Papers in Press. Published January 14, 2010 as doi:10.1373/clinchem.2009.140202
The latest version is at http://www.clinchem.org/cgi/doi/10.1373/clinchem.2009.140202

Clinical Chemistry 56:3
000–000 (2010)

Brief Communications

C-Reactive Protein Uptake by Macrophage Cell Line via Class-A Scavenger Receptor

Yoshiko Fujita, Akemi Kakino, Mariko Harada-Shiba, Yuko Sato, Kazunori Otsui, Ryo Yoshimoto, and Tatsuya Sawamura*

Department of Vascular Physiology, National Cardiovascular Center Research Institute, Suita, Osaka, Japan; *address correspondence to this author at: Department of Vascular Physiology, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. Fax +81–6-6835–5329; e-mail t-sawamura@umin.ac.jp.

BACKGROUND: C-reactive protein (CRP) increases in response to inflammation and is purported to be a risk factor for atherogenesis. We recently demonstrated that a scavenger receptor, lectin-like oxidized LDL receptor (LOX-1), is a receptor for CRP. In light of the overlapping ligand spectrum of scavenger receptors such as modified LDL, bacteria, and advanced glycation end products, we examined whether other scavenger receptors recognize CRP.

METHODS: We analyzed the uptake of fluorescently labeled CRP in COS-7 cells expressing a series of scavenger receptors and in a monocytic cell line, THP-1, differentiated into macrophage with phorbol 12-myristate 13-acetate (PMA). We applied small interfering RNA (siRNA) against class-A scavenger receptor (SR-A) to THP-1 cells to suppress the expression of SR-A. We also analyzed the binding of nonlabeled CRP to immobilized recombinant LOX-1 and SR-A in vitro using anti-CRP antibody.

RESULTS: COS-7 cells expressing LOX-1 and SR-A internalized fluorescently labeled CRP in a dose-dependent manner, but cells expressing CD36, SR-BI, or CD68 did not. The recombinant LOX-1 and SR-A proteins recognized nonlabeled purified CRP and native CRP in serum in vitro. THP-1 cells differentiated into macrophage-like cells by treatment with PMA-internalized fluorescently labeled CRP. siRNA against SR-A significantly and concomitantly inhibited the expression of SR-A ($P < 0.01$) and CRP uptake ($P < 0.01$), whereas control siRNA did not.

CONCLUSIONS: CRP is recognized by SR-A as well as LOX-1 and taken up via SR-A in a macrophage-like cell line. This process might be of significance in the pathogenesis of atherosclerotic disease.

C-reactive protein (CRP),[1] which is synthesized by hepatocytes in response to inflammation and tissue damage *(1)*, binds to various ligands exposed on damaged tissues or bacteria promoting phagocytosis and complement activation with C1q *(1, 2)*. Plasma CRP concentrations may rise as much as 1000-fold during infection or inflammation *(3)*. In addition, CRP concentrations, within the reference range, can predict cardiovascular diseases *(4, 5)*, and there is a good correlation between plasma CRP concentrations and the degree of atherosclerosis in hypercholesterolemic rabbits *(6)*.

Fcγ receptors CD16, CD32, and CD64 have been reported as the receptors for CRP *(7–9)*. In addition, we recently demonstrated that CRP increases vascular permeability through a direct binding to lectin-like oxidized LDL receptor (LOX-1), which is expressed in endothelial cells *(10)*. Members of the scavenger receptor family, such as class A scavenger receptor (SR-A), CD36, LOX-1, and scavenger receptor B-I (SR-BI), recognize common ligands such as modified LDL, bacteria, and advanced glycation end products, and they are thought to affect the progression of atherosclerosis *(11, 12)*. In this study, to further elucidate the atherogenic properties of CRP, we addressed whether other scavenger receptors are involved in the recognition of CRP.

Human sera with high and normal concentrations of CRP were obtained from Dako. Human CRP purified from pleural fluid was purchased from Chemicon (AG723). Sodium azide in the solution was extensively removed by dialyzing 3 times against a 3000-fold volume of Dulbecco's PBS (Wako). Gram-negative bacterial endotoxins were undetectable by limulus amebocyte lysate (Associates of Cape Cod), which can detect as little as 0.03 endotoxin units per mL endotoxins. CRP was fluorescently labeled with CypHer5E (GE Healthcare) and dialyzed 3 times against a 3000-fold volume of PBS.

COS-7 cells maintained with Dulbecco's modified Eagle's medium (DMEM; Invitrogen)/10% fetal bovine serum (FBS) were seeded 1 day before transfection. After reaching 80%–90% confluency, we transfected the cells with the plasmid using Lipofectamin 2000 transfection reagent (Invitrogen). We used the following cDNAs: human LOX-1 (GenBank NM002543), SR-A (GenBank NM002445), CD36 (GenBank NM000072), SR-BI (GenBank NM005505), CD68 (GenBank NM001251), and

[1] Nonstandard abbreviations: CRP, C-reactive protein; LOX-1, lectin-like oxidized LDL receptor; SR, scavenger receptor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; siRNA, small interfering RNA; PMA, phorbol 12-myristate 13-acetate; SRA-C6, anti–SR-A antibody; DAPI, 4′,6-diamidino-2-phenylindole.

dectin-1 (GenBank NM197947), which were subcloned into pcDNA6.2/V5/GW/D-TOPO expression vector (Invitrogen). We used pcDNA3.1/V5-His/lacZ (Invitrogen) as a control. After 48 h, we washed the cells with DMEM:1% antibiotics and antimycotic (AbAm; Invitrogen). We replaced the medium with CypHer5E-CRP-containing DMEM/1% AbAm and incubated the cells for 2 h at 37 °C. After washing with PBS, the cells were fixed with phosphate-buffered formalin (Wako) and permeabilized with 0.1% Triton X-100/PBS. We detected the expression of each receptor by immunostaining with anti-V5 antibody (Nacalai Tesque) combined with Alexa 488 antimouse IgG (Invitrogen). The nuclei of the cells were counterstained with 0.5 mg/L 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). We divided the fluorescence intensities of CypHer5E and Alexa 488 by the cell number in a field, then divided the CypHer5E-CRP fluorescence intensity in the field by the Alexa 488 fluorescence value. These quantitative analyses were performed with an IN Cell Analyzer 1000 system (GE Healthcare).

