Proinflammatory Cytokines Regulate LOX-1 Expression in Vascular Smooth Muscle Cells

Oliver Hofnagel, Birgit Luechtenborg, Katrin Stolle, Stefan Lorkowski, Heike Eschert, Gabriele Plenz, Horst Robenek

Objective—Atherogenesis represents a type of chronic inflammation and involves elements of the immune response, e.g., the expression of proinflammatory cytokines. In advanced atherosclerotic lesions, lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is expressed in endothelial cells, macrophages, and smooth muscle cells (SMCs). In vitro, the expression of LOX-1 is induced by inflammatory cytokines like TNF-α and transforming growth factor (TGF)-β. Therefore, LOX-1 is thought to be upregulated locally in response to cytokines in vivo.

Methods and Results—We determined by reverse-transcription polymerase chain reaction (PCR) and Western blot analysis whether the mediators of the acute phase response in inflammation, IL-1α, IL-1β, and TNF-α, regulate LOX-1 expression in cultured SMCs, and whether this regulation is influenced by peroxisome proliferator-activated receptor γ (PPARγ). We studied by immunohistochemistry whether these cytokines are spatially correlated with LOX-1 expression in advanced atherosclerotic lesions. We found upregulation of LOX-1 expression in SMCs in a dose- and time-dependent manner after incubation with IL-1α, IL-1β, and TNF-α. Simultaneous incubation with these cytokines at saturated concentrations had an additive effect on LOX-1 expression. The PPARγ activator, 15d-PGJ2, however, inhibited IL-1β-induced upregulation of LOX-1. In the intima of atherosclerotic lesions regions of IL-1α, IL-1β, and TNF-α expression corresponded to regions of LOX-1 expression.

Conclusion—We suppose that upregulated LOX-1 expression in SMCs of advanced atherosclerotic lesions is a response to these proinflammatory cytokines. Moreover, the proinflammatory effects of these cytokines can be decreased by the antiinflammatory effect of PPARγ. (Arterioscler Thromb Vasc Biol. 2004;24:1789-1795.)

Key Words: atherosclerosis ■ LOX-1 ■ scavenger receptor ■ smooth muscle cell ■ interleukin

Atherosclerosis is a type of chronic inflammation involving elements of the immune response and cells of the vessel wall. The major cell types involved in atherogenesis, macrophages (Mφ), and SMCs, are activated by proinflammatory stimuli like modified low-density lipoprotein (LDL). LDL modulated by oxidation induces inflammatory responses in Mφ and migration and proliferation in SMCs. Oxidized LDL also triggers foam cell formation of these cells. Scavenger receptors play a key role in foam cell formation by mediating the uptake of modified LDL. In recent years, several newly identified members of the scavenger receptor family have been cloned on the basis of their ability to recognize modified LDL. These include lectin-like oxidized LDL receptor-1 (LOX-1), which was initially identified in endothelial cells (EC). Subsequent studies showed that LOX-1 is also expressed in Mφ and SMCs.

Information concerning the role of LOX-1 in vascular disease is accumulating, and it is clear that proatherogenic conditions, such as hypertension, hyperlipidemia, and diabetes, induce LOX-1 expression. LOX-1 expression was detected in EC of early atherosclerotic lesions of human carotid arteries. Advanced lesions showed LOX-1 expression not only in EC but also in Mφ and more frequently in SMCs. Thus, LOX-1 may be involved in foam cell transformation in Mφ and SMCs. In vitro LOX-1 binds oxidized LDL, and this modified lipoprotein upregulates LOX-1 expression in ECs and SMCs, but downregulates this receptor in Mφ.

Atherosclerotic lesions containing lipid-laden foam cells are the hallmark of the inflammatory state, and recent investigations showed that inflammatory stimuli modulate LOX-1 expression in vitro. IL-4 induces LOX-1 expression in Mφ and tumor necrosis factor (TNF)-α and transforming growth factor (TGF)β upregulate LOX-1 expression in ECs and SMCs. Activation of protein kinase C (PKC), which plays a central role in mediating inflammatory responses, upregulates LOX-1 expression in ECs and SMCs. Further, it is known that peroxisome proliferator-activated receptors (PPARs), which influence the transcriptional regulation of a large number of genes affecting inflammation, modulate LOX-1 expression in vitro: PPARα...
activators increase LOX-1 expression in EC, and PPARγ activators inhibit TNF-α induced LOX-1 expression in EC. These modulators of inflammation may be of great significance in the regulation of LOX-1 in vivo.

