehydes. However, this reactive phospholipid also called "aldehyde phospholipid core" may also form adducts with Schiff-base lysine residues of apolipoprotein B and presumably also with other proteins and amines-containing phospholipids, such as phosphatidylethanolamine and phosphatidylserine (Figure 2).Finally, the authors proved that when LDL presents substantial oxidative modifications, a great number of neoepitopes are generated transforming it in a highly immunogenic LDL. Indeed, there are a variety of autoantibodies directed to epitopes of OxLDL derived from specific oxidation in animals and human, that appear to increase in individuals with clinical and morphological signs of atherosclerosis [28].

On the contrary, OxLDL is thought to promote atherosclerosis through complex inflammatory and immunologic mechanisms that lead to lipid dysregulation and foam cell formation. Matsuura et al (2006) proposed that in the intima of atherosclerotic lesions, the OxLDL forms complex with the Beta 2 glycoprotein I (beta2GPI) and / or C-reactive protein (CRP). In patients with systemic lupus erythematosus (SLE) and/or antiphospholipid syndrome (APS), anti-OxLDL/beta2GPI complex autoantibodies have been found which has been significantly related to arterial thrombosis. In a non-immunized animal model of APS (NZWxBXSB F1 mice), it was demonstrated that anti-OxLDL/beta2GPI complex IgG autoantibodies can emerge spontaneously. Moreover, a monoclonal autoantibody (WB-CAL-1; IgG2a) against a complex beta-2-GPI was derived from the same mice. WB-CAL-1 significantly increased the

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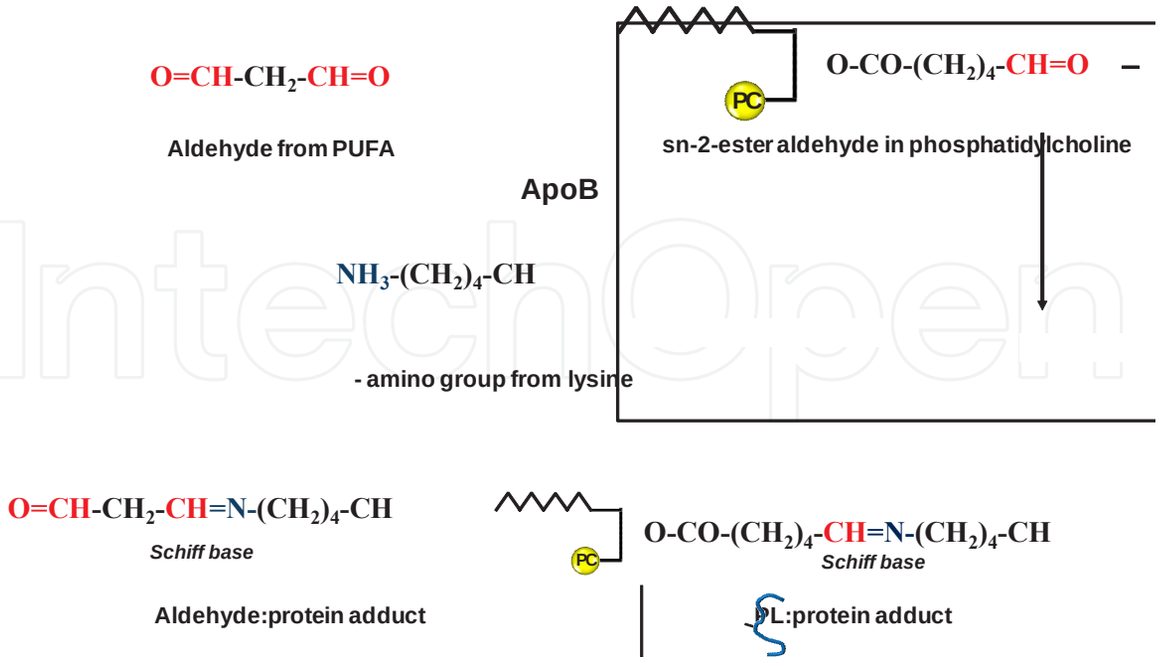

Figure 2. Oxidative modifications in ApoB present in lipoproteins.

in vitro uptake of OxLDL/beta-2-GPI complex by macrophages, suggesting that these IgG auto antibodies are pro-atherogenic. As opposed, IgM antibodies to OxLDL found in pro- atherogenic mice ApoE ($^{-/-}$) and LDL -R ($^{-/-}$) seemed to be protective. In human beings it has been widely reported the presence of IgG anti-Ox - LDL antibodies, but their clinical significance is not clear yet [29].

In the beginning, OxLDL was characterized by its biological properties, specifically for being a ligand for acetyl LDL receptor instead of a native LDL receptor. The Acetyl LDL receptor, present in macrophages, uptakes the OxLDL much faster than the native receptors, favoring the excessive intracellular accumulation of cholesterol. LDL oxidation and its uptake can be accomplished *in vitro* by an overnight incubation with macrophages cultured on an appropriate medium with 5 - 10 μM Cu^{2+} (oxidant) for 8-16 h allowing the study of the mechanism and kinetics of OxLDL.[30]

LDL oxidative modification, produces numerous structural changes, resulting in an increment of electrophoretic mobility, higher density, a polipoprotein B degradation, hydrolysis of phosphatidylcholine, changes on the amino groups of lysine residues and generation of fluorescent adducts caused by the covalent binding of lipid oxidation products to Apo B[31].

