Inducible Expression of Lectin-like Oxidized LDL Receptor-1 in Vascular Endothelial Cells

Noriaki Kume, Takatoshi Murase, Hideaki Moriwaki, Takuma Aoyama, Tatsuya Sawamura, Tomoh Masaki, Toru Kita

Abstract—Endothelial dysfunction, or activation, elicited by oxidized LDL (Ox-LDL) or its lipid constituent, has been implicated in the initiation and progression of atherosclerosis. We have recently identified a C-type lectin-like molecule, designated lectin-like Ox-LDL receptor-1 (LOX-1), which acts as a cell-surface receptor for Ox-LDL in cultured vascular endothelial cells. In this study, we provide evidence that LOX-1 expression can be upregulated by tumor necrosis factor-α (TNF-α) and phorbol 12-myristate 13-acetate (PMA) in cultured bovine aortic endothelial cells. TNF-α and PMA upregulated LOX-1 protein and mRNA in a time- and dose-dependent manner. Nuclear runoff assay revealed that TNF-α stimulates transcription of the LOX-1 gene. Chinese hamster ovary K1 cells stably expressing LOX-1 internalized 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-labeled Ox-LDL but did not significantly internalize acetylated LDL (Ac-LDL), which was effectively suppressed by excess amounts of unlabeled Ox-LDL but not by Ac-LDL. Upregulated expression of LOX-1 by TNF-α and PMA was associated with increased uptake of Dil-Ox-LDL that cannot be blocked by excess amounts of unlabeled Ac-LDL. Taken together, LOX-1 is a receptor specific for Ox-LDL, and enhanced uptake of Ox-LDL via this novel receptor on vascular endothelial cells may play an important role in endothelial activation in atherogenesis. (Circ Res. 1998;83:322-327.)

Key Words: atherosclerosis ■ tumor necrosis factor-α ■ phorbol myristate acetate ■ cytokine

Endothelial dysfunction, or activation, elicited by oxidized LDL (Ox-LDL) or its lipid constituent, has been implicated in the initiation and progression of atherosclerosis.1–5 Ox-LDL, or its lipid constituent, has been shown to transcriptionally induce endothelial genes relevant to atherogenesis6–10 in addition to stimulating the production of hydrogen peroxide11 and impairing NO12 and cell migration.13 Although molecular mechanisms involved in endothelial dysfunction elicited by Ox-LDL and its lipid constituents have not been fully clarified, cultured vascular endothelial cells, as well as liver sinusoidal endothelial cells, have been shown to internalize and degrade modified forms of LDL, including Ox-LDL.14–16

In macrophages, several different molecules have been shown to support the cellular uptake of Ox-LDL.17–23 In vascular endothelial cells, however, previous studies have suggested that endothelial uptake of modified forms of LDL, including Ox-LDL, appears to depend on cell-surface receptors, which may be encoded by genes different from those expressed in macrophages.17,24 We have recently identified a C-type lectin-like molecule, designated lectin-like Ox-LDL receptor-1 (LOX-1), which acts as a cell-surface receptor for Ox-LDL in cultured vascular endothelial cells.25

Inflammatory cytokines, including tumor necrosis factor-α (TNF-α), have been implicated in atherogenesis as well as inflammatory responses that affect plaque stability.26–28 In the present study, therefore, we have examined the regulation of LOX-1 expression by inflammatory stimuli in cultured vascular endothelial cells. Here, we provide evidence that expression of LOX-1 can be transcriptionally induced by TNF-α and phorbol ester in cultured bovine aortic endothelial cells (BAECs).

Materials and Methods

Reagents and Cells

Recombinant human TNF-α was obtained from Genzyme, and phorbol 12-myristate 13-acetate (PMA) was from Sigma Chemical Co. FBS was purchased from Irvine, and DMEM was from Nissui. Cultured BAECs were isolated by scraping with a razor blade and cultured in DMEM supplemented with 10% (vol/vol) FBS. Cells used for experiments were at passage levels between 10 and 20.

