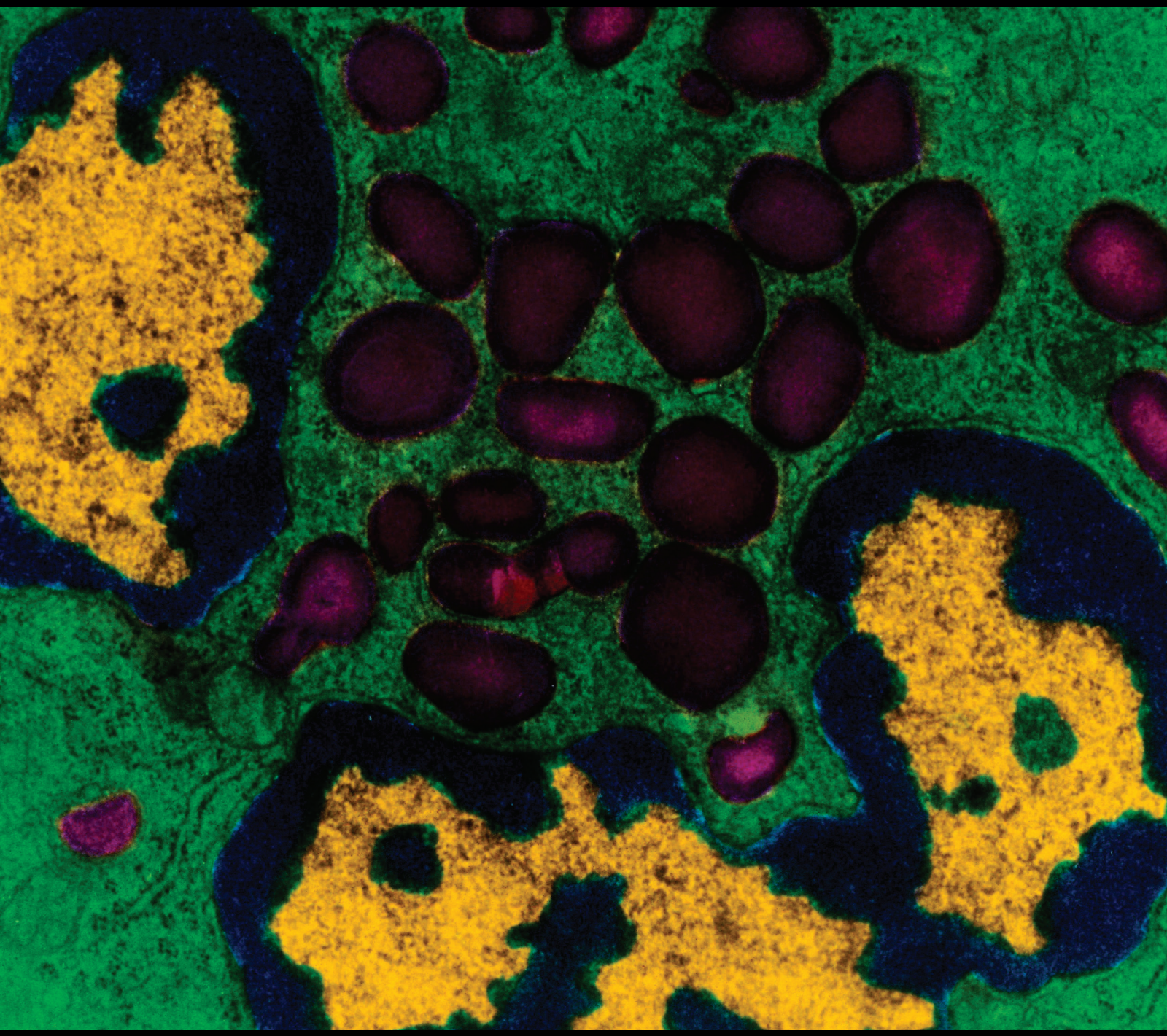
Mediators of Inflammation

Oxidized LDL in Inflammation: From Bench to Bedside

Guest Editors: Ishak Ozel Tekin, Asım Orem, and Ronit Shiri-Sverdlov



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Editorial Board

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Editorial

Oxidized LDL in Inflammation: From Bench to Bedside

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Oxidation of LDL is considered a marker of inflammation. Indeed, numerous studies have documented that oxidized lipids as well as products derived from their decomposition have deleterious biological properties. As such a better understanding of the underlying pathological mechanisms is necessary for the development of novel therapeutic agents and diagnostic tools. Multiple experimental data carried out in cellular lines and animal models of atherosclerosis support the proatherogenic role of oxidized LDLs through several mechanisms which include chemotactic and proliferating actions on macrophages, stimulation of smooth muscle cells, and eliciting apoptosis. While the role of oxLDL in relation to atherosclerosis has been investigated thoroughly, its role in other inflammatory diseases is recently emerging as well.

In this special issue, we report how findings regarding oxLDL in inflammation at the benchside can be translated to the bedside and how these observations may help clinical practice. The papers have been contributed by a number of experts in the field and include both review articles that provide an overview of the work conducted to date and original articles reporting recent discoveries and innovations. In order to highlight the translational relevance, several papers are focused on novel basic mechanisms as well as clinical evidence related to atherosclerosis. In addition, papers describing the novel link between oxLDL to COPD, endometriosis, and preeclampsia are included. We hope that this series of papers will be beneficial for clinicians and researchers in their diagnostic and therapeutic approaches towards inflammatory diseases. Each of the papers in this series is briefly highlighted as follows.

G. Maiolino et al. in the review "*The role of oxidized low-density lipoproteins in atherosclerosis: the myths and the facts*" examine the role played by oxidized LDLs in atherogenesis taking into account data derived by studies based on molecular and clinical approaches. The authors provide available data from animal models and patients cohorts that validate the oxidative modification hypothesis of atherosclerosis. Several experimental papers in this special issue support this view. The paper by F. Mascarenhas-Melo et al. "*Implication of low HDL-c levels in patients with average LDL-c levels: a focus on oxidized LDL, large HDL subpopulation, and adiponectin*" demonstrates that in a patient population with cardiovascular risk factors low HDL-c levels are associated with a poor cardiometabolic profile, despite the average levels of LDL-c. This data suggests that therapeutic interventions directed to inhibition of Ox-LDL levels and raising HDL-c levels and functionality are advisory preventive measures in this type of cardiovascular risk populations.

Furthermore, J.-F. Zhao et al. in "*Activation of TRPV1 prevents OxLDL-induced lipid accumulation and TNF-α-induced inflammation in macrophages: role of liver X receptor α*" demonstrate that suppression of oxLDL and activation of LXRα by TRPV1 is protective in macrophages. These findings may help in developing novel pharmacological targets for treating atherosclerosis-related cardiovascular diseases.

Finally, J. Li et al. in "*Minimally modified LDL upregulates endothelin type A receptors in rat coronary arterial smooth muscle cells*" investigated the effects of mmLDL on the expression of endothelin type A (ETA) receptors in coronary arteries. The authors demonstrated that mmLDL contributes

to the development of ischemic cardiovascular diseases by inducing an upregulation of ETA receptors in the coronary artery. The molecular mechanisms involve the activation of PKC and ERK1/2 MAPK pathways and the downstream NF-κB signalling pathways.

The lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is the main OxLDL receptor of endothelial cells, and it is expressed also in macrophages and smooth muscle cells. A. Pirillo et al. in "*LOX-1, OxLDL, and atherosclerosis*" describe the evidence from mouse and humans data, suggesting that LOX-1 might be an attractive therapeutic target for the management and the prevention of atherosclerosis. Inhibition of the LOX-1 receptor is currently being investigated and represents an emerging approach for controlling OxLDL-LOX-1-mediated proatherogenic effects. The important role of oxidized LDL and LOX-1 in inflammation is further discussed in the review "*Oxidized LDL and LOX-1 in experimental sepsis*" by N. Al-Banna and C. Lehmann. This review highlights the evidence relating oxLDL and LOX-1 to proinflammatory disease mechanisms. Situations in which oxLDL is involved in disease resolution due to exposure time, dose, or degree of oxidization are described. The emerging role of LOX-1 in the migration capacity of MSCs is demonstrated by F. Zhang et al. in "*Ox-LDL promotes migration and adhesion of bone marrow-derived mesenchymal stem cells via regulation of MCP-1 expression*." The authors investigated the effects of ox-LDL on bone marrow-derived mesenchymal stem cells (bmMSCs) migration and adhesion as well as the related mechanisms. They concluded that ox-LDL-induced bmMSC migration and adhesion are dependent on LOX-1 activation and MCP-1 expression. This study is important since the migration capacity of MSCs is one of the determinants of the efficiency of MSC-based transplant therapy.

The dual biological effect of electronegative low-density lipoprotein (LDL (−)) which is the minor modified fraction of LDL found in blood is presented in the review "*Electronegative LDL: a circulating modified LDL with a role in inflammation*" by M. Estruch et al. This review compares LDL (−) to oxLDL, updates findings on the inflammatory and anti-inflammatory effects of LDL (−) on cells, and discusses its putative physiological role.

M. Gursel et al. in "*Plasmacytoid dendritic cell response to CpG ODN correlates with CXCL16 expression and is inhibited by ox-LDL*" report that ox-LDL presence significantly inhibited D-ODN mediated IFNα production by plasmacytoid dendritic cells. Moreover the authors demonstrated that CXCL16/CXCR6 interaction can modify the response of pDCs to environmental danger signals.

F.J. Rios et al. in "*Oxidized LDL induces alternative macrophage phenotype through activation of CD36 and PAFR*" examined the effect of oxLDL on macrophage phenotype. The authors concluded that oxLDL induced macrophage differentiation and activation towards the alternatively activated (M2) phenotype. Furthermore, they showed that this profile of macrophage activation is dependent on the engagement of both CD36 and PAFR.

Among the different models of LDL oxidation that have been studied, the one using myeloperoxidase (MPO) is thought to be more physiopathologically relevant. K. Z. Boudjeltia et al. in "*Myeloperoxidase-dependent LDL modifications in bloodstream are mainly predicted by angiotensin II, adiponectin, and myeloperoxidase activity: a cross-sectional study in men*" suggest that the combination of blood MPO activity, angiotensin II, and adiponectin explains, at least partially, serum Mox-LDL levels. This is significant since serum Mox-LDL levels are involved in the pathogenesis of atherosclerosis. Another example for the important role of LDL oxidation by myeloperoxidase (Mox-LDL) in the context of atherogenesis is presented by C. Delporte et al. in the review "*Low-density lipoprotein modified by myeloperoxidase in inflammatory pathways and clinical studies.*" The review focuses on activation of endothelial cells and monocytes/macrophages, induction of proinflammatory molecules, and inhibiting of fibrinolysis as the main mechanisms by which Mox-LDL increases the risk of thrombus formation. Moreover, the role of Mox-LDL in the physiopathology of several diseases linked to atherosclerosis is also discussed. In addition to atherosclerosis, the involvement of oxLDL in other inflammatory diseases has recently emerged. G. Polak et al. in "*Low-density lipoproteins oxidation and endometriosis*" found that concentrations of oxLDL in peritoneal fluid of endometriotic women were significantly higher compared to women with serous but not dermoid ovarian cysts. Their results indicate that disrupted oxidative status in the peritoneal cavity of women with endometriosis may play a role in the pathogenesis of advanced stages of the disease. The possible relationship between COPD and ox-LDL has been investigated recently by Y. Shen et al. in "*Increased serum ox-LDL levels correlated with lung function, inflammation, and oxidative stress in COPD.*" The authors describe that serum levels of ox-LDL are increased in COPD patients and are correlated with reduced lung function, inflammation, and oxidative stress in COPD. Ş. Açikgöz et al. in "*Levels of oxidized LDL, estrogens, and progesterone in placenta tissues and serum paraoxonase activity in preeclampsia*" demonstrate that the events leading to fetoplacental insufficiency lead to a reduction in the levels of estriol limit deposition of OxLDL in placental tissues. These observations point towards the importance role of serum PON1 activity in the inhibition of OxLDL in preeclampsia.

Finally, "*Paraoxonase-1 inhibits oxidized low-density lipoprotein-induced metabolic alterations and apoptosis in endothelial cells: a nondirected metabolomic study*" summarized by A. García-Heredia et al. showed alterations in carbohydrate and phospholipid metabolism and increased apoptosis in cells incubated with oxidized LDL. These results extend current knowledge on the protective role of HDL and PON1 against oxidation and apoptosis in endothelial cells.

We sincerely hope that the present special issue may provide useful information to understand the mechanisms, the clinical effects, and the novel treatments of oxLDL-induced inflammation. We hope that the reader will find some novel input for future researches.

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Research Article

Myeloperoxidase-Dependent LDL Modifications in Bloodstream Are Mainly Predicted by Angiotensin II, Adiponectin, and Myeloperoxidase Activity: A Cross-Sectional Study in Men

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The present paradigm of atherogenesis proposes that low density lipoproteins (LDLs) are trapped in subendothelial space of the vascular wall where they are oxidized. Previously, we showed that oxidation is not restricted to the subendothelial location. Myeloperoxidase (MPO), an enzyme secreted by neutrophils and macrophages, can modify LDL (Mox-LDL) at the surface of endothelial cells. In addition we observed that the activation of the endothelial cells by angiotensin II amplifies this process. We suggested that induction of the NADPH oxidase complex was a major step in the oxidative process. Based on these data, we asked whether there was an independent association, in 121 patients, between NADPH oxidase modulators, such as angiotensin II, adiponectin, and levels of circulating Mox-LDL. Our observations suggest that the combination of blood angiotensin II, MPO activity, and adiponectin explains, at least partially, serum Mox-LDL levels.

1. Introduction

Atherosclerosis is an inflammatory disease involving a crosstalk between vascular cells, monocytes, proinflammatory cytokines, chemokines, and growth factors [1–3]. The current paradigm of early atherosclerosis claims that low-density lipoprotein (LDL) particles are trapped in the subendothelial space of the vascular wall where they can be oxidized. The precise physiological process for LDL oxidation *in vivo* is still largely unknown and the occurrence of LDL

oxidation outside the lesion sites has not definitively been ruled out yet.

Evidence accumulated during the last decade has suggested implication of myeloperoxidase (MPO) in inflammation leading to atherogenesis. MPO is produced by macrophages and neutrophils [4] and via its chlorination activity, MPO produces hypochlorous acid (HOCl) from hydrogen peroxide (H_2O_2) and chloride anion (Cl^-). HOCl can oxidize protein-bound amino acid residues among which the formation of 3-chlorotyrosine is considered as specific of the activity of MPO as the latter is the only human enzyme able to produce HOCl. In the context of atherogenesis, MPO, 3-chlorotyrosine, and MPO-dependent modified LDL (Mox-LDL) have all been detected in human atherosclerotic lesions and in the bloodstream [5–8].

We previously demonstrated that Mox-LDL generation could occur *in vitro* at the surface of the endothelial cells suggesting that it was not restricted to the subendothelial space *in vivo* [9]. The triad made up by endothelial cell, circulating LDL and MPO, allowed a synergic mechanism for producing Mox-LDL. The starting point of this reaction is the generation of superoxide anion (O_2^-) by the membrane bound nicotinamide-adenine-dinucleotide phosphate (NADPH) oxidase. O_2^- is further dismutated into H_2O_2 a substrate for MPO to produce HOCl. We recently reported, in two different clinical situations, that this indeed enables MPO to rapidly modify LDL and serum proteins by oxidations [8, 10]. Furthermore, NADPH oxidase is activated and upregulated by angiotensin II (ANGII) via the ANGII type I (AT1) receptor present at the surface of endothelial cells [11]. This enzymatic complex therefore plays a central role in the Mox-LDL generation [9].

Based on these data, we wondered whether there was an independent association between NADPH oxidase modulators, such as ANGII, adiponectin [12], and levels of circulating Mox-LDL. To test this hypothesis, we report the data observed in a cohort of male patients ($n = 121$) consulting for lower urinary tract symptoms (LUTS). Indeed, LUTS is associated with the erectile dysfunction, which is an early predictive sign for atherosclerotic cardiovascular events [13].

2. Material and Methods

2.1. Patients. Subjects were 121 males with a mean age of 58.8± 10.8 who consulted for lower urinary tract symptoms (LUTS) at the Erasme University Hospital. This study conforms with the Declaration of Helsinki and its protocol was approved by the Ethics Committee of the Erasme University Hospital. Finally, all subjects gave their written informed consent.

2.2. Standard Analyses. Blood samples were centrifuged for 10 minutes at 4000 g and the supernatant was collected and frozen. Blood tests were performed at the Laboratory of Experimental Medicine of the University Hospital of Charleroi, Site A. Vésale, Unit 222, ULB. The following parameters were measured: C-reactive protein (CRP), blood glucose, total cholesterol, triglycerides, HDL-cholesterol (standard laboratory techniques PLC), and adiponectin. LDL-cholesterol levels were calculated using the Friedewald formula (LDL-chol (mg/dL) = T-chol-HDL-chol-TG/5).

2.3. MPO and Mox-LDL Analyses. Mox-LDL was measured using a sandwich ELISA kit [9]. The specificity of the antibody was further assessed by analyzing LDL oxidized with peroxynitrite (0, 10, 100, and 1000 μM) and comparing with LDL oxidized by MPO/hydrogen peroxide/chloride. Other oxidants produced by MPO such as HOSCN/$^-$OSCN, HOBr/$^-$OBr, and HOI/$^-$OI (from MPO/hydrogen peroxide/corresponding halide) were also used to oxidize LDL and to test the specificity of LDL. It resulted in the fact that the antibody is highly specific for Mox-LDL.

The active and total MPO contents in plasma were measured using the licensed SIEFED and ELISA (ELIZEN MPO, Zentech SA, Belgium) methods [14]. By using these two techniques, we are able to distinguish active and total MPO contents in plasma and to determine the specific activity of MPO (MPO activity/MPO antigen ratio).

2.4. Angiotensin II, Adiponectin, and Interleukin-8 Analyses. ANGII was determined in plasma by a radioimmunoassay kit (BioSource, Nivelles, Belgium). IL-8 and adiponectin concentrations were quantified using ELISA tests (Becton Dickinson).

2.5. Statistic. Data were analyzed using the SigmaPlot 12.0 software (Systat, San Jose, CA). Results were considered statistically significant with a two-tailed $P < 0.05$. Two models of multiple linear regression analysis were tested using a backward stepwise selection of explicative variables.

3. Results and Discussion

The purpose of the present study was to explore whether there is an independent association between ANGII (an NADPH modulator), adiponectin, and levels of circulating Mox-LDL. In this context we analyzed various parameters within 121 male subjects who consulted for the first time for LUTS. Table 1 shows the means and SD of the parameters measured or calculated within patients.

Table 2 describes two models of multivariate analysis of backward regression in these subjects. The standardized regression coefficients are given for each model. As shown in Table 2, in the first model (Model 1) we set Mox-LDL as the dependent variable, while the independent variables included the parameters described above. Significant linear correlations were found between Mox-LDL levels and ANGII, and MPO activity (both positively) and also adiponectin content (negatively). In the second model (Model 2) the Mox-LDL/ApoB ratio (an estimation of the proportion of MPO-modified LDL in the bloodstream) was set as dependent variable and the same set of above parameters as independent variables. The same variables as in Model 1 were found to predict the Mox-LDL/ApoB ratio.

Our observations suggest that the combination of blood ANGII, MPO, activity and adiponectin explains at least

Table 1: Patient parameters.

$N = 121$	Mean	SD
Age (years)	59	11
BMI (kg/m^2)	26	4
Glycemia (mg/dL)	107	48
Total cholesterol (mg/dL)	209	39
Triglycerides (mg/dL)	184	142
HDL cholesterol (mg/dL)	53	18
LDL cholesterol (mg/dL)	122	35
Interleukin-8 (ng/mL)	17	10
Mox-LDL (μg/mL)	9	13
Mox-LDL/ApoB (μg/mg)	0.1	0.2
Angiotensin II (pmol/L)	14	11
Adiponectin (ng/mL)	5252	4197
MPO activity (MPOA) (mU/mL)	27	18
MPO antigen (MPOAg) (ng/mL)	50	36
MPOA/MPOAg (mU/ng)	0.6	0.2

Conversion for lipids: total cholesterol, HDL-c and LDL-c: 1 mmol/L = 38.67 mg/dL.
Triglycerides: 1 mmol/L = 38.67 mg/dL.
BMI: body mass index.

Table 2: Multivariate analysis of backward regressions in the total population.

$N = 121$	Standardized regression coefficient	P value
Model 1 $R = 0.52$, $F = 9.7$, $P < 0.001$		
ANGII	0.425	<0.001
MPO activity	0.236	0.018
Adiponectin	−0.196	0.05
Model 2 $R = 0.58$, $F = 12.6$, $P < 0.001$		
ANGII	0.446	<0.001
MPO activity	0.315	0.001
Adiponectin	−0.201	0.03

Parameters introduced in the stepwise multiple regression analysis: age, BMI, adiponectin, IL-8, total cholesterol, HDL-c, LDL-c, triglycerides, glycaemia, MPO antigen, MPO activity, and the ratio MPO activity/MPO antigen. Model 1: the Mox-LDL is the dependent variable. Model 2: the Mox-LDL/ApoB is the dependent variable.

partially the serum Mox-LDL levels. They corroborate and extend our previous data showing that oxidation could also take place at the surface of endothelial cells [9, 15] and that plasma level of Mox-LDL follows the level of MPO in patients during a hemodialysis process [15, 16]. They are underpinned by established physiopathological mechanisms as endothelial cells express NADPH oxidase, the activity and expression of which are increased by ANGII binding to the AT1 receptor [12]. In support of our proposal, we previously observed that hypertensive COPD patients treated by angiotensin-converting enzyme inhibitors had reduced levels of circulating Mox-LDL (our unpublished data). This is an alternative and complementary explanation to the common model positing that the presence of modified LDL in the circulation is due to the back diffusion of modified LDL from the vessel to the circulation and is a marker of plaque instability in patients with coronary artery disease. Furthermore, it recently arose that human peroxidasin 1, also called vascular peroxidase 1 (VPO1), might be involved in the *in vivo* production of HOCl and so potentially contributes to the oxidation of LDL [17]. Moreover, VPO1 was also suggested as an inductor of vascular smooth muscle cell proliferation [18]. However, further experiments are needed as the formation of HOCl by VPO1 is low at physiological pH.

We also uncovered a negative linear correlation between oxidative stress and adiponectin in our multiple linear regression models (Table 2). This is in agreement with the observation that adiponectin reduced *in vitro* and *in vivo* the NADPH oxidase activity and hence oxidative stress [12]. It is also in support of the general agreement that adiponectin, which is secreted by fat tissue, is antiatherogenic by modulating cytokine inflammatory cascades and inhibiting cholesterol incorporation.

In sum, our study suggests that the combined action of ANGII, MPO, and adiponectin might explain the serum Mox-LDL levels. A definitive validation or invalidation of the proposed role of ANGII in the generation of serum Mox-LDL will request a double blind randomized crossover study comparing subjects receiving an angiotensin-converting enzyme inhibitor or an angiotensin II receptor antagonist and a placebo.

Abbreviations

ANGII: Angiotensin II
ApoB-100: Apolipoprotein B-100
LDL: Low-density lipoprotein
LUTS: Lower urinary tract symptoms
MPO: Myeloperoxidase
Mox-LDL: Low-density lipoprotein modified by the MPO-H_2O_2-chloride system
NADPH: Nicotinamide-adenine-dinucleotide phosphate.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Karim Zouaoui Boudjeltia and Cédric Delporte equally contributed to this paper.

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Research Article

Plasmacytoid Dendritic Cell Response to CpG ODN Correlates with CXCL16 Expression and Is Inhibited by ox-LDL

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Structurally distinct classes of synthetic CpG oligonucleotides (ODN) differentially activate human immune cells. K-type ODN trigger plasmacytoid dendritic cells (pDCs) to differentiate and produce TNFα. In contrast, D-type ODN stimulate large amounts of IFNα secretion from pDCs. The cell-surface receptor CXCL16 was previously shown to influence the nature and specificity of CpG ODN-induced immune activation. Here, we evaluated the expression and function of CXCL16 on pDC from healthy volunteers. We report that increased CXCL16 expression correlated with enhanced in vitro response exclusively to D-type CpG ODN. Conversely, enzymatic digestion of the receptor resulted in a decrease in IFNα production. Moreover, ox-LDL presence significantly inhibited D-ODN mediated IFNα production by pDCs. Coculture of enriched pDCs with the CXCR6 expressing Jurkat T cells decreased the activation threshold of these cells responding to D-ODN, suggesting that CXCL16/CXCR6 interaction may play an important role in modifying the response of pDCs to environmental danger signals.

1. Introduction

Synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs stimulate an innate immune response characterized by the production of cytokines, chemokines, and Ig by B cells, dendritic cells (DC), NK cells, and macrophages [1–4]. In humans, these motifs are recognized by B cells and plasmacytoid dendritic cells (pDC) that express Toll-like receptor 9 (TLR9) [5, 6]. Human PBMC recognize and respond to structurally distinct classes of CpG motifs [7–13]. Of these ODN classes, K type (also known as B class) phosphorothioate ODN express multiple TCGTT and/or TCGTA motifs that stimulate B cells to proliferate and secrete IL-6 and IgM and promote the survival, activation, and maturation of pDC in the absence of IFNα production [7, 8, 10]. In contrast, D type ODN (also known as A class) contain a phosphodiester purine/pyrimidine/CG/purine/pyrimidine motif capped at each end by a phosphorothioate polyG tail are poor stimulators of B cells [8–10]. However, D type ODN stimulate pDCs to produce high levels of IFNα [8–10]. Previous work has established that D but not K type ODN bind to the chemokine and scavenger receptor CXCL16 expressed on the surface of pDCs [14]. This interaction directs D type ODN into the recycling endosomal compartment, where TLR9-MyD88-IRF7 signaling pathway is activated, leading to robust IFNα production [15]. The 3rd class of CpG ODN, designated as C-type, contain one or two CpG motifs with a phosphodiester backbone at the 5′ end [11, 12]. C ODN also contains a palindromic sequence on a phosphorothioate backbone at the 3′ end and can induce proliferation of B cells and production of low amounts of IFNα from pDCs [11, 12].

CXCL16 functions as a scavenger receptor [16], a chemokine [17], and an adhesion [18] molecule, playing a prominent role in the pathogenesis of atherosclerosis [19] and psoriasis [20]. CXCL16 binds to the chemokine receptor CXCR6/Bonzo, expressed on the surface of CD4+ and CD8+ T cells [21]. Soluble to cell-surface expressed CXCL16 is controlled by the metalloproteinase ADAM10 that actively cleaves the membrane bound receptor [22]. This activity can be inhibited by the metalloproteinase inhibitor GM6001, thereby increasing the amount of the cell surface

expressed CXCL16 [14, 22]. Conversely, treatment with o-sialoglycoprotease selectively digests the membrane-bound CXCL16 [14, 21]. In order to clarify the role of CXCL16 expression on human pDCs during CpG ODN-mediated immune activation, we modified the expression levels of this protein in human peripheral blood mononuclear cells prior to stimulation. Results indicate that preventing the cleavage of membrane-bound CXCL16 increased both the number of pDC expressing CXCL16 and their response to D-ODN. In contrast, digesting the membrane-bound CXCL16 reduced the number of pDC expressing CXCL16 and their response to D-ODN. Interestingly, our data indicated that circulating ox-LDL may have detrimental effect on pDC derived D-ODN mediated IFNα production, suggesting an adverse role during viral infection for individuals with elevated ox-LDL levels. Furthermore, we also show for the first time that coculture of purified pDCs with CXCR6 expressing Jurkat T cells decreased the threshold concentration of D-ODN mediated IFNα production. This effect was specific to CXCR6 as CCR5 expressing Jurkat cells proved to be ineffective. These results suggest an important role for CXCL16/CXCR6 interaction in modifying the response of pDCs to environmental danger signals.

2. Methods

2.1. Reagents. Endotoxin free ODN were purchased from IDT. Sequences of ODN used ($5' \rightarrow 3'$) were K3 CpG ODN, ATCGACTCTCGAGCGTTCTC; K3-flip control ODN, ATGCACTCTGCAGGCTTCTC; D35 CpG ODN, GGtgcatcgatgcaggggGG; D35-flip control ODN, GGtgcatgcatgcaggggGG; C type CpG ODN, TCGTCGTTTTCGGCGCGCGCCG. Bases shown in capital letters are phosphorothioate, and those in lower case are phosphodiester. All FITC-, phycoerythrin (PE)-, and PE-Cy5 conjugated antibodies except for BDCA-2 were purchased from Biolegend (London, UK). FITC- or PE-conjugated BDCA-2 was from Miltenyi Biotech (CA, USA). BDCA-4 magnetic bead based pDC isolation kit was from Miltenyi Biotec. Polyclonal goat anti-human CXCL16 (purified and biotin labeled) and its isotype matched control were from R&D Systems.

2.2. Cells and Culture Conditions. PBMC ($2–4 \times 10^6$/mL) from healthy volunteers were obtained following informed consent and were cultured in RPMI 1640 containing 5% fetal calf serum (FCS), 50 U/mL penicillin, 50 μg/mL streptomycin, 0.3 μg/mL L-glutamine, 1 μM nonessential amino acids, 1 μM sodium pyruvate, 10 mM HEPES, and 10^{-5} M 2-mercaptoethanol. Cells were stimulated for 24–48 h with 1–3 μM ODN depending on the assay. In some experiments, magnetic bead enriched pDCs (100,000/well) were plated in 96-well flat-bottom plates. Cell-surface CXCL16 expression was modified/blocked by treating cells with 50 μM GM6001 or 25 μg/mL O-sialoglycoprotein endopeptidase (from Pasteurella haemolytica), 10 μg/mL OxLDL, 10 μg/mL LDL, recombinant IFNγ and TNFα (20 ng/mL each), or PMA/ionomycin (250 pg/mL/100 pg/mL) for 30 min at 37°C. Jurkat cells stably expressing CCR5 or CXCR6 were a kind gift from Dr. Keith Peden (FDA, CBER, Section of Retroviral Immunology) and were cocultured with enriched pDCs in 96-well U-bottom plates (1 : 1 ratio; 100,000 cells/well).

2.3. Flow Cytometric Analysis. Cultured cells were washed in cold PBS, fixed, and stained as previously described [14]. Data was acquired (20,000–50,000 events) on a FACScalibur flow cytometer, and data were analyzed using the CELLQuest software (both from Beckton Dickenson, San Jose, CA). The following combination of antibodies were used in identification of pDCs: CD123(+)/BDCA-2(+).

2.4. ELISA. Ninety-six well microtiter plates (Millipore, Bedford, MA) were coated with antibodies that recognize human IFNα (PBL Biomedical Laboratories, New Brunswick, NJ), IP-10 (e-biosciences), or IL-6 (Biolegend) [14]. The plates were blocked with PBS-5% BSA. Supernatants from cultured cells were added, and their cytokine content quantitated by the addition of biotin-labeled anti-cytokine antibody followed by phosphatase-conjugated avidin and phosphatase-specific colorimetric substrate. Standard curves were generated using known amounts of recombinant human IFNα2a IP-10 or IL-6. All assays were performed in duplicate.

3. Results and Discussion

3.1. Metalloproteinase Inhibitor GM-6001 Enhances Cytokine Production Induced by D-ODN. Membrane-bound CXCL16 is expressed by professional antigen presenting cells (APC) such as pDCs and macrophages [14, 23]. Previous studies have demonstrated that the ratio of soluble to cell-surface expressed CXCL16 is controlled by the disintegrin-like metalloproteinase ADAM10 that actively cleaves the membrane bound receptor and that this activity can be inhibited by the metalloproteinase inhibitor GM6001, thereby increasing the amount of the cell surface expressed CXCL16 [22, 23]. To assess whether inhibition of ADAM10 would affect the response to the three classes of CpG ODN, PBMC from 6 donors were pretreated with GM6001 for 30 min and then stimulated with the D, C, or K type ODN. Exposure to the metalloproteinase inhibitor resulted in increased CXCL16 expression on pDCs (Figure 1(a)), whereas 24.6 ± 2.3 of untreated pDCs expressed cell-surface associated CXCL16 metalloproteinase inhibitor treatment caused a ~72% increase in surface expression levels for the protein (Table 1). GM6001 pretreatment also resulted in a significant increase in D-ODN responsiveness (~2-fold for individual donors, $P < 0.05$) (Figure 1(b) and Table 1) but had no effect on cells stimulated with C (Figure 1(c)) or K-ODN (Figure 1(d)). These results support the previous findings [14] and strengthen the case for CXCL16 involvement in D-ODN specific cellular activation.

3.2. Cleavage of CXCL16 on the Surface of pDCs Reduces Cytokine Production Induced by D-ODN. Treatment of cells with the enzyme O-sialoglycoprotease was shown to cleave cell-surface expressed CXCL16 [22]. Treatment of PBMCs with this enzyme caused a significant reduction in pDC

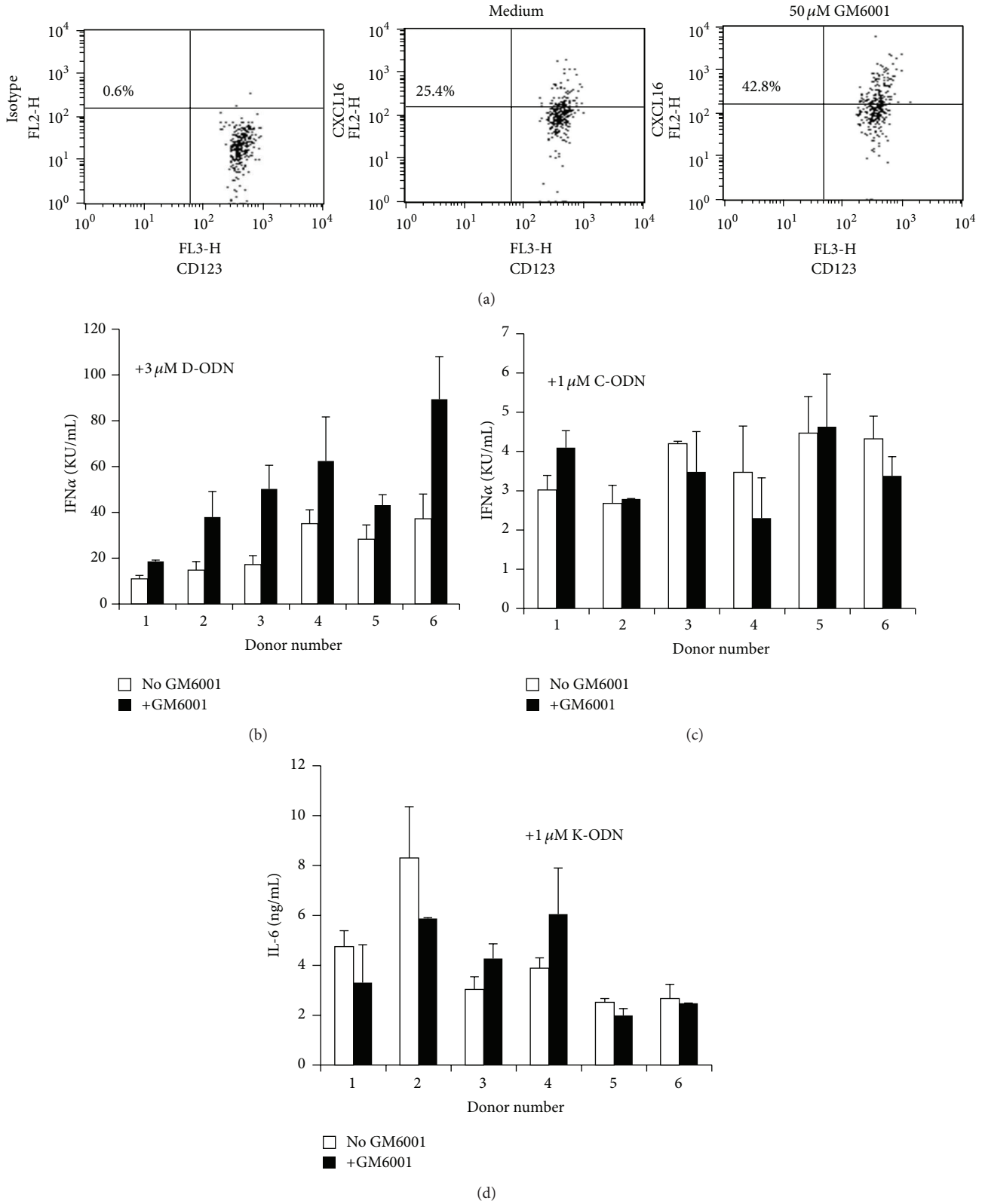

Figure 1: Metalloproteinase inhibitor GM-6001 enhances cytokine production induced by D-ODN. (a) PBMC (4×10^6/mL) were preincubated in the absence or presence of GM6001 (50 μM) for 30 min, fixed, and stained for CXCL16 expression. Cells treated as in (a) were washed and then stimulated with 3 μM D-ODN (b), 1 μM of C ODN (c), or 1 μM of K-ODN (d) for 24 h. Cytokine production (IL-6 for K-ODN and IFNα for D and C ODN) was assessed from culture supernatants using ELISA. Response of 6 individual donors is shown.

Table 1: Altering CXCL16 expression influences "D" ODN induced cytokine production.

	pDC expressing CXCL16		D-ODN induced IFNα production	
Treatment	% of all pDC	% change	KU/mL	% change
Untreated	24.6 ± 2.3		26.3 ± 17.8	
GM6001	43.1 ± 6.2*	↑ 72 ± 2*	50.3 ± 24.1*	↑ 115 ± 56*
O-sialoglycoprotease	9.6 ± 3.7*	↓ 55 ± 1*	6.6 ± 4.0*	↓ 70 ± 12*

PBMC from 5-6 different donors were treated with 50 μM of GM6001 or 25 μg/mL of o-sialoglycoprotein endopeptidase (from Pasteurella haemolytica) for 30 min at 37°C. The cells were then stimulated in vitro with 3 μM "D" ODN for 24 h, and the production of IFNα determined by ELISA. The fraction of CD123/BDCA-2 double positive pDC expressing CXCL16 was determined before and after Rx. Treatment-induced changes were calculated for each donor independently and then averaged.
* $P < 0.05$.

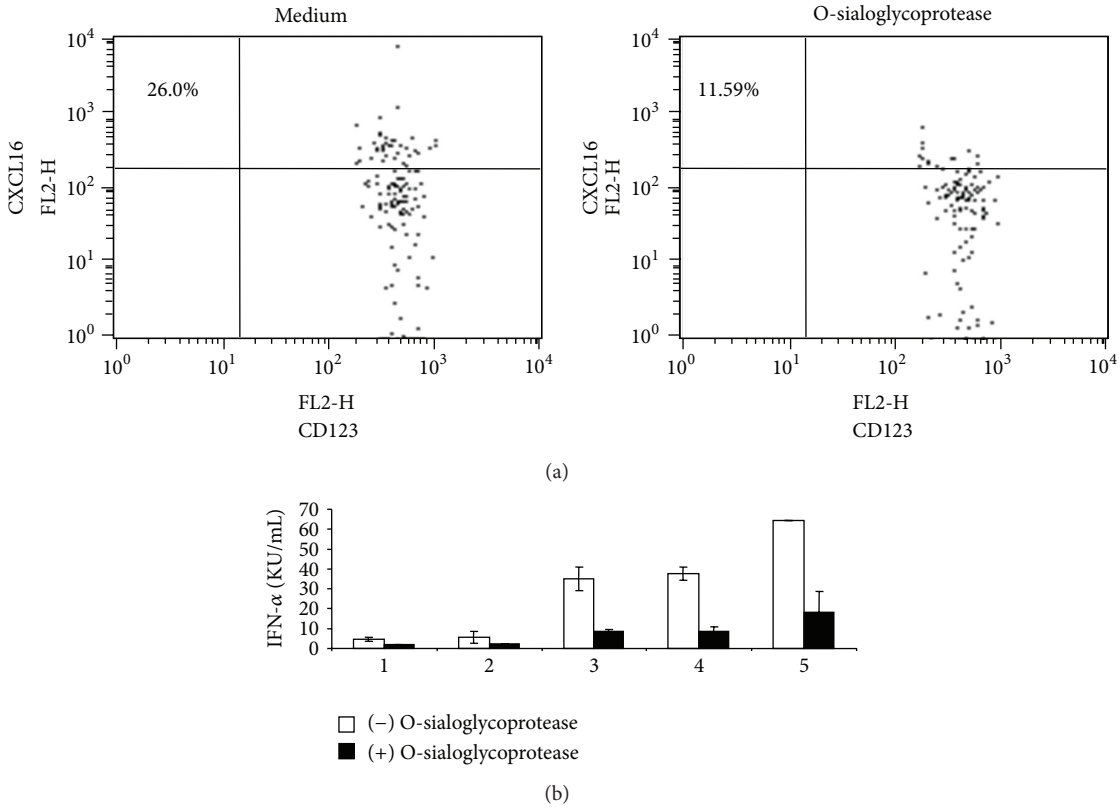

Figure 2: Digestion of CXCL16 on the surface of pDCs reduces cytokine production induced by D-ODN. (a) PBMC (4×10^6/mL) were preincubated in the absence or presence of O-sialoglycoprotein endopeptidase (25 μg/mL) for 30 min, fixed, and stained for CXCL16 expression. (b) Cells treated as in (a) were washed and then stimulated with 3 μM D-ODN for 24 h. Cytokine production was assessed from culture supernatants using ELISA. Response of 6 individual donors is shown.

surface-associated CXCL16 protein levels (Table 1, $P < 0.05$ and Figure 2(a)). This decrease correlated with a significant reduction in D-ODN responsiveness ($P < 0.05$, Table 1 and Figure 2(b)).

These results indicate that factors that can modulate pDC associated cell-surface CXCL16 can affect the magnitude of the immune response to D-ODN. It remains to be seen whether CXCL16 also plays a role in modifying the immune response of pDCs during viral infections.

3.3. CXCL16/CXCR6 Interaction Reduces the Threshold of Activation in pDCs Responding to D-ODN. Transmembrane CXCL16 is composed of three domains: the chemokine domain that interacts with the receptor CXCR6, the glycosylated mucin-like stalk, and the cytoplasmic domain that contains a potential tyrosine phosphorylation and SH2-protein-binding site [21, 24]. Thus, the cytoplasmic domain may also play a role in cell signaling. To assess whether CXCL16 engagement on pDCs can modify the immune response to CpG ODN mediated immune activation, pDCs from 3 different donors were enriched using immunomagnetic separation (at least 80% pure as determined by pDC-specific marker staining, Figure 3(a)). Enriched pDCs were then incubated with CXCR6 expressing Jurkat T cells as a source to provide

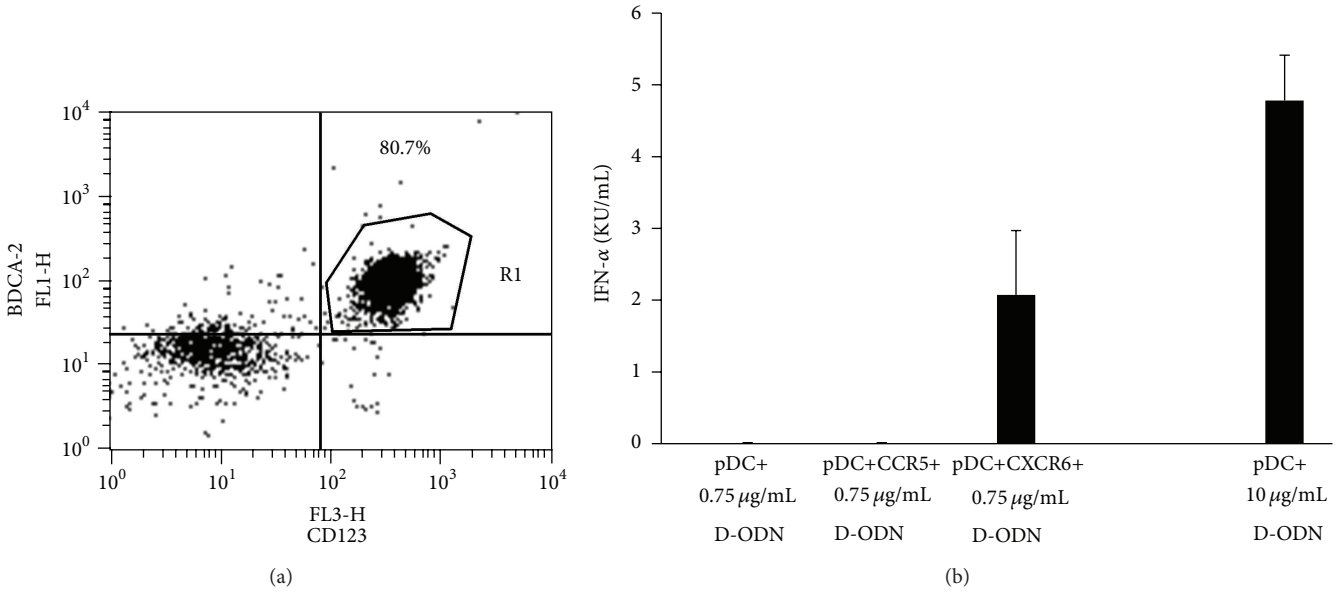

Figure 3: CXCL16/CXCR6 interaction reduces the threshold of activation in pDCs responding to D-ODN. (a) pDCs were enriched from human PBMCs using the BDCA-4 isolation kit as recommended by the manufacturer. Purity of cells was established by flow cytometry following staining with pDC associated cell-surface markers. (b) Enriched pDCs (100,000 cells/well) were incubated with CXCR6 or CCR5 expressing Jurkat T cells (1 : 1 ratio) in 96-well U-bottom plates. Cocultures were then stimulated with a suboptimal concentration of D-ODN (0.75 μg/mL). pDC stimulated with optimal concentration of D-ODN (10 μg/mL) served as a positive control. Cytokine production was assessed from culture supernatants 24 h later using ELISA. Average response of 3 different pDC preparations is shown (mean ± S.D).

CXCL16 engagement. A separate set of pDCs were incubated with CXCR6 negative CCR5 positive Jurkat cells as negative control. The cocultures were then stimulated with suboptimal concentration of D-ODN that did not yield detectable levels of IFNα production (0.75 μg/mL as determined in preliminary experiments). Results showed that coculture of purified pDCs with CXCR6 expressing Jurkat cells decreased the threshold concentration of D-ODN mediated IFNα production, enabling the cells to respond to an otherwise nonstimulating concentration of this ODN (Figure 3(b)). This effect was specific to CXCR6 since coculture with CCR5 expressing cells showed no such effect (Figure 3(b)). This result suggests that CXCL16/CXCR6 interaction may modify pDC responsiveness to environmental danger signals.

3.4. Oxidized LDL and Recombinant IFNγ/TNFα Modify Cytokine Production Induced by D-ODN. We further examined the effect of scavenger receptor ligand oxidized low density lipoprotein (oxLDL) on D-ODN mediated immune activation. Preincubation of enriched pDCs with oxLDL resulted in ~50% reduction in IFNα production in samples stimulated with D-ODN, while native LDL showed very weak or undetectable inhibitory effect (Figure 4(a)). Hyperlipidemia and hypercholesterolemia are two prime risk factors in the development of atherosclerosis, and accumulating evidence suggests that DC functions may be hampered [25]. We demonstrated that increased levels of oxLDL may influence signaling pathways of pDC, thereby altering immune responses against pathogens. This result suggests that oxLDL levels may be of importance in modifying the pDC response during host resistance to microbial infections.

Expression of CXCL16 is induced by the inflammatory cytokines IFN-gamma and TNF-alpha. These two cytokines synergize to upregulate both the soluble and the cell-surface associated CXCL16 [26]. To assess whether pDC response to D-ODN would be affected under conditions replicating an ongoing chronic inflammation, enriched pDCs were stimulated with the CpG ODN in the absence or presence of the recombinant cytokines IFNγ/TNFα. Results show that similar to GM6001, recombinant cytokine pretreatment increased the D-ODN dependent IFNα production ~2-fold (Figure 4(b), $P < 0.05$). In contrast, PMA/ionomycin pretreatment which strongly downregulates CXCL16 expression [27] failed to induce cytokine production following D-ODN stimulation (Figure 4(c)).

K versus D-ODN differentially activate human cells to produce distinct cytokines (TNFα versus IFNα). To assess whether CXCL16 expression is altered during CpG ODN stimulation, PBMC were stimulated with K or D-ODN for 48 h, followed by staining for cell-surface expressed CXCL16. Cells stimulated with K-ODN expressed 3.4-fold more membrane-associated CXCL16 when compared to control ODN treated cells (Figure 4(c), $P < 0.05$). Conversely, D-ODN stimulated cells showed a modest increase (1.4-fold), suggesting that CXCL16 expression is not controlled by type I interferons.

In conclusion, we show that factors that can alter the extent of cell-surface CXCL16 expression can profoundly alter the immune response induced specifically by D-ODN

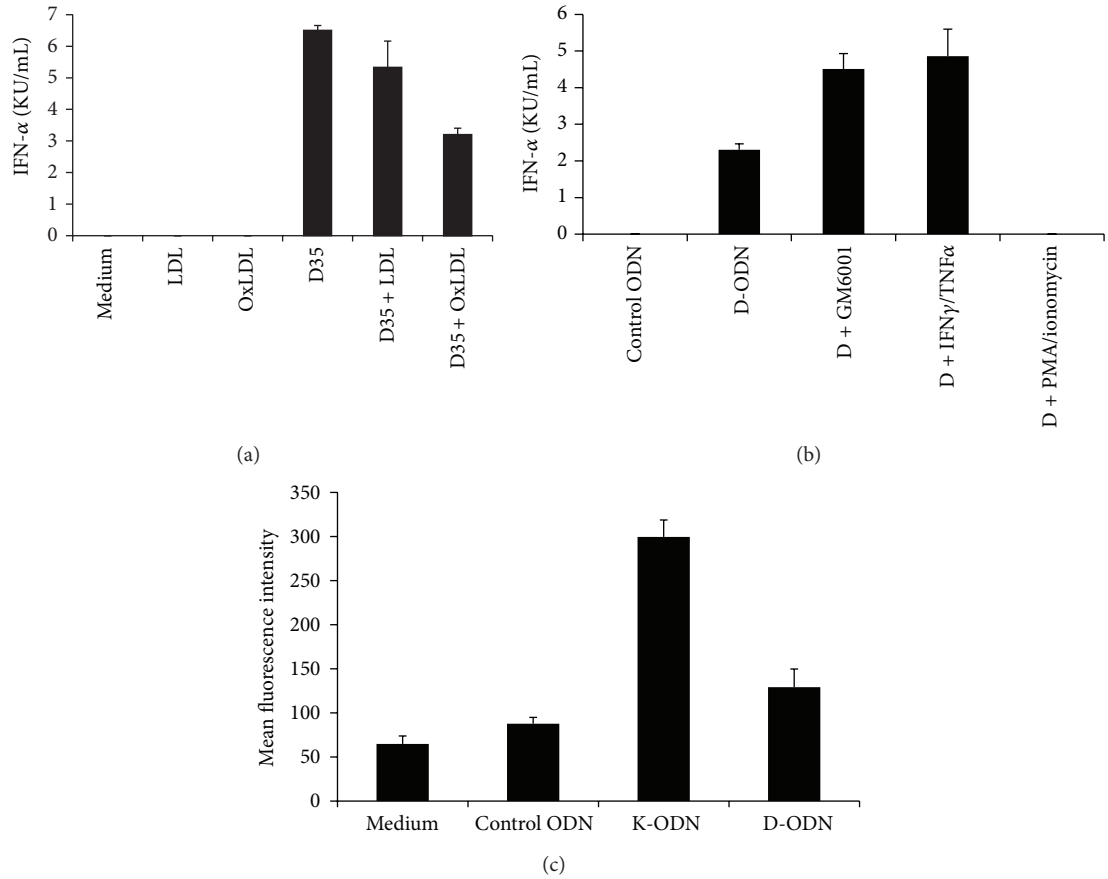

Figure 4: Oxidized LDL and recombinant IFNγ/TNFα modify cytokine production induced by D-ODN. (a) Enriched pDCs (100,000 cells/well) were preincubated with LDL or OxLDL (10 μg/mL each) and then stimulated with 3 μM of D-ODN. Cytokine production was assessed from culture supernatants 24 h later using ELISA. Average response of 3 different pDC preparations is shown (mean ± S.D). (b) Enriched pDCs were preincubated in the absence or presence of GM6001 (50 μM), recIFNγ/TNFα (20 ng/mL each), or PMA/ionomycin (250 pg/mL/100 pg/mL) for 30 min at 37°C. Cytokine production was assessed from culture supernatants 24 h later using ELISA. Average response of 3 different pDC preparations is shown (mean ± S.D). (c) PBMC (4×10^6/mL) were preincubated in the absence or presence of 1 μM Control ODN, 1 μM K-ODN, or 3 μM D-ODN for 48 h. Cells were then fixed and stained for CXCL16 expression. MFI of CXCL16 stained cells ± S.D of 3 different donors is shown.

and that CXCL16/CXCR6 interaction decreases the threshold concentration of D-ODN mediated IFNα production. This D-ODN sensitivity probably depends on the synergistic action of CXCL16/CXCR6 signaling cascade and more efficient delivery of D-ODN to endosome where TLR9 resides.

Conflict of Interests

Mayda Gursel, Dennis M. Klinman, and Ihsan Gursel have patents related to the use of CpG ODN. All rights to such patents have been assigned to the U.S. Federal Government. The authors declare no further conflict of interests.

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Research Article

Implication of Low HDL-c Levels in Patients with Average LDL-c Levels: A Focus on Oxidized LDL, Large HDL Subpopulation, and Adiponectin

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To evaluate the impact of low levels of high density lipoprotein cholesterol (HDL-c) on patients with LDL-c average levels, focusing on oxidative, lipidic, and inflammatory profiles. Patients with cardiovascular risk factors ($n = 169$) and control subjects ($n = 73$) were divided into 2 subgroups, one of normal HDL-c and the other of low HDL-c levels. The following data was analyzed: BP, BMI, waist circumference and serum glucose Total-c, TGs, LDL-c, oxidized LDL, total HDL-c and subpopulations (small, intermediate, and large), paraoxonase-1 (PON1) activity, hsCRP, uric acid, TNF-α, adiponectin, VEGF, and iCAM1. In the control subgroup with low HDL-c levels, significantly higher values of BP and TGs and lower values of PON1 activity and adiponectin were found, versus control normal HDL-c subgroup. However, differences in patients' subgroups were clearly more pronounced. Indeed, low HDL-c subgroup presented increased HbA1c, TGs, non-HDL-c, Ox-LDL, hsCRP, VEGF, and small HDL-c and reduced adiponectin and large HDL. In addition, Ox-LDL, large-HDL-c, and adiponectin presented interesting correlations with classical and nonclassical markers, mainly in the normal HDL-c patients' subgroup. In conclusion, despite LDL-c average levels, low HDL-c concentrations seem to be associated with a poor cardiometabolic profile in a population with cardiovascular risk factors, which is better evidenced by traditional and nontraditional CV biomarkers, including Ox-LDL, large HDL-c, and adiponectin.

1. Introduction

Dyslipidemia is recognized as one of the major risk factors for the development of cardiovascular disease (CVD), which is a major clinical problem worldwide. Large prospective cohort studies, such as the Framingham Heart Study and the Seven Countries Study, have been recognizing the importance of reducing major risk factors, including cholesterol levels, in particular low-density lipoprotein cholesterol (LDL-c), as a pivotal strategy to prevent the development/evolution of cardiovascular disease and related events [1–3]. However, it is now accepted that the current lipid-lowering therapies, in particular those directed to reduce LDL-c levels, such as statins, are insufficient to prevent part of the cardiovascular events; indeed, residual cardiovascular risk remains elevated even in clinical trials in which LDL-c levels have been aggressively reduced [4–6]. In fact, it has been accepted that a considerable proportion of cardiovascular events occur in

individuals who do exhibit normal LDL-c levels, and there is a residual cardiovascular risk that has been the focus of a great deal of interest [7–10]. Furthermore, this fact reinforces the idea that traditional risk factors, including lipidic, might not tell the whole story about CVD progression and prevention of CV events and, thus, there has been an increasing interest in identifying novel biomarkers that might improve the global risk prediction of CVD [11, 12]. In addition to the critical role that LDL-c plays, several lines of evidence have shown the contribution of other lipid fractions/components, such as oxidized LDL (Ox-LDL) and high-density lipoprotein cholesterol (HDL-c), to overall cardiovascular health [3, 13–15].

LDL oxidation is associated with coronary artery disease (CAD) as well as with other disorders, as recently emerged from experimental and clinical studies [16–20]. Concerning the CAD, Ox-LDL is a promoter of key steps in the onset and evolution of atherosclerosis, including stimulation of monocyte infiltration and smooth muscle cell migration and proliferation; conversely, high levels of HDL-c prevent the development of atherosclerosis and CAD, in particular due to the transport of reserve cholesterol and the inhibition of Ox-LDL induced monocyte infiltration; Ox-LDL and HDL are indeed antagonists in the development of CVD [21]. Removal and/or inactivation of circulating Ox-LDL has been increasingly viewed as a promising therapeutic strategy against atherosclerosis, but more research is mandatory to clarify some discrepant data [22, 23]. Concerning the management of HDL-c, clinical and epidemiologic data illustrate the need to expand the scope of therapies to reduce the residual cardiovascular risk associated with low HDL-c levels, even when LDL-c is managed successfully [24–26]. In fact, low plasma levels of HDL-c have been largely recognized as a risk factor for coronary heart disease (CHD) [27, 28].

It has been suggested that monitoring the type of HDL particles, which carry distinct and specific proteins or lipids and are differentiated by density and size (large, intermediate and small), rather than their total quantity, is a more reasonable way of determining the CV risk, suggesting that different subpopulations may have a different role in reverse cholesterol transport (RCT) and CVD risk protection [29]. Actually, some recent studies have been reporting that large HDL levels are reduced in patients with CAD compared to healthy subjects and inversely related to both disease severity and progression of coronary lesions [30]. Although the most widely known mechanism behind the antiatherogenic function of HDL is the RCT, other important protective properties have been described, including anti-inflammatory, antioxidant, antithrombotic and vasorelaxant [31–34]. Although the benefit of high HDL-c contents appears to be obvious, most clinical trials that aimed at increasing HDL-c concentrations failed to generate convincing results. Therefore, the question arises as to whether the quantification of HDL-c level or perhaps rather more the HDL function and subpopulations is of considerable therapeutic relevance [35]. In fact, variations in HDL subfraction levels and functions have been observed in CVD populations, suggesting that large HDL particles are inversely associated with atherosclerosis development while small HDL particles are positively connected with CVD, which is also observed for Ox-LDL contents [36–39]. These considerations indicate that beside the measurement of standard lipids (such as HDL-c and LDL-c levels), the measurement of specific HDL subfractions and Ox-LDL might help to better evaluate the risk of cardiovascular events in specific populations.

Improved characterization of the impact of low-HDL-c levels in populations with normalized LDL-c concentrations and the relevance of HDL subpopulations and Ox-LDL contents will be an important step to develop strategies better directed to reduce dyslipidemia-associated cardiovascular risk. Thus, this study aimed to evaluate the influence of low HDL-c levels on the cardiometabolic profile of patients with cardiovascular risk factors but average LDL-c contents, using both traditional and new nontraditional markers, including Ox-LDL, HDL subpopulations, and inflammatory and angiogenesis mediators.

2. Materials and Methods

2.1. Subjects and Ethical Consideration. Two hundred and forty-two subjects were enrolled in the study, aged 33 to 75 years, divided in two major groups: control volunteers and patients with cardiovascular risk factors (designed as control and as patients, resp.). The control volunteers were randomly recruited during the performance of routine laboratory analysis in a clinical laboratory and were selected after not expressing any diagnosis or taking medication for cardiovascular disease and no family history of CVD. The group included 73 subjects, 39 males and 34 females. The patients group involved 169 volunteers, 88 males and 81 females, defined as having cardiovascular risk factors in terms of previous diagnosis and/or pharmacological treatment for hypertension and/or for type 2 diabetes mellitus (T2DM) and/or for dyslipidemia. T2DM was diagnosed in the Diabetes and Metabolic Diseases Unit from the Coimbra Hospital Centre (EPE), according to the European Guidelines. Patients were recruited during the performance of routine laboratory analysis on the basis of previous diagnosis and/or treatment for hypertension and dyslipidemia, performed according to the International Society of Hypertension/World Health Organization and the Seventh Joint National Committee on Hypertension and National Cholesterol Education Program-Adult Treatment Panel III (NCEP-ATP III) for hypertension and dyslipidemia, respectively. Other cardiovascular disorders of the patients' population include coronary artery and heart disease, ischemic heart failure, angina pectoris, arrhythmias, atrial fibrillation, cardiac valvulopathies, and peripheral vascular disease. Five patients reported a previous cerebrovascular event and other 5 a stroke episode. The patients were under the following medication: (a) insulin and/or oral antidiabetic drugs (OAD)—60.95%: in detail, 41 patients with insulin, 37 patients with sulfonylureas, 47 patients with biguanides, 51 patients with modulators of incretins, and 10 patients with alpha-glucosidase inhibitors; (b) lipid-lowering drugs—63.91%, being 90 patients under statins therapy, 21 under fibrates, 3 using an inhibitor of cholesterol absorption (ezetimibe), and 1 patient with omega-3; (c) antihypertensive drugs—70.41%, in particular 73 patients with diuretics (44 thiazides and analogues, 26 of the loop, 3 potassium-sparing),

47 with angiotensin-converting-enzyme inhibitors, 56 with angiotensin II receptor antagonists, 35 with calcium channel blockers, 32 with beta blockers, and 5 with central alpha-2 agonists. Some of the patients were under combined therapies.

The control subject did not take any drug for cardiovascular disease. Pregnant women and people with age <16 or >75 years were excluded. Each group was divided into two subgroups of normal HDL-c and of low-HDL-c levels, using the cutoffs of 1.03 mmol/L for men and 1.29 mmol/L for women, according to NCEP-ATP III guidelines. The smoking habits of the populations were (i) nonsmokers—46 normal-HDL-c control volunteers (90.20%), 19 low-HDL-c control volunteers (86.36%), 107 normal-HDL-c patients (89.92%), and 46 low-HDL-c patients (92.00%); (ii) ≤10 cigarettes a day—5 control-HDL-c control volunteers (9.80%), 3 low-HDL-c control volunteers (13.64%), 8 normal-HDL-c patients (6.72%), and 3 low-HDL-c patients (6.00%); (iii) >10 cigarettes a day—0 normal-HDL-c control volunteers (0.00%), 0 low-HDL-c control volunteers (0.00%), 4 normal-HDL-c patients (3.36%), and 1 low-HDL-c patients (2.00%). The study was performed in agreement with the code of ethics of the World Medical Association (Declaration of Helsinki) for human studies and received authorization from the local ethics committee, as well as from all the participants by signing a written informed consent.

2.2. Data and Blood Collection. The following data was obtained from each subject by trained personnel: weight and height (without shoes and wearing light outdoor clothing) were measured in order to calculate body mass index (BMI); waist circumference (WC) was assessed, as well as systolic and diastolic blood pressure (SBP and DBP) the latter of which was assessed in the sitting position after a 5 min rest. Blood samples were collected by venipuncture from the subjects after an overnight fasting period, via both EDTA-containing tubes and tubes without anticoagulant, in order to obtain plasma, buffy-coat, and serum, and processed within 2 hours of collection. Aliquots were immediately stored at −80°C until assayed.

2.3. Assays

2.3.1. Lipid Profile. Serum total cholesterol (Total-c), HDL cholesterol (HDL-c), LDL cholesterol (LDL-c), and triglycerides (TGs) were analysed on a Hitachi 717 analyser (Roche Diagnostics) using standard laboratorial methods. Total-c reagents and TGs kit were obtained from bioMérieux sa (Lyon, France). HDL-c Plus and LDL-c Plus tests were obtained from F. Hoffmann-La Roche Ltd. (Roche Diagnostics Div., Basel, Switzerland). Serum glucose levels were measured using a Glucose Oxidase commercial kit (Sigma, St. Louis, Mo, USA). Plasma concentration of Ox-LDL was evaluated by using a standard commercial enzyme-linked immunoassay (Oxidized LDL ELISA, Mercodia, Uppsala, Sweden).

2.3.2. HDL Subpopulations Assay. Subpopulations were separated and quantified using a Lipoprint kit from Quantimetrix Corp. (Redondo Beach, CA, USA). The assay involves a polyacrylamide gel electrophoresis assay and a complete Lipoprint System for data acquisition and quantification of large, intermediate, and small subpopulations of HDL.

2.3.3. PON1 Paraoxonase Activity. This was assessed spectrophotometrically and expressed in nmol of pnitrophenol/mL/min. In brief, paraoxonase activity was measured by adding serum to 1 mL Tris/HCl buffer (100 mmol/L, pH 8.0) containing 2 mmol/L $CaCl_2$ and 5.5 mmol/L paraoxon (O,O-diethyl-O-p-nitrophenylphosphate; Sigma Chemical Co.). The rate of generation of p-nitrophenol was determined at 412 nm, 37°C, via the use of a continuously recording spectrophotometer (Beckman DU-68).

2.4. Serum Inflammatory and Angiogenic and Endothelial Markers. Serum adiponectin, TNF-α, and VEGF contents were assessed using Quantikine enzyme-linked immunoassays kits from R&D Systems (Minneapolis, USA); serum intercellular adhesion molecule 1 (iCAM1) levels were evaluated by using an Elisa kit from Abcam (Cambridge, MA, USA); high-sensitivity C-reactive protein (hsCRP) was evaluated by immunoturbidimetry, using commercially available kits (CRP (latex) High-Sensitivity, Roche Diagnostics); uric acid was analysed on a Hitachi 717 analyser (Roche Diagnostics) using standard laboratory methods.

2.5. Statistical Analysis. Statistical analysis was performed by using the IBM Statistical Package for Social Sciences (SPSS) for Windows, version 20.0, (SPSS, Inc., Chicago, IL, USA). The distribution of continuous variables was analyzed using Kolmogorov-Smirnov tests to assess significant departures from normality. Results for normal distribution samples are presented as mean SD and lower and upper bound 95% confidence interval for mean; P values were obtained using independent samples t-test. Results for non-normal distribution samples are presented as median SD and interquartile range; P values obtained using Mann-Whitney test. The association between categorical variables was analyzed using Pearson's test. Statistical significance was accepted at P less than 0.05.

3. Results

3.1. Anthropometric Data. The demographic and anthropometric data of controls and patients are summarized in Table 1. Both populations were divided according to the HDL-c levels: normal-HDL-c (men > 1.03 mmol/L and women > 1.29 mmol/L) and low HDL-c (men ≤ 1.03 mmol/L and women ≤ 1.29 mmol/L), which were then compared (normal HDL-c versus low HDL-c) for each population under study (control and patients). Seventy-three control volunteers were enrolled in the study: 51 (69.86%) normal HDL-c and 22 (30.14%) low HDL-c. One hundred and sixty-nine patients were recruited: 119 (70.41%) normal HDL-c and 50 (29.59%) low HDL-c. Normal and low HDL-c groups presented no differences concerning age and obesity (BMI and waist circumference), in both study populations (Table 1). Blood pressure (systolic and diastolic) was significantly higher in low HDL-c when compared with normal HDL-c in the control group, while no differences were found between

Table 1: Anthropometric data and general characterization of the study groups.

Parameters	Control group			Patients group		
	Normal HDL ($n = 51$)	Low HDL ($n = 22$)	P	Normal HDL ($n = 119$)	Low HDL ($n = 50$)	P
Age (years)	57.6 ± 8.3 [55.2–59.9]	57.9 ± 9.3 [53.8–62.0]	0.893	62.0 ± 9.9 (13.0)	60.0 ± 9.1 (14.0)	0.629
BMI (Kg/m^2)	27.0 ± 3.6 [26.0–28.0]	28.8 ± 5.7 [26.2–31.3]	0.195	29.2 ± 4.8 [28.3–30.1]	29.9 ± 4.4 [28.6–31.1]	0.391
WC (cm)	96.5 ± 11.7 [93.1–99.8]	98.7 ± 12.9 [93.0–104.5]	0.468	103.5 ± 12.9 [101.0–105.9]	102.4 ± 13.5 [98.2–106.5]	0.644
SBP (mmHg)	140 ± 20 [135–146]	155 ± 21 [145–164]	0.007	140 ± 23 [136–144]	135 ± 22 [129–141]	0.164
DBP (mmHg)	84.9 ± 10.2 [82.0–87.9]	93.0 ± 10.3 [88.5–97.6]	0.003	78.0 ± 12.9 [75.7–80.4]	75.4 ± 13.6 [71.5–79.2]	0.234
Glucose (mmol/L)	5.42 ± 0.61 [5.24–5.59]	5.31 ± 0.45 [5.11–5.52]	0.494	6.38 ± 3.74 (4.86)	8.49 ± 4.12 (6.66)	0.228
HbA1c (%)	6.04 ± 0.49 [5.59–6.50]	6.30 ± 0.30 [5.55–7.05]	0.641	8.12 ± 1.92 [7.68–8.57]	9.38 ± 2.20 [8.58–10.17]	0.004

Results are presented as mean ± SD, lower and upper bound 95% confidence interval for mean, and P values obtained using independent samples t-test in the normal distribution samples and as median ± SD, interquartile range, and P values obtained using Mann-Whitney test in the non-normal distribution samples. BMI: body mass index; HbA1c: glycated hemoglobin; SBP: systolic blood pressure; DBP: diastolic blood pressure; WC: waist circumference.

Table 2: Lipid profile and markers of inflammation, angiogenesis, and endothelial lesion of the study groups.

Parameters	Control group			Patients group		
	Normal-HDL ($n = 51$)	Low-HDL ($n = 22$)	P	Normal-HDL ($n = 119$)	Low-HDL ($n = 50$)	P
Lipid profile						
Total-c (mmol/L)	5.67 ± 0.92 [5.41–5.93]	5.17 ± 0.83 [4.80–5.53]	0.030	4.88 ± 1.07 [4.68–5.07]	5.13 ± 1.14 [4.81–5.46]	0.175
TGs (mmol/L)	1.07 ± 0.37 [0.96–1.17]	1.47 ± 0.50 [1.25–1.69]	0.000	1.24 ± 0.84 (0.90)	2.32 ± 1.31 (2.24)	0.000
Total HDL-c (mmol/L)	1.58 ± 0.30 [1.49–1.66]	1.10 ± 0.16 [10.3–1.17]	0.000	1.48 ± 0.30 [1.42–1.53]	1.01 ± 0.17 [0.97–1.06]	0.000
Large HDL-c (%)	34.7 ± 14.4 (13.1)	31.5 ± 11.3 (11.4)	0.176	35.1 ± 11.8 [32.9–37.2]	27.5 ± 11.1 [24.3–30.6]	0.000
Interm HDL-c (%)	46.5 ± 7.6 (6.9)	48.3 ± 5.6 (7.1)	0.133	45.4 ± 6.3 (8.1)	48.7 ± 7.1 (6.4)	0.000
Small HDL-c (%)	18.2 ± 9.00 [15.7–20.7]	18.5 ± 7.9 [15.0–22.0]	0.886	19.8 ± 8.2 [18.3–21.3]	24.5 ± 11.9 [21.2–27.9]	0.001
LDL-c (mmol/L)	3.60 ± 0.90 [3.35–3.85]	3.40 ± 0.80 [3.04–3.75]	0.359	2.74 ± 0.95 [2.56–2.91]	3.02 ± 1.00 [2.73–3.31]	0.096
Ox-LDL (U/L)	45.7 ± 18.7 [40.4–51.1]	39.2 ± 11.5 [34.1–44.3]	0.202	35.7 ± 12.7 [33.2–38.2]	40.1 ± 14.2 [35.6–44.5]	0.043
Ox-LDL/LDL-c	12.6 ± 3.9 [11.5–13.8]	11.7 ± 3.0 [10.4–13.1]	0.344	13.1 ± 3.5 [12.4–13.8]	14.00 ± 4.1 [12.7–15.3]	0.300
Non-HDL-c (mmol/L)	4.10 ± 0.94 [3.83–4.36]	4.07 ± 0.83 [3.70–4.44]	0.915	3.40 ± 1.08 [3.21–3.60]	4.12 ± 1.11 [3.80–4.44]	0.000
Total-c/HDL-c	3.72 ± 0.91 [3.47–3.98]	4.81 ± 1.07 [4.33–5.28]	0.000	3.42 ± 0.96 [3.25–3.60]	5.18 ± 1.34 [4.80–5.56]	0.000
LDL-c/HDL-c	2.39 ± 0.80 [2.16–2.61]	3.17 ± 0.96 [2.74–3.59]	0.001	1.94 ± 0.80 [1.79–2.08]	3.04 ± 1.16 [2.70–3.38]	0.000
PON1 activity	505 ± 131 [469–542]	443 ± 109 [394–491]	0.042	494 ± 173 [462–525]	510 ± 236 [443–578]	0.774
Markers of inflammation, angiogenesis, and endothelial lesion						
hsCRP (μg/mL)	0.25 ± 0.36 [0.13–0.37]	0.37 ± 0.44 [0.11–0.64]	0.103	0.22 ± 0.54 (0.41)	0.50 ± 0.56 (0.71)	0.034
TNF-α (pg/mL)	3.56 ± 3.23 [2.65–4.48]	3.29 ± 3.35 [1.80–4.78]	0.880	3.12 ± 2.69 [2.59–3.66]	3.28 ± 2.53 [2.49–4.07]	0.560
Adiponectin (μg/mL)	10.8 ± 6.9 [8.9–12.8]	8.0 ± 5.5 [5.6–10.5]	0.069	8.9 ± 6.6 [7.6–10.2]	7.1 ± 5.6 [5.3–8.9]	0.041
Uric acid (mmol/L)	0.32 ± 0.09 [0.30–0.35]	0.29 ± 0.10 [0.24–0.35]	0.283	0.36 ± 0.38 (0.44)	0.40 ± 0.39 (0.59)	0.539
VEGF (pg/mL)	385 ± 388 [274–497]	408 ± 329 [262–554]	0.531	405 ± 295 [346–464]	520 ± 321 [420–620]	0.019
iCAM-1 (ng/mL)	413 ± 318 (160)	486 ± 586 (176)	0.122	572 ± 216 [512–631]	471 ± 137 [411–532]	0.070

Results are presented as mean ± SD, lower and upper bound 95% confidence interval for mean, and P values obtained using independent samples t-test in the normal distribution samples and as median ± SD, interquartile range, and P values obtained using Mann-Whitney test in the non-normal distribution samples. CRP: C-reactive protein; HDL-c: high-density lipoprotein cholesterol; iCAM-1: intercellular adhesion molecule 1; LDL-c: low-density lipoprotein cholesterol; Ox-LDL: oxidized low-density lipoprotein; TGs: triglycerides; TNF-α: tumour necrosis factor alpha; Total-c: total cholesterol; VEGF: vascular endothelial growth factor.

the subgroups of patients. Concerning the glucidic profile, no differences were found for glycemia and HbA1c between normal and low HDL-c subgroups of control subjects, while a significantly increased value of HbA1c was found in the subgroups of patients with low HDL-c levels when compared with the normal HDL-c subgroup of patients (Table 1).