We prepared recombinant human SR-A (amino acids 76–358) as described for LOX-1 *(10)*. Recombinant human SR-A (0.1 μg) or BSA (0.1 μg, Sigma) was immobilized to each well of 384-well plates (High Bind; Corning) by incubating at 4 °C in PBS overnight. After 2 washes with PBS, the plates were blocked with 80 μL of 20% ImmunoBlock (DS Pharma)/PBS at 4 °C for 8 h. After washing twice with PBS, we added CRP in the reaction buffer (10 mmol/L HEPES, 150 mmol/L NaCl, 2 mmol/L $CaCl_2$, 1% BSA, pH 7.0) to each well and incubated them at 4 °C overnight. We detected the binding of CRP with a TMB Peroxidase EIA Substrate kit (Bio-Rad) as described for LOX-1 *(10)*. We obtained small interfering RNA (siRNA) duplex oligoribonucleotides targeting the SR-A coding region (GenBank NM002445) from Invitrogen and used stealth RNAi duplex (Invitrogen) as a negative control. The siRNA sequences were as follows: 5′-GAUAUAACUCAAAGUCUCACGGGAA-3′, 5′-UUCCCGUGAGACUUUGAGUUAUAUC-3′ and 5′-CAGACCUUGAGAAAUAUCACUUUAA-3′, 5′-UUAAAGUGAUAUUUCUCAAGGUCUG-3′.

THP-1 cells were maintained with 10% FBS/1% AbAm/20 μmol/L mercaptoethanol:RPMI 1640 and differentiated with 100 nmol/L phorbol 12-myristate 13-acetate (PMA) (Sigma) for 48 h. We transfected the cells with siRNA oligos or control siRNA using Lipofectamin 2000 transfection reagent (Invitrogen) according to the manufacturer's instruction. After incubation at 37 °C for 24 h, we washed the cells with RPMI 1640/1% AbAm and replaced the medium with CypHer5E-CRP–containing RPMI 1640/1% AbAm, and the cells were incubated for 2 h. After washing with PBS, the cells were fixed with phosphate-buffered formalin (Wako) and permeabilized with 0.1% Triton X-100/PBS. We detected the effects of downregulation of SR-A gene expression by immunostaining with anti–SR-A antibody (SRA-C6; Trans Genic Inc) combined with Alexa 488 antimouse IgG. For detection of Fcγ receptors, we used anti-CD32 antibody (AT10; Santa Cruz) and anti-CD64 antibody (10.1; Santa Cruz). For CRP detection, we used anti-CRP antibody (Bethyl). The nuclei of the cells were counterstained with 0.5 mg/L DAPI. We divided the fluorescence intensities of CypHer5E and Alexa 488 by the cell number in a field. Quantitative analysis was performed with an IN Cell Analyzer 1000 system. All transfections were performed in triplicate.

All data are presented as mean (SE). Statistical analysis was performed with Student *t*-test. A P value <0.05 was considered statistically significant.

We examined whether CRP binds to scavenger receptors: LOX-1, SR-A, CD36, SR-BI, CD68, and dectin-1. Dectin-1 has the closest structural similarity to LOX-1 and belongs to C-type lectin-like molecule, although it is not a member of scavenger receptors.

Alexa546-labeled CRP at the concentration of 1 mg/L at 4 °C bound significantly to LOX-1–expressing cells ($P < 0.01$) but bound poorly to the cells expressing the other receptors (Supplemental Fig. 1, which accompanies the online version of this article at www.clinchem.org/content/vol56/issue3). Cellular uptake of CypHer5E-labeled CRP, which shows fluorescence after endocytosis, was significantly higher in SR-A–expressing cells, in a dose-dependent manner (1–30 mg/L), as well as in LOX-1–expressing cells, compared with cells expressing the other receptors (Fig. 1). Immunostaining with anti-V5 antibody revealed that all the receptors were expressed at a similar level in the respective cells.

Using anti-CRP antibody, we confirmed that nonlabeled CRP was also taken up by SR-A–expressing COS-7 cells. We further observed a significant binding of nonlabeled CRP (0.1–1 mg/L) to immobilized recombinant SR-A ($P < 0.01$) (online Supplemental Fig. 2). The binding was not affected by polymyxin B (5 mg/L), suggesting that it did not depend on the presence of endotoxin. Importantly, native CRP contained in human serum showed significant binding to SR-A, as well as to LOX-1 ($P < 0.01$) (online Supplemental Fig. 3). The binding was dependent on the concentration of CRP in the serum, suggesting that SR-A and LOX-1 have a capacity to bind to a native form of CRP in serum in the presence of other plasma proteins. These results indicate that SR-A and LOX-1 are the receptors for CRP among the examined receptors.

Because SR-A works in the monocyte-macrophage system, we assessed whether CRP is taken up by macrophages via SR-A. We used a human monocytic cell line,

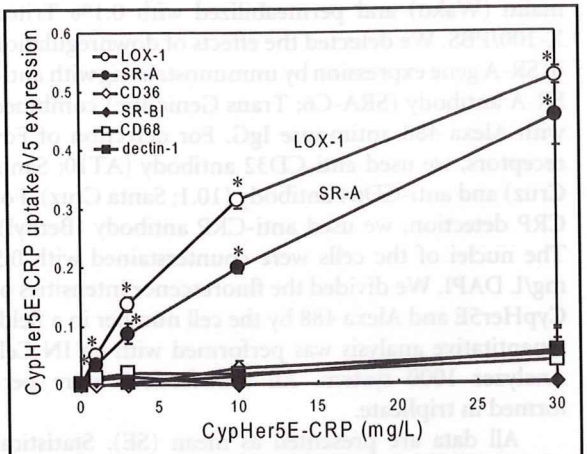


Fig. 1. Quantitative analyses of CypHer5E-CRP taken up by COS-7 cells expressing LOX-1, SR-A, CD36, SR-BI, and dectin-1.

Signals observed in the cells transfected with pcDNA3.1/V5-His/lacZ were considered as non-specific background. *Significant difference vs. negative control ($P < 0.01$).

THP-1, after inducing differentiation into macrophage by the treatment of PMA *(13)*. In PMA-treated THP-1 cells, CypHer5E-CRP was taken up in a dose-dependent manner (0.3–30 mg/L). SR-A expression and CRP uptake were concomitantly suppressed by 2 different siRNAs targeting SR-A, but not by control siRNA (Table 1). The siRNA targeting SR-A did not affect the expression of Fcγ receptors (data not shown), indicating that CRP is taken up mainly via SR-A in a macrophage cell line, at least under these conditions.

The ligand specificity of the scavenger receptor family overlaps considerably *(11, 12)*, and while all can bind to oxidized LDL, only SR-A or LOX-1 bound to CRP. Interestingly, dectin-1, the most structurally similar molecule to LOX-1, did not bind to CRP.

Using a monoclonal antibody, a previous report suggested the presence of an unknown receptor other than Fcγ receptors in macrophages *(14)*. It has been reported that fucoidin, a ligand for SR-A, inhibits the in vivo CRP-promoted uptake of oxidized LDL *(15)*. SR-A might be the unidentified CRP receptor. Fcγ receptors and SR-A are under different regulation of gene expression. In fact, in response to differentiation stimulus of PMA, the expression of SR-A is strongly induced, whereas the expression of Fcγ receptors is suppressed *(16, 17)*. Conversely, stimulation by interferon-γ enhances the expression of Fcγ receptors but suppresses the expression of SR-A *(18)*. These results suggest that Fcγ receptors and SR-A would work in the cells stimulated by different molecules.

