LOX-1 mediates lysophosphatidylcholine-induced oxidized LDL uptake in smooth muscle cells

Takuma Aoyama[a], Mingyi Chen[b], Hisayoshi Fujiwara[a], Tomoh Masaki[b], Tatsuya Sawamura[b,c,*]

[a] *Second Department of Internal Medicine, Gifu University School of Medicine, Gifu, Japan*
[b] *Department of Bioscience, National Cardiovascular Center Research Institute, Suita, Osaka 565-8565, Japan*
[c] *Department of Molecular Pathophysiology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan*

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Abstract A novel receptor for oxidized low-density lipoprotein (OxLDL), lectin-like OxLDL receptor (LOX-1), was cloned from endothelial cells. Since OxLDL is taken up by vascular smooth muscle cells (VSMC) in atheroma, we analyzed the inducible expression of LOX-1 in VSMC in the present study. Incubation of cultured bovine VSMC with lysophosphatidylcholine (LPC), an atherogenic component of OxLDL, increased the level of mRNA for LOX-1 in a dose- and time-dependent manner. Since LPC did not significantly change the half-life of LOX-1 mRNA, the induction seemed to occur at the transcriptional level. The induction accompanied an increase in the protein level of LOX-1 and activity of OxLDL uptake. Blocking antibody against LOX-1 significantly suppressed the enhanced uptake of OxLDL. Thus, LOX-1 is a major receptor for OxLDL in VSMC as in endothelial cells. The enhanced expression of LOX-1 by LPC suggests that OxLDL and LPC would progressively change the function of VSMC and accelerate atherogenesis in vivo.

Key words: Lectin-like oxidized low-density lipoprotein receptor; Oxidized LDL; Vascular smooth muscle cell; Lysophosphatidylcholine; Atherosclerosis

1. Introduction

Oxidized low-density lipoprotein (OxLDL) is believed to be a key atherogenic component which induces endothelial dysfunction and accumulation of foam cells [1]. A number of 'scavenger receptors' characterized by binding to OxLDL have been identified [1]. Among them, lectin-like OxLDL receptor-1 (LOX-1) identified in our laboratory is uniquely expressed in the endothelial cells of large arteries [2]. LOX-1 is a type II membrane protein with a C-type lectin-like structure at the C-terminus. Besides OxLDL, LOX-1 binds aged/apoptotic cells, suggesting potential physiological functions [3]. The expression of LOX-1 in endothelial cells in vitro and in vivo is highly regulated. LOX-1 expression is induced by TNF-α, phorbol ester, shear stress, LPS, angiotensin II and OxLDL in cultured endothelial cells, and by hypertension in vivo [4–10]. Recently, LOX-1 was found to be expressed in macrophages and vascular smooth muscle cells (VSMC) in atheromatous intima and culture [5,11–13]. Since macrophages and smooth muscle cells transform into foam cells in atheroma, a potential role for LOX-1 in foam cell formation has been suggested.

Lysophosphatidylcholine (LPC) is a well characterized atherogenic component of OxLDL, being generated during oxidation of LDL and accumulated in atheroma. LPC causes endothelial dysfunction characterized by impairment of release of nitric oxide, expression of adhesion molecules, and expression of growth factors [14–16]. LPC acts on VSMC as well, and induces proliferation and the release of fibroblast growth factor-2 [17,18].

To address the significance of the expression of LOX-1 in VSMC in atheroma, we explored whether LOX-1 constitutes a significant part of OxLDL receptors in LPC-stimulated VSMC.

2. Materials and methods

2.1. Cells

Bovine thoracic aortas were obtained from a local slaughterhouse. Endothelial cells were removed by scraping with a knife the internal surface of aortas. Small pieces (~5 mm×5 mm) of the medial layer cut from the aorta were explanted on a culture dish, and were then maintained for a week in Dulbecco's modified Eagle's medium (DMEM, Nissui, Japan) containing 10% (v/v) fetal calf serum (JRH Bioscience, Lenexa, KS, USA), ampicillin (50 μg/ml), streptomycin (50 μg/ml) and amphotericin B (0.25 μg/ml) under a humidified atmosphere of 5% CO_2/95% air at 37°C [2]. Migrated smooth muscle cells from the explants were used as VSMC and maintained with the above medium.

2.2. Preparation of lipoproteins

LDL (density: 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation from human plasma. LDL was oxidatively modified by exposure to 7.5 μM $CuSO_4$ in phosphate-buffered saline (PBS) without calcium and magnesium for about 20 h at 37°C as described [2]. Oxidation was monitored by measuring the amount of thiobarbituric acid-reactive substances. The degree of the oxidation was approximately 10 nmol malondialdehyde equivalent/mg protein. Agarose gel electrophoresis showed increased electrophoretic mobility and minimal aggregation of OxLDL particles. Labeling of LDL with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Eugene, OR, USA) was performed as previously described [2].