3.2. Classical Lipid Profile and Oxidized LDL Content. The subjects entering in the control group were without any cardiovascular therapy, including lipid-lowering agents, while the majority of subjects from the patients group were under antidyslipidemic therapy, which justify some of the data obtained for the classic lipid profile. In the control group, lower values of Total-c were found in the low HDL-c subgroup when compared with normal HDL-c one, accompanied by significantly increased contents of TGs. In addition, while no differences were found for LDL-c, Ox-LDL, and non-HDL-c, there were significantly higher values of Total-c/HDL-c and LDL-c/HDL-c ratios (Table 2). However, the differences between the subgroups of patients (normal versus

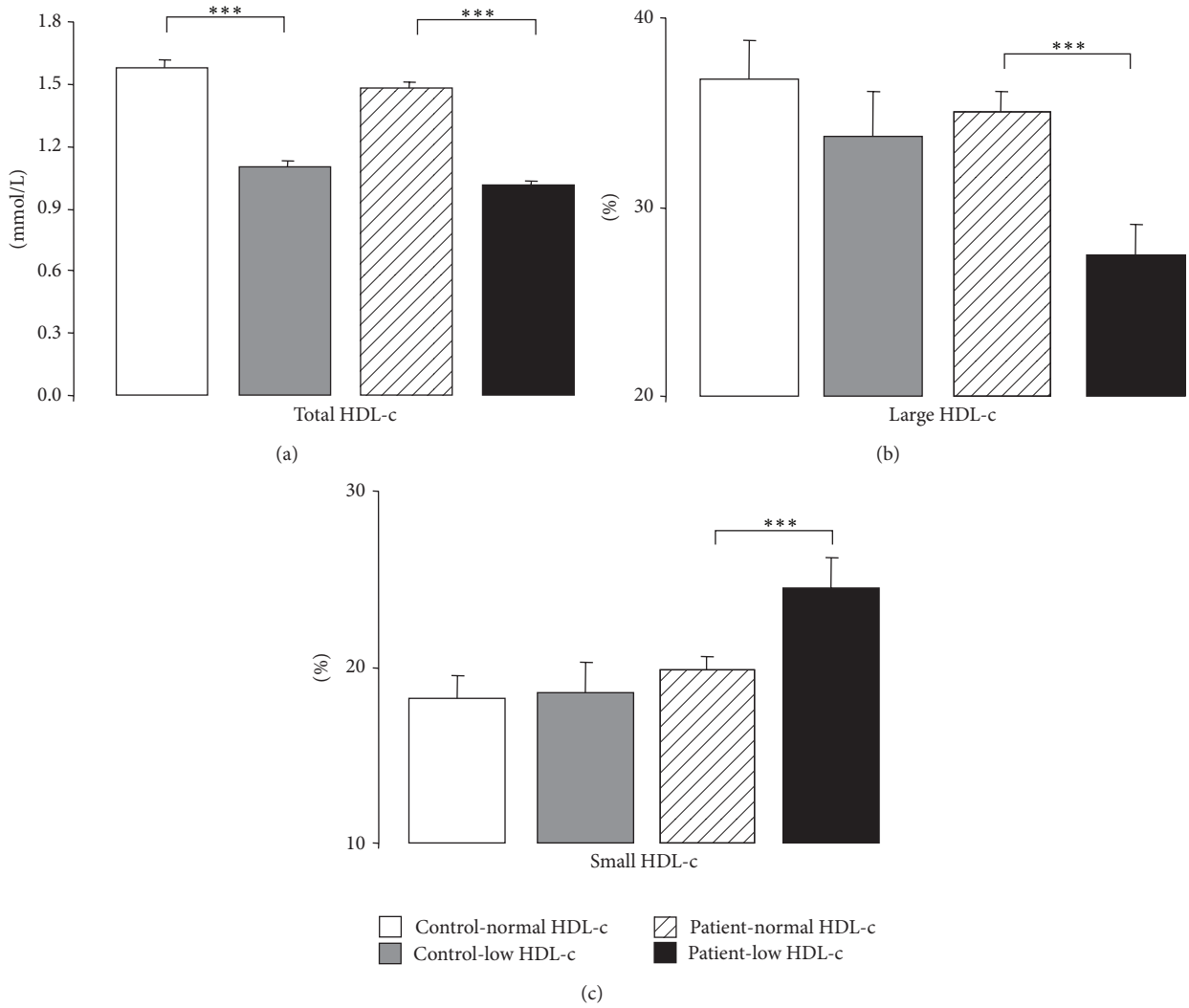

Figure 1: Serum total-HDL-c (a), large HDL-c (b), and small HDL-c (c), in the study groups. Results are presented as mean ± SEM. $^{***}P < 0.001$.

low-HDL-c levels) were more expressive. Indeed, the subgroups of patients with low-HDL-c levels presented a trend to increased values of Total-c and LDL-c, but statistically significant higher of TGs, Ox-LDL, and non-HDL-c, as well as of Total-c/HDL-c and LDL-c/HDL-c ratios (Table 2).

3.3. HDL Subpopulations and Paraoxonase Activity. Regarding the content of HDL subpopulations, despite the lower levels in both low HDL-c groups (which is obvious by definition of the study groups) (Table 2 and Figure 1(a)), only in the patients population there was a significantly decreased percentage of large HDL-c and increased percentage of small HDL-c, while no differences of HDL subpopulations percentages were found between the two subgroups of controls (normal versus low HDL-c) (Table 2 and Figures 1(b) and 1(c), resp.). Concerning PON1 activity, in the control group, there was a reduced value in the low HDL-c subgroup, while unchanged values were encountered between the two subgroups of patients (Table 2).

3.4. Markers of Inflammation, Angiogenesis, and Endothelial Lesion. Regarding other putative markers of cardiovascular disease, in the control individuals, the reduced content of HDL-c was associated only with a significantly reduced concentration of adiponectin (Figure 2(a)), when compared with controls subjects with normal HDL-c levels; all the other parameters were unchanged, including hsCRP, TNF-α, uric acid, iCAM-1, and VEGF (Table 2 and Figure 2). However, in the patients' population, the reduced content of HDL-c was associated not only with an additional reduction of adiponectin (Figure 2(a)) but also with significantly increased concentrations of VEGF and hsCRP (Figures 2(b) and 2(c), resp.), when compared with patient subgroup with normal HDL-c levels (Table 2 and Figure 2).

3.5. Analysis of Correlations between Markers of CV Risk in Patients Subgroups. The values of large-HDL in the normal HDL-c patients' subgroup were negatively and significantly correlated with Ox-LDL ($r = -0.355$, $P = 0.000$)

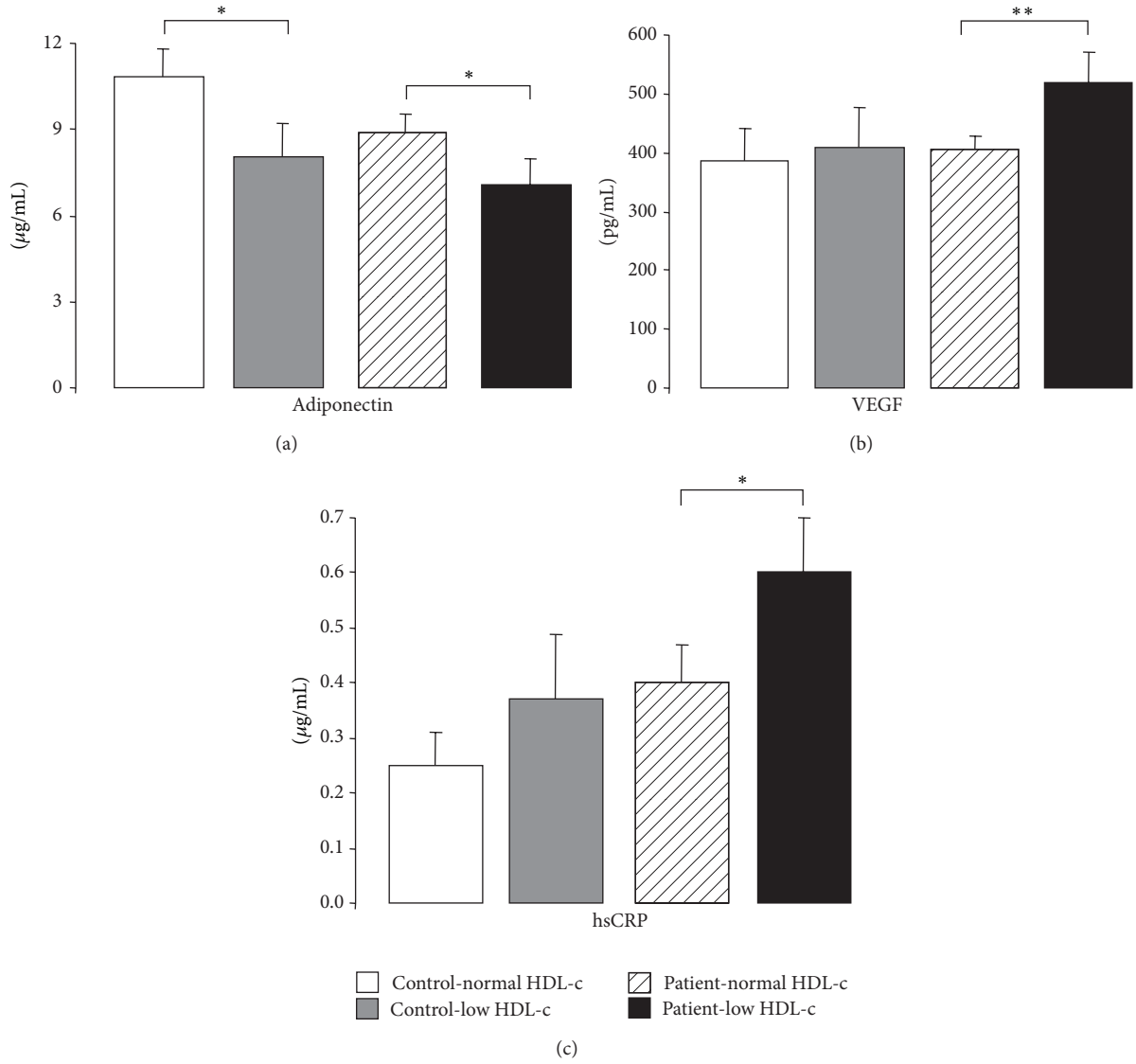

Figure 2: Serum adiponectin (a), VEGF (b), and hsCRP (c) levels, in the study groups. Results are presented as mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$.

(Figure 3(a)), LDL-c ($r = -0.696$, $P = 0.000$) (Figure 3(b)), non-HDL-c ($r = -0.348$, $P = 0.000$) (Figure 3(c)), TNF-α ($r = -0.198$, $P = 0.049$) (Figure 3(e)), and TGs ($r = -0.336$, $P = 0.000$) (Figure 3(f)) levels and positively and significantly correlated with adiponectin ($r = 0.173$, $P = 0.046$) (Figure 3(d)) but not in the low-HDL-c patients' subgroup (versus Ox-LDL: $r = -0.215$, $P = 0.172$; versus LDL-c: $r = -0.175$, $P = 0.235$; versus non-HDL-c: $r = -0.209$, $P = 0.149$; versus adiponectin: $r = 0.129$, $P = 0.429$; versus TNF-α: $r = 0.117$, $P = 0.460$; versus TGs: $r = -0.045$, $P = 0.758$) (Figure 3(a) to Figure 3(f), resp.). In addition, in the normal HDL-c patients' subgroup, Ox-LDL was negatively and significantly correlated with large HDL-c ($r = -0.355$, $P = 0.000$) (Figure 4(a)) and positively and significantly correlated with small HDL-c ($r = 0.437$, $P = 0.000$) (Figure 4(b)), TNF-α ($r = 0.235$, $P = 0.019$) (Figure 4(d)), DBP ($r = 0.314$, $P = 0.001$) (Figure 4(e)), and TGs ($r = 0.307$, $P = 0.002$) (Figure 4(f)), together with a trend to correlation with PON1 activity ($r = 0.179$, $P = 0.072$) (Figure 4(c)). These correlations showed statistically unchanged values in the low HDL-c patients' subgroup (versus large HDL-c: $r = -0.215$, $P = 0.172$; versus small HDL-c: $r = 0.121$, $P = 0.444$; versus PON1 activity: $r = 0.237$, $P = 0.131$; versus TNF-α: $r = -0.095$, $P = 0.551$; versus DBP: $r = 0.222$, $P = 0.157$; versus TGs: $r = 0.092$, $P = 0.569$) (Figure 4(a) to Figure 4(f), resp.). Finally, also in normal-HDL-c patients' subgroup, adiponectin was positively and significantly correlated with large HDL-c ($r = 0.363$, $P = 0.000$) (Figure 5(a)) and negatively and significantly correlated with TGs ($r = -0.235$, $P = 0.019$) (Figure 5(c)), waist circumference ($r = -0.320$, $P = 0.002$) (Figure 5(d)), hsCRP ($r = -0.268$, $P = 0.042$) (Figure 5(e)), and uric acid ($r = -0.376$, $P = 0.002$) (Figure 5(f)) but not in low HDL-c patients' subgroup (with

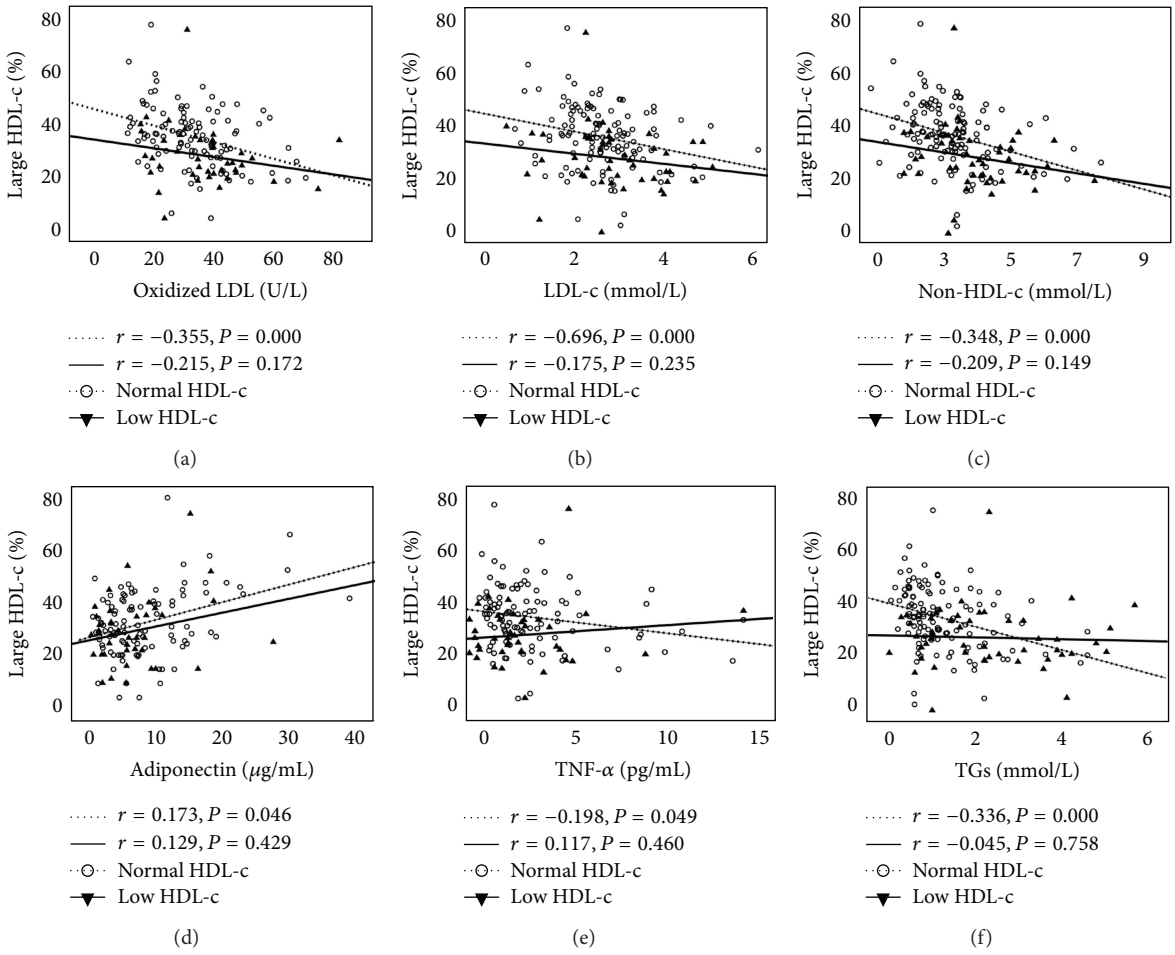

Figure 3: Main correlations in normal and low-HDL-c patients. Correlation between large HDL-c and Ox-LDL (a), LDL-c (b), non-HDL-c (c), adiponectin (d), TNF-α (e), and TGs (f).

the exception of TGs and uric acid) (versus large HDL-c: $r = 0.240$, $P = 0.136$; versus TGs: $r = -0.410$, $P = 0.010$; versus waist circumference: $r = -0.232$, $P = 0.180$; versus hsCRP: $r = 0.037$, $P = 0.852$; versus uric acid: $r = -0.423$, $P = 0.028$, resp.) (Figure 5). In opposition with large HDL-c, no significant correlation was found between adiponectin and small HDL-c in both normal ($r = -0.048$, $P = 0.637$) and low HDL-c ($r = -0.049$, $P = 0.763$) patients' subgroups (Figure 5(b)).

4. Discussion

The main finding of this study is that low HDL-c levels are associated with a poor cardiometabolic profile in a population of patients with cardiovascular risk factors, which is better diagnosed when analyzed in terms of nontraditional markers, including large HDL subpopulation, Ox-LDL, adiponectin, and VEGF. Although in a lesser extent, the impact of low-HDL-c levels is also manifest in the control population, viewed by an increase of blood pressure (SBP and DBP) and TGs concentration, and by a decrease of PON1 activity, adiponectin, and large HDL-c levels. However, when analyzing the patient population, the low HDL-c subgroup presents a notorious worse cardiometabolic profile when compared with normal HDL-c subgroup of patients, being the differences clearly more pronounced than those encountered in the control subjects. The impact of low-HDL-c levels is seen by some classical parameters but mainly by nonclassical markers. Indeed, patients with low concentration of HDL-c present increased contents of HbA1c, TGs, non-HDL-c, Ox-LDL, hsCRP, VEGF, and small HDL, as well as decreased concentration of adiponectin and of large-HDL.

Despite the recognition of an association between low levels of HDL-c with increased risk for CAD [40, 41], it has been suggested that a better indicator of HDL functionality may be their quality [42, 43], which depends on its subpopulation's type (large versus small) and constituents, including PON 1 activity [44, 45]. Our results are in agreement with this theory; indeed, the beneficial HDL profile found in normal HDL-c subgroups, relative to the corresponding low HDL-c ones, was reinforced by significantly increased content of large HDL-c and decreased of small HDL-c (specially in the patients population). Thus, low HDL-c values are

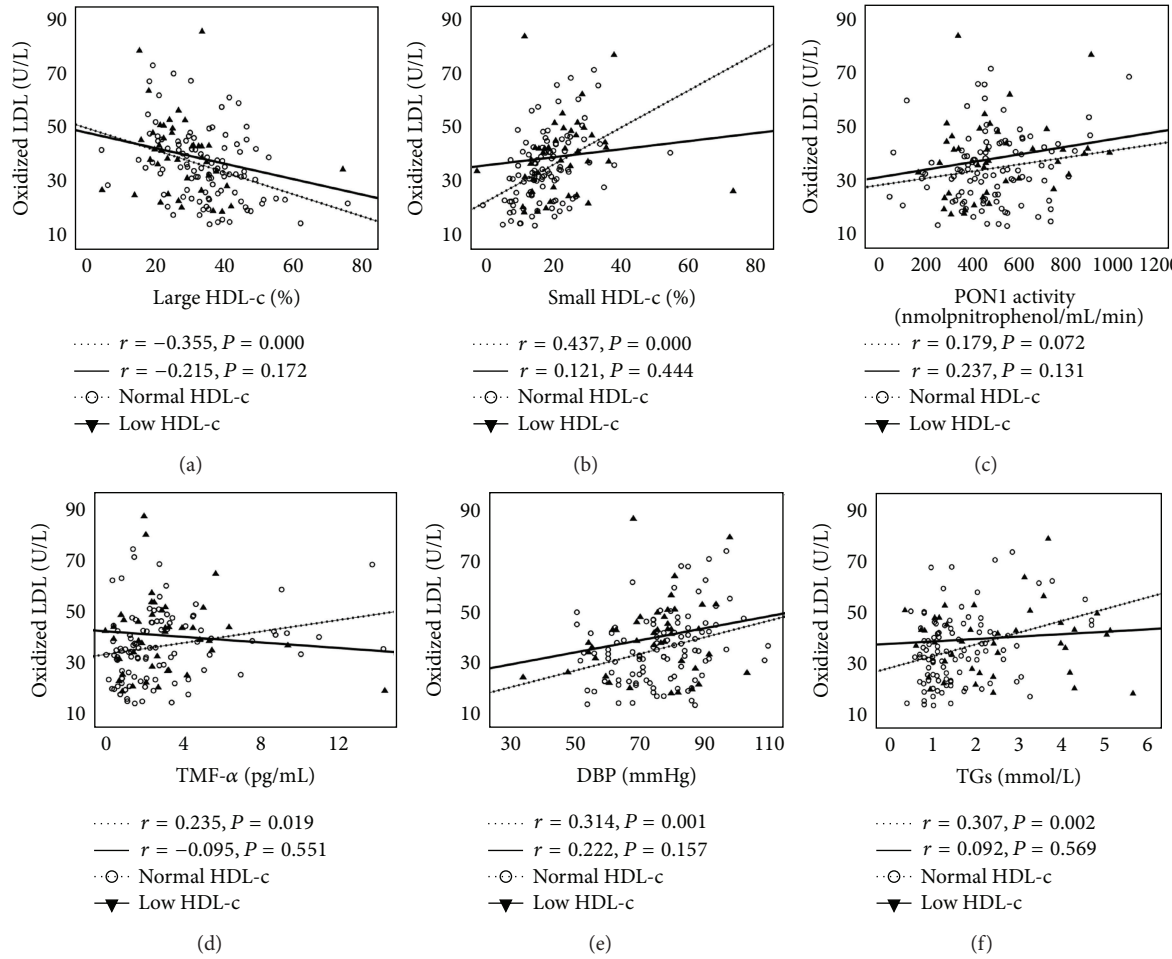

Figure 4: Main correlations in normal and low HDL-c patients. Correlation between Ox-LDL-c and large HDL-c (a), small HDL-c (b), PON1 activity (c), TNF-α (d), DBP (e), and TGs (f).

associated with a seemingly less protective subpopulation typology. Genest et al. [46] reported that although 34% of patients with premature heart disease had LDL-c levels >160 mg/dL, more than half of the patients with premature heart disease (57%) had low HDL-c levels. Additionally, it has been reported that in patients with premature CAD the greatest risk factor is actually low HDL-c levels, though these individuals often possess high TG concentration as well [47]. These studies are in agreement with our results showing a poor cardiometabolic profile in the subgroups with low HDL-c levels, both being accompanied by increased amounts of triglycerides.

Concerning the blood pressure, the values of both SBP and DBP in the control population are indeed higher when compared with the patients' population, no matter the normal or low HDL-c levels, which might be analyzed in terms of the antihypertensive therapy of the patients' population. In these subjects, medication is able to normalize blood pressure in both subgroups (normal and low HDL-c). However, in the control nonmedicated population, the subgroup with low DHL-c levels presents significantly increased values of both SBP and DBP. Several aspects related with HDL functionality might contribute to explain the differences of BP in the low versus normal HDL-c subgroups. In fact, as mentioned above, HDL has distinct properties that contribute to a healthy vasculature, such as antioxidant and anti-inflammatory action and inhibition of expression of cell adhesion molecules in endothelial cells, as well as antithrombotic and vasorelaxant effects, including the promotion of nitric oxide and prostacyclin release by vascular cells [31–34], which is expected to have a beneficial impact on arterial stiffness and blood pressure, in agreement with the suggestion of Woodman et al. [48]. In the presence of antihypertensive medication (as occurs in the patients' population) these differences were absent, but the data from the control nonmedicated subjects seems to be important per se. In fact, according to the South-West Seoul (SWS) study, performed in an elderly Korean population, prehypertension is not associated with increased risk of mortality, but individuals with high-normal blood pressure, when combined with low HDL-c, showed a significantly increased risk of all-cause mortality [49], reinforcing the relevance of our findings in this population of subjects with low-HDL-c levels and high-normal blood pressure, which were yet not diagnosed nor medicated for any

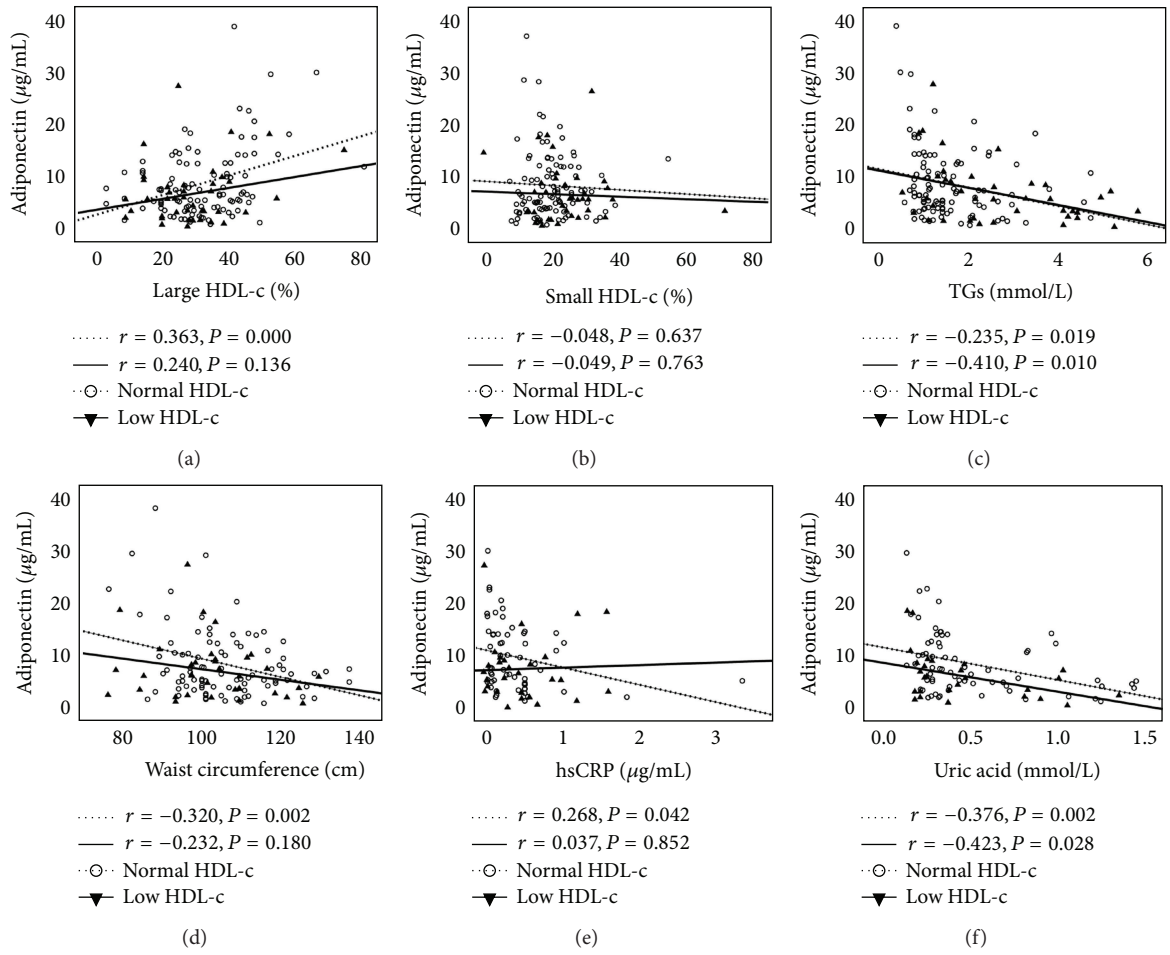

Figure 5: Main correlations in normal and low-HDL-c patients. Correlation between adiponectin and large HDL-c (a), small HDL-c (b), TGs (c), waist circumference (d), hsCRP (e), and uric acid (f).

cardiovascular disease, including for blood pressure and/or lipids.

In relation to the markers of inflammation, our study is in agreement with the study of Khan et al. [50] which has reported that a decrease in serum HDL levels and an increase in hsCRP values strongly predispose the risky individuals to an acute myocardial infarct (AMI) event; in addition, the reduction of serum total cholesterol does not prevent the risk of AMI. In addition, inflammation seems to have a deleterious impact on the antiatherogenic properties of HDL, suggesting that HDL function assessment is of particular importance when predicting CV risk in patients with chronic inflammatory diseases [51]. In our study, adiponectin levels also indicated an interesting association with the values of HDL cholesterol. Adiponectin is a novel adipocyte-specific protein which plays a role in the development of insulin resistance and atherosclerosis [52]. In our study, in both low-HDL-c subgroups the levels of adiponectin were decreased, which is in agreement with Fernandez et al. [53] which reported that individuals with low HDL-c concentrations present an increased risk for diabetes, as they show increased insulin resistance and lower levels of adiponectin.

Inflammation and oxidative stress are key pathways in the development of atherosclerosis, with oxidized LDL being one of the major players in this process, together with several mediators of inflammation [13, 14, 54]. Oxidized LDL induces atherosclerosis by stimulating monocyte infiltration and smooth muscle cell migration and proliferation. It contributes to atherothrombosis by inducing endothelial cell apoptosis and thus plaque erosion, by impairing the anticoagulant balance in endothelium, stimulating tissue factor production by smooth muscle cells, and inducing apoptosis in macrophages [55]. HDL cholesterol levels are inversely related to risk of CAD and prevent atherosclerosis by reversing the stimulatory effect of oxidized LDL on monocyte infiltration [21, 56]. The HDL-associated enzyme paraoxonase inhibits the oxidation of LDL and its effects [44, 45, 57]. In our study, the levels of Ox-LDL are increased in the subgroup of patients with low HDL-c, although there are no changes in the values of PON1 activity. On the contrary, in the control population, a decreased PON1 activity was found in the subgroup with low HDL-c group, without changes on Ox-LDL contents. There seems to be a correlation between these three parameters, which most probably is related with the above-mentioned

functionality (quality) of HDL in the control and patients populations, as well as with the previously reported effects of antidyslipidemic therapy, namely, statins, on Ox-LDL and PON1 activity [58, 59].

Endothelial dysfunction is thought to play a critical role in the development and progression of atherosclerosis and several recent studies have suggested that HDL exerts direct endothelial-protective effects, such as stimulation of endothelial production of the anti-atherogenic molecule nitric oxide, as well as antioxidant, anti-inflammatory, and antithrombotic effects [31–34]. Furthermore, it has been observed that HDL may stimulate endothelial repair processes, involving mobilization and promotion of repair capacity of endothelial progenitor cells [60]. In addition, VEGF has been viewed as a stimulating factor for the progenitor cells and the cell migration response [61]; in our study, although there were no significant changes in serum iCAM-1 levels, significantly higher serum VEGF levels were obtained in the low HDL-c patients' subpopulation, which might be a mechanism for promotion of endothelial repair under these circumstances.

Among the factors studies, some of them seem to have particular relevance, as the correlations analysis indicates. In fact, in the patients' population, but in particular in the subgroup with normal HDL-c levels, interesting correlations were found between three parameters (large HDL-c, Ox-LDL, and adiponectin) and several classical and nonclassical markers/risk factors. Large HDL-c contents showed a significant inverse correlation with Ox-LDL, TGs, non HDL-c and TNF-α, and direct with adiponectin. Ox-LDL values presented inverse significant correlation with large HDL-c and direct and significant with small HDL-c, TGs, TNF-α, and DBD. In addition, adiponectin concentrations presented statistically significant direct correlation with large HDL-c and inverse with waist circumference, hsCRP, uric acid, and TGs contents. These important associations were more evident for the normal HDL-c patients' subgroup and appear less associated in the subgroup with low HDL-c levels, which seem to indicate that when HDL-c levels are below the average values there is a deregulation of the factors (lipidic, oxidative, inflammatory, and angiogenic), with a putative important impact on the evolution of vascular disease.

Considering the cardiometabolic impact of low HDL-c levels on this type of patients with previous cardiovascular risk factors, even when LDL-c concentrations are adequately managed by antidyslipidemic therapy, therapeutic measures able to improve HDL-c levels and their quality/functionality, as well as inhibit LDL oxidation, might be of key importance to reduce the residual risk previously identified on this type of populations, namely, by reducing the oxidative, inflammatory, and angiogenic mechanisms underlying the evolution of disease. Since the current therapeutic arsenal is of limited impact on HDL-c levels, in particular the most popular medication, such as statins, nonpharmacological measures might deserve more attention, as well as new and more effective agents that might prove efficacy to improve HDL and their beneficial effects, including reduction of Ox-LDL as well as of deleterious inflammatory mediators. In fact, current data increasingly recommends aggressive measures to raise HDL-c levels and functionality as part of the prevention and treatment of CHD, while the novel HDL and Ox-LDL-directed pharmacotherapeutic strategies under discovering and evaluation will be able to help on this battle [22, 23, 62–64].

The study has some limitations that deserve further research in a near future: (a) the possibility of bias related with the inclusion of control subjects (defined as not having previous diagnosis for CV disease and not taking any medication for that) with risk factors, such as increased blood pressure and with overweight; (b) a more ample anthropometric and biochemical characterization would strength the results and findings; (c) the influence of other factors in this type of patients, such as menopausal status, lifestyle habits, and medication taken, will improve the data.

5. Conclusions

In a patient population with cardiovascular risk factors, low HDL-c levels are associated with a poor cardiometabolic profile, despite the average levels of LDL-c. This condition is better viewed by nontraditional lipid markers, including HDL subpopulations (in particular the large HDL-c one) and oxidized LDL, as well as markers of inflammation and angiogenesis, such as hsCRP, adiponectin, and VEGF. The existence of average HDL-c levels, with improvement of HDL quality/functionality, reduction of Ox-LDL and hsCRP, and increment of adiponectin, might prevent the evolution of cardiovascular disease in this type of individuals. In fact, despite called patients with residual cardiovascular risk, they often have non-fatal and fatal cardio and cerebrovascular events. Proper pharmacological and nonpharmacological therapeutic interventions directed to raise HDL-c levels and functionality and to inhibit Ox-LDL levels are advisory preventive measures in this type of CV risk populations.

Conflict of Interests

The authors report no conflict of interests.

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Review Article

The Role of Oxidized Low-Density Lipoproteins in Atherosclerosis: The Myths and the Facts

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The oxidative modification hypothesis of atherosclerosis, which assigns to oxidized low-density lipoproteins (LDLs) a crucial role in atherosclerosis initiation and progression, is still debated. This review examines the role played by oxidized LDLs in atherogenesis taking into account data derived by studies based on molecular and clinical approaches. Experimental data carried out in cellular lines and animal models of atherosclerosis support the proatherogenic role of oxidized LDLs: (a) through chemotactic and proliferating actions on monocytes/macrophages, inciting their transformation into foam cells; (b) through stimulation of smooth muscle cells (SMCs) recruitment and proliferation in the tunica intima; (c) through eliciting endothelial cells, SMCs, and macrophages apoptosis with ensuing necrotic core development. Moreover, most of the experimental data on atherosclerosis-prone animals benefiting from antioxidant treatment points towards a link between oxidative stress and atherosclerosis. The evidence coming from cohort studies demonstrating an association between oxidized LDLs and cardiovascular events, notwithstanding some discrepancies, seems to point towards a role of oxidized LDLs in atherosclerotic plaque development and destabilization. Finally, the results of randomized clinical trials employing antioxidants completed up to date, despite demonstrating no benefits in healthy populations, suggest a benefit in high-risk patients. In conclusion, available data seem to validate the oxidative modification hypothesis of atherosclerosis, although additional proofs are still needed.

1. Introduction

Recent postulates on atherosclerosis designate the appearance of qualitative changes on endothelial cells, triggered by "irritative" stimuli (e.g., hypertension, dyslipidemia, and cigarette smoking), as an early pathogenic event [1]. This process occurs at specific segments of the arterial tree, mainly branching points and bifurcations, characterized by disturbed laminar blood flow, probably owing to differences in arteries regional development [2] and to the loss of the atheroprotective effect of laminar shear stress [3]. In this setting, the endothelium expresses adhesion and chemotactic molecules and acquires an increased permeability to macromolecules, which modifies the composition of the subendothelial extracellular matrix. Hence, the entry of low-density lipoprotein (LDL) particles in the arterial wall followed by their retention through the binding of apolipoprotein B100 to proteoglycans of the extracellular matrix [4] is held to be a key-initiating factor in early atherogenesis [5]. The LDL particles trapped in the subintimal extracellular matrix are mildly oxidized by resident vascular cells [6]. They retain the capability of binding to the LDL receptor [6, 7] and to exert their proatherogenic effects [8–10], including stimulation of the resident vascular cells to produce monocyte chemotactic protein-1, granulocyte, and macrophage colony-stimulating factors. These molecules promote monocytes recruitment and their differentiation into macrophages, which are able to further promote the oxidation of LDLs [11] through myeloperoxidase and reactive oxygen species. Completely oxidized LDLs, characterized by an increased apolipoprotein B100 negative charge, are recognized by scavenger receptors on macrophages and internalized to form foam cells [12], the hallmark of the atherosclerotic lesion. Furthermore, macrophages play a key role in atherogenesis

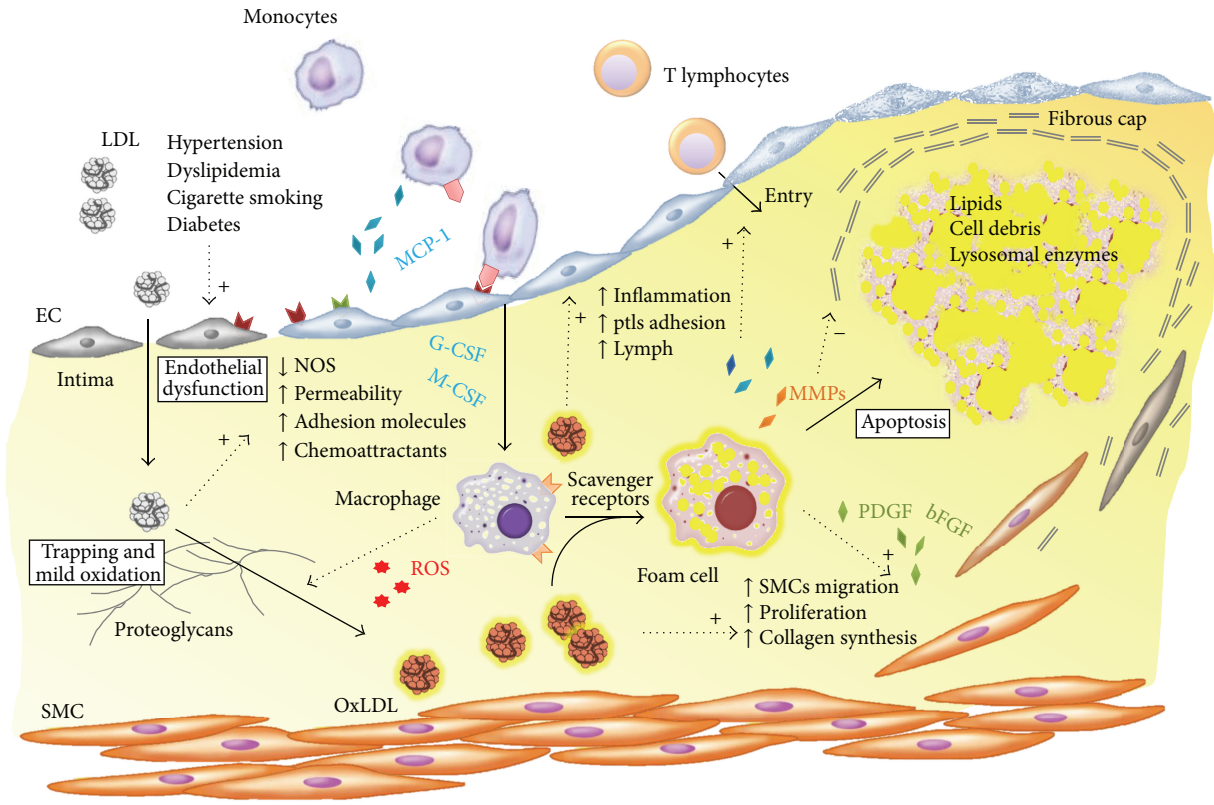

Figure 1: Putative pathway of oxidized low-density lipoprotein (oxLDL) in the atherogenetic process according to the oxidative hypothesis of atherosclerosis.

through their proinflammatory action, which involves the production of interleukin-1β and tumor necrosis factor (Figure 1).

Other main effectors in the development of atherosclerotic lesions are smooth muscle cells (SMCs), which are recruited from the tunica media to the subendothelial space, where they proliferate in response to mediators such as the platelet-derived growth factor. SMCs residing in the tunica intima produce extracellular matrix molecules, for example, interstitial collagen and elastin, and build the fibrous cap that overlies the growing atherosclerotic plaque. The latter entails macrophage-derived foam cells, cellular debris, and extracellular lipids, which are inefficiently cleared due to defective efferocytosis and thereby form the so-called necrotic core of the plaque [13].

The atherosclerotic plaque becomes clinically manifest when it reaches an advanced stage due to its blood flow-limiting effects or its destabilization with ensuing thrombosis. Unfortunately, the latter complication, which is responsible for ischemic events, is not strictly related to the degree of stenosis at angiography [14, 15] as its occurrence stands mostly on the cellular features of the plaque and particularly on the thickness of the overlying fibrous cap [16, 17]. In fact, atherosclerotic plaques prone to rupture are characterized by accumulation of inflammatory cells, mostly at the shoulder regions. These cells degrade collagen through release of collagenolytic enzymes, mainly matrix metalloproteinases (MMPs), and also reduce its synthesis by inducing SMCs apoptosis [18].

Many excellent reviews on the current knowledge of atherosclerosis are available, but few are focused on oxidized LDLs. Hence, this review examines the role played by oxidized LDLs in atherogenesis taking into account data derived by studies based on molecular and clinical approaches.

2. Evidence Linking Oxidized LDLs to Atherosclerosis

The oxidative modification hypothesis designates the oxidative change of LDLs as a crucial, if not mandatory, step in atherogenesis [19]. This theory originated from studies demonstrating that LDLs modified by endothelial cells, transformation entailing an oxidation process [20], could be internalized and accumulated avidly by macrophages [21, 22], leading to foam cell formation, although these cells could also be generated from macrophages internalizing native LDLs from the medium through micropinocytosis [23], or by uptake of aggregated LDLs or LDL immune complexes.

Several potential mechanisms can explain how LDL oxidative modification occurs within the arterial wall *in vivo*. A major role has been proposed for the 12/15-lipoxygenase [24, 25] because (1) it is expressed in atherosclerotic plaques but not in normal vessels [26] and (2) its inhibition was

associated with decreased oxidation of LDLs [27] and reduced atherosclerosis in animal models [25, 28, 29]. Myeloperoxidase, a heme enzyme secreted by neutrophils and monocytes/macrophages, is another suggested agent. It was found in human atherosclerotic plaques [30] and modifies LDLs, thus increasing their affinity for CD36 and SR-A [31, 32], the scavenger receptors mediating the uptake of oxidized LDLs by macrophages. Nitric oxide synthase (NOS) and nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase are other putative players as their products nitric oxide and superoxide anion, respectively, can combine to form the strong oxidizing agent peroxynitrite.

Native LDLs are internalized by macrophages at a pace too low to account for foam cells formation [33] owing to LDL receptor downregulation. Oxidative modification of LDLs increases their uptake by macrophages [20], via scavenger receptors. The latter are not downregulated in response to increased intracellular cholesterol, which explains why foam cells formation is held to occur with oxidized LDLs and not with native LDLs.

Besides contributing to the formation of lipid-laden macrophages, oxidized LDLs exhibit a wide array of biological properties, which are deemed to promote atherosclerosis.

(i) Oxidized LDLs exert chemotactic activity for monocytes [34], stimulate their binding to endothelial cells [35] by inducing the expression of intercellular adhesion molecule-1 and vascular-cell adhesion molecule-1 [36], are mitogenic for macrophages [37], and promote their trapping in the intima, while limiting their egress from the arterial wall [38]. Hence, oxLDL is key for recruitment, activation, and proliferation of monocytes/macrophages in the arterial wall.

(ii) Oxidized LDLs increase the expression of growth factors, such as platelet-derived growth factor (PDGF) and basic fibroblast growth factor (FGF) by endothelial cells and macrophages. The former stimulates migration of SMCs [39–41], while the latter induces SMCs proliferation [42].

(iii) Oxidized LDLs stimulate collagen production by SMCs [43], thus contributing to the fibrous cap lining the atherosclerotic plaque and the expansion of the lesion size. However, they could also promote fibrous cap thinning by increasing secretion of matrix metalloproteinase 1 [44] and matrix metalloproteinase 9, decreasing production of the tissue inhibitor of metalloproteinase 1 [45], and inducing SMCs apoptosis [46]. Therefore, they can contribute to the occurrence of vulnerable plaques [16, 17]. Hence, taken together, this evidence involved oxidized LDLs in the progression of the atherosclerotic plaque and the development of its complications.

(iv) Oxidized LDLs are cytotoxic to vascular cells [47, 48] and promote their apoptosis [49, 50] with ensuing release in the subendothelial space of lipids and lysosomal enzymes, enhancing the progression of the atherosclerotic plaque [47] and the production of the necrotic core.

(v) Oxidized LDLs stimulate platelet adhesion and aggregation, by decreasing endothelial production of nitric oxide, increasing prostacyclin production [51, 52], and stimulating the synthesis of prostaglandins and prostaglandin precursors [53]. Moreover, they decrease the secretion of the tissue-type plasminogen activator and increase that of plasminogen activator inhibitor-1 followed by a reduction of the fibrinolytic activity of endothelium [54–56]. Ultimately, they may determine vasoconstriction by inhibiting nitric oxide [57] and increasing endothelin production [58]. Taken together, these findings may explain the thrombotic complications of advanced atherosclerotic plaques.

3. *In Vivo* Models Supporting the Oxidized LDL Role in Atherosclerosis

Several studies were carried out *in vivo* in animal models where either a modulation of oxidative stress or manipulation of the scavenger receptor was undertaken, in order to prove the role of oxidized LDLs in the pathogenesis of atherosclerosis.

In an animal model of increased oxidative stress obtained through the overexpression of 15-lipoxygenase in the vascular wall, larger atherosclerotic lesions were found in LDL receptor-deficient mice [59]. However, a decreased atherosclerosis in cholesterol-fed rabbits and WHHL rabbits whose macrophages overexpressed human 15-lipoxygenase was also reported [60]. Animal models of reduced oxidative stress, instead, were obtained through knockout of oxidative stress-related genes or increasing the antioxidants: in three different knockout mouse models for 12/15-lipoxygenase, a decreased severity of atherosclerosis was seen [25, 61–64]. However, in apoE-deficient mice, the knocking out of the macrophage-specific 12/15-lipoxygenase increased the extension of atherosclerotic lesions [65].

The knockout in atherosclerosis-prone mice models of either SR-A or CD36 scavenger receptors, accounting for almost 90% of macrophage oxidized LDLs uptake [66], was demonstrated to be efficacious in decreasing the atherosclerotic burden [67, 68]. However, these results were not confirmed in another mice model with a CD36 and SR-A double knockout [69].

The results of these studies proved to be highly contradictory, due to the different animal models used, the different genetic background, and the unexpected consequences of gene deletions [70].

Finally, in spite of these conflicting data, support to the oxidative theory comes from extensive literature on the treatment of atherosclerosis-prone animals with antioxidants (reviewed by Witztum and Steinberg [71]). Most of these studies were carried out with probucol and demonstrated a protection from atherosclerotic lesions with the exception of the murine models, possibly secondary to a peculiar toxicity of this molecule in mice. In fact, in apoE-deficient mice fed with vitamin E, decreased atherosclerosis, paralleled with a decrease of aortic wall, plasma, and urinary F_2 isoprostanes, a marker of oxidative stress, was observed [72].

4. Human Findings Supporting the Oxidized LDLs Role in Atherosclerosis

There is a wealth of literature on the association between oxidized LDLs and cardiovascular events. An important premise needs to be made beforehand, however, in that oxidation of LDLs induces immunogenic epitopes in their particles [73] with ensuing generation of antibodies against them (oxLDL Abs). Since these autoantibodies are detectable in the sera of the majority of patients with advanced atherosclerotic lesions [74], they can be viewed as *in vivo* markers of LDL oxidation. Oxidized LDLs and their involvement in atherosclerosis can therefore be assessed by two ways: (1) using murine monoclonal antibodies directed toward different oxidized LDLs epitopes and (2) determining the immunogenic response to oxidized LDLs. Both approaches have advantages and pitfalls, as reviewed in detail by Tsimikas [75].

Human studies on the association of oxidized LDLs with atherosclerosis or cardiovascular events have been highly conflicting (for rev. [76]). Some cross-sectional studies suggested a direct association of oxidized LDLs or oxLDL Abs with atherosclerosis in different vascular beds [74, 77, 78], whereas others found no association with coronary atherosclerotic burden in coronary artery disease patients [79–81]. Owing to these contradictory results, we focused on cohort studies, which are more solid and less prone to serendipitous findings, because of a lower chance for selection and recall bias [82].

Among the twenty-two cohort studies reporting on cardiovascular events, fourteen were positive [81, 83–94] (Table 1) and eight were negative [80, 95–101] (Table 2). Due to potential publication bias, the preponderance of positive results clearly does not provide a proof of the strength of the association [102]. However, it is important to highlight that three [95, 99, 100] out of eight negative studies were completed in healthy people. This carries a limitation in that the robustness of cohort studies depends on the assumption that the control group—in this case those exposed to low levels of oxidized LDLs—has features as close as possible to the group exposed to high levels of oxidized LDLs [103, 104]. Theoretically, this goal can be better accomplished in selected populations made of patients with similar risk profile, rather than in studies recruiting healthy persons. Among these negative studies, the first one reported on cardiovascular events in a large cohort of more than three thousand elderly patients who had 420 cardiovascular events after 5 years of followup [95]. Oxidized LDLs were predictive of cardiovascular events only if a multivariate analysis was not adjusted for the presence of metabolic syndrome. In the second study, which enrolled almost three thousand healthy subjects, *malonyldialdehyde*-LDL autoantibodies were not associated with cardiovascular events [99]. In the latter, similarly performed in a healthy population, *malonyldialdehyde*- and Cu-LDL autoantibodies and oxidized LDLs were not predictive of progression of carotid atherosclerosis [100].

The lack of association between oxidized LDLs and cardiovascular events, possibly due to lack of statistical power, was also reported in two small cohorts of high-risk end-stage renal disease [101] and diabetes mellitus patients [96].

Other negative studies enrolling patients with coronary heart disease [80, 97, 98] were either too small [80, 97] and with a number of cardiovascular events too low to allow detection of any effect of oxidized LDLs or had an endpoint not appropriate to study atherosclerosis because most of the cardiovascular events were coronary artery restenosis (75% of total events) [98]. Moreover, it is worth highlighting that the negative study published by Tsimikas et al. [80] was on the same cohort where an association between coronary artery atherosclerosis and oxidized LDLs was demonstrated [78].

Among the positive studies four out of fourteen were carried out in a healthy cohort [88, 90, 92, 94], thus exposing them to the same considerations expressed above. Moreover, it has to be pointed out that three of these studies were completed in the same cohort, the Brunick study, at different time points of follow up, that is, 5 [90], 10 [92], and 15 years [94], and all demonstrated a predictive value of oxidized LDLs on cardiovascular events, contradicting the results on carotid artery atherosclerosis [100] on the same population.

Other studies demonstrating an association between oxidized LDLs and cardiovascular events enrolled small cohorts of either high-risk patients [83–86] or coronary artery disease patients [87, 89, 105]. Therefore these positive results could be secondary to serendipitous findings, as suggested by the opposite results on similar cohorts of end-stage renal failure patients where high oxLDL Abs titer was associated to either low [84] or high [86] cardiovascular mortality.

Two studies reported an association of oxidized LDLs with cardiovascular events in diabetics [93] and acute coronary syndrome patients [91]. Finally, using a prospective cohort study design and an unequivocal definition of the coronary artery disease phenotype, we reported the association of oxLDL Abs with cardiovascular mortality and cardiovascular events in more than 700 coronary artery disease patients [81].

In conclusion, most cohort studies reported an association between oxidized LDLs and cardiovascular events or mortality, in particular those including either a very high-risk population, that is, with end-stage renal disease and diabetes, or coronary artery disease patients. However, despite being an appealing hypothesis, the oxidation theory of atherosclerosis is not conclusively corroborated by observational studies, which have conflicting results, probably owing to the enrolment selection criteria and low statistical power.

5. Clinical Trials on Antioxidants and the Oxidized LDL Hypothesis

The oxidative theory of atherosclerosis would be conclusively proven by the beneficial effects of oxidative stress decrease on cardiovascular events. Therefore, the analysis of controlled randomized trials on antioxidant therapy in this setting is crucial. The first report on efficacy of antioxidants on cardiovascular events in coronary artery disease patients [106] was later confirmed by further studies [107–110] (Table 3), but numerous subsequent randomized clinical trials failed to prove any benefit [111–126] (Table 4). Moreover, meta-analyses on this issue are discordant [127, 128].

Table 1: Cohort studies demonstrating an association between oxidized low-density lipoprotein measurement and cardiovascular events.

Oxidative oxLDL test	Population under study	CV endpoints	Number of events	Followup	Findings	Reference
OxLDL Abs 4E06	326 men	IMT	na	3 years	OxLDL predicted IMT and carotid plaque progression	Wallenfeldt et al. [88]
OxLDL Abs 4E06	765 subjects	CV events	77 CV events	5 years	OxLDL predicted CV events	Tsimikas et al. [90]
OxLDL Abs 4E06	765 subjects	CV events	82 CV events	10 years	OxLDL predicted CV events	Kiechl et al. [92]
OxPL/apoB, AutoAbs MDA-/Cu-oxLDL	765 subjects	CV events	138 CV events	15 years	OxPL/apoB predicted CV events and stroke; AutoAbs predicted CV events, stroke, and ACS	Tsimikas et al. [94]
MDA-LDL	907 NIDDM	CV events, MI	152 CV events, 43 MI	3.7 years	MDA-LDL predicted MI and CV events	Lopes-Virella et al. [93]
AutoAbs Cu-oxLDL	249 ESRD	CV mortality	74 CV deaths	63 months	AutoAbs predicted CV mortality	Shoji et al. [84]
AutoAbs Cu-oxLDL	94 ESRD on hemodialysis	Total mortality	32 deaths	24 months	AutoAbs predicted mortality	Bayés et al. [85]
AutoAbs Cu-oxLDL	94 ESRD on hemodialysis	CV mortality	33 CV deaths	4 years	AutoAbs predicted CV mortality	Bayés et al. [86]
OxLDL Abs DLH3	246 pts with coronary angiography	CV events: cardiac death, MI, PTCA, and CABG	76 CV events	38 months	OxLDL predicted CV events	Shimada et al. [87]
AutoAbs MDA-oxLDL	734 IHD pts	CV mortality, MI, ACS, and CV events	65 CV deaths, 153 CV events	7.2 years	OxLDL predicted CV death and events	Maiolino et al. [81]
AutoAbs Cu-oxLDL	74 PTCA pts, 14 ctr	Restenosis	34 restenosis	6 months	AutoAbs predicted restenosis	Lee et al. [107]
OxLDL Abs DLH3	102 primary PTCA pts, 86 ctr	Restenosis	25 restenosis	6 months	OxLDL predicted restenosis	Naruko et al. [89]
OxLDL Abs 4E06	433 ACS pts	CV death, MI	17 CV deaths, 57 MI	2 years	OxLDL predicted MI	Johnston et al. [91]
OxLDL Abs FOH1a/DLH3	84 CHF pts (EF < 45%), 18 ctr	CV death, CV hospitalization, and fatal arrhythmia	26 CV events	780 days	OxLDL predicted CV events	Tsutsui et al. [83]

Abs: antibodies; ACS: acute coronary syndrome; AutoAbs: autoantibodies; CABG: coronary artery by-pass surgery; CHF: congestive heart failure; Crt: controls; CV: cardiovascular; ESRD: end-stage renal disease; IHD: ischemic heart disease; IMT: intima-media thickness; MI: myocardial infarction; oxLDL: oxidized low-density lipoproteins; OxPL/apoB: oxidized phospholipids on apolipoprotein B-100; PTCA: percutaneous transluminal coronary angioplasty; pts: patients.

An in depth analysis of these studies, however, highlighted that most of the negative studies were completed in either healthy or high-risk individuals, whereas results of clinical trials completed in patients with cardiovascular disease demonstrated the benefit conferred by antioxidants in some cases [106, 108, 109], with notable exceptions [112, 113, 117, 125]. The fact that treatment was likely given to the wrong patients, that is, with very low risk profile, can explain the failure of antioxidants trials in preventing cardiovascular events in the aforementioned negative reports [129].

Moreover, as in cohort studies, positive effects of antioxidants were witnessed in randomized trials enrolling very high-risk populations, as end-stage renal disease patients in hemodialysis, characterized by an increased oxidative stress, possibly secondary to hemolysis and hemoglobin-induced LDL oxidation [130, 131]. In these patients, with vitamin E supplementation, as tested in the SPACE trial randomizing patients to vitamin E or placebo [108], cardiovascular events were reduced by 54% and myocardial infarction by 70%. Accordingly, the potent antioxidant N-acetylcysteine showed a significant 40% decrease in the combined primary endpoint of cardiovascular events in another study [109]. After these rewarding results, we proposed the use of vitamin E coated dialysis membrane in these patients, which effectively reduces oxidative stress markers [132, 133]. Finally, in another high-risk population of diabetics carrying the haptoglobin 2-2 genotype, which is associated with inferior antioxidant protection [134], vitamin E was able to reduce the primary composite end point of cardiovascular death, nonfatal myocardial infarction, or stroke [110], even on top of statin therapy [135].

Thus, most controlled randomized trials involving the use of antioxidants provided negative results. However,

Table 2: Cohort studies demonstrating no association between oxidized low-density lipoprotein measurement and cardiovascular events.

Oxidative oxLDL test	Population under study	CV endpoints	Number of events	Followup	Findings	Reference
OxLDL Abs 4E06	3033 elderly	CV events	418 IHD, 120 MI	3 years	OxLDL did not predict CV events at MV analysis	Holvoet et al. [95]
AutoAbs MDA-oxLDL	2619 subjects	IHD (angina, ACS, and IHD death); CV events (IHD + TIA/stroke)	151 IHD, 234 CV events	8 years	AutoAbs did not predict CV events	Wilson et al. [99]
OxLDL Abs 4E06, AutoAbs MDA-/Cu-oxLDL	919 subjects	Carotid atherosclerosis progression	na	5 years	AutoAbs and oxLDL did not predict CV events	Mayr et al. [100]
AutoAbs Cu-oxLDL	92 NIDDM, 80 ctr	CV events	34 CV events, 15 CV deaths	10 years	AutoAbs did not predict CV events	Uusitupa et al. [96]
OxLDL Abs 4E06	69 ESRD on hemodialysis, 33 ctr	CV events	18 CV events	43 months	OxLDL did not predict CV events	Lee et al. [101]
AutoAbs Cu-oxLDL	415 IHD	CV events	35 CV deaths/MI, 33 PTCA/CABG	5 years	AutoAbs did not predict CV events	Erkkilä et al. [97]
OxLDL Abs 4E06	687 PTCA pts	Restenosis, CV events	135 restenosis, 181 CV events	1 year	OxLDL did not predict CV events	Braun et al. [98]

Abs: antibodies; AutoAbs: autoantibodies; CABG: coronary artery by-pass surgery; CHF: congestive heart failure; Crt: controls; CV: cardiovascular; ESRD: end-stage renal disease; IHD: ischemic heart disease; IMT: intima-media thickness; MI: myocardial infarction; NIDDM: noninsulin dependent diabetes mellitus; oxLDL: oxidized low-density lipoproteins; PTCA: percutaneous transluminal coronary angioplasty; pts: patients.

Table 3: Randomized controlled trials demonstrating a beneficial effect of antioxidant therapy.

Source	Patients	Inclusion criteria	Antioxidant agent	Dose	Route	Endpoints	Followup	Events
CHAOS [106]	2002	Angiographically demonstrated CAD	Vit E	400/800 IU	PO	CV death + MI; nonfatal MI	510 d	CV death: 27 vit E, 23 pl; nonfatal MI: 14 vit E, 41 pl
WHS [107]	39876	Healthy women	Vit E	600 IU q48 h	PO	Composite endpoint (CV death, MI, and stroke)	10.1 y	CV events: Vit E 482, pl 517; CV death: Vit E 106, pl 140; MI: Vit E 196, pl 195
SPACE [108]	196	Hemodialysis CV disease pts	Vit E	800 IU	PO	Composite endpoint (MI, ACS, PAD, and stroke)	519 d	Composite endpoint: Vit E 15, pl 33; CV death: vit E 9, pl 15; nonfatal MI: vit E 8, pl 18
Tepel et al. [109]	134	Hemodialysis CV disease pts	Acetylcysteine	1200 mg	PO	Composite endpoint (CV death, MI, PTCA/CABG, PAD, and stroke)	14.5 m	Composite endpoint: acetylcysteine 18, pl 33
Milman et al. [110]	1434	Diabetes mellitus Hp 2-2 genotype	Vit E	400 IU	PO	Composite endpoint (CV death, MI, and stroke)	18 m	Composite endpoint: Vit E 16, pl 33

CAD: coronary artery disease; CV: cardiovascular; d: days; DM: diabetes mellitus; HR: hazard ratio; HTN: arterial hypertension; m: months; MI: myocardial infarction; MLD: minimal luminal diameter; na: not available; PAD: peripheral artery disease, pl: placebo; PO: per os; pts: patients; RF: risk factor; vit: vitamin; y: years.

Table 4: Randomized controlled trials demonstrating no effect of antioxidant therapy.

Source	Number of patients	Inclusion criteria	Antioxidant agent	Dose	Route	Endpoints	Followup	Events
Virtamo et al. [111]	27271	Male smokers	Vit E, beta-carotene	50 mg, 20 mg	PO	Major coronary events (CV death, MI)	6.1 y	CV events: Vit E 519, beta-carotene 547, Vit E + beta-carotene 511, and pl 534; CV death: Vit E 212, beta-carotene 235, Vit E + beta-carotene 222, and pl 238; non-fatal MI: Vit E 307, beta-carotene 312, Vit E + beta-carotene 289, and pl 296
Rapola et al. [112]	1862	Previous MI	Vit E, beta-carotene	50 mg, 20 mg	PO	Major coronary events (CV death, MI)	5.3 y	CV events: Vit E 94, beta-carotene 113, Vit E + beta-carotene 123, and pl 94
HATS [113]	80	CAD	Vit E/C, beta-carotene, and selenium	800 IU, 1 g, 25 mg, and 100 g	PO	Composite endpoint (CV death, MI, and PTCA/CABG)	38 m	CV events: antiox 9, pl 9; CV death: antiox 0, pl 1; nonfatal MI: antiox 1, pl 4
PHS II [114]	14641	Male physicians	Vit E/C	400 IU 500 mg	PO	Composite endpoint (CV death, MI, and stroke)	8 y	CV events: Vit E 620, pl 625; Vit C 619, pl 626; CV death: Vit E 258, pl 251; Vit C 256, pl 253; MI: Vit E 240, pl 271; Vit C 260, pl 251
WACS [115]	8171	High CV risk women	Vit E/C, beta-carotene	600 IU q48 h, 500 mg, and 50 mg q48 h	PO	Composite endpoint (CV death, MI, PTCA/CABG, and stroke)	9.4 y	CV events: Vit E 708, pl 742; Vit C 731, pl 719; beta-carotene 731, pl 719; CV death: Vit E 193, pl 202; Vit C 206, pl 189; beta-carotene 211, pl 184; MI: Vit E 131, pl 143; Vit C 140, pl 134; beta-carotene 135, pl 139
PPP [116]	4495	Subjects ≥ 1 RF	Vit E	300 mg	PO	Composite endpoint (CV death, MI, and stroke)	3.6 y	CV events: Vit E 56, pl 53; CV death: Vit E 22, pl 26; MI: Vit E 22, pl 25
GISSI-prevenzione [117]	5660	Recent MI	Vit E	300 mg	PO	Composite endpoint (CV death, MI, and stroke)	3.5 y	CV events: Vit E 371, pl 414; CV death: Vit E 155, pl 193; MI: Vit E 22, pl 25
Greenberg et al. [118]	1720	Skin cancer	beta-carotene	50 mg	PO	CV death	4.3 y	CV death: beta-carotene 68, pl 59
PHS [119]	22071	Male physicians	beta-carotene	50 mg q48	PO	Malignant neoplasm; composite endpoint (CV death, MI, and stroke)	12 y	CV events: beta-carotene 967, pl 972; CV death: beta-carotene 338, pl 313; MI: beta-carotene 468, pl 489
SUVIMAX [120]	13017	Adult subjects	Vit E/C, beta-carotene, selenium, and zinc	30 mg, 120 mg, 6 mg, 100 g, and 20 mg	PO	CV ischemic events	7.5 y	CV events: antiox 134, pl 137
HPS [121]	20536	CAD, PAD, DM, and HTN	Vit E/C, beta-carotene	600 mg, 250 mg, 20 mg	PO	Composite endpoint (CV death, and MI)	5 y	CV death: antiox 878, pl 840; MI: antiox 1063, pl 1047; CV events: antiox 2306, pl 2312
HOPE [122]	9541	CV disease or DM + additional CV RF	Vit E	400 IU	PO	Composite endpoint (CV death, MI, and stroke)	7 y	CV events: Vit E 1022, pl 985; CV death: Vit E 482, pl 475; MI: Vit E 724, pl 686

Table 4: Continued.

Source	Number of patients	Inclusion criteria	Antioxidant agent	Dose	Route	Endpoints	Followup	Events
Mark et al. [123]	3318	Esophageal dysplasia	Vit E/C, beta-carotene	60 IU, 180 mg, and 15 mg	PO	CV death	6 y	CV death: antiox 22, pl 35
CARET [124]	1845	Exposure to asbestos or smoke	Vit E/A	15/30 mg, 25000 IU	PO	Lung cancer incidence	5.5 y	CV death: HR 1.26 (0.99–1.61)
WAVE [125]	213	Postmenopausal women with CAD	Vit E/C	400 IU, 500 mg	PO	Change in MLD	2.8 y	CV events: antiox 10, pl 5; CV death: antiox 4, pl 2; nonfatal MI: antiox 3, pl 1
HOPE [126]	9541	CV disease or DM + additional CV RF	Vit E	400 IU	PO	Composite endpoint (CV death, MI, stroke)	4.5 y	CV events: Vit E 772, pl 739; CV death: Vit E 342, pl 328; MI: Vit E 532, pl 524

CAD: coronary artery disease; CV: cardiovascular; d: days; DM: diabetes mellitus; HR: hazard ratio; HTN: arterial hypertension; m: months; MI: myocardial infarction; MLD: minimal luminal diameter; na: not available; PAD: peripheral artery disease, pl: placebo; PO: per os; pts: patients; RF: risk factor; vit: vitamin; y: years.

administration of antioxidants to patients with known cardiovascular disease or with a very high-risk profile proved to be beneficial in a nontrivial number of studies.

6. Conclusions

Evidence supports on a molecular ground the oxidative hypothesis of atherosclerosis. The translation of experimental evidence in humans with studies aimed at the demonstration of the association of oxidative stress with cardiovascular events proved to be difficult and resulted in contrasting findings, particularly with administration of antioxidant therapy. However, the selection of patients either at higher risk or with cardiovascular disease provided much rewarding outcomes with numerous positive studies. It seems therefore that although this theory still needs further proofs to be definitely clarified, data available so far strengthen the pivotal role for oxidative stress in atherosclerosis.

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Clinical Study

Increased Serum ox-LDL Levels Correlated with Lung Function, Inflammation, and Oxidative Stress in COPD

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Chronic obstructive pulmonary disease (COPD) is associated with abnormal inflammation and high oxidative stress. Studies suggest that oxidized low density lipoprotein (ox-LDL) is involved in diseases associated with oxidative stress and inflammation. However, no data on the possible relationship between COPD and ox-LDL are available. This study compared serum levels of ox-LDL in 48 COPD patients and 32 health controls and correlated them with lung function, systematic inflammation, and oxidative stress. Serum levels of ox-LDL, C-reactive protein (CRP), and oxidative stress (measured by reactive oxygen species, ROS) were analyzed using commercial kits. Mean levels of serum ox-LDL were significantly higher in COPD patients than in controls (18.62 ± 7.56 versus 12.57 ± 5.90 mU/L, $P < 0.05$). Serum levels of CRP and ROS were also significantly higher in COPD patients. Serum levels of ox-LDL in COPD patients correlated inversely with FEV_1% predicted, an index of lung function ($r = -0.347$, $P = 0.016$), while they correlated positively with CRP and ROS levels. These results suggest that serum levels of ox-LDL are increased in COPD patients and that these levels are associated with lung function, inflammation, and oxidative stress in COPD. Future studies are needed to determine whether and how ox-LDL plays a role in COPD.

1. Introduction

Chronic obstructive pulmonary disease (COPD) remains a worldwide health care burden. Currently the fourth leading cause of death, COPD, is projected to become the third leading cause by 2030 [1, 2]. This disease is characterized by progressive, partially reversible airflow obstruction associated with abnormal airway inflammation and oxidant/antioxidant imbalance [3]. Recent studies suggest that in a subset of COPD patients, the inflammation may also "spread" into the circulatory system and cause systematic inflammatory injuries and organ damage [4, 5]. Thus, COPD can no longer be considered a disease only of the lungs. Instead, increased systemic inflammation may contribute to complex chronic comorbidities often observed in COPD patients, such as coronary artery disease, which is associated with poor clinical outcomes in COPD [6]. Despite the seriousness of COPD and its comorbidities, how systemic inflammation occurs in patients is unclear.

A possible explanation for the systemic inflammation in COPD is oxidized low density lipoprotein (ox-LDL), a marker of oxidative stress that links the immune system with the abnormal inflammation. This lipoprotein has a proinflammatory effect, and it enhances inflammatory cytokine production, as well as cell adhesion, migration, proliferation, and apoptosis [7]. Circulating ox-LDL is elevated in several diseases associated with high oxidative stress and inflammation, such as arteriosclerosis and sepsis [8, 9]. Numerous in vitro and animal studies, as well as epidemiological and correlational studies in humans, have proposed that ox-LDL may contribute to atherosclerosis. These findings raise the question of whether COPD, which is characterized by systemic inflammation and high risk of arteriosclerosis [4, 5], is also associated with elevated ox-LDL levels. If so, it may mean that ox-LDL may play a role in the pathophysiology of the disease.

Despite the importance of these questions, we are unaware of any studies examining a possible relationship between COPD and ox-LDL. Therefore, we carried out a preliminary clinical study to determine whether a relationship exists between COPD and ox-LDL.

2. Patients and Methods

2.1. Study Protocol. The study protocol conforms to the principles of the Declaration of Helsinki and was approved by the Institutional Review Board for Human Studies of West China Hospital of Sichuan University, China. Written consent was obtained from all subjects. Between October 2012 and January 2013, patients with COPD were recruited from the Outpatient Department of West China Hospital, and healthy controls were recruited from the hospital's physical examination center. All subjects underwent a standard lung function test, and COPD was diagnosed prospectively for this study on the basis of Global Initiative for Chronic Obstructive Lung Disease criteria [1].

To be included in the study, patients had to show (a) a ratio of forced expiratory volume in the first second to forced vital capacity (FEV_1/FVC) below 70% after bronchodilation and (b) an increase in FEV_1 below 12% after inhalation of β2-agonist (200 mg salbutamol), and they had to have remained (c) clinically stable for at least 3 months prior to the study. No COPD patients had received standard COPD treatments, such as inhaled corticosteroids. Patients were excluded if they had conditions known to affect serum levels of ox-LDL, including coronary heart disease and metabolic syndrome, the presence of which was determined based on medical history and fasting blood-glucose tests.