Table 1. Suppression of the uptake of fluorescently labeled CRP by siRNA against SR-A in differentiated THP-1 cells.[a]

siRNA	SR-A expression, %	CypHer5E-CRP, %
None	103 (3.2)	108 (9.1)
Control siRNA	100 (2.7)	100 (9.8)
siRNA1 for SR-A	17 (1.6)[b]	31 (3.9)[b]
siRNA2 for SR-A	13 (0.0)[b]	32 (1.1)[b]

[a] Data are as mean (SE).
[b] Significant difference vs control siRNA groups ($P < 0.01$).

Interestingly, the activity of SR-A as CRP receptor was more pronounced in the uptake of CRP, whereas LOX-1 showed strong activity in both binding and uptake. Because SR-A works in phagocytes, the CRP uptake activity of SR-A is reasonable. CRP was originally identified as a binding protein for bacterial component C-polysaccharide *(3)*. SR-A may function to engulf bacteria, viruses, and harmful substances opsonized by CRP in a context of innate immunity.

Related to epidemiological risk factors for cardiovascular disease, the presence of CRP in atheroma has been reported in both rabbits and humans *(6)*. Furthermore, the colocalization of CRP and SR-A in macrophages in atheromas has been reported *(19)*. Taking these reports together with the present results, SR-A-mediated CRP uptake by macrophages in atheromas might affect the foam cell formation and progression of atherosclerotic disease.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures of Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Ministry of Health, Labour and Welfare of Japan; the National Institute of Biomedical Innovation; and Japan Science and Technology Agency.
Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

References

1. Black S, Kushner I, Samols D. C-reactive protein. J Biol Chem 2004;279:48487–90.
2. Volanakis JE, Kaplan MH. Interaction of C-reactive protein complexes with the complement system. II. Consumption of guinea pig complement by CRP complexes: requirement for human C1q. J Immunol 1974;113:9–17.
3. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. N Engl J Med 1999;340:448–54.
4. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. N Engl J Med 1997;336:973–9.
5. Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM Jr, Kastelein JJ, et al. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. N Engl J Med 2008; 359:2195–207.
6. Sun H, Koike T, Ichikawa T, Hatakeyama K, Shiomi M, Zhang B, et al. C-reactive protein in atherosclerotic lesions: its origin and pathophysiological significance. Am J Pathol 2005;167: 1139–48.
7. Khreiss T, Jozsef L, Hossain S, Chan JS, Potempa LA, Filep JG. Loss of pentameric symmetry of C-reactive protein is associated with delayed apoptosis of human neutrophils. J Biol Chem 2002; 277:40775–81.
8. Bharadwaj D, Stein MP, Volzer M, Mold C, Du Clos TW. The major receptor for C-reactive protein on leukocytes is Fcgamma receptor II. J Exp Med 1999;190:585–90.
9. Stein MP, Mold C, Du Clos TW. C-reactive protein binding to murine leukocytes requires Fc gamma receptors. J Immunol 2000;164:1514–20.
10. Fujita Y, Kakino A, Nishimichi N, Yamaguchi S, Sato Y, Machida S, et al. Oxidized LDL receptor LOX-1 binds to C-reactive protein and mediates its vascular effects. Clin Chem 2009;55:285–94.
11. Moore KJ, Freeman MW. Scavenger receptors in atherosclerosis: beyond lipid uptake. Arterioscler Thromb Vasc Biol 2006;26:1702–11.
12. Greaves DR, Gordon S. The immune system and atherogenesis: recent insights into the biology of macrophage scavenger receptors. Thematic review series. J Lipid Res 2005;46:11–20.
13. Nishimura N, Harada-Shiba M, Tajima S, Sugano R, Yamamura T, Qiang QZ, Yamamoto A. Acquisition of secretion of transforming growth factor-beta 1 leads to autonomous suppression of scavenger receptor activity in a monocyte-macrophage cell line, THP-1. J Biol Chem 1998; 273:1562–7.
14. Tebo JM, Mortensen RF. Characterization and isolation of a C-reactive protein receptor from the human monocytic cell line U-937. J Immunol 1990;144:231–8.
15. Singh U, Dasu MR, Yancey PG, Afify A, Devaraj S, Jialal I. Human C-reactive protein promotes oxidized low density lipoprotein uptake and matrix metalloproteinase-9 release in Wistar rats. J Lipid Res 2008;49:1015–23.
16. Fleit HB, Kobasiuk CD. The human monocyte-like cell line THP-1 expresses Fc gamma RI and Fc gamma RII. J Leukoc Biol 1991;49:556–65.
17. Morganelli PM, Kennedy SM, Mitchell TI. Differential effects of interferon-gamma on metabolism of lipoprotein immune complexes mediated by specific human macrophage Fcgamma receptors. J Lipid Res 2000;41:405–15.
18. Geng YJ, Hansson GK. Interferon-gamma inhibits scavenger receptor expression and foam cell formation in human monocyte-derived macrophages. J Clin Invest 1992;89:1322–30.
19. Turk JR, Carroll JA, Laughlin MH, Thomas TR, Casati J, Bowles DK, Sturek M. C-reactive protein correlates with macrophage accumulation in coronary arteries of hypercholesterolemic pigs. J Appl Physiol 2003;95:1301–4.

Previously published online at
DOI: 10.1373/clinchem.2009.140202

LOX-1 mediates vascular lipid retention under hypertensive state

Atushi Nakano[a], Nobutaka Inoue[a], Yuko Sato[a], Norihisa Nishimichi[b], Kenji Takikawa[b], Yoshiko Fujita[a], Akemi Kakino[a], Kazunori Otsui[a], Saburo Yamaguchi[a], Haruo Matsuda[b] and Tatsuya Sawamura[a]

Objectives **Hypertension is a powerful independent risk factor for atherosclerotic cardiovascular diseases; however, the precise molecular mechanisms whereby hypertension promotes atherosclerotic formation remain to be determined. The interaction between oxidized low-density lipoprotein (oxLDL) and its receptor lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) plays a critical role in atherogenesis. To clarify how hypertension promotes atherosclerosis, we investigated specific roles of LOX-1 in acceleration of lipid deposition under a hypertensive state.**

Methods **We employed a model of stroke-prone spontaneously hypertensive rats (SHR-SP) that exhibits acute lipid deposition in mesenteric artery induced by high fat and salt loading. These vascular lipid deposition lesions share similar characteristics with the initial lesions of human atherosclerosis.**

Results **The enhanced LOX-1 expression in SHR-SP was associated with oxidized LDL deposited in vascular wall. Anti-LOX-1 neutralizing antibody dramatically suppressed the lipid deposition *in vivo* in SHR-SP. Vitamin E decreased serum oxLDL-like LOX-1 ligands, and suppressed the vascular lipid deposition. The vascular permeability, evaluated by the leakage of Evans blue, was markedly enhanced by pretreatment of oxLDL. The enhancement of vascular permeability induced by oxLDL was suppressed by anti-LOX-1 antibody.**