Development of a Monoclonal Antibody for Bovine LOX-1

A fragment of LOX-1 cDNA covering the extracellular domain (amino acids 61 to 270) was amplified by polymerase chain reaction with a pair of primers tagged with the BamHI restriction site. The amplified fragment was digested with BamHI and subcloned into the BamHI site of the pQE10 vector (Qiagen). The protein corresponding to the extracellular domain of LOX-1 was synthesized and purified using the QiAexpress system (Qiagen) and was used as an

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antigen to immunize mice. A monoclonal antibody was developed by screening hybridomas by an ELISA as previously described.25

**Fluorescence Immunobinding Assay**

After BAECs were washed twice with PBS containing 10% (vol/vol) FBS, they were incubated with anti-bovine LOX-1 antiserum (100-fold dilution) on ice for 1 hour, washed 3 times with PBS/10% FBS, and subsequently incubated with FITC-conjugated F(ab’), fragments of anti-mouse IgG (Caltag Laboratories) on ice for 1 hour. After they were washed 4 times with PBS/10% FBS, cells were lysed with 0.01% (wt/vol) NaOH containing 0.1% SDS, and the fluorescence (485-nm excitation and 538-nm emission) was determined using Titertek Fluoroscan II (Flow Laboratories).6

**Immunoblot Analysis**

BAECs were lysed in boiled Laemmli sample buffer (2% SDS, 10% glycerol, 60 mmol/L Tris [pH 6.8], and 0.001% bromophenol blue), and the whole-cell lysates were passed through 25-gauge needles 10 times. After they were heated at 98°C for 10 minutes, samples were subjected to SDS–polyacrylamide (10% to 20% gradients) gel electrophoresis in a nonreducing condition and transferred onto nitrocellulose filters (Hybond ECL, Amersham) by electroblotting. After preincubation with TBST (0.1% Tween 20, 500 mmol/L NaCl, 35 mmol/L Tris–Cl [pH 7.4], and 5% [wt/vol] nonfat dry milk for 1 hour at room temperature), filters were incubated with an anti-bovine LOX-1 monoclonal antibody or subclass-matched nonimmune mouse IgG diluted in TBST for 2 hours at room temperature, followed by 2 washes with TBST and nonfat dry milk. Filters were then incubated with a horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG (Amersham) diluted in TBST for 2 hours at room temperature, washed twice in TBST without nonfat dry milk, and visualized by chemiluminescence reagents (ECL kit, Amersham).

**Northern Blot Analysis**

Total RNA, isolated from BAECs by the acid-guanidinium phenol-chloroform method, was electrophoresed through 1% agarose gels containing formaldehyde and transferred onto nitrocellulose membranes (Schleicher & Schuell). Northern blots were hybridized with bovine LOX-1 cDNA probe-labeled with [α-32P]dCTP (DuPont-New England Nuclear) using random hexanucleotide primers (DNA labeling kit, Pharmacia). An XhoPstI fragment of pBLOX-1, which includes the entire coding region of bovine LOX-1, was used. Densitometric scanning was performed to measure the amounts of mRNA using an Image laser densitometer (Pharmacia).

**Nuclear Runoff Assay**

Nuclear runoff assay was performed as described previously with minor modification.3 In brief, nuclei were isolated from BAECs by NP-40 lysis, followed by centrifugation. Nascent transcription in vitro was performed with [32P]UTP (Amersham) and other unlabeled nucleotides (Pharmacia) at 30°C for 30 minutes. Transcribed RNA was isolated by an RNA isolation reagent (Isogen LS, Wako Pure Chemical), followed by denaturation with sodium hydroxide. Target cDNAs were denatured and immobilized onto nylon membranes (Hybond N+, Amersham) with the use of a slot-blot apparatus (Schleicher & Schuell, Inc.) and were then hybridized with transcribed RNA.

**Cellular Uptake of Dil-Labeled Ox-LDL**

LDL (density, 1.019 to 1.063 g/mL) was isolated by sequential ultracentrifugation from human plasma, and oxidative modification of LDL was carried out with cupric ion in vitro as previously described.14 Oxidation was monitored by measuring the amount of thiobarbituric acid–reactive substances (∼10 nmol malondialdehyde equivalent/mg protein in Ox-LDL). Oxidized LDL particles. Labeling of LDL with 1,1-dioctadecyl-3,3,3,3-tetrachlorofluorescein diacetate (DiI, Molecular Probes) was performed as described.25 To examine cellular uptake of Ox-LDL, BAECs were incubated with DiI-labeled Ox-LDL (5μg/mL), with or without unlabeled competitors, in DMEM/10% FBS for 2 hours and washed 3 times with the culture media. Fluorescence microscopy was performed to detect Dil-Ox-LDL internalized in cells. To measure amounts of Dil-Ox-LDL accumulated in cells, Dil was extracted by isopropanol, and the fluorescence was counted in Fluoroscan II (Flow Laboratories). Acetylation of LDL was carried out using acetic anhydride as previously described.25