2.2. Measurement of ox-LDL, CRP, and ROS. Subjects were asked to fast overnight from 21:00 the night before, after which venous blood samples were collected and serum was separated immediately and stored at −80°C until analysis. Levels of ox-LDL and C-reactive protein (CRP) were analyzed using an enzyme-linked immunosorbent assay (ELISA; Xitang Bio-Technology Co., Ltd., Shanghai, China), and oxidative stress was assessed by measuring levels of reactive oxygen species (ROS) using a colorimetric kit (Xitang Bio-Technology Co., Ltd., Shanghai, China). The ROS kit measures $OH^{\bullet}$ and other free radicals in the serum. All the measurements were carried out strictly according to the manufacturer's instructions. Manufacturer-specified limits of detection were 1 mU/L for ox-LDL, 0.3 ng/mL for CRP, and 15 nmol/mL for ROS. The other biochemical tests, including assays for high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride (TG), total cholesterol (TC), and other indices, were carried out by the Department of Laboratory Medicine of West China Hospital. Technicians performing tests were blinded to the clinical details of the subjects.

2.3. Statistical Analysis. Results are presented as mean ± standard deviation (SD). Differences between groups were statistically analyzed using an unpaired Student's t-test after confirming that data were normally distributed. Correlations were performed using the bivariate Pearson's correlation test. The threshold of significance was set at 5%. Data were analyzed using SPSS 18.0 for Windows (IBM, Chicago, IL, USA).

Table 1: Characteristics of patients with COPD and healthy subjects.

Characteristic	COPD (n = 48)	Control (n = 32)	P value
Age (year)	62 ± 10	58 ± 11	0.177
Sex (m/f)	33/15	22/10	0.626*
BMI (kg/m^2)	22.57 ± 3.25	23.04 ± 2.62	0.494
TG (mmol/L)	1.12 ± 0.46	1.31 ± 0.45	0.071
TC (mmol/L)	4.66 ± 0.73	4.81 ± 0.78	0.376
HDL (mmol/L)	1.60 ± 0.31	1.55 ± 0.37	0.489
LDL (mmol/L)	2.75 ± 0.58	2.51 ± 0.59	0.078
Smoking (pack years)	13.93 ± 16.34	12.08 ± 19.69	0.457
FEV_1 (L)	2.06 ± 0.78	2.66 ± 0.61	0.000
FVC (L)	3.40 ± 1.13	3.21 ± 0.77	0.367
FEV_1/FVC%	59.73 ± 8.19	83.30 ± 5.05	0.000
FEV_1% predicted	86.87 ± 21.64	115.22 ± 15.91	0.000
oxLDL (mU/L)	18.62 ± 7.56	12.57 ± 5.90	0.000
CRP (ng/mL)	18.49 ± 5.79	15.21 ± 4.91	0.01
ROS (nmol/mL)	293.11 ± 90.44	242.86 ± 70.15	0.01

Data are presented as mean ± SD. *The chi-squared test was used to test the significance of the difference in gender proportions.
Abbreviations: COPD: chronic obstructive pulmonary disease; CRP: C-reactive protein; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity; HDL: high-density lipoprotein; LDL: low-density lipoprotein; ox-LDL: oxidized low-density lipoprotein; ROS: reactive oxygen species; TC: total cholesterol; TG: triglyceride.

3. Results

3.1. Clinical Characteristics of Subjects. A total of 48 COPD patients and 32 healthy controls participated in this study. Table 1 summarizes their demographic and clinical characteristics, smoking pack-years, and serum levels of TG, TC, HDL, LDL, ox-LDL, CRP, and ROS. Patients and controls were similar in terms of age, sex, and smoking pack-years. FEV_1% predicted among COPD patients was 86.87 ± 21.64%, suggesting that most had mild airflow limitation.

3.2. Serum Levels of ox-LDL, CRP, and ROS. Mean serum levels of ox-LDL were significantly higher in COPD patients (18.62 ± 7.56 mU/L) than in controls (12.57 ± 5.90 mU/L, P = 0.000; Figure 1(a)). The same was true of serum levels of CRP, a marker of systemic inflammation (18.49 ± 5.79 versus 15.21 ± 4.91 ng/mL, P = 0.01), and serum levels of ROS, a marker of oxidative stress (293.11 ± 90.44 versus 242.86 ± 70.15 nmol/mL, P = 0.01).

3.3. Correlations between ox-LDL and Indicators of Lung Function, Inflammation, and Oxidative Stress. Serum levels of ox-LDL in COPD patients correlated inversely with lung function, based on FEV_1% predicted (r = −0.347, P = 0.016; Figure 1(b)). In addition, serum levels of ox-LDL in patients also correlated inversely with the ratio of FEV_1/FVC (r = −0.412, P = 0.004). Conversely, ox-LDL levels in patients correlated positively with CRP (r = 0.365, P = 0.011; Figure 2(a)) and ROS (r = 0.346, P = 0.016; Figure 2(b)).

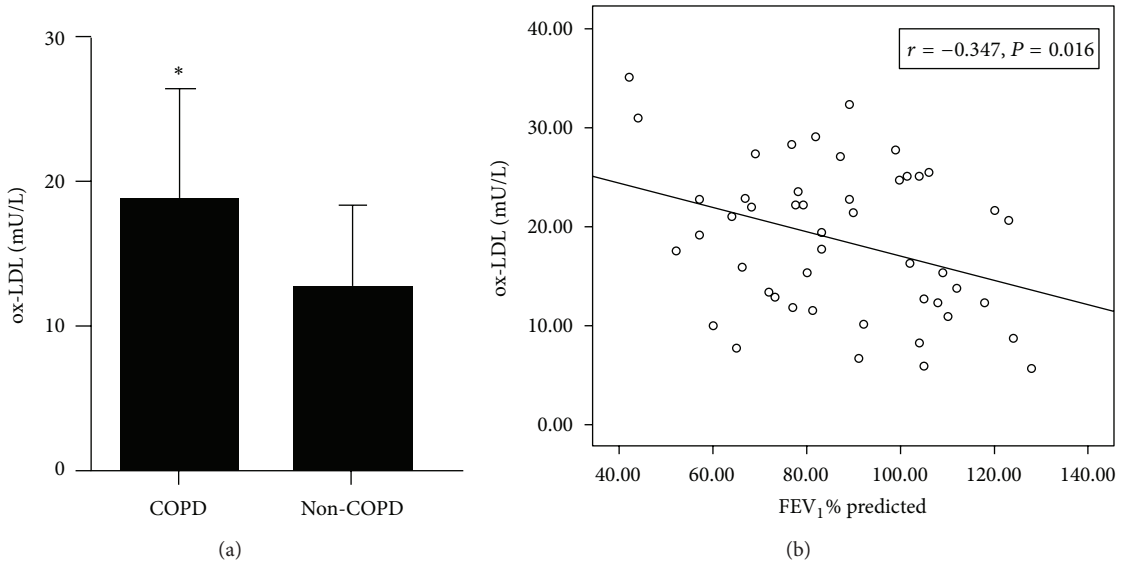

Figure 1: Serum levels of ox-LDL and correlation with lung function. (a) Levels of ox-LDL in COPD patients and healthy controls (*, $P < 0.05$). (b) Correlation between serum ox-LDL levels and lung function (FEV_1% predicted) in patients.

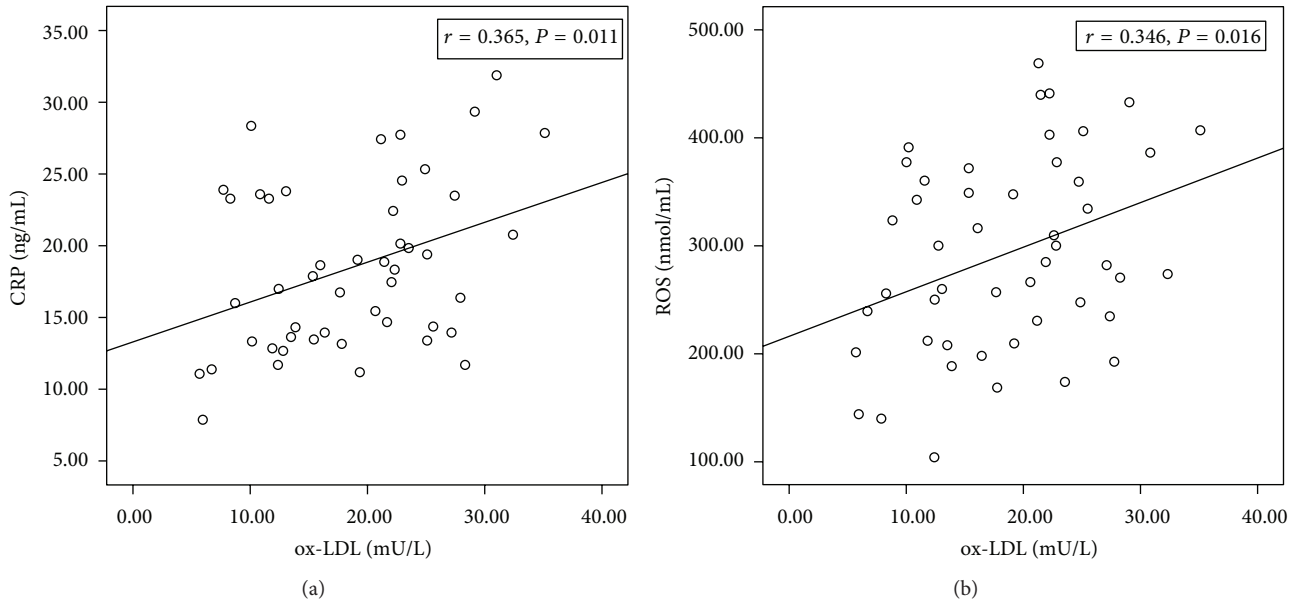

Figure 2: Correlations between serum levels of ox-LDL and (a) CRP or (b) ROS in COPD patients.

4. Discussion

To our knowledge, this is the first study to examine correlations between serum levels of ox-LDL levels in COPD patients and indicators of lung function, inflammation, and oxidative stress. We found significant correlations between ox-LDL levels and lung function decline (measured as FEV_1% predicted), inflammation (CRP), and oxidative stress (ROS). These findings justify further detailed work into the mechanistic basis of these correlations in order to determine whether and how ox-LDL levels contribute to the pathogenesis of COPD.

Circulating ox-LDL has been proposed to play a critical role in cardiovascular diseases such as atherosclerosis and to help upregulate the inflammatory response. Our findings suggest that it may also play a role in COPD. Such a role would be consistent with numerous lines of indirect evidence linking ox-LDL with this disease. First, ox-LDL activates several transcription factors involved in the pathogenesis of COPD, including activator protein 1, NF-κB, signal transducer and activator of transcription (STAT), and hypoxia-inducible factor 1 [10]. Second, an increase in ox-LDL levels activates chemotaxis of neutrophil or eosinophil granulocytes and monocytes in studies in vitro designed to mimic worsening airway inflammation [8, 11]. Third, ox-LDL has been shown to increase the expression or secretion of chemokines such as monocyte chemotactic protein-1,

macrophage inflammatory protein- (MIF-)1α, and MIF-2, as well as pro-inflammatory cytokines such as interleukin (IL)-1β, IL-12, and tumor necrosis factor-α [7, 12]. All these inflammatory mediators are known to contribute to COPD pathogenesis. Fourth, oxidized lipids are thought to promote a shift from acute to chronic inflammation [13], and this transition is a key characteristic of COPD. Fifth, ox-LDL increases intracellular ROS production [14], thereby increasing oxidative stress and potentially activating stress kinases and redox-sensitive transcription factors that enhance inflammation. This signaling increases the expression of a battery of distinct pro-inflammatory mediators that contribute to COPD [3]. Indeed, our laboratory has shown that ox-LDL up-regulates TGF-β1 production, Smad3 phosphorylation, and Ras/extracellular signal-regulated kinase activity in a dose- and time-dependent manner in A549 human alveolar epithelial cells [15]. These signaling molecules are known to be involved in the development of COPD. The present study extends the literature by showing that serum levels of ox-LDL are increased in COPD, suggesting for the first time that ox-LDL may be involved in the disease.

Our study also identified elevated serum levels of CRP and ROS in COPD patients relative to controls, which is consistent with previous studies [16, 17]. Circulating CRP levels are elevated in COPD patients and may thus be regarded as a valid biomarker of systemic inflammation [17]; in addition, serum CRP levels are associated with FEV_1 decline [18]. These results suggest that CRP and ROS may contribute to the chronic inflammation and oxidative stress characteristic of COPD. Another important observation in our study is the inverse correlation between ox-LDL and lung function (FEV_1% predicted) in patients, suggesting that ox-LDL may correlate with the severity of airway obstruction. In addition, ox-LDL in patients correlated positively with CRP and ROS, suggesting that ox-LDL may function as a biomarker of the severity of systematic inflammation and oxidative stress in this disease. Future studies should examine how ox-LDL is produced in COPD patients and what signaling pathways it triggers. Previous work suggests that smoking over the clinical course of COPD may lead to the production of ox-LDL [19], but our two groups showed similar levels of smoking. Larger, more detailed studies are needed to clarify whether and how smoking may contribute to ox-LDL levels and to COPD in general.

COPD exerts significant extrapulmonary effects that may contribute to its severity. A major comorbidity of COPD is coronary heart disease (also called atherosclerotic heart disease), which is the leading cause of death in patients with the disease [20]. Why these two diseases are so frequently found in combination remains unclear. Studies have implicated systematic inflammation and oxidative stress as pathophysiological links between COPD and coronary heart disease [21]. We speculate that the elevated serum levels of ox-LDL in COPD patients not only induce inflammation and oxidative stress but also play a role in promoting arteriosclerosis. In this way, ox-LDL may function as a bridge between COPD and coronary heart disease.

The primary goal of this study was to determine whether levels of ox-LDL correlate with COPD. While we achieved this goal by showing that there is indeed a correlation, the fact that we used a limited number of subjects and only two indicators to analyze systemic inflammation (CRP) and oxidative stress (ROS) means that we cannot gain detailed insights into whether and how ox-LDL contributes to the disease. Our small number of subjects also means that we cannot definitively conclude whether smoking contributed or not to our results. Given the strong evidence for smoking as a risk factor for COPD, larger studies specifically designed to assess the role of smoking in COPD-related inflammation and oxidative stress are needed. Furthermore, most of our patients presented with relatively mild COPD, so it would be important to verify our findings in patients with more severe disease.

To avoid the limitations of the present work, future studies should use computed tomography to detect the presence of emphysema, since our results may be biased by the relative numbers of subjects with "blue bloater" versus "pink puffer" phenotypes [22]. Future studies should also examine additional markers of systemic inflammation and oxidative stress in order to gain a more detailed and reliable picture of the role of ox-LDL in COPD. Given that ox-LDL is known to be a risk factor for coronary heart disease, subjects should be analyzed using echocardiography in order to correlate cardiac and lung function.

5. Conclusion

In this prospective study, serum levels of ox-LDL were higher in COPD patients than in healthy controls. Levels of ox-LDL correlated inversely with lung function and positively with serum levels of CRP and ROS. Larger, more detailed studies are needed to confirm our findings and determine whether and how high ox-LDL levels play a role in COPD.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Yongchun Shen and Ting Yang contributed equally to this work and are joint first authors.

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Research Article

Oxidized LDL Induces Alternative Macrophage Phenotype through Activation of CD36 and PAFR

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OxLDL is recognized by macrophage scavenger receptors, including CD36; we have recently found that Platelet-Activating Factor Receptor (PAFR) is also involved. Since PAFR in macrophages is associated with suppressor function, we examined the effect of oxLDL on macrophage phenotype. It was found that the presence of oxLDL during macrophage differentiation induced high mRNA levels to IL-10, mannose receptor, PPARγ and arginase-1 and low levels of IL-12 and iNOS. When human THP-1 macrophages were pre-treated with oxLDL then stimulated with LPS, the production of IL-10 and TGF-β significantly increased, whereas that of IL-6 and IL-8 decreased. In murine TG-elicited macrophages, this protocol significantly reduced NO, iNOS and COX2 expression. Thus, oxLDL induced macrophage differentiation and activation towards the alternatively activated M2-phenotype. In murine macrophages, oxLDL induced TGF-β, arginase-1 and IL-10 mRNA expression, which were significantly reduced by pre-treatment with PAFR antagonists (WEB and CV) or with antibodies to CD36. The mRNA expression of IL-12, RANTES and CXCL2 were not affected. We showed that this profile of macrophage activation is dependent on the engagement of both CD36 and PAFR. We conclude that oxLDL induces alternative macrophage activation by mechanisms involving CD36 and PAFR.

1. Introduction

The stimulation of monocytes/macrophages by modified low density lipoprotein (LDL), such as oxidized LDL (oxLDL), is an early event in atherosclerosis development. These macrophages accumulate in the subendothelial space and differentiate into foam cells [1], which contribute to a chronic inflammatory response in the arterial wall and atherosclerotic plaque progression [2]. Several pattern recognition receptors are involved in oxLDL recognition by macrophages; CD36 is one of the most studied.

Macrophages can acquire distinct phenotypes according to stimuli from the microenvironment. M1 or classically activated macrophages are induced by Th1 cytokines and exhibit high microbicidal activity and induce inflammation. In contrast, M2 or alternatively activated macrophages are induced by Th2 cytokines and contribute to the resolution of inflammation and tissue remodeling [3]. The atherosclerotic plaque provides a complex microenvironment for macrophages, and both populations, M1 and M2, have been found in human lesions [4]. It has been demonstrated that M1 macrophages are predominant in regions prone to rupture, while M2 macrophages are mostly detected in the adventitia and in stable plaque areas [5]. However, it is not known which role macrophages with such opposing functions play in the progression of atherosclerosis. M2 macrophages express high levels of CD36 and SR-A1 and are thus able to efficiently take up oxLDL and are more prone to differentiating into foam cells [6]. Macrophages also express receptors for platelet-activating factor (PAFR), and our previous work suggested that PAFR works in conjunction with CD36 for optimal oxLDL uptake and cytokine gene expression [7]. Moreover, preferential production of IL-10 over IL-12 was observed (submitted article).

In the present study, we investigated the effect of oxLDL on macrophage differentiation, activation, and phenotype and the role played by CD36 and PAFR. We found that oxLDL increases the expression of alternative activation markers and that both CD36 and PAFR are involved.

2. Methods

2.1. Purification and Oxidation of LDL. The study was approved by the ethics committee of the Institute of Biomedical Sciences, University of São Paulo. Plasma was obtained from normolipidemic volunteers and treated with benzamidine (2 mM), gentamicin (0.5%), chloramphenicol (0.25%), phenyl-methyl-sulfonyl-fluoride (PMSF) (0.5 mM), and aprotinin (0.1 units/mL). LDL (1.019–1.063 g/mL) was isolated by sequential ultracentrifugation at 100,000 g at 4°C, using a P90AT-0132 rotor (CP70MX ultracentrifuge; Hitachi Koki Co., Ltd., Tokyo, Japan). LDL was dialyzed at 4°C against PBS (pH 7.4) with 1 mM EDTA, filtered (0.22 μm), and stored at 4°C. The protein concentration was determined by the BCA kit (Pierce, USA). Oxidized LDL (oxLDL) was obtained by incubation of LDL (2 mg/mL) with 20 μM $CuSO_4$ for 18 h at 37°C. The oxidation of both protocols was terminated by the addition of 0.5 mM EDTA.

2.2. Human THP-1 Monocytic Cells. The monocytic line THP-1 was cultured in RPMI-1640 medium supplemented with 5% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 15 mM HEPES, and 11 mM sodium bicarbonate. Cell cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. The differentiation of THP-1 monocytes into macrophages was induced by 150 nM phorbol 12-myristate-13-acetate (PMA) for 24 h. Nonadherent cells were removed by aspiration of the supernatant followed by replacement with fresh medium. The experiments proceeded for 24 h according to the protocol.

2.3. Culture of Murine Macrophages. Male C57Bl/6 7–10 weeks old mice were acquired from the Department of Immunology Animal Facility at the University of São Paulo and kept in microisolator cages under specific pathogen-free conditions. Animal care and research protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Biomedical Sciences Institute/USP-Ethical Committee for Animal Research (CEEA). Peritoneal exudate cells were obtained by lavage of the peritoneal cavity 4 days after injection of 1 mL of 3% thioglycolate medium. The fluid lavage was centrifuged (100 ×g, 10 min, 4°C). The cellular concentration was adjusted to 2×10^6 cells/mL with RPMI 1640 medium, supplemented with 5% FCS, streptomycin (100 μg/mL), penicillin (60 U/mL), sodium bicarbonate (11 mM), l-glutamine (2 mM), and HEPES (20 mM), referred to hereafter as RPMI/FBS. Cells were left to adhere on microplates for 2 h at 37°C in a 5% CO_2. Nonadherent cells were removed by aspiration of the supernatant and replacement with fresh medium. The experiments proceeded according to the protocol.

Bone marrow-derived macrophages (BMDM) were obtained as previously described by Davies and Gordon [8], with minor modifications. In brief, femurs were flushed with DMEM medium containing 2 mM L-glutamine, 100 U/mL penicillin G, and 100 mg/mL streptomycin (all from Gibco, Long Island, NY, USA), using a $26G \times 1/2''$ needle. Cells were grown in DMEM containing 20% L-929 cell-conditioned medium (LCM) and 15% heat-inactivated fetal calf serum (FCS), incubated at 37°C in 5% CO_2. On day 3, fresh DMEM with LCM was added. A monolayer of macrophages was scrapped on day 6 (96% of the cells were positive for CD11b and F4/80). Macrophages were cultured in DMEM with 5% FCS for one day before further experiments.

2.4. Quantification of Nitric Oxide. NO production was assessed by nitrite production in culture supernatants using the Griess reaction. Briefly, culture supernatants were incubated with the Griess reagent (0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 2.5% phosphoric acid, v/v) at room temperature for 10 min. Absorbance was determined using a Dinatech microplate reader at 540 nm. A standard curve of sodium nitrite was used to determine the concentration.

2.5. Western Blot for iNOS and COX-2 Expression. Thioglycolate-elicited macrophages were treated with different concentrations of LDL or oxLDL for 24 h and then stimulated with LPS (1 μg/mL). Cells were washed with cold PBS, and lysates were obtained in lysis buffer (10% Nonidet P-40, 150 mM NaCl, 10 mM Tris-HCl, pH 7.6, and 2 mM 0.1% SDS) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO, USA) and phosphatase inhibitors (10 mM sodium fluoride and 1 mM sodium orthovanadate). Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of proteins were separated by 10% SDS-PAGE, transferred to a Hybond nitrocellulose membrane (GE Healthcare, NJ, USA), and incubated with rabbit-anti-COX-2 or rabbit-anti-iNOS (both from Cayman Chemical, Ann Arbor, MI, USA), and with mouse anti-β-actin (Sigma-Aldrich, Saint Louis, MO, USA). As secondary antibodies, we used antirabbit IgG-HPR (1 : 2000) and antimouse-HRP (1 : 2000) (Cell Signaling Technology, Beverly, MA, USA). Expression was visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). The resulting autoradiograms were analyzed with the AlphaEaseFC software V3.2 beta (Alpha Innotech, San Leandro, CA, USA).

2.6. MTT Assay. The mitochondrial-dependent reduction of methylthiazolyldiphenyl-tetrazolium bromide (MTT) into insoluble formazan crystals was used to evaluate cell viability. Briefly, 500 mg/mL of MTT in RPMI was added to the cells after treatment. Cells were incubated for 3 h at 37°C in a 5% CO_2 atmosphere. Next, a solution of 10% SDS in 0.01 M HCl was added to the cells to dissolve the crystals, and the absorbance was measured after 14 h using a Dynatech microplate reader at 570 nm.

2.7. Real-Time RT-PCR. Cells were harvested at various time points, and total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was generated from total RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific Fermentas, Vilnius, Lithuania). Real-time PCR was performed with the Stratagene MxPro3005PTM QPCR Systems (Santa Clara, CA, USA), using SYBR Green (SYBR Green Master Mix, Applied Biosystems, Warrington, UK) and specific primers for *IL-10, MR, Arg-1, PPAR-γ, iNOS, IL-12p40, TGF-β1, RANTES,* and *GAPDH* (Table 1). Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method as previously described [9]. Data are shown as the fold change in expression of the target gene relative to the internal control gene (*GAPDH*).

2.8. Detection of Cytokines. Human IL-6, IL-8, IL-10, TGF-β, IL-1β, and TNF-α and murine IL-10 and IL-12p40 production were determined by ELISA (ELISA Kits, BD Biosciences, San Diego, CA, USA) according to the manufacturer's specifications.

3. Results and Discussion

3.1. oxLDL Preferentially Increases LPS-Induced Anti-Inflammatory Cytokines. Oxidized LDL was obtained according to a previous publication and presented high values for both negative charge and TBARS compared to non-modified LDL [10]. Human monocytic THP-1 cells were differentiated into macrophages using PMA, followed by treatment with oxLDL (20 μg/mL); 24 h later, they were stimulated with LPS (100 ng/mL) for an additional 24 h. We found that pretreatment with oxLDL decreased the LPS-induced production of IL-8 and IL-6 (29% and 34% inhibition, resp.), potentiated the production of IL-10 (2-fold increase) and TGF-β (3-fold increase), and did not significantly affect TNF-α and IL-1β production (Figure 1). Although pretreatment with oxLDL did not significantly affect the LPS-induced production of TNFα and IL-1β, the fact that it decreased IL-6 and IL-8 and increased IL-10 and TGF-β production suggests that oxLDL stimulates macrophages towards an alternative activation phenotype.

It is known that depending on the degree of LDL oxidation, different products are formed, and distinct biological effects have been attributed to the LDL preparations subjected to high and low oxidation [11–13]. Here, we used oxLDL with high oxidative degree. In this situation, phospholipids, triacylglycerol, and cholesterol esters are transformed into hydroperoxides which react with ApoB-100, resulting in modification and fragmentation of amino acid side chains [14, 15].

3.2. oxLDL Inhibits LPS-Induced NO, iNOS, and COX-2. We next examined the effect of oxLDL on thioglycolate-elicited murine macrophages that already express a pro-inflammatory phenotype. Macrophages were treated with different concentrations of oxLDL for 24 h and then stimulated with LPS (1 μg/mL) for an additional 24 h. Figure 2(a) shows that oxLDL inhibits nitric oxide (NO) production induced by LPS in a dose-dependent manner. No effect was observed when nonoxidized LDL was used. Treatment with oxLDL also inhibited the expression of iNOS and COX-2 induced by LPS stimulation (52% and 55% for iNOS and COX-2, resp.) (Figures 2(b) and 2(c)). The inhibitory effects of oxLDL were not related to a decrease in cell viability, evaluated by measuring mitochondrial activity by the MTT assay, which was actually increased in oxLDL-treated macrophages (Figure 2(d)).

Although oxLDL particles have been associated with proinflammatory mechanisms related to the development of atherosclerosis [2], our data indicate that oxLDL increases anti-inflammatory and reduces pro-inflammatory markers induced by LPS, favoring macrophage differentiation toward the M2 phenotype. Compounds present in the oxLDL particle, such as sphingosine 1-phosphate (S1P) and oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (oxPAPC) have been shown to inhibit TLR2 and TLR4 activation, respectively [16, 17]. The complexity involved in this mechanism could be explained by interactions between different compounds formed after the oxidative process with different receptors present on macrophages.

3.3. The Presence of oxLDL during Macrophage Differentiation Induces the M2 Phenotype. Murine bone marrow-derived cells were differentiated to macrophages (BMDM) with L929 supernatant for six days in the presence of oxLDL. Cells were treated with oxLDL (20 μg/mL) on the first day of culture and supplemented every two days. We found that macrophages treated with oxLDL expressed high levels of mRNA for the M2 macrophage markers IL-10, arginase-1 (Arg-1), mannose receptor (MR), and PPARγ and decreased expression of IL-12 mRNA with no effect on iNOS mRNA (Figure 3). This indicates that the presence of oxLDL during macrophage differentiation shifts the phenotype toward the M2 profile.

3.4. Engagement of PAFR and CD36 is Required for the oxLDL-Induced M2 Phenotype. In a previous study, we found that costimulation of PAFR and CD36 is needed for optimal macrophage activation induced by oxLDL [7]. Here, we investigated if both receptors are involved in the induction of the M2 phenotype. Murine BMDM were treated with PAFR antagonists (WEB2086 or CV3988) alone or in combination with blocking antibody to CD36 for 30 min and then treated with oxLDL (20 μg/mL) for 5 h. Figure 4(a) shows that oxLDL induced the expression of TGF-β and Arg-1 mRNA and that this was reversed by treatment with PAFR antagonists (TGF-β: 36% and 45% inhibition; Arg1: 57% and 50% inhibition for WEB and CV, resp.). Blockage of CD36 reduced only the mRNA expression of TGF-β (56%). Simultaneous blockage of CD36 and PAFR did not further reduce Arg-1 and TGF-β mRNA expression. The oxLDL also induced the expression of RANTES and CXCL2, but this was not affected by treatment with the PAFR antagonists or CD36 blocking antibody. Next, we examined the requirement of PAFR and CD36 for IL-10 and IL-12 production. To ensure the production of detectable levels of these cytokines, the cells were activated with LPS. BMDM were pretreated with the CD36 blocking antibody alone, or in combination with the PAFR antagonists 30 min

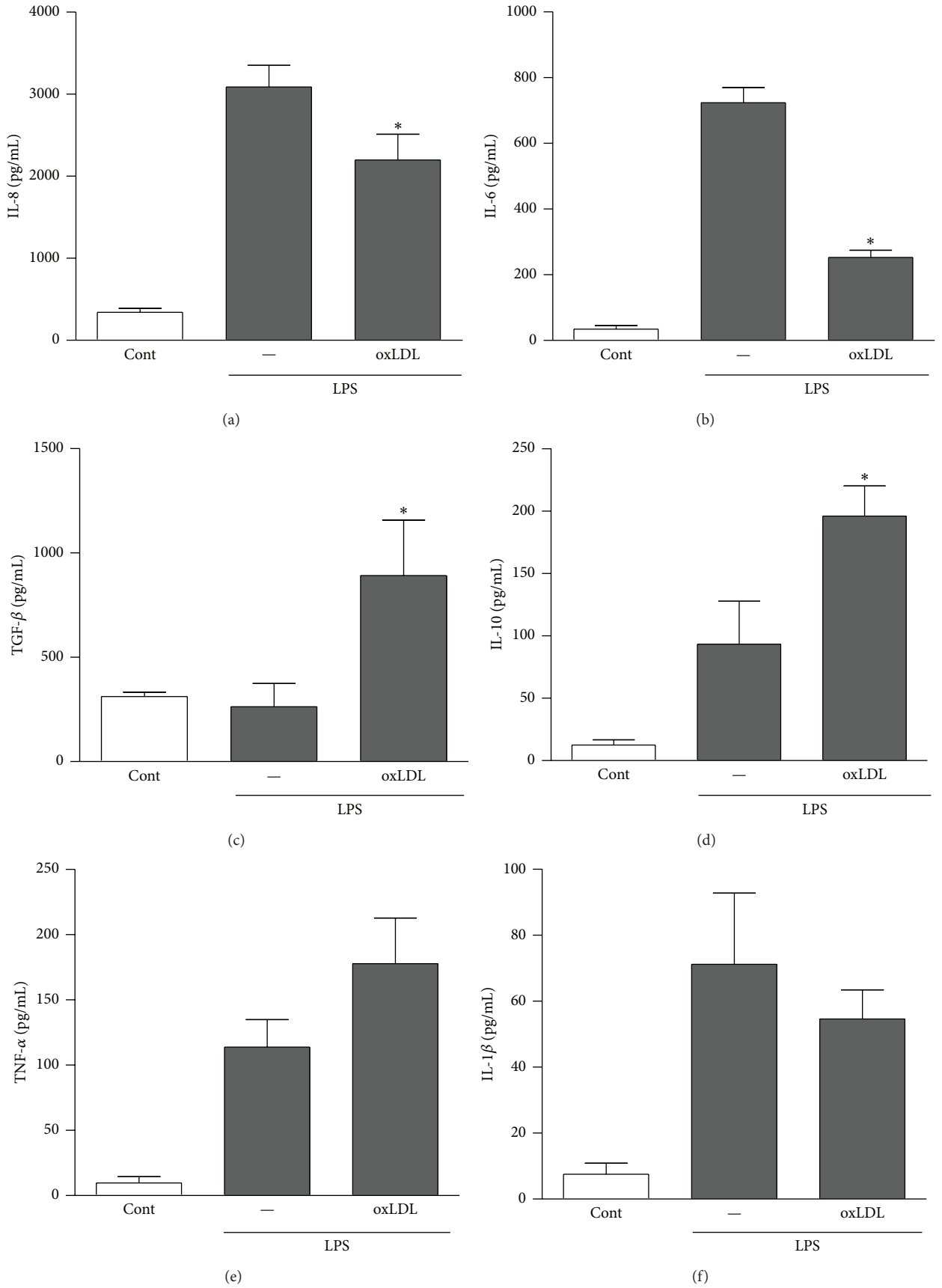

Figure 1: oxLDL preferentially increases LPS-induced anti-inflammatory cytokines. THP-1 monocytes were differentiated into macrophages with PMA, followed by treatment with oxLDL (20 μg/mL) for 24 h, and then stimulated with LPS (100 ng/mL) for an additional 24 h. Cytokines concentration in the supernatant was measured by ELISA. Data are presented as mean ± SEM of four independent experiments. $^{*}P < 0.05$ versus LPS-stimulated cells.

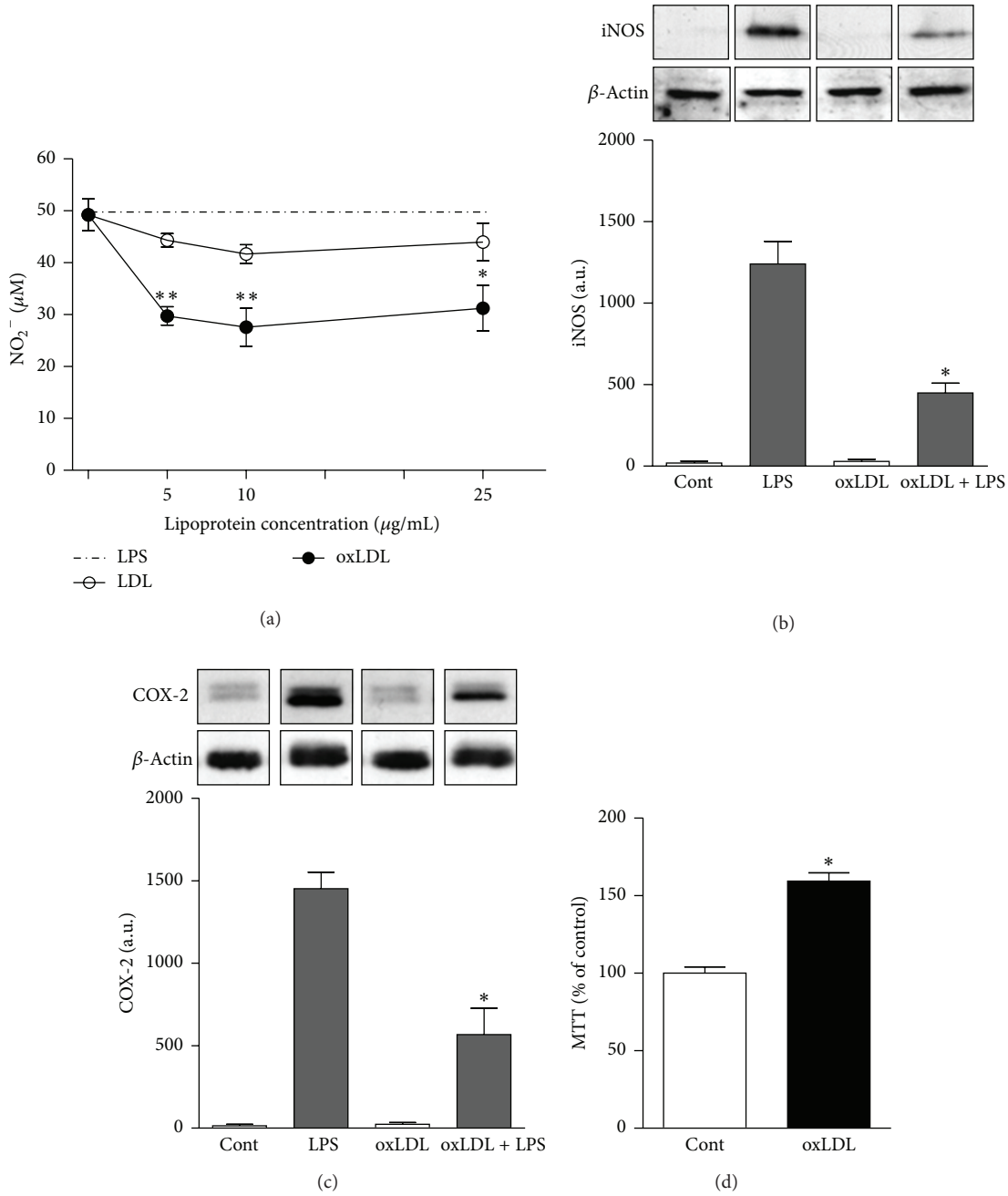

Figure 2: oxLDL treatment inhibits LPS-induced NO, iNOS, and COX-2. Thioglycolate-elicited murine macrophages were treated with different concentrations of LDL or oxLDL for 24 h and then stimulated with LPS (1 μg/mL) for an additional 24 h. (a) Nitric oxide production was analyzed by the Griess assay. (b) iNOS and COX-2 expressions were analyzed by western blot, and the protein expression was quantified by AlphaEase FC software, V3.2 beta (Alpha Innotech). The autoradiographs show one representative experiment, $^*P < 0.05$ versus LPS stimulated cells. (c) Cell viability was measured by MTT assay, $^*P < 0.05$ comparing oxLDL-treated with the nontreated cells. Data are presented as mean ± SEM of six independent experiments.

before overnight stimulation with oxLDL (20 μg/mL) followed by activation with LPS (10 ng/mL). Figure 4(b) shows that oxLDL increased the LPS-induced production of IL-10 but did not affect IL-12. Furthermore, the IL-10 concentration was strongly reduced by the PAFR antagonists WEB and CV and by the CD36 blocking antibody.

Our results show that both CD36 and PAFR are involved in IL-10 production. Although several receptors may be involved in oxLDL recognition [18], the upregulation of IL-10 induced by oxLDL depends mainly on CD36 and PAFR since, in the present study, the production of this cytokine was almost completely blocked by treatment with PAFR antagonists and antibodies to CD36. It has been described that oxLDL induces Arginase expression and activity in macrophages and in endothelial cells [19, 20]. We showed here that PAFR antagonists decreased Arg-1 and TGF-β1

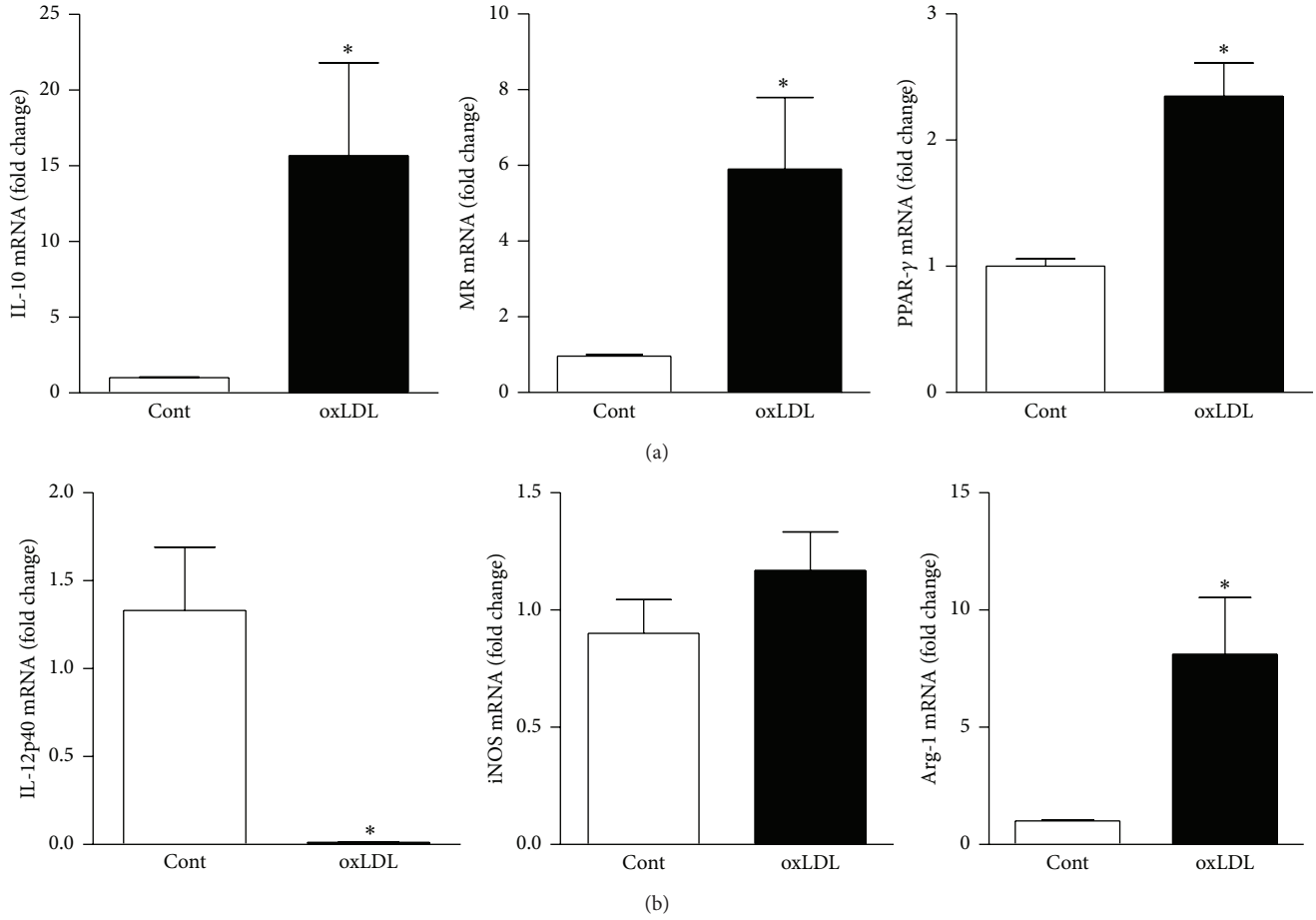

Figure 3: oxLDL induces macrophages differentiation towards M2 phenotype. Murine bone marrow-derived cells were differentiated to macrophages (BMDM) with L929 supernatant for six days in the presence of oxLDL. Cells were treated with oxLDL (20 μg/mL) on the first day of culture and supplemented every two days. The mRNA expression of IL-10, arginase-1 (Arg-1), mannose receptor (MR), PPARγ, IL-12p40, and iNOS was assessed by real-time PCR. Data are presented as mean ± SEM of independent experiments and expressed in fold change. $^{*}P < 0.05$ comparing oxLDL-treated with nontreated cells.

Table 1: List of primer sequences used for real-time RT-PCR analysis in this study.

Name	Forward	Reverse
IL-10	5′-CAGAGCCACATGCTCCTAGA-3′	5′-TGTCCAGCTGGTCCTTTGTT-3′
MR	5′-GATATGAAGCCATGTACTCCTTACTGG-3′	5′-GGCAGAGGTGCAGTCTGCAT-3′
Arg-1	5′-TTCTCAAAAGGACAGCCTCG-3′	5′-AGCTCTTCATTGGCTTTCCC-3′
PPAR-γ	5′-TCCTGTAAAAGCCCGGAGTAT-3′	5′-GCTCTGGTAGGGGCAGTGA-3′
iNOS	5′-GTTCTCAGGCCAACAATACAAGA-3′	5′-GTGGACGGGTCGATGTCAC-3′
IL-12p40	5′-TGGTTTGCCATCGTTTTGCTG-3′	5′-ACAGGTGAGGTTCACTGTTTCT-3′
TGF-β1	5′-TGGAGCAACATGTGGAACTC-3′	5′-CAGCAGCCGGTTACCAAG-3′
RANTES	5′-TTTGCCTACCTCTCCCTCG-3′	5′-CGACTGCAAGATTGGAGCACT-3′
CXCL2	5′-CGCCCAGACAGAAGTCATAGCC-3′	5′-TCTTCCGTTGAGGGACAGCAGC-3′
GAPDH	5′-AGGTCGGTGTGAACGGATTTG-3′	5′-TGTAGACCATGTAGTTGAGGTCA-3′

mRNA expression induced by oxLDL, indicating that PAFR activation is required for the induction of alternative activation markers in macrophages. Atherosclerosis is characterized by a chronic inflammatory reaction in the arterial wall, and both forms of activated macrophages, classical and alternative, have been found in atherosclerotic lesions [4]. M1 macrophages are mostly present in areas prone to rupture, while M2 macrophages are found in the adventitia, and both M1 and M2 are present in the fibrous cap [5]. However, the real function of each macrophage population still needs to be elucidated. In the atherosclerotic plaque, there are different forms of modified LDL. Here, we found that LDL

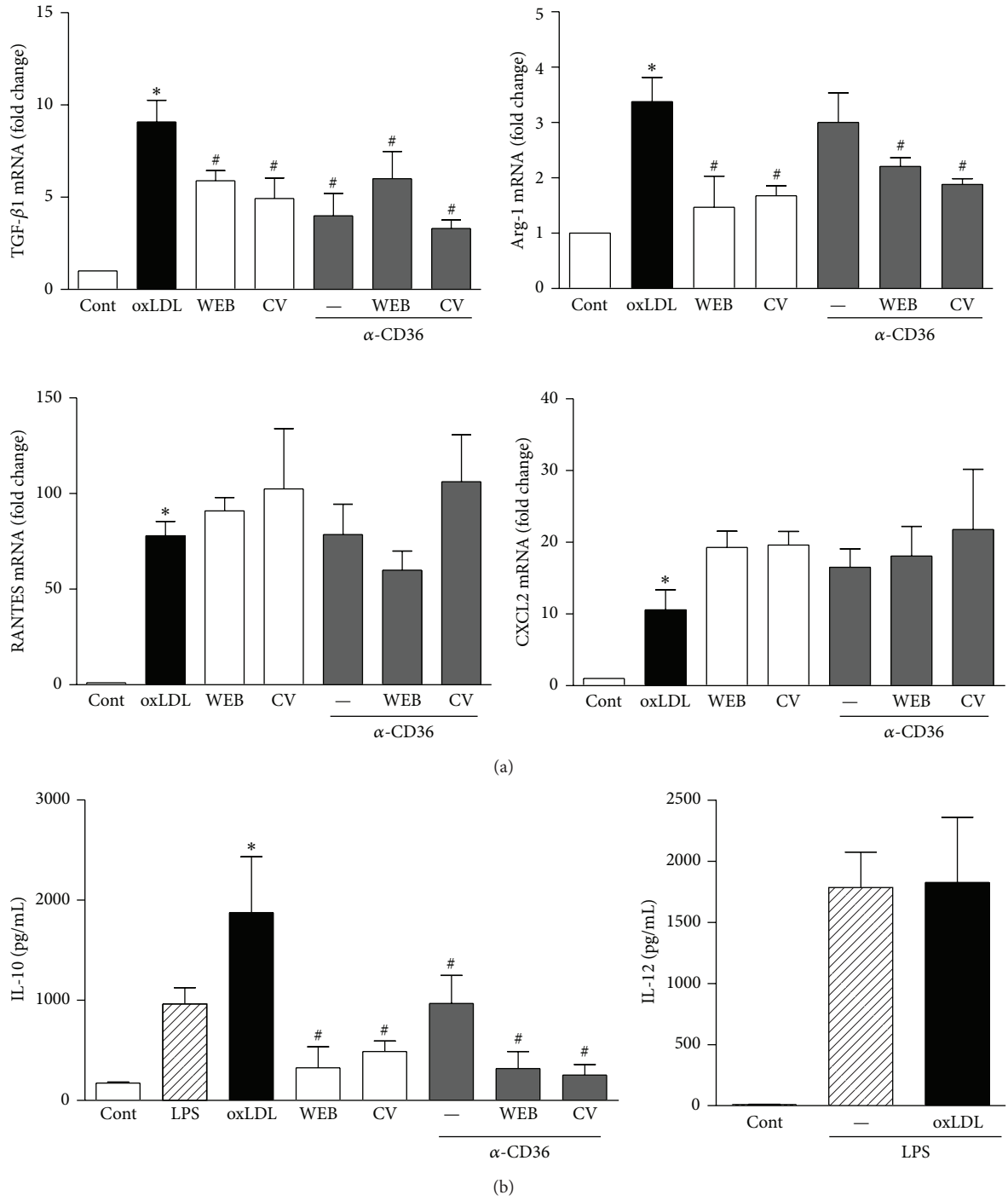

Figure 4: Engagement of PAFR and CD36 is required for the oxLDL-induced M2 phenotype. Murine BMDM were pretreated with PAFR antagonists WEB2086 (50 μM) or CV3988 (10 μM) alone or in combination with blocker antibody to CD36 (1 : 500) for 30 min and then treated with oxLDL (20 μg/mL). (a) The mRNA expression of TGF-β, Arg-1, RANTES and CXCL2 was analyzed by real-time PCR after 5 h. (b) BMDM were treated overnight with oxLDL and then stimulated with LPS (10 ng/mL). IL-10 and IL-12 production was assessed in the supernatants by ELISA assays. $^{*}P < 0.05$ comparing oxLDL-treated with nontreated cells and $^{\#}P < 0.05$ versus oxLDL-treated cells.

with a high degree of oxidation increased anti-inflammatory markers in a PAFR- and CD36-dependent manner. We do not exclude the idea that additional mechanisms may contribute to oxLDL-induced macrophage differentiation into the M1 or M2 phenotype. It can be speculated that the presence of M2 macrophages in the plaque is an attempt of the organism to control the inflammatory response; in this case, the PAFR would be involved in atheroprotection. However, M2 macrophages express more CD36 [4, 6] and are more likely to become foam cells, which contribute to the

pathophysiology of atherosclerosis [21]. Moreover, these cells are more sensitive to death in a high lipid environment, such as the lipid core of the atherosclerotic plaque [6].

4. Conclusions

In the present study, we demonstrated that oxLDL induces macrophage differentiation towards an alternative phenotype and that this requires the engagement of PAFR and CD36. As the real function of M2 macrophages in the development of atherosclerosis remains unclear, *in vivo* studies using PAFR antagonists are needed before suggesting their use as a therapeutic approach for the treatment of atherosclerosis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Electronegative LDL: A Circulating Modified LDL with a Role in Inflammation

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Electronegative low density lipoprotein (LDL(−)) is a minor modified fraction of LDL found in blood. It comprises a heterogeneous population of LDL particles modified by various mechanisms sharing as a common feature increased electronegativity. Modification by oxidation is one of these mechanisms. LDL(−) has inflammatory properties similar to those of oxidized LDL (oxLDL), such as inflammatory cytokine release in leukocytes and endothelial cells. However, in contrast with oxLDL, LDL(−) also has some anti-inflammatory effects on cultured cells. The inflammatory and anti-inflammatory properties ascribed to LDL(−) suggest that it could have a dual biological effect.

1. Introduction

The inflammatory properties of modified LDLs are a main topic in atherosclerosis research. In addition to their inflammatory properties, modified LDLs are recognized by the scavenger receptor (SR), leading to the formation of lipid-loaded foam cells, typical of atherosclerotic lesions. LDL can be modified in arterial intima and in plasma circulation by several mechanisms, such as glycation, lipolysis, aggregation, and oxidation [1]. Oxidized LDL (oxLDL) and minimally modified LDL (mmLDL), a mild oxidized LDL, are the most widely studied modified LDLs in the literature. The involvement of oxLDL and mmLDL in atherogenesis and inflammation in the arterial wall is well established [2], but they have been detected in blood only at a very low concentration [3].

Electronegative LDL (LDL(−)) is a modified circulating form of LDL found in blood. It is an LDL subfraction with a high negative charge that constitutes about 3–5% of the total LDL in normolipidemic (NL) subjects. Its existence was first reported by Avogaro in 1988 [4]. Numerous studies focusing on LDL(−) have since been performed, and the most widely accepted idea is that LDL(−) is a pool of LDL particles modified by several mechanisms.

LDL(−) has several physicochemical characteristics that differ from native LDL (hereafter referred to as LDL(+)) [5, 6]. Regarding lipid and protein composition, LDL(−) has a higher content of triglycerides [7], nonesterified fatty acids (NEFA) and lysophosphatidylcholine (LPC) [8], and ceramide (CER) [9] than LDL(+). It also shows associated phospholipolytic activities that are absent in LDL(+) [10, 11]. LDL(−) has an abnormal apolipoprotein B (apoB) conformation, which seems to play a role in both its greater binding to proteoglycans (PG) and greater susceptibility to aggregation than LDL(+) [12]. These physicochemical properties are likely responsible for its biological effects in different cell types that participate in the atherosclerotic process.

Early studies regarding the biological effects of LDL(−) were performed in endothelial cells. It was found that LDL(−) promoted cytotoxicity [13, 14] and release of inflammatory cytokines [7]. The cytokine release effect has since been reported in monocytes and lymphocytes [15]. These observations support an atherogenic role for LDL(−). Nevertheless,

recent data suggest that LDL(−) may not only have such an inflammatory role as was first thought. Studies in mononuclear cells have shown that LDL(−) has the ability to induce anti-inflammatory cytokine IL10 [15] and counteract the inflammatory effect promoted by lipopolysaccharide (LPS) [16].

This review focuses on the biological effect of LDL(−) on cells, emphasizing its role in monocytes, which are pivotal to the inflammatory response in atherosclerotic lesions. We discuss the dual function of LDL(−), inflammatory and anti-inflammatory, and its physiological role.

2. A Heterogeneous LDL

Although LDL(−) was first considered an oxidized particle in the circulation, it is now widely accepted to be a pool of modified LDLs with different properties but sharing the common feature of increased electronegativity. Nowadays, LDL(−) heterogeneity is considered a consequence of its different origins.

The oxidative origin of LDL(−) is controversial. Avogaro et al. and Sevanian et al. reported that LDL(−) has a lower vitamin E content [17], a higher amount of lipoperoxides and oxidized cholesterol [14, 17], and a higher susceptibility to oxidation [18] than LDL(+). However, other studies do not replicate these findings [19, 20]. Chen and coworkers focused their research on the most electronegative LDL subfraction, the so-called L5, detected in dyslipidemic patients but not in NL subjects [21]. They described that L5 is a mild oxLDL subfraction contained in the whole pool of LDL(−). The observation that L5 is a minor LDL(−) subfraction is in agreement with the oxLDL proportion found in blood (0.1–0.5% of total LDL) [3] compared to the LDL(−) proportion (3–5%) [5].

It has been suggested that LDL modifications other than oxidation contribute to the generation of LDL(−). Such modifications include nonenzymatic glycosylation, NEFA enrichment, and modification by phospholipolytic enzymes: phophospholipase A2 (PLA2) and sphingomyelinase (SMase) [1]. These modifications are known to increase the negative charge of LDL and likely to occur not only in blood but also in the arterial intima. It is described that in the arterial intima of atherosclerotic lesions there is an overexpression of PLA2 and SMase [22, 23], which could generate LDL(−).

LDL(−) is heterogeneous in size and density. This heterogeneity seems to depend on the mechanism involved in the generation of the particle. LDL(−) are small-dense particles in NL subjects and large-buoyant particles in familial hypercholesterolemic (FH) subjects, whereas hypertriglyceridemic patients can present both dense and light particles [24].

LDL(−) is also heterogeneous in its lipid and protein content. Compared to native LDL, it has an increased content of several non-apoB apolipoproteins: apoE, apoCIII, apoAI, apoAII, apoD, apoF, and apoJ [25]. Besides apolipoproteins, LDL(−) has a higher content in platelet-activating factor acetylhydrolase (PAF-AH) than LDL(+), leading to an increase in its enzymatic activity. Another enzymatic activity found in LDL(−) is the phospholipase C (PLC)-like activity [11]; its origin in LDL(−) is unknown, and it is absent in LDL(+). Both enzymatic activities in LDL(−) could be responsible for the altered lipid content in LDL(−), including its higher content in NEFA, LPC, and CER than LDL(+). These three lipid components are related to the inflammatory effect of LDL(−) on cultured cells [8, 9, 26]. The increased NEFA and LPC content in LDL(−) seems to be generated by hydrolysis of choline-containing phospholipids by PAF-AH activity [5] and the increased CER content by hydrolysis of sphingomyelin by PLC-like activity [9].

Finally, the heterogeneity of LDL(−) is also suggested by the presence of a minor proportion of an aggregated subfraction (agLDL(−)). AgLDL(−) seems to be responsible for the PLC-like activity of LDL(−), since such activity is mainly present in agLDL(−) [27]. It has been described that the heterogeneity in the aggregation level is responsible for LDL(−) populations with a normal or high binding affinity to PG compared to native LDL [12]. A relationship between aggregation and the abnormal apoB conformation of LDL(−) also exists [12].

3. An Atherogenic LDL

Several inflammatory effects have been ascribed to LDL(−), and they are probably a consequence of the combination of the different LDL(−) physicochemical properties (Figure 1). These inflammatory effects and other evidence described in this section suggest that this modified LDL could play an atherogenic role and be a putative biomarker of cardiovascular risk, as suggested elsewhere [28, 29]. The usefulness of LDL(−) as a biomarker in the diagnosis of cardiovascular risk should be determined in large cohorts of patients, but methods to do this are still under development [28].

3.1. Increased LDL(−) Proportion in Inflammation. The first evidence of the relationship between LDL(−) and atherosclerosis is the increased proportion of LDL(−) in subjects with pathologies known to be associated with cardiovascular risk and inflammation. These pathologies include FH [30], hypertriglyceridemia [24], type 1 and type 2 diabetes mellitus (DM) [31, 32], chronic kidney disease requiring hemodialysis [33, 34], and rheumatoid arthritis [35]. LDL(−) is also increased in patients with acute myocardial infarction [36] and angiographically documented coronary artery disease [37]. In each pathology, the mechanisms involved in LDL(−) generation likely depend on the individual characteristics and the underlying disease of the patients. Some drugs administered to treat DM and FH, such as insulin and statins, decrease the proportion of LDL(−), besides decreasing the cardiovascular risk [30, 32].

Moreover, a high LDL(−) proportion has been associated with a worse lipid profile since there is a positive correlation of LDL(−) proportion with nonhigh density lipoprotein cholesterol (non-HDLc) and a negative correlation with HDLc [38].

3.2. Immunological Response Induced by LDL(−). It has been described that LDL(−) can trigger an adaptative immune response, leading to the production of anti-LDL(−)-autoantibodies and immunocomplexes, which can be quantified by ELISA [39]. The presence of these autoantibodies is increased in DM [40] and in acute coronary syndromes [41].

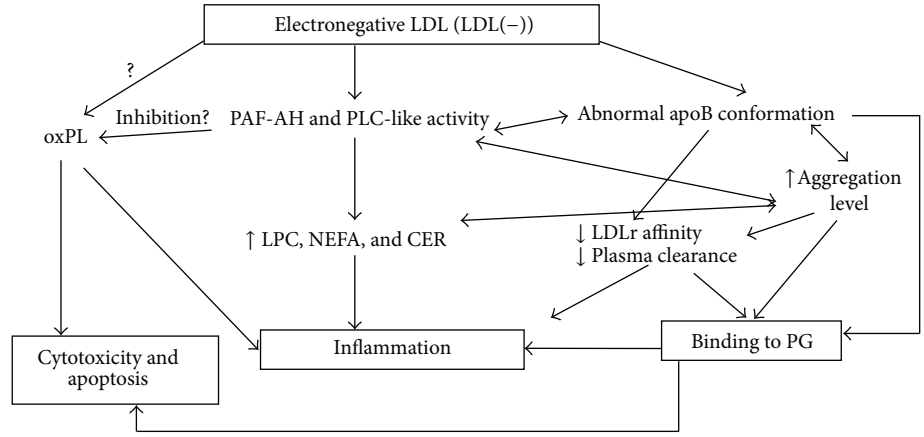

Figure 1: Putative relationships between the physicochemical properties of LDL(−) and its inflammatory actions. Phospholipolytic activities contained in LDL(−) increase its LPC, NEFA, and CER content. These compounds are involved in the inflammatory action of the particle. Phospholipolytic activities could also be related to the abnormal apoB conformation and high aggregation of LDL(−), which may contribute to its decreased plasma clearance and increased binding to PGs. The retention of LDL(−) to endothelium by PG would favor the inflammatory action of LDL(−) on the arterial wall cells. Some authors have suggested that the presence of oxPL in LDL(−) is responsible for the inflammatory, cytotoxic, and apoptotic effects of this particle. LDL(−): electronegative LDL, oxPL: oxidized phospholipids, PAF-AH: platelet-activating factor acetylhydrolase, PLC: phospholipase C, LPC: lysophosphatidylcholine, NEFA: nonesterified fatty acids, CER: ceramide, apoB: apolipoprotein B, LDLr: LDL receptor, PG: proteoglycans.

Grosso et al. reported that anti-LDL(−)-autoantibodies administered intravenously in mice can play a protective role in atherosclerosis [42]. Taken together, it seems that anti-LDL(−)-autoantibodies could be useful biomarkers in patients with high risk for coronary events [39, 41].

3.3. Apoptotic and Cytotoxic Effects of LDL(−). Some authors have reported that LDL(−) has cytotoxic properties in cultured endothelial cells. This was considered due to its high content of oxidized cholesterol [14, 43]. In contrast, other authors have reported that LDL(−) has no cytotoxic effect [7, 15] or that its cytotoxic effect is due to mechanisms other than oxidation [13]. The divergence in results is probably a consequence of the LDL(−) heterogeneity.

There is an agreement that LDL(−) induces apoptosis. Chen and colleagues reported that the highly electronegative LDL subfraction L5 promoted apoptotic effects on endothelial cells through a decrease in fibroblast growth factor 2. This induction of apoptosis was found for L5 isolated from FH [44, 45], DM [46, 47], and smokers [48]. The apoptotic effect was suppressed in the presence of low concentration of aspirin [36]. These authors attributed the apoptotic ability of L5 to oxidation. However, the apoptotic effect could be due to the increased CER content in LDL(−) since CER is an inductor of apoptosis [49]. An apoptotic effect of LDL(−) was also shown in macrophages [50] and in cardiomyocytes [51]. In the latter study, it was found that apoptosis was induced by culture-conditioned medium of endothelial cells incubated with LDL(−). In addition, LDL(−) has been described to induce in lymphocytes and macrophages the gene expression and membrane-bound protein of Fas [50, 52], a factor that triggers extrinsic pathway of apoptosis [53].

At subapoptotic concentrations, however, L5 impairs differentiation of endothelial progenitor cells and inhibits endothelial cell regeneration and neovascularization [48]. In endothelial cells, L5 also inhibits reendothelization [46], growth, and survival signaling [54] and activates cell stress by promoting inflammation and mitochondrial dysfunction [55].

3.4. Inflammatory Properties of LDL(−). There is consensus that LDL(−) induces an inflammatory response on cells participating in the atherosclerotic process. The most important effect induced by LDL(−) is the release of cytokines, particularly in endothelial and mononuclear cells. Figure 2 summarizes the role of LDL(−) in atherogenesis in relation to the inflammatory effects promoted on cells.

3.4.1. Effects on Endothelial Cells. The endothelium is the physical barrier between blood and the vessel wall. Endothelial cells control important physiological processes, including cellular trafficking. They also control the recruitment of circulating monocytes and lymphocytes to the arterial endothelium. Infiltration of these circulating cells to sites of inflammation is one of the earliest events in atherosclerosis. It has been described that LDL(−) attracts monocytes and lymphocytes to endothelial cells [21, 56], suggesting its participation in the early phases of atherosclerosis. It has been reported that LDL(−) promotes this attraction by inducing adhesion molecules and chemokine release in endothelial cells. In relation to adhesion molecules, LDL(−) induces vascular cell adhesion molecule (VCAM) [56, 57]. The induction of chemokine release by LDL(−) was first reported by De Castellarnau et al. who observed that LDL(−) promotes monocyte chemotactic protein 1 (MCP1) and interleukin 8 (IL8) release in human umbilical vein endothelial cells (HUVEC) [7]. MCP1 and IL8, respectively, induce the recruitment of monocytes and T lymphocytes to the endothelium. The release of

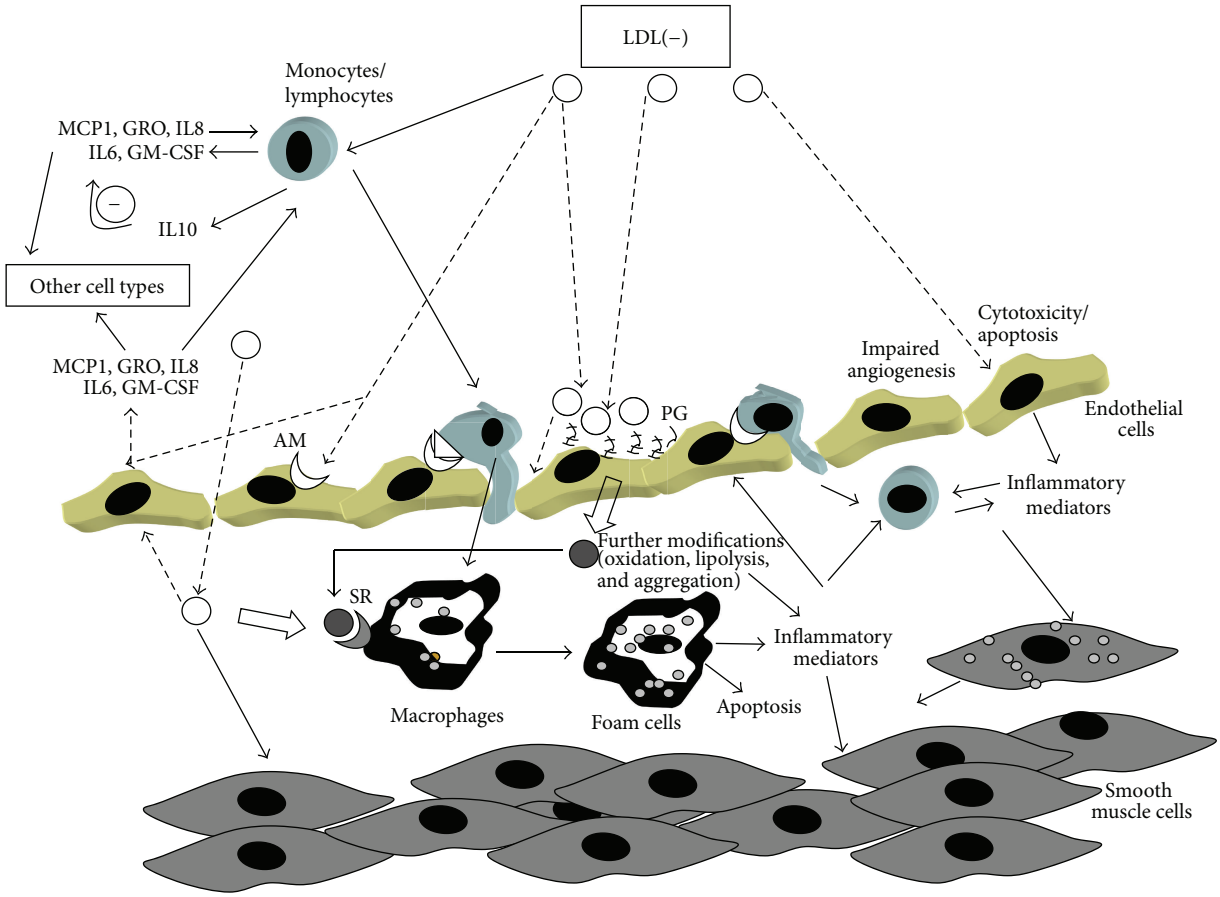

Figure 2: Biological actions of LDL(−) on circulating mononuclear cells (monocytes/lymphocytes) and arterial wall cells (endothelial cells, macrophages and smooth muscle cells) in relation to atherogenesis. LDL(−) can activate circulating leukocytes, mainly monocytes, and lymphocytes. LDL(−) also induces chemokine and adhesion molecules in endothelial cells, promoting the recruitment of more circulating leukocytes to endothelium. Cytokines released by endothelial cells can also act on other cell types of the arterial wall. LDL(−) retained in the subendothelial space by its increased binding to PG can also stimulate arterial wall cells. In this environment, LDL(−) could be further modified, leading to additional inflammatory actions on cells. It could also be uptaken by SR, promoting the formation of foam cells. LDL(−): electronegative LDL, MCP1: monocyte chemoattracting-protein 1, GRO: growth-related oncogen, IL6, IL8, and IL10: interleukin 6, 8, and 10, GM-CSF: granulocyte monocyte-colony stimulating factor, SR: scavenger receptor, AM: adhesion molecule, PG: proteoglycans.

these chemokines in HUVEC has been reported for LDL(−) isolated from NL [7], FH [20], and DM subjects [58]. As the LDL(−) proportion is higher in FH and DM than in NL, the inflammatory effect promoted by LDL(−) should be greater in these patients than in NL subjects.

Further studies in HUVEC have shown that LDL(−) induces other inflammatory cytokines, such as interleukin 6 (IL6), growth-related oncogen (GRO), granulocyte-monocyte-colony stimulating factor (GM-CSF) [59], and epithelial cell-derived neutrophil-activating peptide 78 [56]. The cytokine release promoted by LDL(−) has been reproduced in cultured human endothelial cells of arterial origin [60]. In bovine arterial endothelial cells, the most electronegative subfraction L5 also induces secretion of matrix metalloproteinases and vascular endothelial growth factor expression [45].

3.4.2. Effects on Monocytes and Lymphocytes. Besides endothelial cells, lymphocytes and particularly monocytes play a pivotal role in atherogenesis and inflammation by secreting cytokines and growth factors. As they are present in blood, it is highly feasible that they interact with LDL(−). For this reason, the interaction between mononuclear cells and LDL(−) has been a focus for study in recent years. It has been observed that LDL(−) induces the release of the same cytokines in mononuclear cells, monocytes, and lymphocytes, as in endothelial cells [15]. However, LDL(−) induces anti-inflammatory IL10 in mononuclear cells [15], but not in endothelial cells [59]. The putative physiological role of the IL10 production and other theoretically anti-inflammatory actions promoted by LDL(−) will be discussed further on.

Cytokine induction by LDL(−) in monocytes and lymphocytes occurs both at RNA and protein levels [15]. In a genomic study it was shown that LDL(−) modifies the transcription of other genes related to inflammation and atherosclerosis in mononuclear cells. Among these modifications, LDL(−) promotes Fas upregulation, colony stimulating factor 1 receptor (CSF1R), and CD36 downregulation [52]. Fas

has been reported to be involved in apoptosis and in cytokine induction [53, 61]. Therefore, Fas induction could be related to these biological effects of LDL(−).

3.4.3. Increased Affinity to Proteoglycans. LDL(−) presents higher affinity to PG than LDL(+). Aggregation of LDL(−), mediated by its PLC-like activity, seems to be important in its binding to PG since agLDL(−) is the LDL(−) subfraction that has the highest affinity to PG [12]. It has been hypothesized that alterations in the N-terminal extreme of apoB could be responsible for this increased binding [12]. LDL(−) could also act as a seeding factor since its aggregation stimulates aggregation of native lipoproteins. This process could promote the subendothelial retention of lipoproteins in vivo. The higher LDL(−) binding to PG and subendothelial retention could favor LDL(−) exerting its inflammatory action locally in the microenvironment of the arterial wall, besides acting on circulatory cells. Moreover, LDL(−) retention in the arterial intima would allow induction of cytokine release for a longer period of time.

3.4.4. Global Inflammatory Effect of LDL(−). It is noteworthy that LDL(−) promotes an inflammatory action on several cell types that participate in the atherosclerotic process. The biological effects found in "in vitro" experiments with a cell type cannot be considered individually because in a physiological context all the cell types interact. These interactions enhance the effect promoted by LDL(−) since some cytokines can induce the release of other cytokines, and, moreover, cytokines induced in a cell type can act on other cell types, as shown in Figure 2.

LDL(−) in the circulation induces cytokine release in monocytes and lymphocytes. LDL(−) also promotes chemokine and adhesion molecule expression in endothelial cells, and these molecules promote the recruitment of circulatory leukocytes to endothelium. In addition, cytokine released by endothelial cells can act on cells that are already in the subendothelial space, such as recruited monocytes, macrophages, and smooth muscle cells. These cell types are also exposed to LDL(−) retained in the subendothelial space by PG. In addition, LDL(−) retained in the arterial wall could be further modified by oxidation since it is not protected by the plasma antioxidants and by enzymatic hydrolysis. These modifications of LDL(−) could lead to additional inflammatory actions on cells or to further aggregation of LDL(−). This latter effect could favor LDL(−) recognition by SR, promoting the formation of foam cells.

The biological effects described for LDL(−) are, in part, similar to that for mmLDL/oxLDL, whose involvement in the atherosclerotic process has been extensively reported. Nevertheless, there are several differences between the biological properties of these modified LDLs, shown in Table 1.

4. An Antiatherogenic LDL?

Early observations regarding the cytotoxic effect of LDL(−) on endothelial cells typecasted this modified LDL as a "bad guy" in the atherosclerotic process. Further findings describing an apoptotic and inflammatory effect for LDL(−) also supported this idea. However, in recent years, other studies ascribed some putative anti-inflammatory and regulatory properties to LDL(−), questioning whether LDL(−) is really so "bad".

