LOX-1 Mediates Oxidized Low-Density Lipoprotein–Induced Expression of Matrix Metalloproteinases in Human Coronary Artery Endothelial Cells

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Background—Oxidized LDL (ox-LDL) accumulation in the atherosclerotic region may enhance plaque instability. Both accumulation of ox-LDL and expression of its lectin-like receptor, LOX-1, have been shown in atherosclerotic regions. This study was designed to examine the role of LOX-1 in the modulation of metalloproteinases (MMP-1 and MMP-3) in human coronary artery endothelial cells (HCAECs).

Methods and Results—HCAECs were incubated with ox-LDL (10 to 80 μg/mL) for 1 to 24 hours. Ox-LDL increased the expression of MMP-1 (collagenase) and MMP-3 (stromelysin-1) in a concentration- and time-dependent manner. Ox-LDL also increased collagenase activity. Ox-LDL did not significantly affect the expression of tissue inhibitors of metalloproteinases. Native LDL had no effect on the expression of MMPs. The effects of ox-LDL were mediated by its endothelial receptor, LOX-1, because pretreatment of HCAECs with a blocking antibody to LOX-1 (JTX92, 10 μg/mL) prevented the expression of MMPs in response to ox-LDL (P<0.01). In parallel experiments, ox-LDL caused the activation of protein kinase C (PKC), which was inhibited by LOX-1 antibody. The PKC-β isoform played a critical role in the expression of MMPs, because the PKC-β inhibitor hispidin reduced ox-LDL–induced activation of PKC and the expression of MMPs. Other PKC subunits (α, γ, and ε) did not affect the expression of MMPs.

Conclusions—These findings indicate that ox-LDL, via LOX-1 activation, modulates the expression and activity of MMPs in HCAECs. In this process, activation of the PKC-β subunit plays an important signaling role. (Circulation. 2003;107:612-617.)

Key Words: endothelium ■ metalloproteinases ■ receptors, oxidized LDL ■ lipoproteins ■ kinases

Oxidized LDL (ox-LDL) changes the secretory activities of endothelium and causes it to become dysfunctional.1 Ox-LDL inhibits the expression of constitutive nitric oxide synthase,2 induces expression of adhesion molecules, and facilitates inflammatory cells to adhere to the intima.3 Recent studies show that LOX-1, a novel lectin-like receptor for ox-LDL expressed primarily in endothelial cells, facilitates the uptake of ox-LDL and mediates several of the biological effects of ox-LDL, such as apoptosis.4–7 Expression of LOX-1 is upregulated by angiotensin II, free radicals, inflammatory cytokines, and shear stress.8–10 Recent studies show that LOX-1 expression is upregulated in atherosclerotic tissues in rabbits and humans.11,12 Matrix metalloproteinases (MMPs) are an endogenous family of enzymes that are responsible for vascular remodeling.13,14 Increased MMP expression and activity have been identified in human and animal models of atherosclerosis and heart failure.15–17 Recent studies18,19 have shown that an increase in the activity and expression of MMPs plays a central role in the composition of atherosclerotic plaques.

Ox-LDL, LOX-1, and MMPs have been found to be colocalized in atherosclerotic plaques.11,20 Their interaction may lead to the instability of atherosclerotic plaques. In the present study, we investigated the role of the ox-LDL receptor LOX-1 in the expression of MMPs and their tissue inhibitors (TIMPs) and the related intracellular mechanism in human coronary artery endothelial cells (HCAECs).

Methods

Cell Culture

The methodology for culture of HCAECs has been described previously.5,6 The initial batch of HCAECs was purchased from Clonetics Corp. The endothelial cells were pure on the basis of morphology and staining for factor VIII–related antigen and acety-
lated LDL. These cells were 100% negative for α-actin smooth muscle expression.

Study Design
HCAECs were incubated with ox-LDL (10, 20, 40, and 80 μg/mL) for 1, 3, 6, or 24 hours to determine the expression and activity of MMP-1 and -3 and TIMP-1 and -2. The concentration and time point for maximal effect of ox-LDL were used in subsequent experiments.

To examine the receptor specificity of ox-LDL action, HCAECs were pretreated with human LOX-1–blocking antibody (JT902, 10 μg/mL) and then exposed to ox-LDL. The details of preparation of the antibody and its specificity were presented earlier. To the harvested cells were used to measure expression and activity of MMPs and TIMPs.