2.3. Treatment of VSMC with LPC

Stearoyl LPC was obtained from Sigma and dissolved in DMEM containing 0.3% bovine serum albumin (BSA). VSMC were cultured in DMEM containing 0.3% BSA for 24 h before treatment with LPC. The viability of cells was confirmed by the trypan blue exclusion assay and by the measurement of the activity of lactate dehydrogenase as described [19].

*Corresponding author. Department of Bioscience, National Cardiovascular Center Research Institute, Suita, Osaka 565-8565, Japan. Fax: (81)-6-6872 7485. E-mail: sawamura@ri.ncvc.go.jp

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2.4. Northern blotting

Total RNA was isolated from VSMC by the acid guanidium-phenol-chloroform method and electrophoresed through 1.1% agarose gels containing formaldehyde. RNA was then transferred to nylon membranes. The membranes were hybridized with a cDNA fragment of bovine LOX-1 or human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described previously [2]. To determine the half-life of LOX-1 mRNA, serum-starved VSMC were treated for different periods with actinomycin D (5 μg/ml) in the absence or presence of LPC. The logarithm of percent RNA remaining was plotted against the time after the actinomycin D addition, and the half-life was calculated by linear regression analysis.

2.5. Uptake of DiI-OxLDL by VSMC

VSMC were cultured on a glass-bottom culture dish (Meridien, Ashland, MA, USA). The cells were incubated with LPC for 18 h, and then 5 μg/ml DiI-OxLDL was added to the medium and incubation continued for 2 h under absence or presence of anti-LOX-1 antibody, JTX20. The cells were fixed with 4% formaldehyde and 0.2% glutaraldehyde in PBS and examined by fluorescence microscopy.

2.6. Statistics

To test for the differences between experimental groups, Student's *t* test (unpaired and two-tailed) was performed.

3. Results

Treatment of cultured bovine VSMC with LPC for 12 h increased the level of LOX-1 mRNA at as low as 3 μM and dose-dependently increased the mRNA level within a range of 0–100 μM, whereas the mRNA level of GAPDH was not affected by LPC (Fig. 1). Similarly, Western blot analysis showed that this induction was accompanied by an increase in the protein level of LOX-1, detecting LOX-1 as a 50 kDa protein (data not shown). Time course analysis of the induction showed a significant increase of the mRNA level for LOX-1 within 3 h. The induction had a peak at 12 h and

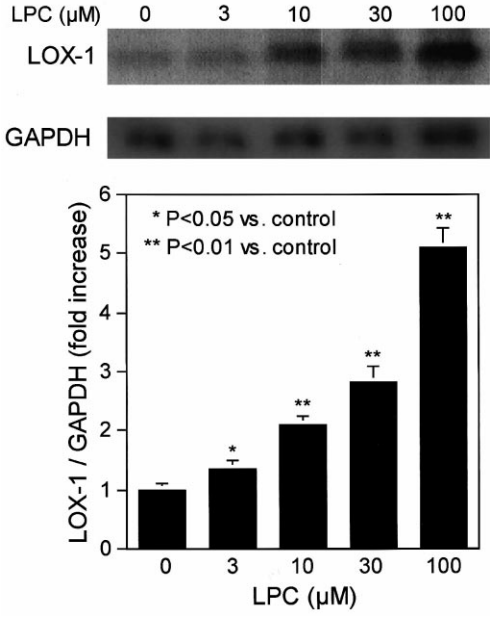

Fig. 1. Dose-dependent induction of LOX-1 mRNA by LPC. Total RNA was prepared from VSMC after treatment with the indicated concentrations of stearoyl LPC in DMEM containing 0.3% BSA for 12 h. RNA (3 μg/lane) was separated by electrophoresis and hybridized with bovine LOX-1 cDNA probe. Hybridization of the GAPDH cDNA probe to the same membrane was done to show the uniformity in quantity of RNA among samples. The upper panel shows a representative of three reproducible results. The lower panel shows a semi-quantitative analysis of the results. Data are the mean ± S.E.M.