The main modulatory property promoted by LDL(−) is the induction of the anti-inflammatory cytokine IL10 in monocytes and lymphocytes. The relationship between IL10 and protection against atherosclerosis has been widely established in human clinical studies and in mice [62, 63]. The protective role of IL10 has also been demonstrated in studies with cultured cells, in which IL10 regulates the production of proinflammatory cytokines [64]. All data support a physiological function of IL10 as a controller of inflammatory response, as it seems to be the role of IL10 induced by LDL(−). IL10 diminishes the release of the inflammatory cytokines promoted by LDL(−) in monocytes and lymphocytes [15]. The addition of exogenous IL10 and blocking of IL10 action with antibodies, respectively, inhibit and increase the cytokine release promoted by LDL(−). Therefore, if LDL(−) does not induce IL10 in mononuclear cells, its inflammatory response will be higher. IL10 also promotes its inhibition by negative feedback to avoid the absence of an inflammatory response [15]. Taken together, these data show that LDL(−) counteracts its inflammatory cytokine induction in leukocytes through IL10 to avoid an excessive inflammatory response. Otherwise, this counteracting mechanism does not occur in endothelial cells because they do not produce IL10 in response to LDL(−) [59].

Another modulatory action promoted by LDL(−) is the induction of nuclear translocation of the transcription factor Nrf2 in macrophages [50]. Nrf2 decreases apoptotic activity and modulates the metabolic response to oxidative stress. Accordingly, LDL(−) promotes cell survival and adaptation to oxidative stress in macrophages and endothelial cells [65]. Nrf2 production by LDL(−) in macrophages attenuates their LDL(−)-induced apoptosis [50]. IL10 production by LDL(−) could also be involved in the regulation of apoptosis since IL10 promotes antiapoptotic effects in macrophages [66]. However, Nrf2 activation does not overcome the proapoptotic effect of LDL(−), and IL10 induction does not avoid inflammatory cytokine release either. These compensatory mechanisms could limit the atherogenic effects of LDL(−) but could not inhibit them altogether.

A study by Bancells et al. showed that LDL(−) could avoid monocyte differentiation to macrophages [52], in contrast to oxLDL [67, 68]. LDL(−) downregulates the expression of molecules involved in monocyte differentiation: CSF1R, CD36, and peroxisome proliferator-activated receptor γ (PPARγ) [52]. The inhibition of PPARγ by LDL(−) could promote the CD36 downregulation since PPARγ is a transcription factor that induces CD36 expression [69]. In contrast to these results, Pedrosa et al. observed that LDL(−) induces CD36 in macrophages [50]. On the other hand, it has been described that LPS downregulates the expression of CD36 and CSF1R in inflammatory situations, hindering excessive cell activation [70].

It has been proposed that the combination of PAF-AH and phospholipase C-like enzymatic activities associated with LDL(−) could play a role in the inactivation of oxidized

Table 1: Differences in the properties of oxLDL/mmLDL and LDL(−).

oxLDL/mmLDL	LDL(−)
(i) Oxidized particle	(i) Resistance to oxidation. Oxidized LDL?
(ii) 0.1–0.5% of total plasma LDL	(ii) 3–5% of total plasma LDL (increased in some pathologies)
(iii) No increased PG affinity	(iii) Increased PG affinity
(iv) No phospholipolytic activity	(iv) Associated phospholipolytic activities
(v) Recognition by SRA, EC accumulation	(v) No recognition by SRA, no EC accumulation
(vi) TNF induction, no IL10 induction	(vi) No TNF induction, IL10 induction.
(vii) CD36 upregulation and PPARγ upregulation	(vii) CD36 downregulation (and PPARγ) in monocytes, CD36 upregulation in macrophages
(viii) Cytotoxicity	(viii) Discrepances in cytotoxic effect
(ix) No induction of LDL fusion	(ix) Induction of LDL fusion
(x) Altered immunoreactivity to antibodies anti-apoB	(x) Altered immunoreactivity to antibodies anti-apoB, but different than oxLDL
(xi) No competition with LDL(−) for binding to monocytes	(xi) No competition with oxLDL for binding to monocytes, competition with LPS

(oxLDL/mmLDL) and LDL(−). oxLDL: oxidized LDL, mmLDL: minimally modified LDL, PG: proteoglycans, SRA: type A scavenger receptor, TNFα: tumor necrosis factor α, IL10: interleukin 10, EC: esterified cholesterol, PPARγ: peroxisome proliferator-activated receptors, LPS: lipopolysaccharide.

phospholipids (oxPL), inflammatory components of oxLDL, and mmLDL [6]. PAF-AH activity hydrolyzes PAF-like phospholipids, which could prevent LDL oxidation, but it yields LPC that is an inflammatory molecule. Therefore, LPC could be hydrolyzed by the PLC-like activity of LDL(−) since it is the main substrate. According to this theory, LDL(−) develops a protective function since it avoids the presence of oxLDL or mmLDL, which have greater atherogenic effects than those of LDL(−) [6].

Finally, the most recent observation showing an anti-inflammatory action for LDL(−) is the counteraction of LPS-induced inflammation in monocytes [16]. This counteracting action of LDL(−) seems to be a consequence of the competition between LPS and LDL(−) for the same pathway in monocytes. Both LPS and LDL(−) promote cytokine release in monocytes through the activation of two receptors, CD14 and toll-like receptor 4 (TLR4) [16]. This observation suggests a putative protective action of LDL(−) by decreasing systemic LPS toxicity in cases of overwhelming inflammation, such as a sepsis syndrome arising from bacterial infection.

There is controversy regarding a putative competition between modified LDLs and LPS. Some authors describe an inhibitory action of oxLDL on the LPS effect in monocytes [71, 72]. In contrast, others have reported that native LDL [73] and oxLDL [74] present a synergic proinflammatory effect on monocytes when incubated with LPS. These discrepancies are probably related to the concentrations of LPS and LDL and to the type and degree of LDL modification. OxPL have been described to compete with LPS in the inflammatory effect [75]. In spite of TLR4 binding to small amounts of oxPL [76], oxPL are considered weak agonists for TLR4. The most accepted idea is that oxPL could inhibit TLR signaling by preventing LPS interaction with accessory proteins involved in TLR4 binding [75, 77, 78]. In the atherosclerotic lesion there could be oxPL and mmLDL. However, their presence in plasma is not so feasible, whereas circulating LDL(−) is a likely physiological TLR-ligand.

5. Molecular Mechanisms Involved in LDL(−) Effect on Cells

As reviewed above, several LDL(−) actions on cells have been described. Nevertheless, the components or the physicochemical characteristics of LDL(−) responsible for its effect on cells are not totally understood. The receptors that bind and mediate the biological effects of LDL(−) are reasonably well established, but the intracellular pathways activated by LDL(−), which would lead to its inflammatory and anti-inflammatory effects on cells, are not well known.

5.1. Inflammatory Components of LDL(−). Some authors suggest that oxidation is the mechanism responsible for the inflammatory and cytotoxic effects of LDL(−) [13, 14]. Other authors do not attribute an oxidative origin to LDL(−) [20] and do not find a cytotoxic effect either [7, 15]. They suggest other explanations for the atherogenic properties of LDL(−), such as the increased content in LPC, NEFA, and CER.

The increased PAF-AH activity associated with LDL(−) [10] might be the origin of the increased amount of LPC and NEFA in LDL(−). Both components are involved in the cytokine release promoted by LDL(−) in endothelial cells [8]. The increased NEFA content of LDL(−) is also involved in the induction of cytokine release promoted by LDL(−) in monocytes [26]. In these cells, the presence of HDL caused a diminution in both the NEFA content in LDL(−) and the cytokine release induced by LDL(−) [26], thereby supporting a relationship between NEFA and inflammation promoted by LDL(−).

PLC-like activity of LDL(−) seems to be involved in the cytokine release promoted in monocytes through the generation of CER. PLC-like activity, CER content, and cytokine release are reduced by preincubation of LDL(−) with HDL, suggesting a relationship between these LDL(−) properties [26]. PLC-like activity hydrolyzes the polar head of choline-containing phospholipids and preferentially

degrades LPC, with intermediate medium efficiency for sphingomyelin (SM) and with lower efficiency for phosphatidylcholine (PC). The products of this hydrolysis are CER, monoacylglycerol (MAG), diacylglycerol (DAG), and phosphorylcholine (Pchol). Pchol is water soluble and presumably leaves the LDL particle, but the other products are hydrophobic and remain retained in the LDL particle. Even though LPC is rapidly degraded by the PLC-like activity, MAG would be scarce in LDL since the amount of LPC is much lower (2-3% of total phospholipids in LDL) than PC (70%) and SM (20%). For this reason, CER and DAG are more abundant products of PLC-like activity than MAG in LDL(−). CER and DAG are considered as bioactive and inflammatory molecules that promote cell signal transduction. A relationship between PLC-like activity and increased CER and DAG content in LDL(−) has been shown. The involvement of CER content in LDL, but not of DAG, in cytokine release in monocytes has been demonstrated [9].

The role of CER and NEFA in the cytokine release promoted by LDL(−) in monocytes could be explained by the fact that both compounds can bind to CD14 [79]. It is well known that CD14 binds to inflammatory ligands and afterwards interacts with TLR4 to mediate cytokine release. However, apart from CER and NEFA, other factors seem to contribute to the inflammatory effects of LDL(−). LDL modified "in vitro" to increase its content of CER or NEFA to a similar or higher degree than LDL(−) promotes a lower inflammatory action than LDL(−). This suggests that a combination of several LDL(−) properties contributes to its inflammatory effect.

LDL(−) presents a higher aggregation level than LDL(+), probably as a consequence of its increased CER and NEFA content. However, the high aggregation of LDL(−) as a cause of its inflammatory properties has been ruled out. In vitro aggregation of LDL does not promote cytokine release in monocytes compared to native LDL [9]. But as discussed previously, aggregation is responsible for the increased binding to PG of LDL(−), where it would remain retained favoring its inflammatory action.

5.2. LDL(−) Cell Receptors. The first step in the knowledge of the mechanisms involved in the biological effects for LDL(−) is to determine the receptor or receptors that recognize LDL(−) and mediate the starting signals in the activation of intracellular pathways. Several physicochemical properties ascribed to LDL(−), such as electronegative charge, higher aggregation level, conformational changes in apoB, and increased content in inflammatory lipids, suggest that LDL(−) interacts with different cell receptors than LDL(+). This would influence the clearance of LDL(−) from the circulation and the activation of certain intracellular pathways involved in the induction of cytokine release promoted by LDL(−).

Early studies regarding cell binding focused on LDL receptor (LDLr). LDL binds to LDLr through its apoB lysine residues. As LDL(−) has a higher negative charge than LDL(+), it was expected that LDL(−) would bind to LDLr with lesser affinity. The first study performed in this regard observed that LDL(−) presented loss of affinity for LDLr [4]. These results concur with those of Benitez et al. who found that LDLr affinity was 3-fold lower for LDL(−) than for LDL(+) [80]. The lower affinity for LDLr could be partly explained by the higher NEFA content in LDL(−) [80], its increased degree of aggregation [27], and the abnormal conformation of its apoB [12]. The global consequence of the loss of affinity would be a diminished clearance of LDL(−) from plasma circulation, making this particle susceptible to further modifications. In contrast, other studies reported that LDL(−) binds to LDLr with a similar or increased affinity compared to LDL(+) [13, 19, 81]. The increased binding was attributed to the increased content in apoE of LDL(−).

As LDL(−) possesses an electronegative charge, some SR could uptake this subfraction, as occurs in the case of other modified LDL, such as oxLDL or acetylated LDL [82]. Once again, there is no concensus on this point as some authors describe no differences in the uptake through type A SR [4, 80, 83] while others suggest that LDL(−) could be recognized by SRs [84, 85]. In any case, LDLr and SR should not be related to cytokine release but to plasma cholesterol uptake and accumulation of intracellular cholesterol, respectively. So which cell receptor or receptors are involved in the inflammatory effects of LDL(−)?

Chen et al. suggested that the PAF receptor plays a role in mediating apoptotic effects of L5 in endothelial cells [44]. However, as LDL(−) presents high PAF-AH activity [10], its PAF content can be expected to be low. More recently, Chen and coworkers also reported that lectin-like oxidized LDL receptor (LOX-1) plays a role in L5 recognition. As a consequence of binding to LOX-1, L5 induces several biological effects in endothelial cells, including apoptosis and LOX-1 upregulation [46, 48, 54]. LOX-1 is the main SR in endothelial cells, whereas low LOX-1 expression can be found in monocytes [86]. Moreover, oxLDL, the typical ligand for LOX-1, does not compete with LDL(−) for its binding to monocytes [16]. For these reasons, it is unlikely that LOX-1 is the mediator of the cytokine release promoted by LDL(−) in monocytes. Other SRs, such as SRA, are expressed in low amounts in monocytes, increasing its expression during the differentiation of this cell type to macrophages.

The involvement of TLRs in the biological effects of LDL(−) had been suggested [87] and recently demonstrated [16]. TLRs are immune response receptors against pathogens, which are related to atherosclerosis [88]. TLR ligands, such as LPS, bind to CD14, a differential marker of monocytes, which associates with TLR2 or TLR4 to induce intracellular signal transduction [89]. TLR2 and TLR4 can bind directly to LPS and also modified lipoproteins. The activation of the system CD14-TLR4 by mmLDL has been studied in depth by Miller and coworkers, particularly in macrophages. They found that CD14 binds to mmLDL, the binding site being different from that for LPS [90]. This binding promotes CD14 and TLR4 association and leads to stimulation of phagocytosis [90], macropinocytosis, and cholesterol accumulation [91]. mmLDL also induces inflammatory cytokines in macrophages, such as MCP1, IL6, and tumor necrosis factor α (TNFα), in a TLR4-dependent or -independent manner [92]. Studies by Chávez-Sánchez et al. show that, in monocytes and macrophages, mmLDL induces IL1, IL6,

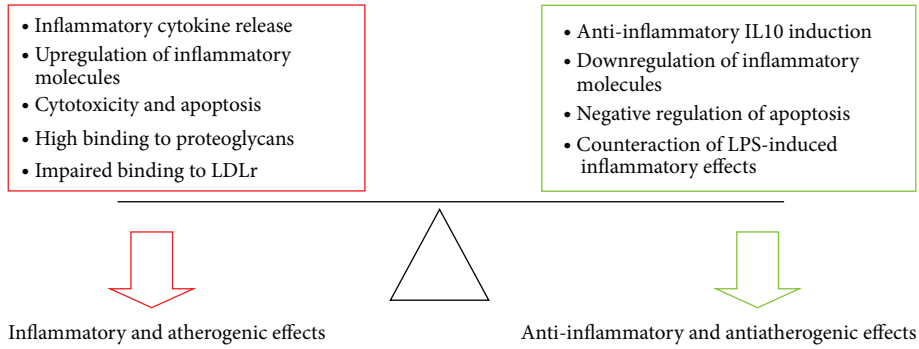

Figure 3: Balance of inflammatory and anti-inflammatory effects of LDL(−) on cells. LDLr: LDL receptor, IL10: interleukin 10, LPS: lipopolysaccharide.

IL10, and TNFα secretion through CD14, TLR4, and TLR2 [93, 94]. Other authors have reported that oxLDL promotes MCP1 and IL8 release and upregulates TLR4 in monocytes [95], and mmLDL also induces TLR4 in macrophages [96]. Because of the role of CD14-TLR4 in the inflammatory action of mmLDL, the involvement of TLRs in the LDL(−) effects on cells seems to be feasible. According to this, recent findings from our group have demonstrated that CD14 is the main receptor of LDL(−) in monocytes. CD14 association with TLR4 triggers the subsequent intracellular machinery leading to cytokine release [16]. The fact that LDL(−) shares the CD14-TLR4 pathway with LPS explains the previously mentioned cross-competition between LDL(−) and LPS in binding to monocytes and in cytokine release.

5.3. Intracellular Mechanisms Activated by LDL(−). Knowledge about intracellular signaling pathways activated by LDL(−) that lead to cell response is scarce. In contrast, the activation of several signaling pathways by mmLDL is better known, particularly in macrophages. Some of these pathways could also be activated by LDL(−).

In macrophages, mmLDL activates phosphoinositide-3-kinase (PI3k) by TLR4-dependent or -independent pathways, [90, 92] initiating Akt signaling [92]. It has also been suggested that LDL(−) activates PI3k and nuclear factor κB (NFκB) in cardiomyocytes leading to induction of apoptosis [51]. However, these findings contrast with those reported for the electronegative L5 subfraction in endothelial cells and endothelial progenitor cells, where the PI3k-Akt pathway is inhibited via LOX-1 [46, 48, 54]. As endothelial progenitor cells derive from circulating monocytes, LDL(−) could also have an inhibitory effect on the PI3k-Akt pathway in monocytes.

It has been described that mmLDL induces the recruitment of spleen tyrosine kinase to TLR4 in macrophages [91, 97, 98]. This leads to phosphorylation of endothelial cell signal-regulated kinase (ERK1/2) and of c-Jun N-terminal kinase, which finally induces activating-protein 1 (AP1) [98]. In endothelial cells, the stimulation of TLR4 by oxLDL is described to induce the activation of ERK and p38 mitogen-activated protein kinase [99]. The involvement of these kinases on the biological effects of LDL(−) has not yet been studied.

Several observations show that AP1 and NFκB seem to be involved in the inflammatory effects of LDL(−). In HUVEC, an increased nuclear translocation of some components of these transcription factors was observed (p65 and p50 for NFκB and c-jun, cfos, and ATF2 for AP1) [100]. AP1 and NFκB have also been reported to be involved in VCAM induction by LDL(−) [57]. A gene expression study in leukocytes suggests the activation of NFκB and downregulation of PPARγ [52]. The involvement of NFκB and AP1 activation in the inflammatory effect of LDL(−) in monocytes has also been recently reported [16].

6. Physiological Effects of LDL(−)

It is difficult to ascertain the physiological effects that LDL(−) could exert in vivo, where other factors can contribute to modify its action on cells. The role displayed by LDL(−) will probably depend on the cell environment in each particular situation. The presence of other lipoproteins or cell activators, such as HDL and LPS, could modulate the biological action of LDL(−). Moreover, LDL(−) can promote different biological effects depending on the cell type. For example, LDL(−) downregulates CD36 expression in monocytes, probably to inhibit activation of these cells and differentiation to macrophages [52]. In contrast, LDL(−) upregulates CD36 in macrophages [50] to eliminate toxic compounds, including oxidized lipids, leading to foam cell formation.

The fact that LDL(−) is recognized by innate immune receptors on monocytes suggests, a priori, that it could be a "self-pathogen" particle that the immune system has to eliminate. This is supported by the detection of antiLDL(−)-autoantibodies and immunocomplexes [39]. Although some anti-inflammatory actions on cells have been ascribed to LDL(−), the abundant atherogenic properties would lead to a global inflammatory effect rather than to an atheroprotective effect, as shown in Figure 3. Probably, it would be more appropriate to consider the anti-inflammatory actions described for LDL(−) as regulatory/modulatory mechanisms to minimize the inflammatory effect of this modified LDL.

Thus, the classification of the biological effect of LDL(−) as positive or negative is not so categorical since it would depend on the situation. Cytokine release promoted by LDL(−) could be considered as an atherogenic action, but,

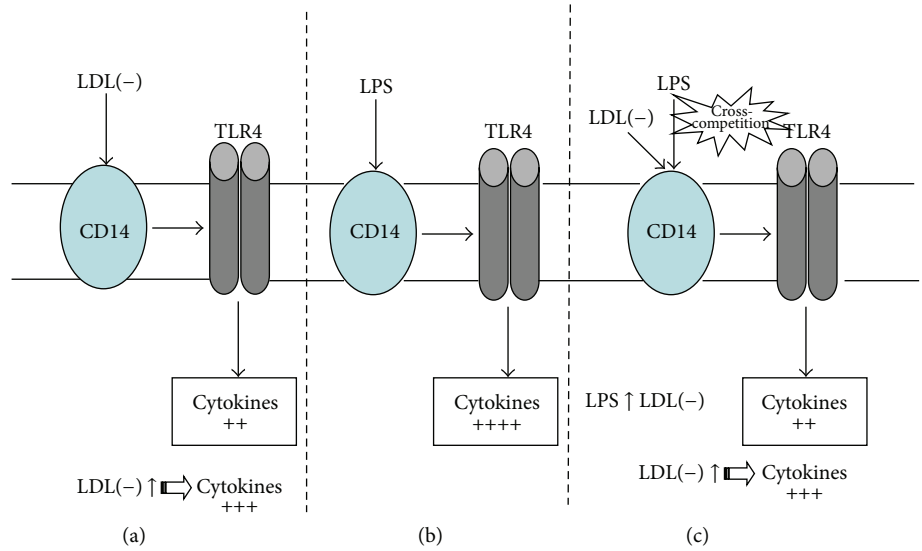

Figure 4: Cytokine induction through CD14-TLR4 by LPS and LDL(−). In the absence of bacterial infection, CD14-TLR4 activation mediated through LDL(−) triggers an inflammatory response that would be deleterious in case of high LDL(−) concentration (a). This effect would be lower than that induced by LPS at high concentrations (infection) in the absence of LDL(−) (b). When LPS and LDL(−) coexist, there is a competition between the two stimuli. The global effect will depend on the relative concentration of both molecules (c). TLR4: toll-like receptor 4, LPS: lipopolysaccharide, LDL(−): electronegative LDL.

in turn, this inflammatory response would be beneficial in counteracting an external aggression. Regarding the physiological role of LDL(−)-induced apoptosis, it is not so clear whether this is an atherogenic effect. Apoptosis could be considered detrimental in late atherosclerotic lesions, but, in early atherosclerotic lesions, the clearance of apoptotic cells is associated with decreased lesion progression [101]. Therefore, these two "atherogenic" properties may not be so bad, and, only when these processes are uncontrolled or excessive, they became detrimental. On the other hand, a putative protective action may not be so good. The counteraction by LDL(−) of the LPS-induced inflammatory effect could be protective. Nevertheless, LDL(−) exerts an inflammatory action that could also be harmful when LDL(−) concentrations increase, even though it is less deleterious than LPS, as shown in Figure 4.

LDL(−) could play a role as a modulator of the inflammatory response to avoid detrimental and inappropriate immune responses. The proportion of LDL(−) is increased in inflammatory situations, such as rheumatoid arthritis or DM. In such events, it could modulate the immune response to some degree. It can be hypothesized that LDL(−) would emerge as a negative feedback to counteract an excessive/overwhelming inflammatory response and play a protective role. It thus seems likely that LDL(−) is more of a consequence of inflammatory situations than a cause.

7. Conclusions

In summary, LDL(−) is a heterogeneous modified LDL which promotes several inflammatory actions on cells. LDL(−) also promotes some anti-inflammatory actions to control an excessive inflammatory response. The global effect of LDL(−) will be the result of the combination of its inflammatory/anti-inflammatory properties. The importance of each individual property in the global action of LDL(−) depends on the physicochemical characteristics of LDL(−) and the cell milieu. Taken together, all data concur that, depending on the context, LDL(−) promotes or inhibits inflammation, playing a dual role in atherogenesis.

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Review Article

Oxidized LDL and LOX-1 in Experimental Sepsis

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Oxidized low-density lipoproteins (oxLDL) and the lectin-like oxLDL receptor-1 (LOX-1) are upregulated in inflammation. Because of the importance of inflammation and capillary leakage in the impairment of the microcirculation, which in turn contributes to the development of sepsis and multiorgan failure, the role of oxidized LDL and LOX-1 as players of intestinal inflammation is of great interest. In fact, the blockade of LOX-1 during experimental endotoxemia was effective in reducing leukocyte activation. There are several mechanisms by which oxLDL can participate in local and systemic inflammation, including cell proliferation, apoptosis, capillary perfusion, leukocyte-endothelial cell interactions, and endothelial activation. This review highlights the evidence relating oxLDL and LOX-1 to proinflammatory disease mechanisms. We also indicate situations when oxLDL, because of exposure time, dose, or degree of oxidization, is involved in disease resolution. Modulation of LOX-1 response could be utilized for the treatment of local and systemic inflammation, but the successful use of this target requires further understanding of its broad effects.

1. Introduction

Recently, the contribution of oxidized LDL (oxLDL) to leukocyte activation and microvascular perfusion disturbances in experimental endotoxemia has been reported [1]. It is thought that oxidized LDL and LOX-1 may play a role in the increased inflammation and capillary leakage, two factors which induce disturbances in the microcirculation. Because of the importance of impaired microcirculation in the development of sepsis, and the ensuing organ failure, it is important to consider oxidized LDL and LOX-1 as players of intestinal inflammation. In this review, our goal is to introduce the literature on oxLDL in local and systemic inflammation and its relation to sepsis development. We also discuss the variety of mechanisms by which oxLDL can participate in inflammation, including cell proliferation/apoptosis, capillary perfusion status, leukocyte-endothelial cell interaction, neutrophil recruitment, leukocyte activation, and the endothelial cell response. While considering its potential for promoting proinflammatory disease mechanisms, we also highlight situations when it can be involved in the disease resolution. It seems obvious that this axis should be utilized for the treatment of intestinal inflammation and sepsis, but significant literature gaps need to be addressed beforehand.

2. Lectin-Like oxLDL Receptor

The lectin-like oxLDL receptor-1 (LOX-1) binds the protein moiety of oxLDL [2]. This receptor was first studied in vascular endothelial cells [3], and it was later shown to be expressed in human intestinal cell lines [4], endothelial cells, macrophages, and smooth muscle cells [5]. Several factors are known to upregulate LOX-1, including endotoxin (lipopolysaccharide; LPS), shear stress and oxidative stress [5], and the presence of oxLDL itself [4]. In addition, *in vitro* cultured smooth muscle cells were shown to upregulate the expression of LOX-1 in the presence of inflammatory cytokines, including IL-1alpha, IL-1beta, and TNF-alpha [6].

The oxidation of LDLs can occur by activated leukocytes, whether activated polymorphonuclear cells [7], macrophages, or neutrophils [8]. In the presence of oxLDL, lipid body formation is enhanced in leukocytes, as shown for mouse peritoneal macrophages after 1 hour of *in vitro* culture [9]. This was also shown *in vivo*; the intraperitoneal injection of phospholipid fractions of oxLDL into mice resulted in the development of lipid body formation within 3 hours [9]. In addition, the upregulation of LOX-1 on intestinal cell lines was associated with the enhanced uptake of other factors (e.g., pancreatic bile salt-dependent lipase) into the cells [4], reflecting the overall lipid dysfunction that occurs as part of the inflammatory process.

3. LOX-1 in Experimental Sepsis

Oxidation of LDL was illustrated in animals with endotoxemia. In fact, LPS-injected hamsters were found to have higher levels of oxidized fatty acids in serum, namely, a 4- to 6-fold increase in the levels of conjugated diene and lipid hydroperoxide in LDL fraction and 17-fold increase in LDL content of lysophosphatidylcholine. In our own study, we reported the increased expression of LOX-1 in the intestinal tissues of animals with endotoxemia, both at the mRNA and the protein level [1]. These studies indicate the oxidation of LDL after LPS injection, as would occur during acute infection and during inflammatory conditions.

Mice that lacked the expression of LOX-1 (i.e., LOX-$1^{-/-}$ mice) were found to have improved survival in a cecal ligation and puncture (CLP) sepsis model. LOX-$1^{-/-}$ mice also had lower inflammatory response to CLP, in terms of pro-inflammatory cytokine levels (e.g., TNF-alpha) in the serum and the extent of lung damage (e.g., edema). These mice were also able to clear the bacteria from peritoneum, blood, and lungs more than wild mice [10]. The contribution of LOX-1 in the development of lung tissue damage in sepsis was also observed in other models of sepsis. Intraperitoneal injection of LPS increased LOX-1 expression in mouse lung. When LOX-1 is blocked in mice with endotoxemia, the lung tissue damage was significantly reduced, as indicated by the reduced numbers of neutrophils, lower expression of adhesion molecule (intracellular adhesion molecule-1; ICAM-1), and reduced activation of transcription factor NF-κB in the lungs [11]. These effects may explain why animals with endotoxemia pretreated with anti-LOX-1 antibody had partially recovered from the LPS-induced leukopenia and showed improved survival [12].

OxLDL and LOX-1 are also involved in several cardiovascular diseases, for example, atherosclerosis [13] and autoimmune diseases, for example, antiphospholipid syndrome [14]. For instance, peripheral blood mononuclear cells (PBMCs) from patients with antiphospholipid syndrome were shown to produce more pro-inflammatory cytokines (e.g., IFN-gamma and IL-2) in the presence of oxLDL [14]. Since oxLDL can be formed by leukocytes themselves [7, 8], this mechanism represents a vicious cycle that augments the inflammatory process in several diseases.

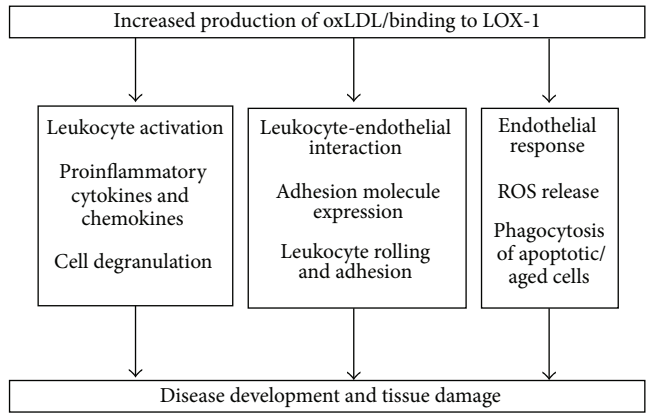

Figure 1: Schematic presentation of selected cellular effects of oxidized LDL (oxLDL) and/or Lectin-like oxLDL receptor (LOX-1) activation in systemic inflammation.

4. Proposed Mechanisms

Because of the broad expression of LOX-1 on several cells [3, 5] and the ability of macrophages, neutrophil, and other immune cells to oxidize LDL [7, 8], there are several possible ways by which oxLDL can influence the inflammatory process. This is summarized in Figure 1.

4.1. Cell Proliferation/Apoptosis. OxLDL and LOX-1 has been associated with cell death. The growth of *in vitro* activated blood lymphocytes was partially inhibited by treatment of oxLDL after 48 h [15]. OxLDL treated T cell lines [15] and cardiomyocytes [16] were shown to undergo apoptosis. *In vitro* studies have suggested that cell apoptosis is enhanced by the presence of other inflammatory signals, for example, the expression of chemokine receptors, and the production of cytokines TNF-alpha and IL-1beta. This explains why these effects were abolished by the blockade of LOX-1 [16].

It is important to note that the ability of oxLDL to induce apoptosis is influenced by the degree of oxidation, dose of oxLDL, and the exposure time. Apoptosis was induced in macrophage cell lines by incubation with extensively oxidized LDL (at 100 mg/mL) or with a higher dose (200 mg/mL) of light or extensively oxidized LDL [17]. In contrast, macrophage activation and proliferation were induced by low dose (100 mg/mL) of the lightly oxidized LDL. Interestingly, the use of shorter incubation time induced cell proliferation, even if the LDL was highly oxidized LDL and at a lower dose (100 mg/mL) [17].

4.2. Capillary Perfusion. Studies examined whether the microhemodynamic parameters are modified by oxLDL [18] or LOX-1 expression [1]. In hamsters treated with oxLDL or endotoxemic rats treated with LOX-1 blockade, the capillary microperfusion was not affected [1, 18]. When endothelial-dependent vasodilation in isolated microvessels exposed to oxLDL was observed, the effect was related to the oxidative stress, since it was restored by incubation with oxygen radical scavengers [19].

4.3. Leukocyte-Endothelial Cell Interactions

4.3.1. Expression of Adhesion Molecules on Endothelial Cells. There is an increased expression of adhesion molecules on endothelial cells in the presence of oxLDL or in association with LOX-1 expression. OxLDL treatment was shown to stimulate the adherence of THP-1 (a human acute monocytic leukemia cell line) to human umbilical vein endothelial cells, in conjunction with an increased expression of LOX-1 and several adhesion molecules (namely, Intracellular cell adhesion molecule-1 (ICAM-1), Vascular cell adhesion molecule-1 (VCAM-1) and E-selectin) on the endothelial cells [20].

In another study, both xanthoma tissue-modified LDL (x-LDL) and copper sulfate oxidized-LDL (Cu-LDL) were able to induce the adhesion of monocyte leukemic cell lines (U937) to human dermal microvascular endothelial cells (HDMEC), but this was related to upregulation in the expression of different adhesion molecules depending on the kind of oxLDL. For instance, x-LDL induced upregulation of VCAM-1 and E-selectin, and Cu-LDL upregulated VCAM-1, E-selectin, and ICAM-1 on endothelial cells [21]. This mechanism may be responsible for the effects of other inflammatory mediators. For example, C-reactive protein was shown to induce the expression of ICAM-1 and VCAM-1 on human endothelial cells, but this was inhibited when LOX-1 was blocked (using LOX-1 small-interfering RNA) [22].

4.3.2. Leukocyte Rolling and Adhesion. The interaction between leukocytes and endothelium in the presence of oxLDL or LOX-1 has been studied both *in vitro* and *in vivo*. Surface coating with LOX-1 was sufficient to induce the adhesion of polymorphonuclear cells (PMNs) under physiologic shear conditions [12]. Platelets were also shown to bind to endothelium using the LOX-1 and oxLDL interaction. In fact, the negative phospholipids present on surface of platelets were suggested to be ligands for LOX-1. Therefore, the presence of oxLDL on endothelium was shown to enhance platelet aggregation and to lead to an induction of endothelin-1 in the endothelial cells (a sign of endothelial dysfunction) [23]. When the interaction of copper-oxidized LDL treated endothelium with flowing erythrocytes and platelets was examined using intravital microscopy, there was a transient increase in the number of platelet-endothelial cell adhesions. It is important to note that this effect was inhibited by the administration superoxide dismutase and catalase, suggesting the participation of oxygen-derived free radicals in the oxLDL-induced effects [24].

Several studies utilized oxLDL to induce leukocyte rolling and/or adhesion on endothelium. Local intra-arterial infusion of oxLDL was shown to increase the leukocyte rolling across rat mesenteric venules [25]. It was also reported to promote the adhesion of neutrophils and leukocytes to endothelium *in vitro*, in a manner that was dependent on the degree of lipid peroxidation. Local intra-arterial infusion of oxLDL elicited significant increases in leukocyte adherence and albumin leakage in rat mesenteric venules, without affecting the venular shear rate [25, 26].

The administration of oxLDL in rats was shown to increase leukocyte-endothelial interactions by increasing the number of rolling cells and reducing their rolling velocity [27]. It is interesting to note that these effects were not observed in pregnant rats [27]. Using another model, the skinfold chamber model in hamsters, oxLDL administration induced leukocyte rolling and adhesion to the endothelium of venules and arterioles [18]. This may be related to the action of PAF on leukocyte adhesion, as shown by the ability of PAF receptor antagonism to attenuate oxLDL-induced leukocyte adhesion *in vivo* [18]. In addition, they may be related to the action of inflammatory mediators (e.g., leukotrienes) and oxidative stress determinants (e.g., superoxide radicals).

The role of LOX-1 was specifically tested in the context of endotoxemia. Our group has modeled the increased adhesion of leukocyte to intestinal venules in animals with endotoxemia. Blocking LOX-1 had an inhibitory effect on leukocyte adhesion. Using intravital microscopy, we demonstrated that blocking LOX-1 was sufficient to reverse the LPS-induced increase in leukocyte adhesion to intestinal venules [1]. Likewise, Honjo et al. [12] demonstrated that LOX-1 blockade suppresses leukocyte infiltration and protein exudation in animals with endotoxin-induced uveitis. In addition, the retinal microvessels appear to have reduced interaction with leukocytes in animals with endotoxemia after LOX-1 blockade, with regard to the reduced number of rolling cells and increased velocity of rolling. This study therefore suggested LOX-1 as a vascular tethering ligand specifically [12].

4.4. Neutrophil Recruitment.

Since LOX-1 is expressed in neutrophils, the function of LOX-1 was important to examine. LOX-1 deletion was found to increase the activation and recruitment of neutrophils to infection sites, for example, peritoneum in CLP-induced sepsis [10]. The mechanism of this phenotype is not clear to us at this point.

4.5. Leukocyte Activation

4.5.1. Inflammatory Signaling Pathways. OxLDL can modulate the NF-κB pathway. Treatment of human PBMCs with oxLDL leads to an increased NF-κB induction (p65 nuclear translocation) [28], and the same was reported for human umbilical vein endothelial cells (HUVECs) [20] and aortic endothelial cells that express LOX-1 [29]. This effect was correlated with an increased production of IL-6 and expression of toll-like receptor (TLR)-2 and TLR-4 in oxLDL-treated PBMCs [28], and an increased level of reactive oxygen species [29].

Moreover, the treatment of minimally oxidized LDL on LPS-activated macrophages was reported to upregulate their NF-κB and AP-1 pathways, in conjunction with an increased expression of inflammatory chemokines [30]. In contrast, oxLDL can delay the activation of NF-κB in LPS-treated peritoneal macrophages [31], and therefore it may have immunosuppressive effects during immune response. These contrasting reports are likely due to the differences in concentrations of ox-LDL [32] or the degree of oxidation of the LDL used [30].

It should be noted that the effect of oxLDL on the activation of signaling pathways can be cell specific. For instance, the addition of copper oxidized-LDL to *in vitro* cultures of vascular smooth muscle cells and human monocyte-derived macrophages was shown to induce the phosphorylation of MAP kinase, but this was not the case for bovine endothelial cells [33].

4.5.2. Cytokine and Chemokine Production. Similar to what was reported on the effect of oxLDL in the activation or inhibition of inflammatory signaling pathway, its impact on cytokine production also varies. On one hand, the treatment of oxidized LDL can increase the production of IL-8 from freshly isolated monocytes and monocytic THP-1 cell lines [34], HUVECs [20], and LOX-1 overexpressing human aortic endothelial cell line [35]. OxLDL administration was also shown to induce the production of IL-6 from HUVECs [20], chemokines CXCL2 and CXCL3 from LOX-1 overexpressing human aortic endothelial cell line [35], MCP-1 from human articular chondrocytes [36], and the intestinal tissue of animals with endotoxemia [1]. In one study, the capacity of oxLDL to induce the production of IFN-γ and TNF-alpha from PBMCs was suggested to be related to the PAF-like lipids in oxLDL, since this function was at least partially inhibited by PAF-receptor antagonist [37]. In striking contrast, oxLDL was reported to inhibit the ability of LPS-activated peritoneal macrophages to produce a number of cytokines and chemokines [31].

4.5.3. Mast Cell Activation/Degranulation. It is interesting to note that oxLDL can induce mast cell degranulation, as shown when oxLDL was locally infused in rat mesentery [26]. This effect was suggested to promote leukocyte-endothelial cell adhesion and albumin leakage responses to copper-oxidized LDL, that were inhibited by pretreatment with the mast cell-stabilizing agents [26]. It is worthwhile to consider the vast breadth of immune cells that can be affected by oxLDLs; it is important to note that the variation in the effect of oxLDL/LOX-1 on cytokine production and inflammatory signaling pathways that were mentioned above may also impact the cell activation status.

4.6. Endothelial Cell Response

4.6.1. Reactive Oxygen Species (ROS) and Free Radical Production. The generation of ROS, inducible NO synthase, NO, and oxygen radicals by HUVECs [20] and aortic endothelial cells [38] was found to increase after oxLDL treatment, even as early as 30 seconds of incubation [29]. This is important, since the blockade of superoxides, using ellagic acid, in oxLDL treated HUVECs inhibited many of the downstream effects of LOX-1, including the phosphorylation of MAPK and NF-κB pathways, the production of cytokines, and expression of adhesion molecules to HUVECs [20].

4.6.2. Matrix Metalloproteinase (MMP). When human endothelial cells were cultured *in vitro* and treated with oxLDL, they increased the mRNA and protein level of membrane type 1-matrix metalloproteinase (MT1-MMP), an enzyme, that is known to promote the activation of matrix degradation (by activating pro-MMP2). This effect occurred within 6 hours of exposure and was even further enhanced when cells were treated with TNF and oxLDL in combination [39].

4.6.3. Phagocytosis of Apoptotic/Aged Cells. The expression of LOX-1 by endothelial cells and Chinese hamster ovary cells was associated to their ability to bind aged RBC and apoptotic cells. As such, the treatment of mAB to LOX-1 was found to inhibit the binding of aged RBC to these cells. In fact, the study by Oka et al. suggested that the ability of oxLDL to bind to aged RBCs was due to the PS part of the surface of apoptotic cells, and therefore this effect increases coagulation and inhibits the removal of apoptotic cells [40]. The function of LOX-1 as a scavenger receptor may extend to several other usages. In fact, IFN-alpha-activated dendritic cells were found to upregulate LOX-1, where it appears to participate not only in the uptake of apoptotic cells but also for CD8 T cell priming [41].

5. Concluding Remarks

Sepsis and intestinal inflammation involve significant changes in oxLDL levels and LOX-1 expression. Studies have demonstrated the efficacy of blocking or knocking out LOX-1 in decreasing the inflammatory process and tissue damage that occur in models of sepsis. Oxidized LDL is produced by a variety of cells, and it can bind to intestinal endothelial cells, as shown by cultured *in vitro* and *in vivo* studies. Research has provided evidence for a wide range of effectors that are modulated by the upregulation of oxLDL and the activation of LOX-1 (summarized in Figure 1). These include enhancing the leukocyte-endothelial interactions, whether by increasing the expression of adhesion molecules on endothelial cells or by increasing the rolling and adherence of leukocytes to the microcirculation. They were also shown to activate leukocytes, evident as activation of NF-κB pathway, production of cytokines and chemokines, and degranulation. In addition to these, oxLDL and LOX-1 can influence the endothelial cell responses, in terms of ROS production, MMP, levels and the phagocytosis of apoptotic cells.

This review summarizes the significant efforts that aim to understand the breadth of effects induced by oxLDL and LOX-1. However, it is important to note that the contribution of oxLDL to the development of sepsis is a relatively newer domain, in comparison to the literature available on the lipid dysfunction in cardiovascular diseases. There is still a significant need for examining this key mediator in more detail, in order to be able to target it specifically to control the development of sepsis. Challenges include the fact that, under certain situations, oxLDL was shown to have some anti-inflammatory roles. Thus, it is crucial to consider the dose, time of exposure, and the degree of oxidation when studying this mediator. In the future, the specific ability to control these elements will determine the effectiveness of

using LOX-1 blockade strategies to alleviate the inflammatory response in sepsis.

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Review Article

Low-Density Lipoprotein Modified by Myeloperoxidase in Inflammatory Pathways and Clinical Studies

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Oxidation of low-density lipoprotein (LDL) has a key role in atherogenesis. Among the different models of oxidation that have been studied, the one using myeloperoxidase (MPO) is thought to be more physiopathologically relevant. Apolipoprotein B-100 is the unique protein of LDL and is the major target of MPO. Furthermore, MPO rapidly adsorbs at the surface of LDL, promoting oxidation of amino acid residues and formation of oxidized lipoproteins that are commonly named Mox-LDL. The latter is not recognized by the LDL receptor and is accumulated by macrophages. In the context of atherogenesis, Mox-LDL accumulates in macrophages leading to foam cell formation. Furthermore, Mox-LDL seems to have specific effects and triggers inflammation. Indeed, those oxidized lipoproteins activate endothelial cells and monocytes/macrophages and induce proinflammatory molecules such as TNFα and IL-8. Mox-LDL may also inhibit fibrinolysis mediated via endothelial cells and consecutively increase the risk of thrombus formation. Finally, Mox-LDL has been involved in the physiopathology of several diseases linked to atherosclerosis such as kidney failure and consequent hemodialysis therapy, erectile dysfunction, and sleep restriction. All these issues show that the investigations of MPO-dependent LDL oxidation are of importance to better understand the inflammatory context of atherosclerosis.

1. Introduction

Atherosclerosis is an inflammatory process involving vascular cells, monocytes, T lymphocytes, proinflammatory cytokines, chemoattractant cytokines (chemokines), and growth factors [1–3]. Specific arterial regions are favorable to atherosclerosis development [4], and these areas have been linked to shear stress abnormalities [5]. More recently, it was shown in apoE−/− mice that smooth muscle cells display a different transcriptome at locations where atherogenesis is prone even before the development of the lesion [6]. The accumulation of foam cells in intima leads to primary lesions characterized by fatty streaks in the artery wall and by thickening of the wall. Early lesions are found in the aorta of healthy 10-year-old children, in coronary arteries of 20-year-old adults, and later in cerebral arteries [7]. These lesions can naturally disappear without causing any disorder to the patient or progress of advanced lesions with smooth muscle cell migration and proliferation, foam cell accumulation, and can even lead to plaque rupture and thrombus formation.

Among the factors associated with this process, modification and particularly oxidation of low-density lipoproteins

(LDLs) have been of major interest since Steinberg et al. showed that native LDL does not accumulate in macrophages, whereas modified lipoprotein does [8, 9]. However, the exact mechanisms of LDL oxidation are still not completely understood, and researchers continue to argue about them [10]. Several mechanisms have been described including reactive oxygen species (ROS) produced by endothelial cells and monocytes/macrophages [11], metal ions [12], lipoxygenase [13], or myeloperoxidase [14, 15]. Each oxidative mechanism of lipoprotein is characterized by targeting either lipid, protein, or both moieties [8].

Highly oxidized LDL (ox-LDL) cannot bind to the LDL receptor and is taken up by monocytes which transform into macrophages. Indeed, these cells express scavenger receptors such as (SR) such as CD36, SR-A, SR-B1, and LOX-1 at their surface, which bind ox-LDL and enable scavenger receptor-mediated endocytosis [16]. This reaction is the best way for removing excess of ox-LDL in the arterial wall. Conversely, this process could worsen, and ox-LDL continues to accumulate in the subendothelial space. Macrophages continue to engulf the modified lipoproteins and evolve to a state where high quantities of lipids are intracellularly accumulated leading to foam cell formation [17]. Resistance of ox-LDL to acidic lysosomal proteolysis via cathepsins has also been observed [18]. The latter phenomenon increases the risk of LDL accumulation in macrophages and therefore foam cell formation. Foam cells themselves have a proinflammatory effect by producing cytokines and growth factors such as interleukins (IL) 1β and -8, interferon-γ, tumor-necrosis factor-α (TNFα), and macrophage colony stimulating factor (M-CSF).

Ox-LDL is widely described as a key component of atherogenesis and triggers the inflammatory processes of the disease. Ox-LDL induces a number of potentially proatherogenic activities such as the production of proinflammatory cytokines and chemokines by monocytes, endothelial cells, and smooth muscle cells *in vitro* [19, 20]. In this paper, we focused on a particular and frequent LDL oxidation mechanism involving myeloperoxidase (MPO). MPO is an important enzyme of neutrophils which combat pathogen invasion in the body. Indeed, MPO catalyzes the production of oxidative reagents which damage pathogens and aid in their elimination. Unfortunately, in chronic inflammation syndromes, MPO is also released into the extracellular space due to neutrophil activation where MPO-derived oxidants can in turn cause tissue damage. One of the targeted components is LDL, leading to MPO-dependent oxidized LDL, commonly named Mox-LDL.

In this paper, we first review LDL, apolipoprotein B-100, the unique protein of LDL, and its oxidation sensitive components. MPO and its enzymatic mechanism are then briefly described. Following this, modifications of LDL are discussed with particular focus on MPO-dependent oxidation mechanisms and the specificity of MPO to modify LDL. *In vitro* experiments on inflammation involving Mox-LDL are then addressed. In this section, we will show that Mox-LDL has a key role in triggering the inflammatory response during atherogenesis and has effects on monocytes, macrophages, and endothelial cells and that those effects are different than LDL modified by other systems. Finally, clinical aspects of Mox-LDL are illustrated, focusing on several conditions such as atherosclerosis, erectile dysfunction, dialysis, nonalcoholic fatty liver disease, and sleep disorders.

2. Low-Density Lipoprotein and Apolipoprotein B-100

LDL is one of the major carriers of cholesterol in the human body and plays a role in cholesterol metabolism, as well as other lipoproteins such as high-density, intermediate-density, or very-low-density lipoproteins. LDL is generally considered to be a spherical particle of about 22 nm in diameter [21]. It includes two major groups of compounds: (i) lipids and (ii) protein representing 80% and 20% of total lipoprotein weight, respectively.

The lipid moiety contains approximately 3000 molecules including cholesterol esters mainly but also free cholesterol, phospholipids, and triglycerides. In LDL, lipids are separated into two parts: (i) a monolayer of phospholipids and free cholesterol at the surface and (ii) a core majorly composed of cholesterol esters but also free cholesterol and triglycerides.

The protein moiety of LDL includes a unique protein which is an exception among lipoproteins. This protein is apolipoprotein B-100 (apoB-100) and was completely sequenced for the first time in 1986 by several labs thanks to genetic investigations [22–25]. Mature apoB-100 consists of 4536 amino acid residues with 19 putative N-glycosylation sites making it one of the largest monomeric proteins in the human body with a molecular mass estimated to be 550 kDa. ApoB-100 is usually divided into 5 domains as first described by Yang et al. in 1989 [26] and later summarized by Segrest et al. [27]. This division of apoB-100 follows α- and β-domain characteristics. The consensus structure is as follows: NH_2-$\beta\alpha$1-β1-α2-β2-α3-COOH. ApoB-100 is distributed both at the surface and in the core of the lipoprotein where the NH_2-$\beta\alpha$1 domain (the first 1000 residues) is generally described as being a highly hydrophilic domain located outside the particle. Although the latter domain is hydrophilic, apoB-100 has several hydrophobic segments which enable strong interactions with lipids (core and surface lipids) and stabilize the lipoprotein structure.

ApoB-100 is also a key player in LDL recognition and binding to the LDL receptor, which is present at the surface of most human cells. When LDL binds to the receptor, uptake occurs followed by degradation of the lipoprotein and release of cholesterol for the cell's needs. Modifications to the apoB-100 structure can lead to decrease in affinity or even the inability of LDL to bind the receptor [28, 29]. More than 50 variants are currently described in UniProt Database, many with no effect on LDL function although others have harmful properties resulting in hypercholesterolemia and its deleterious effects. It is also assumed that a receptor-binding site is present at the surface of apoB-100. Many studies have tried to determine the exact binding site, but it is still controversial [22, 30–33]. The sequence between residues 3345 and 3381 would include the receptor-binding site and sometimes the 3359–3369 segment is mentioned [28], but it

should be kept in mind that mutations on apoB-100 close to this binding site also disrupt the ability of LDL to bind to LDL receptor (e.g., R3500Q mutation which is the major cause of familial defective apoB-100 disease). Finally, oxidized apoB-100 also has a decreased affinity for the LDL receptor.

The characteristics of apoB-100 make its analysis a very complex and difficult task. Since its sequencing in 1986, apoB-100 has been the subject of several studies to elucidate its exact structure [27, 34–37]. In 1989, Yang et al. used a specific methodology to distinguish segments of the protein which are hydrophobic from those which are hydrophilic [38]. This approach is useful for prediction and supposition of segments which could be more sensitive to post-translational modifications (PTMs).

The best experimental procedures to study the modifications that occur to apoB-100 are those that utilize mass spectrometers (MS/MS or MS^n) [39]. Indeed, many of these instruments have a high-resolution mass analyzer coupled to the ability to fragment the peptides in order to sequence them. Such instruments include quadrupole-time of flight (QToF) mass spectrometers which can detect peptide and PTMs with high accuracy which can be coupled to separation techniques such as liquid chromatography. This represents a powerful strategy to discover PTMs on proteins.

Another strategy has been recently described to analyze modifications of apoB-100 taking advantage of known modifications and their specific product ions to monitor PTMs by LC-MS/MS [40]. However, the huge sequence of apoB-100 also makes it important to optimize all parameters of the analysis. For this purpose, we developed and optimized an LC-MS/MS method capable of recovering up to 80% of apoB-100, and we have shown that this is required to detect the maximum of PTMs currently achievable (4 times more modifications were recovered thanks to the optimized protocol) [41].

In summary, LDL and apoB-100 investigations are important to understand their implications in the processes of disease. However, LDL/apoB-100 complexity make these studies particularly difficult, but recent improvements in instrumentation, such as those for mass spectrometry, are very helpful. In the following paragraphs, we principally discuss MPO-dependent oxidation of LDL and its roles in inflammation *in vitro* as well as *in vivo*.

3. Myeloperoxidase and MPO/H_2O_2/Halide System

MPO is a key enzyme in innate immunity and defense against pathogens [42]. Hereinafter, we will describe the major points of interest of MPO devoted to LDL oxidation and inflammation. For reviews on molecular mechanisms as well as physiological and physiopathological aspects of MPO, see Klebanoff [43], Davies [44], Davies et al. [45], and van der Veen et al. [46]. MPO expression is limited to myeloid cells, and its synthesis in neutrophils starts at the promyelocyte stage and terminates at the beginning of the myelocyte stage. Mature MPO is packed in azurophilic granules of neutrophils and accounts for 5% of the total dry cell weight, making MPO the major protein of neutrophils. It is also present in monocytes but to a lesser extent [47, 48].

MPO is a hemeglycoprotein with a mass of 140–155 kDa [49]. Its biosynthesis is a complex process including proteolytic events, heme and glycan additions, and a final dimerization step [50, 51]. Briefly, nascent MPO, called preproMPO, undergoes a first proteolytic event and N-glycan addition to make apoproMPO in the endoplasmic reticulum. The latter lacks the hememoiety which is then inserted due to the activity of chaperones (calreticulin and calnexin) which interact with MPO oligosaccharides. This forms proMPO which leaves the endoplasmic reticulum and travels to the Golgi apparatus and granules where MPO undergoes several new proteolytic events. The final monomer of MPO consists of a light chain of 106 residues and a heavy chain of 467 residues. The two chains are linked by a disulfide bond and also via the heme group. In the mature form, MPO is a dimer of two monomers linked by a disulfide bond on position cysteine 369 of each heavy chain. Each monomer is enzymatically active and can produce oxidants. MPO also contains a calcium binding site contributing to the stabilization of the structure. N-glycans play a key role in protein synthesis and also in enzymatic activity as recently shown by our experiments [52]. Furthermore, MPO is a highly cationic protein with a pI ≈ 11 enabling its binding to electronegative surfaces such as endothelial wall, lipoprotein, or proteoglycans [53, 54].

In the azurophilic granules, MPO is kept in an inactive state as long as the neutrophil is not activated and hydrogen peroxide (H_2O_2) is absent [43]. After phagocytosis, activation of neutrophils leads to the release of the contents of the azurophilic granules (including MPO) into phagosomes and to the assembly of the NADPH oxidase enzyme complex (NOX_2) that produces superoxide radicals ($O_2^{\bullet -}$). This radical is highly reactive and unstable and is rapidly converted into H_2O_2 spontaneously or by the action of superoxide dismutase. H_2O_2, which has a lower oxidation potential, can reach the ingested pathogen and contributes to its destruction by oxidizing vital molecules [55]. However, the reactivity of H_2O_2 alone does not produce optimal antimicrobial efficacy.

Using H_2O_2 and chloride ion (Cl^-), MPO produces a more powerful oxidant molecule, namely, hypochlorous acid (HOCl). MPO can also use other (pseudo-) halide anions including Br^-, I^-, and SCN^- to give the corresponding hypo-(pseudo-) halogenous oxidants (Figure 1). The first reaction of MPO is its oxidation by H_2O_2 to give Compound I. In the halogenation cycle, MPO is then reduced back to its native form in a two-electron reaction. The latter enables the generation of hypo- (pseudo-) halogenous acid. Although Cl^- has the lowest reactivity to MPO among (pseudo-) halide anions [56], it is considered to be the major physiological substrate of MPO due to its high *in vivo* concentration [57–60]. HOCl is a strong oxidant, and it is thought to be more efficient than H_2O_2 in killing pathogens [61]. HOCl effectively attacks biomolecules of the ingested pathogen resulting in the death of pathogen in the phagosome.

It is worth noting that MPO also has a peroxidase cycle in which electron donors can be oxidized and native MPO

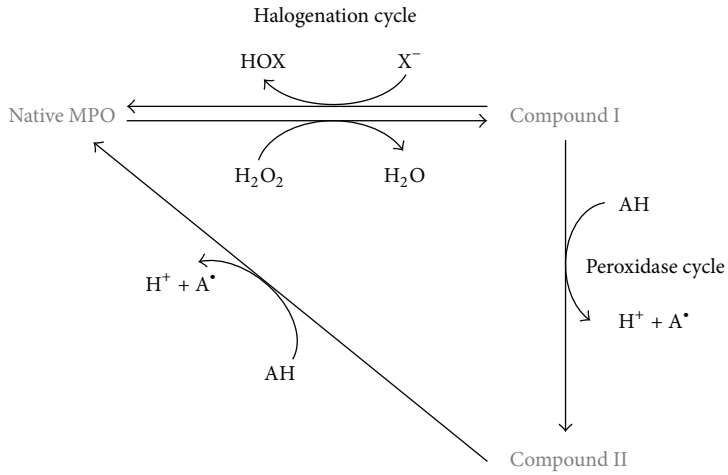

Figure 1: Scheme of the interconversion between different oxidized states of myeloperoxidase. The first reaction is the oxidation of native MPO to Compound I by a two-electron reaction. In the halogenation cycle, Compound I is backconverted to native MPO, and a two-electron oxidation of (pseudo-) halide generates hypo- (pseudo-) halogenous acid. In the peroxidase cycle, Compound I can oxidize an electron donor via 1-electron process transforming Compound I to Compound II and the electron donor to a radical product. Compound II can be reduced to native MPO by using 1 electron from other electron donors.

is regenerated in a two-step reaction (via the formation of Compound II: see Figure 1).

Due to its powerful oxidation products, MPO would be required to give the neutrophil optimum antimicrobial activity. Although neutrophils retain normal phagocytosis activity when MPO is inhibited or deficient, they cannot kill all types of ingested pathogens [62].

Despite its key role in host defense, MPO has also been involved in pathologic states. Indeed, during chronic inflammation or acute oxidative stress, MPO is released into the extracellular space where oxidants can be produced and host tissues damaged. Among biomolecular targets of MPO, LDL has been pointed out, and MPO is considered to be a major contributor of ox-LDL generation *in vivo* [8]. Moreover, clinical studies have highlighted serum MPO levels as a prognosis factor in patients with acute coronary syndromes or chest pain. These data support the necessity to understand the *in vivo* impact of Mox-LDL [63, 64], resulting from the reaction of MPO in the presence of LDL.

4. Modification of LDL, Myeloperoxidase, and Mox-LDL

4.1. Introduction. One of the primary steps of atherogenesis is the activation of the immune and vasculature systems leading to endothelial dysfunction and infiltration of immune cells and LDL into the vascular wall. This also leads to an oxidative burst and production of reactive oxygen species (ROS). The latter play a key role in the disease by inducing LDL modification (oxidation) resulting in the accumulation of lipids in macrophages, and the formation of foam cells and atherosclerotic plaques. LDL can be subjected to a variety of PTMs [40] among which oxidation [12, 14, 65], glycation [66, 67], and glycosylation [68, 69] are described. The oxidation mechanism is described from here on, focusing on MPO-dependent oxidation.

4.2. Oxidation of LDL. Increased plasma cholesterol level is a well-documented proatherogenic factor, and hypocholesterolemic therapy is the only approved pharmacological treatment. However, around 50% of patients experiencing a cardiovascular event have a normal level of cholesterol. This fact leads researchers and physicians to consider that the quality of lipoproteins might be more important than the quantity. In this context, it is largely admitted that the modifications of lipoproteins, particularly LDL and HDL, are of major importance in the development of atherosclerosis. This was first highlighted by Steinberg et al. who observed that native LDL is not extensively taken up by macrophages and does not lead to foam cell formation even though modified lipoproteins accumulate in these cells [9, 16]. Among the modifications, oxidation of LDL has certainly been the most studied for the last number of decades, and many studies have described the presence of ox-LDL in atheromatous lesions [14, 70–72].

Several mechanisms of oxidation exist involving metal cations as well as many different enzyme systems such as lipoxygenase, myeloperoxidase, xanthine oxidase, and NADPH oxidase. See Yoshida and Kisugi 2010 [10] for a review on major mechanisms of LDL oxidation. Numerous oxidants preferentially target the lipid moiety (i.e., Cu^{2+}, lipoxygenases, and RNS; [72–74]), whereas others target the protein moiety of lipoproteins (HOCl and MPO; [59, 75]). From here on, we summarize some of the mechanisms involved in LDL oxidation, starting with an introduction to metal cation- and lipoxygenase-dependent LDL oxidation, followed by a complete description of the MPO-dependent process.

4.2.1. Oxidation of LDL by Metal Cations and Lipoxygenases. The exact process of oxidation of LDL *in vivo* is controversial. Since the discovery of the impact of LDL oxidation in atherosclerosis, metal ion-dependent oxidation of LDL has been extensively used for *in vitro* experiments. Iron (Fe^{3+}) and copper (Cu^{2+}) are the two major metal ions described to catalyze LDL oxidation. Copper had sometimes been preferred due to its ability to bind to apoB-100 and form a complex with it [12]. However, copper cations seem to target the lipid moiety of LDL and not apoB-100 [76]. Recently, Kriško et al. studied the impact of Cu^{2+} on the apoB-100 structure and observed conformational modifications early in the oxidation process, principally in β-sheet regions [77]. Metal ion-dependent oxidation mechanisms assume a high concentration of cations at the site of oxidation, a subject which is controversial [10]. Nevertheless, Stadler et al. quantified both copper- and iron-free cations using a technique that did not release transition metals from proteins during the reaction mechanism [78]. Furthermore, in this study, the authors showed an increase in both copper and iron levels in the intima of lesions compared with healthy controls. In addition, they correlated the iron levels, but not copper levels, with cholesterol levels. Whereas these cations are present, their implication in atherosclerosis remains a point of contention. Indeed, studies have sometimes positively correlated metal ion levels with cardiovascular risk, whereas others have negatively correlated them [79]. Nevertheless, epidemiological studies as well as *in vitro* experiments with iron agree on its potential impact on atherogenesis, whereas copper might be ambiguous [80–82].

Lipoxygenase-dependent LDL oxidation is also a contentious hypothesis because they are intracellular enzymes. However, 15-lipoxygenase mRNA and protein, as well as epitopes of ox-LDL, have been colocalized in human lesions [83, 84]. Lipoxygenase could migrate from the cytoplasm to the membrane surface of macrophages where LDL could be oxidized without phagocytosis/endocytosis of the lipoprotein. Lipoxygenases would be able to promote lipid peroxidation either directly by action on LDL lipids or indirectly by triggering ROS formation and subsequent LDL oxidation [10].

Several groups, like ours, have thus focused their research on the MPO-dependent mechanism of LDL oxidation which might be more physiologically relevant than the copper-dependent oxidation of LDL.

4.2.2. Oxidation of LDL by Myeloperoxidase

Background. The first evidence of MPO implication in atherogenesis was highlighted in 1994 by Daugherty et al. when they observed that MPO was expressed in atherosclerotic lesions [65]. Since then, many clues have arisen such as the fact that fingerprints for *in vivo* modification by the $MPO/H_2O_2/Cl^-$ system of apoB-100 were observed by immunohistological analyses [15] and later confirmed by gas chromatography-mass spectrometry [85, 86]. Other reports also showed that MPO deficiency or low plasma levels of MPO decrease cardiovascular risk in patients [87, 88] strengthening the case that MPO is a key element for oxidative damages in atherosclerosis.

Targets of MPO on LDL and Products of Oxidation. It is important to keep in mind that HOCl is the most abundant product of MPO *in vivo,* and traces of HOCl-modified epitopes have been found in acute and chronic vascular inflammatory diseases such as atherosclerosis [14, 89]. MPO produces HOCl by the enzymatic system $MPO/H_2O_2/Cl^-$. However, to facilitate the experimental scheme, HOCl reactant is commonly used instead of the enzymatic system.

Modification of LDL by HOCl has been studied by different groups, and Malle et al. have recently reviewed the effects of that reactant on LDL [90]. From these works, it clearly appeared that the protein moiety apoB-100 is the major target of HOCl although the production of hydroperoxides, chlorohydrins, chlorinated sterols or fatty acids, and lysophospholipids has also been described in the presence of HOCl [76, 91, 92]. These lipid oxidations occur in strict reaction conditions such as an acidic pH (3–5) and with a large excess of reactant.

Specificity of MPO to Oxidize LDL. As mentioned above, HOCl added as a reactant has been usually used to mimic the $MPO/H_2O_2/Cl^-$ system. However, this model may not be a perfect model to mimic MPO action on LDL/apoB-100. The main reason for this is the fact that MPO rapidly adsorbs at the surface of LDL and seems to have a strong interaction with the protein moiety of LDL [93, 94]. This adsorption phenomenon is due to the cationic characteristic of MPO, and it has been described on lipoproteins and also on endothelial cells [95]. LDL-MPO bound was first shown by Carr et al. who observed a coprecipitation of apoB-100-containing lipoproteins and MPO. The authors demonstrated that lipoprotein-deficient plasma did not permit MPO precipitation, whereas a dose-dependent MPO precipitation was observed by addition of LDL [93]. More recently, Sokolov et al. studied the specificity of MPO to bind different lipoproteins and concluded that MPO binds LDL more avidly and more specifically than HDL [94]. They also demonstrated that ceruloplasmin, which is a human plasmatic protein and a physiological MPO inhibitor [96], is able to inhibit MPO activity when HDL is present but not in the presence of LDL. The same group then studied the binding site for MPO on apoB-100 [97]. The authors predicted that MPO should bind to the NH_2-$\beta\alpha$1 domain of apoB-100 because this domain is exposed on the outside of the lipoprotein. Furthermore, because of the cationic property of MPO, these authors speculated that the MPO binding site on apoB-100 may not include any positively charged residues (lysine or Arginine) but at the opposite include negatively charged ones (aspartic or glutamic acid). They therefore proposed that MPO might bind one of the three following apoB-100 sites: ^{1}EEEMLEN^{7}, ^{53}VELEVPQ^{59}, or ^{445}EQIQDDCTGDED^{456}. They synthesized these three peptides and studied their affinity for MPO. Only the ^{445}EQIQDDCTGDED^{456} peptide was able to form a complex with MPO, and the authors concluded that it might be the binding site of MPO on apoB-100. However, experiments should be performed to confirm

this binding site. Experiments with site-specific mutations on apoB-100 to disable MPO-LDL complex formation would be a good experimental procedure, in this respect and this has previously been done to reveal the binding site of MPO on apolipoprotein A-I of HDL [98]. Furthermore, Carr et al. described MPO : LDL ratio of 3 : 1, whereas Sokolov et al. found a ratio of 1 : 1. The study of MPO-LDL interactions and LDL oxidation by MPO thus remains a challenge for the future.

Oxidation of LDL by MPO-Dependent Species Other than Hypochlorous Acid. Whereas HOCl is the major oxidant formed by MPO, others might also be produced *in vivo*. Among them, tyrosyl radical, nitrogen dioxide, hypobromous acid, cyanate, and hypothiocyanous acid are often mentioned as potential species that may target LDL.

Tyrosine is a substrate of MPO, and tyrosyl radical can be produced via the one-electron reaction of the peroxidase cycle. The latter rapidly oxidizes lipids and also forms dityrosine residues in proteins, and protein-bound dityrosine residues have been identified in atherosclerotic lesions [99].

Nitration of LDL has been also studied in several works. Recently, Hamilton et al. have shown that LDL nitration leads to unfolded protein and deleterious effects [100]. These authors studied the phenomenon using Sin-1 as a peroxynitrite generator for protein nitration, and MPO has also been used as a catalyzer of nitration [101–103]. However, MPO mediates protein nitration via the formation of nitrogen dioxide (NO_2^-) from nitric oxide [104, 105].

MPO can also produce hypobromous and hypothiocyanous acids via the halogenous cycle. The former would be a minor product of MPO activity [49], but several studies have been performed on its reactivity on lipoproteins [106]. The latter work concluded that hypobromous acid attacks both lipid and protein moieties, but it has less deleterious effects than hypochlorous acid. On the other hand, hypothiocyanous formation might be present *in vivo* to a larger extent; however, there are no clear data showing definitive alteration produced by this species [59]. However, cyanate ($^-$OCN), a product of decomposition of hypothiocyanous acid, reacts with the terminal amino group of lysine, forming a carbamylated residue, also named homocitrulline. MPO can also use thiocyanate ($^-$SCN) and produce $^-$OCN [107]. MPO might therefore indirectly result in protein carbamylation, and this phenomenon was observed in lesions and lipoproteins [107, 108]. It is also worth noting that $^-$SCN concentration may change the efficiency of MPO to produce HOCl [59]. These data illustrate how complex the process of LDL oxidation is *in vivo,* and the latter should be the subject of future experiments.

4.2.3. Localization of LDL Oxidation by Myeloperoxidase. The consensus model of atherogenesis describes the first step of the disease as migration of native LDL from plasma to the subendothelial space where it can be oxidized [17]. However, the mechanism and localization of *in vivo* LDL oxidation is still not fully understood. The model of early LDL oxidation in the circulation is often ruled out by the fact that blood contains lots of antioxidant molecules. It is further thought that the presence of ox-LDL in the plasma is due to backdiffusion from lesions. However, evidence has recently emerged to strengthen the possibility of LDL oxidation in the circulation. Our group has shown that LDL can be oxidized at the surface of activated endothelial cells in the presence of MPO. Circulating MPO is indeed known to adsorb on LDL and also on endothelium where this oxidation process could happen when the cells are activated and NADPH oxidase complex produces $O_2^{\bullet -}$. To this end, *in vitro* experiments were performed using endothelial cells (Ea.hy926) which were incubated for 24 h in the presence of native LDL, MPO, and angiotensin II, a modulator of $O_2^{\bullet -}$ production by the NADPH oxidase complex. Mox-LDL production was monitored using a specific Mox-LDL antibody [109] and increased dependently of MPO and LDL concentrations. These data showed that LDL oxidation is possibly not restricted to intima. The plasma level of Mox-LDL is potentially a marker of plasma MPO activity in the field of cardiovascular disease. In this context, we showed that patients exposed to hemodialysis therapy due to kidney failure have higher blood levels of Mox-LDL, and this could be linked to their high cardiovascular risk [110].

To summarize, Figure 2 illustrates a revised scheme of the LDL oxidation by MPO in atherogenesis taking into account the model of oxidation in the circulation.

5. Impacts of Mox-LDL on Inflammation: *In Vitro* Experiments

As mentioned previously in this paper, Mox-LDL is very specific and differs from LDL oxidized by Cu^{2+}. In this context, monoclonal antibodies against Mox-LDL were developed for immunochemical studies. In our research group, several antibodies were generated by immunizing mice and collecting and analyzing clones. Four antibodies were specific for Mox-LDL and did not crossreact with Cu-LDL, LDL oxidized by H_2O_2, or albumin oxidized by the MPO system. Three of these antibodies recognize the protein moiety of LDL (AG948, EB2E9, and EB2G3), and one (14A2G6) is dependent on the presence of the lipid moiety. Furthermore, the three protein-sensitive antibodies appear to be conformation dependent [109]. These antibodies react with atherosclerotic plaques showing that they can be used for immunohistochemistry studies. Other monoclonal antibodies against Mox-LDL have also been developed by other groups [14].

A large number of transcription factors have been observed to be activated by ox-LDL [111], and many of them have particular impacts on the inflammatory effect of atherosclerosis. In the following paragraphs, we report the major effects observed on monocytes, macrophages, and endothelial cells with a particular interest on Mox-LDL induction.

5.1. Effects of Mox-LDL on Monocytes/Macrophages. Atherosclerosis is a complex process involving inflammatory and oxidative stress pathways [112]. Ox-LDL is involved in monocyte/macrophage activation and in the inflammatory

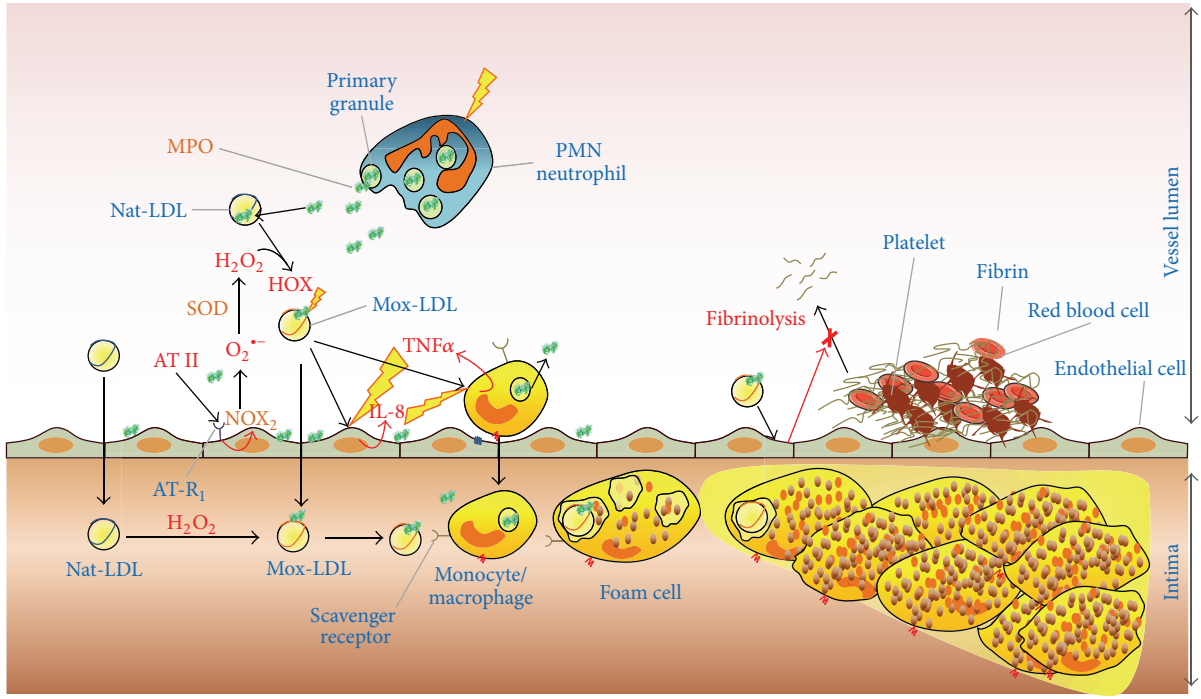

Figure 2: Role of myeloperoxidase and Mox-LDL in triggering inflammation and atherosclerosis plaque formation. Activation of neutrophils and monocytes leads to MPO release in the extracellular space, that is, the circulation. Due to its cationic properties, free MPO rapidly adsorbs at the surface of endothelial cells or native LDL (Nat-LDL). Angiotensin II (AT II) activates endothelial cells via angiotensin receptor 1 (AT-R1), which in turn produces superoxide anion ($O_2^{\bullet-}$) via the NADPH oxidase complex, (NOX2). $O_2^{\bullet-}$ is rapidly transformed into hydrogen peroxide (H_2O_2) spontaneously or by the enzyme superoxide dismutase (SOD). Nat-LDL can be so directly oxidized by MPO/H_2O_2/chloride system in the circulation and form the so-called Mox-LDL. The latter can in turn pass through the endothelium (due to endothelial dysfunction) to the subendothelial space where it will be recognized by macrophages and eliminated. Accumulation of oxidized lipoproteins leads to foam cell formation and lipid accumulation in the subendothelial space. Nat-LDL, can also directly pass through the endothelial wall where they are oxidized by MPO in the subendothelial space. Finally, LDL oxidized by myeloperoxidase (Mox-LDL) activates endothelial cells and induces interleukine-8 (IL-8) secretion by these cells. Mox-LDL effects on monocyte are similar and activate tumor-necrosis factor-α (TNFα) secretion by these cells. In turn, IL-8 and TNFα activate monocytes and endothelial cells, respectively. Mox-LDL also inhibits fibrinolysis process via endothelial cell interaction.

response [113]. Monocytes are one of the first cells that reach the site of inflammation such as in nascent atherosclerotic lesions. When activated, this cell type expresses leukocyte adhesion molecules [114], and it also produces ROS and RNS, partly due to MPO activity, and causes the transformation of LDL into a high-uptake form for macrophages [115–117]. Cu-LDL has the capacity to activate monocytes and increases expression of peroxisome proliferator-activated receptor-γ (PPAR-γ), a regulator of cell proliferation, inflammation, monocyte/macrophage differentiation, and CD36 scavenger receptor expression at the cell surface [118, 119]. In 2005, Westendorf et al. have shown that HOCl-LDL has the same proinflammatory properties *in vitro* [120].