In vitro assays have shown that oxidative modification of LDL can be mutated by cultured endothelial cells or by cupric ions, which results in an increase of the lipoprotein uptake into macrophages [32, 33]. Therefore, it seems to be obvious that LDL oxidation is a crucial step for macrophage-derived foam cells formation in early stages of an atherosclerotic lesion. Moreover, LDL can be oxidized by specific enzymes such as lipooxygenase and phopholipase A2, even when these modifications are not necessarily identical to the endothelial cells-dependent

modifications, they are still useful for studying oxidative alterations of LDL. In fact, in 1998, it was demonstrated that the oxidative modification of LDL by specific enzymes leads to an increased recognition by macrophages [32]. In conclusion, it is possible to say that oxidation of LDL in cells depends on at least three possibilities: (a) lipid oxidation by the action of lipoxygenase within the cells followed by the LDL exchange on its medium; (b) direct lipoxygenase-dependent lipid oxidation during cell contact with LDL and (c) both possibilities mentioned above [34].

It is has been reported that the *in vitro* addition of acetyl groups to LDL (acetylation), generates a modified LDL which can induce cholesterol accumulation in macrophages. Indeed, acetylated LDL is incorporated by "scavenger receptors" (SRA), which in contrast to the normal LDL receptor, are not "down regulated", so they induce a great intracellular lipid accumulation. Thus, acetylated LDL increases the formation of foam cells [35].

Another process that needs to be taken into account is the autoxidation of glucose or the early glycation products (carbonyl compounds) generated by oxygen free radicals (superoxide and hydroxyl) and hydrogen peroxide that can cause oxidative damage. Baynes et al in 1991 introduced the "glycoxidation hypothesis", which proposes that oxidative stress concomitant to glycation plays an important role in the stage of advanced glycation of proteins. Modifications of lipoproteins by glycation and oxidation alter their structure to make them sufficiently immunogenic. In type 2 diabetes, high titles of antibodies have been found against glicosylated-LDL and glicosylated-OxLDL. Immunogenic properties of glycosilated-OxLDL induce immune complex formation. It has been shown that glycosilated-OxLDL is trapped in the artery wall *in situ* [36-40].

Various pathologies can be originated by oxidative stress-induced apoptotic signaling which is a consequence of an increase of ROS and a decrease of other oxidative species and/or antioxidants, disruption of intracellular redox homeostasis and irreversible oxidative modifications of lipids, proteins or DNA. A better understanding of redox control over the development of apoptotic process in the cell, could better guide the course of the therapeutic strategies associated with disorders related to oxidative stress [25].

A great number of diseases have been related to oxidative stress and generation of free radicals, for this reason, antioxidant therapies and diets (such as Mediterranean diet) rich or enriched with antioxidants are thought to be a promising way to prevent or at least to attenuate the organic deterioration originated by the excessive oxidative stress.

3. OxLDL in atheromatous plaque formation

Atherosclerosis is a chronic inflammatory disease of the arterial wall that culminates with the atheromatous plaque formation. At present, there is a consensus that oxidation of LDL in the endothelial wall is an early event in atherosclerosis, according to the oxidative hypothesis [24]. First, the circulating LDL particles are transported from the vascular space into the arterial wall, mainly across trancytosis[41]. LDL is retained in the extracellular matrix of subendothe-

lial space, through the binding of basic aminoacids in a polipoprotein B100 to negatively charged sulphate groups of proteoglycans in the extracellular matrix (ECM) [42, 43], where it is prone to be oxidized by oxidative stress, generating OxLDL[21], as we previously mentioned in this article.

It is known that OxLDL participates actively in atheromatous plaque formation, where it is retained. Multiple studies provide evidence suggesting OxLDL contribute in atherosclerotic plaque formation in several ways. In fact, at least four mechanisms have been proposed, being they complementary to each other: a) endothelial dysfunction, b) foam cell formation, c) SMCs migration and proliferation and c) induction of platelet adhesion and aggregation.

3.1. Endothelial dysfunction

The Endothelial dysfunction is a pathological condition in which the endothelium presents an impairment of anti-inflammatory, anti-coagulant and vascular regulatory properties. Nowadays, it is considered a key event in the atherosclerosis development. OxLDL formed and retained in the sub-entothelial space, activates endothelial cells (ECs) through the induction of the cell surface adhesion molecules which in turn, induce the rolling and adhesion of blood monocytes and T cells. It is reported that OxLDL induces the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular-cell adhesion molecule-1 (VCAM-1), increasing the adhesive properties of endothelium in a similar manner to the effects of pro-inflammatory cytokines as interleukin 1 beta [44].

