Identification of Soluble Forms of Lectin-Like Oxidized LDL Receptor-1

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Abstract—Lectin-like oxidized LDL receptor-1 (LOX-1) is a type II membrane protein belonging to the C-type lectin family molecules, which can act as a cell-surface endocytosis receptor for atherogenic oxidized LDL. In this study, we show that soluble forms of LOX-1 are present in conditioned media of cultured bovine aortic endothelial cells (BAECs) and CHO-K1 cells stably transfected with LOX-1 cDNA. Immunoblot analysis of conditioned media from TNF-α-activated BAECs and CHO-K1 cells stably expressing LOX-1 revealed that soluble LOX-1 has an approximate molecular mass of 35 kDa. In TNF-α-activated BAECs, cell-surface expression of LOX-1 precedes soluble LOX-1 production. Cell-surface biotinylation followed by immunoprecipitation and immunoblotting showed that soluble LOX-1 in cell-conditioned media is derived from LOX-1 expressed on the cell surface. Production of soluble LOX-1 was inhibited by PMSF, suggesting that PMSF-sensitive proteases may be involved in this process. Purification of soluble LOX-1 by high-performance liquid chromatography and N-terminal amino acid sequencing of soluble LOX-1 identified the 2 cleavage sites between Arg86-Ser87 and Lys89-Ser90, which were located in the membrane proximal extracellular domain of LOX-1. The data demonstrate that cell-surface LOX-1 can be cleaved at 2 different sites and transformed into soluble forms. Further studies may explore therapeutic and diagnostic applications of soluble LOX-1 in atherosclerotic diseases. (Arterioscler Thromb Vasc Biol. 2000;20:715-720.)

Key Words: LOX-1 ■ oxidized LDL ■ proteolysis ■ atherosclerosis ■ endothelial cells

Endothelial activation elicited by oxidized LDL (ox-LDL) and its lipid constituents has been implicated in the pathogenesis of atherosclerosis.1,2 We recently identified a novel receptor for atherogenic ox-LDL, designated lectin-like ox-LDL receptor-1 (LOX-1), in vascular endothelial cells.3 LOX-1 is a 50-kDa type II membrane protein with a short N-terminal cytoplasmic domain and a long C-terminal extracellular domain, whose structure belongs to the C-type lectin family. Interestingly, LOX-1 does not share any homology with other receptors for ox-LDL, including class A and B scavenger receptors. Functional analysis revealed that LOX-1 supports binding, internalization, and proteolytic degradation of ox-LDL but not significant amounts of acetylated LDL.4 Our recent studies have shown that LOX-1 can also bind oxidized or aged red blood cells, as well as apoptotic cells, thus further suggesting its physiological roles in vivo.5 With regard to the regulation of expression, LOX-1 can be upregulated by inflammatory stimuli, such as tumor necrosis factor (TNF)-α and phorbol ester,6 and by a mechanical stimulus, fluid shear stress,7 in cultured bovine aortic endothelial cells (BAECs). More importantly, expression of LOX-1 is highly upregulated in atherosclerotic lesions of humans.8 These results suggest that LOX-1 may be expressed locally and play important roles in atherogenesis and inflammatory responses in vivo.

In the past decade, a number of membrane proteins have been shown to be converted into soluble molecules by proteolytic cleavage at the membrane proximal site of the extracellular domain.9–12 Elevated levels of soluble forms of membrane proteins in circulating blood in humans may reflect increased expression of membrane proteins and disease activities.10,12–17 In addition, soluble forms of receptors may interact with its ligands, inhibit the binding of its ligands to the cell-surface receptor, and thus modulate the pathophysiology.10,18–20

In the present study, therefore, we explored the possibility that LOX-1, a novel membrane receptor for ox-LDL, may also be proteolytically cleaved and released as soluble forms. We have identified soluble LOX-1 in conditioned media of cultured cells that express LOX-1 on their cell surface and determined the cleavage sites by protein purification and N-terminal amino acid sequencing.

Methods

Reagents
DMEM and Ham’s F12 medium were obtained from Nissui. FCS was purchased from Sanko Junyaku. TNF-α was obtained from...
Boehringer Mannheim, and protease inhibitors were from Wako Pure Chemicals.