As with Cu-LDL [18], HOCl-LDL inhibits lysosomal proteases (e.g., cathepsin B), but the mechanism was identified as dependent on the chloramine content of apoB-100 and oxidized residues that are not present in Cu-LDL [121]. This protease inhibition contributes to lipid accumulation in macrophages and to foam cell transformation.

Furthermore, both Cu- and HOCl-LDL are potent inducers of caspase-dependent apoptosis as shown by Vicca et al. [122] on THP-1 monocytes cell. However, macrophage-differentiated cells seemed to be resistant to apoptosis in these experiments. Nevertheless, this effect is compatible with the idea that macrophages have a prolonged survival and boost atherogenesis.

Considering the literature of LDL oxidation and cell inflammatory processes, studies of ox-LDL effects on monocytes/macrophages have been mainly performed using Cu-LDL, whereas HOCl-LDL and MPO-LDL are more rarely used. We recently investigated Mox-LDL impacts on a THP-1 cell line and observed an intriguing result [123]. Incubation of Mox-LDL with THP-1 cells during 4 h increased 2-folds the secretion of TNFα (a key regulator of the synthesis of acute-phase proteins (i.e., fibrinogen, factor VIII) that are linked to atherogenesis [124]), whereas no increase was detected for native LDL or native and Mox-albumin. These data highlighted the specificity of Mox-LDL as MPO-oxidized proteins did not induce TNFα production. TNFα is itself an activator of other cells such as endothelial cells where it induces among other things the expression of VCAM-1 [125]. We will return to discuss this activation later in this paper in the context of endothelial cell activation by Mox-LDL.

More recently, macrophage reactivity to ox-LDL was investigated by comparing Mox-LDL and Cu-LDL on RAW264.7 cells [126]. This cell line is usually used for metabolic studies with the advantage of interaction with ox-LDL and to induce foam cell formation [127–130]. Cells were incubated with native and ox-LDL for 48 h before analysis. The first set of experiments highlighted that accumulation of lipids is higher in the presence of Mox-LDL than Cu-LDL (Figure 1 of [126]). The same trend was confirmed with macrophages derived from peripheral blood mononuclear cells (PBMCs) differentiated by macrophage-colony stimulating factor. In a second set of experiments, ROS production was explored by monitoring fluorescence of dichloro-dihydro-fluorescein when RAW264.7 and PBMC-derived macrophages were exposed to ox-LDL. Whereas both Mox- and Cu-LDL significantly increased ROS accumulation in RAW264.7, only Mox-LDL seemed to increase this accumulation in PBMC-derived macrophages (Figure 2 of [126]).

In order to combat an excess of ROS, cells have developed several mechanisms. In this context, NF-E2-related factor 2 (Nrf2) is a transcription factor which is upregulated when ROS is increased. In redox homeostatic conditions, Nrf2 is inactive and kept in the cytoplasm bound with a Keap1/Rbx1/Cul3 complex, which promotes its ubiquitination and subsequent degradation by the 26S proteasome. When activated, Nrf2 binds DNA at the "antioxidant response element" and regulates the expression of protective genes. The regulatory subunit of glutamate-cysteine ligase (Gclm) and hemeoxygenase-1 (HO-1) are two examples of Nrf2-regulated genes [131]. Glcm is the limiting component of glutathione production, a strong *in vivo* antioxidant, and HO-1 is responsible for hemedegradation in cells, decreasing potential oxidant formation by heme-enzymes. In the same study, Calay et al. [126] showed that Mox-LDL and Cu-LDL result in overexpression of Gclm and HO-1 induction by Nrf2-dependent activation. However, Mox-LDL triggered a stronger response than Cu-LDL. In addition, Trolox, a well-known water-soluble antioxidant able to quench ROS production, was added to experimental batches in order to confirm that Nrf2 was induced by ROS accumulation. Addition of Trolox led to reduced Nrf2 expression. However, while ROS production was totally inhibited by Trolox, Gclm and HO-1 expression was still higher than basal level, suggesting the implication of other pathways in their overexpression. RNA interference approaches targeting Nrf2 gave the same result of partial abolition of Gclm and HO-1 expression and confirmed the hypothesis that transcription factors other than Nrf2 are implicated in the antioxidant response of macrophages.

Differences observed between Cu- and Mox-LDL could be explained by the fact that their ROS induction is mediated by different pathways. ROS production via NADPH oxidase is activated by both ox-LDL, but only Mox-LDL induced the production of ROS by cytosolic phospholipase A2. This was illustrated by quantifying ROS production in the presence of methylarachidonylfluorophosphonate, an inhibitor of cytosolic phospholipase A2. A 43% decrease was observed in ROS production induced by Mox-LDL where no decrease was observed for Cu-LDL.

Interestingly, when Mox-LDL was generated by a stronger MPO-dependent oxidative process, which is capable of extending the oxidation of LDL to the lipid moiety, the ROS production was decreased but remained higher than Cu-LDL induction. These data suggest that lipid peroxidation levels of ox-LDL could be inversely correlated to ROS production in macrophages.

In summary, Mox-LDL induces ROS production, lipid accumulation, and antioxidant responses in macrophages as with other ox-LDL but by using a different pathway than Cu-LDL. However, Mox-LDL seems to induce a higher responsiveness in monocytes/macrophages than ox-LDL and might be more atherogenic.

5.2. Effects of Mox-LDL on Endothelial Cells. Endothelial dysfunction is potentially the first event of atherosclerosis development. It is still not totally understood why this occurs and when these lesions start, but evidence has been claimed many times. Endothelial permeability is increased at the site of lesions and favors LDL penetration into the artery wall. Furthermore, ox-LDL mediates endothelial dysfunction and is considered as a key event in the initiation of arterial lesions [132].

Endothelial cells also express scavenger receptor (SR) at their surface and can interact with ox-LDL. However, the main SR expressed on endothelial cells is LOX-1, the specific lectin-like endothelial receptor for ox-LDL. However, CD36 and SR-B1 have also been localized at the endothelial cell surface [133]. HOCl-LDL is internalized by CD36 and SR-B1 [134], while the receptor(s) which recognize(s) Mox-LDL and enable(s) endocytosis remain(s) to be documented for monocytes, macrophages, and endothelial cells. The presence of scavenger receptors at the surface of endothelial cells could lead to endocytosis of Mox-LDL. However, endothelial cells are not able to accumulate lipids when incubated with ox-LDL. Nevertheless, Mox-LDL activates endothelial cells, as well as monocytes/macrophages.

Cu-LDL but not native LDL is able to induce interleukin-8 (IL-8) production by endothelial cells [135]. IL-8 belongs to the C-X-C subgroup of chemokines and is a multifunctional cytokine involved in numerous biological processes including atherosclerosis. IL-8 acts as a chemoattractant to inflammatory cells and also to smooth muscle cells and is involved in the migration of the latter in the intima. Furthermore, IL-8 activates monocytes and/or macrophages and up-regulates their production of TNFα. HOCl-LDL induction of IL-8 was also demonstrated but only in monocytes [136].

In order to assess whether Mox-LDL is also an inducer of IL-8 production by endothelial cells, our group performed an experiment where Mox-LDL was incubated with EAhy926 endothelial cells for 48 h [123]. This cell line is a reliable model for studying vascular inflammation, leukocyte-endothelial interactions, and metabolic impacts of ox-LDL [135]. IL-8 was measured in the supernatant, and a dose-dependent response was observed. No response was detected with native LDL and native or Mox-albumin (control experiments). The specificity of Mox-LDL was also confirmed by the absence of a Mox-albumin effect.

With the capability of Mox-LDL to trigger both monocytes and endothelial cells, it appeared that a cycle could be present. Indeed, Mox-LDL activated both monocytes and endothelial cells which secrete TNFα and IL-8 respectively. The latter activates endothelial cells and monocytes/macrophages leading (i) to ROS production, (ii) to MPO release in the extracellular matrix, and (iii) so potentially to new Mox-LDL.

5.3. Mox-LDL and Fibrinolysis. Coagulation and fibrinolysis are continuously in balance at the surface of the endothelial cell wall. These cells contribute to fibrinolysis by secreting tissue-plasminogen activator (t-PA), urokinase-plasminogen activator (u-PA), and plasminogen activator inhibitor-1 (PAI-1), three fibrinolysis regulators, or by expressing specific receptors which bind these fibrinolysis factors [137]. Enhancement of fibrin generation gives a prothrombotic environment on the endothelial cell surface, and fibrin induces the production of IL-8 by endothelial cells. In this context, a dysfunctional fibrinolysis process was reported to be a factor in atherogenesis, complementary to ox-LDL [138]. This was first mentioned as a key factor in 1998 by Sueishi et al. [139] and Mayerl et al. [140], and a recent clinical study from our group further confirmed it [141]. However, until 2012, the interplay between Mox-LDL (and more generally ox-LDL), endothelial cells, and fibrinolysis had not been investigated [142]. Our group documented this intriguing subject with the aid of a device which allows us to monitor fibrinolysis in real time [143]. Briefly, fibrin formation and degradation occurs in adapted circular microcuvettes. To monitor the effect of endothelial cells (EA.hy926) on fibrinolysis taking place at their surface, cells were immobilized on collagen-coated membranes, fixed to the bottom of glass circular microcuvettes, and grown to confluence. The microcuvettes were inserted in the experimental apparatus at 37°C, the euglobuin fraction was added and clot formation started by addition of thrombin. TNFα was used as a positive control as it is known to have antifibrinolytic activity. To test the system, a 24 h TNFα treatment of endothelial cells was performed and effectively showed an increase in fibrinolysis time. Monitoring of native and Mox-LDL showed that Mox-LDL at concentrations of 10 and 50 μg/mL also increased the time of fibrinolysis unlike native lipoprotein, confirming again that Mox-LDL has a physiopathological effect on atherogenesis. Nevertheless, higher concentrations of Mox-LDL (100 μg/mL) showed a decreased effect.

PAI-1, t-PA, annexin II (a t-PA receptor), and uPAR secretion were also analyzed in this study, but there was no effect of native or Mox-LDL. However, as TNFα increased t-PA and PAI-1 in smooth muscle cells, it is suggested that other pathways/factors are involved in fibrinolysis modulation. This raises the question whether the receptor and signal transduction pathways activated by Mox-LDL and TNFα are different. With a view to clarify the underlying biomolecular mechanism, scavenger receptor interactions with Mox-LDL were investigated. Previous data described LOX-1 binding to ox-LDL and mediating effects in endothelial cells. However, our first investigations by neutralization of this receptor using antibodies did not impact IL-8 production induced by Mox-LDL, disproving this pathway [142]. Hence, future research on scavenger receptor is required to extend the understanding of Mox-LDL effects on endothelial cells.

In summary, Mox-LDL disturbs fibrinolysis but in a different pathway than the t-PA- and PAI-1 dependent pathways. Future investigations are needed to solve the mechanism by which Mox-LDL is involved in this pathological process.

5.4. Summary of In Vitro Effects of Mox-LDL. It appears that Mox-LDL plays a crucial role in lipid accumulation in macrophages/foam cells and also in the whole proinflammatory process linked to atherosclerosis lesion development. Figure 2 summarizes the different aspects and effects of MPO/Mox-LDL in the circulation/intima including effects on endothelial cells, monocytes/macrophages, and fibrinolysis. Mox-LDL generates a vicious circle effect, prevents resolution of the nascent lesion, triggers oxidative stress and lipid accumulation in the subendothelial space, and inhibits normal fibrinolysis.

6. Clinical Aspects of Mox-LDL

6.1. Introduction. For a long time, ox-LDL and MPO have been accepted as cardiovascular risk factors and have been largely documented in the literature by *in vivo* experiments or clinical studies [17, 63, 144–146]. However, only a small number of studies have investigated LDL modified by the MPO/H_2O_2/chloride system (Mox-LDL). Our group has contributed to this, and we observed that Mox-LDL is present in atherosclerotic lesions [109]. We have already discussed numerous Mox-LDL effects in atherosclerosis plaque formation in this paper and this subject is not further discussed here. Nevertheless, cardiovascular diseases are linked, notably in relation to atherosclerosis development, to several pathologies such as kidney failure/end stage renal disease, sleep restriction, erectile dysfunction, or chronic obstructive pulmonary disease. Evidences have been growing for Mox-LDL implications in these pathologies, and they are summarized in the following paragraphs.

6.2. Mox-LDL, Kidney Failure, and Hemodialysis. Kidney failure and subsequent uremia has been linked to chronic inflammation and cardiovascular disease [147]. It has also been proposed that hemodialysis triggers inflammation as a result of exposure of blood to the bioincompatible system stimulating monocyte and macrophage cells [148, 149]. These processes induce proinflammatory oxidative stress responses, and MPO has been implicated in the development of cardiovascular and chronic kidney diseases [88, 150, 151]. MPO also directly targets kidneys through HOCl production [152, 153].

Wu et al. reported [154] that MPO concentration could serve as a marker of oxidative stress during hemodialysis, and Himmelfarb et al. [155] showed that MPO concentration increases during hemodialysis sessions. We also recently contributed to MPO investigations in the context of hemodialysis therapy [156]. In this paper, MPO activity was monitored using the SIEFED (specific immunoextraction followed

by enzymatic detection) method developed previously by Franck et al. [157]. We further developed a total protein hydrolysis method assisted by microwave and coupled to LC-MS analyses devoted to the monitoring of protein-bound 3-chlorotyrosine and homocitrulline. In this work, it was observed in 15 patients that an increase of plasma MPO, levels during the hemodialysis, is accompanied (i) by a direct increase of MPO activity and, more interestingly, (ii) by a direct MPO-dependent oxidation of plasma proteins.

Previously, we had shown that Mox-LDL levels in blood are increased during hemodialysis [110]. Together with the recent results, these data highlight that MPO induces direct protein oxidation and potentially targets LDL. Indeed, MPO avidly interacts and adsorbs at the surface of LDL and triggers apoB-100 oxidation with an impact on the cardiovascular risk of the patients [156]. Furthermore, these data strengthen the model of the MPO-dependent oxidation of LDL in the circulation as a contributive mechanism of atherosclerosis. The latter process is also illustrated in Figure 2.

6.3. Mox-LDL and Sleep Restriction. Nowadays, people are more and more sleep deprived due to work pressure and requirement (i.e., shift schedules or extension of hours), family demands, or our 24/7-week lifestyle [158]. According to the 2009 National Sleep Foundation Survey, 20% of Americans sleep less than 6 h per night during the week [159]. This modification of sleep duration is not of minor consequence to our health. Sleep deprivation is harmful and can lead to problems in metabolism [160], immune [161], or cardiovascular systems [162, 163].

Studying sleep-deprived individuals, van Leeuwen et al. have associated the increase of proinflammatory molecules IL-17, C-reactive protein (CRP), IL-1β, and IL-6 with cardiovascular risk [164]. Furthermore, total or severe sleep restriction alters blood cell counts with a particular increasing effect on neutrophils and granulocytes [165, 166]. Neutrophil count has even been proposed as a marker of immune recovery function of sleep [167]. An increase in MPO plasma levels was also detected after acute sleep restriction [166].

In this context, MPO and Mox-LDL levels were recently studied, together with inflammatory markers in 9 mid-twenties men for 11 consecutive days (3 baseline nights followed by 5 restricted-sleep nights (max. 5 hours of sleep) and then 3 recovery-sleep nights) [168]. Results showed that MPO was not increased during the sleep restriction but rose during the first night of recovery sleep. Whereas MPO levels peaked during the first recovery night, Mox-LDL levels were significantly higher during the first and third nights of sleep restriction but not at the recovery period. Mox-LDL/apoB-100 ratio, which expresses the fraction of modified lipoproteins in the total pool, was also statistically increased during the first night of sleep restriction. Speculating on the reason for this temporal discordance between Mox-LDL and MPO levels in blood, it appears from the literature that catecholamines are increased during sleep restriction [169] and can activate the NADPH oxidase complex at the surface of endothelial cells [170]. As a result, the $O_2^{-\bullet}$ formed could be converted into H_2O_2 and react with MPO to form Mox-LDL.

In summary, these data show that the recovery process after sleep restriction is linked to modifications of levels of cardiovascular risk biomarkers in blood. However, future experiments are required to help understand the impact of sleep restriction on human health. Studies including more male and female individuals are also needed but are unfortunately difficult to set up and standardize.

6.4. Mox-LDL and Nonalcoholic Fatty Liver Disease. Nonalcoholic fatty liver disease (NAFLD) includes different liver disorders such as steatosis, steatohepatitis, cirrhosis, and advanced fibrosis. Furthermore, NAFLD has been associated with a high risk of cardiovascular disease (CVD) [171], obstructive sleep apnea [172], or colorectal cancer [173]. MPO has been involved in the progression of non-alcoholic steatohepatitis where neutrophil accumulation is a key component of the inflammatory process. Moreover, it has been shown that NAFLD is associated with increased levels of nitrated proteins that might partly come from MPO activity [174]. More recently, Rensen et al. reported that MPO deficiency decreases hepatic cholesterol accumulation and inflammation in mice that do not express the LDL-receptor ($LDLR^{-/-}$ mice) and that were fed with a high-fat diet, [175]. In these experiments, the authors also observed that, after 3 weeks of high-fat diet, MPO levels were increased in the liver of hyperlipidemic mice that expressed MPO. Furthermore, MPO activity in mouse liver was investigated by monitoring nitrotyrosine levels. The latter product is indeed, at least partially, generated by MPO during the inflammation process. These data demonstrate the important role of MPO in NAFLD, and this might be linked to the oxidation of lipoproteins and particularly of LDL by MPO.

6.5. Mox-LDL and Chronic Obstructive Pulmonary Disease. Patients with chronic obstructive pulmonary disease (COPD) have increased systemic inflammation, increased endothelial dysfunction, and changes in the oxidant/antioxidant ratio. Furthermore, these patients are at a high risk of cardiovascular disease, and 22%–50% of them will die of cardiovascular conditions [176–178]. Long-term oxygen therapy has been observed to prolong survival in hypoxemic COPD patients, but the mechanisms are not completely understood.

In this context, we hypothesized that oxygen therapy could alter systemic inflammation and oxidative stress. As a consequence, several markers, including neutrophils, Mox-LDL, and IL-8, were monitored in 11 patients before starting, after one week and after one month of oxygen therapy [179]. Neutrophils, IL-8, and Mox-LDL were all significantly decreased after one month of oxygen therapy. These data showed that oxygen breathing is favorable to reduce the oxidative stress and inflammatory state in hypoxemic patients with COPD.

At this point only, speculation could explain these observed decreases. In this way, chronic hypoxia is associated with raised sympathetic activity, activation of the renin-angiotensin system, and production of catecholamines [180]. By activating the NADPH oxidase complex, catecholamines

can induce Mox-LDL formation in the circulation. Conversely, when oxygen therapy is provided, catecholamine formation is reduced, which could decrease Mox-LDL formation. Reduction of the sympathetic activity might also decrease neutrophil counts in blood and act on MPO level. Finally, IL-8 released by endothelial cells is also induced by Mox-LDL *in vitro* and suggests that Mox-LDL decrease could partly be explained by the reduction in IL-8 secretion.

6.6. Mox-LDL and Erectile Dysfunction. Erectile dysfunction (ED) is a vascular disorder. Indeed, erection and detumescence of the penis are hemodynamic events controlled by the relaxation and contraction, respectively, of arterial and intracavernous smooth muscle cells. Erectile function is also subject to endothelial cells and their ability to release nitric oxide ($NO^{\bullet}$). $NO^{\bullet}$ is indeed the main neurotransmitter involved in erection and reacts with the enzyme guanylate cyclase that increases cyclic guanosine monophosphate and produces a cascade of events at the intracellular level. This process results in a loss of contractile tone of smooth muscle cells [181, 182].

Clinical studies have identified a link between hypercholesterolemia, atherosclerosis, and erectile dysfunction. ED has been even described as a preliminary event of future major cardiovascular outcomes and as a predictor of coronary heart disease [183–186]. Furthermore, it was reported that HOCl-LDL inhibits $NO^{\bullet}$ synthesis in endothelium [187], and we previously reported effects of Mox-LDL on endothelial cells ([123] and see above: effects of Mox-LDL on endothelial cells).

On this basis, our group has performed an immunohistochemical approach to study the presence of Mox-LDL in penile tissues in patients suffering from ED [188]. Intracavernous tissue was taken from 8 patients undergoing penile implant surgery, and an antibody against Mox-LDL [109] was used to reveal the presence of MPO-dependent oxidized LDL. Among these patients, 7 were known to have vascular ED, and one patient had ED due to neurologic lesions after radical prostatectomy. In the 7 patients with vascular ED, the presence of Mox-LDL was observed, whereas no Mox-LDL was observed in the patient with ED due to prostatectomy. The latter was a good negative control and these data confirmed that, when ED is due to vascular dysfunction, LDL is oxidized and this phenomenon could trigger ED development.

In addition, careful observation of stained slides revealed that Mox-LDL is restricted to the endothelium and subendothelial space in artery, but, conversely, they are deeply diffused and intermingled between the smooth muscle fibers in the intracavernous tissues. Furthermore, Mox-LDL staining revealed the presence of Mox-LDL in the cytoplasm of endothelial cells. This confirms the endocytosis of these lipoproteins into cells.

As cyclic guanosine monophosphate (cGMP) is a key mediator in the $NO^{\bullet}$-dependent pathway of erectile function, it was hypothesized that cGMP levels could be influenced by the presence of Mox-LDL. We showed *in vitro* that a 48 h incubation of Mox-LDL with EA.hy926 endothelial cells induces a decrease of the level of cGMP when compared with control and native LDL [189]. In the cavernosum tissue, cGMP is naturally hydrolyzed by phosphodiesterase 5 (PDE5). Inhibition of this enzyme is considered a first-line therapy for patients with ED and helps maintain higher levels of cGMP [190–192]. Thus, Mox-LDL presence in penile tissue could be an explanation for the resistance of patients to PDE5 inhibitor therapy [189]. Conversely, PDE5 inhibitors were tested to know whether they protect or not against the proinflammatory effects of Mox-LDL on endothelial cells. For this purpose, EA.hy926 endothelial cells were incubated with Mox-LDL and available PDE5 inhibitors (sildenafil, vardenafil and tadalafil), and IL-8 production was then measured [193]. Only one of the PDE5 inhibitors (tadalafil) was shown to have a beneficial effect *in vitro* by significantly decreasing IL-8 production compared with the other two inhibitors. A complementary effect on endothelial cells (in addition to the relaxation) could be also produced by tadalafil. This could potentially be an interesting effect that could be considered in the future when a physician implements a chronic treatment for ED. However, clinical data are required to confirm the *in vitro* experiments before drawing further conclusions.

7. Concluding Remarks and Future Perspectives

In summary and conclusion, *in vitro* experiments and clinical studies support a key role of MPO-dependent oxidized LDL in the process of atherosclerosis and cardiovascular linked diseases. This specifically modified lipoprotein induces proinflammatory effects by stimulating TNFα and IL-8 secretion in monocytes and endothelial cells respectively. In turn, TNFα and IL-8 activate endothelial cells and monocytes, respectively, and promote MPO and ROS release leading to new Mox-LDL formation. Furthermore, Mox-LDL has exhibited inhibitory effects on fibrinolysis, a key process in the release of fibrin.

In vivo, Mox-LDL is present in atherosclerotic lesions, and its level is increased in the circulation of patients with high cardiovascular risk, such as those with kidney failure and those undergoing hemodialysis. The presence of Mox-LDL was also revealed in patients suffering from vascular erectile dysfunction, disease linked to atherosclerosis and endothelial dysfunction. In addition, increased blood levels of Mox-LDL have also been observed during sleep restriction. Mox-LDL is thus a potentially good marker of cardiovascular disorders and/or cardiovascular risk.

In contrast to other types of oxidation (e.g., by copper), MPO-dependent oxidation of LDL primarily targets the protein moiety of LDL, namely, apolipoprotein B-100. Furthermore, MPO oxidation is thought of as a more physiopathological model of LDL oxidation than that involving copper. As Mox-LDL induces much more ROS production and lipid accumulation in macrophages than Cu-LDL, Mox-LDL in particular should be considered more in biological experiments and clinical studies.

Challenges thus remain for the future, and researchers should keep working on the impact of MPO and Mox-LDL on human health. As an example, the exact receptors that

enable Mox-LDL endocytosis in macrophages/monocytes and endothelial cells should be further studied. Last but not least, it has been observed that MPO specifically oxidizes apoB-100, but the exact binding sites of MPO at the surface of apoB-100 remain to be further described, as well as the residues oxidized on apoB-100. Increased understanding of the impact of Mox-LDL on the induction of proinflammatory and oxidative stress processes is also of major importance for the future.

Abbreviations

ApoB-100:	Apolipoprotein B-100
Cu-LDL:	Low-density lipoprotein modified by reagent Cu^{2+}
ED:	Erectile dysfunction
H_2O_2:	Hydrogen peroxide
$HOCl/OCl^-$:	Hypochlorous acid/hypochlorite
HOCl-LDL:	Low-density lipoprotein modified by reagent HOCl
IL:	Interleukin
LDL:	Low-density lipoprotein
M-CSF:	Macrophage colony stimulating factor
Mox-albumin:	Albumin modified by the MPO-H_2O_2-chloride system
Mox-LDL:	LDL modified by the MPO-H_2O_2-chloride system
MPO:	Myeloperoxidase
MS:	Mass spectrometry
NADPHox:	Nicotinamide adenine dinucleotide phosphate oxidase
Ox-LDL:	Oxidized LDL
PTM:	post-translational modification
RNS:	reactive nitrogen species
ROS:	reactive oxygen species
TNFα:	tumor-necrosis factor-α.

Authors' Contribution

Cédric Delporte and Pierre Van Antwerpen equally contributed to this paper.

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Research Article

Ox-LDL Promotes Migration and Adhesion of Bone Marrow-Derived Mesenchymal Stem Cells via Regulation of MCP-1 Expression

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Bone marrow-derived mesenchymal stem cells (bmMSCs) are the most important cell source for stem cell transplant therapy. The migration capacity of MSCs is one of the determinants of the efficiency of MSC-based transplant therapy. Our recent study has shown that low concentrations of oxidized low-density lipoprotein (ox-LDL) can stimulate proliferation of bmMSCs. In this study, we investigated the effects of ox-LDL on bmMSC migration and adhesion, as well as the related mechanisms. Our results show that transmigration rates of bmMSCs and cell-cell adhesion between bmMSCs and monocytes are significantly increased by treatments with ox-LDL in a dose- and time-dependent manner. Expressions of ICAM-1, PECAM-1, and VCAM-1 as well as the levels of intracellular Ca^{2+} are also markedly increased by ox-LDL in a dose-dependent manner. Cytoskeleton analysis shows that ox-LDL treatment benefits to spreading of bmMSCs and organization of F-actin fibers after being plated for 6 hours. More interestingly, treatments with ox-LDL also markedly increase expressions of LOX-1, MCP-1, and TGF-β; however, LOX-1 antibody and MCP-1 shRNA markedly inhibit ox-LDL-induced migration and adhesion of bmMSCs, which suggests that ox-LDL-induced bmMSC migration and adhesion are dependent on LOX-1 activation and MCP-1 expression.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that can differentiate into several types of cells, including osteocytes, adipocytes, chondrocytes, endothelial cells, cardiomyocytes, and neurons when exposed to appropriate conditions [1, 2]. Bone-marrow derived MSCs (bmMSCs) are the most widely used MSCs in tissue regenerative medicine. It has been reported that bmMSC transplantation has therapeutic benefits to many kinds of diseases such as Alzheimer's disease, heart infarction, stroke, and rheumatoid arthritis [3–6]. The migration capacity of bmMSCs is the most important determinant of the efficiency of bmMSC transplant therapy. It has been shown that less than 1.5% bmMSCs can reach the injured tissues after intracoronary injection [7]. The low homing rate of bmMSCs after transplantation severely limits their clinical uses. Another limitation of bmMSC transplant therapy is the poor viability of bmMSCs after transplantation [8]. Cell adhesion is a prerequisite for the survival of the transplanted bmMSCs and is also responsible for bmMSC migration [8–10].

Intracellular Ca^{2+} is an important regulator of cell adhesion and migration. The increase of intracellular Ca^{2+} is required for integrin-mediated cell adhesion [11, 12]. Intracellular Ca^{2+} also participates in regulating organization of cytoskeleton [13]. The dynamic rearrangement of cytoskeleton is required for cell adhesion and migration. Ox-LDL is an important stimulator for inflammation and cell adhesion. Previous studies have shown that ox-LDL induces migration of monocytes and smooth muscle cells [14]. A recent study from our group showed that LOX-1, a receptor of

ox-LDL, is highly expressed in bmMSCs, and its activation by ox-LDL stimulates proliferation of bmMSCs [1]. Actually, LOX-1 itself also serves as an inflammatory and adhesive molecule, and it is involved in migration of leukocytes [15].

Monocyte chemoattractant protein-1 (MCP-1) is an important regulator of the genesis of acute and chronic inflammation. It plays a key role in monocyte activation and recruitment to the injured sites. Previous studies have shown that MCP-1 mediates transmigration of monocytes and THP-1 cells [16]. It has been reported that ox-LDL through activating LOX-1 enhances MCP-1 expression in the cultured chondrocytes, vascular smooth muscle cells, endothelial cells, and macrophages [17–21]. The ox-LDL-mediated MCP-1 upregulation has been involved in expression of adhesion molecules in endothelial cells [19, 20]. Whether ox-LDL affects bmMSC migration and adhesion and MCP-1 expression in bmMSCs has not been examined. In the present study, we investigated the effects of ox-LDL on bmMSC migration and adhesion, as well as their possible mechanisms.

2. Materials and Methods

2.1. Materials. Ox-LDL and Dil-ox-LDL were purchased from Biomedical Technologies, Inc. (Stoughton, MA, USA). Fluo-3/AM, Rhodamine phalloidin, Lipofectamine LTX kit, RNeasy Mini-Kit, SuperScript II 1st-strand DNA synthesis kit and cell tracker were obtained from Invitrogen (Carlsbad, CA, USA). LOX-1 and MCP-1 antibodies were purchased from Abcam (Cambridge, MA, USA); TGF-β, ICAM-1, PECAM-1, VCAM-1, and β-actin antibodies were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA, USA). MCP-1 shRNA kit was purchased from OriGene Technologies (Rockville, MD, USA). ECL Western-blotting substrate was purchased from Thermo Scientific (Rockford, IL, USA). The PVDF membrane was purchased from GE Healthcare (Pittsburgh, PA, USA).

2.2. Isolation and Culture of bmMSCs. BmMSCs were isolated and cultured as previously described [1]. Mice (C57BL/6J, 8-week old) were killed by cervical dislocation. The animals were rinsed in 70% ethanol for 20 seconds to make the bodies sterile, and then the limbs were collected by surgery and put in DMEM medium on ice. After cleaning the muscles, the tibia and femur were cut just below both ends of the marrow cavities. The bone marrow was flushed out using DMEM medium in a 10 mL syringe with a 25-gauge needle and collected in a 15 mL tube on ice. After centrifugation, bone marrow was suspended in DMEM by pipetting several times and filtered through a 70 mm filter mesh to remove the bone spicules and cell clumps. The cell density was calculated by cell counting under a microscope. Then, the cells were plated into 100 mm Petri dishes at the densities of 10×10^6/mL in complete DMEM medium with 15% FBS, 2 mM L-glutamine, 100 μg penicillin, and 100 μg streptomycin, and they cultured for 3 h. After 3 h, the nonadherent cells were removed, and the fresh medium was replaced. Thereafter, the medium was replaced every 2 days. A purified population of bmMSCs can be obtained after 3-week cultureing period.

2.3. Dil-ox-LDL Uptake Measurement. The primary and the 3rd-passage bmMSCs were plated in 24-well plates and incubated with 5 μg/mL Dil-ox-LDL in the dark at 37°C for 30 min. Then, the cells were gently washed with PBS for 3 times, and they were imaged with a fluorescent microscope.

2.4. Transwell Migration Assay. In this study, migration of bmMSCs was measured using Transwell plates (Corning Costar, USA) with 8 μm pore filters. In brief, human umbilical vein endothelial cells (HUVECs) were seeded into the upper inserts of Transwell chamber (4×10^4 cells/well), and they cultured for 24 h. BmMSCs were treated with 0, 5, 10, and 20 μg/mL ox-LDL for 6 h or treated with 10 μg/mL ox-LDL for 0, 3, 6, and 12 h, and then they were washed with PBS. The washed cells (1×10^5) were plated onto HUVECs in the upper inserts of Transwell plates. After 6 h of coculture, the numbers of migrated bmMSCs on the lower side of the filters were counted.

2.5. Cell Adhesion Assay. BmMSCs were plated in 12-well plates. Monocytes were darkly preincubated with cell tracker at 37°C for 30 min and washed with PBS for 3 times. When bmMSCs were nearly 80% confluent, they were incubated with 0, 5, 10, and 20 μg/mL ox-LDL for 6 h. Then, the predyed monocytes (2×10^4) were seeded onto bmMSCs (washed with PBS) and coincubated for 30 min in the dark. And then, the cells were gently washed with PBS for 3 times and randomly imaged with a fluorescence microscope.

2.6. RT-PCR Assay. In this study, LOX-1 expression in bmMSCs was measured by RT-PCR assay. In brief, total RNA was isolated from bmMSCs using RNeasy Mini-Kits according to the kit's instructions; 1 μg RNA was applied to synthesize cDNA with SuperScript II 1st-strand DNA synthesis kits. PCR assay was performed using a 20 μL reaction volume containing 100 ng cDNA, 10 μL 2× PCR reaction mixture, and 0.5 μM primers. The products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide on a UV transilluminator. The primers for LOX-1 were the following: forward: 5′-GAGCTGCAAACTTTTCAGG-3′, reverse: 5′-CTCTTTCATGCGGCAACAG-3′; the primers for β-actin were the following: forward: 5′-TTCTTTGCAGCCCTTCGTTGCCG-3′, reverse: 5′-TGGATGGCTACGTACATGGCTGGG-3′.

2.7. Western-Blotting Assay. Proteins were extracted from bmMSCs and separated by 12% SDS-PAGE. After electrophoresis, proteins were transferred to the PVDF membranes. The membranes were blocked with 5% BSA or 5% no-fat milk (according to the manufacturer's instructions) in TBS-T, and they were then incubated with LOX-1, MCP-1, TGF-β, ICAM-1, VCAM-1, PECAM-1, and β-actin (1 : 2000) primary antibodies at 4°C overnight. Then, the blots

were incubated with HRP-conjugated secondary antibodies (1 : 10000) for 1 h at room temperature. The immunoreactive bands were visualized by enhanced chemiluminescence.

2.8. Immunofluorescence Staining. Immunostaining was performed using standard protocols. In brief, the bmMSCs grown on 10 mm round coverslips were treated with 0, 5, 10, and 20 μg/mL ox-LDL for 6 h. Then, the cells were fixed with 4% buffered paraformaldehyde for 15 min and treated with 0.1% Triton X-100 for 10 min at room temperature. And then, the cells were blocked with 1% BSA for 1 h and incubated with rabbit anti-mouse ICAM-1, VCAM-1, and PECAM-1 antibodies (1 : 200) for 1 h at room temperature. After washing with PBS, the cells were incubated with TR- or FITC-conjugated duck anti-rabbit secondary antibody (1 : 1000) in the dark. After washing, the cells were mounted on slides using ProlongH Gold antifade reagent with DAPI and imaged with a fluorescence microscope. Fluorescent density of ICAM-1, VCAM-1, and PECAM-1 was measured using Image J 1.34 software in several random fields. The average fluorescent density was calculated from 100 cells of each sample.

2.9. Flowcytometry Assay. In this study, intracellular Ca^{2+} of bmMSCs was measured by flowcytometry assay. Briefly, bmMSCs were plated in 6-well plates and treated with 0, 5, 10, and 20 μg/mL ox-LDL for 6 h. Then, the cells were loaded with 5 μM Fluo-3/AM and darkly incubated for 30 min at 37°C. The cells were collected and washed with PBS for 3 times. The washed cells were resuspended in 500 μL PBS and analyzed with a flowcytometer.

2.10. Cytoskeleton Analysis. BmMSCs were plated in 24-well plates and immediately exposed to ox-LDL. After 6 h exposure, the cells were fixed using with 4% buffered formaldehyde, treated with 0.1% Triton-X-100, and then labeled with 2U Rhodamine phalloidin for 30 min in the dark. After washing for 3 times, fluorescence was imaged with laser-inverted confocal microscope.

2.11. MCP-1 shRNA. BmMSCs were plated in 6-well or 12-well plates. When the cells reached 80% confluence, shRNA was performed using Lipofectamine 2000 in Opti-MEM medium and a CCL2 (MCP-1) shRNA kit including CCL2 shRNA duplexes and noneffective 29-mer scrambled shRNA according to the kit's instruction.

2.12. Statistical Analysis. Statistical analysis was performed with SPSS 11.5 software. Data were presented as the mean ± standard deviation (SD). Univariate comparisons of means were evaluated using appropriate Student's t-tests and/or one-way ANOVA with Tukey's post hoc adjustment for multiple comparisons; $P < 0.05$ was considered a statistically significant difference.

3. Results

3.1. Dil-ox-LDL Uptake and LOX-1 Expression in the Primary and the 3rd-Passage bmMSCs. In a recent study, we had identified the characteristics of bmMSCs and found that the primary bmMSCs have a potential to take up ox-LDL and highly express LOX-1 receptors [1]. In the present study, we observed that the passaged (the 3rd passage) bmMSCs have the same potential to take up ox-LDL and express LOX-1 receptors with the primary bmMSCs (Figure 1).

3.2. Ox-LDL Stimulates Transmigration of bmMSCs in a Dose- and Time-Dependent Manner. The migration ability of bmMSCs was measured using a Transwell system. As shown in Figure 2(a), ox-LDL at doses of 5~20 μg/mL significantly increases transmigration rates of bmMSCs ($P < 0.01$) in a dose-dependent manner. From the preliminary data of transmigration of bmMSCs after being exposed to 5~20 μg/mL ox-LDL, we saw that 10 μg/mL ox-LDL exposure caused the medium levels of increase of cell transmigration. So, 10 μg/mL ox-LDL was selected to study the time-dependent transmigration of bmMSCs. When exposed to 10 μg/mL ox-LDL, bmMSCs also exhibit an increased transmigration in a time-dependent manner (Figure 2(b)).

3.3. Ox-LDL Enhances bmMSC Adhesive Ability and Expression of Adhesive Molecules. It is known that cell adhesion is a critical factor for cell transmigration, and the capacity of cell migration is dependent on expression of adhesive molecules [22]. In this study, adhesive ability of bmMSCs was measured by evaluating cell-cell adhesion between bmMSCs and monocytes. As shown in Figures 2(c)–2(f), the numbers of monocytes adhered to bmMSCs (pretreated with 5 ~ 20 μg/mL ox-LDL) were significantly ($P < 0.01$) increased by treatments with ox-LDL in a dose-dependent manner. When bmMSCs were exposed to 10 μg/mL ox-LDL, the numbers of adhered monocytes were also significantly increased ($P < 0.01$) in a time-dependent manner.

Cell-cell adhesion is dependent on expression of adhesive molecules. Our results showed that expression of the adhesive molecules ICAM-1, PECAM-1, and VCAM-1 in bmMSCs was significantly increased ($P < 0.01$) by induction with ox-LDL in a dose-dependent manner (Figure 3).

3.4. Ox-LDL Increases Intracellular Ca^{2+}. Intracellular Ca^{2+} is an important regulator of cell migration. It has been reported that ox-LDL causes an increase of intracellular Ca^{2+} in other cell lineages such as endothelial cells and smooth muscle cells [23, 24]. In the present study, we also found that ox-LDL (5~20 μg/mL) causes an increase of intracellular Ca^{2+} in bmMSCs in a dose-dependent manner (Figure 4).

3.5. Ox-LDL Mediates Reorganization of Cytoskeleton in bmMSCs. Cytoskeleton has been known to regulate cell migration and adhesion [25]. In this study, cytoskeleton organization was studied by staining F-actin using Rhodamine phalloidin. Compared with the control, bmMSCs treated with ox-LDL had better spreading and more integrated networks of F-actin filaments (Figure 5).

3.6. Ox-LDL Induces Expression of LOX-1, MCP-1, and TGF-β. Our previous study has shown that ox-LDL stimulates LOX-1

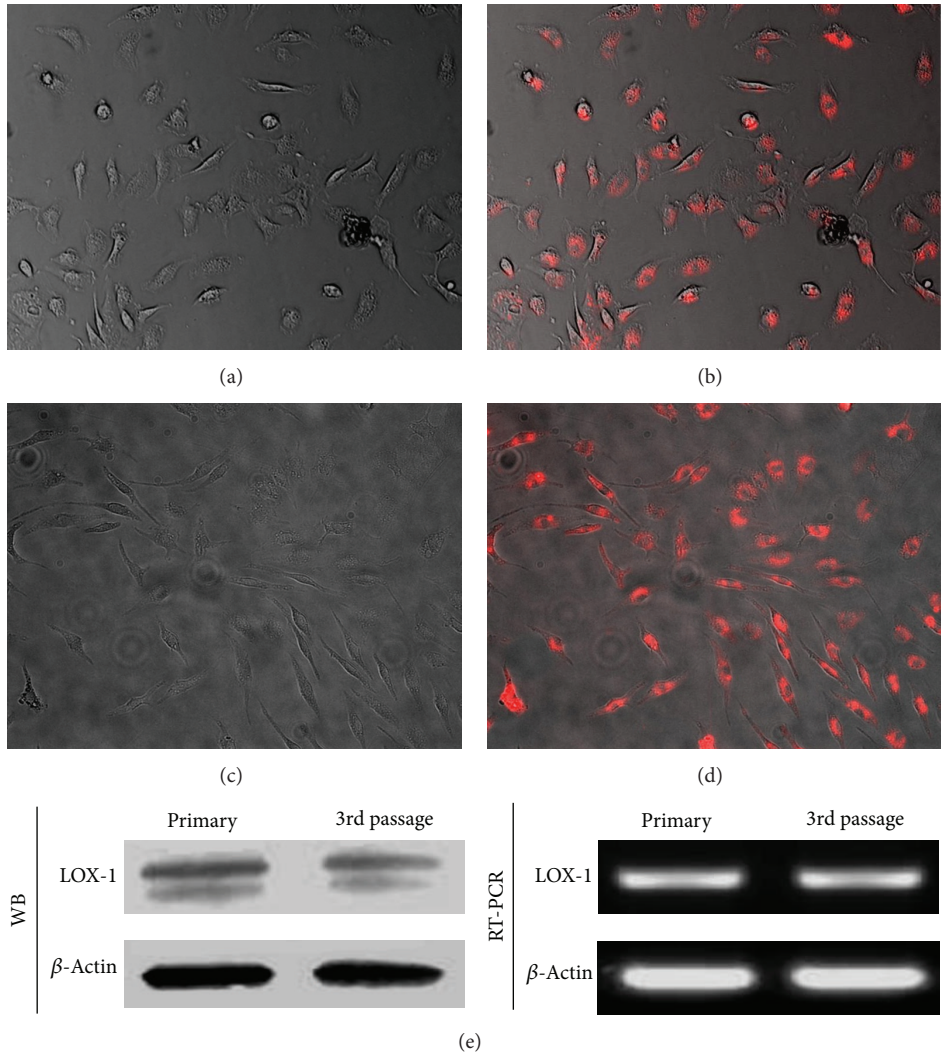

Figure 1: Uptake of Dil-ox-LDL and LOX-1 expression in bmMSCs. (a) Morphology of primary bmMSCs; (b) Dil-ox-LDL uptake in primary bmMSCs; (c) Morphology of the 3rd-passage bmMSCs; (d) Dil-ox-LDL uptake in the 3rd passage bmMSCs; (e) RT-PCR and Western-blotting assays show LOX-1 expression in the primary and the 3rd-passage bmMSCs.

expression in bmMSCs [1]. In accordance with the previous study, we observed in this study that ox-LDL (5~20 μg/mL) induces LOX-1 expression in a dose-dependent manner (Figure 6(a)). Furthermore, ox-LDL also increases MCP-1 and TGF-β expression in bmMSCs in a dose-dependent manner (Figures 6(b) and 6(c)).

More importantly, pretreatment with LOX-1 antibody inhibits ox-LDL-induced MCP-1 expression (Figure 6(d)), cell migration (Figure 6(e)), adhesion (Figure 6(f)), and expression of ICAM-1 (Figure 6(g)), PECAM-1, and VCAM-1 (data not shown). These data suggest that ox-LDL-induced adhesion and migration of bmMSCs are at least partially via activation of LOX-1 receptors.

3.7. MCP-1 Knockdown Inhibits Ox-LDL-Induced Cell Migration and Adhesion. To further investigate the role of MCP-1 in ox-LDL-induced bmMSC migration and adhesion, we performed MCP-1 shRNA in bmMSCs. As shown in Figure 6(h), compared with transfection of noneffective scrambled shRNA, MCP-1 shRNA significantly downregulates MCP-1 expression in bmMSCs ($P < 0.01$). More interestingly, MCP-1 knockdown also significantly decreases ox-LDL-induced bmMSC transmigration and adhesion, as well as expression of adhesive molecules (Figures 6(i)–6(k); $P < 0.01$).

4. Discussion

In this study, we for the first time investigated the effects of ox-LDL on migration and adhesion of bmMSCs. We found that treatment with ox-LDL enhances migration and adhesion capacity of bmMSCs. We also observed that treatment with ox-LDL increases intracellular Ca^{2+} and expression of LOX-1, MCP-1, and TGF-β, and it facilitates cytoskeleton reorganization. More importantly, use of LOX-1 antibody and knockdown of MCP-1 both significantly inhibit

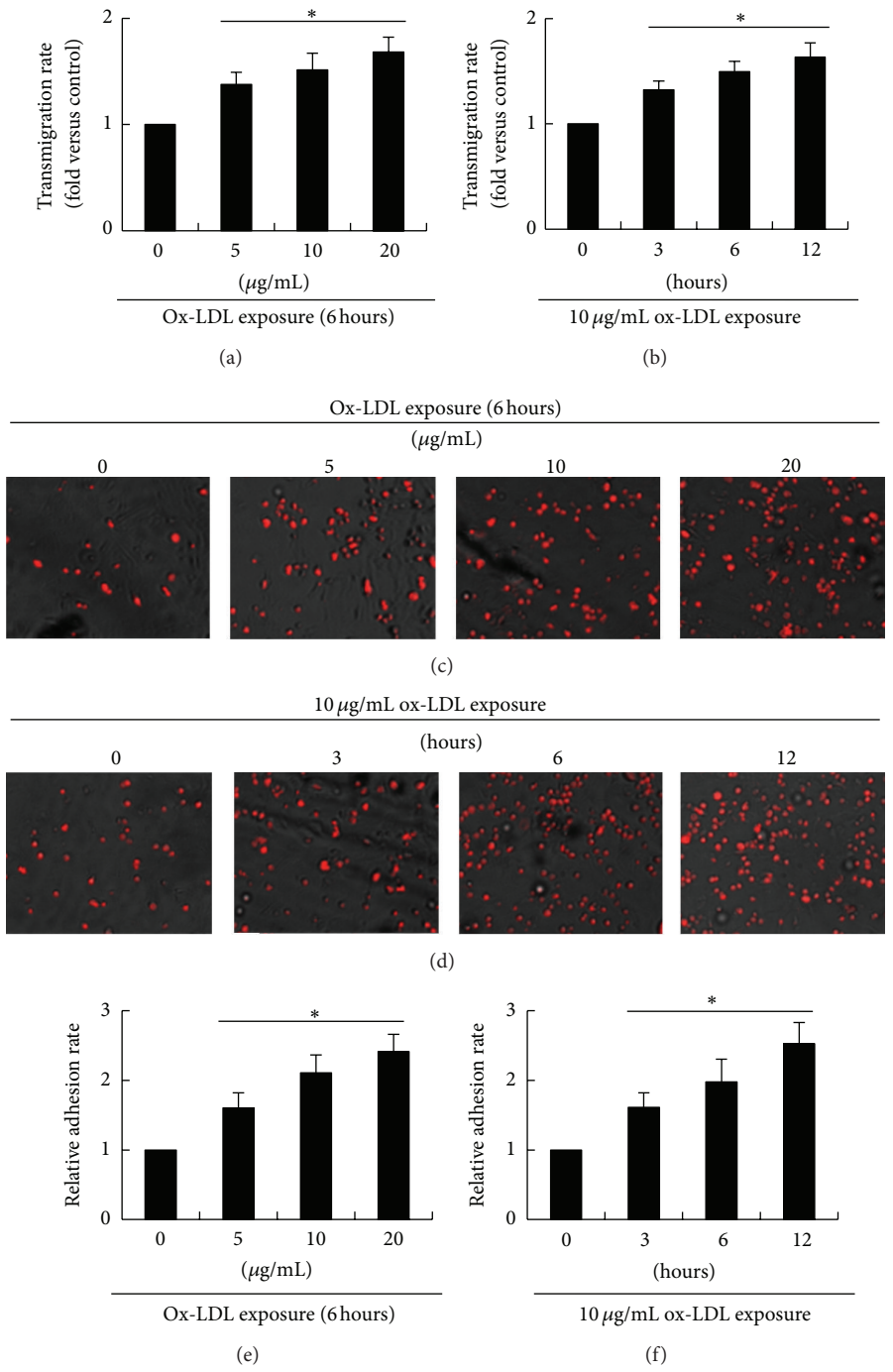

Figure 2: Ox-LDL promotes transmigration of bmMSCs and enhances cell adhesion between bmMSCs (grey color) and monocytes (red color). (a) Transmigration rates of bmMSCs after exposure to 0, 5, 10, and 20 μg/mL ox-LDL for 6 hours; (b) transmigration rates of bmMSCs after exposure to 10 μg/mL for 0, 3, 6, and 12 hours; (c) the merged phase contrast and fluorescence images show adhesion between bmMSCs and monocytes after treatment with 0, 5, 10, and 20 μg/mL ox-LDL for 6 hours; (d) the merged phase contrast and fluorescence images show adhesion between bmMSCs and monocytes after treatment with 10 μg/mL for 0, 3, 6, and 12 hours; (e) the relative adhesive rate of monocytes onto bmMSCs after treatment with 0, 5, 10 and 20 μg/mL ox-LDL for 6 hours; (f) the relative adhesive rate of monocytes onto bmMSCs after treatment with 10 μg/mL ox-LDL for 0, 3, 6, and 12 hours. Bar graphs represent mean ± SD ($n = 4$ per group). $^*P < 0.01$ versus control.

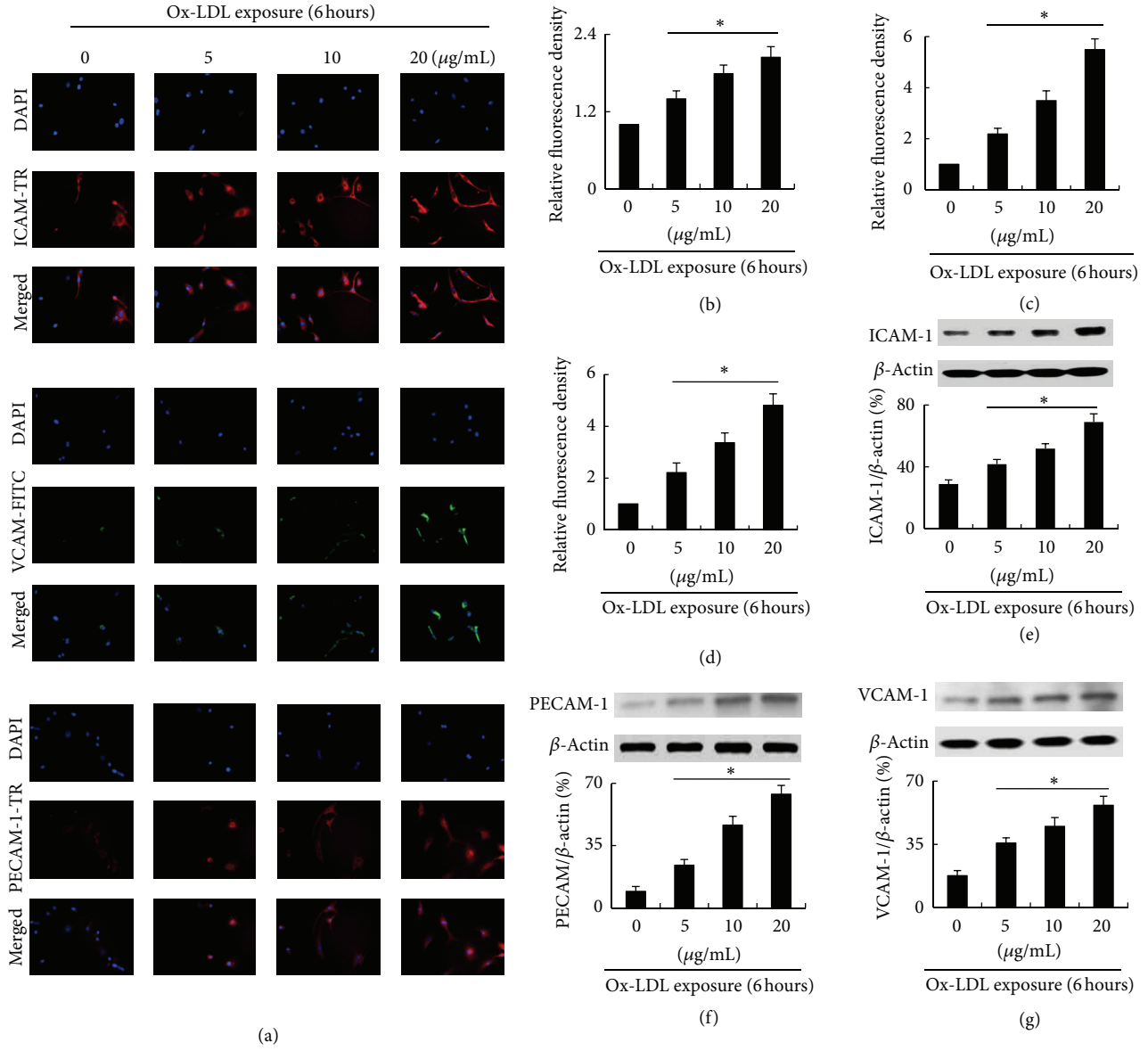

Figure 3: Ox-LDL increases expression of ICAM-1, PECAM-1, and VCAM-1 in a dose-dependent manner in bmMSCs. (a) Immunofluorescence assay shows expression of ICAM-1, PECAM-1 and VACM-1 in bmMSCs exposed to 0, 5, 10 and 20 μg/mL ox-LDL for 6 hour; (b)–(d) Relative fluorescence density of ICAM-1, PECAM-1, and VCAM-1; (e)–(g) Western-blotting assay shows expression of ICAM-1, PECAM-1, and VCAM-1 in bmMSCs exposed to 0, 5, 10, and 20 μg/mL ox-LDL for 6 hours. Bar graphs represent mean ± SD ($n = 4$ per group). $^*P < 0.01$ versus Control.

ox-LDL-induced bmMSC migration and adhesion, as well as expression of adhesive molecules. These findings indicate that ox-LDL can promote migration of bmMSCs, which is dependent on LOX-1 activation and MCP-1 expression.

The migration capacity of bmMSCs is one of the most important determinants of the efficiency of bmMSC-based transplant therapy. It has been reported that the intravenously injected bmMSCs have a steady capacity to migrate back to the bone marrow and home to the injured organs by migrating across the endothelium [26]. But, the homing rates of the injected bmMSCs to the injured tissues are very low (<1.5%) [7]. The low homing rate of bmMSCs would severely affect their therapeutic efficiency in transplant therapy. So, it is necessary to find more effective methods to stimulate migration of bmMSCs. It has been reported that ox-LDL can induce production of inflammatory molecules (MCP-1, IL-6, and adhesive molecules), and subsequently promote migration of macrophages and endothelial cells [27–29].

Cell adhesion is a prerequisite for transmigration of the circulating cells. The first step of the intravenously injected bmMSCs to the injured organs is adhering to the endothelium and overcoming the endothelial barrier. The stable cell adhesion affects cytoskeleton reorganization and actin polymerization, facilitates cell protrusion, and leads to directional cell movement [30]. So, expression of adhesion molecules is critically important for cell migration. It has

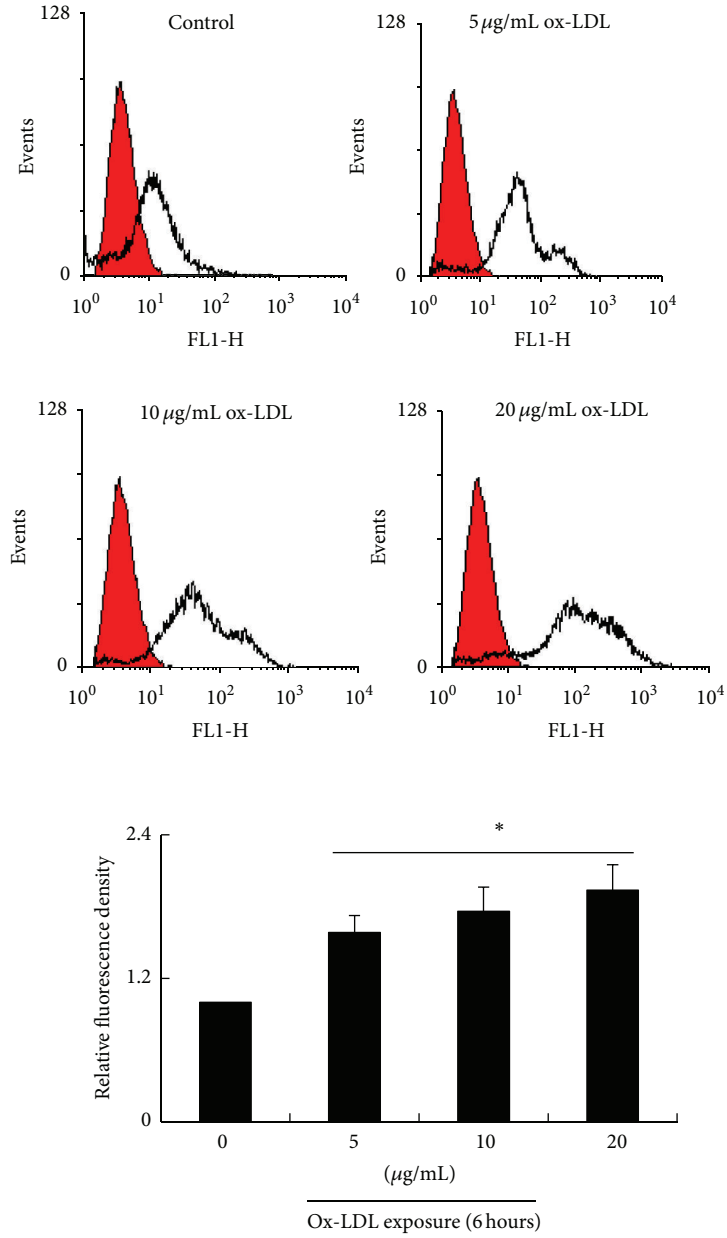

Figure 4: Flowcytometry assay shows the levels of intracellular Ca^{2+} of bmMSCs exposed to 0, 5, 10, and 20 μg/mL ox-LDL for 6 hours. Bar graphs represent mean ± SD ($n = 4$ per group). $^*P < 0.01$ versus control.

been reported that PECAM-1 is required for TNF-α-induced transmigration of leukocytes [31]. Use of PECAM-1 antibody can inhibit migration of leukocytes [31]. Moreover, cell-cell adhesion is also required for survival of the transplanted bmMSCs in the target organs or tissues. In the present study, we found that low concentrations (5~20 μg/mL) of ox-LDL have potential to stimulate bmMSC migration and adhesion and mediate expression of adhesion molecules (ICAM-1, PECAM-1, and VCAM-1).

Calcium ion (Ca^{2+}) is a very important cellular secondary messenger, which plays a prominent role in signal transduction and cell physiology. A number of studies have shown that intracellular Ca^{2+} regulates cell adhesion and migration. The increase of intracellular Ca^{2+} is in parallel with an increase of adhesion of lymphocytes, erythrocytes, macrophages, and cancer cells [32–35]. And, increase of intracellular Ca^{2+} can also cause upregulation of adhesive molecules such as ICAM-1, PECAM-1, VCAM-1, and E-selectin [36–39]. Moreover, the expression of adhesion molecules is also required for transmission of calcium [37, 39]. In this study, we also observed that expression of ICAM-1, VCAM-1, and PECAM-1 is in parallel with an increase of intracellular Ca^{2+} in bmMSCs. A study by Cook-Mills et al. showed that the response of intracellular Ca^{2+} to VCAM-1 stimulation is dependent on the activation of NADPH oxidase in endothelial cells [39]. Not surprisingly, as a strong stimulator of NADPH oxidase,

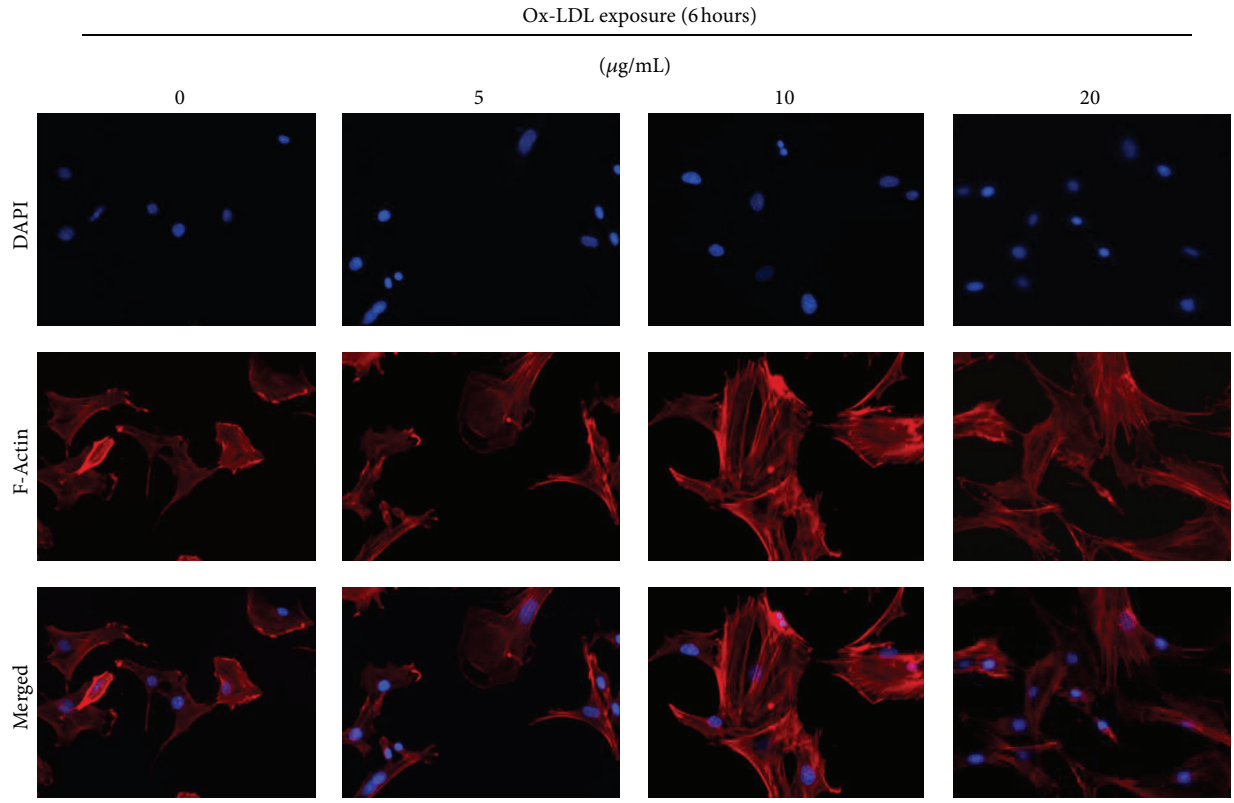

Figure 5: Cytoskeleton (F-actin fibers) organization in bmMSCs after exposure to 0, 5, 10, and 20 μg/mL ox-LDL for 6 hours.

ox-LDL can increase expression of adhesive molecules and intracellular Ca^{2+} in bmMSCs. Treatment with Ca^{2+} or Ca^{2+} ionophore A23187 was also observed to stimulate migration of smooth muscle cells; and use of Ca^{2+} entry blocker nicardipine inhibited cell migration of these cells [40]. The calcium-mediated cell migration is dependent on its role in regulating cytoskeletal rearrangement [36]. It is known that the dynamic organization of cytoskeleton is a prerequisite of cell migration. In the present study, treatment with ox-LDL facilitates bmMSC spreading and organization of F-actin fibers. Previous studies reported that the regulation of ox-LDL in actin organization is involved in activation of Rho GTPases and PI3K/Akt pathway [41]. However, some other studies reported that high concentrations of ox-LDL (100 μg/mL) cause disorganization of cytoskeleton and death of smooth muscle cells [42]. In our other ongoing studies, we also observed that high concentrations of ox-LDL (>40 μg/mL) have toxicity to bmMSCs and cause apoptosis of bmMSCs.

It has been reported that ox-LDL stimulates cell migration via activation of its receptor LOX-1. Our recent study has shown that LOX-1 is highly expressed in primary bmMSCs. In the present study, we also found that LOX-1 is highly expressed in the passaged bmMSCs. More interestingly, blockade of LOX-1 using LOX-1 antibody significantly inhibits ox-LDL-induced MCP-1 expression, cell adhesion, and migration of bmMSCs. This suggests that ox-LDL-induced bmMSC migration is at least partially via activation of LOX-1.

MCP-1 is an important regulator of inflammatory events. Previous studies have shown that ox-LDL via activation of LOX-1 enhances MCP-1 expression in many cell lineages such as human articular chondrocytes, vascular smooth muscle cells, endothelial cells, and macrophages [17–21]. Treatment with exogenous recombinant MCP-1 or increase of endogenous MCP-1 expression can induce transendothelial migration of T cells, monocytes, smooth muscle cells, and adult neural stem cells [43–45]. TGF-β is another important factor for cell migration. TGF-β stimulates cell migration via regulation of MCP-1 expression [44, 46]. In the present study, we also found that ox-LDL stimulates MCP-1 and TGF-β expression in bmMSCs in a dose-dependent manner. More importantly, knockdown of MCP-1 expression significantly inhibits ox-LDL-induced bmMSC transmigration, cell-cell adhesion, and expression of adhesion molecules. These data show that the inflammatory factor MCP-1 plays an important role in ox-LDL-induced bmMSC migration and adhesion.

5. Conclusion

In this study, we investigated the effects of ox-LDL on bmMSC migration and adhesion. Our results show that ox-LDL enhances transmigration and adhesion capacities of bmMSCs, which is mediated by LOX-1 activation and MPC-1 expression. Blockade of LOX-1 receptor using antibody significantly decreases ox-LDL-induced MCP-1 expression and inhibits bmMSC transmigration and adhesion. More importantly, MCP-1 knockdown also significantly inhibits

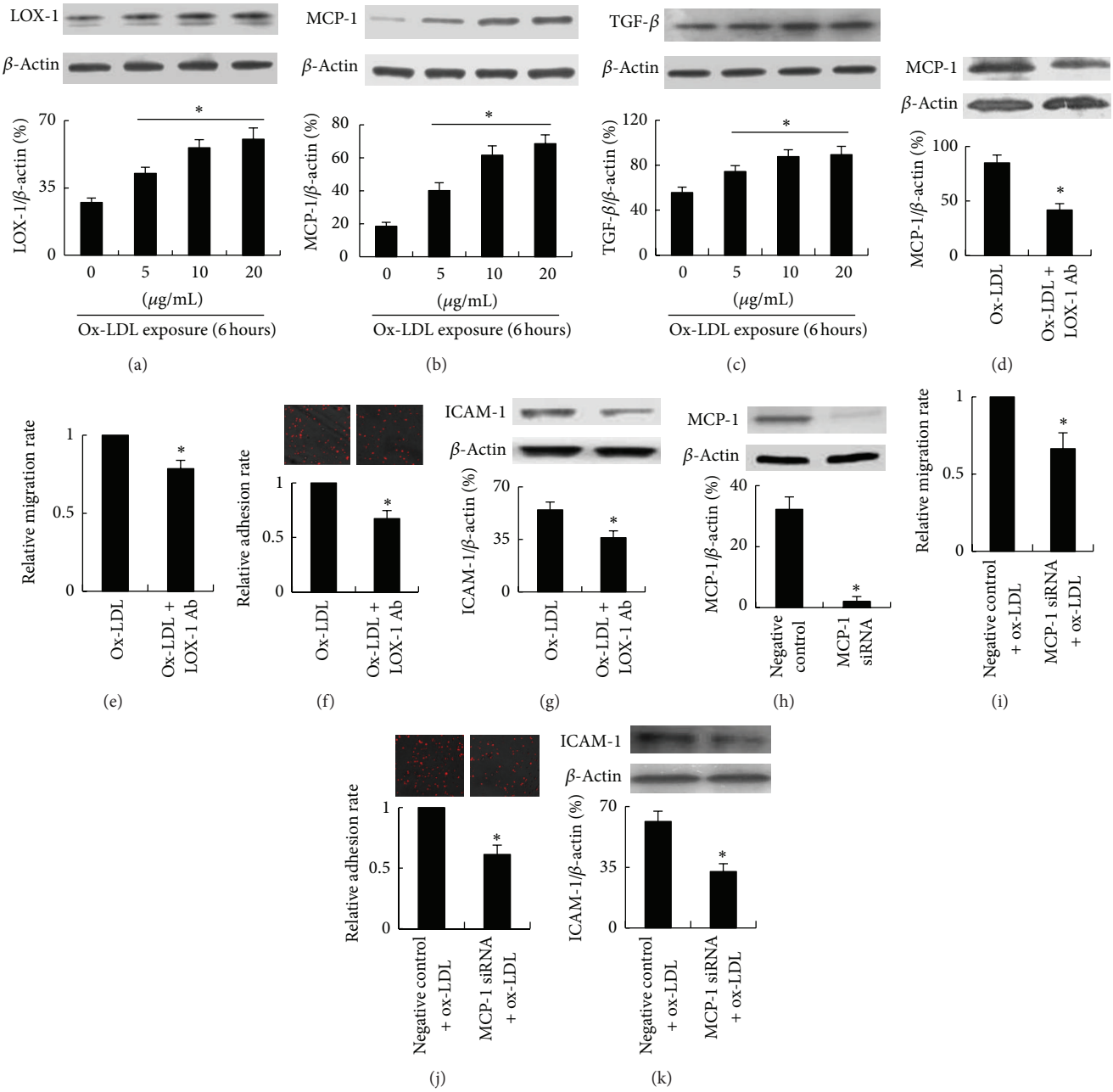

Figure 6: Role of LOX-1 and MCP-1 in ox-LDL-mediated migration and adhesion of bmMSCs. (a)–(c) Western-blotting assay shows LOX-1, MCP-1 and TGF-β expression in bmMSCs after exposure to 0, 5, 10, and 20 μg/mL ox-LDL for 6 hours. (d) Western-blotting assay shows that LOX-1 antibody inhibits ox-LDL-induced MCP-1 expression. (e) Transwell assay shows that LOX-1 antibody inhibits ox-LDL-induced transmigration of bmMSCs. (f) LOX-1 antibody inhibits ox-LDL-induced cell adhesion between bmMSCs and monocytes. (g) Western-blotting assay shows that LOX-1 antibody decreases ICAM-1 expression. (h) Western-blotting assay shows MCP-1 expression after transfection of noneffective shRNA and MCP-1 shRNA. (i) MCP-1 knockdown inhibits ox-LDL-induced migration of bmMSCs. (j) MCP-1 knockdown inhibits ox-LDL-induced cell-cell adhesion between monocytes and bmMSCs. (k) MCP-1 knockdown decreases ox-LDL-induced ICAM-1 expression. Bar graphs represent mean ± SD ($n = 4$ per group). $^*P < 0.01$ versus control, ox-LDL treatment, or negative control transfection.

ox-LDL-induced bmMSC transmigration and cell adhesion. These findings indicate that MCP-1 plays an important role in ox-LDL-mediated migration and adhesion of bmMSCs.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

F. Zhang and C. Wang contributed equally to this work.

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Review Article

LOX-1, OxLDL, and Atherosclerosis

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Oxidized low-density lipoprotein (OxLDL) contributes to the atherosclerotic plaque formation and progression by several mechanisms, including the induction of endothelial cell activation and dysfunction, macrophage foam cell formation, and smooth muscle cell migration and proliferation. Vascular wall cells express on their surface several scavenger receptors that mediate the cellular effects of OxLDL. The lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is the main OxLDL receptor of endothelial cells, and it is expressed also in macrophages and smooth muscle cells. LOX-1 is almost undetectable under physiological conditions, but it is upregulated following the exposure to several proinflammatory and proatherogenic stimuli and can be detected in animal and human atherosclerotic lesions. The key contribution of LOX-1 to the atherogenic process has been confirmed in animal models; LOX-1 knockout mice exhibit reduced intima thickness and inflammation and increased expression of protective factors; on the contrary, LOX-1 overexpressing mice present an accelerated atherosclerotic lesion formation which is associated with increased inflammation. In humans, LOX-1 gene polymorphisms were associated with increased susceptibility to myocardial infarction. Inhibition of the LOX-1 receptor with chemicals or antisense nucleotides is currently being investigated and represents an emerging approach for controlling OxLDL-LOX-1 mediated proatherogenic effects.