The blood leukocytes recruited migrate into the tunica intima, guided by chemokines. Indeed, OxLDL stimulates ECs and SMCs to secrete monocyte chemotactic protein-1 (MCP-1) and monocyte colony stimulating factor (mCSF) that induce the recruitment of monocytes into the endothelial wall [45-47]. On another hand, OxLDL can be chemotactic itself for monocytes and T lymphocytes (since it possesses lyso-phosphatidylcholine) and also for macrophages [48].

Nitric oxide (NO), is recognized as an important cardiovascular protective molecule, because exerts vasodilator properties and inhibits the adhesion of leucocytes and platelets to endothelium. This is generated in the vasculature by endothelial NO sythase (eNOS); the impairment of NO production and secretion by ECs is considered one of the most important characteristic of endothelial dysfunction [49].

The NO production from ECs is inhibited by OxLDL, given that the OxLDL is able to induce cholesterol depletion in the plasma membrane invaginations called caveolae, which causes the translocation of the protein caveolin and eNOS from the membrane domains, inhibiting eNOS activity in ECs [50]. Besides, another mechanisms to explain the inhibitory effect of OxLDL over NO production in ECs, has been proposed. It has been reported that OxLDL leads to an increased oxidative stress in ECs, producing significant amounts of superoxide, which chemically inactivates NO, forming peroxynitrite [51].

Lectin-like oxidized LDL receptor-1 (LOX-1), identified as the mayor OxLDL receptor in ECs, is expressed in several pro-inflammatory conditions and seems to play a crucial role in endothelial dysfunction induced by OxLDL[52]. Indeed, in human atherosclerotic lesions, LOX-1 overexpression in ECs has been reported, especially in the early stage of plaque

formation [53]. It has been observed that the knockdown of LOX-1, inhibits the MCP-1 expression in human ECs stimulated with OxLDL and mitogen-activated protein kinase (MAPK) pathway would play a critical role [54]. Also, up-regulation of endothelial adhesion molecules as ICAM-1 and VCAM-1, can be induced by OxLDL in a LOX-1-dependent manner and this is mediated by the nuclear factor κB (NF-κB) [55]. Furthermore, the inhibitory effects of OxLDL over endothelial NO productions has been associated with LOX-1 function [51, 56]. Finally, it has been proposed that OxLDL can induce endothelial cell death through the activation of NF-κB and AP-1 pathways [57], worsening endothelial dysfunction and promoting the progression of the atherosclerotic plaque.

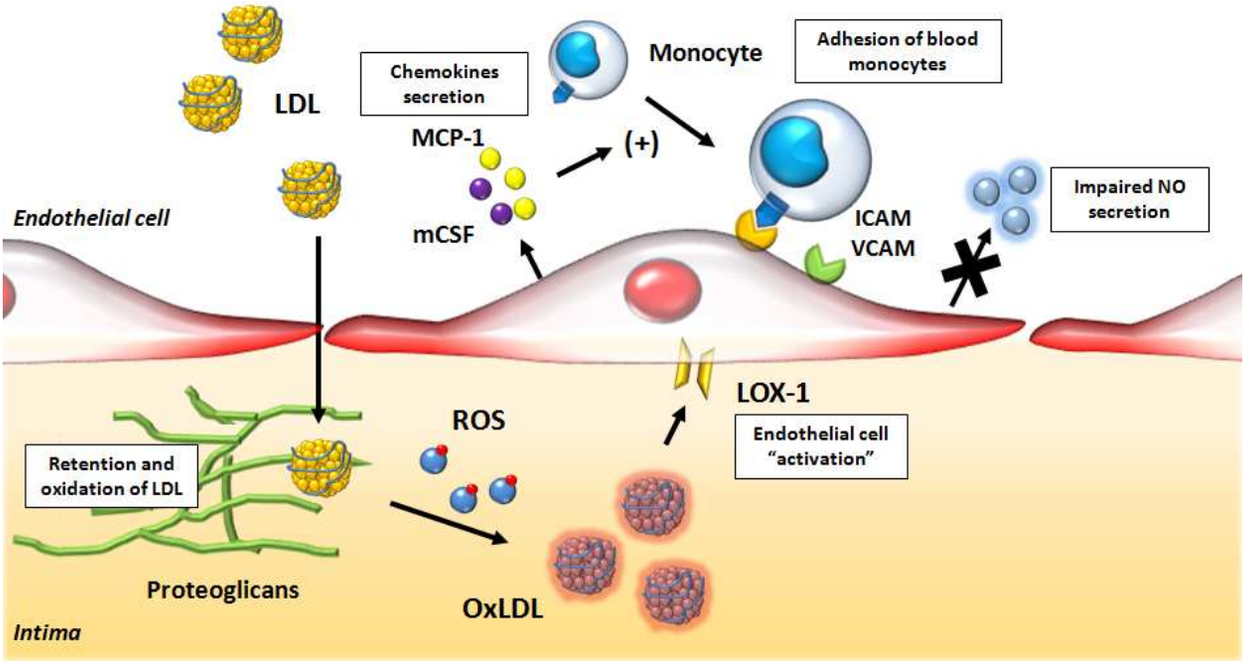

Figure 3. Role of OxLDL in endothelial dysfunction.