**Results**

**TNF-α and PMA Induce Cell-Surface Expression of LOX-1**

Confluent monolayers of BAECs were treated with TNF-α or PMA, and fluorescence immunobinding assay was performed to evaluate cell-surface expression of LOX-1 with the use of anti-bovine LOX-1 monoclonal antibody. As shown in Figure 1A, treatment with TNF-α for 7 hours, as well as 100 nmol/L of PMA, dose-dependently increased levels of LOX-1 cell-surface expression. Time-course experiments showed that cell-surface expression of LOX-1 peaked at 12 or 8 hours after TNF-α or PMA treatment, respectively, and declined after 12 hours (Figure 1B). Immunoblot analyses were also...
performed to measure LOX-1 protein levels. Figure 2 demonstrates that treatment with TNF-α dose-dependently increased the intensities of 50-kDa bands in immunoblotting. Amounts of LOX-1 protein in BAECs were also time-dependently increased by both TNF-α and PMA treatment; amounts of LOX-1 protein were peaked at 12 hours by both TNF-α and PMA treatment and declined after 24 hours (Figure 3).

**TNF-α and PMA Increase LOX-1 mRNA Levels**

To determine whether enhanced cell-surface expression of LOX-1 by TNF-α or PMA depends on increased levels of LOX-1 mRNA, Northern blot analyses were performed. Confluent monolayers of BAECs were incubated with various concentrations of TNF-α for 4 hours, and total cellular RNA was extracted and subjected to Northern blotting. As shown in Figure 4, TNF-α treatment for 4 hours dose-dependently increased the amount of LOX-1 mRNA; a 24.1-fold increase in LOX-1 mRNA was observed at 5000 IU/mL of TNF-α. Time-course experiments showed that LOX-1 mRNA levels peaked at 2 hours (13.2-fold increase) and remained elevated after 21 hours in response to 5000 IU/mL TNF-α. In PMA-treated cells, LOX-1 mRNA levels peaked at 4 hours (15.2-fold increase) and declined after 21 hours (Figure 5). These results appeared to be in parallel with the time-dependent increases in cell-surface protein expression of LOX-1 after both TNF-α and PMA treatments.

**TNF-α Stimulates Transcription of LOX-1 Gene**

To examine whether increased amounts of LOX-1 mRNA depends on enhanced transcription of the LOX-1 gene by TNF-α, a nuclear runoff assay was performed. Treatment with TNF-α for 2 hours dramatically induced transcription of LOX-1, whereas transcription of the GAPDH gene was not significantly altered by TNF-α (Figure 6).

**LOX-1 Takes Up Ox-LDL but Not Ac-LDL**

To examine whether LOX-1 can also take up acetylated LDL (Ac-LDL), we have established a Chinese hamster ovary (CHO)-K1 cell line, which stably expresses bovine LOX-1 (BLOX-1-CHO). As shown in Figure 7, BLOX-1-CHO showed prominent uptake of DiI-Ox-LDL but not DiI-Ac-LDL. Uptake of DiI-Ox-LDL in BLOX-1-CHO was completely inhibited by the 100-fold excess amount of unlabeled Ox-LDL but not by the 100-fold excess amount of unlabeled Ac-LDL (Figure 7). These results indicate that LOX-1 ap-
pears to be a receptor that recognizes Ox-LDL but does not significantly recognize Ac-LDL.

TNF-α and PMA Enhance Specific Ox-LDL Uptake in BAECs
To determine whether upregulated expression of LOX-1 by TNF-α and PMA is correlated with enhanced uptake of Ox-LDL, amounts of DiI-labeled Ox-LDL internalized into BAECs was measured. Since LOX-1 can internalize Ox-LDL but not Ac-LDL, we have measured the amounts of DiI-labeled Ox-LDL taken up via Ox-LDL–specific pathways that cannot be blocked by excess amounts of unlabeled Ac-LDL. After treatment with or without TNF-α or PMA for 12 hours, confluent monolayers of BAECs were incubated with DiI-labeled Ox-LDL in combination with or without the 100-fold excess amount of unlabeled Ox-LDL or Ac-LDL for an additional 90 minutes. Fluorescence microscopy was carried out to evaluate the cellular uptake of DiI-Ox-LDL. As shown in Figure 8, DiI-Ox-LDL, which was taken up via Ox-LDL–specific pathways, was not detectable in untreated BAECs. In TNF-α–treated or PMA-treated BAECs, in contrast, significant amounts of DiI-Ox-LDL were internalized. To measure the amounts of DiI-labeled Ox-LDL incorporated into BAECs, DiI was extracted from BAECs by isopropanol, and the fluorescence was measured. TNF-α and PMA enhanced specific Ox-LDL uptake by Ac-LDL at 6.6-fold and 4.2-fold, respectively (Figure 9). These results demonstrate that increased cell-surface expression of LOX-1 was associated with enhanced Ox-LDL–specific uptake in BAECs.