To explore the molecular basis of the action of LOX-1, we studied the protein kinase C (PKC) signaling pathway. For this purpose, HCAECs were pretreated with the PKC inhibitor bisindolylmaleimide I (1 μmol/L) for 30 minutes, and then the cells were exposed to ox-LDL. The harvested cells were used to measure expression of MMPs and TIMPs and PKC activity.

To further explore the roles of PKC isoforms (α, β, γ, and ε subunits) in this process, HCAECs were pretreated with inhibitors of PKC-α (Ro-32-0432, 20 mmol/L), PKC-β (hispidin, 4 mmol/L), PKC-γ (Ro-31-7549, 0.4 mmol/L), or PKC-ε (Ro-32-0432, 0.2 mmol/L) for 30 minutes, and then the cells were exposed to ox-LDL. The harvested cells were used to measure expression of MMPs and TIMPs and PKC activity.

Concentrations of all reagents and the duration of incubation were chosen on the basis of published studies.

Preparation of Lipoproteins
Native LDL and ox-LDL were prepared as described earlier. The thiobarbituric acid–reactive substance contents of ox-LDL and native LDL were 10.2±0.53 and 0.79±0.26 nmol/100 μg protein, respectively (P<0.01). Ox-LDL was extensively dialyzed against Tris-saline. Ox-LDL was kept in 50 mmol/L Tris-HCl, 0.15 mol/L NaCl, and 2 mmol/L EDTA at pH 7.4 and was used within 10 days of preparation. The level of endotoxin measured by the E-Toxase kit (Sigma) was consistently <0.005 endotoxin units/mL (lowest detection limit).

Semi-quantitative RT-PCR
MMP mRNA was examined by RT-PCR. Total RNA (1 μg) extracted from cultured HCAECs was reverse-transcribed with Oligo dT (Promega) and M-MLV reverse transcriptase (Promega) at 37°C for 1 hour. Then, 1.5 μL of the reverse-transcribed material was amplified with Taq DNA polymerase (Promega) using specific primers (Biomol Research Laboratory, Inc) for human MMP-1 and -3. The products of the PCR-amplified samples were visualized on 1.2% agarose gels by use of ethidium bromide. Each specific mRNA band was normalized with the β-actin mRNA band.

Collagenase Activity Assay
Collagenase zymography was carried out according to the method described by Guarda et al. Essentially, the conditioned culture medium was collected from the dishes, and 10 μL of the medium was subjected to electrophoresis in SDS-polyacrylamide gel containing 0.1% gelatin under nonreducing conditions. The gels were soaked in 2.5% Triton-X100 for 60 minutes and then washed with water for 60 minutes to remove SDS. The gels were then incubated in a developing buffer containing 50 mmol/L Tris, pH 7.4, 5 mmol/L CaCl₂, and 0.02% sodium azide for 18 hours at 37°C. After incubation, the gels were stained with Coomassie blue and photographed.

Western Analysis
HCAEC lysates from each experiment (40 μg per lane) were separated by SDS-PAGE and transferred to nitrocellulose membranes. After incubation in blocking solution (4% nonfat milk, Sigma), membranes were incubated overnight with 1:1000 dilution primary antibody to human MMP-1 or -3 and TIMP-1 and -2 (Oncogene) at 4°C. Membranes were washed and incubated with 1:2000 dilution second antibody (Amersham) for 1 hour. Relative intensities of protein bands were analyzed by Scan-gel-it software.

Measurement of PKC Activity
Cells (5×10⁶ to 10×10⁶ cells/100-mm dish) from different groups were washed twice with PBS and scraped into 1 mL of membrane-bound PKC extraction buffer containing (in mmol/L) 25 Tris-HCl (pH 7.4), 0.5 EDTA, 0.5 EGTA, 0.0% Triton X-100, 10 β-glycerophosphatase, 0.5 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 μg/mL leupeptin, and 1 μg/mL aprotinin. The lysate was homogenized and centrifuged at 14 000g at 4°C for 30 minutes, and the supernatant was used to measure PKC activity. An assay system (Promega) was used to determine PKC activity as described previously. Reactions for each sample were performed separately in the presence of phospholipids (activated PKC reaction) and in the absence of phospholipids (control reaction). Results were expressed as picomoles of phosphate per minute per microgram of protein.