Cell Culture
BAECs were cultured in DMEM containing 10% heat-inactivated FCS in an atmosphere of 95% air, 5% CO₂ at 37°C. Wild-type CHO-K1 cells were maintained in F12/10% FCS. CHO-K1 cells stably expressing bovine LOX-1 (BLOX-1-CHO) were maintained in F12/10% FCS supplemented with 10 μg/mL of leuprolide as previously described.3,6

Immunoblot Analysis
Cells were lysed with 62.5 mmol/L Tris/HCl (pH 7.4), 2% SDS, and 10% glycerol. Cell-conditioned media were concentrated by Centricron 10 (Amicon). Equal protein concentrations of the whole-cell lysates and cell-conditioned media were subjected to SDS-polyacrylamide (12%) gel electrophoresis (SDS-PAGE) under reducing conditions, followed by electroblotting onto an Immobilon polyvinylidene difluoride (PVDF) transfer membrane (Millipore). Membranes were then incubated with a monoclonal antibody (mAb) directed to bovine LOX-13,6,7 and horseradish peroxidase (HRP)–labeled antimouse immunoglobulin (Amersham) as the second antibody. Bands were visualized by chemiluminescence reagents (ECL Western blotting detection reagents, Amersham).

Cell-Surface Biotinylation and Immunoprecipitation
Cells were washed twice with PBS and incubated with 0.5 mg/mL of sulfo-NHS-LC-biotin (Pierce) at room temperature for 15 to 30 minutes. After being washed with PBS, cells were cultured in F12 medium with 5% FCS for 30 minutes at 37°C. The culture media were then replaced with serum-free F12 medium, and the cells were incubated at 37°C for 24 hours. Cell-conditioned media were collected and filtered through a 0.22-μm filter, then incubated overnight at 4°C with anti-biotin agarose (Sigma), which was followed by centrifugation and extensive washing. Immunoprecipitated samples were denatured at 98°C in SDS sample buffer (62.5 mmol/L Tris/HCl [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 1% bromophenol blue) and subjected to immunoblotting with anti–LOX-1 mAb or streptavidin-HRP (Amersham).

Purification of Soluble LOX-1
Cell-conditioned media from BLOX-1-CHO cells cultured in serum-free F-12 media for 2 days were filtered through a 0.22-μm filter and subjected to ammonium sulfate precipitation. Precipitates in 80% saturated ammonium sulfate were then centrifuged at 20 000 g for 20 minutes, after which the pellet was dissolved in PBS and dialyzed against 66.7 mmol/L phosphate buffer (pH 7.4). After centrifugation at 15 000 g for 15 minutes, the supernatant was applied to a Q Sepharose (Pharmacia) column equilibrated with buffer A (20 mmol/L HEPES, 0.1% CHAPS, pH 7.2). The column was washed with 5 column volumes of buffer A and eluted with buffer A containing 250 mmol/L NaCl. Eluted proteins were concentrated by Centricron 10, diluted with buffer A, and then subjected to a Mono Q 5/5 high-performance liquid chromatography (HPLC) ion exchange column (Pharmacia). The column was washed with buffer A and eluted with a linear concentration gradient (0 to 500 mmol/L) of NaCl in buffer A. Aliquots of the fractions (0.5 mL) were subjected to immunoblotting with an anti–LOX-1 mAb. Fractions containing soluble LOX-1 were diluted with buffer C (20 mmol/L MES, 0.1% CHAPS, pH 6.5) and subjected to a Mono S 5/5 HPLC ion exchange column (Pharmacia). The column was eluted with a linear NaCl gradient (0 to 500 mmol/L NaCl) in buffer C. Aliquots of the fractions (0.5 mL) were subjected to immunoblotting with an anti–LOX-1 mAb. Fractions containing soluble LOX-1 were pooled, concentrated with Centricron 10, diluted with buffer A, and applied to a heparin Sepharose CL-6B column (Pharmacia). The column was washed with 5-fold column volumes of buffer A and 80 mmol/L NaCl/buffer A, followed by elution with 160 mmol/L NaCl/buffer A. Aliquots of the fractions (1.5 mL) were subjected to immunoblotting with an anti–LOX-1 mAb and silver staining. Fractions containing soluble LOX-1 were then applied to a blue Sepharose 6 FF column (Pharmacia) column equilibrated with buffer A. Proteins bound to the column were eluted in a stepwise manner with 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, and 0.45 mol/L NaCl.