Conclusion **The enhanced expression and activation of LOX-1 mediated the enhancement of vascular permeability, which contributed to the vascular lipid accumulation under hypertensive states. *J Hypertens* 28:000–000 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

Journal of Hypertension 2010, 28:000–000

Keywords: LOX-1, oxidized low-density lipoproteins, SHR-SP

Abbreviations: ApoB, Apo-lipoprotein B; DiI, 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate; DiI-oxLDL, DiI-labeled oxLDL; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HDL, high-density lipoprotein; KRS, Krebs-Ringer solution; LDL, low-density lipoprotein; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; MBP, mean blood pressure; MFI, mean fluorescence intensity; oxLDL, oxidized low-density lipoprotein; PCR, polymerase chain reaction; SHR, spontaneously hypertensive rats; SHR-SP, stroke-prone spontaneously hypertensive rats; SMC, smooth muscle cells; TC, total cholesterol; Vit-E, Vitamin E; WKY, Wistar–Kyoto rats

[a]Department of Vascular Physiology, National Cardiovascular Center Research Institute, Fujishirodai, Suita, Osaka, Japan and [b]Laboratory of Immunobiology, Department of Molecular and Applied Biosciences, Graduate School of Biosphere Science, Hiroshima University, Hiroshima, Japan

Correspondence to Tatsuya Sawamura, MD, PhD, Director, Department of Vascular Physiology, National Cardiovascular Center Research Institute, 5-7-1, Fujishirodai, Suita, Osaka 565-8565, Japan
Tel: +81 6 6833 5012 x2518; fax: +81 6 6835 5329;
e-mail: t-sawamura@umin.ac.jp

Received 30 July 2009 **Revised** 28 December 2009
Accepted 1 February 2010

Introduction

An elevation of either systolic or diastolic pressure is a powerful independent risk factor for atherosclerotic cardiovascular diseases [1]. Yamori *et al.* [2] presented important findings, soon after the development of spontaneously hypertensive rats (SHR). Namely, SHR rapidly developed arterial lipid deposition in mesenteric artery and the basilar artery as well as a greater hypercholesterolemic response within two weeks when fed a high-fat diet and 1% salt loading in drinking water [2,3]. This rapid accumulation of lipids, which is referred to as acute atherosis, shares similar characteristics with fatty streak of the initial atherosclerotic lesion. This experimental model, therefore, is useful and appropriate for analyzing pharmacological effects of various substances on the process of atherosclerosis.

Since the proposal of 'the response to endothelial injury/dysfunction hypothesis' [4], the role of endothelial cells in the pathogenic mechanisms of atherosclerosis have been well recognized. On the contrary, 'the response-to-retention model' in early atherosclerosis [5,6] proposed that the retention of lipoprotein in the vessel wall is essential for atherogenesis. Indeed, it is reported that the above-mentioned fatty streak lesion can be found even in young children and infants. The lipid retention, so-called acute atherosis, is also well documented in the maternal vasculature of women with preeclampsia [7,8].

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) was identified as the receptor for oxidized low-density lipoprotein (oxLDL) on endothelial cells [9]. Since this discovery, accumulating evidence has

0263-6352 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI:10.1097/HJH.0b013e32833835d4

suggested that the atherogenic properties of oxLDL affecting endothelial cells are mediated mainly via LOX-1. OxLDL induces endothelial dysfunction/apoptosis, a major change in vascular biology observed at the beginning of atherogenesis through LOX-1-mediated pathway [10]. Furthermore, the binding of ligands to LOX-1 induces the activation of NADPH oxidase [11–13], which leads to oxidative stress in the vessel wall. LOX-1 is dynamically up-regulated by various atherogenic substances such as advanced glycated end products, angiotensin II, and oxLDL itself [14–16]. Furthermore, the expression of LOX-1 is enhanced under various proatherogenic conditions, such as diabetes, hypertension [17], and dyslipidemia [18–20]. Reflecting the proatherogenic properties of LOX-1, disruption of LOX-1 gene in mice actually preserved endothelial function and reduced atherogenesis under hyperlipidemia. Recently, Sankaralingam *et al.* [21] reported the enhanced expression of LOX-1 in association with the condition of preeclampsia.

In the present study, we sought to establish the role of LOX-1 in the arterial wall lipid deposition under hypertension, utilizing SHR-SP. Here, we present the experimental results, which link endothelial dysfunction and vascular lipid retention model via LOX-1.

Materials and methods

Animals

All protocols were approved by the Institutional Animal Care and Use Committee of the National Cardiovascular Center. Eight-week-old male SHR-SP and Wistar–Kyoto rats (WKY) (Japan SLC, Hamamatsu, Japan) were fed with high-fat chow (1.25% cholesterol, 0.5% cholate, 20% milk casein, 15% cocoa butter; α-tocopherol: 2 mg/kg chow) and physiological saline (9 g/l NaCl) instead of drinking water. To evaluate the involvement of LOX-1-mediated pathway, anti-LOX-1 antibody (TS20; 10 mg/kg body weight) or nonimmune mouse IgG (I5381; 10 mg/kg body weight, Sigma-Aldrich, St. Louis, Missouri, USA) was administered via a tail vein two times, just before and on the fourth day of a week's loading. TS20 is the antibody formerly named JTX20 [22]. To some animals indicated in the results, the high-fat diet supplemented with α-tocopherol (50 mg/kg chow) was fed. Body weight and blood pressure were measured before loading and every other day during the examination. Blood pressure was determined by a non-invasive tail cuff and pulse transducer system (BP-98A; Softron, Tokyo, Japan).

After the loading period, the rats were euthanized with inhalation of diethyl ether. The abdominal cavity was immediately opened, and a systemic venous blood was sampled from inferior vena cava. Then, the rat was perfused systemically with physiological saline solution. The mesentery was excised out with intestine. Then, the enteric canal and mesenteric fat and vein were removed to isolate mesenteric artery. Serum levels of total cholesterol (TC), triglyceride, phospholipids, and high-density lipoprotein (HDL) were determined using commercially available reagent kits (Wako, Tokyo, Japan).

Quantitative reverse transcription-polymerase chain reaction

Expression of LOX-1 mRNA in rat mesenteric artery was evaluated by quantitative reverse transcription–polymerase chain reaction (QRT-PCR). Complementary DNA was synthesized with total RNA (1 μg/ml per sample) and random hexamers using SuperScript III RNase H-reverse transcriptase (Invitrogen, Carlsbad, California, USA). Aliquots from each cDNA solution were subjected to QRT-PCR using a primer pair and a quencher specific to rat LOX-1 sequence (Rn00591116) using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, California, USA). A GAPDH fragment was amplified as an internal control. Data are expressed as the ratio of LOX-1 to GAPDH mRNA.