1. Introduction

Atherosclerosis is a chronic inflammatory vascular disease, having as ultimate outcome the atheromatous plaque, a focal lesion located within the intima of large and medium sized arteries [1, 2]. Subendothelial retention of low density lipoprotein (LDL) and its oxidative modification represent the initial event in atherogenesis, which is followed by infiltration and activation of blood inflammatory cells. Oxidized LDL (OxLDL) in fact activates endothelial cells (ECs) by inducing the expression of several cell surface adhesion molecules which mediate the rolling and adhesion of blood leukocytes (monocytes and T cells); after adhesion to the endothelium, leukocytes migrate into the intima in response to chemokines. Monocytes then differentiate into macrophages that upregulate both toll-like receptors (TLRs), involved in macrophage activation, and scavenger receptors (SRs), that internalize apoptotic cell fragments, bacterial endotoxins, and OxLDL, leading to lipid accumulation and foam cell formation [2]. Macrophage activation leads to the release of proinflammatory cytokines, reactive oxygen species (ROS), proteolytic enzymes involved in matrix degradation and thus in atherosclerotic plaque destabilization. T cells respond to local peptide antigens present on the surface of antigen-presenting cells, become activated, and release proinflammatory cytokines [2].

OxLDL acts via binding to several SRs, including SR-A, SR-BI, CD36, and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) [3]. LOX-1 is a type II integral membrane glycoprotein consisting of a short N-terminal cytoplasmic domain, a transmembrane domain, a neck region, which regulates receptor oligomerization, and an extracellular C-type lectin-like extracellular domain, involved in ligand binding (Figure 1) [4]. LOX-1 has been identified first in ECs as the major OxLDL receptor [5]; however also macrophages and smooth muscle cells express LOX-1

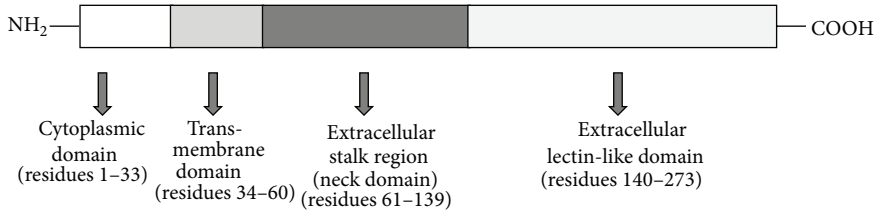

Figure 1: Schematic representation of LOX-1 structure. LOX-1 consists of four domains: a cytoplasmic N-terminal domain, a single transmembrane domain, an extracellular neck domain, and an extracellular lectin-like domain.

together with other SRs [6]. Basal cellular LOX-1 expression is very low, but it can be induced by several proinflammatory and proatherogenic stimuli [7, 8]. *In vitro*, LOX-1 expression is induced by many stimuli related to atherosclerosis, including proinflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1), and interferon-gamma (IFNγ); angiotensin II; endothelin-1; OxLDL and other modified lipoproteins; free radicals; and fluid shear stress [8–10] (Table 1). *In vivo*, the expression of LOX-1 is upregulated in the presence of pathological conditions including atherosclerosis, hypertension, and diabetes [8] (Figure 2). In human atherosclerotic lesions, LOX-1 is overexpressed in ECs especially in the early stage of atherogenesis; in advanced atherosclerotic plaques, LOX-1 is overexpressed in ECs of neovascular formations [11]. Furthermore, LOX-1 is highly expressed by intimal smooth muscle cells (SMCs) and macrophages in human carotid atherosclerotic plaques [11], suggesting the roles for LOX-1 in endothelial activation and in foam cell formation. Finally, the results obtained in LOX-1 knockout or LOX-1 overexpressing mice have suggested a key contribution of LOX-1 in the inflammatory response and lipid deposition in heart vessels [12, 13].

Besides OxLDL, LOX-1 binds multiple ligands, including other forms of modified lipoproteins, advanced glycation end-products, activated platelets, and apoptotic cells [8–10] (Table 2). LOX-1 also binds delipidated OxLDL, suggesting that LOX-1 recognizes the modified apolipoprotein B; furthermore, LOX-1 binds with higher affinity to mildly oxidized LDL rather than extensively oxidized LDL, suggesting the ability to recognize also oxidized lipids (i.e., lipids that are covalently bound to the protein and are not removed by the delipidation process) [3] (Table 2). OxLDL is rapidly internalized into cells following the interaction with LOX-1; this internalization process is inhibited by LOX-1-blocking antibody which acts by preventing the binding of OxLDL with the receptor [14]. After the endocytosis of the complex OxLDL-LOX-1, the receptor is uncoupled from OxLDL, and both are located in separate compartments within the cytosol [14].

LOX-1 is mainly localized in caveolae/lipid rafts (cholesterol-enriched membrane microdomains) in the plasma membrane; its function is modulated by the cholesterol content of membrane: cholesterol depletion induces the mislocalization of LOX-1, which results in a more diffuse distribution of the receptor in the plasma membrane (without a reduction of the amount of receptor exposed on the cell surface) and in a marked reduction of LOX-1-mediated OxLDL binding and uptake [15]. This finding suggests that the clustered distribution of LOX-1 in specific membrane microdomains is essential for an efficient interaction with OxLDL and for the internalization of OxLDL-LOX-1 complexes.

Table 1: LOX-1 inducers.

Proinflammatory cytokines
Tumor necrosis factor α (TNFα)
Interleukin-1 (IL-1)
Interferon γ (IFNγ)
Lipopolysaccharide (LPS)
C-reactive protein (CRP)
Modified lipoproteins
OxLDL (copper-oxidized LDL)
15-Lipoxygenase-modified LDL
15-Lipoxygenase-modified HDL_3
Glycoxidized-LDL
Lysophosphatidylcholine (LPC)
Palmitic acid
Hypertension-related stimuli
Angiotensin II
Endothelin-1
Fluid shear stress
Hyperglycemic stimuli
High glucose
Advanced glycation end-products (AGEs)
Other stimuli
Homocysteine
Free radicals

2. LOX-1-Mediated Endothelial Dysfunction

2.1. LOX-1 Upregulation by OxLDL. Endothelial dysfunction represents a very early stage in the atherogenic process and is a pathological condition characterized by alterations in anti-inflammatory and anticoagulant properties and by impaired ability to regulate vascular tone. LOX-1 is the main receptor for OxLDL in ECs [5], and OxLDL, through LOX-1, contributes to the induction of endothelial dysfunction by several mechanisms (Figure 3). Basal LOX-1 expression is very low, but it can be induced by proinflammatory cytokines

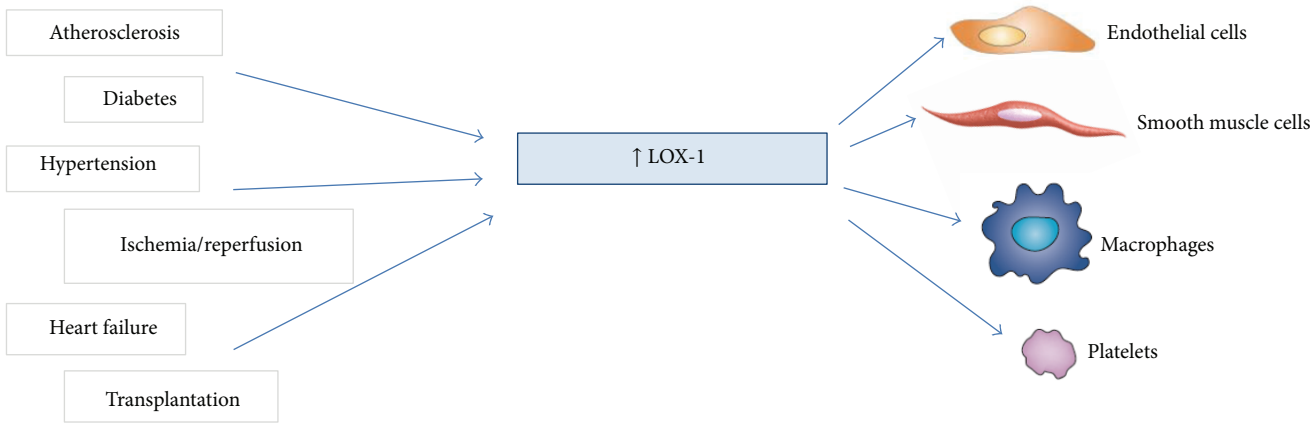

Figure 2: *In vivo* stimuli of LOX-1. Several pathological conditions can upregulate LOX-1 expression resulting in vascular wall cell activation.

Table 2: LOX-1 ligands.

Modified lipoproteins	Other ligands
OxLDL (copper-oxidized LDL)	Apoptotic cells
15-Lipoxygenase-oxidized LDL	Activated platelets
15-Lipoxygenase-oxidized HDL_3	Advanced glycation end-products (AGEs)
Glycoxidized LDL	
Delipidated OxLDL	
HOCl-modified HDL	

generated in a local environment within the arterial wall or by other atherogenic stimuli including OxLDL [8].

OxLDL upregulates LOX-1 mRNA and protein expression in a dose-dependent manner [16]; OxLDL-mediated upregulation of LOX-1 is suppressed by pretreatment of cells with antisense to LOX-1 mRNA [17], suggesting that OxLDL modulates its own receptor through interaction with LOX-1. *In vitro*, LOX-1 expression can be upregulated in ECs also by 15-lipoxygenase-modified LDL, and LOX-1 overexpression increases the association of 15-lipoxygenase-modified LDL to ECs [9], supporting the hypothesis that also minimally oxidized LDL may contribute to LOX-1 induction and to EC activation. LOX-1 upregulation occurs also in ECs exposed to 15-lipoxygenase-modified HDL_3; this modified lipoprotein is also a ligand for LOX-1 [10].

2.2. LOX-1 Mediates OxLDL-Induced EC Activation. After adhesion to the endothelium, monocytes migrate into the intima where they differentiate into macrophages, accumulate lipids, and become foam cells. Recruitment of monocytes involves both chemokines and adhesion molecules. Monocyte chemoattractant protein-1 (MCP-1) is a chemotactic protein for monocytes; incubation of ECs with OxLDL significantly increases MCP-1 expression and monocyte adhesion to ECs; these effects are both suppressed in the presence of an antisense to LOX-1 mRNA [18]. Activation of mitogen-activated protein kinase (MAPK) is required for OxLDL-mediated induction of MCP-1, and antisense to LOX-1 mRNA completely inhibits the OxLDL-induced MAPK activation [18]. Additional chemokines are upregulated by LOX-1 activation in response to OxLDL, including IL-8, chemokine (C-X-C motif) ligands 2 and 3 (CXCL2 and CXCL3) [19].

Upregulation of endothelial adhesion molecules contributes to the leukocyte adhesion; OxLDL significantly increases the expression of E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) in ECs [20]. These effects are mediated by LOX-1, since antisense to LOX-1 mRNA decreases both LOX-1 expression and adhesion molecule upregulation in response to OxLDL [20]. In addition, pretreatment of ECs with a statin or with polyinosinic acid or carrageenan (two known ligands of LOX-1) lowers OxLDL-induced expression of LOX-1 as well as adhesion molecules, confirming that LOX-1 activation plays an important role in OxLDL-induced expression of adhesion molecules [20, 21]. ICAM-1 expression is upregulated in LOX-1-overexpressing ECs exposed to 15-lipoxygenase-modified LDL [9], providing a role for minimally modified LDL (mmLDL) as proinflammatory particle.

Nuclear factor kappa B (NF-κB), which is activated by several proinflammatory cytokines, modulates the expression of proinflammatory genes, including adhesion molecules, cytokines, and chemokines, and is also involved in LOX-1 upregulation [8, 22]. OxLDL, following the binding with LOX-1, activates NF-κB; preincubation of ECs with anti-LOX-1 antibody markedly attenuates the transcription factor activation [23].

The stimulation of CD40/CD40L signaling results in the induction of proatherogenic pathways (including the expression of adhesion molecules and proinflammatory cytokines) and in EC activation [24]. Incubation of human coronary artery endothelial cells (HCAECs) with OxLDL increases the expression of CD40 and CD40L, while the inhibition of LOX-1 with a blocking antibody reduces the OxLDL-mediated increase of CD40 [25].

2.3. OxLDL-LOX-1 Interaction Impairs Endothelium-Dependent Relaxation. ECs, due to their location at the interface between blood and the vessel wall, play a crucial role in

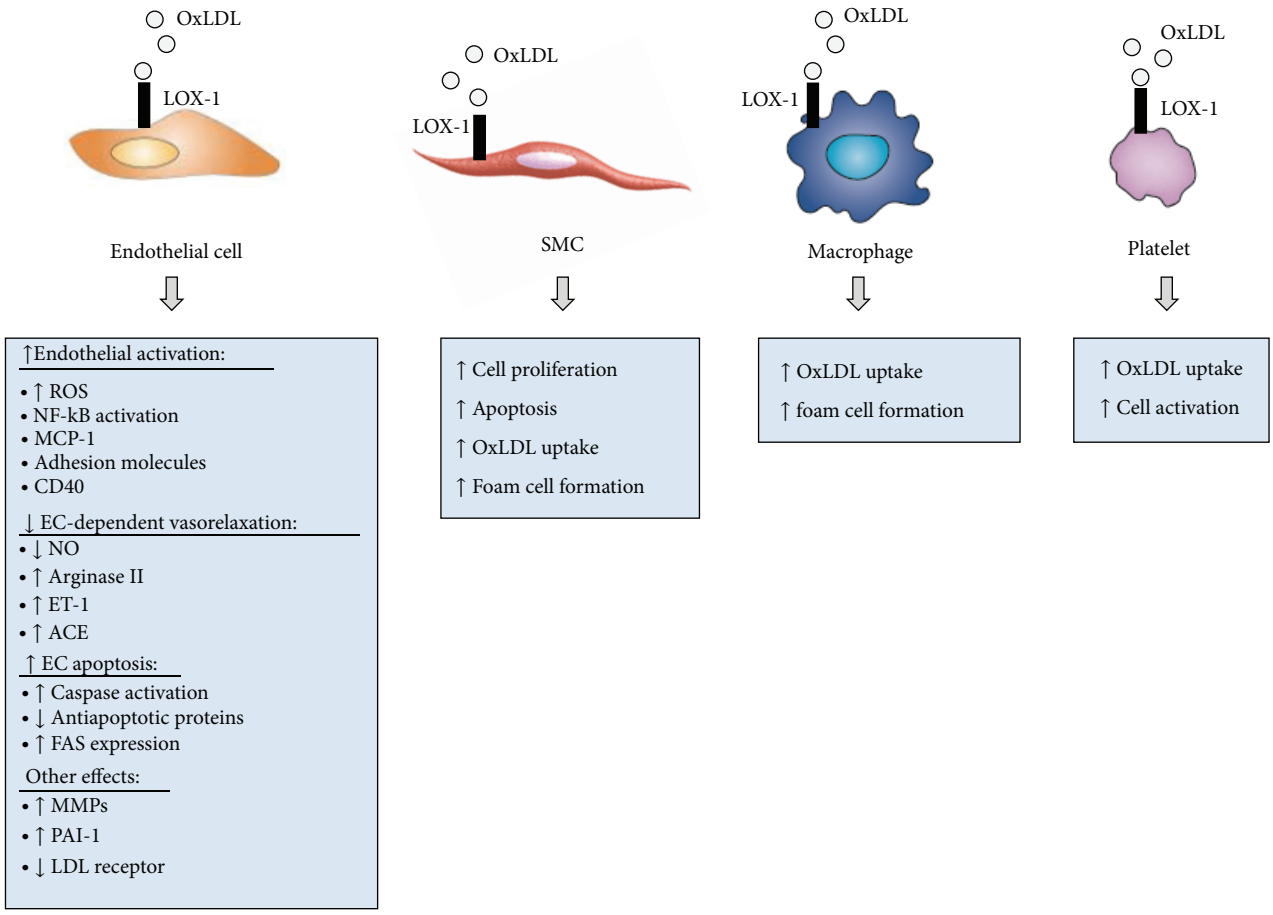

Figure 3: Role of LOX-1 in atherosclerosis. OxLDL binding to LOX-1 induces endothelial activation and dysfunction, supports the recruitment of circulating leukocytes, triggers foam cell formation, and sustains migration and proliferation of smooth muscle cells, thus contributing to the development of the atherosclerotic plaque. Furthermore, OxLDL-LOX-1 interaction may also contribute to plaque destabilization by inducing smooth muscle cell apoptosis and the release of matrix degrading enzymes (MMPs).

the control of vascular tone and homeostasis, through the production of several vasoactive mediators. Among them, nitric oxide (NO) is produced by endothelial NO synthase (eNOS) in response to several stimuli; functions mainly as a vasodilator; inhibits leukocyte-endothelial cell adhesion, platelet adhesion and aggregation, and smooth muscle cell proliferation; and stimulates angiogenesis [26]. In disease conditions, ECs exhibit a reduced NO bioavailability, due to an increased degradation of NO in response to an enhanced oxidative stress [26]. The release of NO by ECs can be reduced by OxLDL: OxLDL displaces eNOS from its caveolae membrane localization by depleting caveolae of cholesterol, thus inhibiting NO generation [27]. Furthermore, OxLDL inactivates NO through an oxidative mechanism by increasing cellular production of reactive oxygen species (ROS), and in particular superoxide [28]. Anti-LOX-1 monoclonal antibody reduces superoxide formation and increases intracellular NO level, suggesting the involvement of LOX-1 in these OxLDL-induced effects [28]. Additionally, OxLDL activates endothelial arginase II (which regulates NO production by competing with eNOS for the common substrate L-arginine) through the dissociation of arginase from the microtubule cytoskeleton, resulting in a decreased NO production and a raise of ROS [29, 30]. The increased arginase activity is inhibited by monoclonal antibody to LOX-1; furthermore, ECs from LOX-1 knockout (KO) mice do not increase arginase activity and exhibit reduced changes in NO and superoxide production in response to OxLDL [30], thus confirming the involvement of LOX-1 in OxLDL-mediated arginase activity. Further, neutralizing antibodies to LOX-1 restore NO-mediated coronary arteriolar dilation in ApoE KO mice [31].

Dysfunctional ECs can also generate vasoconstrictor factors, including endothelin-1 (ET-1) and angiotensin II (Ang II). ET-1 is a potent vasoconstrictor, proinflammatory, and mitogenic peptide produced by injured vascular ECs in response to several stimuli; a tight interaction exists between ET-1 and OxLDL: OxLDL stimulates the generation of ET-1, and ET-1 enhances the uptake of OxLDL in ECs by promoting the expression of LOX-1 [32]. Incubation of ECs with OxLDL increases ET-1 mRNA and protein expression, an effect inhibited by anti-LOX-1 antibody [33].

Renin-angiotensin system may contribute to the endothelial dysfunction; angiotensin-converting enzyme (ACE),

which converts Ang I to Ang II, is mainly expressed in ECs [34]. Ang II induces LOX-1 expression and facilitates OxLDL uptake by ECs [35]; in turn, OxLDL increases the expression of ACE in a concentration- and time-dependent manner. The upregulation of ACE expression in response to OxLDL is mediated by LOX-1, as pretreatment of ECs with LOX-1-blocking antibody significantly reduces the OxLDL-induced expression of ACE [36].

2.4. LOX-1 Mediates OxLDL-Induced Apoptosis of ECs. Incubation with high concentrations of OxLDL induces cellular changes that may result in cell death. OxLDL may induce both necrosis and apoptosis; the last is a highly regulated process and involves multiple pathways, including ROS generation, caspase and protein kinase activation, alteration of calcium homeostasis, and alteration of proapoptotic/antiapoptotic gene expression [37]. EC apoptosis results in increased vascular permeability to cells and lipids, smooth muscle cell proliferation, and increased coagulation, thus contributing to the development of atherosclerotic lesions.

OxLDL induces EC apoptosis through LOX-1; in fact, inhibition with antisense to LOX-1 mRNA or with chemical inhibitors of LOX-1 significantly reduces the number of apoptotic cells in response to OxLDL [17]. NF-κB activation following EC exposure to OxLDL acts as a signal transduction mechanism in LOX-1-mediated apoptosis, and antisense to LOX-1 significantly inhibits OxLDL-induced NF-κB activation [17].

OxLDL also activates caspase-9 and caspase-3 in ECs and induces the release of mitochondrial activators of caspases, while reducing the expression of the antiapoptotic proteins B-cell lymphoma 2 (Bcl-2) and cellular inhibitor of apoptosis protein 1 (c-IAP-1) [38]. These effects are mediated by LOX-1, as pretreatment of cells with antisense to LOX-1 mRNA significantly decreases OxLDL-induced activation of caspases as well as the percentage of apoptotic cells [38]. These findings indicate that OxLDL through its receptor LOX-1 modulates activity and expression of relevant players in apoptosis.

Fas is a death-receptor that triggers apoptosis when activated by its ligand FasL and is involved in OxLDL-induced apoptosis; in fact, OxLDL sensitizes vascular cells to Fas-mediated apoptosis, upregulating Fas surface expression, while OxLDL-induced apoptosis is reduced by FasL-neutralizing antibodies [37]. LOX-1 activation is involved in these effects, as neutralizing LOX-1 antibody prevents OxLDL-induced activation of Fas-mediated apoptosis and inhibits OxLDL-induced modulation of surface Fas expression in ECs [39].

2.5. OxLDL Increases Oxidative Stress through LOX-1 in ECs. High levels of ROS are produced in several disease conditions, including atherosclerosis, and contribute to endothelial dysfunction. ROS are involved in LDL oxidation, and, in turn, OxLDL mediates many of its biological effects by generating more intracellular ROS through the binding to LOX-1; anti-LOX-1 antibody markedly reduces OxLDL-induced ROS formation [23, 40]. OxLDL-induced increase of ROS also results in NF-κB activation [23] and in EC apoptosis [40]; both these effects are mediated by LOX-1, as pretreatment of the cells with LOX-1-blocking antibody significantly reduces ROS production, NF-κB activation, and apoptosis rate in response to OxLDL [23, 40].

Endothelial NADPH oxidase, a multisubunit enzymatic complex, is a major source of ROS in vascular ECs, and OxLDL induces a significant increase of NADPH oxidase-generated ROS in ECs [41]. The binding of OxLDL to LOX-1 activates NADPH oxidase by inducing translocation of specific subunits on the cell membrane, leading to a rapid increase in intracellular ROS such as hydrogen peroxide and superoxide; the latter reacts with intracellular NO, thereby causes intracellular NO level to decrease, and upregulates LOX-1 expression, thus resulting in further increase in ROS production [42].

$p66^{Shc}$ is a redox enzyme involved in mitochondrial ROS generation and the translation of oxidative signals into apoptosis; higher levels of $p66^{Shc}$ have been found under pathological conditions and well correlated with oxidative stress in cardiovascular disease [43]. When exposed to OxLDL, ECs increase phosphorylation of $p66^{Shc}$, an effect that is prevented by blockade or molecular silencing of LOX-1 [43], supporting a role for LOX-1 also in this OxLDL-mediated effect. On the other hand, lysophosphatidylcholine (LPC), a relevant component of OxLDL, induced $p66^{Shc}$ activation independently of LOX-1 [43].

In HCAECs, OxLDL promotes intracellular ROS production and induces DNA oxidative damage [44], resulting in the regulation of several transcription factors, including NF-κB and octamer-binding transcription factor-1 (Oct-1). Oct-1 acts as a transcriptional repressor for endothelial enzymes involved in the production of vasoactive molecules, thus providing a link between oxidative DNA damage and impaired function of endothelium upon exposure to OxLDL. Oct-1 is also involved in OxLDL-induced LOX-1 promoter activation and gene expression [45]. Inhibition of LOX-1 attenuates OxLDL-mediated endothelial DNA damage, Oct-1/DNA binding, and reverses impaired production of vasoactive compound [44].

2.6. Other Effects of OxLDL Mediated by LOX-1 in ECs. Incubation of ECs with OxLDL modulates the expression of several other genes associated with atherosclerosis, and LOX-1 plays a crucial role in mediating such effects.

Metalloproteinases (MMPs) are a family of matrix degrading enzymes involved in vascular remodeling that contribute to the determination of atherosclerotic plaque stability; their expression and activity are increased in atherosclerotic plaques [46]. OxLDL increases the expression of MMP-1 and MMP-3 mRNA and protein in ECs, without affecting the expression of tissue inhibitor of metalloproteinases (TIMPs) [47], suggesting an OxLDL-induced imbalance between MMPs and TIMPs. LOX-1 activation mediates the modulation of MMPs by OxLDL: LOX-1-blocking antibody prevents the increase of MMPs in response to OxLDL [47]. Similarly, OxLDL enhances MMP-9 production in human aortic ECs, and anti-LOX-1 antibody inhibits this effect [48].

Angiogenesis is a highly regulated physiological process involved in several pathological conditions including inflammation and atherosclerosis and requires disruption of cell-cell contact, EC migration, proliferation, and capillary tube formation. Generation of small amount of ROS, as those induced by low concentrations of OxLDL (<5 μg/mL), seems to be involved in this process. Low concentrations of OxLDL stimulate tube formation from ECs *in vitro*. This effect is inhibited by anti-LOX-1 antibody, that also decreases OxLDL-induced ROS generation, NF-κB activation, and upregulation of vascular endothelial growth factor (VEGF) [49].

The plasminogen activator (PA) is involved in the control of fibrinolysis within the vascular lumen; plasminogen activator inhibitor-1 (PAI-1) attenuates fibrinolysis through inhibition of plasminogen activation and modulates cellular responses; increased expression of PAI-1 has been described in atherosclerosis [50]. OxLDL induces PAI-1 upregulation in cultured ECs through LOX-1, as showen by the inhibitory effect of anti-LOX-1-blocking antibody; this finding suggests an involvement of LOX-1 also in OxLDL-induced thrombotic process [51].

The LDL receptor regulates plasma LDL-cholesterol levels. OxLDL decreases the expression of LDL receptor in a concentration- and time-dependent manner in HCAECs [52]. This effect is mediated by LOX-1, as cell pretreatment with LOX-1-blocking antibody or with antisense to LOX-1 mRNA reduces the effect of OxLDL on LDL-receptor expression [52].

2.7. LOX-1 Mediates OxLDL-Induced Effects in Endothelial Progenitor Cells. Endothelial progenitor cells (EPCs) are involved in the regeneration of the injured endothelium and in the neovascularization process, which represents a compensatory mechanism in ischemic disease; atherosclerosis is associated with reduced numbers and impaired functionality of EPCs [53]. OxLDL has a negative effect on EPCs, induces EPC senescence, inhibits VEGF-induced EPC differentiation, decreases EPC number, and impairs their function [54–56]. LOX-1 is expressed in EPCs, and incubation of EPCs with OxLDL increases LOX-1 expression [57], an effect depending on the interaction between OxLDL and LOX-1, as confirmed by the use of an anti-LOX-1 antibody. OxLDL also induces EPC apoptosis in a dose-dependent manner, thus reducing their survival; moreover, OxLDL impairs EPC adhesive, migratory, and tube formation capacities [57]. All these effects are attenuated by pretreatment with a LOX-1 monoclonal antibody. Furthermore, OxLDL reduces eNOS expression and NO production, that may in part explain the inhibitory effect of OxLDL on EPC survival and function [57]. Pretreatment with anti-LOX-1 antibody inhibits all these OxLDL-induced effects.

At low concentrations (<10 μg/mL) OxLDL does not induce apoptosis but accelerates EPC senescence; this effect is significantly attenuated by LOX-1-blocking antibody or by atorvastatin (that reduces LOX-1 expression) [54]. OxLDL significantly reduces telomerase activity (which plays a critical role in cellular senescence) and impairs proliferation and network formation capacity, resulting in cellular dysfunction [54].

3. Role of LOX-1 in OxLDL-Induced Smooth Muscle Cell Proliferation and Apoptosis

LOX-1 is expressed also in smooth muscle cells (SMCs) [6]; *in vitro*, several stimuli, including OxLDL and Ang II, can upregulate LOX-1 expression in SMCs [58–60]; *in vivo*, LOX-1 protein is not detectable in the media of noninjured aorta but is present in SMCs two days after vascular injury or after balloon-angioplasty [61]. OxLDL and LOX-1 colocalize with SMCs of human restenotic lesions, suggesting a role for LOX-1 in OxLDL-induced SMC proliferation and restenosis [61].

MicroRNAs are small noncoding RNAs that negatively modulate gene expression through the binding with their mRNA and play a role also in atherosclerosis [60]. MicroRNA let-7g targets LOX-1 gene and inhibits its expression; OxLDL, by inducing the transcription factor Oct-1, reduces let-7g expression. Let-7g mimic reduces OxLDL-induced LOX-1 and Oct-1 upregulation as well as OxLDL-enhanced SMC proliferation and migration [60].

At higher concentrations, OxLDL upregulates LOX-1 expression and induces apoptosis of vascular SMCs [62] (Figure 3), a process that may contribute to atherosclerotic plaque destabilization. OxLDL-induced apoptosis is a consequence of LOX-1 upregulation, as apoptosis is inhibited by a neutralizing anti-LOX-1 antibody. Furthermore, OxLDL increases the expression of the proapoptotic protein Bcl-2-associated X protein (Bax) and inhibits the expression of the antiapoptotic Bcl2. This effect is mediated by LOX-1, as anti-LOX-1 antibody markedly inhibits OxLDL-induced modulation of these two proteins [62]. LOX-1 colocalizes with Bax in human atherosclerotic plaques, particularly in rupture-prone shoulder region, suggesting a role for LOX-1 in the mechanisms that contribute to plaque destabilization [62].

Since SMCs can transform into foam cells, LOX-1 might be a potential player in this process (Figure 3). Treatment of SMCs with LPC increases LOX-1 expression [63], with consequent LOX-1-mediated increase of OxLDL uptake; anti-LOX-1 antibody markedly inhibits the LPC-enhanced OxLDL uptake [63].

Bone marrow cells can potentially originate smooth muscle progenitor cells (SMPCs) that may differentiate into smooth muscle-like cells (SMLCs) in the damaged vessels [64], thus contributing to the atherogenic process. *In vitro*, long-term culture with platelet-derived growth factor PDGF-BB induces the differentiation of SMPCs into SMLCs that express SMC-specific markers [65]; OxLDL inhibits PDGF-BB-induced differentiation of SMLCs, as revealed by the decrease of SMC marker expression in the presence of OxLDL. Furthermore, OxLDL incubation induces lipid droplet accumulation in the cytoplasm and LOX-1 surface expression [65]. Anti-LOX-1 antibody significantly reduces OxLDL uptake by SMLCs, suggesting a role for LOX-1 in the transdifferentiation of SMLCs into foam-like cells [65].

4. Role of LOX-1 in OxLDL-Induced Effects in Macrophages

Macrophages internalize OxLDL by several SRs including SR-AI/II, SR-BI, cluster of differentiation 36 (CD36), and LOX-1, resulting in lipid accumulation and transformation into foam cells. LOX-1 is not detectable in freshly isolated human monocytes, but its expression increases in differentiated macrophages [66]. LOX-1 expression in macrophages can be upregulated by several stimuli, including OxLDL, LPC, high-glucose levels, and proinflammatory cytokines [8]. The contribution of LOX-1 in macrophage uptake and degradation of OxLDL under normal conditions is small. No significant differences are observed between wild-type and LOX-1 deficient macrophages [67], probably due to the high expression of other SRs that could mask the contribution of LOX-1. However, in cells stimulated with LPC, LOX-1 expression and OxLDL uptake and degradation increase in wilt type cells but not in LOX-1-deficient cells [67]. These observations suggest that LOX-1 gene inactivation does not markedly modify OxLDL uptake in unstimulated macrophages, as LOX-1 accounts for 5–10% of OxLDL uptake by these cells, but, when LOX-1 is upregulated, internalization of OxLDL increases by more than 40% [67] (Figure 2). Proinflammatory cytokines upregulate LOX-1 and downregulate other SRs (SR-AI/II and CD36), suggesting that, in inflamed microenvironments, where these cytokines are relatively abundant (such as in atherosclerotic lesions), LOX-1 might play a significant role in macrophage OxLDL uptake.

Monocytes may also differentiate into dendritic cells (DCs), a specific type of leukocytes that play a key role in the initiation of innate and adaptive immune responses, and OxLDL affects both DC maturation and migration [68, 69]. LOX-1 is highly expressed on mature DCs and significantly contributes to OxLDL uptake, as anti-LOX-1 antibody reduced OxLDL uptake by 48% [70].

5. Role of LOX-1 in Platelet Activation

Platelets express several SRs, some of which are constitutively expressed (including CD36), while LOX-1 appears on the surface of platelets on activation [71]; thus, in resting platelets, CD36 mediates OxLDL binding to platelets, while, in activated platelets, in which OxLDL binding is increased compared to resting cells, binding of OxLDL is mediated by both CD36 and LOX-1 [71]. Activated platelets internalize significant amount of OxLDL compared with resting platelets [72] (Figure 3); OxLDL induces platelet activation, increases their ability to adhere to ECs, induces an inflammatory response, and leads to platelet accumulation after vascular injury. In fact, OxLDL-positive platelets induce adhesion molecule expression on ECs, reduce regeneration of ECs, and induce foam cell formation [72], suggesting that OxLDL-activated platelets may contribute to vascular inflammation by several mechanisms.

Dysfunctional endothelium exhibits procoagulant and adhesive properties to platelets, and endothelial LOX-1 plays a major role in the platelet-endothelium interaction thus enhancing endothelial dysfunction. In fact LOX-1 binds also anionic phospholipids, including those present on the surface of apoptotic cells or activated platelets [73], thus working as an adhesion molecule for platelets. OxLDL partially inhibits the binding of platelets to LOX-1, indicating a high affinity of platelets for this receptor [73]. The binding of activated platelets to endothelial LOX-1 induces the release of ET-1, further supporting the induction of endothelial dysfunction [73]. Furthermore, the binding of activated platelet to endothelial LOX-1 induces ROS generation followed by a reduction of NO bioavailability; all effects are prevented by anti-LOX-1 antibody [74].

6. LOX-1 and Atherosclerosis: Experimental Evidences

As previously described, LOX-1 mediates many of the effects of OxLDL, that is, EC growth, dysfunction, adhesion, and activation of monocytes/macrophages, and all critical features of atherosclerosis. LOX-1 mRNA has also been found in human atherosclerotic plaques, while negligible amounts are detectable in unaffected aortas [11]. The key relevance of LOX-1 in atherosclerosis has been demonstrated by key experiments in LOX-1 KO mice crossed to a mouse model prone to develop atherosclerosis such as the LDL-R KO mouse. Feeding these mice with a cholesterol-rich diet results in a reduced binding of OxLDL to the aortic endothelium and a preservation of endothelium-dependent vasorelaxation after treatment with OxLDL compared to wild type animals [12]. More importantly, LOX-1 KO/LDL-R KO animals show a significant reduction in atherosclerosis compared with LDL-R KO mice [12]. This is associated with reduced NF-κB expression as well as decreased inflammatory markers and also with an increased eNOS expression, suggesting an improved endothelial function [12]. Conversely, mice overexpressing LOX-1 on ApoE KO background show a dramatic increase in atherosclerosis compared to the nontransgenic mice [13]. In hypercholesterolemic rabbits, LOX-1 expression is detected mainly in atherosclerotic plaques with a thinner fibromuscular cap and is localized to the lipid core where it correlates with tissue factor expression and apoptosis [75], potentially connecting LOX-1 with plaque destabilization and rupture. In line with this, LOX-1 expression is increased in unstable plaques when the AMI-prone Watanabe heritable hyperlipidemic rabbit and control rabbits are injected with an antibody tracing LOX-1 [76].

Among the critical player in atherogenesis, the activation of the rennin-angiotensin system and the consequent generation of Ang II are thought to be critical factors. Ang II via its type 1 receptor (AT1 receptor) also upregulates the expression of LOX-1 mRNA, and OxLDL via LOX-1 upregulates the expression of AT1 receptor [77]. Of note hypertensive rats present an upregulation of LOX-1 expression mainly in vascular ECs [78–80]. The mutually facilitatory cross-talk between LOX-1 and AT1 receptors may explain the coexistence of multiple risk factors in the same patient and the increase in atherosclerosis risk with the presence of multiple risk factors. In agreement with this assumption, LOX-1 expression

is increased in several animal models of cardiometabolic disorders, including streptozotocin-induced diabetes [81] or ischemia-reperfusion (I/R) injury [82]. LOX-1 deficiency is associated with a more preserved left ventricular function following I/R injury which resulted in a reduced oxidative stress, collagen deposition, and fibronectin expression thus resulting in a significant decrease in myocardial injury as well as in the accumulation of inflammatory cells [83, 84].

Altogether, the results from experimental atherosclerosis support a proatherogenic role for LOX-1.

7. Genetics of LOX-1 and Atherosclerosis

LOX-1 is encoded by the oxidized low-density lipoprotein (lectin-like) receptor 1 (OLR1) gene, mapped to chromosome 12p13 [85]. The association of polymorphisms in the human OLR1 gene with the susceptibility to myocardial infarction (MI) has been reported [85, 86]. In particular, six single nucleotide polymorphisms (SNPs), located within introns 4, 5, and 3′ UTR (untranslated region), that are comprised in a linkage disequilibrium (LD) block are strongly associated with the elevated risk to develop MI [86]. The observation that the SNPs related to an increased risk of MI do not affect the coding sequence of the gene suggests the possibility that the SNPs could give rise to a functional product such as messenger RNA (mRNA) isoforms as a consequence of alternative splicing. Indeed it has been shown that the SNPs located in the LD block regulate the level of the new fully functional transcript by modulating the retention of exon 5 of the OLR1 gene [85]. The alternative splicing of OLR1 mRNA leads to different ratios of LOX-1 full receptor and LOXIN (lack of exon 5), an isoform lacking part of the C-terminus lectin-like domain which is a functional domain. LOXIN, through heterooligomerization with LOX-1 [87], blocks the negative effects of LOX-1 activation, and this variant has a functional role on plaque instability and therefore in the pathogenesis of MI [86]. *Ex vivo* data show that macrophages from subjects carrying the "nonrisk" allele at OLR1 gene display increased expression of LOXIN resulting in protection against OxLDL-mediated apoptosis [87].

Later, an SNP on exon 4, rs11053646 (G501C), which leads to an amino acidic substitution (lysine to asparagine at position 167, K167N) has been studied. Of note, basic residues in the lectin domain are important for strengthening the ligand binding; substitution of this residue (K167N) causes a change on the positive isopotential surface and thereby results in reduced binding and internalization of OxLDL [88]. This study, performed on CV-1 (simian) in Origin and carrying the SV40 (COS-1) cells overexpressing either GG (KK) or CC (NN) LOX-1, showed that GG (KK) COS-1 cells bind and internalize less OxLDL than CC (NN) [88].

The K167N SNP has been identified among others in the *ORL-1* gene to be associated with acute MI and coronary artery disease (CAD) [89–91]. The frequencies of the KK genotype and the K allele are higher in the CAD group than in controls ($P < 0.05$), while the opposite is true for NN genotype ($P < 0.05$) [92]. The relevance of LOX-1 SNPs has been tested also in relation to markers of atherosclerosis. Among them, ultrasound detection and quantification of the common carotid artery wall thickness (intima-media thickness, IMT) are considered a surrogate marker of subclinical atherosclerosis [93, 94]. The association between the nonsynonymous substitution K167N (rs11053646) and IMT has been tested in 2,141 samples from the Progression of Lesions in the Intima of the Carotid (PLIC) study (a prospective population-based study) [95]. Significantly increased IMT has been observed in male carriers of the minor C (N) allele compared to GC and GG (KN and KK) genotypes [95]. A gender-specific association has been also described between the C (N) allele and prevalence of carotid plaque also in a cohort of Dominican-Hispanic origin [96].

Functional analysis on macrophages suggests a decreased association to OxLDL in NN carriers compared to KN and KK carriers which is also associated with a reduced OLR1 mRNA expression [95]. Macrophages from NN carriers present also a specific inflammatory gene expression pattern compared to cells from KN and KK carriers [95]. How these data, obtained with human primary macrophages, relate with those obtained in a transfected fibroblast-like cell line derived from monkey kidney tissue is unclear, and further studies are warranted to clarify this issue [88].

In summary, genetic alterations favoring LOXIN isoform production coupled to the observation that this isoform exerts a dominant-negative effect on LOX-1 function make it an attractive new target for prevention and treatment of initiation, progression, and clinical consequences of atherosclerosis such as plaque instability, acute myocardial infarction, and ischemia reperfusion injury.

8. Concluding Remarks

OxLDL plays multiple roles in atherosclerosis; LOX-1 scavenger receptor mediates many of the OxLDL-induced effects, and the OxLDL-LOX-1 interaction can alter the expression of several genes, resulting in the induction of cellular dysfunction, proliferation, and apoptosis. A recent study showed that LOX-1 inhibition in ApoE KO mice using a schizophyllan-based antisense oligonucleotide therapy resulted in reduced LOX-1 expression in the arterial wall [97]. Blocking the expression and/or function of LOX-1 results in the improvement of cellular functions and reduction of atherosclerotic lesion formation, suggesting that LOX-1 might be an attractive therapeutic target for the management and the prevention of atherosclerosis.

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Research Article

Activation of TRPV1 Prevents OxLDL-Induced Lipid Accumulation and TNF-α-Induced Inflammation in Macrophages: Role of Liver X Receptor α

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The transient receptor potential vanilloid type 1 (TRPV1) is crucial in the pathogenesis of atherosclerosis; yet its role and underlying mechanism in the formation of macrophage foam cells remain unclear. Here, we show increased TRPV1 expression in the area of foamy macrophages in atherosclerotic aortas of apolipoprotein E-deficient mice. Exposure of mouse bone-marrow-derived macrophages to oxidized low-density lipoprotein (oxLDL) upregulated the expression of TRPV1. In addition, oxLDL activated TRPV1 and elicited calcium (Ca^{2+}) influx, which were abrogated by the pharmacological TRPV1 antagonist capsazepine. Furthermore, oxLDL-induced lipid accumulation in macrophages was ameliorated by TRPV1 agonists but exacerbated by TRPV1 antagonist. Treatment with TRPV1 agonists did not affect the internalization of oxLDL but promoted cholesterol efflux by upregulating the efflux ATP-binding cassette (ABC) transporters ABCA1 and ABCG1. Moreover, the upregulation of ABC transporters was mainly through liver X receptor α- (LXRα-) dependent regulation of transcription. Moreover, the TNF-α-induced inflammatory response was alleviated by TRPV1 agonists but aggravated by the TRPV1 antagonist and LXRα siRNA in macrophages. Our data suggest that LXRα plays a pivotal role in TRPV1-activation-conferred protection against oxLDL-induced lipid accumulation and TNF-α-induced inflammation in macrophages.

1. Introduction

Complications of atherosclerosis are the leading cause of death in Western society. Atherosclerosis starts with increased circulating cholesterol levels and involves several events leading to chronic vascular inflammation [1, 2]. The initiation and progression of atherosclerosis largely depend on the function of macrophage foam cells, which focally accumulate within the artery wall and release various cytokines and chemokines to induce inflammation [2–4]. The formation of foam cells primarily results from uncontrolled uptake of modified low-density lipoprotein (LDL) by macrophages, which leads to excess lipoprotein-derived lipid accumulation inside cells and induction of proinflammatory mediators [3, 4]. Cellular lipid levels of foam cells are dynamically regulated by macrophage scavenger receptors (SRs) and cholesterol efflux transporters. The internalization of oxidized LDL (oxLDL) by macrophages is controlled by several types of SRs, including SR-A and CD36. Conversely, cholesterol efflux is mediated by SR-BI and ATP-binding cassette (ABC) transporters such as ABCA1 and ABCG1 [5–10]. Growing evidence indicates that preventing the formation of foam cells can impede the progression of atherosclerosis [11–13], but much remains to be explored.

The transient receptor potential (TRP) vanilloid type 1 (TRPV1), a ligand-gated nonselective cationic channel, is mainly expressed in primary nociceptive sensory neurons

[14, 15]. Neuronal TRPV1 is activated by heat, protons, endogenous lipid molecules and oxidative stimuli [14–19] and exogenous agonists such as evodiamine (an active ingredient of the evodia fruit) and capsaicin (an active ingredient of hot pepper) [16, 20]. Activation of TRPV1 permits calcium (Ca^{2+}) entry, which results in an elevated level of intracellular Ca^{2+} and serves as a Ca^{2+} signal to elicit responses in neurons [14, 15].

TRPV1 expresses in certain types of nonneuronal cells such as epithelial and endothelial cells and regulates several of their pathophysiological functions [21–24]. Emerging evidence suggests that TRPV1 may be an important sensor and regulator of cardiovascular homeostasis and a protector against certain cardiovascular diseases such as hypertension, endothelial dysfunction, and stroke [25, 26]. Recently, we reported that deletion of TRPV1 worsened atherosclerotic lesions in apolipoprotein E-deficient mice ($ApoE^{-/-}$, a model of atherosclerosis-prone mice), so TRPV1 may have a role in atherogenesis [27]. However, the expression of TRPV1 in macrophages is still unclear and its functional significance in macrophage foam cells remains largely unknown.

In this study, we aimed to address the role of TRPV1 in the pathophysiological functions of macrophage foam cells. We first examined TRPV1 expression in macrophages and its distribution in mouse atherosclerotic lesions, then, investigated the therapeutic effects of 2 TRPV1 agonists, evodiamine, and capsaicin, on the deregulation of lipid metabolism and inflammation in macrophages. Finally, we explored the molecular mechanisms underlying the protection conferred by TRPV1 agonists in macrophages.

2. Materials and Methods

2.1. Reagents. Evodiamine, human LDL, capsaicin, capsazepine, apolipoprotein AI (apoAI), high-density lipoprotein (HDL), EGTA, Oil-red O, and mouse antibody for α-tubulin were from Sigma-Aldrich (St. Louis, MO, USA). Mouse antibody for TRPV1 was from Abnova (Taoyuan, Taiwan). Rabbit antibodies for ABCG1, CD36, histone H1, goat antibody for SR-A, control small interfering RNA (siRNA), and LXRα siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse antibody for ABCA1, α-actin, and rabbit antibodies for SR-BI, LXRα, and F4/80 were from Abcam (Cambridge, MA, USA). Macrophage colony-stimulating factor (MCSF), tumor necrosis factor α (TNFα), and ELISA kits were from R&D systems (Minneapolis, MN, USA). Dil-labeled oxLDL was from Biomedical Technologies (Stoughton, MA, USA). NBD-cholesterol and T0901317 were from Cayman Chemical (Ann Arbor, MI, USA). The Fluo-8 Ca^{2+} assay kit was from ABD BioQuest (Sunnyvale, CA, USA). Cholesterol and triglyceride assay kits were from Randox (Crumlin, Co. Antrim, UK).

2.2. Mice. The investigation conformed to the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and all animal experiments were approved by the Animal Care and Utilization Committee of National Yang-Ming University. Wild-type (WT) C57BL/6 mice were purchased from the National Laboratory Animal Center, National Science Council (Taipei)

2.3. Cell Culture. Bone-marrow-derived macrophages (BMDMs) were prepared as described [28]. Briefly, WT mice were killed by CO_2 exposure, and mononuclear cells from femurs were harvested by Percoll (1.073 g/cm^3) density gradient centrifugation. The cells were seeded in minimum essential medium α (MEMα) supplemented with 50 ng/mL MCSF, 10% fetal bovine serum (FBS), and penicillin (100 U/mL)/streptomycin (100 g/mL) at 37°C for 5 d. Mouse macrophage-like J774.A1 cells (Bioresource Collection and Research Center; Hsinchu, Taiwan) were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL).

2.4. LDL Modification. LDL was exposed to $CuSO_4$ (5 μmol/L) for 24 h at 37°C and Cu^{2+}, then, removed by extensive dialysis. The extent of modification was determined by measuring thiobarbituric acid-reactive substances (TBARs). OxLDL containing approximately 30–60 nmol of TBARs defined as malondialdehyde equivalents per milligram LDL protein was used for experiments.

2.5. Western Blot Analysis. BMDMs were rinsed with PBS, then, lysed in immunoprecipitation lysis buffer (50 mmol/L Tris pH 7.5, 5 mmol/L EDTA, 300 mmol/L NaCl, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin). Aliquots (50 μg) of cell lysates were separated on 8% SDS-PAGE. After transfer to membranes, samples were immunoblotted with primary antibodies, then, horseradish peroxidase-conjugated secondary antibodies. Bands were revealed by use of an enzyme-linked chemiluminescence detection kit (Perkin Elmer, Waltham, MA, USA), and density was quantified by use of ImageQuant 5.2 (Healthcare Bio-Sciences, Philadelphia, PA, USA).

2.6. Measurement of $[Ca^{2+}]_i$ Level. Ca^{2+} assay was performed according to the manufacturer's protocol (ABD BioQuest, Sunnyvale, CA, USA). Briefly, BMDMs were seeded in 24-well plates and grown for 24 h. Cells were then washed and Fluo-8 NE dye-loading solution was added for 1 hr at room temperature. Medium was then replaced with fresh medium containing test compounds. Fluorescence was measured by fluorometry (Molecular Devices, Sunnyvale, CA, USA) with 490 nm excitation and 525 nm emission.

2.7. Oil-Red O Staining. Cells were fixed with 4% paraformaldehyde and then stained with 0.5% Oil-red O. Hematoxylin was used for counterstaining.

2.8. Dil-OxLDL Binding Assay. Dil-oxLDL, labeled with green fluorescence, has been used to measure oxLDL binding to SRs of macrophages [29]. Briefly, BMDMs were treated with concentrations of evodiamine or capsaicin for 24 h, then, incubated with Dil-labeled oxLDL (10 μg/mL) for an

additional 4 h at 4°C. After a washing with phosphate-buffered saline (PBS), cell lysates were analyzed by fluorometry (Molecular Devices, Sunnyvale, CA, USA) at 540 nm excitation and 590 nm emission.

2.9. Cholesterol Efflux Assay. BMDMs were treated with concentrations of evodiamine or capsaicin for 12 h, then, underwent equilibration with NBD-cholesterol (1 μg/mL) for an additional 6 h. NBD-cholesterol-labeled cells were washed with PBS and incubated in MEMα for 6 h with apoAI (10 μg/mL) or HDL (50 μg/mL). Fluorescence-labeled cholesterol released from cells into the medium was measured by use of a multilabel counter (PerkinElmer, Waltham, MA, USA) at 485 nm excitation and 535 nm emission. Cholesterol efflux was expressed as percentage fluorescence in the medium relative to total fluorescence (cells and medium).

2.10. Preparation of Nuclear Extracts. Nuclear extracts were prepared as described [30]. BMDMs were lysed in 10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5% Nonidet P-40, 1 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Nuclei were pelleted at 5000 g for 5 min at 4°C, and the resulting supernatant was used as the cytosolic fraction. Nuclei were resuspended in lysis buffer, sheared for 15 sec by microprobe sonication, and incubated on ice for 5 min. After centrifugation at 12000 g for 5 min at 4°C, supernatant was collected as the nuclear extract.

2.11. Chromatin Immunoprecipitation (ChIP). ChIP assays were performed as described [12]. BMDMs were cultured in MEMα with or without pretreatment with evodiamine (0.5 μM) or capsaicin (10 μM) for 6 h and fixed by formaldehyde for 15 min at room temperature. After cells were lysed and sonicated, chromatin solution was diluted and cells were incubated overnight with rabbit anti-LXRα Ab or rabbit IgG at 4°C. Immunocomplexes were precipitated with salmon sperm DNA/protein A agarose and collected by centrifugation. After cells were washed, chromatin DNA was eluted, purified, and subjected to PCR analysis. An amount of 1% chromatin solution was used as an input control. The mouse *ABCA1* gene promoter containing LXR binding element was amplified by PCR with the following primer sequences: 5′-CCA CGT GCT TTC TGC TGA GT-3′ and 5′-TGC CGC GAC TAG TTC CTT TT-3′. PCR products were resolved on a 2% agarose gel and visualized by ethidium bromide staining.

2.12. Transient Transfection and Luciferase Reporter Assay. Cells were transfected with the plasmids phABCA1 (-928)-Luc, a reporter plasmid for the human ABCA1 promoter with LXRα responsive element (LXRE, 3′-AAACTGGC TATCATTGGA GACGCG-5′) or phABCA1-DR4 m-Luc, a reporter plasmid with a mutation in the LXRE (3′-AAACACAC TATCATTGAT GACGCG-5′), by use of TurboFect. The pGL3-renilla plasmid was cotransfected as a transfection control. After transfection for 24 h, cells were treated with evodiamine (500 nM), capsaicin (10 μM), or T0901317 (10 μM), an LXR agonist, for another 24 h. Cells were then lysed for Luc and renilla activity assays.

2.13. Small Interfering RNA Transfection. Macrophages were transfected with control or LXRα siRNA with use of TurboFect for 24 h and then treated with evodiamine or capsaicin for another 24 h before further experiments.

2.14. Measurement of Inflammatory Cytokines. The levels of proinflammatory cytokines, including monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), and macrophage inflammatory protein-2 (MIP-2), in culture medium were measured by use of ELISA kits.

2.15. Statistical Analysis. Results are presented as mean ± SD from 5 independent experiments. Mann-Whitney test was used to compare 2 independent groups. The Kruskal-Wallis test followed by Bonferroni post-hoc analysis was used to account for multiple testing. SPSS v20.0 (SPSS Inc., Chicago, IL) was used for analysis. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Expression of TRPV1 in Macrophages and Atherosclerotic Lesions. To study the possible role of TRPV1 in atherogenesis, we first investigated the expression of TRPV1 in atherosclerotic lesions. The protein level of TRPV1 was markedly higher in $ApoE^{-/-}$ than wild-type mouse aortas (Figure 1(a)). In addition to the expression of TRPV1 in aortic ECs, immunohistochemical staining for TRPV1 demonstrated positive signals confined mainly to areas of macrophages in atherosclerotic lesions of $ApoE^{-/-}$ mice (Figure 1(b)). Because neuronal TRPV1 can be activated by several oxidative stimuli and lipids [14, 18, 19, 24], we next examined the effect of oxLDL on the expression of TRPV1 in macrophages. Treating BMDMs with 50 μg/mL oxLDL for up to 24 h time-dependently increased the expression of TRPV1 (Figure 1(c)) as early as 3 h after treatment or up to 24 h. Thus, TRPV1 may play an important role in the development of atherosclerosis.

3.2. OxLDL Upregulates and Activates TRPV1 in BMDMs. We next investigate the stimulatory effect of oxLDL on the channel activity of TRPV1 in BMDMs. In response to oxLDL, the intracellular level of Ca^{2+} ($[Ca^{2+}]_i$) in BMDMs, as reflected by intensity of Ca^{2+}-sensitive Fluo-8 fluorescence, rapidly peaked at 30 sec, slightly decreased at 1 min, and gradually increased to peak again at 4 h (Figure 2(a)). Importantly, the oxLDL-induced increase in $[Ca^{2+}]_i$ level at 30 sec and 4 h poststimulation were prevented by pretreatment with capsazepine (a TRPV1 antagonist) (Figures 2(b) and 2(c)). We then checked the specificity of capsazepine and found that exposure of BMDMs to evodiamine or capsaicin (TRPV1 agonists) also increased $[Ca^{2+}]_i$ level at 30 sec, which was abolished by capsazepine pretreatment (Figure 2(d)).

3.3. Activation of TRPV1 by Agonists Suppresses Lipid Accumulation in Macrophage Foam Cells. We then determined the functional significance of TRPV1 in foam-cell formation in BMDMs. Pretreatment with evodiamine or capsaicin decreased oxLDL-induced lipid accumulation, as revealed

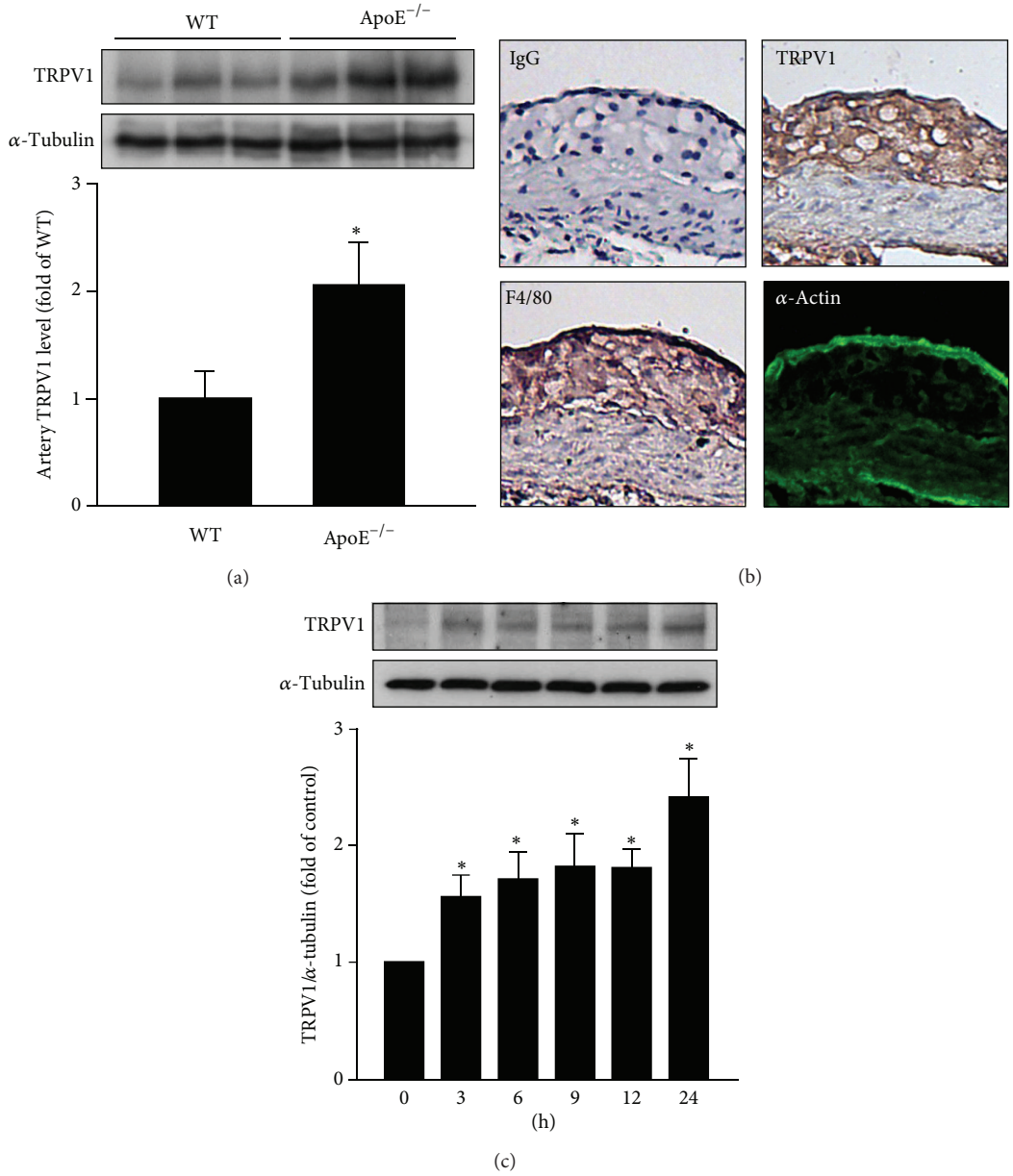

Figure 1: Expression of TRPV1 is increased in atherosclerotic lesions of ApoE$^{-/-}$ mice. (a) Western blot analysis of protein expression of TRPV1 in aortas from 5-month-old ApoE$^{-/-}$ and wild-type (WT) mice. α-Tubulin was a normalization control. Data are mean ± SD from 6 animals. $^{*}P < 0.05$ versus WT mice. (b) Immunohistochemical staining for TRPV1, F4/80 (macrophage marker), and α-actin (smooth-muscle-cell marker) in atherosclerotic lesions of aortas from 5-month-old ApoE$^{-/-}$ mice. Specificity of immunostaining was confirmed with an IgG-negative control. Hematoxylin was used as counterstaining. Magnification = 100 x. (c) Western blot analysis of protein expression of TRPV1 induced by oxLDL (50 μg/mL) relative to that induced by vehicle (PBS) for 0–24 h. Data are mean ± SD from 5 independent experiments. $^{*}P < 0.05$ versus vehicle-treated group. α-Tubulin was a normalization control.

by Oil-red O staining (Figure 3(a)) and cellular levels of cholesterol and triglycerides (Figures 3(b) and 3(c)). In contrast, capsazepine treatment augmented oxLDL-induced lipid accumulation (Figures 3(a)–3(c)).Thus, activation of TRPV1 by agonists may protect against foam-cell formation.

3.4. Activation of TRPV1 by Agonists Enhances Cholesterol Efflux without Altering OxLDL Internalization. We then elucidated the effect of TRPV1 agonists on oxLDL internalization and cholesterol efflux. Pretreating BMDMs with the TRPV1 agonists evodiamine (0.5 μM) or capsaicin (10 μM) did not alter the cholesterol binding but dose-dependently increased the apoAI- or HDL-dependent cholesterol efflux (Figures 4(a)–4(c)). SR-A, CD36, SR-BI, ABCA1, and ABCG1 have crucial roles in cholesterol homeostasis during foam-cell formation [5–10]. We therefore delineated the mechanisms of TRPV1 agonists in attenuating lipid accumulation by examining the alteration in expression of these receptors

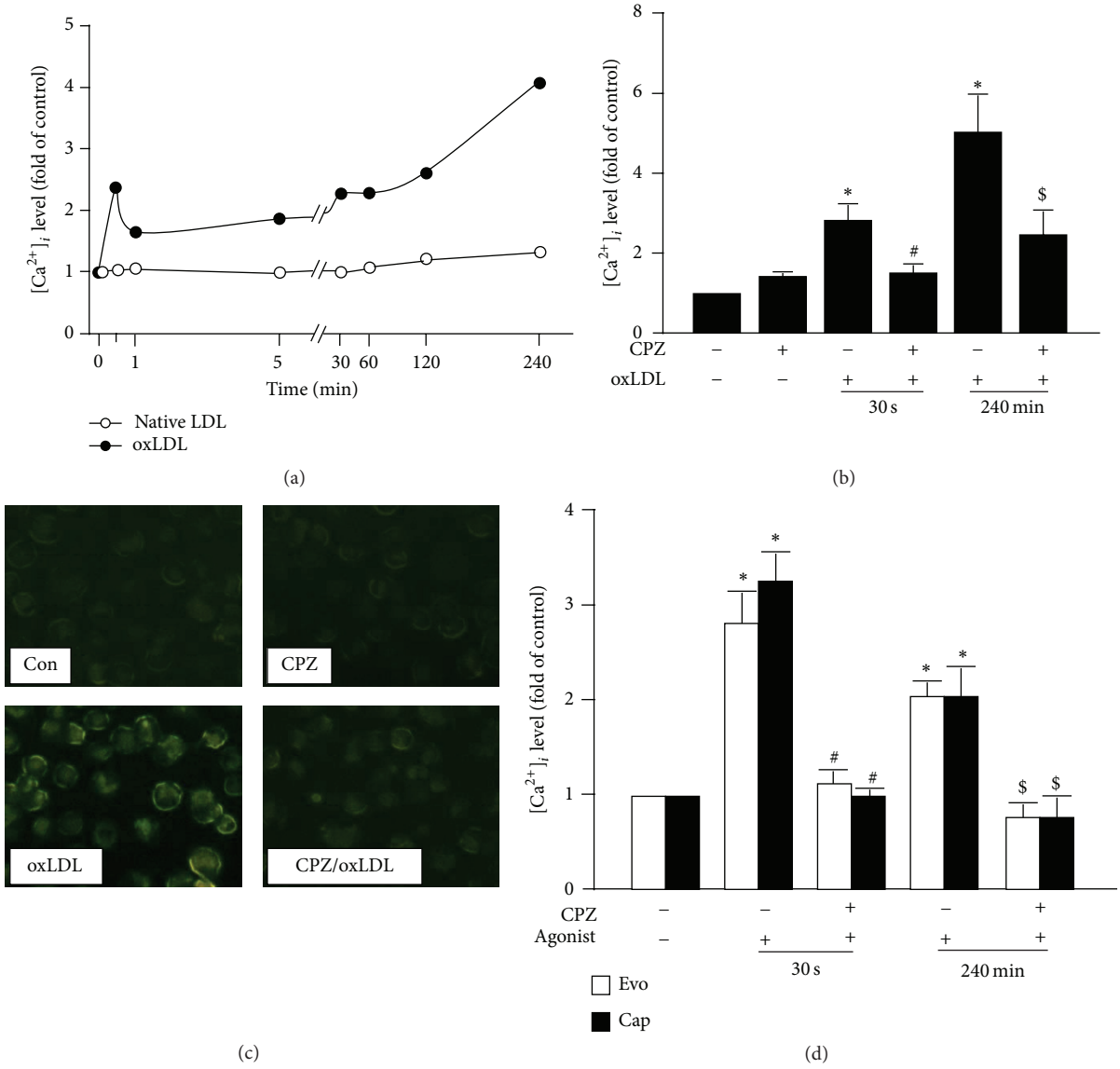

Figure 2: Treatment with oxLDL upregulates and activates TRPV1 in macrophages. (a) Intracellular levels of Ca^{2+} ($[Ca^{2+}]_i$) in response to incubation with oxLDL (50 μg/mL) or native LDL (50 μg/mL). $[Ca^{2+}]_i$ was quantified by measuring the intensity of Ca^{2+}-sensitive Fluo-8 fluorescence. (b) $[Ca^{2+}]_i$ level at 30 sec and 240 min after incubation with oxLDL in BMDMs pretreated or not with capsazepine (CPZ; 10 μM). Data are mean ± SD from 5 independent experiments. $^*P < 0.05$ versus vehicle, $^\#P < 0.05$ versus 30 sec/oxLDL, and $^\$P < 0.05$ versus 240 min/oxLDL. (c) Representative microscopy images of Ca^{2+}-binding Fluo-8 fluorescence at 240 min after incubation with or without oxLDL in BMDMs pretreated or not with capsazepine. (d) $[Ca^{2+}]_i$ level at 30 sec and 240 min after incubation with evodiamine (Evo; 0.5 μM) or capsaicin (Cap; 10 μM) in BMDMs pretreated or not with capsazepine. Data are mean ± SD from 5 independent experiments. $^*P < 0.05$ versus LDL-treated group or vehicle, $^\#P < 0.05$ versus 30 sec/Evo- or Cap-treated group, and $^\$P < 0.05$ versus 240 min/Evo- or Cap-treated group.

and transporters. Macrophages treated with evodiamine or capsaicin showed increased protein levels of ABCA1 and ABCG1, with no change in protein levels of SR-A, CD36, and SR-BI (Figures 5(a) and 5(b)). Therefore, TRPV1 activation suppressing intracellular lipid accumulation is likely due to an increase in reverse- cholesterol- transporter- (RCT-) dependent cholesterol efflux but not inhibition of SR-mediated oxLDL uptake in macrophages.

3.5. LXRα Mediates the Suppressive Effect of TRPV1 Agonists on Foam-Cell Formation. To address whether the LXRα is involved in TRPV1-agonist-induced expression of ABCA1 and ABCG1, we examined the nuclear protein level of LXRα in evodiamine- or capsaicin-treated macrophages. Evodiamine, capsaicin, or T0901317 (LXRα agonist) treatment increased the nuclear level of LXRα (Figure 6(a)) and enhanced binding of LXRα to LXRE in the ABCA1 promoter (Figure 6(b)). Furthermore, to explore the transcriptional regulation of LXRα in evodiamine-treated macrophages, we performed LXR activation assays by transfecting with phABCA1 (-928)-Luc or phABCA1-DR4 m-Luc (reporter plasmid with a mutation in LXRE), followed by evodiamine

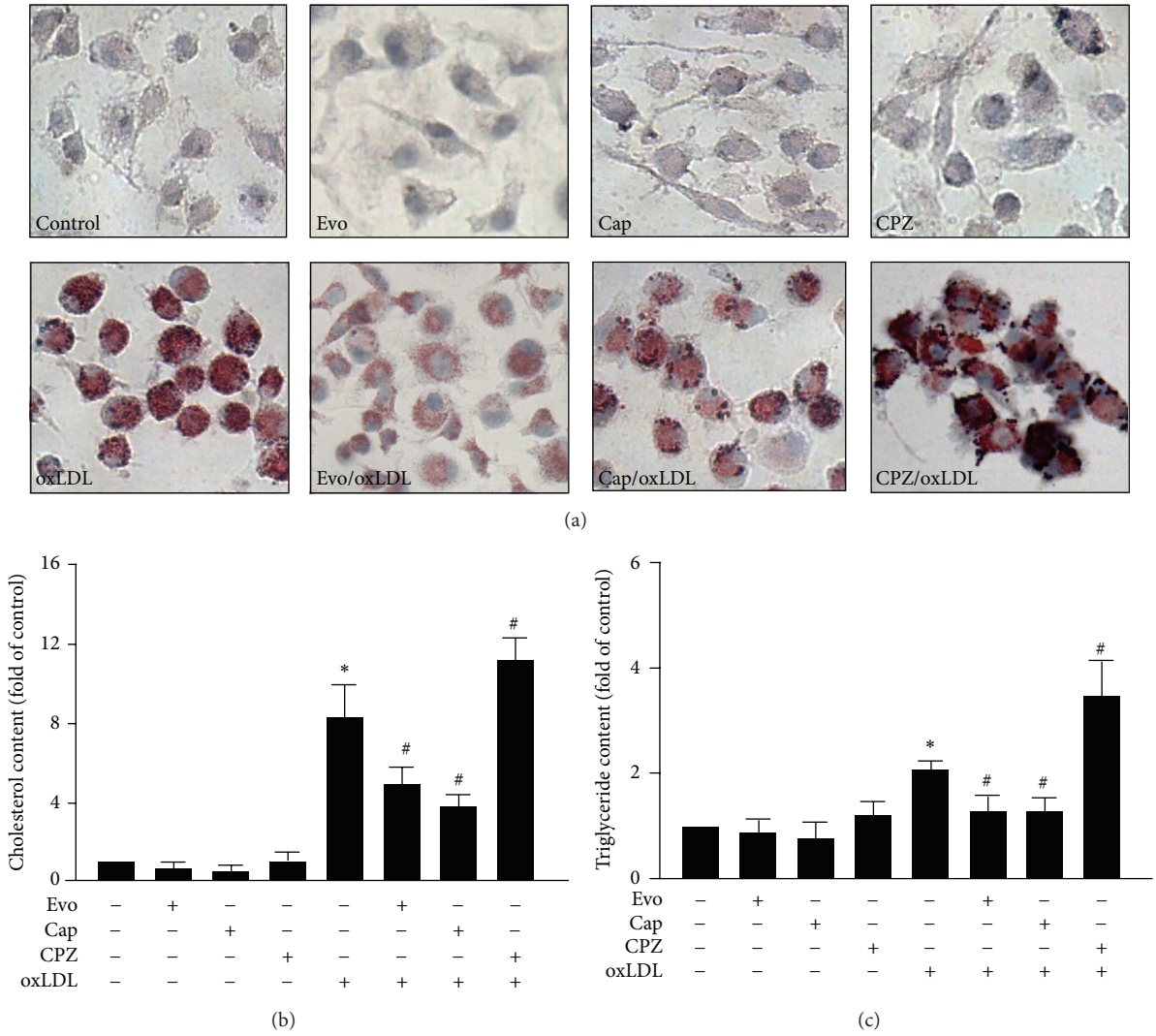

Figure 3: Activation of TRPV1 by agonists alleviates oxLDL-induced foam-cell formation.Cells were incubated with vehicle (DMSO), evodiamine (0.5 μM), capsaicin (10 μM), or capsazepine (10 μM) with or without oxLDL (50 μg/mL). (a) Representative microscopy images of cells with intracellular lipids stained by Oil-red O. Hematoxylin was used as counterstaining. Magnification = 400 x. ((b) and (c)) Intracellular levels of cholesterol (b) and triglycerides (c) were extracted by use of hexane/isopropanol (3/2, v/v) and analyzed by colorimetric assay kits. Data are mean ± SD from 5 independent experiments. $^{*}P < 0.05$ versus vehicle-treated cells, $^{\#}P < 0.05$ versus oxLDL-treated cells.

or capsaicin treatment. As compared with the control group, treatment with evodiamine, capsaicin, or T0901317 as a positive control markedly increased the promoter activity of phABCA1 (-928)-Luc (Figure 6(c)). In contrast to phABCA1-Luc, phABCA1-DR4 m-Luc showed blunted induction with evodiamine, capsaicin, or T0901317 treatment (Figure 6(c)). In addition, transfection with LXRα siRNA decreased the protein expression of LXRα and abolished the mRNA expression of ABCA1 and ABCG1 induced by evodiamine or capsaicin (Figures 7(a) and 7(b)). Moreover, siRNA inhibition of LXR activation abrogated the beneficial effect of evodiamine or capsaicin on apoAI- or HDL-dependent cholesterol efflux (Figure 7(c)). These results indicate the essential role of LXRα activation in evodiamine- or capsaicin-regulated gene expression of ABCA1 and ABCG1, which may contribute to the suppressive effect of the agonists in the transformation of macrophage foam cells.