Data Analysis
All data represent the mean of 6 separately performed experiments. Data are presented as mean±SD. Data were analyzed by ANOVA, followed by post hoc Scheffé’s F test. A probability value of P<0.05 was considered to be statistically significant.

Results
Ox-LDL and Expression of MMPs and TIMPs
Incubation of HCAECs with ox-LDL (10 to 80 μg/mL) for 24 hours increased the expression of MMP-1 and -3 (mRNA and protein) in a concentration-dependent manner (Figure 1A). Expression of MMP-1 and -3 (mRNA and protein) was also time-dependent (1, 3, 6, and 24 hours), with maximal expression at 24 hours (Figure 1B). The summarized data from 6 independent experiments are shown in Figure 1, right. Each protein band was normalized with a positive band in the control group. Each mRNA band was normalized with a β-actin mRNA band.

Incubation of HCAECs with ox-LDL (10 to 80 μg/mL) for 24 hours increased the expression of TIMP-1 and -2 protein slightly but not significantly (Figure 2).

Role of LOX-1 in the Expression of MMPs
In these experiments, HCAECs were incubated with ox-LDL (80 μg/mL) for 24 hours. HCAECs in parallel experiments were incubated with the LOX-1–blocking antibody (10 μg/mL) for 30 minutes before exposure to ox-LDL. Ox-LDL increased the expression of MMPs, whereas native LDL had no effect. LOX-1 antibody reduced the effect of ox-LDL on the expression of both MMP-1 and MMP-3 (P<0.01, n=6). LOX-1 antibody alone had no effect on the basal expression of MMPs (Figure 3).

Collagenase Activity
Incubation of HCAECs with ox-LDL increased collagenase activity markedly in a concentration-dependent manner (n=6). The increase in collagenase activity paralleled MMP-1 expression (mRNA and protein) in response to ox-LDL (Figure 4).

Intracellular Mechanism of LOX-1–Mediated Expression of MMPs
To determine one of the intracellular mechanisms of the action of LOX-1 in the expression of MMPs, we examined
the PKC signaling pathway. Ox-LDL increased PKC activity in HCAECs (*P* < 0.01), and the ox-LDL–induced PKC activation was decreased by pretreatment of HCAECs with the LOX-1 antibody (*P* < 0.01). As expected, pretreatment of cells with the PKC inhibitor also inhibited ox-LDL–mediated PKC activation (Figure 5). Antibody alone or the PKC inhibitor alone had no effect on PKC activity.

Concomitantly, pretreatment of cells with the PKC inhibitor attenuated ox-LDL–induced MMP-1 and MMP-3 protein expression. Notably, the PKC inhibitor alone did not affect the basal expression of MMPs (Figure 6).

### Role of PKC Subunits in the Expression of MMPs

To further identify the role of PKC isoforms in ox-LDL–induced MMP expression, HCAECs were pretreated with different inhibitors of PKC isoforms (α, β, γ, or ε) and exposed to ox-LDL. Again, ox-LDL consistently increased the activation of PKC and the expression of MMP-1 and -3 in HCAECs. Importantly, PKC-β played a critical role in the expression of MMP-1 and -3, because the PKC-β inhibitor hispidin inhibited ox-LDL–induced PKC activation and MMP expression. Other PKC subunit inhibitors did not alter ox-LDL–induced MMP expression, although they reduced PKC activation (Figure 7).

### Discussion

The present study shows that ox-LDL upregulates the expression of MMP-1 and -3 in HCAECs. We found that these effects of ox-LDL are mediated via activation of its endothelial receptor LOX-1, because inhibition of LOX-1 action by a specific blocking antibody JTX92 reduced the upregulation of MMPs. Ox-LDL had no significant effect on the expression of TIMPs. Therefore, ox-LDL, via its receptor LOX-1, alters the MMP/TIMP ratio in HCAECs. PKC-β activation appears to play a critical role in this process, because inhibition of PKC-β activation attenuated the expression of MMPs in response to ox-LDL.