Whole-mount immunohistochemistry

The segments of mesenteric artery were fixed with 2% formalin, and incubated consecutively with 1 mg/ml of dispase/PBS, 2% formalin/0.2% Triton X-100/PBS, methanol at −20°C, and 0.1% Triton X-100 in Tris-buffered saline. After blocking, the tissues were stained with Cy3-labeled antibovine LOX-1 antibody (TS20, 5 μg/ml) or Cy3-labeled mouse IgG as a negative control. The tissues were dehydrated in graded alcohols, and cleared in xylene. A whole-mount immunohistochemical image was obtained by a confocal laser microscope (Fluoview; Olympus, Tokyo Japan).

Oil Red O staining of accumulated lipid in mesenteric arteries

Oil Red O staining was used to detect lipid deposition in mesenteric arteries. The isolated mesenteric artery was fixed with 10% formalin, and washed with PBS. After flushing with 60% isopropanol for 5 min at room temperature, the blood vessel was incubated for 15 min at 55°C with 0.6% Oil Red O (Merck KGaA, Darmstadt, Germany) in 60% isopropanol. Then, the vessel was serially rinsed with 60 and 30% isopropanol, and PBS. The number of lipid deposits was manually counted under the stereomicroscope (Stemi 2000; Carl Zeiss, Göttingen, Germany).

Immunohistochemistry

Freshly frozen cross-sections of mesenteric artery were fixed with 10% formalin. The sections were incubated with 25% Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) and 5% donkey serum at 4°C overnight. The sections were incubated with anti-LOX-1 antibody (1 μg/ml, TS20), rabbit anti-oxLDL antiserum (1/100, AB3232; Chemicon, Temecula, California, USA), anti-smooth muscle actin (1 μg/ml, 1A4; DAKO, Carpinteria,

California, USA) or anti-rat macrophage (1 μg/ml, RM-4; TransGenic, Kumamoto, Japan) at 4°C overnight. The sections were then incubated with Biotinylated Link (DAKO), and visualized with Alexa 633 or 546 streptavidin (Invitrogen), or with streptavidin-horseradish peroxidase in combination with diaminobenzidine (DAB).

Measurement of serum LOX-1 ligands

Serum concentration of LOX-1 ligands was determined by sandwich enzyme-linked immunosorbent assay, as described previously [23].

In vivo DiI-oxLDL uptake analysis

Oxidized LDL and DiI-oxLDL were prepared as described previously [9]. One hour after the treatment with anti-LOX-1 antibody (TS20; 10 mg/kg body weight) or mouse IgG (10 mg/kg body weight), SHR-SP were administrated with DiI-oxLDL (10 mg/kg body weight) via tail vein. After 1 h, the whole mesenteric artery was isolated, and fluorescence of the deposited DiI-oxLDL was observed with a macro fluorescence microscope (MVX10; Olympus) equipped with a cooled CCD camera (ORCA-1394-ER; Hamamatsu Photonics, Hamamatsu, Japan). The distribution of DiI-oxLDL was quantitatively estimated by integration of fluorescence of DiI in the vessels. The data are expressed as mean fluorescence intensity (MFI) per unit area of mesenteric artery in the photograph.

Ex-vivo perfusion experiment of isolated mesenteric artery

Segments of mesenteric artery with 100–150 μm internal diameter were isolated in a length of 1–1.5 cm each. The arterial segments were cannulated at both ends and the side branches were ligated. They were then perfused with Krebs-Ringer solution (KRS) (NaCl 155 mmol/l, KCl 3 mmol/l, $CaCl_2$ 2 mmol/l, $MgCl_2$ 1 mmol/l, NaH_2PO_4 3 mmol/l, HEPES 5 mmol/l, glucose 10 mmol/l) containing 10 μg/ml TS20 or control IgG for 30 min, and with the same buffer with or without oxLDL (30 μg/ml) for 30 min. Then, the arterial segments were perfused with 1 μg/ml of DiI-oxLDL/KRS for 15 min, or with 0.1 μg/ml of Evans blue/KRS for 10 min. The vessels were washed with PBS for 5 min and fixed with 4% paraformaldehyde, and subjected to the microscopic analysis. The uptake of DiI-oxLDL or leakage of Evans blue of the arterial segments was semi-quantitatively estimated by integration of fluorescence of DiI or Evans blue in arterial segments. The data are expressed as MFI per unit area of arterial segment in the photograph.

Statistical analysis

All data are presented as mean ± SEM. Statistical analysis between two groups was performed by the Mann–Whitney's *U*-test. Multiple comparisons were done using analysis of variance (ANOVA) with Bonferroni post-hoc analysis. *P* value less than 0.05 was considered as significant.

Results

Enhanced expression of LOX-1 in SHR-SP

QRT-PCR demonstrated that LOX-1 mRNA expression in the mesenteric artery of SHR-SP was 3.5 times higher than that of WKY rats before high-fat diet and saline loading (Fig. 1a). Whole-mount immunostaining using Cy3-labeled anti-LOX-1 antibody further showed abundant LOX-1 expression in SHR-SP, whereas only negligible expression was observed in WKY rats (Fig. 1b).

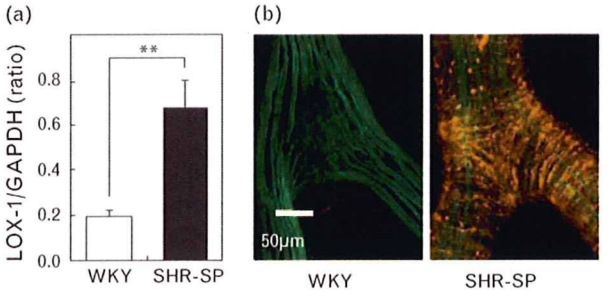
High-fat and salt diet-induced lipid deposition in SHR-SP

Loading with high-fat diet and saline dramatically induced lipid deposition in the mesenteric arteries of SHR-SP, whereas no deposition was observed in control WKY rats. Interestingly, the lipid deposition observed in mesenteric artery was regionally localized and dotted throughout the branches (Fig. 2a). High-fat diet and saline loading resulted in elevation of total serum cholesterol in both rat strains, whereas arterial blood pressure was increased in SHR-SP, but not in WKY. Lipid deposition was observed in SHR-SP in as early as 1 week of high-fat diet and saline loading, whereas WKY showed negligible lipid deposition (Fig. 2b). Thus, the mesenteric arteries of SHR-SP were highly susceptible to lipid deposition.