N-Terminal Amino Acid Sequence Analysis of Soluble LOX-1
Sequence analysis of purified soluble LOX-1 was performed with automated Edman degradation on a Hewlett Packard G1005A protein sequencing system.

Results
Production of Soluble LOX-1 in TNF-α-Activated BAECs and BLOX-1-CHO
To examine whether BAECs produce soluble forms of LOX-1, cell-conditioned media and whole-cell lysates from BAECs cultured in the presence or absence of TNF-α were subjected to immunoblotting with a mouse mAb directed to the extracellular domain of LOX-1. Figure 1 demonstrates that both expression of membrane-anchored LOX-1 and production of soluble LOX-1 were induced by TNF-α.

![Figure 1. Immunoblotting with anti-LOX-1 mAb in whole-cell lysates and conditioned media of TNF-α-activated BAECs. After BAECs were treated with or without TNF-α (5 ng/mL) for 24 hours, equal protein concentrations of cell-conditioned media and whole-cell lysates were subjected to immunoblotting with anti–LOX-1 mAb. Cont indicates control.](image)

Soluble LOX-1 has an approximate molecular mass of 35 kDa, as shown by immunoblotting of the cell-conditioned media. In untreated BAECs, in contrast, neither the membrane-bound nor the soluble form of LOX-1 was detectable by immunoblotting. Soluble LOX-1 was detectable in conditioned media of BAECs treated for 24 hours with 1 to 5 ng/mL of TNF-α (Figure 2). In response to 5 ng/mL of TNF-α, soluble LOX-1 was detectable as early as 8 hours after the treatment and kept increased for ≥24 hours (Figure 3). Conversely, cell-surface expression of LOX-1 was detectable within 4 hours after TNF-α treatment and remained elevated for ≥36 hours, as previously reported (Figure 3).6 These results indicate that expression of membrane-bound LOX-1 appeared to precede release of soluble LOX-1 in BAECs.

We also examined whether soluble LOX-1 was produced in CHO-K1 cells transfected with bovine LOX-1 cDNA

![Figure 2. Concentration dependency in TNF-α-induced production of soluble LOX-1. After BAECs were exposed to indicated concentrations of TNF-α for 24 hours at 37°C, equal protein concentrations of cell-conditioned media were subjected to immunoblotting with anti–LOX-1 mAb.](image)
As shown in Figure 4, large amounts of soluble LOX-1 with an approximate molecular mass of 35 kDa were detectable in conditioned media from BLOX-1-CHO but not in those from untransfected CHO-K1 cells.

Soluble LOX-1 in Cell-Conditioned Media Derives From Cell-Surface LOX-1

To determine whether soluble LOX-1 in cell-conditioned media results from proteolytic cleavage of membrane-anchored LOX-1 expressed on the cell surface, we carried out immunoprecipitation of cell-conditioned media after cell-surface biotinylation. After cell-surface biotinylation of BLOX-1-CHO, cell-conditioned media were harvested and immunoprecipitated with anti-biotin agarose. Immunoprecipitates were subsequently analyzed by Western blotting with anti–LOX-1 mAb or streptavidin-HRP. As shown in Figure 5, biotinylated 35-kDa bands, which appear to correspond to soluble LOX-1, were detectable in conditioned media of BLOX-1-CHO. This clearly demonstrates that soluble LOX-1 in cell-conditioned media is derived from LOX-1 expressed on the cell surface.

Cleavage of the Membrane-Anchored LOX-1 Requires Protease Activities Sensitive to PMSF

To gain insights into mechanisms responsible for the release of soluble LOX-1 from the cell surface, we tested the effects of various protease inhibitors on soluble LOX-1 release from TNF-α–activated BAECs and BLOX-1-CHO cells. Protease inhibitors were used at concentrations that are known to specifically inhibit the protease activities without exhibiting cytotoxicity. As shown in Figure 6A, PMSF, a potent serine protease inhibitor, markedly inhibited soluble LOX-1 release. Leupeptin, TLCK, and other serine protease inhibitors partially blocked the soluble LOX-1 release. Conversely, neither E64 (cysteine protease inhibitor), aprotinin (serine protease inhibitor), pepstatin (acid protease inhibitor), nor calpain inhibitor II affected soluble LOX-1 release. In TNF-α–activated BAECs, PMSF similarly inhibited the soluble LOX-1 release as shown in BLOX-1-CHO (Figure 6B). None of these protease inhibitors caused morphological changes in BLOX-1-CHO or TNF-α–activated BAECs (data not shown). Taken together, soluble LOX-1 is produced and released by proteolytic cleavage of cell-surface LOX-1 by proteases sensitive to PMSF.