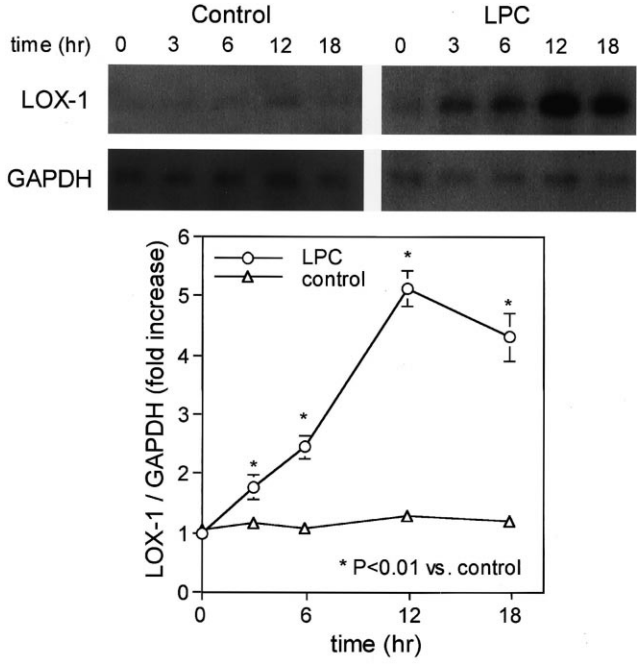

Fig. 2. Time course of LOX-1 mRNA induction by LPC. VSMC were treated with 100 μM stearoyl LPC in DMEM with 0.3% BSA for the period indicated. The amount of LOX-1 mRNA was analyzed as in Fig. 1.

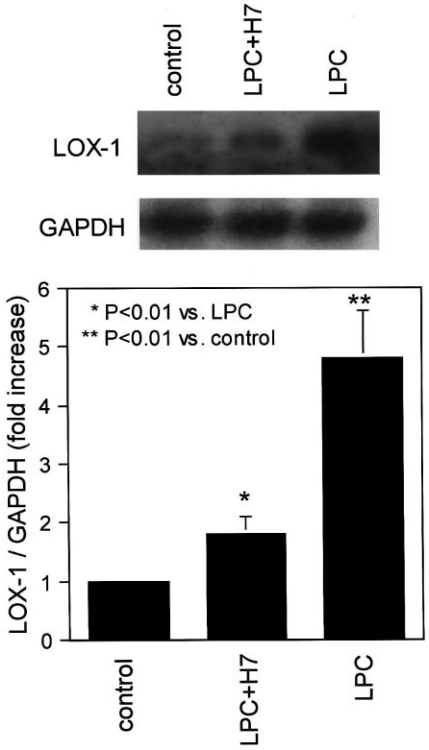

Fig. 3. VSMC were treated with 100 μM stearoyl LPC in DMEM with 0.3% BSA for 12 h under the presence or absence of 50 μM H-7. The amount of LOX-1 mRNA was analyzed as in Fig. 1.

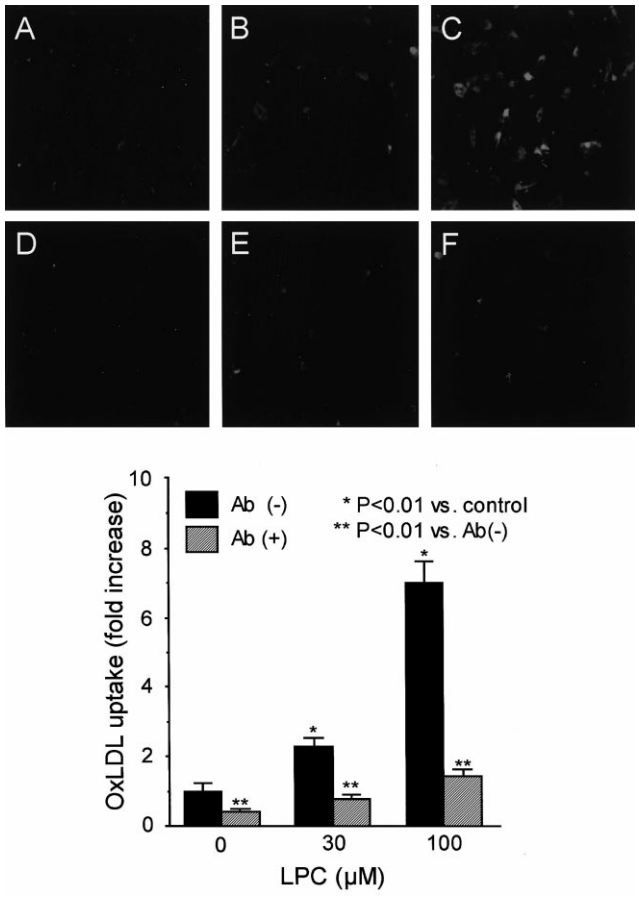

Fig. 4. Uptake of DiI-OxLDL by LPC-treated VSMC. VSMC were treated with 0 (A, D), 30 (B, E) or 100 μM (C, F) stearoyl LPC for 18 h in the absence (A–C) or presence (D–F) of anti-LOX-1 antibody, then incubated with 5 μg/ml DiI-OxLDL for 2 h. The upper panel shows a representative of three reproducible results. The lower panel shows a semi-quantitative analysis of the results. Data are the mean ± S.E.M.