3.2. Foam cell formation

Once inside the sub-endothelial ECM, monocytes differentiate into macrophages that express several scavenger receptors (SRs) such as SR-AI/II, SR-BI, cluster of differentiation 36 (CD36) and LOX-1, and toll-like receptors (TLRs). It is important to remark that this phenotypic change, since the internalization of native LDL, occurs at a very low rate to account for foam cells formation and this process is prone to suffer down regulation of LDL receptor. In contrast, scavenger receptors have high affinity for OxLDL and they are not down regulated, leading to a massive intracellular lipid accumulation [20], which results in the foam cells formation [58, 59]. This differentiation into macrophages that promotes pro-inflammatory milieu, is part of a "macrophage trapping", a vicious circle that involves cell retention, oxidation of new LDL and the recruitment of more monocytes [18].

OxLDL also induce the expression of a number of genes associated to inflammation in macrophages: MCP-1, serum amyloid A, ceruloplasmin and hemeoxigenase-1 [60]. Moreover, macrophage activation induces the release of pro-inflammatory cytokines (interleukin 1-β, tumor necrosis factor), reactive oxygen species (ROS) and metalloproteases, which are associated with progression of inflammation [58].

Internalized OxLDL provides oxidized lipids as ligands for PPAR-γ pathway, upregulating CD36 expression, facilitating in turn, the internalization of more OxLDL[61, 62]. This internalization activates the macrophage, inducing the secretion of cytokines that recruits immune cells to intima and the secretion of the enzymes myeloperoxidase and 12/15-lipoxigenase, which are thought to participate in the oxidization of new LDL, increasing the local pool of OxLDL[63, 64]. Also, the internalization of OxLDL by CD36 seems to induce the inhibition of macrophage migration, favoring cell spreading and the activation of focal adhesion kinase, in a process mediated by src-kinases and oxidative stress [65]. Besides, OxLDL-CD36 interaction induces the loss of cell polarization in macrophages, an essential process to cell migration [66]. Thus, the evidence suggests that OxLDL not only participates in monocyte differentiation and macrophage activation, but also macrophage retention.

As mentioned, LOX-1 is one of the SRs expressed in macrophages and when it occurs by the influence of pro inflammatory cytokines, OxLDL or other stimuli, the OxLDL uptake increases significantly favoring the foam cells formation [67, 68]. The accumulation of OxLDL can lead to foam cell apoptosis or necrosis, forming cellular debris deposited in the core of the atherosclerotic plaque and contributing to inflammatory progression.

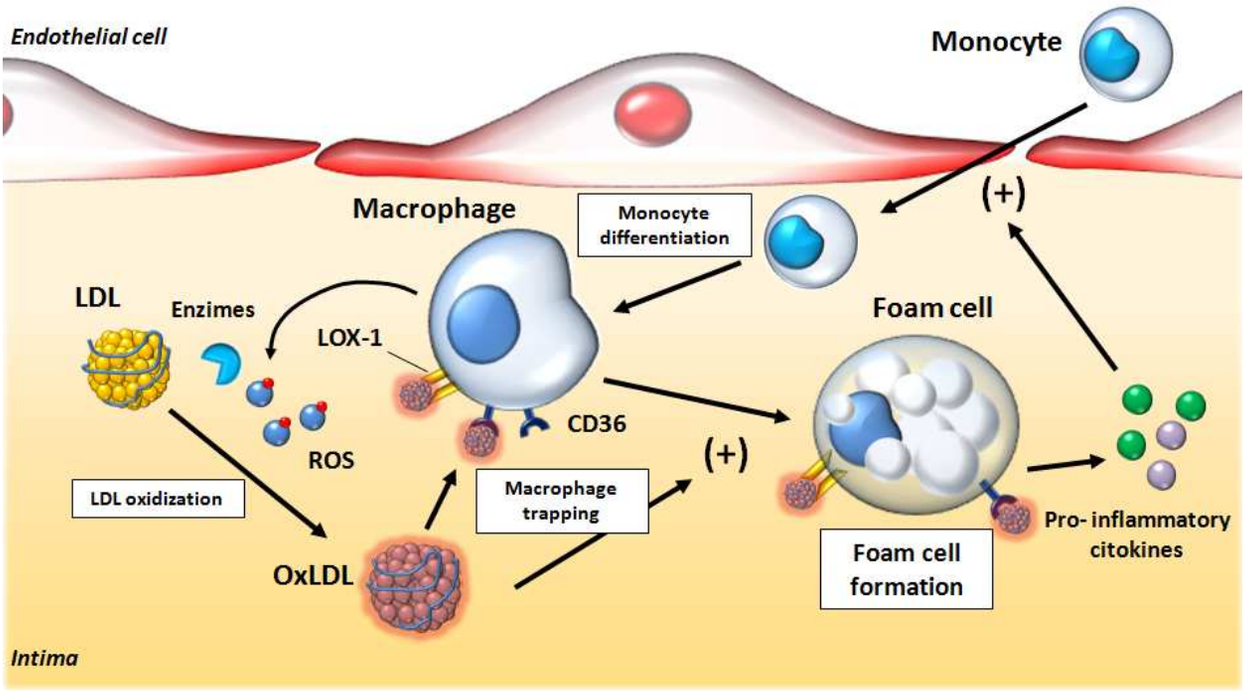

Figure 4. Role of OxLDL in foam cell formation.