3.6. Knockdown of LXRα Abolishes TRPV1 Activation-Conferred Protection in the TNF-α-Induced Inflammatory Response. TNF-α is a key proatherogenic mediator for the progression of atherosclerotic lesions [31]. We next examined the effect of TRPV1 agonists on the TNF-α-induced inflammatory response in macrophages. TNF-α-increased production of MCP-1, IL-6, and MIP-2 in BMDMs was significantly attenuated by pretreatment with the 2 TRPV1 agonists; moreover, pretreatment with capsazepine exacerbated the TNF-α-induced production of MCP-1 and IL-6 but not MIP-2 (Figure 8(a)). Additionally, evodiamine or capsaicin suppressing the TNF-α-induced increase in MCP-1, IL-6,

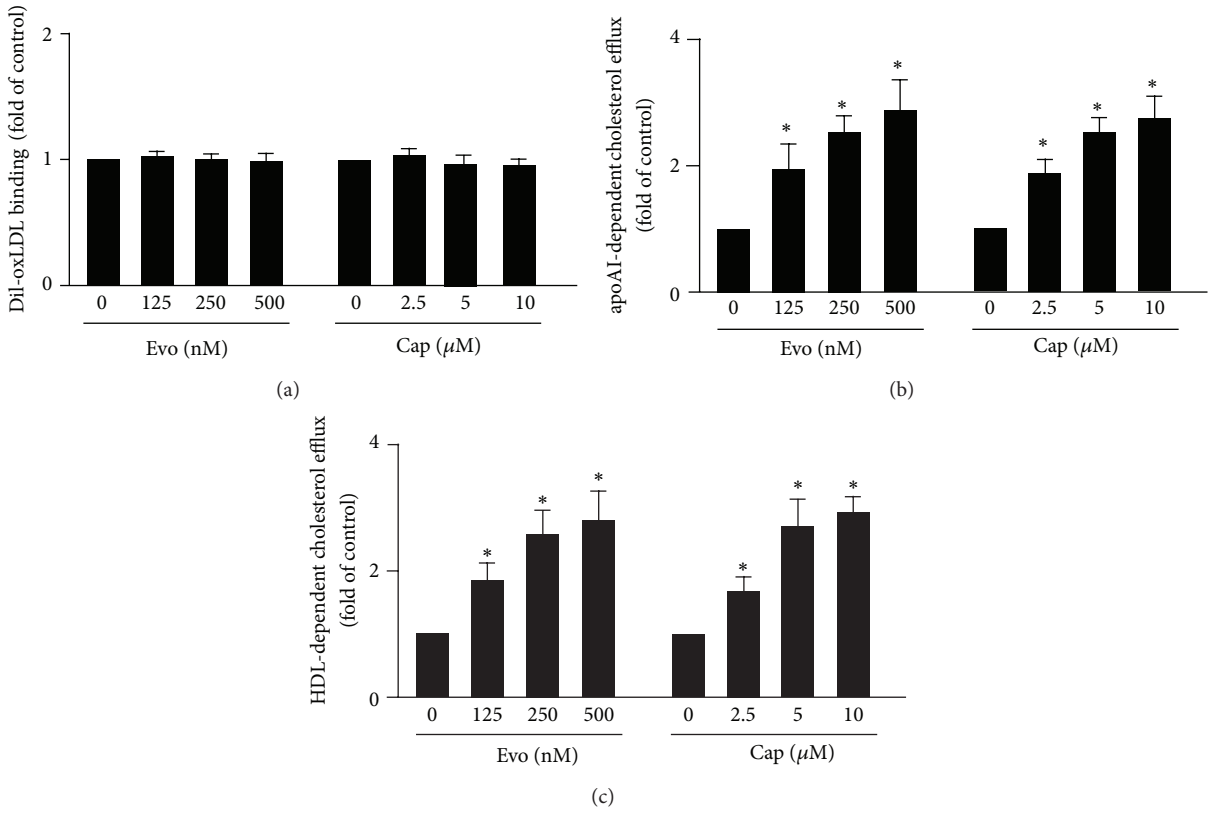

Figure 4: TRPV1 activation by agonists promotes apoAI- and HDL-dependent cholesterol efflux in macrophages. (a) For Dil-oxLDL binding assay, BMDMs were treated with vehicle, evodiamine (125, 250, 500, 500 nM), or capsaicin (2.5, 5, 10 μM) for 12 h and then incubated with 10 μg/mL Dil-oxLDL at 4°C for 4 h. Cellular lysates were analyzed by fluorimetry. ((b) and (c)) BMDMs were treated with indicated concentrations of evodiamine (125, 250, 500, 500 nM) or capsaicin (2.5, 5, 10 μM) for 12 h, followed by NBD-cholesterol (1 μg/mL) for another 6 h in the presence of (b) apoAI (10 μg/mL) or (c) HDL (50 μg/mL). The medium and cell lysates were collected for the measurement of fluorescence. Cholesterol efflux was defined as fluorescence in the medium relative to total amount of fluorescence. Data are mean ± SEM from 5 independent experiments. $^*P < 0.05$ versus vehicle treatment.

and MIP-2 production was reversed by siRNA inhibition of LXRα activation (Figure 8(b)). These results suggest that LXRα activation is required for the anti-inflammatory action of TRPV1 agonists in macrophages.

4. Discussion

Here we characterized a new effect of TRPV1 activation and its underlying molecular mechanism in suppressing oxLDL- or TNF-α-induced deregulation of lipid metabolism and inflammation in macrophages. We first validated TRPV1 expression in atherosclerotic aortas and in particular regions of macrophage-foam cells. The accumulation of macrophage-derived foam cells in the intima and subsequent release of inflammatory cytokines from these cells are 2 critical steps in the initiation and progression of atherosclerosis [1–4]. This cellular localization implies the possible role of TRPV1 in regulating the pathophysiological functions of such cells. We thus used an *in vitro* model to study the role of TRPV1 in macrophage-foam cells. Incubation with evodiamine or capsaicin, TRPV1 agonists, alleviated the oxLDL-induced lipid accumulation and TNF-α-induced inflammation in BMDMs, so the function of TRPV1 is linked to the lipid metabolism and inflammatory response of macrophage-foam cells. Interestingly, the protective effects of TRPV1 agonists may be due to the activation of LXRα. Our *in vitro* data suggest that TRPV1 has a novel effect in maintaining lipid homeostasis and the inflammatory response in macrophages.

We then investigated the molecular mechanisms underlying the beneficial function of TRPV1 activation in macrophages by use of this experimental cell culture model. Treatment with oxLDL, the most important modulator in the development of atherosclerosis, increased TRPV1 channel activity in BMDMs, as evidenced by a TRPV1-mediated increase in $[Ca^{2+}]_i$ level to a profile similar to that evoked by TRPV1 agonists. In addition, oxLDL-induced foam-cell formation, as evidenced by increased cellular levels of cholesterol and triglycerides, was suppressed by TRPV1 agonists but exacerbated by a TRPV1 antagonist. Removal of extracellular Ca^{2+} by EGTA aggravated the oxLDL-induced lipid accumulation and abrogated the protective effect of TRPV1 agonist in BMDMs (data not shown), which agrees with previous studies that the increase in $[Ca^{2+}]_i$ level induced by oxLDL may play a key role in the formation

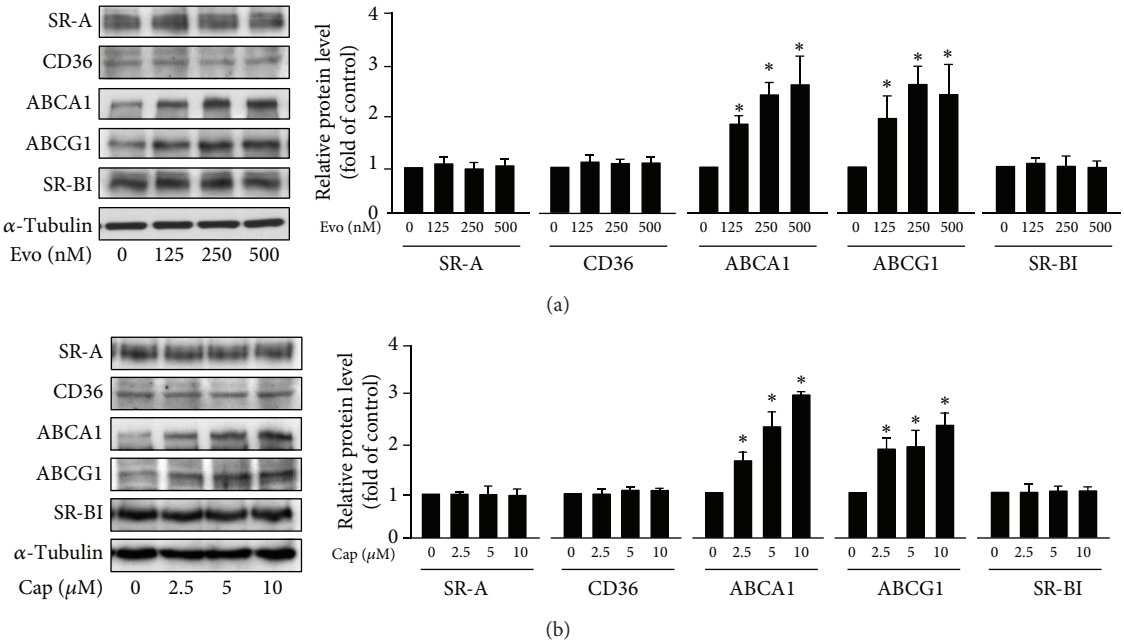

Figure 5: Effect of TRPV1 activation on expression of SR-A, CD36, SR-BI, ABCA1, and ABCG1 in macrophages. BMDMs were incubated with vehicle, (a) evodiamine (125, 250, 500 nM), or (b) capsaicin (2.5, 5, 10 μM) for 24 h. Western blot analysis of protein levels of SR-A, CD36, ABCA1, ABCG1, SR-BI, and α-tubulin. Data are mean ± SD from 5 independent experiments. $^*P < 0.05$ versus vehicle-treated cells.

of macrophage-foam cells [32]. Therefore, activation of TRPV1/Ca^{2+} signaling may inhibit the formation of foam cells *in vitro*.

SR-dependent oxLDL uptake and RCT-mediated cholesterol efflux are 2 key regulatory mechanisms in the intracellular lipid homeostasis of macrophage-foam cells [5–10]. Several lines of evidence indicate that reduced expression of SRs or elevated function of RCTs in macrophages leads to reduced deposition of cholesterol in macrophages [12, 30, 33]. Interestingly, TRPV1 agonist treatment did not alter the binding of Dil-oxLDL to SRs or the protein expression of SR-A, CD36, and SR-BI in BMDMs but promoted cholesterol efflux. Moreover, TRPV1 agonist treatment upregulated both ABCA1 and ABCG1, 2 major types of ABC transporters responsible for cholesterol efflux from macrophage-foam cells to apoAI and HDL, respectively. The critical role of ABCA1 and ABCG1 in maintaining cholesterol homeostasis in macrophages has been well defined [34, 35]. Loss or impaired function of ABCA1 or ABCG1 in human or experimental rodents leads to hyperlipidemia, excessive cholesterol accumulation in peripheral tissues, and an overwhelming inflammatory response [34, 36]. Thus, our *in vitro* results strongly support that the TRPV1-mediated suppression of foam-cell formation was solely due to an increase in RCT-dependent cholesterol efflux, which is consistent with the previous studies that cytokine- or flavonoid-induced upregulation of ABCA1 or ABCG1 contributes to alleviated lipid accumulation in foam cells [11–13]. The detailed mechanism by which activation of TRPV1 leads to upregulation of ABC transporters is not clear. However, an increase in $[Ca^{2+}]_i$ level evoked by other interventions may regulate the expression of ABC transporters in macrophages [37].

Additionally, we showed that the TRPV1 agonist-induced upregulation of ABCA1 and ABCG1 was accompanied by an increase in nuclear levels of LXRα and its DNA binding ability. This notion is further supported by findings that TRPV1-agonist-induced increase in promoter activity was abrogated by transfection with the LXRE mutant (phABCA1-DR4 m-Luc). Inhibition of LXRα activation by siRNA diminished the TRPV1-agonist-mediated upregulation of ABCA1 and ABCG1. Thus, LXRα-mediated transcriptional regulation may be required for induction of ABCA1 and ABCG1 expression by TRPV1 agonists. Although we found a unique pathway for TRPV1 activity, the detailed molecular mechanisms of TRPV1 agonists affecting cholesterol efflux merit further investigation. In functional analysis to inhibit LXRα activation, the suppressive effect of TRPV1 agonists on intracellular lipid accumulation was abolished. LXRα may be required for the TRPV1 activation-induced gene expression of ABCA1 and ABCG1, which may contribute to suppressing the transformation of macrophage foam cells *in vitro*. In addition to the LXRα-mediated transcriptional regulation, increasing evidence suggests that ABCA1 is also regulated by the posttranscriptional modification [38, 39]. For example, calmodulin is known to prevent protein degradation of ABCA1 by interacting with calmodulin-binding motif (1244 to 1257 amino acids within ABCA1 protein), which is located near the PEST sequence (1283 to 1306 amino acids) and thus prevents the binding of calpain to ABCA1, leading to the inhibition of ABCA1 degradation [38]. However, whether the posttranscriptional regulation is engaged in the TRPV1

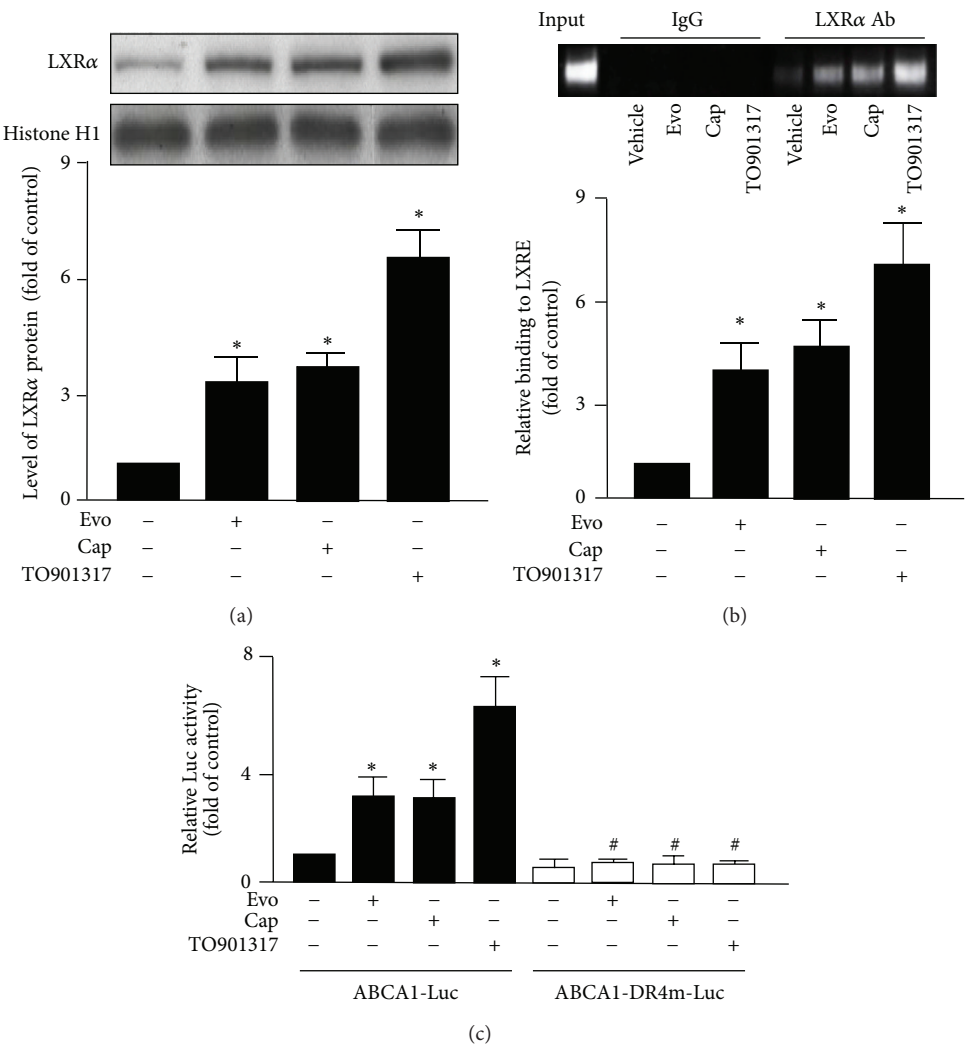

Figure 6: Treatment with TRPV1 agonists increases the activation of LXRα in macrophages. (a) BMDMs were pretreated with capsazepine (10 μM) for 1 h then incubated with evodiamine (500 nM), capsaicin (10 μM), or T0901317 (10 μM) for 6 h. Western blot analysis of nuclear protein level of LXRα and Histone H1 as a normalization control. (b) Macrophages were treated with vehicle, evodiamine (500 nM), capsaicin (10 μM), or T0901317 for 6 h, then immunoprecipitated with anti-LXRα or rabbit IgG. PCR amplification involved specific primers for the ABCA1 gene promoter. The amplified DNA products were separated by electrophoresis with 2% agarose gel. (c) Macrophages were transfected with plasmid phABCA1-Luc or phABCA1-DR4 m-Luc for 24 h, treated with vehicle, evodiamine (500 nM), capsaicin (10 μM), or TO901317 (10 μM) for 24 h, then lysed for Luc activity assays with renilla activity as an internal control. Data are mean ± SD from 5 independent experiments. $^{*}P < 0.05$ versus vehicle-treated cells, $^{\#}P < 0.05$ versus phABCA1-Luc-transfected cells with evodiamine or capsaicin treatment.

agonist-mediated upregulation of ABCA1 remain the further investigations.

Emerging evidence suggests that in addition to its action on cholesterol metabolism, ABCA1 also functions as a critical modulator in regulating the inflammatory response [39, 40]. Patients with Tangier disease and ABCA1 mutation or mice with functional ablation of ABCA1 show an irregular inflammatory response [41–43]. Moreover, growing evidence demonstrates that the expression and activity of ABCA1 is impaired during inflammation *in vivo* [44, 45]. Treatment with pro-inflammatory cytokines or lipopolysaccharide (LPS) decreases the expression of ABCA1 and its related function in various types of cells [44, 45]. More importantly, viral or bacterial infection decreases the expression of LXRα and that of its target genes, including ABCA1 [46]. In addition, activation of LXRα by its ligands inhibits the LPS-induced production of pro-inflammatory mediators including TNF-α, MCP-1, IL-6, and MIP-2 in macrophages [46–48]. We found that TNF-α-induced production of MCP-1 and IL-6 was inhibited by treatment with TRPV1 agonists but augmented by the TRPV1 antagonist. siRNA knockdown of LXRα expression abolished the anti-inflammatory effect of TRPV1 agonists on TNF-α-treated BMDMs. Activation of TRPV1 by its agonists also suppressed the inflammatory response of macrophages *in vitro*, which both may explain the protective role of TRPV1 in reducing atherosclerotic lesions *in vivo* [49–51]. Conceivably, the anti-inflammatory property of LXRα may play a pivotal role in the crosstalk between

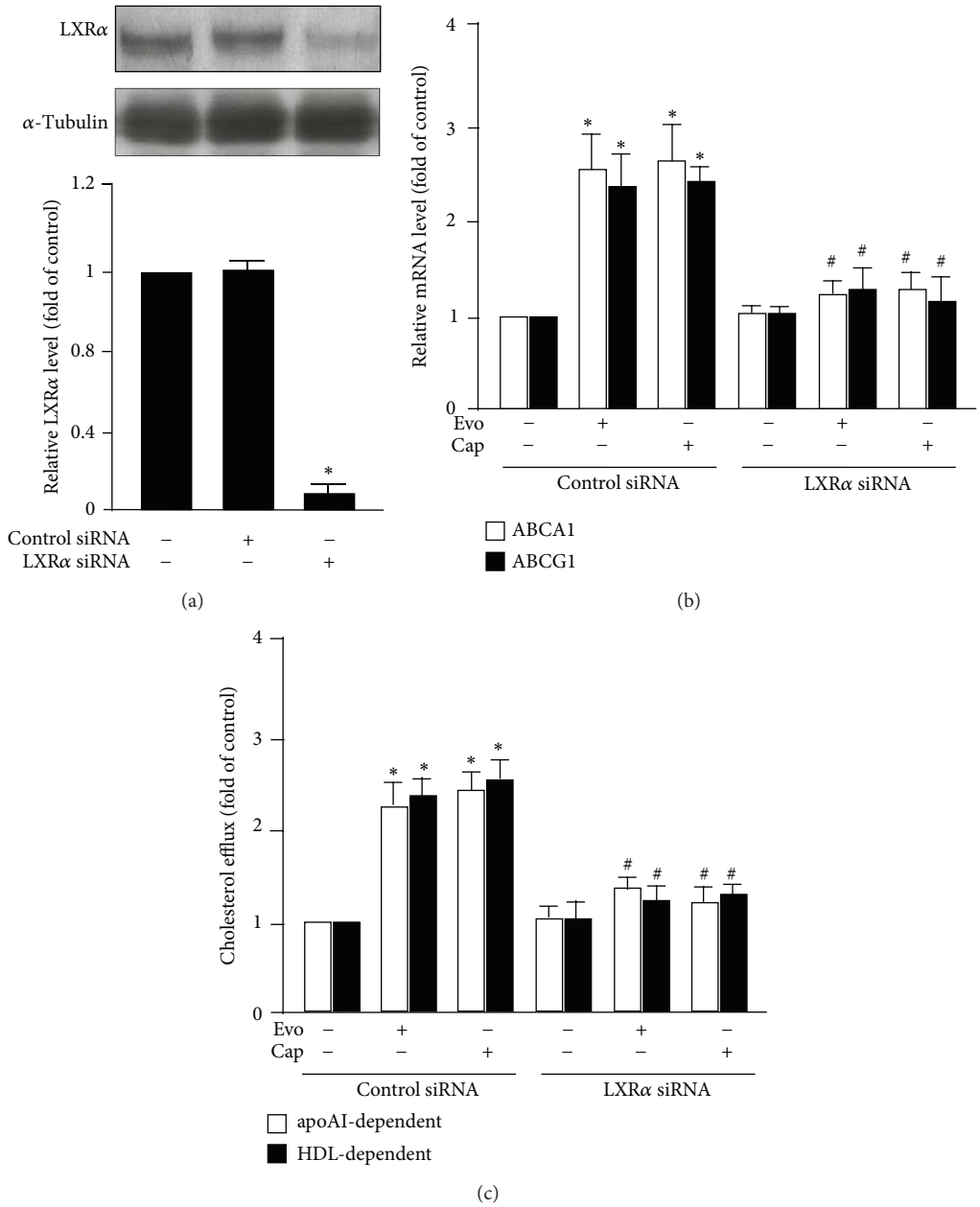

Figure 7: Knockdown of LXRα abolishes the protein expression of ABCA1 and ABCG1 and attenuates lipid accumulation by TRPV1 agonists. BMDMs were preincubated with control siRNA (50 nmol/L) or LXRα siRNA (50 nmol/L) for 24 h, followed by evodiamine or capsaicin treatment for additional 24 h. (a) Western blot analysis of protein expression of LXRα. (b) RT-PCR analysis of mRNA expression of ABCA1 and ABCG1. (c) ApoAI- and HDL-dependent cholesterol efflux was evaluated by use of NBD-cholesterol. Data are mean ± SD from 5 independent experiments. $^{*}P < 0.05$ versus control siRNA-treated cells, $^{\#}P < 0.05$ versus control siRNA-treated cells with evodiamine or capsaicin treatment.

reverse cholesterol transport and immunity. However, the detailed molecular mechanism underlying this interaction needs further investigation.

TRPV1 is originally found expressed in primary nociceptive sensory neurons and plays an important role in detecting irritative, inflammatory, and oxidative substances by somatic and visceral afferents [14, 15]. However, increasing evidence suggests that TRPV1 is expressed in several types of non-neuronal cells, including macrophages [52], endothelial cells (ECs) [27], and preadipocytes [53, 54] and vitally regulates their functions. Recently, convergent sets of evidence support a physiological role for TRPV1 as a crucial integrator in the functions of the cardiovascular system and in cardiovascular diseases [25, 27, 55]. For example, TRPV1 activation by capsaicin increased the activity of endothelial NO synthase (eNOS), promoted vasorelaxation, and thereby reduced blood pressure in experimental hypertensive rats [25]. Additionally, we previously reported that TRPV1 activation by evodiamine or capsaicin triggered Ca^{2+}-dependent PI3 K/Akt/CaMKII/AMPK signaling, thus leading to eNOS

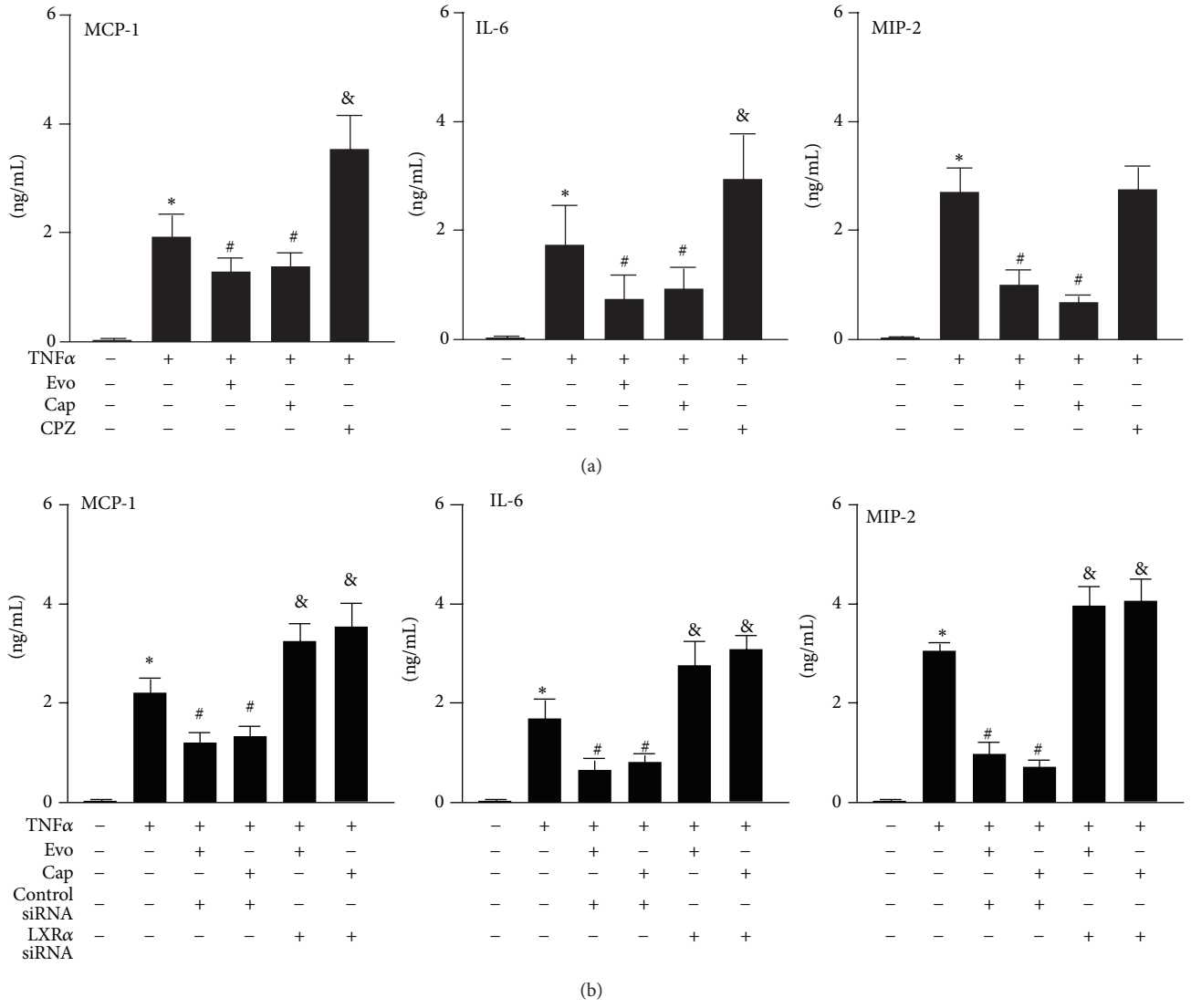

Figure 8: Knockdown of LXRα diminishes the protective effect of TRPV1 agonists against TNF-α-induced inflammation in macrophages. (a) BMDMs were pretreated with capsazepine (10 μM) for 1 h or (b) transfected with control siRNA (50 nmol/L) or LXRα siRNA (50 nmol/L) for 24 h, then incubated with vehicle (DMSO), evodiamine (500 nM), and capsaicin (10 μM) with or without TNF-α (10 ng/mL) for an additional 18 h. ELISA of levels of MCP-1, IL-6, and MIP-2 in the culture medium. Data are mean ± SD from 5 independent experiments. $^{*}P < 0.05$ versus vehicle-treated cells, and $^{\#}P < 0.05$ versus TNF-α-treated cells, and $^{\&}P < 0.05$ versus TNF-α-treated group with evodiamine or capsaicin treatment.

activation in ECs [27, 56]. As well, inactivation of TRPV1 accelerates the development of metabolic disorders such as hypertension, atherosclerosis, obesity, and fatty liver, whereas TRPV1 agonist activation protects against these metabolic diseases [25, 55, 57, 58]. Our previous study demonstrated that chronic treatment of ApoE$^{-/-}$ mice with the TRPV1 agonist evodiamine alleviated hyperlipidemia, systemic inflammation, hepatic macrovesicular steatosis, and atherosclerosis [59]. Moreover, our recent data demonstrated that chronic treatment with evodiamine upregulated the hepatic levels of LDLR, ABCG5, ABCG8, and CYP7A1 in ApoE$^{-/-}$ mice, which may promote bile acid synthesis and fecal excretion. Interestingly, activation of LXRα potentiated cholesterol excretion by increasing the transcriptional regulation of LDLR, ABCG5, ABCG8, and CYP7A1 in liver [60]. These lines of evidence indicate the potential role of TRPV1 in lipid metabolism. This notion is substantiated by our findings that activation of TRPV1 by evodiamine increased $[Ca^{2+}]_i$ level, which activated the LXRα signaling pathway and led to the upregulation of ABCA1 and ABCG1, ultimately reducing the lipid accumulation of macrophage-foam cells. Altogether, although the target cells for evodiamine have not yet been identified, our studies strongly suggest that collaboration of multiple physiological pathways in different types of cells may be required for the beneficial effects of TRPV1 agonists in treating metabolic disorders.

5. Conclusions

In conclusion, we demonstrate a novel protective function of TRPV1 in macrophages, whereby activation by TRPV1 agonists could suppress the oxLDL-induced deregulation of lipid metabolism and inflammation *in vitro* by an increase in LXRα activation, thus leading to the promotion of cholesterol efflux and attenuation of the inflammatory response. Our findings may help in developing novel pharmacological targets for treating atherosclerosis-related cardiovascular diseases.

Conflict of Interests

The authors declared that they have no conflict of interests.

Author's Contribution

Jin-Feng Zhao and Li-Chieh Ching equally contributed to this work.

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Research Article

Minimally Modified LDL Upregulates Endothelin Type A Receptors in Rat Coronary Arterial Smooth Muscle Cells

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Minimally modified low-density lipoprotein (mmLDL) is a risk factor for cardiovascular disease. The present study investigated the effects of mmLDL on the expression of endothelin type A (ET_A) receptors in coronary arteries. Rat coronary arteries were organ-cultured for 24 h. The contractile responses were recorded using a myographic system. ET_A receptor mRNA and protein expressions were determined using real-time PCR and western blotting, respectively. The results showed that organ-culturing in the presence of mmLDL enhanced the arterial contractility mediated by the ET_A receptor in a concentration-dependent and time-dependent manner. Culturing with mmLDL (10 μg/mL) for 24 h shifted the concentration-contractile curves toward the left significantly with increased E_{max} of 228% ± 20% from control of 100% ± 10% and significantly increased ET_A receptor mRNA and protein levels. Inhibition of the protein kinase C, extracellular signal-related kinases 1 and 2 (ERK1/2), or NF-κB activities significantly attenuated the effects of mmLDL. The c-Jun N-terminal kinase inhibitor or the p38 pathway inhibitor, however, had no such effects. The results indicate that mmLDL upregulates the ET_A receptors in rat coronary arterial smooth muscle cells mainly *via* activating protein kinase C, ERK1/2, and the downstream transcriptional factor, NF-κB.

1. Introduction

Oxidized low-density lipoprotein (oxLDL) is not limited to atherosclerotic plaques but can circulate as minimally modified LDL (mmLDL) that is formed when only the lipid region of LDL is oxidized. mmLDL is a potential biomarker for cardiovascular disease. It enhances cytokine production and expression of CD14 and toll-like receptor, induces proinflammatory activities in monocytic cells [1], damages endothelial function, promotes the formation of oxLDL and foam cells, and enhances vascular cell migration and proliferation [2]. These effects contribute to atherosclerotic lesion formation [3], which occurs through a mechanism involving the stimulation of receptor-mediated signal transduction pathways [4].

Endothelin peptides are produced in the endothelium of vessels [5]. Endothelin-1 (ET-1) stimulates vascular smooth muscle cell proliferation [6], migration [7], contraction [8], matrix remodeling [9, 10], synthesis of extracellular matrix components [11], and the expression of other proatherogenic growth factors, such as platelet-derived growth factor and transforming growth factor-beta [12]. There are two types of endothelin receptors in the vasculature of mammals, the endothelin type A (ET_A) and endothelin type B (ET_B) receptors, which are involved in ischemic cardiovascular disease by enhancing the contraction and proliferation of smooth muscle cells [13]. The expression of ET-1 and its receptors is upregulated in experimental models of atherosclerosis and in human atherosclerotic lesions [14, 15].

We have developed an organ culture model that mimics the upregulation of receptors in cardiovascular disease [16, 17]. This organ culture allows in-depth investigation of the intracellular mechanisms underlying the alteration in the expression of the ET receptors in rat coronary arteries. Using this model, we have demonstrated that mmLDL upregulates

ET_B receptors in both the rat coronary artery and basilar artery via activation of signal transduction pathways [18, 19].

The mitogen-activated protein kinases (MAPK) include the extracellular signal-regulated proteins 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), and the p38 cascade proteins [20] and play an important role in the intracellular signaling that occurs in response to extracellular stimuli [20], which causes phosphorylation and activation of transcription factors in the cytoplasm or the nucleus [21]. Protein kinase C (PKC) participates in signal transduction events in response to specific hormonal, neuronal, and growth factor stimuli [22]. NF-κB is a pivotal transcription factor downstream of the MAPK and PKC pathways [18, 19]. Activation of NF-κB is essential for controlling the inducible expression of several genes involved in inflammation and cell proliferation.

It is well known that both mmLDL and ET_A receptors upregulation is involved in inflammation and the pathogenesis of atherosclerosis; however, their relationship is unclear. The present study was designed to investigate the hypothesis that mmLDL upregulates ET_A receptor in rat coronary arterial smooth muscle cells and the possible intracellular mechanisms.

2. Materials and Methods

2.1. Reagents. mmLDL and LDL were obtained from the Xiehe Research Institute (Beijing, China). ET-1 and sarafotoxin 6c were purchased from Auspep, Parkville, Australia and dissolved in 0.9% saline with 0.1% bovine serum albumin. DMSO was used to dissolve staurosporine, SB386023, U0126, SP600125, SB203580, and wedelolactone (Sigma, St. Louis, MI, USA). BQ-788 (Sigma, St. Louis, MO, USA) was dissolved in 0.9% saline. Analytical grade chemicals and double-distilled water were used throughout the experiments. All of the drugs were further diluted in buffer solution immediately before being used in the experiments. The concentrations were expressed as the final molar concentration in the tissue baths.

2.2. Animals. Three hundred and thirty Sprague-Dawley rats (300–350 g) were obtained from the Animal Center of Xi'an Jiaotong University College of Medicine, China, and handled according to the guidelines provided by the Animal Care and Use Committee at Shaanxi Province. The experimental protocols were approved by the animal ethics committee at Xi'an Jiaotong University.

2.3. Organ Culture of Coronary Arteries. Rats were anaesthetized with CO_2 and decapitated to prepare artery samples. The hearts were removed and immersed into cold buffer solution. Under a dissection microscope, the left anterior descending coronary artery was gently excised from the myocardium [23, 24] and freed from the adhering tissue. The arteries were then cut into approximately 1-2 mm long ring segments. For organ culture, the coronary artery ring segments were placed in 24-well plates, two segments in each well containing 1 mL of Dulbecco's Modified Eagle's Medium [17]. The arterial segments were cultured with mmLDL (10 μg/mL) or LDL (10 μg/mL). An organ culture group was added as a control to eliminate the impact of organ culture *per se* on the experimental results. To examine the mechanism of the effects, the specific inhibitors of different signal transduction pathways were used. The inhibitors and mmLDL were added to Eagle's medium simultaneously at the beginning of the organ culture process.

In order to study the effect of the intracellular signaling pathways on the upregulation, we used some pathway inhibitors such as the PKC pathway inhibitor, staurosporine (0.1 μM) [22]; the inhibitors selected to target the different kinases leading to ERK1/2 activation, U0126 (10 μM) and SB386023 (10 μM); the specific JNK and p38 MAPK inhibitors, SP600125 (10 μM) and SB203580 (10 μM) [18, 19]; the NF-κB-inhibitor, wedelolactone (10 μM) [25, 26]. Each inhibitor was present for 24 h. Thereafter, the artery segments were mounted in myography baths. For analysis by real-time PCR or western blotting, the vessels were frozen in liquid nitrogen for 3 h and then stored at −80°C until they were processed.

2.4. Myographic Studies. The isometric tension in the isolated coronary arterial segments was recorded using a myography system [23, 24]. The artery segments were threaded on two 40 μm diameter stainless steel wires and mounted in myography baths. One wire was connected to a force-displacement transducer attached to a computer. The other wire was attached to a movable displacement device allowing fine adjustments of the vascular tension. The arterial segments were immersed in baths containing Krebs solution (37°C) [27]. The contractile capacity was determined by exposure to a potassium-rich (63.5 mM) Krebs solution. The segments were used only if potassium elicited reproducible responses greater than 0.8 mN. Concentration-response curves were obtained by cumulative addition of the agonists to the baths. A specific ET_A receptor agonist was not found. To study ET_A receptor-mediated contraction, sarafotoxin 6c was added to the baths to a final concentration of 1 μM to induce a contraction, and the segments remained in the sarafotoxin 6c (1 μM) supplemented solution for an additional 1 h to desensitize the ET_B receptor. Concentration-response curves for the agonist ET-1 (an ET_A and ET_B receptor agonist) were obtained by cumulative application of the substance (10^{-10} M–10^{-7} M). During this period, the contractile response to sarafotoxin 6c faded to the baseline level even though sarafotoxin 6c was still present in the bath with the segments. After the ET_B receptors had been desensitized, the concentration-effect curve induced by ET-1 was obtained. Thus, the contractile response to ET-1 was mediated only by the ET_A receptors [28, 29]. To confirm the desensitization of the ET_B receptors, the effect of the selective ET_B receptor antagonist, BQ-788 (0.1 μM), on the ET-1-induced contractions after sarafotoxin 6c desensitization was examined. The ET-1-induced contractions were similar in the presence and absence of BQ-788, suggesting the activation of only the ET_A receptors.

2.5. Real-Time PCR. A RNAfast200 Kit (Shanghai Flytech Biotechnology Co., Ltd., Shanghai, China) was used to

extract the total RNA. The resulting pellet was washed with 75% ethanol, air-dried, and redissolved in 40 μL of diethylpyrocarbonate-treated water. The OD_{260}/OD_{280} ratios were between 1.9 and 2.1. The concentration of the total RNA was approximately 3 μg/μL. Reverse transcription of total RNA to obtain cDNA was conducted using a GeneAmp RNA PCR Kit (Applied Biosystems, Beijing, China) and a Perkin-Elmer DNA thermal cycler. First-strand cDNA was synthesized from total RNA in a 40 μL reaction volume using random hexamers as primers. The reaction mixture was incubated at 25°C for 10 minutes, heated to 42°C for 15 minutes, heated further to 99°C for 5 minutes, and chilled to 5°C for 5 minutes. Real-time PCR was performed in a GeneAmp 5700 sequence detection system using the GeneAmp SYBR Green Kit (Toyobo Co., Ltd., Osaka, Japan) with the previously synthesized cDNA as the template in a 25 μL reaction volume. A no-template control was included in all of the experiments. The primers were designed using Primer Express 2.0 software and were synthesized by Beijing Sunbiotech Co., Ltd. (Beijing, China). The specific primers for the rat ET_A receptor (GenBank accession number NM_012550) were as follows:

ET_A *receptor*

forward: 5′-GCTCAACGCCACGACCAAG-3′

reverse: 5′-GTGTTCGCTGAGGGCAATCC-3′.

The housekeeping gene β-actin (GenBank accession number NM_031144) was used as the internal control. The primers used were as follows:

β-actin

forward: 5′-ACTATCGGCAATGAGCGGTTCC-3′

reverse: 5′-CTGTGTTGGCATAGAGGTCTTTACG-3′.

Real-time PCR was performed using the following profile: 95°C for 1 minute, followed by 40 cycles at 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 45 seconds. Dissociation curves were run after the real-time PCR was complete to identify the specific PCR products.

2.6. Western Blotting. Cultured or fresh coronary arterial segments were stored at −80°C. The total proteins were quantified using a BCA Protein Assay Kit (Pierce Biotechnology, Shanghai, China) according to the manufacturer's instructions, separated on SDS-PAGE gels, transferred to a polyvinylidene difluoride (PVDF) membrane, and blocked with 5% nonfat dry milk. The immunoblots were incubated with primary antibodies directed against endothelin receptor A (1 : 100) (Millipore, CA, USA). The immunolabeled protein bands were detected using Super Signal Chemiluminescent Substrate after incubation with horseradish peroxidase-conjugated secondary antibody (1 : 5000). β-actin was used as the internal loading control. Densitometric analysis was performed using Image Gauge verion 4.0 software (Fuji Photo Film Co., Ltd., Japan).

2.7. Calculations and Statistics. The maximum contraction (E_{max}) value was calculated as the percentage of the contraction induced by 63.5 mM K^+, and the pEC_{50} value refers to the negative logarithm of the molar concentration of a drug that produces half-E_{max}. The concentration-effect curve of each agonist was fitted to the Hill equation using an iterative, least square method (GraphPad Prism 5) to provide estimates of the E_{max} and pEC_{50} values. All of the real-time PCR experiments were performed in duplicate, and the mean values were used. The amount of ET_A receptor mRNA was calculated relative to the level of the mRNA expression of the β-actin housekeeping gene in the same sample. The following formula was used to calculate the amount of ET_A receptor mRNA: $X_0/R_0 = 2^{C_{tR}-C_{tX}}$, where X_0 = the original amount of endothelin ET_A receptor mRNA, R_0 = the original amount of β-actin mRNA, C_{tR} = the C_t-value for β-actin, and C_{tX} = the C_t-value for the ET_A receptor. The amount of receptor protein relative to the amount of the internal control is expressed as a percentage of the value for the control group.

The statistical analyses of the myography experiments and real-time PCR experiments were based on one measurement per rat. When the number of arterial segments was more than one in an individual, the average was used for that individual in myograph experiment. In the Western blotting experiments, each sample was a pool of 4 coronary arterial segments. All of the data are expressed as the mean values ± SEM. Student's t-test was used to compare two sets of data, and a one-way analysis of variance (ANOVA) or a two-way ANOVA followed by Dunnett's test (GraphPad Prism) was used for comparisons of more than two data sets. A P value of less than 0.05 was considered significant.

3. Results

3.1. Upregulation of ET_A Receptors in the Coronary Artery. The Krebs solution containing 63.5 mM K^+ was used to examine the viability and contractility of the arteries during organ culture. There was no significant difference in the E_{max} of the contractile responses induced by K^+ among the groups (i.e., freshly isolated: 1.90 ± 0.11 mN, organ-cultured: 2.12 ± 0.17 mN, organ-cultured in the presence of mmLDL: 2.05 ± 0.13 mN, organ-cultured in the presence of LDL: 1.98 ± 0.15 mN, $n = 8$, $P > 0.05$). ET-1 induced concentration-dependent contractions in freshly isolated coronary arteries. After 24 h of culture, the ET-1-induced concentration-contraction curve was not significantly different from that of freshly isolated coronary arteries. Culturing for 24 h with mmLDL at 5, 10, or 20 μg/mL shifted the contractile curves induced by ET-1 toward the left in a concentration-dependent manner (Figure 1(a)). The E_{max} of the 10 and 20 μg/mL mmLDL groups was increased to 228% ± 20% and 257% ± 23% compared to the control group value ($P > 0.05$). After organ culture for 6 h with 10 μg/mL mmLDL, the ET-1-induced concentration-contractile curve was not significantly affected. Culturing with 10 μg/mL mmLDL for 12, 24, or 48 h shifted the contractile curves induced by ET-1 toward to the left in a time-dependent manner (Figure 1(b)). The E_{max} of 24 h mmLDL-supplemented cultures (228% ± 20%) was significantly higher than that of 12 h mmLDL-supplemented cultures (151% ± 15%, $P < 0.01$) but not

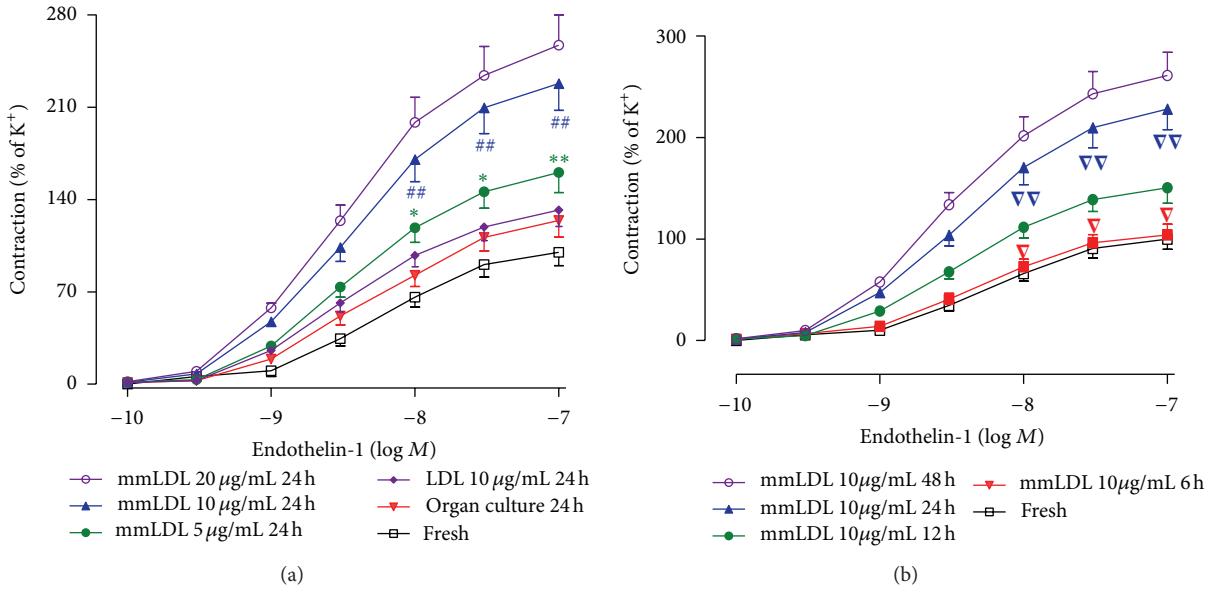

Figure 1: Culturing with mmLDL enhanced the ET-1-induced contraction of rat coronary artery segments. (a) The concentration-dependent effect of culturing with mmLDL for 24 h; $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* organ culture for 24 h; $^{\#\#}P < 0.01$ *versus* 5 μg/mL of mmLDL for 24 h. (b) The time-dependent effect of culturing with 10 μg/mL of mmLDL. $^{\triangledown}P < 0.05$, $^{\triangledown\triangledown}P < 0.01$ *versus* 10 μg/mL of mmLDL for 12 h. The data are presented as the mean ± SEM. $n = 8$ coronary arteries, from that number of animals.

significantly lower than that of 48 h mmLDL-supplemented cultures (261% ± 23%, $P > 0.05$). mmLDL was used at a concentration of 10 μg/mL with one time point, for 24 h, in the present study. Organ culture for 24 h *per se* did not increase the contractile responses of the arterial segments to ET-1, which could be obviously enhanced by exposure to 10 μg/mL mmLDL. However, 10 μg/mL native LDL did not affect the concentration-contractile curves of coronary artery segments induced by ET-1 (Figure 1(a)).

The levels of expression of ET_A receptor mRNA and protein in coronary artery segments were determined using real-time PCR and western blotting, respectively. Organ culture did not elevate the mRNA and protein levels of the ET_A receptor compared to those of freshly isolated coronary artery segments. Culturing with mmLDL significantly elevated the levels of ET_A receptor mRNA and protein compared to those of the control cultures (Figure 2).

3.2. Effect of a PKC Inhibitor on the mmLDL-Induced Upregulation. The presence of staurosporine, a specific inhibitor of PKC, markedly inhibited the mmLDL-induced enhancement of the contractile response to ET-1 and decreased the E_{max} from 228% ± 20% in the mmLDL-supplemented group to 178% ± 18% ($P < 0.05$) (Figure 3(a), Table 1). In addition, the expression of ET_A receptor mRNA and protein in the coronary arterial smooth muscle cells cocultured with staurosporine was lower than that of mmLDL group (Figures 3(b) and 6).

3.3. Effect of MAPK Inhibitors on the mmLDL-Induced Upregulation. After culture for 24 h with mmLDL and specific inhibitors for ERK1/2, the concentration-response curves

Table 1: Contractile effects of endothelin-1 (ET-1) in coronary artery.

	n	Endothelin-1	
		E_{max} (%)	pEC_{50}
Fresh	8	100 ± 10**	7.88 ± 0.10**
24 h culture	8	124 ± 13**	8.04 ± 0.09**
24 h culture + mmLDL	8	228 ± 20	8.46 ± 0.08
mmLDL + staurosporine	8	178 ± 18*	8.11 ± 0.10*
mmLDL + SB386023	8	163 ± 15**	7.92 ± 0.09**
mmLDL + U0126	8	156 ± 14**	8.04 ± 0.11*
mmLDL + SP600125	8	192 ± 16	8.37 ± 0.12
mmLDL + SB203580	8	194 ± 18	8.29 ± 0.10
mmLDL + wedelolactone	8	141 ± 13**	7.96 ± 0.09**

Responses to endothelin-1 are expressed as E_{max} in percent of 63.5 mM K^+-induced contraction and in pEC_{50} values (negative logarithm of the molar concentration that produces half-maximum contraction). The data are expressed as mean ± SEM. Statistical analyses were performed using two-way ANOVA followed by Dunnett's test and Student's t-test with Welch's correction. n = number of animals examined in rats. $^{*}P < 0.05$, $^{**}P < 0.01$ versus 24 h culture + mmLDL.

of ET-1-induced contractions in the SB386023- and U0126-treated groups were markedly shifted toward the right compared to the mmLDL group, in a nonparallel manner (Figures 4(a) and 4(b)). The E_{max} and pEC_{50} of contraction in the groups coincubated with mmLDL and SB386023 or U0126 were significantly lower than those of the group incubated with mmLDL ($P < 0.01$ and $P < 0.05$, resp., Table 1). However, the JNK inhibitor SP600125 and the p38 inhibitor SB203580 did not modify the mmLDL effects on the ET-1-induced responses ($P > 0.05$) (Figures 4(c) and 4(d); Table 1). The levels of expression of ET_A receptor

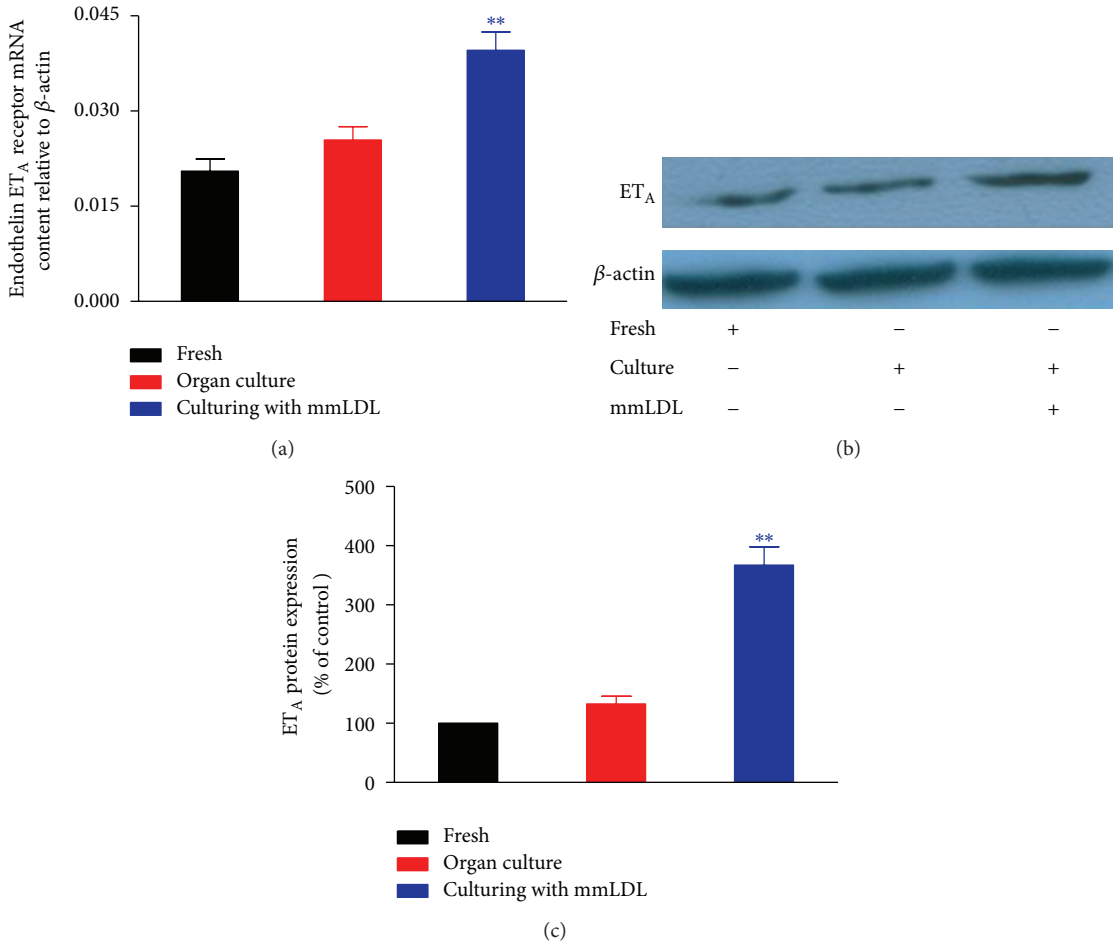

FIGURE 2: Culturing with mmLDL-induced increase of the level of expression of ET_A receptor mRNA ((a) n = 5-6 coronary arteries, from that number of animals) and protein ((b and c) n = 4 samples, each sample being a pool of 4 coronary arteries). The data are presented as the mean ± SEM. $^{**}P < 0.01$ *versus* organ culture.

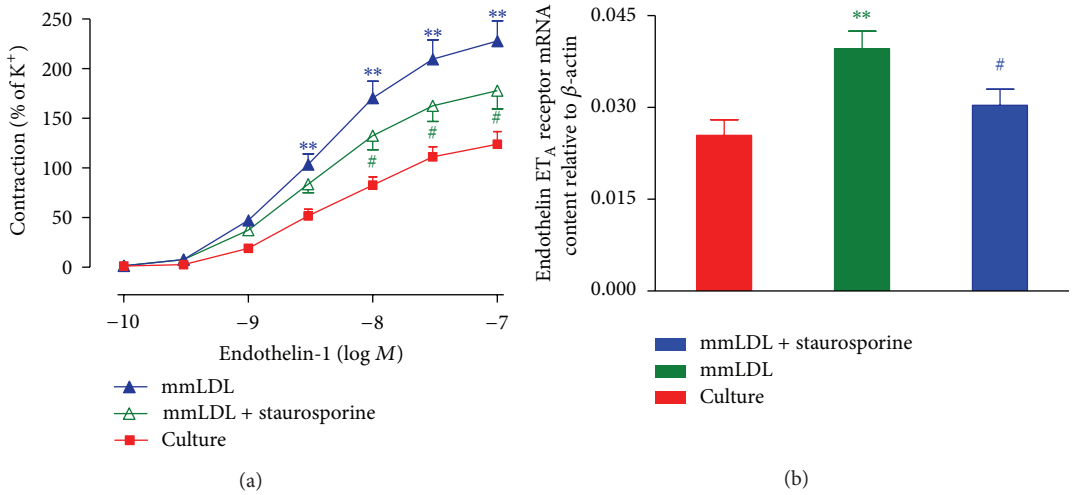

FIGURE 3: Effect of a PKC inhibitor on the mmLDL induced increase in contractile function and mRNA levels of ET_A receptor in the rat coronary artery. After the coronary artery rings were cultured for 24 h with mmLDL (10 μg/mL) in the presence of the PKC inhibitor staurosporine (0.1 μM), the concentration-contraction curves of the rings mediated by ET_A receptor ((a) n = 8 coronary arteries, from that number of animals) and the levels of the ET_A receptor mRNA ((b) n = 5-6 coronary arteries, from that number of animals) were determined. Staurosporine inhibited the mmLDL-induced increase in ET_A receptor contractile function and mRNA expression. The data are presented as the mean ± SEM. $^{**}P < 0.01$ *versus* culture, $^{\#}P < 0.05$, *versus* mmLDL.

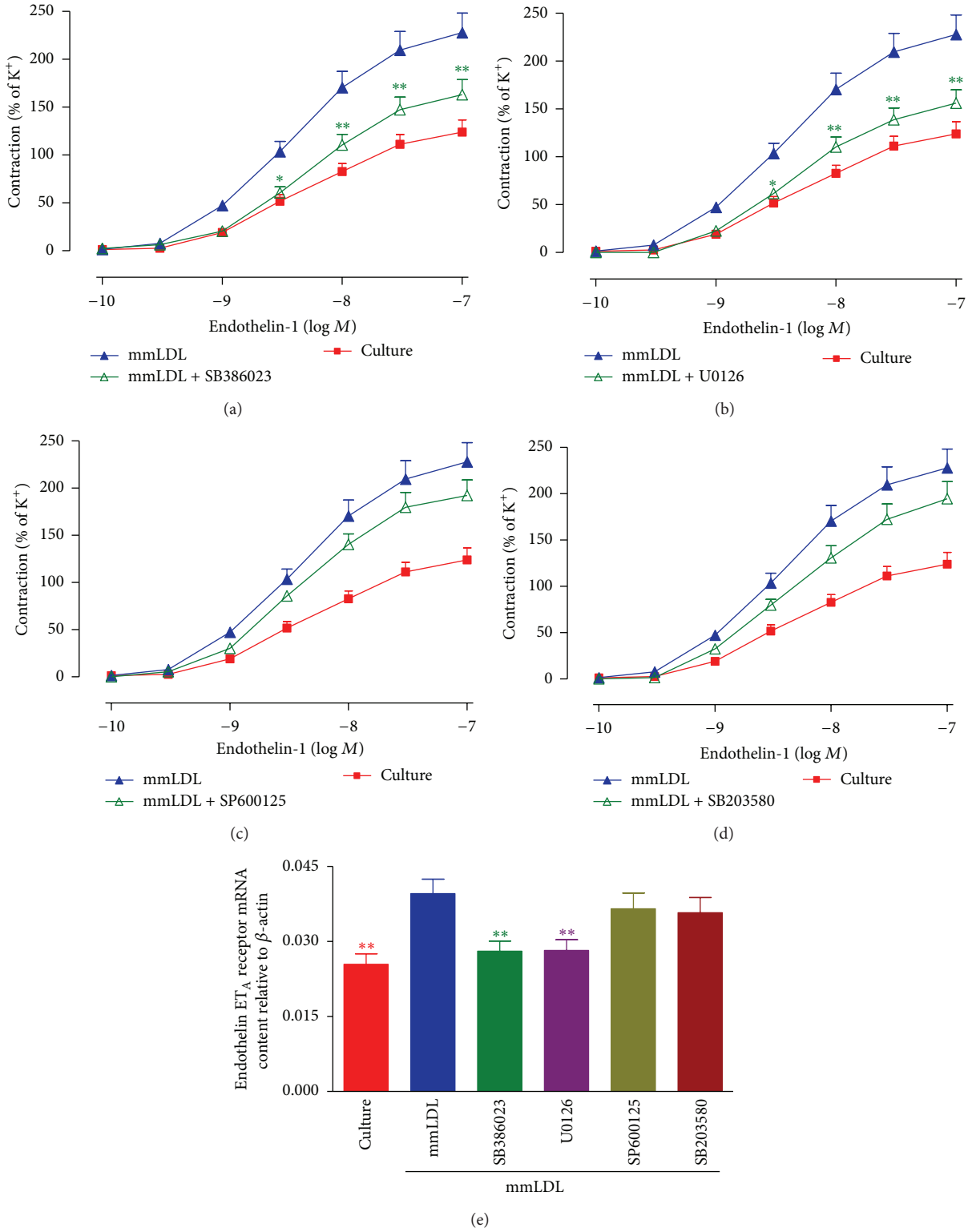

Figure 4: Effect of the MAPK pathway inhibitors on the mmLDL-induced increase in contractile function and levels of ET_A receptor mRNA in the coronary artery. After rat coronary arteries were cultured for 24 h with mmLDL (10 μg/mL) in the presence of MAPK inhibitors, including the ERK1/2 inhibitors SB386023 (a), U0126 (b), the JNK inhibitor SP600125 (c), or the p38 inhibitor SB203580 (d), the ET-1-induced concentration-contraction curves were constructed (n = 8 coronary arteries, from that number of animals). The effects of the MAPK inhibitors on the level of ET_A receptor mRNA are shown ((e) n = 5-6 coronary arteries, from that number of animals). The data are presented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* mmLDL.

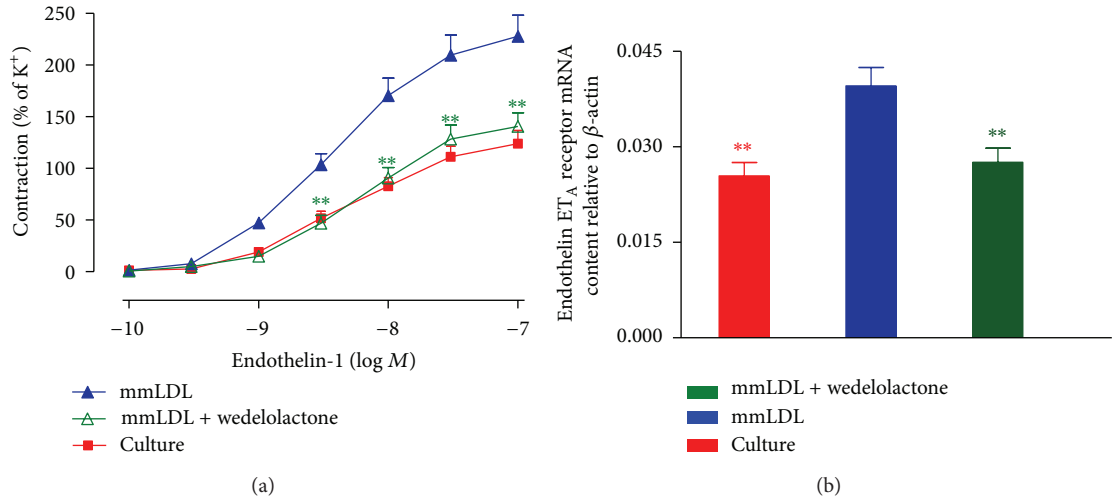

FIGURE 5: Effect of the NF-κB pathway inhibitor on the mmLDL-induced upregulation of ET_A receptors in the coronary artery. Rat coronary artery segments were cultured with mmLDL (10 μg/mL) in the presence of the NF-κB inhibitor wedelolactone (10 μM) for 24 h. The concentration-contraction curves mediated by the ET_A receptor ((a) $n = 8$ coronary arteries, the number of animals) and the levels of ET_A receptor mRNA ((b) $n = 6$ coronary arteries, from that number of animals) are shown. The data are presented as the mean ± SEM. $^{**}P < 0.01$ *versus* mmLDL.

mRNA and protein in the vascular smooth muscle cells were determined. The results showed that the ERK1/2 inhibitors SB386023 and U0126 significantly attenuated the mmLDL-induced increase of the expression of ET_A receptor mRNA and protein. This was paralleled by the decreased ET_A receptor-mediated contraction. However, inhibition of JNK and p38 MAPK did not have these effects (Figures 4(e) and 6).

3.4. Effect of Inhibition of NF-κB on the mmLDL-Induced Upregulation of ET_A *Receptors.* A specific inhibitor of the NF-κB pathway, wedelolactone, shifted the mmLDL-enhanced concentration-contraction curve of the coronary artery induced by ET-1 treatment toward the right, with significantly decreased E_{max} and pEC_{50} ($P < 0.01$) (Figure 5(a); Table 1). The levels of ET_A receptor mRNA and protein in the coronary artery samples showed that wedelolactone significantly inhibited the mmLDL-enhanced expression of the ET_A receptor (Figures 5(b) and 6).

4. Discussion

ET-1 is the strongest known vasoconstrictor. The upregulation of the ET_A receptor leads to enhanced contraction and reduced blood flow, which exacerbates inflammation and contributes to ischemic disease [14, 30, 31]. The present work has contributed to the elucidation of the intracellular signal transduction pathways involved in the mmLDL-induced regulation of the ET_A receptor. Culturing rat coronary arteries with mmLDL resulted in the upregulation of ET_A receptor-mediated contraction. Likewise, the ET_A receptor immunostaining intensity and mRNA levels were increased. The inhibition experiments revealed that the PKC and ERK1/2 MAPK pathways and the downstream NF-κB transcriptional factor signaling pathway were involved in the mmLDL-mediated process of upregulating the ET_A receptor.

Previous studies used organ cultures of coronary arteries and other vessels as an experimental model for the detailed delineation of the regulation of endothelin receptors because the changes that occur in this model are similar to those frequently observed in cardiovascular disease. This is the first time that culturing rat coronary arteries in the presence of mmLDL was evaluated as an experimental model for the regulation of the ET_A receptor. Culturing rat coronary artery segments with mmLDL for 24 h resulted in increased ET-1-induced contraction which was mediated by the ET_A receptor. Furthermore, the real-time PCR results demonstrated that mmLDL treatment elevated the level of ET_A receptor mRNA, and western blot analysis showed that the mmLDL treatment increased the expression of ET_A receptor protein. The present study showed that organ culture *per se* did not affect the contractile response mediated by the ET_A receptor, which agrees well with the results of the previous study [22]. LDL treatment did not significantly increase the contractility of coronary arterial segments, suggesting that LDL might not affect the regulation of the ET_A receptor. Taken together, these results suggest that the mmLDL-supplemented organ culture model is suitable for stimulating the upregulation of the ET_A receptor in rat coronary arteries. The changes that occur during culture with mmLDL might be comparable to those that occur in cardiovascular disease. Studies using porcine models found large numbers of ET_A receptors in the tunica media and neointima of porcine saphenous vein grafts [32]. *In vivo*, secondhand smoke increased the contractile response of the mouse airway to ET-1 [33]. Exposure to secondhand smoke also upregulated the level of ET_A receptors in rat cerebral and coronary arteries via the Raf/ERK/MAPK pathway [23, 24]. In human studies, the ET_A receptors are localized at the regions of the saphenous vein

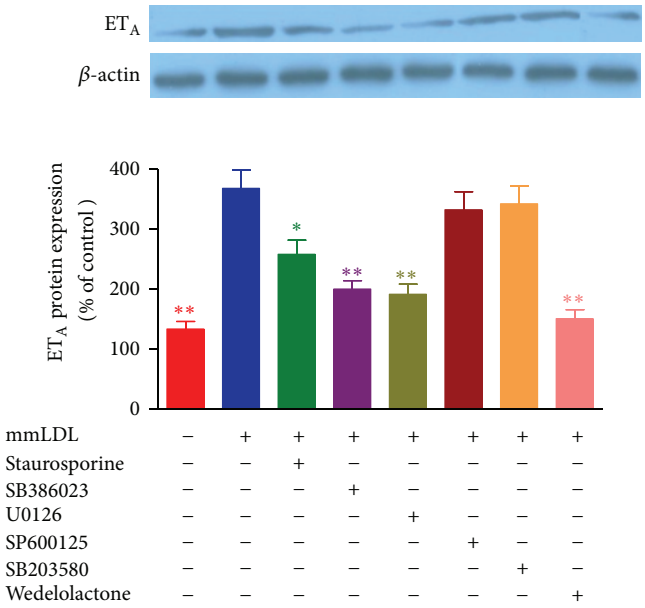

FIGURE 6: Effect of mmLDL and the intracellular signaling inhibitors on the level of expression of ET_A receptor protein in the coronary artery. Rat coronary arteries were cultured with mmLDL (10 μg/mL) in the presence of the PKC inhibitor staurosporine, ERK1/2 inhibitors SB386023 and U0126, JNK inhibitor SP600125, p38 inhibitor SB203580, and NF-κB inhibitor wedelolactone for 24 h. The levels of ET_A receptor protein were determined by western blotting. The results are expressed as the mean ± SEM. n = 3-4 (each sample being a pool of 4 coronary arteries). $^*P < 0.05$, $^{**}P < 0.01$ *versus* mmLDL.

where cellular proliferation occurs [34]. The upregulation of contractile response receptors in arterial smooth muscle cells is observed in ischemic vascular diseases [35, 36], and suppressing receptor upregulation or blocking the receptors has been shown to be beneficial in the case of vascular damage [37, 38]. Thus, upregulation of these receptors is a key event in the development of vascular diseases.

There is a significant relationship between receptor upregulation and its stimulatory factors. This study aimed to elucidate the role of the PKC and MAPK intracellular signal transduction pathways and the downstream NF-κB transcriptional factor signaling pathway in the mmLDL-induced upregulation of the ET_A receptor. PKC takes part in signal transduction events in response to certain stimuli. PKC has been reported to be activated by ET-1 in diabetic vascular smooth muscle cells and to increase extracellular matrix deposition, cellular hypertrophy, and cell proliferation [39]. In the present experiments, inhibition of PKC by staurosporine reduced the mmLDL-enhanced contraction mediated by the ET_A receptor and the mmLDL-induced increase of the ET_A receptor mRNA and protein levels. This result suggests that the PKC pathway is involved in the process of upregulating the ET_A receptor in coronary arteries. This suggestion is supported by studies in rats that demonstrated that upregulation of the endothelin receptors involves PKC [40–42]. Furthermore, *in vivo* rat studies demonstrated that PKC inhibitors prevent the upregulation of vascular endothelin receptors and reverse the reduction of cerebral blood flow subsequent to subarachnoid hemorrhage [43]. PKC has previously been reported to contribute to the vascular remodeling that occurs during hypertension [44]. In addition, PKC has been implicated in the induction of hypertrophy of cardiomyocytes, and PKC activation has also been shown to aggravate hypoxic myocardial injury and to be proarrhythmic [45].

PKC activates the MEK/ERK pathways at several levels [46]. U0126, a noncompetitive inhibitor of the MEK substrates [47], blocks the enzymatic activity of MEK1/2 and subsequently inhibits the activation of ERK1/2. SB386023 inhibits the MAPKKK upstream of MEK, namely, the Raf family [48]. Raf binds to and activates MEK and no other MAPKK, which makes it specific for the ERK pathway [49]. Previous studies have shown that ERK1/2 is involved in the upregulation of the ET receptors that mediate the contraction of the rat cerebral artery, coronary artery and superior mesenteric artery [18, 19, 22, 23, 28]. In the present study, both of the ERK1/2 inhibitors used, U0126 and SB386023, significantly decreased the mmLDL-enhanced contraction mediated by the ET_A receptor and significantly attenuated the mmLDL-induced increase of the ET_A receptor mRNA and protein levels. These results indicate that the ERK1/2 pathway is involved in the mmLDL-induced upregulation of the ET_A receptor in coronary arteries. ERKs mediate cellular responses that are initiated by growth factors [50] and have been implicated in cardiovascular and cerebrovascular disease. Inhibition of ERK1/2 attenuates the lipoprotein (α)-induced growth of human vascular smooth muscle cells, which is an independent risk factor for cardiovascular disease [51]. *In vivo* studies have shown that ERK1/2 inhibitors prevent the upregulation of the vascular ET_B receptor and reverse the reduction of cerebral blood flow after subarachnoid hemorrhage in rats [52].