### Role of Ox-LDL in Vascular Injury

There is ample evidence that ox-LDL plays an important role in steps leading to atherosclerosis.2,3 It impairs endothelial...
function and activates platelets. It has been suggested that the composition of atherosclerotic plaque is the most important determinant of plaque disruption and development of acute coronary syndromes. The unstable and rupture-prone plaque is rich in monocytes/macrophages and shows intense immunopositivity for ox-LDL. Immunocytochemistry studies have demonstrated that MMP-1 and MMP-3 are expressed in endothelial cells, macrophages, and smooth muscle cells in the atherosclerotic regions. Release of MMPs and enhanced collagenase activity may degrade various components of the fibrous caps and contribute to the vulnerability of plaques to rupture. The present study provides direct evidence that ox-LDL but not native LDL upregulates MMP-1 and -3 expression as well as collagenase activity. In contrast to its effect on MMPs, ox-LDL did not significantly increase TIMP-1 and TIMP-2 expression. It may therefore be speculated that an imbalance between MMPs/TIMPs is a basis for the central role of ox-LDL in converting the stable atherosclerotic plaque into an unstable one.
Vascular endothelial cells secrete MMPs through both the luminal and the basolateral surfaces. MMP-1 and -3 secreted from the basolateral surface could be involved in the separation of endothelial cells from each other and lead to endothelial dysfunction in the early stage of atherosclerosis. Release of MMPs in the late stage could be a basis for disruption of the basement membrane and rupture of the fibrous cap. The function of MMPs secreted from the luminal surface of endothelial cells, particularly in the early stage of atherosclerosis, into the blood circulation remains unknown. MMP-1 and -3 belong to the family of MMPs, with each member having some distinct and some overlapping function.

Role of LOX-1 in MMP Expression

Ox-LDL exerts its biological effects via activation of its receptors on the surface of endothelial cells, macrophages, and smooth muscle cells. LOX-1, found predominantly on endothelial cells, has a different biochemical structure from the scavenger receptors. Several investigators have demonstrated that the uptake of ox-LDL by endothelial cells is mediated by LOX-1 activation. Whereas the uptake of ox-LDL by endothelial cells does not result in foam cell formation, ox-LDL uptake in vascular endothelium causes endothelial activation and changes its biological characteristics. For example, we have shown that LOX-1 mediates ox-LDL-induced apoptosis, adhesion molecule expression, and monocyte adhesion to endothelial cells.

In the present study, we demonstrate that ox-LDL upregulates the expression of MMP-1 and -3 via LOX-1 activation. The confirmatory evidence for the role of LOX-1 came from experiments in which a specific LOX-1–blocking antibody decreased the expression of MMPs in response to ox-LDL. It is noteworthy that LOX-1 is highly expressed in atherosclerotic human and animal tissues. In rabbits with hypercholesterolemic atherosclerosis, we showed upregulation of LOX-1 primarily in the endothelial lining and the proliferating intima. In these animals, there is evidence for increased expression of MMPs. The present study provides direct evidence linking LOX-1 and MMP expression in HCAECs.

Intracellular Mechanism of LOX-1–Mediated MMP Expression

Experimental studies have shown that ox-LDL causes injury to the endothelial cells via activation of different signal transduction pathways, such as PKC and mitogen-activated protein kinase. We recently showed that LOX-1 activation is associated with changes in protein kinase B activity. Cominacini et al showed that ox-LDL increases intracellular free radical generation and activates the transcription factor nuclear factor-κB in bovine endothelial cells. It should be noted that ox-LDL might activate different signal pathways, which interact each other. Signaling in response to ox-LDL may also be different in different tissues. Reuben et al demonstrated that basic calcium phosphate crystal stimulates PKC-α signal transduction pathway in the expression of MMP-1 and -3. Ren et al suggested that PKC-β may mediate ox-LDL–induced PAI-1 gene expression. In the present study, we demonstrate that ox-LDL induces the activation of PKC, which plays an important role in the expression of MMPs and collagenase activity. Pretreatment of HCAECs with the PKC-β inhibitor attenuated ox-LDL–induced expression of MMPs. In contrast, other PKC isoforms did not change ox-LDL–induced MMP expression.

In summary, this study shows that ox-LDL upregulates the expression and activity of MMP-1 and -3 through LOX-1 activation. In this process, PKC-β activation plays an important role.

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References

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