3.3. Smooth muscle cell migration and proliferation

The migration and subsequent focal proliferation of SMCs in tunica intima are some of the hallmarks of the atheromatous phenomenon and they play a critical role on it. The SMCs migrate from tunica media to the subendothelial space, where they proliferate in response to growth factors. The proliferation of SMCs can be stimulated by OxLDL, since these particles enhance platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) expression and secretion [69, 70] by ECs and macrophages. On the other hand, OxLDL also induces the secretion of a variety of other growth factors and their receptors: insulin-like growth factor-1(IGF-1) and epidermal growth factor (EGF), all with mitogenic effects inducing SMCs proliferation [71].

OxLDL has also been shown to induce changes directly in SMCs. OxLDL increases migration and leads to changes in SMCs phenotype making them to produce large amounts of ECM [72]. The production of interstitial collagen and elastin leads to the building of a fibrous cap that covers the developing atherosclerotic plaque, forming a "necrotic core" containing foam cells, cellular debris, extracellular lipids and lysosomal enzymes [73]. Thus, OxLDL participate in the expansion of the atherosclerotic lesion size.

OxLDL also induce LOX-1 expression in SMCs and recently, it has been proposed that many of the named effects of OxLDL are mediated by LOX-1[71]. Another important effect mediated by LOX-1 is the increment of ROS generation induced by OxLDL in SMCs, which can induce the cell death, contributing to plaque instability and rupture in the final stage of atherosclerosis [74]. Taken together, the evidence suggests that OxLDL has a crucial role in the plaque instability and hence, in the development of its complications.

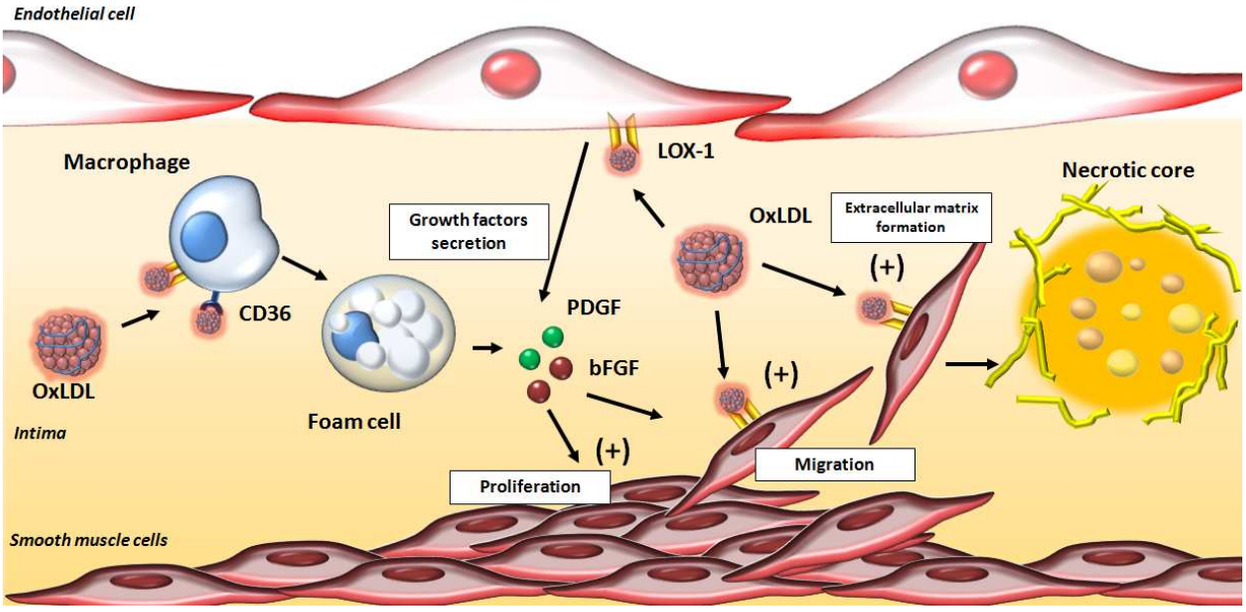

Figure 5. Role of OxLDL in smooth muscle cells proliferation and migration.

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3.4. Induction of platelet adhesion and aggregation

Platelets are important players in atherosclerosis plaque development, especially after the plaque rupture, where they promote thrombus formation. In this process, OxLDL also is implicated. The impairment of endothelial NO production by OxLDL has been associated with an increase in prostaglandin secretion and thus, platelet aggregation [73]. CD36 is expressed in resting platelets and its interaction with OxLDL has been implicated with platelet activation, evidenced by P-selectin expression and the activation of integrin $\alpha_{IIb}\beta_3$[75].