decreased at 18 h although the level remained higher than that at time 0 (Fig. 2). Protein levels of LOX-1 also showed a significant increase within 6 h and peaked at 18 h, several hours after the mRNA induction (data not shown). Since LOX-1 has a cluster of unstabilizing signals in the 3′-untranslated region of mRNA, the effect of LPC on the half-life of mRNA for LOX-1 in VSMC was examined. The half-life of LOX-1 mRNA in the presence and absence of LPC was 4.4 ± 1.3 and 4.3 ± 1.2 h, respectively, showing no significant difference. This result suggests that the induction by LPC occurs at the transcriptional level. Furthermore, treatment of VSMC with H-7, an inhibitor for protein kinase C, significantly suppressed the induction of LOX-1 mRNA, suggesting protein kinase C might be involved in the signal transduction triggered by LPC (Fig. 3). Then, we confirmed that the induction of LOX-1 by LPC was accompanied by activity of VSMC to take up OxLDL. The LPC-treated VSMC showed intense fluorescence of DiI as a result of uptake of OxLDL in a dose-dependent manner (Fig. 4A–C). The enhanced uptake was significantly hampered by the introduction of anti-LOX-1 neutralizing antibody into the medium for taking up OxLDL (Fig. 4D–F), indicating most of the induced activity can be ascribed to LOX-1.

4. Discussion

Functions of cells that constitute atherosclerotic blood vessels are modified by the factors present in atheroma. Some of these factors are released by the cells, others are derived from plasma. The former include TNF-α, TGF-β, IL-8, G-CSF etc., the latter, OxLDL and its lipid constituents. Among them, LPC is the best-characterized atherogenic molecule. It constitutes about 40% of the total lipid component of OxLDL and is accumulated in atherosclerotic tissue at about 1 mM. In endothelial cells, LPC impairs the release of nitric oxide and promotes the expression of leukocyte adhesion molecules, leukocyte chemotactic factors and growth factors. In VSMC, LPC works as a growth factor and chemoattractant. Here, we showed that LPC induces expression of LOX-1 in VSMC. This suggests that LPC strengthens the effect of OxLDL on VSMC and further promotes functional change of VSMC. There are reports that lipid components other than LPC also mediate the action of OxLDL. 25-Hydroxycholesterol was reported to mediate apoptosis of endothelial cells and 13- and 15-hydroxyoctadecadienoic acid, agonists for peroxysome proliferator-activated receptor-γ, to mediate the induction of CD36 expression in macrophage [20,21]. Although we did not examine the effect of these substances in the present study, it is possible that they change the expression level of LOX-1 together with LPC as components of OxLDL.

One study described that the phorbol ester induced the expression of another type of OxLDL receptor, class A scavenger receptor, in cultured rabbit VSMC [22]. Despite the possible expression of other types of OxLDL receptors, the neutralizing antibody for LOX-1 significantly inhibited the LPC-enhanced uptake of OxLDL in VSMC in the present study. This result indicates that the part of the activity to uptake OxLDL can be ascribed to LOX-1 at least in some conditions as demonstrated in endothelial cells. Various effects of OxLDL on VSMC have been reported. OxLDL alters the expression of the proteins which constitute the contractile machinery of cultured mouse and rat aortic smooth muscle cells, i.e. it decreases the expression of α-actin, smooth muscle myosin heavy chain-1 and calponin [23]. OxLDL is chemotactic for cultured bovine smooth muscle cells [24]. It induces the proliferation of cultured smooth muscle cells by stimulating the release of fibroblast growth factor-2 [18]. OxLDL stimulates synthesis of collagen from porcine cultured aortic smooth muscle cells [25]. It stimulates the extracellular matrix and induces apoptosis under some conditions in cultured human coronary artery smooth muscle cells [26]. These in vitro effects of OxLDL on VSMC well illustrate the in vivo behavior of VSMC in atheroma, specifically, the change from a contractile to synthetic phenotype, migration from media to intima, proliferation in intima, synthesis of matrix proteins, and eventual death in acute coronary syndrome. VSMC are also well-known to participate in the formation of foam cells as well as macrophages, probably taking up OxLDL in atheroma [1]. LOX-1 expressed in VSMC as a major part of the OxLDL receptor may mediate these various actions of OxLDL on VSMC, and blockade of the action of LOX-1 might delay the progression of atherosclerosis and related diseases.

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