Cytokines of the acute phase response in inflammation, like IL-1α, IL-1β, and TNF-α, are expressed in the intima of atherosclerotic lesions and promote migration and proliferation of SMC. SMC in the intima of advanced atherosclerotic lesions have enhanced LOX-1 expression, and we supposed that LOX-1 expression is locally high in response to these proinflammatory cytokines. Therefore, the present study investigates whether primary mediators of the acute phase response in inflammation, IL-1α, IL-1β, and TNF-α, regulate LOX-1 expression in SMC. We examined the influence of these cytokines on LOX-1 expression in cultured aortic SMC and the expression of the cytokines and LOX-1 in advanced atherosclerotic lesions.

**Methods**

**Reagents and Cells**

Recombinant human IL-1α, IL-1β, and TNF-α (1×10^6 UI/mg each) were obtained from R&D Systems, phosphor 12-myristate 13-acetate (PMA) was from Sigma Chemicals, and 15-deoxy-Delta12,14-prostaglandin (PG) J2 (15d-PGJ2) was from the Cayman Chemical Company. PMAs and 15d-PGJ2 were dissolved in dimethyl sulfoxide. Human aortic SMC (third passage) were purchased from BioWhittaker and cultured in Smgm-2 medium, also from BioWhittaker, under a humidified atmosphere of 5% CO2/95% air at 37°C. Cells used for experiments were between passages 4 and 6.

**Cell Treatments**

Subconfluent cells were treated with the indicated concentrations of IL-1α, IL-1β, and TNF-α or PMA for the times shown. In some experiments, the cells were cultivated with or without 15d-PGJ2 for 24 hours before adding or not adding 10 ng/mL IL-1β for a further 16 hours.

**Reverse-Transcription Polymerase Chain Reaction**

Total RNA from cultured cells was isolated with RNeasy Mini Kit (Qiagen), including digestion of genomic DNA with DNase I. 0.5 μg of total RNA was reverse transcribed into cDNA (cDNA) using Superscript II (Invitrogen). Polymerase chain reaction (PCR) was performed with specific primers for human LOX-1 (forward: 5′-TTACTCTCCATGTTGTTGC-3′, reverse: 5′-AGCTTTCTCTGTCTTTGGC-3′, and human β-actin primers (forward: 5′-GGCATCTCAATGAGAAGTA-3′, reverse: 5′-GGGTTTGGAT-3′)

**Western Blot Analysis**

Cells were treated with lysis buffer (50 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; 2% sodium dodecyl sulfate [SDS]; protease inhibitor cocktail from Boehringer). The lysates were ultrasonicated and centrifugated at 5000g for 10 minutes at 4°C. Protein concentration was determined using a Lowry protein assay (BioRad). Samples containing 20 μg of total protein were mixed with 6× loading buffer (100 mmol/L Tris-HCl, pH 6.8; 30% glycerol; 10% SDS; 600 mmol/L dithio-threitol; 0.012% bromphenol blue), boiled for 2 minutes, and loaded onto a 10% SDS-polyacrylamide gel. Proteins were transferred to Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore) by electroblotting (BioRad), stained with Ponceau S to determine amount of total protein, photographed, washed with distilled water, and blocked with ECL blocking agent (ECL kit; Amersham Biosciences) in 200 mmol/L Tris-HCl, pH 7.5; 300 mmol/L NaCl; 0.1% Tween 20 (TBS-T) at room temperature (RT) for 1 hour. Membranes were incubated with a polyclonal goat anti–LOX-1 antibody (Research Diagnostics) at RT for 2 hours, washed in TBS-T, and incubated with peroxidase-conjugated anti–goat antibody (Vector Laboratories) at RT for 1 hour. Bound antibodies were detected by enhanced chemiluminescence using the ECL kit. Densitometric analysis was performed to measure the amount of LOX-1 protein. The LOX-1 protein per lane was normalized to the total protein amount determined by Ponceau S staining.

**Immunohistochemistry**

For fluorescence microscopic studies serial cryosections, 5- to 8-μm thick, were made from thoracic aorta of 8-month-old Watanabe heritable hyperlipidemic rabbits whose vessels have advanced atherosclerotic lesions. The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health Publication 85-23, revised 1996), and the rabbits were euthanized by administration of intravenous sodium pentobarbital (25 mg/kg). The sections were fixed with ice-cold methanol, preincubated with 1% bovine serum albumin, and incubated with monoclonal mouse antimuscle actin antibody HHF35 (DAKO), monoclonal mouse anti-αMβ antibody RAM11 (DAKO), polyclonal goat anti–LOX-1 antibody (Research Diagnostics), polyclonal goat anti–IL-1β antibody (R&D Systems), polyclonal goat anti–TNF-α antibody (BD Pharmingen) at RT for 1 hour. Secondary antimouse or antigoat antibodies conjugated with fluorochrome Cy3 (Dianova) were used. Sections incubated with mouse IgG (Dianova) served as controls. Nuclear staining was performed with Hoechst dye 33258. Sections were analyzed with a Zeiss Axioskop II.
Results