OxLDL seems to induce a hyperactive state in platelets, since when they are cultured with OxLDL, they show more sensitivity to the classic platelet-activator ADP, in a process mediated by JNK and Vav family members [76, 77]. OxLDL is able to promote shape change and fast platelet activation through the action of Src kinases and Rho kinase-signaling pathways [78]. The effects of OxLDL over platelets could account also for additional pro-atherogenic phenomena. Platelets exposed to OxLDL release chemokines that favors atherosclerotic development [79] and promote endothelial dysfunction and foam cells formation [80, 81].

LOX-1 is expressed in platelets once they are activated [82], where it contributes to OxLDL internalization together with CD36. Since LOX-1 is able to binds anionic phospholipids as the present in the surface of activated platelets, has been proposed that endothelial LOX-1 mediates platelet adhesion to ECs [68]. Indeed, platelet binding to LOX-1 enhances endothelin-1 (ET-1) release from ECs [83] and induces oxidative inactivation of NO in ECs [56], suggesting that LOX-1 participates in endothelial dysfunction also through activated platelets. Thus, OxLDL seems to play a pivotal role in the pro-atherosclerotic behavior of activated platelets.

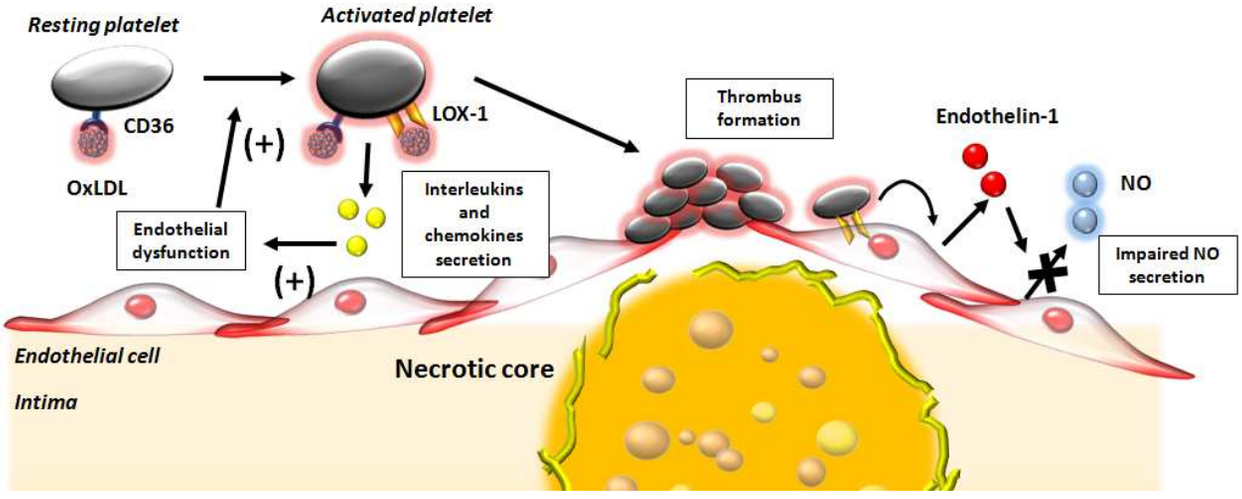

Figure 6. Role of OxLDL in pro-atherosclerotic function of platelets.

4. Mouse models for atherosclerosis

Given the importance of knowing the role of oxidized LDL in the process of atherosclerotic plaque formation, the study of animal models has been an important tool, where the examination of genetically modified mice has significantly contributed towards a better understanding of the mechanisms involved in this pathology.

It is worth noting that the use of small animals in research benefits from easy availability and low cost compared to large animals like primates. In addition, working with small animals reduces ethical concerns and limits the quantity of new agents needed for *in vivo* studies.

Transgenic and knockout mouse models for atherosclerosis have also been instrumental in evaluating existing, finding and testing new atherosclerotic drugs [84]. Small-animal models have the advantage of a well-defined genetic characterization which opens the possibility to transform them into transgenic and gene knockout animals [85].

Atherosclerosis is it not developed by wild-type mice; in fact they have high levels of anti-atherogenic high-density lipoprotein (HDL) and low levels of pro-atherogenic LDL and very-low-density lipoprotein (VLDL). Furthermore, mice do not express cholesteryl ester transfer protein (CETP), a plasma protein known to transfer cholesteryl ester from HDL particles and other lipoprotein fractions to pro-atherogenic apolipoprotein-B-containing lipoproteins LDL, VLDL and intermediate low-density lipoproteins (ILDL).