Relationship of LOX-1 expression with lipid accumulation

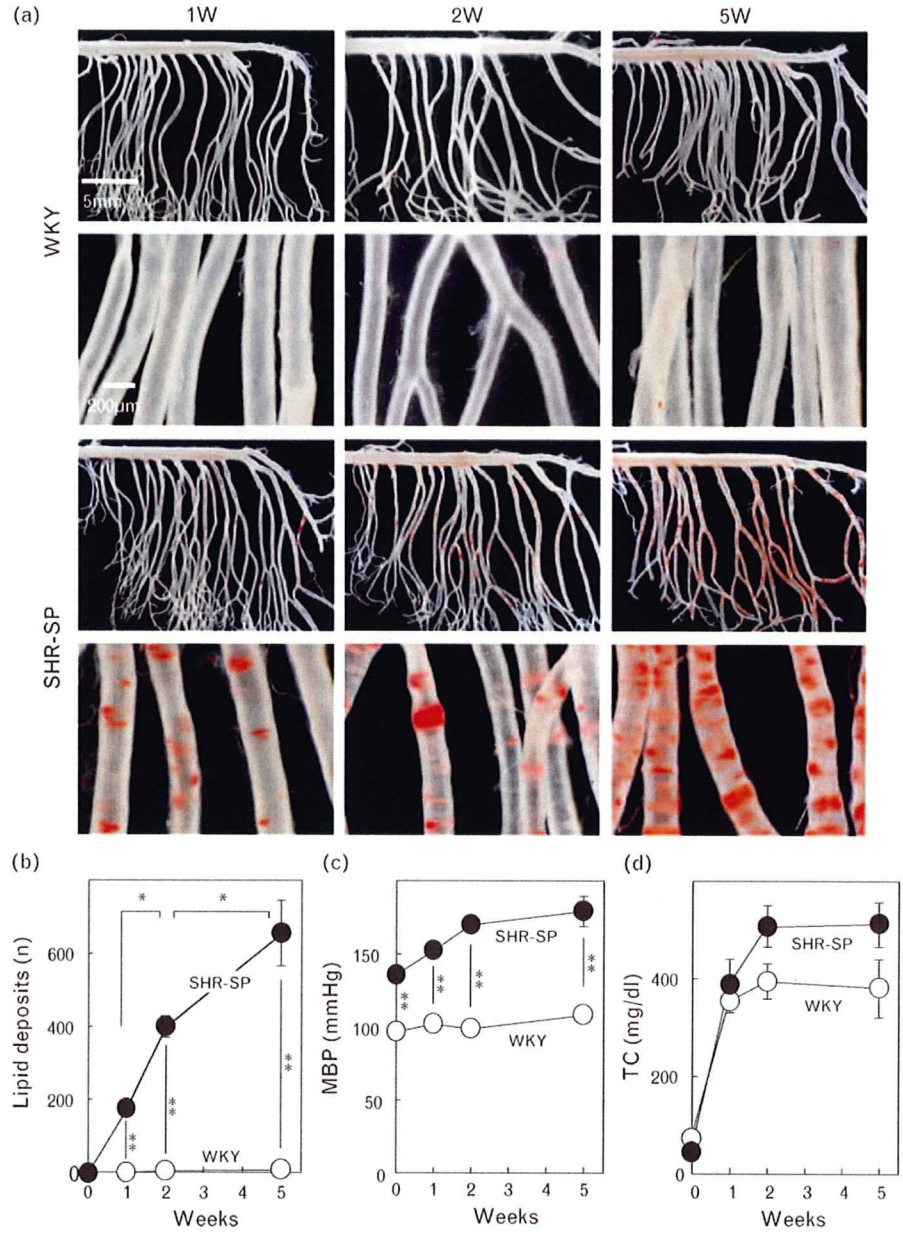
Next, we investigated spatial relationship between LOX-1 and oxLDL in the mesenteric arteries. OxLDL was accumulated in medial smooth muscle layer (Fig. 3a), and associated with LOX-1 expression (Fig. 3b). The expression of LOX-1 was observed in endothelium as well as smooth muscle cells (Fig. 3b-d). Immunoreactivity of macrophages was not observed in these lesions (Fig. 3e).

Fig. 1



Enhanced expression of LOX-1 in mesenteric arteries of SHR-SP. (a) Comparison of LOX-1 mRNA expression in mesenteric arteries between SHR-SP and WKY prior to high-fat diet and saline loading ($n = 11$, $^{**}P < 0.005$). (b) LOX-1 protein expression in mesenteric artery of SHR-SP and WKY rats detected by anti-LOX-1 antibody. Orange and green fluorescence represent the expression of LOX-1 and auto-fluorescence of internal elastic layer, respectively. LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; SHR-SP, stroke-prone spontaneously hypertensive rats; WKY, Wistar–Kyoto rats.

Fig. 2



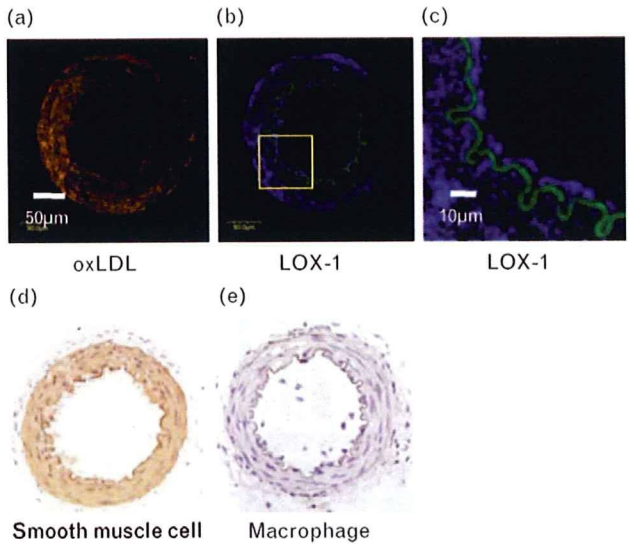
Lipid deposition in mesenteric artery of SHR-SP and WKY after high-fat (HF) diet and saline loading. (a) Oil Red O staining of lipid deposition in mesenteric artery of SHR-SP and WKY after 1, 2 and 5 weeks of HF diet and saline loading. Each upper panel shows the whole branches of mesenteric artery at low magnification and each lower panel shows arteries at high magnification. (b) Time-dependent increase in the number of vascular lipid deposits during HF diet and saline loading. (c) Comparison of mean blood pressure (MBP) between SHR-SP and WKY after HF diet and saline loading. Mean blood pressure of SHR-SP was significantly higher compared with WKY. (d) Comparison of serum cholesterol between SHR-SP and WKY after HF diet and saline loading. The levels of total serum cholesterol in both groups were increased by HF diet and saline loading ($n = 5$, $^{*}P < 0.05$, $^{**}P < 0.005$). SHR-SP, stroke-prone spontaneously hypertensive rats; TC, total cholesterol; WKY, Wistar–Kyoto rats.

Suppression of lipid accumulation by the inhibition of LOX-1

The aforementioned histological findings prompted us to investigate whether LOX-1 mediates the vascular lipid accumulation in SHR-SP. Lipid deposition in SHR-SP was significantly reduced by treatment with anti-LOX-1 antibody (Fig. 4a, b). Blood pressure was not significantly different between the two groups (Fig. 4c), although TC concentration notably increased in the group of anti-LOX-1 antibody treatment (Fig. 4d).