IL-1α, IL-1β, and TNF-α Stimulate LOX-1 Expression in Cultured SMC

All studied cytokines upregulated LOX-1 expression in a dose- and time-dependent manner. After 3 hours, IL-1α (10 ng/mL) significantly increased expression of LOX-1 mRNA (5.3-fold) and LOX-1 protein (2.7-fold) in cultured human aortic SMC (Figure 1). IL-1β (50 ng/mL) increased expression of LOX-1 mRNA (2.2-fold) and protein (2.4-fold) after 16 hours (Figure 2). Incubation with 10 ng/mL TNF-α also caused upregulation of LOX-1 mRNA and LOX-1 protein expression (Figure 3). After 8 hours, LOX-1 mRNA expression increased 2.4-fold, and after 16 hours LOX-1 protein expression increased 2.3-fold.

Figure 4a and 4b demonstrate that simultaneous incubation with combinations of IL-1α, IL-1β, and TNF-α at saturated concentrations (10 ng/mL) of each of these cytokines in-
creased LOX-1 mRNA and protein expression in an additive manner.

Further, we found as much as a 3-fold upregulation of LOX-1 mRNA and LOX-1 protein expression after incubating the cells with PMA, an activator of PKC. This upregulation also was dose- and time-dependent, revealing a peak after incubation with 63 ng/mL PMA for 16 hours (data not shown).

PPARγ Activator 15d-PGJ2 Inhibits IL-1β-Stimulated LOX-1 Expression in Cultured SMC

Before incubation with IL-1β, human aortic SMC were also pretreated with the PPARγ activator 15d-PGJ2. Upregulation of LOX-1 mRNA and LOX-1 protein expression by IL-1β was inhibited by pretreatment of the cells with 15d-PGJ2 (Figure 4c and 4d). Moreover, treatment with 15d-PGJ2...
without IL-1β incubation decreased LOX-1 mRNA and protein expression (Figure 4c and 4d).

**LOX-1 mRNA Expression Is Higher in Laser-Microdissected Intimal SMC Than in Medial SMC**

To examine the LOX-1 expression in vivo, we isolated total RNA from laser-microdissected intimal and medial SMC of human coronary arteries with intimal thickenings. The subsequent real-time reverse-transcription PCR revealed 6.7-fold higher LOX-1 mRNA expression in intimal SMC than in medial SMC (Figure 5).

**Discussion**

In the present study, we demonstrate for the first time to our knowledge a strong codistribution of the proinflammatory cytokines IL-1α, IL-1β, and TNF-α with LOX-1 expression in advanced atherosclerotic lesions. We also show that these cytokines upregulate LOX-1 expression in cultured SMC.
Therefore, we assume that LOX-1 expression is regulated by IL-1α, IL-1β, and TNF-α in intimal SMC of advanced atherosclerotic lesions.

LOX-1 Expression Is Stimulated in All Stages of Atherogenesis

LOX-1 expression has been reported in EC of early atherosclerotic lesions, but not in EC of nonatherosclerotic vessels, suggesting a potential role of LOX-1 in the initiation of atherosclerosis. Increased expression of LOX-1 in EC is most prominent at arterial bifurcations exposed to complex shear forces and circumferential strain, indicating that LOX-1 expression in EC might be upregulated by hemodynamic factors. In advanced atherosclerotic lesions expression of LOX-1 was reported by Kataoka et al to be found not only in EC but also in a few round subendothelial cells with morphology consistent with that of MΦ and frequently in spindle-shaped cells with morphology consistent with that of intimal SMC. Our study confirmed in vivo expression of LOX-1 in SMC of atherosclerotic lesions. Reverse-transcription PCR of mRNA from laser-microdissected human SMC and immunohistochemistry of rabbit aorta showed higher LOX-1 mRNA and protein expression in intimal SMC than in medial SMC. Therefore, we suggest that LOX-1 expression not only is involved in the initiation of atherogenesis but also plays an important role as atherosclerotic lesions progress.