Purification of Soluble LOX-1 From Conditioned Media of BLOX-1-CHO

Because BLOX-1-CHO produces larger amounts of soluble LOX-1 with the same molecular mass on SDS-PAGE than TNF-α–activated BAECs, we used BLOX-1-CHO to isolate and purify soluble LOX-1. Proteins in cell-conditioned media were concentrated by ammonium sulfate precipitation, applied to a Q Sepharose anion exchange column, and subsequently subjected to a Mono Q 5/5 anion-exchange HPLC column. Proteins bound to the column were eluted with a linear concentration gradient of NaCl. Soluble LOX-1 was
eluted between 0.08 and 0.15 mol/L of NaCl, as detected by immunoblotting (Figure 7A). Fractions containing soluble LOX-1 were then applied to a Mono S cation exchange HPLC column and eluted with a linear concentration gradient of NaCl. Soluble LOX-1 was eluted between 0.16 and 0.27 mol/L of NaCl (Figure 7B). After application to a heparin column chromatograph, fractions containing soluble LOX-1 were applied to a blue Sepharose column. Soluble LOX-1 was eluted at 0.2 mol/L NaCl when the column was eluted with a stepwise concentration gradient of NaCl. SDS-PAGE and silver staining of the purified protein showed that 1 major protein, which corresponds to soluble LOX-1, was present in the purified fraction (Figure 7C).

Figure 6. Effects of protease inhibitors on soluble LOX-1 release. A, After BLOX-1-CHO cells were incubated in the presence or absence of various protease inhibitors for 24 hours at 37°C, equal volumes of cell-conditioned media were subjected to immunoblotting with an anti–LOX-1 mAb. Lane 1, control (cont); lane 2, E64 (40 μg/mL); lane 3, PMSF (2 mmol/L); lane 4, aprotinin (5 MIU/mL); lane 5, pepstatin (10 μmol/L); lane 6, leupeptin (10 μg/mL); lane 7, calpain inhibitor II (50 μmol/L); lane 8, TLCK (100 μmol/L). B, After preincubation with or without PMSF (2 mmol/L) for 30 minutes at 37°C, BAECs were cultured in the presence or absence of TNF-α (5 ng/mL) for 24 hours. Cell-conditioned media were concentrated by Centricon 10 and subjected to immunoblotting. Lane 1, control; lane 2, TNF-α; lane 3, TNF-α and PMSF.

Figure 7. Purification of soluble LOX-1. A, Soluble LOX-1–containing fractions from Q-Sepharose column were applied to a Mono Q ion exchange HPLC column, and the bound proteins were eluted as described in Methods. Each elution fraction (0.5 mL) was subjected to immunoblotting with anti–LOX-1 mAb and silver staining to determine soluble LOX-1–positive fractions. B, Soluble LOX-1–containing pooled fractions from Mono Q column were applied to Mono S ion exchange HPLC column, and the bound proteins were eluted. Soluble LOX-1–positive fractions were determined by immunoblotting with anti–LOX-1 mAb. C, Soluble LOX-1–containing fractions from Q Sepharose column (lane 1), Mono Q HPLC column (lane 2), Mono S HPLC column (lane 3), heparin column (lane 4), and blue Sepharose column (lane 5) were subjected to SDS-PAGE, followed by silver staining.

N-Terminal Amino Acid Sequencing of Purified Soluble LOX-1 and Identification of the Cleavage Sites

To determine the cleavage site of soluble LOX-1, the N-terminal amino acid sequence of purified soluble LOX-1 was analyzed with an automated protein sequencer after SDS-PAGE and transfer onto a PVDF membrane. Two different N-terminal amino acid sequences, SAQES and SEKSA, were found. These amino acid sequences correspond
to the amino acid numbers between 87 and 91 and between 90 and 94 of LOX-1, respectively. These results thus demonstrate that purified 35-kDa soluble LOX-1 consists of 2 forms of the proteins resulting from proteolytic cleavage of the Arg^{86} - Ser^{87} and Lys^{89} - Ser^{90} bonds of LOX-1 (Figure 8), although SDS-PAGE analysis of the purified soluble LOX-1 was not able to distinguish these 2 proteins with similar molecular weights (Figure 7C).