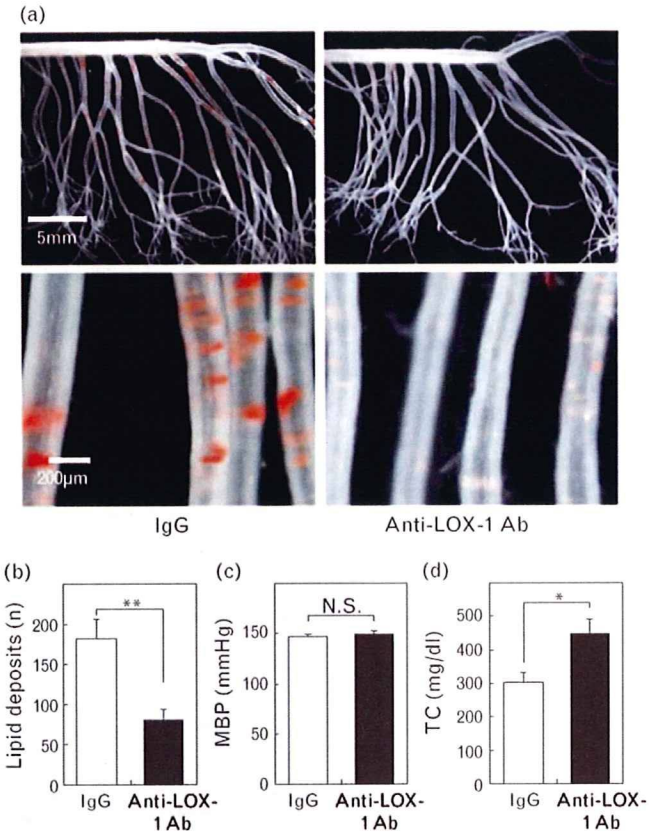
To clarify the significance of oxidative stress, the effects of vitamin E, a lipophilic antioxidant, were examined. As shown in Fig. 5a and b, vitamin E potently suppressed vascular lipid deposition. Concomitantly, the increase in serum levels of LOX-1 ligands induced by a high-fat diet

Fig. 3



Immunostaining analysis of the frozen section at a fat deposit in mesenteric artery of SHR-SP after one week of HF diet and saline loading. (a) Accumulation of oxLDL detected by anti-oxLDL antiserum. OxLDL accumulation was visualized with Alexa546-streptavidin (orange). (b) Expression of LOX-1 detected by anti-LOX-1 antibody. LOX-1, visualized with Alexa633-streptavidin (blue) was expressed in medial smooth muscle layer and intima. Green fluorescence indicates auto fluorescence of the internal elastic layer. (c) Image in high magnification of the yellow square area of (b). The expression of LOX-1 was observed in endothelial cells as well as smooth muscle layer. (d, e) Immunochemical staining of smooth muscle (d) and macrophages (e) of serial cryosection visualized with DAB (brown). Nuclei were counter-stained with Mayers hematoxyline. DAB, diaminobenzidine; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; oxLDL, oxidized low-density lipoprotein; SHR-SP, stroke-prone spontaneously hypertensive rats.

Fig. 4



Effects of anti-LOX-1 antibody on vascular lipid deposition induced by high-fat diet and saline loading. (a) Suppression of vascular lipid deposition by anti-LOX-1 antibody. (b) Effects of anti-LOX-1 antibody on the number of lipid deposits. (c) Effects of anti-LOX-1 antibody on arterial blood pressure. (d) Effects of anti-LOX-1 antibody on serum cholesterol concentration ($n = 11$, $^{*}P < 0.05$, $^{**}P < 0.005$). LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; MBP, mean blood pressure; TC, total cholesterol.

was also significantly reduced by vitamin E (Fig. 5e). Taken together, functional blocking of LOX-1 or reduction in oxidative stress efficiently suppressed the arterial lipid deposition. It is noteworthy that the levels of arterial blood pressure were not significantly reduced by the treatment of anti-LOX-1 antibody, or vitamin E.

Enhanced uptake of oxLDL in mesenteric artery of SHR-SP

The involvement of LOX-1 in lipid accumulation was further examined by analyzing the distribution of DiI-oxLDL. DiI-oxLDL administered intravenously to SHR-SP was regionally taken up in mesenteric artery (Fig. 6a, b). This acute distribution of DiI-oxLDL was suppressed by pretreatment with anti-LOX-1 antibody (Fig. 6a, b). These findings indicate that the oxLDL distributed via LOX-1-mediated pathway possibly contributes to localized lipid deposition in the vessel wall.

To further examine the accumulation of oxLDL via LOX-1-mediated pathway, ex-vivo perfusion experiment was also performed. Perfusion of isolated SHR-SP mesenteric artery with DiI-oxLDL induced accumulation of DiI-oxLDL in the vessel wall (Fig. 6c, d, control). Pretreatment with oxLDL increased the accumulation of DiI-oxLDL, which was suppressed with anti-LOX-1 antibody (Fig. 6c, d, oxLDL).

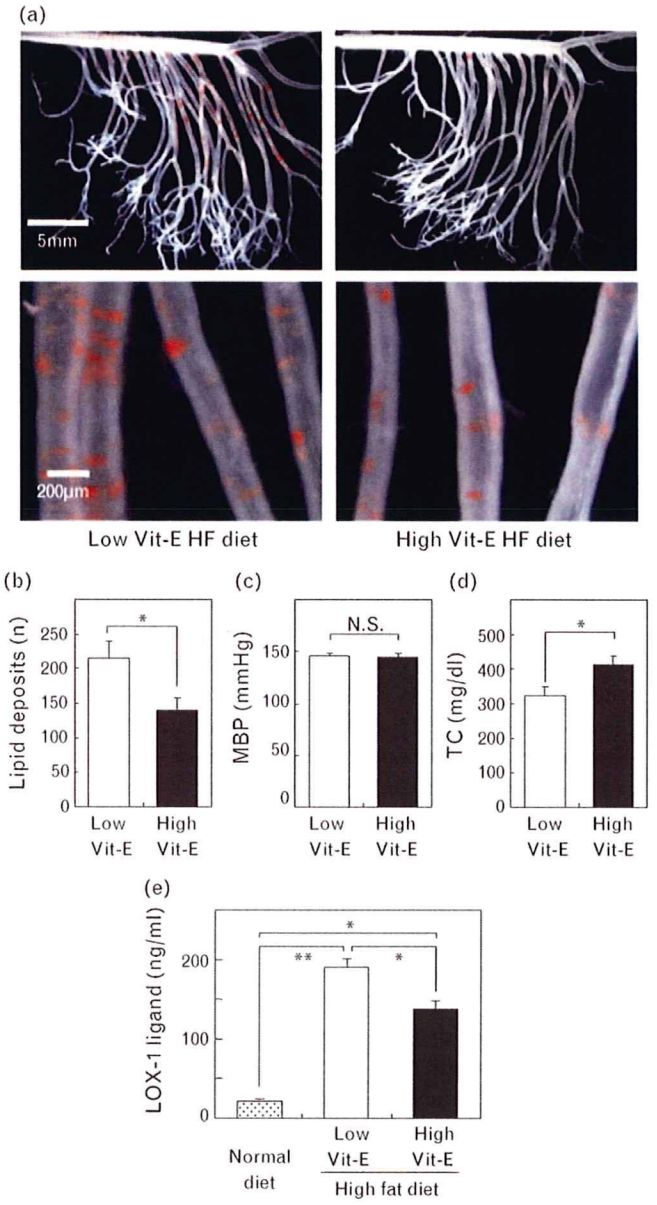
Enhancement of vascular permeability via LOX-1-mediated pathway