The current mouse models of atherosclerosis are based on disorders on the metabolism of lipoproteins through diets or genetic manipulations [84]. These perturbations have been made thanks to the current availability of genetic information, a variety of inbred strains and the development of molecular biology technics [86]. Atherosclerotic mice were first reported by Thompson et al., 1969, [87] using C57BL/6 inbred mice fed for five weeks with a diet containing a 50% of fat, whereas control mice were fed with a regular diet of 5% of fat. Nevertheless, this diet had a high percentage of mortality [86]. Paigen et al. modified the diet proposed by Thompson supplementing it with a regular diet containing 1.25% of cholesterol, 0.5% of cholic acid and 15% of fat. Nowadays, this diet is named the "Paiged diet" [88]. However, Ignatowski et al., [89] reported in 1980 the first evidence of atherosclerosis in the aorta of a rabbit model fed with a diet containing animal proteins like meat, eggs and milk.

Nowadays, the most used model of atherosclerosis in mice is based on the alteration of genes that codify the low-density lipoprotein receptor (LDLr) and the apolipoprotein E (ApoE), being both key elements for the lipid metabolism.

5. Apolipoprotein E-deficient (*Apo E* $^{-/-}$) mice

The ApoE, is a glycoprotein found in almost every lipoprotein with the exception of LDL. The purpose of this glycoprotein mainly synthesized in the brain and liver is to serve as a ligand for receptors that removes the VLDL and chylomicrons remnants. Since the ApoE can also be

synthesized by macrophages and monocytes in the atherosclerotic vessels, is thought to have an important role on inflammatory processes and on the cholesterol homeostasis [90]. Moreover, it has been reported that ApoE may function in the biliary excretion and in dietary absorption of cholesterol [91].

Plump et al., [92] in 1992, produced the first mice models deficient in apolipoprotein E (*ApoE*$^{-/-}$). These animals were fed with a diet of 4.5% fat to develop a strong atherosclerosis model. This became an important tool in the research of atherosclerosis.

To inactivate the mice's *ApoE* gene, a homologous recombination of genes was made in embryonic stem cells. Two plasmids (pNMC109 and pJPB63) with a neomycin-resistance gene were used to disrupt the structure of the *ApoE* gene. Chimeric mice were generated by blastocyst injection with targeted lines [93]. The fact that homozygous animals were born at the expected frequency and that they appeared to be healthy, was of significant importance.

Currently, the *ApoE*$^{-/-}$mice are available on Jackson Laboratories which are direct descendants of the original *ApoE*$^{-/-}$ mouse created by the Maeda group (002052 B6.129P2-Apoe^{tm1Unc}).

Under a normal chow-fed diet, the mice developed a fatty streak observed in the aorta as early as a 3-month-old [93]. Foam cells at 10 weeks of age under the same diet were observed using a light microscopy. At 15 weeks of age, lesions containing SMCs and foam cells were observed, and at 20 weeks of age, fibrous plaques could be seen. It is worth mentioning that when a Western diet is used, the process is accelerated [94].

Although it is known that this model of *ApoE*$^{-/-}$ has considerable limitations, it has been used widely, because of the rapid development of atherosclerosis. A major drawback of the complete absence of ApoE protein is that most plasma cholesterol is confined to VLDL and not to LDL particles as in humans.

6. LDL receptor-deficient (*LDLr*$^{-/-}$) mice

In humans, mutations in the *LDLr* gene cause familial hypercholesterolemia. The *LDLr*$^{-/-}$mouse has a milder lipoprotein alteration than *ApoE*$^{-/-}$mice when fed standard low-fat chow, with plasma cholesterol levels around 250 mg/dL due mainly to the accumulation of LDL [95].

In 1993, *LDLr*$^{-/-}$ mice were created by gene targeting of embryonic stem cells [96]. By feeding them with a 10% fat diet, an increase of total cholesterol level (2-fold) was observed on these mice, due mainly to the high levels of VLDL and LDL. When fed on a high-fat/high-cholesterol diet, *LDLr*$^{-/-}$ mice showed a rapid increase in the severity of hypercholesterolemia and atherosclerotic lesion development throughout the coronary arteries, aortic root, and aorta [85, 97]. The plasma lipoprotein profile of *LDLr*$^{-/-}$ mice resembled the one of humans, with the cholesterol being confined mainly to the LDL fraction. Nevertheless, this mice model of atherosclerosis is very responsive to the diet. In fact, their cholesterol levels rose up to 1500 mg/dL when they were under the Paigen diet [98]. The lesions produced in *LDLr*-/- mice were similar to the lesions produced in the *ApoE*-/- mice, in terms of their development of plaques

in a time-dependent manner. On the contrary, the *LDLr-/-* mouse produced a more moderate murine model of atherosclerosis than the *ApoE-/-* mouse. This characteristic is produced mainly due to a milder degree of hyperlipidemia [99].

In 1998, a mouse model deficient in the Apo B mRNA editing activity (*Apobec1*$^{-/-}$) and LDL receptor (*LDLr*$^{-/-}$) were generated by Powell-Braxton et al [100]. The lipoprotein profile of this animal model resembles the human familial hypercholesterolemia and when fed with a chow diet, exhibited atherosclerosis at its 8-month of age. The characteristics of this animal model provided an advantage to study the interactions between the environment (high fat diet) and the gene response in the onset of atherosclerosis [101].