Three different JNK pathways (JNK1, -2, and -3) have been identified in humans. JNK1 and JNK2 have a broad tissue distribution, whereas JNK3 is primarily localized in neuronal tissues and cardiac myocytes. SP600125 inhibits the JNK1, -2, and -3 pathways [53]. The JNK inhibitor SP600125 and the p38 inhibitor SB203580 had no obvious effect on the mmLDL-induced increase of contractile function and the expression of ET_A receptor mRNA and protein. These results suggested that the JNK and p38 MAPK signaling pathways might not be involved in the upregulation of ET_A receptors induced by mmLDL. In previous studies, p38 MAPK was found to not be involved in the ET_B receptor-mediated elevation of the contractility of organ-cultured rat middle cerebral arteries and porcine coronary arteries [54, 55], and we also demonstrated that the p38 MAPK pathway was not involved in the mmLDL-induced upregulation of the ET_B receptor in the rat coronary artery [18]. It was reported that the JNK pathway is not involved in either the mmLDL-induced or the DSP-induced elevation of vascular contraction or the expression of the ET_B receptor of the rat coronary artery and basilar artery [18, 22].

Wedelolactone, an inhibitor of the NF-κB signal transduction pathway, prevents the phosphorylation and degradation of IκB, blocking the translocation of NF-κB

to the nucleus. The present study showed that the ET_A receptor-mediated vascular contraction was upregulated during culture in the presence of mmLDL. Moreover, the real-time PCR and western blotting analyses showed ET_A receptor-mediated upregulation of the levels of expression of ET_A receptor mRNA and protein. The results suggest that the mmLDL-induced alteration in ET_A receptor expression involves increased transcription. Treatment with wedelolactone almost abolished the mmLDL-induced increase in the ET_A receptor-mediated function and ET_A receptor expression in the coronary artery. These results strongly suggested that the NF-κB pathway is involved in the mmLDL-induced upregulation of the ET_A receptor. This result is consistent with the previous finding that NF-κB appears to be involved in the upregulation of the level of the ET_B receptor [18, 19, 22, 25].

mmLDL is a risk factor for coronary artery disease. Recent results obtained by our group showed that the increased endothelin-induced contraction in organ cultures containing mmLDL could be attributed to the upregulation of endothelin receptors on the vascular smooth muscle cells. Using organ cultures of cerebral arteries in mmLDL-supplemented solutions, we demonstrated that the expression of the ET_B receptor was upregulated in the vascular smooth muscle cells and that PKC, MAPK, and NF-κB were involved in the intracellular mechanisms leading to this upregulation [19]. mmLDL also upregulates the level of ET_B receptors in coronary arteries by activating ERK1/2 MAPK and the NF-κB transcription factor [18]. In the present work, we demonstrated that culturing coronary arteries with mmLDL increased their ET-1-induced contraction in a concentration-dependent and time-dependent manner and increased the expression of ET_A receptor mRNA and protein. The activation of the upstream intracellular PKC and ERK1/2 MAPK pathways and the downstream NF-κB inflammatory signaling pathway is mainly responsible for this upregulation.

In conclusion, mmLDL induces an upregulation of ET_A receptors in coronary artery, which may contribute to the development of ischemic cardiovascular diseases. The molecular mechanisms involve the activation of PKC and ERK1/2 MAPK pathways and the downstream NF-κB signaling pathways. Understanding the upregulation and underlying molecular mechanisms may lead to novel treatments of cardiovascular disease.

Abbreviations

ERK1/2: Extracellular signal-regulated proteins 1 and 2
ET-1: Endothelin-1
ET_A: Endothelin type A
MAPK: Mitogen-activated protein kinase
mmLDL: Minimally modified low-density lipoprotein
oxLDL: Oxidized low-density lipoprotein
PKC: Protein kinase C.

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Research Article

Low-Density Lipoproteins Oxidation and Endometriosis

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The etiopathogenesis of endometriosis still remains unknown. Recent data provide new valuable information concerning the role of oxidative stress in the pathophysiology of the disease. It has been proved that levels of different lipid peroxidation end products are increased in both peritoneal fluid (PF) and serum of endometriotic patients. We assessed the concentration of oxidized low-density lipoproteins (oxLDL) in PF of 110 women with different stages of endometriosis and 119 women with serous ($n = 78$) or dermoid ($n = 41$) ovarian cysts, as the reference groups. PF oxLDL levels were evaluated by ELISA. We found that concentrations of oxLDL in PF of endometriotic women were significantly higher compared to women with serous but not dermoid ovarian cysts. Interestingly, by analyzing concentrations of oxLDL in women with different stages of the disease, it was noted that they are significantly higher only in the subgroup of patients with stage IV endometriosis as compared to women with ovarian serous cysts. In case of minimal, mild, and moderate disease, PF oxLDL levels were similar to those noted in reference groups. Our results indicate that disrupted oxidative status in the peritoneal cavity of women with endometriosis may play a role in the pathogenesis of advanced stages of the disease.

1. Introduction

Despite many years of research efforts and impressive progress in knowledge of mechanisms of endometriosis development, the etiopathogenesis of the disease and exact cause of infertility in patients suffering from endometriosis still remain poorly understood. None of the theories and models of endometriosis pathogenesis provide definitive explanation of the disease development, considering its different manifestations and various localizations. Recently published studies present new data on potential role of free radicals in endometriosis pathophysiology. Although the origin of the oxidative stress occurring in the peritoneal cavity in endometriotic patients is unknown, accumulating data suggest that increased iron levels, together with apoptotic endometrial fragments and activated macrophages, may promote prooxidant environment. In addition, oxidative stress in endometriotic patients may potentially be induced by environmental factors, including dioxins or heavy metals [1, 2].

In our preliminary work we found significantly increased levels of ox-LDL in peritoneal fluid of women with stage III/IV endometriosis compared to patients with follicle ovarian cysts. However, peritoneal fluid oxLDL concentrations did not differ significantly between patients with minimal/mild endometriosis and women from the reference group [3]. Murphy et al. [4] showed increased low-density lipoprotein (LDL) oxidation in peritoneal fluid of patients with endometriosis, which may be a result of peritoneal cavity macrophages hyperactivity. It was also proved that oxidized LDLs stimulate monocyte chemotactic protein-1 (MCP-1) expression in mesothelial and endometrial cells which provides direct evidence of oxidative stress role in etiopathogenesis of the disease [5]. Increased concentrations of lipid peroxidation end products, malondialdehyde (MDA), 8-isoprostane, and 25-hydroxycholesterol, were found in peritoneal fluid of infertile women with endometriosis [6–10]. Serum of patients with endometriosis, compared to healthy women, contains also significantly higher 8-isoprostane levels [11]. Murphy et al. [12] showed that peritoneal fluid of patients with endometriosis contains increased concentration of lysophosphatidylcholine, another lipid peroxidation product with confirmed chemotactic properties for monocytes. MDA and 7-hydroxynonenal (HNE-7) expression were increased in endometriosis implants tissue; however, both

lipid proteins are also expressed in eutopic endometrium [4]. Concentrations of antibodies against lipid peroxidation products were found to be increased in serum of women with endometriosis, with no immunoglobulins detected in their peritoneal fluid [13]. Serum of women with endometriosis contains also elevated concentration of lipid hydroperoxide (LOOH), and its levels correlate positively with the stage of the disease according to revised American Fertility Society classification [14]. Peritoneal fluid of endometriotic patients contains oxidatively modified protein-lipid complexes, showing both chemotactic properties and ability to stimulate selected cytokines production [15]. However, there are relevant data published in the literature according to which the concentrations of MDA and MDA-Cu complexes demonstrate no significant differences, being comparable in peritoneal fluid of patients with and without endometriosis, showing also no significant correlation with the stage of the disease [16–18]. No differences were also found in peritoneal fluid concentration of another lipid peroxidation product, cholest-3,5-dien-7-one [18].

The objective of the study was to assess concentrations of oxidized low-density lipoproteins (oxLDL) in peritoneal fluid (PF) of women with endometriosis.

2. Material and Methods

We examined 229 women, aged 15–53, who underwent diagnostic or therapeutic laparoscopy. Clinically and histologically confirmed diagnosis established the following groups: women with endometriosis (E, $n = 110$) and as the reference groups: patients with simple serous (R1, $n = 78$) and dermoid (R2, $n = 41$) ovarian cysts. In each case, endometriosis was staged according to the American Society for Reproductive Medicine classification [19]. The disease was found to be minimal (E1) in 23 cases, mild (E2) in 25 patients, moderate (E3) in 39 women, and severe (E4) in 23 cases.

Subjects were not given hormonal therapy and/or anti-inflammatory medications for at least 3 months before laparoscopy. Medical history of the patients and basic clinical examination showed no general chronic diseases, except for the condition, which was the indication for laparoscopy. Mean age of women did not differ significantly between the studied groups. Similarly, no significant difference was found in the phase of menstrual cycle of the time of laparoscopic procedures between women in all study groups.

All patients signed an informed consent, and the Lublin Medical University Ethics Committee approval was obtained for the study.

All visible PFs were aspirated during laparoscopy from the anterior and posterior cul-de-sacs, under direct vision to avoid blood contamination. Samples were immediately centrifuged at 500 ×g for 5 minutes, and the supernatants were aspirated and stored at −70°C until analysis. OxLDL concentration in the PF was measured in duplicate using a commercially available enzyme-linked immunoassay kit (Immundiagnostik AG, Cat. no. K7810). The concentration of oxLDL was expressed in nanograms per milliliter.

For further analysis all data were tested with the Shapiro-Wilk test for normality. Because data were not normally distributed, statistical significance between E and R groups was determined with the Mann-Whitney U test. The Kruskal-Wallis H test was used for comparisons between the E groups. P value less than 0.05 was considered statistically significant. Data are presented as medians (Me), minima (Min), maxima (Max), and lower and upper quartiles.

Table 1: OxLDL concentrations (ng/mL) in the PF of women with and without endometriosis.

	Me	Min	Max	Lower quartile	Upper quartile
R1	57,4	1,2	1470	36,6	103,7
R2	71,6	17,9	1420	44,8	157,6
E1	75,9	22,1	1134	37,9	157,1
E2	61,6	30,1	1347	47,2	119,4
E3	85,1	16,7	1723	51,4	270,9
E4	270,6	20,8	1870	71,5	1361
E	85,1	16,7	1870	47,3	270,7

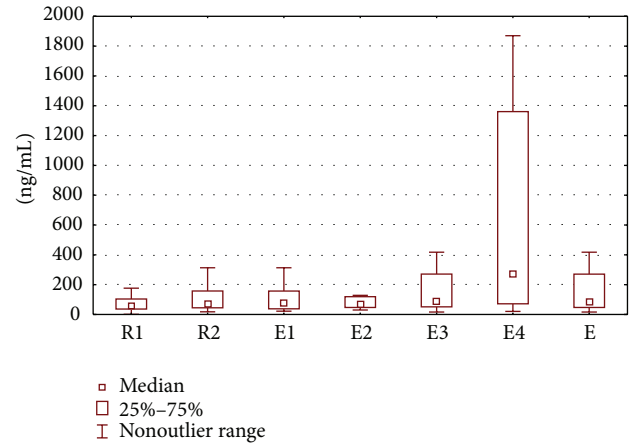

Figure 1: Concentrations of oxLDL in PF of studied women.

3. Results

Oxidized low-density lipoprotein levels were detectable in all PF samples. Concentrations of oxLDL in PF of patients with endometriosis were significantly higher compared to women with serous ovarian cysts ($P = 0.03$). However, no significant difference in the PF oxLDL levels was found between patients with endometriosis and women with dermoid ovarian cysts ($P = 0.4$). Levels of oxLDL in PF of women with serous ovarian cysts were similar to those noted in patients with dermoid cysts (Figure 1, Table 1).

By analyzing concentrations of oxLDL in PF of women with different stages of the disease, it was noted that they were higher only in the subgroup of patients with stage IV endometriosis as compared to women with ovarian serous cysts. No significant differences were found between concentrations of oxLDL in PF of women with different stages of the disease (Table 2).

PF oxLDL concentration did not differ significantly between the subgroups of women in the follicular and the

Table 2: *P* values for comparisons of oxLDL PF concentrations between study groups.

Variable: oxLDL	*P* values for multiple comparisons (two-sided comparisons) The Kruskal-Wallis test					
	R1	R2	E1	E2	E3	E4
R1		1,000000	1,000000	1,000000	1,000000	*0,001582*
R2	1,000000		1,000000	1,000000	1,000000	0,212814
E1	1,000000	1,000000		1,000000	1,000000	0,147871
E2	1,000000	1,000000	1,000000		1,000000	0,111283
E3	1,000000	1,000000	1,000000	1,000000		0,466813
E4	*0,001582*	0,212814	0,147871	0,111283	0,466813	

luteal phase of the menstrual cycle (Me, range: 71.5, 1.2–1470 ng/mL versus 80.3, 16.7–1870 ng/mL, $P = 0.5$).

4. Discussion

In our work, we demonstrated that peritoneal fluid oxLDL concentration was significantly higher in patients with endometriosis than in women with serous ovarian cysts; however, it did not differ significantly as compared to subjects with dermoid cysts. After analysis of data obtained in women with different stages of the disease, it was noted that these results are found only in patients with severe endometriosis. In case of minimal, mild, and moderate disease, peritoneal fluid oxLDL levels were similar to those noted in reference groups. To our knowledge, only Murphy and colleagues investigated the possible role of oxLDL in the pathogenesis of endometriosis. Based on a small number of cases, they found increased oxidation of low-density lipoprotein in women with pelvic endometriosis and increased levels of oxLDL in the PF of patients with this disease [20]. Our results agree with these findings. However, our data suggest that only the severe stage of endometriosis is associated with increased oxidation of low-density lipoprotein in the peritoneal cavity, probably as the result of an imbalance in prooxidant/antioxidant PF systems.

Shanti et al. [13] demonstrated that women with endometriosis had increased serum concentrations of autoantibodies to markers of oxidative stress including oxLDL. Data from our work indirectly confirm these results. Based on many findings, there is an emerging concept of treating endometriosis as an autoimmune disease. An increased incidence of endometriosis was observed in the group of women with autoimmune diseases such as multiple sclerosis, lupus erythematosus, psoriasis, Crohn's disease, hypothyroidism, hyperthyroidism, and rheumatoid arthritis. Endometriosis shares many similarities with other auto-immune diseases including elevated levels of cytokines, decreased cell apoptosis, and T- and B-cell abnormalities. A variety of autoantibodies have been detected in patients with endometriosis patients, which suggests a polyclonal activation of B cells. The most commonly reported types are antiendometrial, antiovarian antibodies, and autoantibodies against phospholipids, histones, and nucleotides. Other similarities between endometriosis and autoimmune diseases include familial occurrence, tissue damage, preponderance of females, and multiorgan involvement [21, 22]. Lipid peroxidation processes are closely associated with the pathophysiology of autoimmune diseases. Therefore, increased levels of oxidized LDL in PF of women with endometriosis support the theory of treating endometriosis as an autoimmune disease.

Oxidized LDL induces secretion of numerous proinflammatory cytokines including macrophage colony-stimulating factor (M-CSF), interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α) [23]. Concentrations of these cytokines were found to be elevated in the PF of patients with endometriosis. We can speculate that increased levels of oxidized LDL in the peritoneal cavity of women with severe endometriosis may be one of the factors responsible for increased levels of M-CSF, IL-6, and TNF-α in PF. Elevated PF concentrations of these cytokines promote adhesion, invasion, proliferation, and angiogenesis of ectopic endometrium and create an inflammatory environment in the peritoneal cavity in women with endometriosis. Rong et al. [5] demonstrated that oxLDL caused an increase in accumulation of monocyte chemotactic factor-1 (MCP-1) in the medium of cultured mesothelial and endometrial cells. They also found that cells cultured in the presence of PF from endometriosis patients secreted more MCP-1 than those cultured with PF from subjects without the disease. Therefore, we hypothesize that increased concentration of oxLDL may be responsible for, as demonstrated in other studies, higher concentrations of MCP-1 in the PF of women with endometriosis. Stimulation of MCP-1 production by increased PF levels of oxidized LDL may hypothetically be another factor responsible for the creation of proinflammatory environment in the peritoneal cavity of patients with endometriosis.

Unfavorable changes in lipid profile are present not only in the PF of women with endometriosis. It has been recently shown that plasma of patients with this disease contains higher concentrations of total cholesterol, low-density lipoproteins, high-density lipoproteins (HDL) and triglycerides as compared to healthy women. Although all lipoproteins were significantly elevated in endometriosis patients, the difference was most substantial for LDL levels, which were 38% higher in women with endometriosis [24]. Verit et al. [14] found that patients with endometriosis displayed significantly lower serum levels of HDL and higher levels of triglycerides, total cholesterol, and LDL than women without the disease. They also demonstrated that serum of

women with endometriosis is characterized by significantly lower activity of paraoxonase-1 (PON-1), which negatively correlated with the progression of the disease [14]. This HDL-associated antioxidant enzyme with paraoxonase activity prevents LDL and HDL oxidation and is also responsible for the antioxidant effect of HDL. Therefore, authors speculated that unfavorable lipid profile combined with lower PON-1 activity in women with endometriosis may contribute to the increased susceptibility for the development of atherosclerosis.

In conclusion, oxLDL levels were significantly higher in the peritoneal fluid of patients with severe endometriosis than in women with serous ovarian cysts. This suggests that disrupted oxidative status in the peritoneal cavity of women with endometriosis may play a role in the etiopathogenesis of the more advanced stages of the disease. However, it cannot be excluded that high oxLDL levels are the result of more oxidant environment in the peritoneal cavity of patients with severe endometriosis than with less advanced disease. Further experiments are required to specify the potential role of oxLDL in disease progression.

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Research Article

Paraoxonase-1 Inhibits Oxidized Low-Density Lipoprotein-Induced Metabolic Alterations and Apoptosis in Endothelial Cells: A Nondirected Metabolomic Study

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We studied the influence of PON1 on metabolic alterations induced by oxidized LDL when incubated with endothelial cells. HUVEC cells were incubated with native LDL, oxidized LDL, oxidized LDL plus HDL from wild type mice, and oxidized LDL plus HDL from PON1-deficient mice. Results showed alterations in carbohydrate and phospholipid metabolism and increased apoptosis in cells incubated with oxidized LDL. These changes were partially prevented by wild type mouse HDL, but the effects were less effective with HDL from PON1-deficient mice. Our results suggest that PON1 may play a significant role in endothelial cell survival by protecting cells from alterations in the respiratory chain induced by oxidized LDL. These results extend current knowledge on the protective role of HDL and PON1 against oxidation and apoptosis in endothelial cells.

1. Introduction

Atherosclerosis, one of the major causes of morbidity and mortality in the Western world, involves complex interactions among endothelial cells of the arterial wall, blood cells, and circulating lipoproteins [1]. Oxidative stress, which is mainly derived from mitochondrial dysfunction, decreases nitrous oxide (NO) synthesis, upregulates the secretion of adhesion molecules and inflammatory cytokines, and is responsible for the oxidation of low-density lipoproteins (LDLs) [2, 3]. These events play a key role in the pathogenesis of atherosclerosis [4, 5].

Paraoxonase-1 (PON1) is an enzyme found in the circulation associated with high-density lipoproteins (HDLs) [6, 7]. The original function attributed to PON1 was that of a lactonase, and lipophylic lactones constitute its primary substrates [8]. PON1 also degrades oxidized phospholipids and, as such, plays a role in an organism's antioxidant system [7]. In the atherosclerosis process, PON1 accumulates in the artery wall [9], and PON1$^{(-/-)}$ mice have been shown to have greater levels of oxidized LDL and larger atheromatous plaques when fed a proatherogenic diet [10]. PON1 also inhibits the production of the proinflammatory chemokine monocyte chemoattractant protein-1 (MCP-1), induced by oxidized LDL in endothelial cells [11].

Despite its potential clinical and biochemical relevance, there is a paucity of studies investigating the influence of PON1 on metabolic alterations when oxidized LDL is incubated with endothelial cells. We reasoned that metabolomics might be a useful tool to evaluate the effects of this enzyme. The study was complemented with an evaluation of oxidative stress and apoptosis in this cell line.

2. Materials and Methods

2.1. Experimental Design. We employed primary cultures of human umbilical vein endothelial cells (HUVECs), cultured according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). HUVECs were grown in medium 200 supplemented with low serum growth, 10 mg/L gentamicin and 0.25 mg/L amphotericin (all these reagents were from Invitrogen), and maintained in a humidified incubator at 37°C, with 5% CO_2. Cells were subcultured when 80%–90% confluent. In all the experiments, cells were plated in 10 cm Petri dishes at a density of 2.5×10^3 cells per dish, and at passage 3. Petri dishes at 70% confluence were incubated over 24 h with isolated human LDL (50 mg/L), oxidized LDL (50 mg/L), oxidized LDL (50 mg/L) + HDL (40 mg/L) from wild type mice, oxidized LDL (50 mg/L) + HDL (40 mg/L) from PON1$^{(-/-)}$ mice, or with serum-free media as controls. All incubations were performed in serum-free media.

Normal human sera were obtained from healthy individuals participating in a population-based study being conducted in our institution. The study was approved by the Ethics Committee (Institutional Review Board) of the Hospital Universitari Sant Joan de Reus. Sera were pooled and used for lipoprotein fractionation and LDL isolation by sequential preparative ultracentrifugation [12, 13]. Human oxidized LDL was prepared by incubation of native LDL with 5 μM $CuSO_4$, as described previously [11]. Increased thiobarbituric acid-reactive substances levels were detected in LDL after oxidation (45 versus <0.5 mmol/g protein).

Normal mice were from the C57BL/6J strain (Charles River Labs., Wilmington, MA, USA), and PON1$^{(-/-)}$ mice were the progeny of those provided by the Division of Cardiology of the University of California in Los Angeles and were of a C57BL/6J genetic background [10]. Animals were housed under standard conditions and given a commercial mouse diet (14% Protein Rodent Maintenance Diet, Harlan, Barcelona, Spain) in accordance with our institutional guidelines. At 16 weeks of age, they were sacrificed and approximately 30 mL of sera were pooled for HDL isolation [12, 13].

2.2. Metabolomics Analyses. The metabolomics platform employed in the present study has been previously described in detail [14]. Briefly, small molecule metabolites from an equivalent amount of cell cytoplasm homogenates were extracted with methanol, and the resulting extract divided into equal fractions for analysis by ultrahigh performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS; separately under positive mode and negative mode) and gas chromatography-mass spectrometry (GC-MS). Metabolites were identified by comparing the ion data obtained to a reference library of ~2,800 chemical standard entries. Comparisons included retention times, mass (m/z), and MS or MS/MS spectra. Results of metabolomics measurements are expressed as the mean quotients between the areas under the peak of the different experimental conditions.

Differences between groups were assessed with Welch's t-test for group comparisons. Statistical analyses were performed with the program "R" http://cran.r-project.org/.

2.3. Caspase 9 Western Blot. We analyzed caspase 9 expression in endothelial cell homogenates as a marker of apoptosis pathways. The cytoplasmic homogenates were prepared with a Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) [15]. Denaturing electrophoresis was performed in polyacrylamide gels (4–12%) from Invitrogen (Carlsbad, CA, USA). Transfer was performed with the iBlot Gel Transfer Device (Invitrogen). Blotting was performed with the ECL Advanced Western Blotting Detection Kit (GE Healthcare, Fairfield, CT, USA) using a rabbit anticaspase 9 antibody at 1 : 2000 dilution (Abcam, Cambridge, UK) [13].

2.4. Measurement of Apoptosis by Flow Cytometry. Cells (300 μL of cell suspension at approximately 10^9 cells/L) were stained with annexin V conjugated with fluorescein isothiocyanate in the presence of propidium iodide. This enables the detection of phosphatidylserine on the surface of apoptotic cells. We used the Annexin-FITC Kit (Beckman-Coulter, Fullerton, CA, USA) according to the manufacturer's instructions, in a Coulter Epics XL-MLC flow cytometer (Beckman-Coulter).

2.5. Measurement of PON1 Activities and Total Peroxide Concentrations. PON1 lactonase activity in the culture's supernatant was measured as the hydrolysis of 5-thiobutyl butyrolactone (TBBL), as described [16]. The assay reagent contained 1 mmol/L $CaCl_2$, 0.25 mmol/L TBBL, and 0.5 mmol/L 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) in 0.05 mmol/L Tris-HCl buffer (pH = 8.0). The change in absorbance was monitored at 412 nm. Activities were expressed as U/L (1 U = 1 mmol of TBBL hydrolyzed per minute). The concentration of total peroxides in the supernatant was determined by a colorimetric enzymatic assay (Immundiagnostik, AG, Bensheim, Germany).

3. Results and Discussion

PON1 lactonase activity remained relatively low in supernatants of those cultures not containing added HDL. PON1 lactonase activity significantly increased in those cultures with normal HDL and returned to low levels in those cultures with HDL from PON1$^{(-/-)}$ mice. These results were as expected and provide a quality control of the HDL preparations obtained (Figure 1(a)). Total peroxide concentrations in the supernatants were maximal in the cultures with added oxidized LDL and showed a significant decrease following the addition of normal HDL. This decrease was not as marked following the addition of HDL from PON1$^{(-/-)}$ mice (Figure 1(b)).

We analyzed 124 biochemical compounds by nondirected metabolomics, corresponding to carbohydrate, lipid, amino acid, and nucleotide metabolism, as well as vitamins and xenobiotics. We obtained statistically significant variations in 37 metabolites (Table 1). The main findings corresponded to

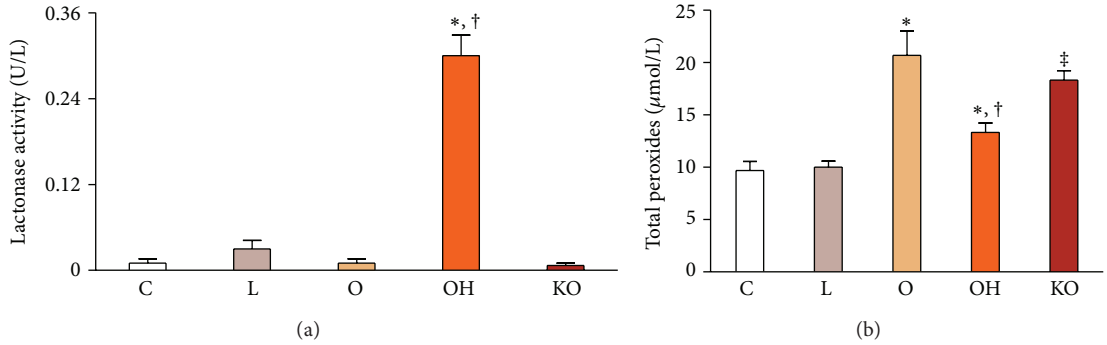

Figure 1: PON1 lactonase activity (a) and total peroxide concentrations (b) in the supernatant of the HUVEC cell culture ($n = 3$, for each experiment). Endothelial cells were incubated over 24 h with 50 mg/L isolated human LDL (L); 50 mg/L oxidized LDL (O); 50 mg/L oxidized LDL + 40 mg/L HDL from wild type mice (OH); 50 mg/L oxidized LDL + 40 mg/L HDL from PON1$^{(-/-)}$ mice (KO); or with serum-free media as controls (C). $^{*}P < 0.05$, with respect to C; $^{\dagger}P < 0.05$, with respect to O; $^{\ddagger}P < 0.01$, with respect to C.

carbohydrate and phospholipid metabolism and are summarized in the following sections.

3.1. Hexose Metabolism. The addition of LDL to cultured endothelial cells decreased the levels of gluconate, galactose, and phosphorylated hexose intermediates. These molecules are important entrance intermediates in energy- and biomass-generating pathways such as glycolysis, pentose phosphate, and protein glycosylation. Their decreases suggest that these pathways were activated to a greater extent in endothelial cells treated with LDL, compared to control-treated cells. In contrast, increased levels of gluconate, galactose, and phosphorylated hexose intermediates were seen in all cells that were treated with oxidized LDL, relative to LDL alone, and regardless of whether HDL was also added to the cultures (Figure 2).

3.2. Glycolysis and Tricarboxylic Acid (TCA) Cycle. Relative to control cultures, the addition of LDL resulted in increased levels of 3-phosphoglycerate and 2-phosphoglycerate (which are 3-carbon glycolytic intermediates). This same treatment also increased the levels of the TCA cycle intermediates (fumarate and malate) relative to control cultures. An interpretation of these data is that uptake of LDL by endothelial cells results in the generation of acetyl-CoA, which drives flux through the TCA cycle. Increased levels of LDL-generated acetyl-CoA may have relieved the need for carbohydrate-derived precursors, thereby inhibiting glycolytic flux into the TCA cycle and elevating the 3-carbon intermediates.

By comparison, treatment of endothelial cells with oxidized LDL may have induced levels of oxidative stress that were sufficient to impair normal energy pathways. For example, in cells treated with oxidized LDL, 6-carbon glycolytic intermediates accumulated, whereas the 3-carbon intermediates were reduced. This may be due to changes in glyceraldehyde-3-phosphate dehydrogenase (GADPH; levels or activity) in response to oxidized LDL, since superoxide overproduction inhibits GADPH through a mechanism that involves poly (ADP-ribose) polymerase (PARP) activation [16]. Likewise, TCA cycle intermediates were lower in oxidized LDL-treated cells due, most likely, to the attenuated conversion of 6-carbon glycolytic intermediates to 3-carbon compounds that feed into this cycle through pyruvate and acetyl-CoA. These changes suggest that energy production through glycolysis is impaired, since ATP generation occurs downstream of GADPH activity.

The addition of normal HDL to oxidized LDL-treated cells partially reverses its impact on energy metabolism pathways, since levels of the 3-carbon glycolytic intermediates as well as TCA cycle intermediates are more similar to levels observed after LDL treatment alone. It is of note that the impact of addition of HDL from PON1$^{(-/-)}$ mice on these molecules was intermediate between the effects produced by treatment with PON1-containing HDL and of no HDL (Figure 3). This observation is of considerable importance, because PARP activation and its consequent metabolic changes have been associated with endothelial dysfunction in diseases such as atherosclerosis and diabetes [17, 18]. Indeed, the levels of circulating endothelial cells are increased in patients with diabetes mellitus [19], and PON1 has been shown to attenuate diabetes development in mice [20, 21]. Our results suggest that the beneficial role of PON1 may involve, at least in part, a protection against the biochemical changes leading to endothelial dysfunction.

3.3. Phospholipid Metabolism. Levels of choline, ethanolamine, and glycerol-3-phosphate—key building blocks for phospholipids—were similar in endothelial cells following treatment with LDL, when compared to levels in control cells. By comparison, oxidized LDL reduced levels of phospholipid precursors and increased the levels of at least one phospholipid breakdown product. This could indicate that oxidized LDL induces membrane damage, breakdown, or remodeling. As was observed for the energy metabolism pathways, coadministration of normal HDL to oxidized LDL-treated cells reversed, or partially reversed, these deleterious effects. However, the addition of HDL from PON1$^{(-/-)}$ mice only generated subtle changes in phospholipid-related

Table 1: Heat map of metabolites showing statistically significant differences between groups.

Pathway	Metabolite	L/C*	O/C*	O/L*	OH/O*	KO/O*	KO/OH*
Glycine, serine, and threonine metabolism	Threonine	**1.40**	0.80	*0.57*	0.96	1.10	1.16
Glutamate metabolism	N-acetylglutamate	1.14	0.84	0.73	1.10	0.92	*0.83*
Phenylalanine and tyrosine metabolism	Phenylalanine	1.12	**0.63**	*0.56*	***1.41***	**1.19**	0.84
	Tyrosine	1.11	0.62	*0.56*	**1.57**	1.37	0.87
Valine, leucine, and isoleucine metabolism	Isoleucine	1.36	0.65	*0.48*	**1.57**	**1.38**	0.88
	Leucine	1.09	**0.70**	*0.64*	1.25	1.07	0.85
	Valine	1.26	**0.72**	*0.57*	1.18	1.08	0.91
Urea cycle; arginine-, proline-, metabolism	Praline	1.18	0.89	*0.75*	0.92	0.97	1.05
Gamma-glutamyl peptides	Gamma-glutamyl-leucine	*0.73*	0.87	1.19	1.09	1.48	1.36
Amino-sugar metabolism	Fucose	*0.72*	**0.76**	1.05	1.25	1.06	0.85
Fructose, mannose, galactose, starch, and sucrose metabolism	Galactose	*0.37*	0.94	***2.58***	1.04	0.73	0.7
	Mannose-6-phosphate	0.64	2.21	***3.44***	1.01	0.87	0.87
	Glucose-6-phosphate	0.33	2.06	***6.20***	1.22	1.17	0.96
	Fructose-6-phosphate	0.50	**2.50**	***5.01***	1.13	0.94	0.83
	2-phosphoglycerate	**2.28**	0.67	*0.29*	***2.17***	1.05	*0.48*
	3-phosphoglycerate	1.62	**0.42**	*0.26*	***2.92***	***1.94***	*0.67*
	1,3-dihydroxyacetone	0.85	0.98	1.15	0.80	*0.60*	0.75
	Phosphoenolpyruvate	1.06	*0.23*	*0.22*	***4.66***	***3.05***	0.66
Nucleotide sugars, pentose metabolism	Gluconate	*0.43*	0.89	***2.07***	1.06	0.86	0.81
TCA cycle	Fumarate	1.35	0.84	*0.62*	1.10	1.12	1.02
	Malate	1.31	0.89	*0.68*	**1.21**	1.07	0.88
Oxidative phosphorylation	Acetyl phosphate	1.00	1.12	1.12	0.84	*0.59*	0.70
	Phosphate	0.96	1.45	***1.52***	0.89	*0.70*	*0.78*
Medium chain fatty acid	Laurate (12 : 0)	0.98	1.15	**1.17**	0.92	*0.74*	*0.81*
Fatty acid, dicarboxylate	Undecanedioate	1.28	**1.52**	1.19	2.55	*0.70*	0.28
Glycerolipid metabolism	Ethanolamine	1.00	0.68	*0.68*	1.28	***1.55***	1.21
	Choline	1.07	0.84	*0.78*	***1.29***	1.25	0.96
	Glycerol 3-phosphate	1.54	0.31	*0.20*	***4.13***	***2.87***	*0.69*
	Glycerophosphorylcholine	*0.60*	1.17	***1.97***	0.80	**0.78**	0.97
Purine metabolism, adenine containing	Adenosine 3′-monophosphate	**2.38**	0.73	*0.31*	***1.70***	1.09	*0.64*
Pyrimidine metabolism, uracil containing	Uracil	1.22	0.48	*0.40*	***1.94***	***1.91***	0.99
	Uridine 5′-monophosphate	*0.50*	1.19	***2.38***	0.84	0.92	1.10
Pantothenate and CoA metabolism	Pantothenate	0.98	*0.87*	0.89	***1.19***	1.13	0.95
Riboflavin metabolism	Riboflavin (Vitamin B2)	*0.68*	0.76	1.11	1.15	1.11	0.97
Benzoate metabolism	4-hydroxy catechol	1.23	1.37	1.11	0.79	*0.43*	*0.54*
Chemicals	Glycolate (hydroxyacetate)	1.12	**1.55**	**1.38**	*0.47*	0.77	**1.64**
	Glycerol 2-phosphate	0.98	0.65	*0.67*	1.96	1.11	0.57

Endothelial cells were incubated over 24 h with 50 mg/L isolated human LDL (L); 50 mg/L oxidized LDL (O); 50 mg/L oxidized LDL + 40 mg/L HDL from wild type mice (OH); 50 mg/L oxidized LDL + 40 mg/L HDL from PON1$^{(-/-)}$ mice (KO); or with serum-free media as controls (C). Bold italic and italic cells in the Table indicate $P \leq 0.05$. Bold italic indicates that the mean values are significantly higher; italic indicates significantly lower. Bold text indicates $0.05 < P < 0.10$.
*Results are expressed as the mean quotients of the areas under the peak of the different experimental conditions. For example, galactose values are, on average, 2.58 times higher when endothelial cells are incubated with oxidized LDL than when incubated with native LDL. All measurements were performed in triplicate.

compounds, when compared to treatment with normal HDL (Figure 4).

3.4. Apoptosis. The observation of alterationsin phospholipids levels and the suggested membrane damage channeled us towards investigating the possibility of an increased apoptosis in endothelial cells incubated with oxidized LDL, and a possible protection by introducing HDL as coincubation. Hence, we analyzed caspase 9 protein expression. The activation of this enzyme is a good indicator of apoptosis induction, since caspase 9 plays a determinant role in

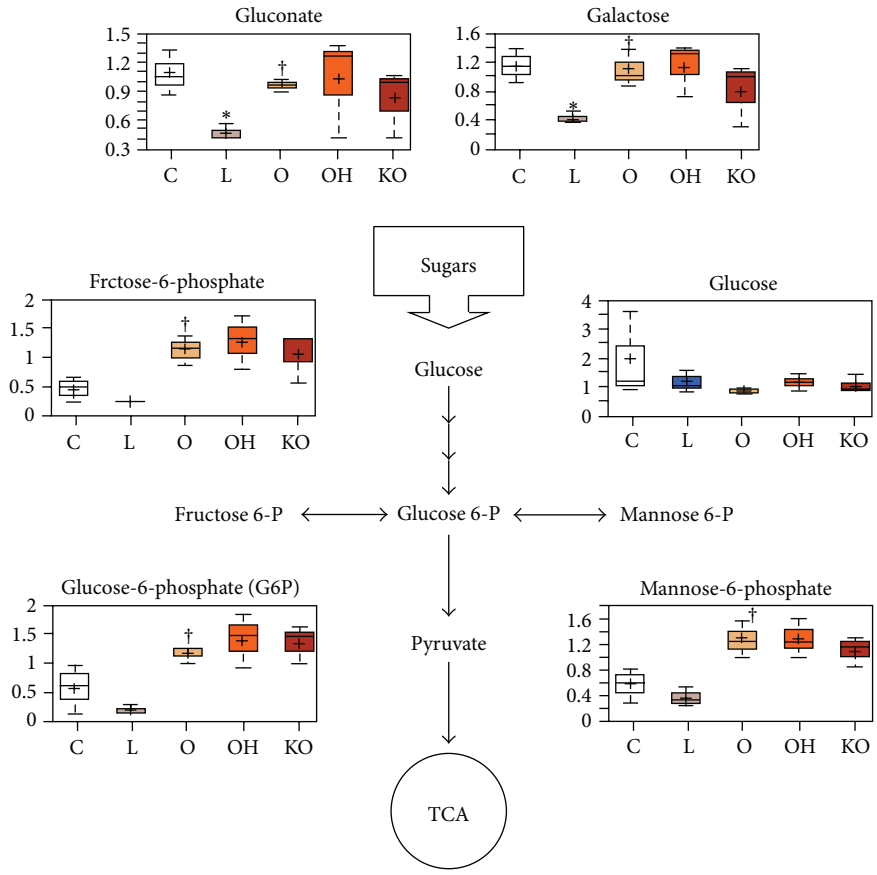

FIGURE 2: Variations in the hexose metabolites in HUVEC cell homogenates ($n = 3$, for each experiment). Endothelial cells were incubated over 24 h with 50 mg/L isolated human LDL (L); 50 mg/L oxidized LDL (O); 50 mg/L oxidized LDL + 40 mg/L HDL from wild type mice (OH); 50 mg/L oxidized LDL + 40 mg/L HDL from PON1$^{(-/-)}$ mice (KO); or with serum-free media as controls (C). $^{*}P < 0.05$ with respect to C; $^{\dagger}P < 0.05$ with respect to L.

apoptosome formation [22]. Also, we measured the numbers of apoptotic cells by flow cytometry. We observed that oxidized LDL addition increased caspase 9 expression and the percentage of apoptotic endothelial cells, when compared to control cells and cells treated with normal LDL. Coincubation with normal HDL completely preempted this effect. However, the influence of HDL from PON1$^{(-/-)}$ animals was much lower (Figures 5(a) and 5(b)). We observed a strong direct correlation ($r = 0.91$; $P < 0.001$) between total peroxides concentrations and the percentage of apoptotic cells (Figure 5(c)). Previous studies had shown that increased lipid peroxidation in HDL particles from coronary artery disease patients was associated with an impaired capacity of this particle to stimulate endothelial NO production [23]. Notably, PON1 has been reported to prevent lipid peroxidation in HDL particles and to promote HDL-mediated inactivation of oxidized lipids in LDL. Its activity was shown to be decreased in patients with coronary disease [7]. Further, HDL and PON1 decreased the formation of malondialdehyde-like epitopes and the formation of apoptotic particles in monocytes [24]. A very recent study showed that HDL from healthy people induced the expression of endothelial antiapoptotic protein Bcl-xL and reduced endothelial cell apoptosis *in vitro* as well as *in vivo* in apoE-deficient mice. In contrast, HDL from coronary artery disease patients did not inhibit endothelial apoptosis, failed to activate endothelial Bcl-xL, and stimulated endothelial proapoptotic pathways [25]. Our findings of a decreased oxidized LDL-induced apoptosis by normal HDL, but not by HDL from PON1$^{(-/-)}$ mice, together with a significant association between lipid peroxidation (as measured by total peroxides concentrations) and the percentage of the apoptotic cells would tend to confirm this very recent information.

Our results suggest that PON1 may play a significant role in cell survival by improving mitochondrial function. Indeed, mitochondria regulate apoptosis in response to cellular stress signals and, hence, determine whether cells live or die. As such, it is probable that peroxides constitute important candidates in the regulation of cell death, and that mitochondria act as both sensor and effector sites [26]. This could explain the influence of apoptosis-related proteins on mitochondrial respiration. Whether or not this finding has any impact on the atherosclerosis process warrants further exploration.

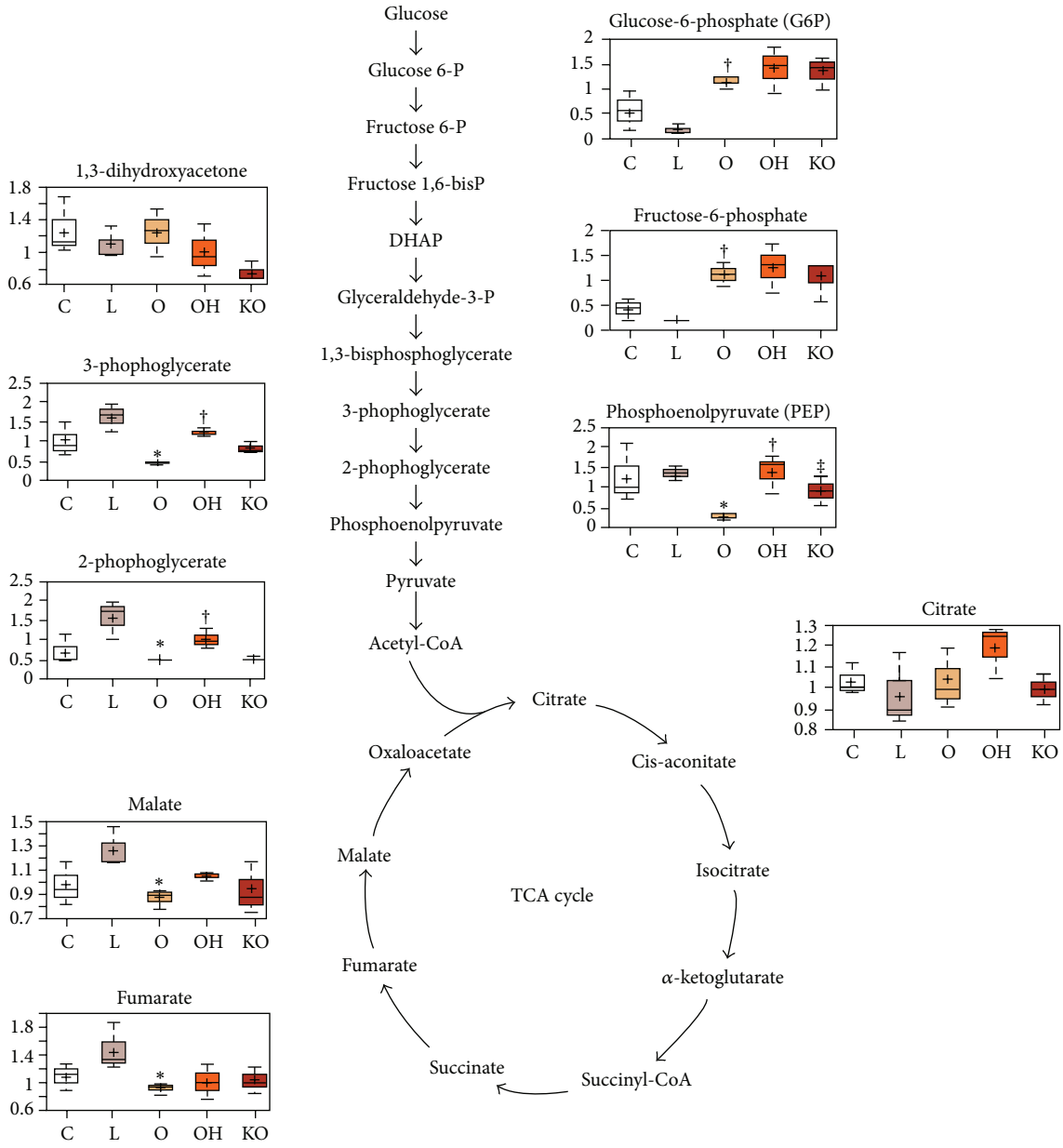

FIGURE 3: Variations in the metabolites of the glycolytic pathway and tricarboxylic acid cycle in HUVEC cell homogenates ($n = 3$, for each experiment). Endothelial cells were incubated over 24 h with 50 mg/L isolated human LDL (L); 50 mg/L oxidized LDL (O); 50 mg/L oxidized LDL + 40 mg/L HDL from wild type mice (OH); 50 mg/L oxidized LDL + 40 mg/L HDL from PON1$^{(-/-)}$ mice (KO); or with serum-free media as controls (C). $^{*}P < 0.05$ with respect to L; $^{\dagger}P < 0.05$ with respect to O; $^{\ddagger}P < 0.05$ with respect to OH.

4. Conclusion

Epidemiological studies have shown that the risk of atherosclerosis is inversely associated with HDL concentrations. The protective effect of this lipoprotein has been attributed, in part, to the antioxidant and anti-inflammatory action of PON1 [27]. We have showed, previously, that PON1 inhibits MCP-1 induction in endothelial cells [11], which suggested a protective role against liver inflammation mediated by MCP-1 [28]. More recent studies indicated that the anti-inflammatory effect of PON1 depends on its association with HDL [29], and that PON1 stimulates HDL antiatherogenicity [30], and macrophage response [31], and increases the duration over which HDL is able to prevent LDL oxidation [32].

The present study is novel in using a metabolomic approach to investigate the protective effect of PON1 on endothelial cells incubated with oxidized LDL. We observed important metabolic alterations in human endothelial cells incubated with oxidized LDL. These include an impaired glycolysis, TCA cycle, phospholipids, and activation of apoptotic pathways. These changes were ameliorated by incubation

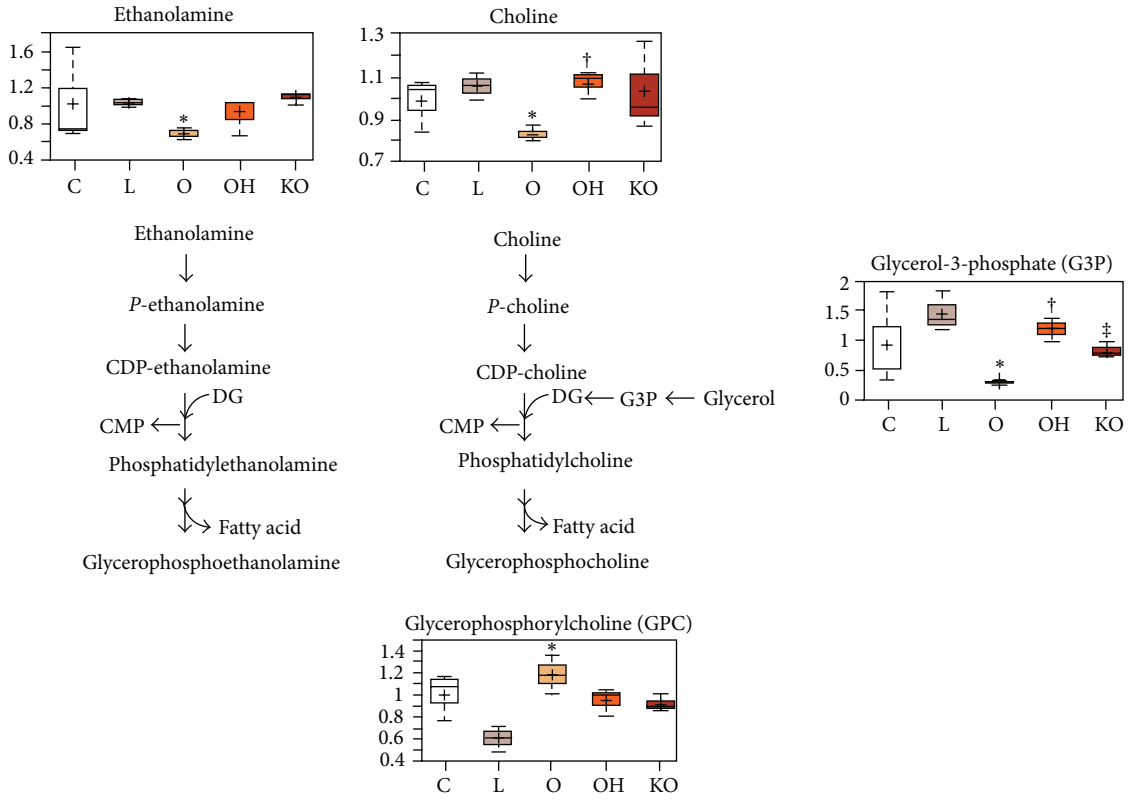

FIGURE 4: Variations in phospholipid metabolites in HUVEC cell homogenates ($n = 3$, for each experiment). Endothelial cells were incubated over 24 h with 50 mg/L isolated human LDL (L); 50 mg/L oxidized LDL (O); 50 mg/L oxidized LDL + 40 mg/L HDL from wild type mice (OH); 50 mg/L oxidized LDL + 40 mg/L HDL from PON1$^{(-/-)}$ mice (KO); or with serum-free media as controls (C). $^{*}P < 0.05$ with respect to L; $^{\dagger}P < 0.05$ with respect to O; $^{\ddagger}P < 0.05$ with respect to OH.

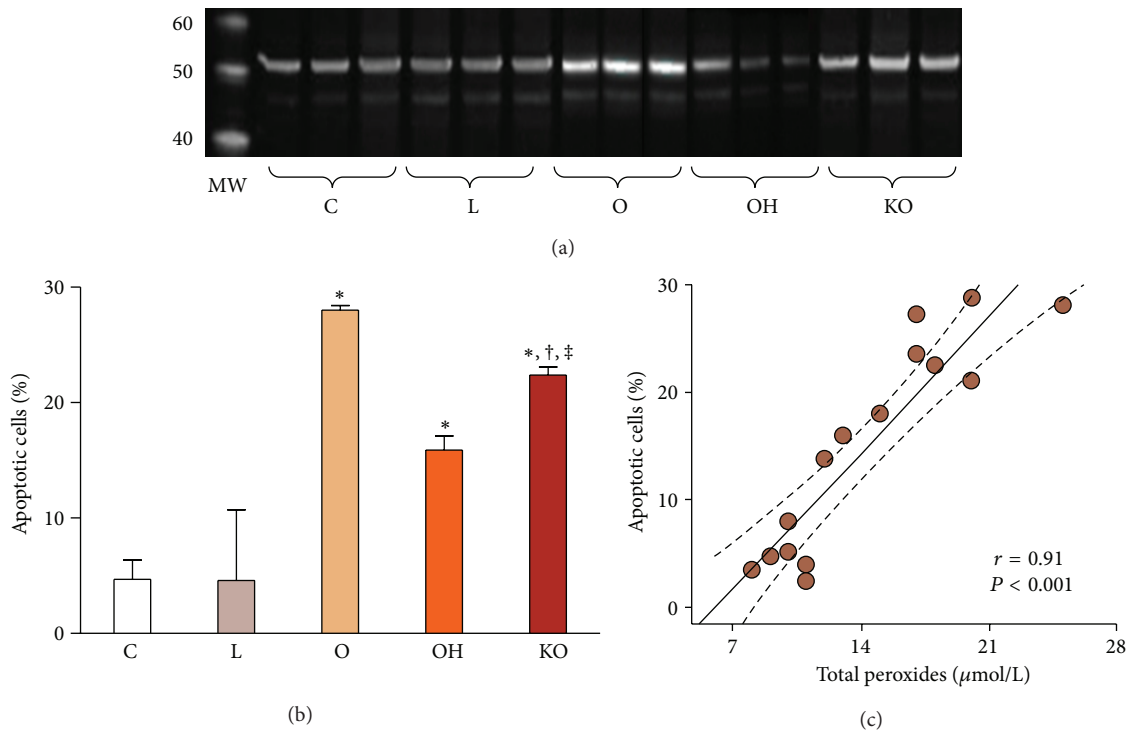

FIGURE 5: (a) Western blot analyses for caspase 9; (b) percentage of apoptotic cells; (c) relationship between total peroxide concentrations and the percentage of apoptotic cells in HUVEC cell homogenates ($n = 3$, for each experiment). Endothelial cells were incubated over 24 h with 50 mg/L isolated human LDL (L); 50 mg/L oxidized LDL (O); 50 mg/L oxidized LDL + 40 mg/L HDL from wild type mice (OH); 50 mg/L oxidized LDL + 40 mg/L HDL from PON1$^{(-/-)}$ mice (KO); or with serum-free media as controls (C). MW: molecular weight marker. $^{*}P < 0.01$ with respect to C; $^{\dagger}P < 0.05$ with respect to OH; $^{\ddagger}P < 0.01$ with respect to O.

with normal HDL, while HDL isolated from PON1$^{(-/-)}$ mice showed an impaired efficiency to protect against the oxLDL-induced changes. These results extend the current knowledge on the protective role of HDL and PON1 against oxidation in endothelial cells.

Abbreviations

DTNB:	5,5′-Dithio-bis-2-nitrobenzoic acid
GADPH:	Glyceraldehyde-3-phosphate dehydrogenase
GC-MS:	Gas chromatography-mass spectrometry
HDL:	High-density lipoproteins
HUVEC:	Human umbilical vein endothelial cells
LDL:	Low-density lipoproteins
PARP:	Poly (ADP-ribose) polymerase
PON1:	Paraoxonase-1
TBBL:	5-thiobutyl butyrolactone
UPLC-MS/MS:	Ultrahigh performance liquid chromatography-tandem mass spectrometry.

Conflict of Interests

The authors declare that they do not have conflict of interests.

Acknowledgments

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Clinical Study

Levels of Oxidized LDL, Estrogens, and Progesterone in Placenta Tissues and Serum Paraoxonase Activity in Preeclampsia

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In vitro literature studies have suggested that atherosclerotic oxidized low density lipoprotein (OxLDL) inhibits trophoblast invasion. The objective of this study was to determine the levels of OxLDL and to examine the relationship between antioxidative estradiol, estriol, and prooxidative progestin in normal and preeclamptic placental tissues and measure the serum activity of antioxidative paraoxonase (PON1). The study included 30 preeclamptic and 32 normal pregnant women. OxLDL was determined with ELISA, estradiol, unconjugated estriol, and progesterone that were determined with chemiluminescence method in placental tissues. Serum PON1 activity was determined with spectrophotometric method. Levels of OxLDL ($P = 0.027$), estriol ($P < 0.001$), estradiol ($P = 0.008$), and progesterone ($P = 0.009$) were lower in the placental tissues of preeclamptic group compared to the normal pregnant women. Serum PON1 activity was higher in preeclamptic group ($P = 0.040$) and preeclamptic group without intrauterine growth restriction ($P = 0.008$) compared to normal pregnant women. Tissue estriol of preeclamptic group without/with IUGR ($P < 0.001$, $P = 0.002$) was lower than the normal group. Results of our study suggest that the events leading to fetoplacental insufficiency lead to a reduction in the levels of estriol limit deposition of OxLDL in placental tissues. The serum PON1 activity is probably important in the inhibition of OxLDL in preeclampsia.

1. Introduction

Preeclampsia is a syndrome characterized by hypertension and proteinuria leading to maternal and perinatal mortality [1]. The etiopathogenesis of preeclampsia has not been fully understood to date. It is believed that in preeclampsia, the physiological remodeling of the uterine spiral arteries into dilated uteroplacental vessels observed in normal pregnancies is disrupted [2].

Indeed, medial hyperplasia and atherosis of the spiral arteries have been reported [2]. Acute atherosis is an occlusive lesion of the spiral arteries with an unknown time of onset characterized with infiltration by foam cells that store cytoplasmic fat [3]. Oxidation of low density lipoproteins (LDL) to oxidized LDL (OxLDL) is a triggering and accelerating factor in the development of atherosclerotic lesions [4]. Oxidative stress is one of the stimulating factors taking part in the inflammatory response of pregnancy [3].

The placenta is the major source of prooxidant agents, antioxidant enzyme systems and hormones, and is able to keep the lipid peroxidation under control in normal pregnancy. The balance between prooxidative and antioxidative metabolites affects the lag phase of LDL oxidation [5].

Estradiol inhibits LDL oxidation, whereas progesterone increases LDL oxidation in primary tissue cultures of term human placenta [6]. PON1 is an antioxidative enzyme found in the high density lipoprotein and acts as protective against in vitro oxidative modification of LDL [7].

Since spiral artery disease due to atherosis is suggested to be involved in the pathogenesis of preeclampsia, it seemed reasonable to examine levels of OxLDL because of its atherogenic role.

Our review of the literature failed to find a report that has done OxLDL measurements directly in placental tissues in preeclampsia. We hypothesized that atherogenic OxLDL might be increased in preeclamptic placental tissues. The objective of our study was to measure the levels of OxLDL and investigate the relationship between OxLDL and prooxidant progestin and antioxidant estrogens in preeclamptic placental tissues and placental tissues of normal pregnant women. Furthermore, PON1 activity was investigated in preeclamptic and normal serum because of its antioxidative property.

2. Material and Methods

The patients in this study were all Turkish women. Thirty women with preeclampsia and 32 women with normotensive, uncomplicated pregnancies who delivered at the Department of Obstetrics and Gynecology, Bülent Ecevit University, were included in the study after obtaining approval from the Faculty Ethics Committee. Written informed consent was obtained from each patient prior to the performance of any study procedures.

The diagnosis of preeclampsia was established in accordance with the definition of the American College of Obstetricians and Gynecologist [8].

After delivery, fetal intrauterine growth restriction (IUGR) was defined as birthweight <10th percentile as assessed by gestational age at delivery with the definition of Alexander et al. [9].

Samples were taken from the central region of the placenta and placed on ice, transported to the laboratory, processed within 30 min, rinsed in ice cold PBS, and immediately frozen at −80°C for analysis at a later date. Tissue samples were minced and homogenized at 1/1 rate with phosphate buffer saline at pH 7.4 using a homogenizer (Ultra Turrax IKA T18 Basic). The homogenates were centrifuged at 9000 ×g at 4°C for 30 minutes and the supernatants were immediately analyzed. The levels of OxLDL, estradiol, unconjugated estriol, and progesterone were measured in supernatant, following centrifugation, and the results were calculated as per gram tissue. Blood samples were obtained prior to the onset of labor. After the blood clotted, the samples were centrifuged and aliquots of serum were immediately stored at −80°C until assayed for PON1 activity. All analyses completed on blinded samples and they ran in duplicate.

Levels of oxLDL were determined using the Human OxLDL ELISA kit (Cusabio Biotech Catalog no. CSB-E07931 h, China) with the sandwich method in EPSON LX-300 ELISA device (BIO-TEC Instruments, Winooski, USA) with an intra-assay CV of <3% and interassay CV of <10%. ELISA kit was specific for total OxLDL.

Levels of estradiol (intra-assay CV of <4.9%, interassay CV of <7.1%), unconjugated estriol (intra-assay CV of <5.2%, interassay CV of <7.3%), and progesterone (intra-assay CV of <7.0%, interassay CV of <9.5%) were assayed using an Immulite 2000 device (Siemens, CA, USA), with the solid phase, competitive chemiluminescent enzyme immunoassay.

PON1 activity was measured using Shimadzu UV 1601 spectrophotometer (Shimadzu Co., Kyoto, Japan) according to the definition of Gan et al. with and intra-assay CV of <8.8%. One mMol of paraoxon was used as substrate in the presence of 1 mMol of $CaCl_2$ in 100 mMol Tris-HCl buffer (pH 8.0); serum was added to start the reaction, and the increase in absorbance at 412 nm was recorded. The amount of p-nitrophenol was calculated from the molar extinction coefficient (17.100 M^{-1} cm^{-1}). Results were expressed as U/L [10].

3. Statistical Analysis

The statistical package for social sciences 13.0 (SPSS Inc, Chicago, IL, USA) was used in all data analyses. The Kolmogorov-Smirnov test was used to test the normality of distributions. Student t-test was used to test the significance of differences between preeclamptic and normal study groups with normal distribution.

Three group comparisons of the variables with normal distribution were done with one way analysis of variance (ANOVA). Bonferroni test was used as a post-hoc test for significant variables. For the variables without normal distribution, Kruskal-Wallis test was used for three group comparisons and Mann Whitney U-test with Bonferroni adjustment used for two group comparisons of significant variables. Pearson's and Spearman's correlation analyses were used to determine the relationship between continuous variables state measurement.

Power calculations for the related comparison tests were done with PASS 2008 package programme. P values less than 0.05 were considered statistically significant.

4. Results

Demographic (maternal age, BMI), clinical (systolic and diastolic blood pressures, urinary protein excretion per day, infant's birth weight, scores of Apgar at 1 and 5 minutes) and biochemical characteristics (tissue and serum), and correlation coefficients of tissue markers and clinical data are shown in Tables 1, 2, 3, and 4, respectively. Biochemical characteristics of normal pregnant women (group 1), preeclamptic pregnant women without IUGR (group 2), and preeclamptic pregnant women with IUGR (group 3) are shown in Tables 5 and 6.

Mean gestational weeks (GW) were 35.1 ± 4.4 in the preeclamptic group and 37.4 ± 1.5 in the control group. The rates of vaginal delivery/C-section were 9/23 in normal group, 2/28 in preeclamptic patients. Nulliparity/multiparity were 18/14 in normal group and 18/12 in preeclamptic group. Pregnant women in both groups had quit smoking throughout their pregnancies.

The OxLDL ($P = 0.027$), estriol ($P < 0.001$), estradiol ($P = 0.008$), and progesterone ($P = 0.009$) levels of the placental tissues in preeclamptic patients were significantly lower than in the healthy pregnant women using student t-test ($P < 0.05$). After Bonferroni adjustment, tissue estriol, estradiol, and progesterone levels were found to be statistically significant in the preeclamptic patients compared

Table 1: Demographic and clinical data of the study subjects.

	Normal pregnant women ($n = 32$)	Preeclamptic pregnant women ($n = 30$)	P value
	Mean ± SD		
Maternal age, years	27 ± 6	30 ± 6	0.078
Gestational age, weeks	37.4 ± 1.5	35.1 ± 4.4	**0.009**
Infant's birth weight, g	3172 ± 369	2429 ± 907	**0.001**
BMI	27.6 ± 1.5	28.6 ± 1.1	**0.008**
	Median (min-max)		
Systolic BP, mm Hg	110 (90–140)	150 (140–210)	**0.001**
Diastolic BP, mm Hg	70 (50–100)	90 (90–120)	**0.001**
Urine mg protein/day	37.6 (7.90–200)	921 (307–6923)	**0.001**
Apgar 1 min	9 (3–10)	9 (0–10)	**0.038**
Apgar 5 min	10 (5–10)	10 (0–10)	**0.023**

Statistically significant $P < 0.05$.

Table 2: Levels of OxLDL, estriol, estradiol, and progesterone in the placenta of preeclamptic and normal pregnant women.

Placental tissue	Normal pregnant women ($n = 32$)	Preeclamptic pregnant women ($n = 30$)	P value
OxLDL, μMol/mL/g tissue	9.85 ± 6.82	6.54 ± 4.42	0.027
Estriol, ng/mL/g tissue	67.6 ± 23.1	41.9 ± 23.2	**0.001**
Estradiol, pg/mL/g tissue	25.9 ± 18.0	14.6 ± 14.2	**0.008**
Progesterone, ng/mL/g tissue	0.34 ± 0.31	0.16 ± 0.21	**0.009**

Mean ± SD
Statistically significant $P < 0.01$.

Table 3: Serum PON1 activity in preeclamptic and normal pregnant women.

Serum	Normal pregnant women ($n = 32$)	Preeclamptic pregnant women ($n = 30$)	P value
PON1, U/L	121 ± 80	174 ± 115	**0.040**

Mean ± SD
Statistically significant $P < 0.05$.

Table 4: Correlation coefficients of tissue markers and clinical data ($n = 62$).

	Correlation coefficient (r)	P value
Tissue estriol-tissue OxLDL	0.36	**0.004**
Tissue estriol-GW	0.25	>0.05
Tissue OxLDL-GW	0.04	>0.05
Tissue estriol-infant's birth weight	0.39	**0.002**
Tissue estriol-systolic BP	−0.44	**0.001**
Tissue estriol-diastolic BP	−0.48	**0.001**

Statistically significant $P < 0.05$.

to those of the normal pregnant women, whereas the levels of OxLDL were not different significantly (Table 2).

Serum PON1 activity ($P = 0.040$) was significantly higher in the preeclamptic group compared to that of the normal pregnant women (Table 3).

A positive correlation between the tissue OxLDL-tissue estriol levels ($r = 0.36$, $P = 0.004$) and between tissue estriol-infant's birth weight ($r = 0.39$, $P = 0.002$) was given in Table 4. A negative correlation between the tissue estriol levels and systolic BP ($r = -0.44$, $P < 0.001$) and diastolic BP ($r = -0.48$, $P < 0.001$) was given in Table 4.

The estriol ($P < 0.001$), estradiol ($P = 0.007$), and progesterone ($P = 0.007$) levels of the placental tissues and serum PON1 ($P = 0.025$) were different significantly between normal pregnant group, preeclamptic group without IUGR, preeclamptic group with IUGR (Tables 5 and 6).

Comparison between the groups is shown in Tables 5 and 6.

Power calculation of the biochemical data revealed 62.6% for OxLDL, 99% for estriol, 79% for estradiol, 76.9% for progesterone, and 54.4% for PON 1.

5. Discussion

Several pathological processes occurring in the preeclamptic placenta are thought to be involved in the pathogenesis, including impaired spiral artery remodeling [2], endothelial dysfunction, impaired placental trophoblastic implantation, and inadequate perfusion of uterine-placental unit [11]. Atherosclerotic endothelial alterations triggered by oxidative stress have also been stressed in the pathogenesis [1]. Oxidation of LDL secondary to oxidative stress and transformation

Table 5: Levels of OxLDL, estriol, estradiol, and progesterone in the placenta of preeclamptic without/with and normal pregnant women.

Placental tissue	Normal pregnant women ($n = 32$) group 1	Preeclamptic pregnant women without IUGR ($n = 21$) group 2	Preeclamptic pregnant women with IUGR ($n = 9$) group 3	P value
		Mean ± SD		
OxLDL, μMol/mL/g tissue	9.85 ± 6.82	5.88 ± 4.41	8.09 ± 4.26	0.058
Estriol, ng/mL/g tissue	67.6 ± 23.1	43.5 ± 24.6*	38.4 ± 20.6*	**0.001**
		Median (min-max)		
Estradiol, pg/mL/g tissue	20.3 (6.2–62.5)	8.2 (5.6–37.2)*	11.4 (5.3–61.3)	**0.007**
Progesterone, ng/mL/g tissue	0.22 (0.06–1.18)	0.08 (0.06–0.45)*	0.11 (0.05–0.97)	**0.007**

Statistically significant $P < 0.05$
*Differ from the normal group: (group 1-2: estriol $P < 0.001$, group 1–3: estriol $P = 0.002$, group 1-2: estradiol $P = 0.001$, group 1-2: progesterone $P = 0.001$).

Table 6: Serum PON1 activity in preeclamptic without/with and normal pregnant women.

Serum	Normal pregnant women ($n = 32$) group 1	Preeclamptic pregnant women without IUGR ($n = 21$) group 2	Preeclamptic pregnant women with IUGR ($n = 9$) group 3	P value
PON1, U/L	116 (22–288)	185 (50–520)*	86 (70–213)	**0.025**

Median (Min–Max)
Statistically significant $P < 0.05$
*Differ from the normal group: group 1-2: PON1 $P = 0.008$.

of monocytes into foam cells following the uptake of OxLDL are significant steps towards endothelial dysfunction [11].

5.1. OxLDL. Pavan et al. have demonstrated the presence of OxLDL in villous and extravillous cytotrophoblasts of human placenta in the first trimester using immunohistochemistry, and reported in a cell culture study that OxLDL leads to defective trophoblast invasion [12].

There is no consensus on the levels of OxLDL in preeclamptic women in the literature. Kim et al. [13] and Genç et al. [14] have reported that serum OxLDL levels were higher in the preeclamptic pregnant women compared to the normal pregnant women, whereas others Pecks et al. [15] and Raıjmakers et al. [16] have reported lower levels.

Our results are in conjunction with those of Pecks et al. and Raıjmakers et al. In our study, differing from the previous studies, we examined the levels of OxLDL in placental tissues.

Raıjmakers et al. have determined lower OxLDL levels in the serum of preeclamptic pregnant women compared to the controls and suggested that autoantibodies against OxLDL could accelerate the serum clearance of OxLDL [16].

Pecks et al. did not find increased levels of serum OxLDL in preeclamptic patients contrary to their hypothesis. They found lower serum OxLDL levels in preeclampsia and significantly lower serum OxLDL levels in IUGR (normotensive IUGR+IUGR with preeclampsia) patients compared to normals. Their measurements were done in serum samples; although they could not demonstrate a systemic alteration, they underlined the importance of probable factors (local effects) within the placenta tissues and maternal vessels. They speculated the diminished fetal cholesterol supply as a probable mechanism responsible from the low OxLDL levels [15]. In our study, we failed to demonstrate increased levels of OxLDL within the placental tissues.

In our study, there were not significant differences between the levels of OxLDL in the preeclamptic pregnant women with IUGR in comparison with normal pregnant women and preeclamptic patients without IUGR.

5.2. Steroid Hormones. Several studies in the literature have demonstrated the presence of OxLDL in placenta [12] and the effects of antioxidant estrogens and prooxidant progesterone on LDL oxidation [5, 6, 17, 18]. These sex hormones are synthesized in placental tissue during gestation [5], and cholesterol is the precursor of both. Muraguchi et al. have reported that estradiol indicates placental function, whereas estriol indicates placental and fetal function [19]. Zhu et al. have reported that progestin inhibits the antioxidant effect of estradiol in the absence and presence of aortic endothelial cell culture [17, 18] and that this effect is similar to that observed in trophoblastic cell cultures [6]. Mueller et al. have reported that estriol is an antioxidant that increases the lag phase of LDL oxidation in vitro [5].

In our study, levels of estriol, estradiol, and progesterone were reduced in the placental tissues of preeclamptic patients compared to normal pregnant women. Those gave the impression that the in vivo reduction in OxLDL in preeclamptic placenta tissues had a different mechanism from the antioxidant effect of estrogens.

Estriol was significantly reduced in the placental tissues of preeclamptic patients and preeclamptic patients without/with IUGR compared to normal pregnancy group. This implied

significancy of estriol in evaluating placental and fetal growth as reported by Muraguchi et al. [19].

We suggest that the reduction in steroid hormones synthesized from cholesterol in preeclamptic placental tissues might be associated with the reduction in fetal cholesterol maintenance as reported by Pecks et al. [15].

5.3. PON1. Genç et al. [14], Sarandöl et al. [20], Baker et al. [21], and Yaghmaei et al. [22] have measured PON1 activity using the same method as in our study (hydrolysis of paraoxon by PON1).

Yaghmaei et al. have determined that serum PON1 activity was significantly increased in preeclampsia compared to the normal subjects [22].

Baker et al. have determined higher paraoxonase activity in preeclamptic pregnant women compared to controls. Authors have also reported higher paraoxonase activity in severe compared to mild preeclampsia and stressed the importance of paraoxonase in the pathogenesis of preeclampsia [21].

Genc has reported lower PON1 in preeclamptic pregnant women compared to the controls [14].

Sarandöl et al. have measured paraoxonase activity in normal, mild, and severe preeclamptic pregnant women. Authors have reported no differences between the groups [20].

The results of our study were compatible with those of Yaghmaei and Baker and suggested that elevated serum PON1 activity in preeclampsia might be important in the in vivo inhibition of OxLDL. PON1 activity of preeclamptic pregnant women without IUGR was significantly higher than the normal group. It might be important in the progression of fetus.

Mackness et al. investigated PON 1 and PON2 in several tissues by real-time polymerase chain reaction (PCR) and showed PON 2 in placenta tissues, but they stated that the wide tissue distribution of PON1 would theoretically lead to greater protection for oxidative stress [23]. In preeclampsia regarding our results, the potential role of PON 1 activity on fetal growth can be explained by further studies.

Our literature search failed to find a report that has done OxLDL measurements directly in placental tissues. We hypothesized that atherogenic OxLDL might be increased in preeclamptic placental tissues and aimed to demonstrate the relationship with sex steroids in placental tissue. However, our results demonstrated that levels of OxLDL and steroid hormones were reduced in placental tissues in preeclampsia. Therefore, we believe that reductions in tissue estriol and OxLDL are correlated, and this positive correlation, although not a strong one, might in turn be associated with a reduction in the maintenance of fetal cholesterol. Additionally, these reductions in placental tissues might also be associated with the rapid removal of the possible complexes formed by OxLDL and autoantibodies by RES cells. However, we were unable to demonstrate the increase of OxLDL in preeclamptic placental tissues. Further studies should be performed to investigate the role of other factors acting in spiral artery atherosis and multifactorial endothelial dysfunction.

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