LOX-1 Expression Is Upregulated by Proinflammatory Cytokines IL-1α, IL-1β, and TNF-α

Some authors supposed that inflammatory cytokines might be responsible for increased expression of LOX-1 in the intima of atherosclerotic lesions. TGF-β is known to upregulate LOX-1 expression in cultured bovine SMC, and an induction of LOX-1 expression in cultured SMC by TNF-α has been mentioned. We found upregulated LOX-1 expression in human SMC after incubation with IL-1α, IL-1β, and TNF-α. These findings point to IL-1α and IL-1β as important factors in the cascade of events resulting in chronic inflammation and vascular disease. No expression of IL-1β has been detected in nonatherosclerotic rabbit aortas, whereas, consistent with our results, strong expression of IL-1β has been reported in intimal SMC of aorta of cholesterol-fed endothelia-denuded rabbits with intimal thickening. Our results showed codistribution of this strong IL-1α and IL-1β expression with strong LOX-1 expression. Therefore, we assume that LOX-1 expression is regulated by IL-1α and IL-1β in SMC of atherosclerotic lesions.

TNF-α is also expected to have a major role in pathogenesis of atherosclerosis, and inflammatory effects of TNF-α and IL-1β during atherogenesis, eg, induction of expression of colony-stimulating factors and matrix metalloproteinases, are nearly identical. Nevertheless, TNF-α and IL-1β seem to act independently in upregulating LOX-1 expression, because the effects of TNF-α and IL-1β are additive in cell cultures exposed to both cytokines at saturated concentrations. These results are in accordance with findings of Seelentag et al, who reported additive effects of TNF-α and IL-1 on gene expression of colony-stimulating factors in endothelial cells. A study of Lei and Buja describes strong expression of TNF-α in both intimal and medial SMC of WHHL rabbit aorta with advanced atherosclerotic lesions. Our results revealed strong expression of TNF-α in intimal SMC, and this expression colocalized with strong LOX-1 expression. In contrast to Lei and Buja, we found low TNF-α expression in medial SMC. The low amount of TNF-α found by us may cause the low basal expression of LOX-1 mRNA found in laser-microdissected medial SMC. However, expression of LOX-1 protein could not be detected in medial SMC. We assume this is because of the lower sensitivity of the immunohistochemical method compared with real-time reverse-transcription PCR. The colocalization of strong TNF-α expression with strong LOX-1 expression in the intima, on the one hand, and low TNF-α expression with low LOX-1 expression in the media, on the other hand, indicate that TNF-α also presumably influences upregulation of LOX-1 expression in vivo.

PKC plays an important role in mediating biological responses, including tumor promotion and inflammation. Studies on the regulation of LOX-1 expression in EC, MΦ, and SMC revealed upregulation of LOX-1 expression after incubation with PMA, an activator of PKC. Our study also showed a 3-fold upregulation of LOX-1 mRNA and protein expression after incubation with PMA, comparable with the detected upregulation of LOX-1 expression after incubation with IL-1α, IL-1β, and TNF. This stimulated LOX-1 expression after PMA incubation indicates involvement of PKC in regulation of LOX-1 expression in SMC.

PPARγ Modulates LOX-1 Expression in Cultured SMC

There is evidence that PPARs are involved in the regulation of LOX-1 expression, but the results are not consistent. Hayashida et al reported upregulated LOX-1 mRNA and LOX-1 protein expression after incubation with the PPARα ligands fenofibric acid and WY14643 in bovine EC, whereas Chiba et al found no effect of either ligand. Findings on the influence of PPARγ are also in conflict. Hayashida et al describe that LOX-1 mRNA and LOX-1 protein expression is not affected in bovine EC after incubation with the PPARγ activators troglitazone and 15d-PGJ2, whereas Chiba et al found decreased LOX-1 mRNA expression after incubation with PPARγ activators pioglitazone and 15d-PGJ2 in the same cell type. Moreover, Chiba et al detected inhibition of TNF-α-stimulated LOX-1 mRNA expression in bovine EC after incubation with the PPARγ activators pioglitazone and 15d-PGJ2. To elucidate the role of PPARγ in human SMC, we studied the influence of 15d-PGJ2 on LOX-1 expression. In agreement with the results of Chiba et al, our experiments showed that 15d-PGJ2 downregulates LOX-1 expression and inhibits IL-1β–induced LOX-1 expression. Because PPARγ activators also inhibit expression of IL-1 and TNF-α in cultured cells, these activators might also reduce LOX-1 expression indirectly by inhibiting cytokine expression. Further studies will have to clarify whether LOX-1 expression within the plaque can be modulated by antiinflammatory factors, eg, PPARγ activators.
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