7. ApoE3-leiden transgenic mice

Mutations in the gene encoding ApoE can lead to a binding-defective ApoE, which mediates the binding of lipoproteins to LDL receptor and is an essential ligand for the receptor-mediated uptake of chylomicron and VLDL remnants by hepatic lipoprotein receptors. This results in a disturbed receptor-mediated clearance of lipoprotein remnants by the liver, as has been described for patients with familial dysbetalipoproteinemia[102]. Premature atherosclerosis is produced by a genetic disorder named familial dysbetalipoproteinemia, which presents high levels of plasmatic triglycerides and cholesterol, mainly due to the increase in the VLDL remnants and chylomicron. The ApoE3-Leiden, a genetic variant of ApoE, is related with an inherited familial dysbetalipoproteinemia [103].

The ApoE3-Leiden mutation it is characterized by a rare dominant-negative tandem duplication of codons 120 to 126 in human *ApoE3* gene. Introducing a human ApoE3-Leiden gene construct into C57BL/6 mice has generated the ApoE3-Leiden transgenic mice. The ApoE3-Leiden gene consists of a construct with the *ApoC1*and *ApoE* genes with a promoter element to regulate the expression. While, this mice model of atherosclerosis still expresses ApoE protein, the clearance of lipoproteins containing ApoE is impaired, being less dramatic than the *ApoE*$^{-/-}$ mice model of atherosclerosis. The introduction of the *ApoC1* gene in transgenic mice has exhibited elevated levels of cholesterol and triglycerides owing to an accumulation of VLDL-size particles in the circulation, increasing plasma lipid levels by diminished lipolysis and VLDL uptake through both the LDLr and low density lipoprotein receptor-related protein (LRP) [84, 104].The ApoE3-Leiden mice have a hyperlipidemic phenotype, develop atherosclerosis on being fed cholesterol, and are more sensitive to lipid-lowering drugs than *ApoE*$^{-/-}$ and *LDLr*$^{-/-}$ mice [105]. The ApoE3-Leiden mice model is very responsive to diets containing sugar, fat and cholesterol, developing high levels of plasma triglycerides and cholesterol, with a prominent increase in LDL and VLDL lipoproteins.

Compared with *ApoE*$^{-/-}$ and *LDLr*$^{-/-}$ mice, ApoE3-Leiden mice represent a moderate mouse model for hyperlipidemia. Therefore, diets and drugs that influence the production of VLDL and chylomicron also show parallel effects on plasma levels of triglycerides and cholesterol. In this sense, the ApoE3-Leiden mice are more responsive to hypolipidemic compounds than the *LDLr*$^{-/-}$ and *ApoE*$^{-/-}$ mice [84, 106].

8. Double knockout mice models

A model that develops severe hyperlipidaemia and atherosclerosis was obtained with an *ApoE* and *LDLr* double knockout (*ApoE*$^{-/-}$/ *LDLr*$^{-/-}$ / DKO) [98]. It has been observed that, even on a regular chow diet, the atherosclerosis progression is generally more considerable than in mice only deficient in ApoE [107, 108]. Hence, the *ApoE*$^{-/-}$/, *LDLr*$^{-/-}$/, DKO mouse is appropriate to study the effect of compounds with anti-atherosclerotic activity without the need of atherogenic diets.

Besides, the role of the ApoE and the LDLr in the development of the atherosclerosis and dysregulation of the NOS system leading to impairment of NO bioavailability, has been documented for some time in atherosclerotic vessels of both experimental animals and humans [109]. To study the contribution of endothelial eNOS to lesion formation, Kuhlencordt et al. [110] created *ApoE*$^{-/-}$/ *eNOS*$^{-/-}$/double knockout mice (*ApoE*$^{-/-}$/ *eNOS*$^{-/-}$ / DKO), which presents a more pronounced atherosclerosis than ApoE-/- mouse model. Besides, eNOS absence favors the development of peripheral coronary disease, chronic myocardial ischemia, heart failure and an array of other vascular complications not detected in *ApoE*$^{-/-}$ mice [111].

Additionally, key structural proteins like apoB100 and apoB48, are needed to assemble lipoproteins rich in triacylglycerol; moreover, these proteins are part of all classes of atherogenic lipoproteins [112]. Veniant et al., 1998, characterized *LDLr*$^{-/-}$ and *ApoE*$^{-/-}$ mice which were homozygous to the ApoB-100 allele, founding that the *LDLr*$^{-/-}$/ApoB$^{100/100}$ mice model develop an extensive atherosclerosis, even when were fed with a normal chow diet, [113]

In summary, the experimental evidences show that the oxidative stress plays a pivotal role in atherogenesis, having OxLDL as a crucial player. Nevertheless, the clinical trials that used antioxidants strategies have shown poor results in relationship to the development of atherosclerosis, besides strong discrepancies between the different studies to establish the correlation between oxidative stress and atherogenic process. Therefore, the achievement of a successful therapy in humans based on the oxidative modification hypothesis is still a major challenge.

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