**Discussion**

LOX-1 has been identified as a cell-surface receptor for ox-LDL in endothelial cells. Our recent study indicated that LOX-1 can also support binding and phagocytosis of oxidized or aged red blood cells and apoptotic cells, suggesting its physiological roles as a “scavenger” receptor. Expression of LOX-1 are present in cell-conditioned media of TNF-α-activated BAECs, as well as CHO-K1 cells transfected with LOX-1 cDNA. We also showed that soluble LOX-1 results from proteolytic cleavage of cell-surface LOX-1 by proteases sensitive to PMSF and further identified the cleavage sites. Among the scavenger receptor family molecules, LOX-1 is the first that has been shown to be converted into soluble forms.

Soluble receptors, in general, can be generated by 2 different mechanisms: alternative splicing out of the exon encoding the transmembrane regions and proteolytic cleavage of the full-length membrane-bound receptor. For instance, soluble forms of cell-surface molecules, such as platelet and endothelial cell adhesion molecule-1, P-selectin, and interleukin (IL)-4 receptor, are generated by alternative splicing of their mRNA. Conversely, soluble forms of L-selectin, vascular cell adhesion molecule-1, E-selectin, and platelet-derived growth factor receptor are produced by proteolytic processing of full-length membrane-bound molecules. Soluble IL-6 receptor can be generated by both alternative splicing and proteolytic cleavage. Our results in this report demonstrated that expression of cell-surface membrane-bound LOX-1 precedes production of soluble LOX-1 in TNF-α-activated BAECs (Figure 3). In addition, large amounts of both membrane-bound and soluble LOX-1 were produced in BLOX-1-CHO but not in untransfected CHO-K1 cells (Figure 4). Finally, cell-surface biotinylation followed by immunoprecipitation provided evidence that production of soluble LOX-1 appears to result from proteolytic cleavage of LOX-1 expressed on the cell surface (Figure 5). Studies with various protease inhibitors indicated that PMSF-sensitive protease activities appear to be involved in the proteolytic cleavage to generate soluble LOX-1 (Figure 6). Furthermore, series of reverse transcription–polymerase chain reaction analyses in TNF-α-activated BAECs did not detect LOX-1 mRNA lacking the transmembrane domain (data not shown). These results thus provide evidence that soluble LOX-1 is produced mainly by proteolytic cleavage of LOX-1 expressed on the cell surface.

Purification and N-terminal amino acid sequencing of soluble LOX-1 identified the 2 different cleavage sites Lys^{89} - Ser^{90} and Arg^{86} - Ser^{87} in the membrane proximal extracellular domain (Figure 7C). One of the amino acid sequences of LOX-1 cleavage sites, Lys^{89} - Ser^{90}, appears to be identical to the L-selectin cleavage site, Lys^{321} and Ser^{322}. Recognition of LOX-1 cleavage sites by the putative converting enzyme, however, may depend on the protein conformation rather than amino acid sequence, because 2 distinct amino acid sequences of LOX-1 can be cleaved by this enzyme. In fact, conformation- or position-dependent recognition of membrane proteins by proteases has been implicated in the shedding of IL-6 receptor and L-selectin. Alternatively, 2 different proteases may specifically shed LOX-1 at 2 different sites, both of which are sensitive to PMSF.

Recent studies have indicated that shedding of L-selectin, as well as proteolytic cleavage of TNF-α, appears to be supported by metalloproteases. Recent studies have indicated that shedding of L-selectin, as well as proteolytic cleavage of TNF-α, appears to be supported by metalloproteases. Recent studies have indicated that shedding of L-selectin, as well as proteolytic cleavage of TNF-α, appears to be supported by metalloproteases.
inhibitory effects of soluble LOX-1 in the ox-LDL binding to the LOX-1 expressed on the cell surface (data not shown). This might result from the lower affinity of soluble LOX-1 in the ox-LDL/binding to the LOX-1 expressed on the cell surface (data not shown).

In summary, the present report provides the first evidence that LOX-1 can be cleaved at the 2 sites located in the membrane proximal extracellular domain and secreted as soluble forms. Further studies related to pathophysiologically functions of soluble LOX-1 and molecular mechanisms of LOX-1 cleavage might elucidate novel therapeutic and diagnostic values of soluble LOX-1 in atherogenesis and vascular diseases.

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