Discussion
Activation of vascular endothelium elicited by Ox-LDL and its lipid constituent appears to play a key role in the initiation and progression of atherosclerosis. LOX-1, a novel cell-surface receptor for Ox-LDL, may play an important role in uptake of Ox-LDL and subsequent endothelial activation. Involvement of proinflammatory cytokines has been implicated in the pathogenesis of atherosclerosis. Increased expression of TNF-α has been shown in atherosclerotic lesions, including an experimental animal model after balloon injury, as well as acute rejection of cardiac allo-
The present study provides evidence, for the first time, that expression of LOX-1 is not constitutive but can dramatically be induced by TNF-α, suggesting that inflammatory cytokines generated in a local milieu in the vascular wall may further amplify Ox-LDL uptake and subsequent endothelial activation or dysfunction in atherosclerosis.

Previous studies have indicated that TNF-α and PMA induced the expression of class A scavenger receptors in cultured vascular smooth muscle cells, although the expression of class A scavenger receptors was suppressed by TNF-α in macrophages. In addition, interferon gamma, a T-lymphocyte--derived cytokine, downregulated the expression of class A scavenger receptors in macrophages but upregulated the expression in smooth muscle cells. CD36, another class of Ox-LDL receptor, has been shown to be inducible by PMA in macrophages. In our preliminary experiments, interferon gamma did not significantly alter LOX-1 expression in BAECs (data not shown); therefore, inflammatory cytokines can differentially affect distinct Ox-LDL–uptake pathways in different cell types in arterial wall and thus may modulate atherosclerotic progression.

Mechanisms involved in endothelial uptake of modified LDLs, such as Ox-LDL and Ac-LDL, appear to depend on multiple molecules, since BAECs can internalize both Ox-LDL and Ac-LDL, although LOX-1 can take up Ox-LDL but cannot sufficiently take up Ac-LDL. As shown in the present study, TNF-α–activated and PMA-activated BAECs showed the increased Ox-LDL uptake that was not blocked by Ac-LDL uptake. These results indicate that LOX-1 plays a more significant role in activated BAECs by the enhancement of Ox-LDL uptake, which may modulate atherosclerotic progression.

TNF-α appears to activate the transcription of LOX-1, since nuclear runoff assays showed enhanced transcription of the LOX-1 gene elicited by TNF-α. In fact, the 5′ flanking region of the LOX-1 gene contains a consensus nuclear factor-κB binding site–like sequence, as well as AP-1 binding site–like sequences (authors’ unpublished data, 1998), suggesting that transcriptional regulation mediated by nuclear factor-κB and/or AP-1 might be involved in LOX-1 gene induction by inflammatory stimuli. Further studies, however, would be necessary to elucidate transcriptional regulatory mechanisms of the LOX-1 gene.

Ox-LDL uptake by LOX-1 in vascular endothelium in vivo may not result in massive lipid accumulation, since foam cells from endothelial cell origin have not been identified. If cholesterol is not efficiently reesterified in cytoplasm by acyl CoA cholesterol acyltransferase activities in vascular endothelium, free cholesterol might be removed by certain acceptors in circulating blood. Ox-LDL uptake via LOX-1 in vascular endothelium, however, may cause endothelial dysfunction, since a variety of biological effects of Ox-LDL and its lipid constituents on endothelial cells have been reported. Alternatively, Ox-LDL uptake by LOX-1 may protect from atherosclerotic progression by removing this atherogenic lipoprotein, if Ox-LDL uptake by LOX-1 does not cause endothelial dysfunction or lipid accumulation in cytoplasm.

In summary, the present study demonstrates that endothelial expression of LOX-1 can be upregulated by inflammatory stimuli. Studies are in progress in our laboratory to explore the inducibility of LOX-1 in vascular endothelium in atherogenesis, as well as inflammatory diseases, in vivo. Further studies related to transcriptional regulation of LOX-1, as well as pathophysiological consequences of Ox-LDL uptake through this novel Ox-LDL receptor, may provide new insights into the pathogenesis of atherosclerosis.

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References


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