Next, the regulation of vascular permeability via LOX-1 pathway was evaluated by exudation of Evans blue in mesenteric artery. *Ex vivo* perfusion experiment demonstrated that the pretreatment with oxLDL increased exudation of Evans blue from luminal surface of the isolated mesenteric arteries of SHR-SP, which was suppressed by anti-LOX-1 antibody (Fig. 7a, b).

Discussion

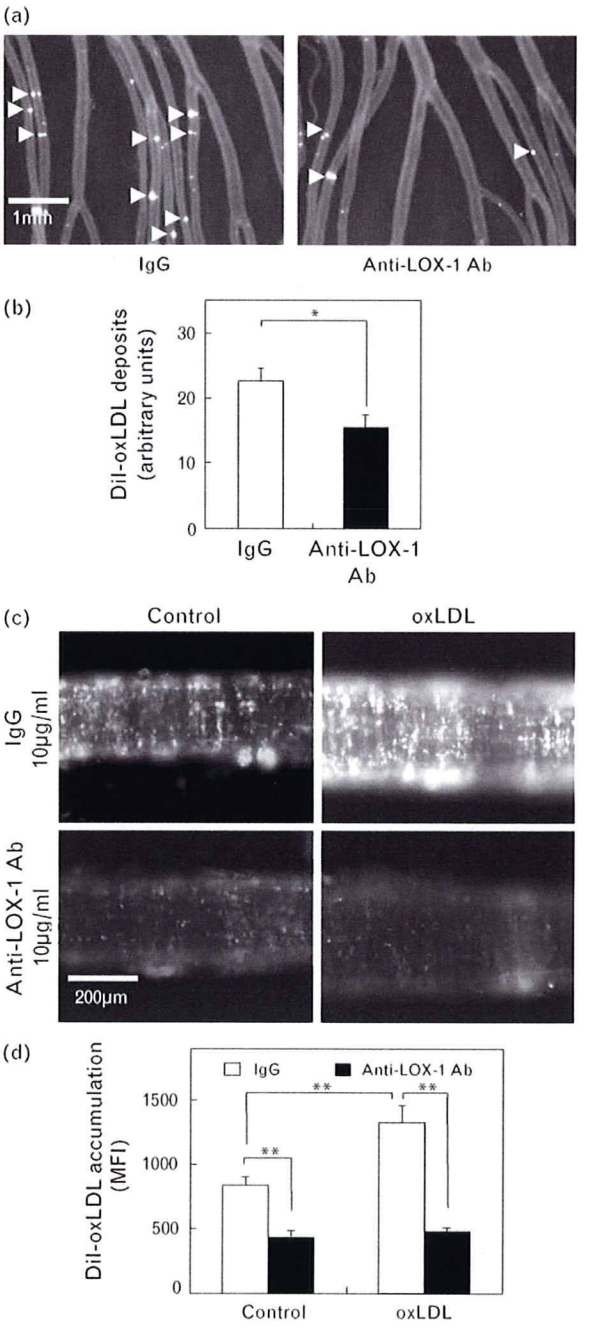
In the hypertensive state, mechanical stress induced by hemodynamic forces such as shear stress and stretch force is one of the most important factors contributing to

Fig. 5



Effects of vitamin E on vascular lipid accumulation. (a) Suppressive effects of high dose of vitamin E on lipid deposition induced by high-fat diet and salt loading. (b) Quantitative analysis of vitamin E effects on vascular lipid deposition induced by high-fat diet and salt loading. (c) Effects of vitamin E on arterial blood pressure. Vitamin E exerted no influence on mean blood pressure. (d) Effects of vitamin E on serum cholesterol levels. Administration of high dose of vitamin E increased total serum cholesterol levels. (e) Changes in LOX-1 ligand by high-fat diet containing low or high dose of vitamin E ($n=6$, $^{*}P<0.05$, $^{**}P<0.005$). LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; MBP, mean blood pressure; TC, total cholesterol.

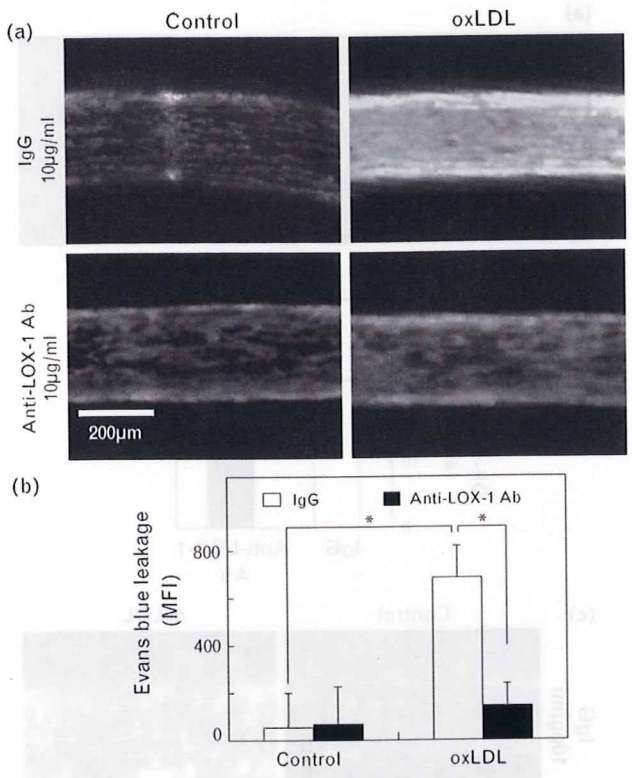
Fig. 6



Distribution of Dil-oxLDL to mesenteric arteries *in vivo*. (a) Suppressive effects of anti-LOX-1 antibody on Dil-oxLDL accumulation (arrowhead) in mesenteric artery *in vivo*. (b) Quantitative analysis of anti-LOX-1 antibody effect on the number of Dil-oxLDL accumulation foci *in vivo* ($n=6$, $^{*}P<0.05$). (c) Accumulation of Dil-oxLDL in isolated mesenteric artery from SHR-SP. The vessels were pretreated with (right) or without oxLDL (left) in the presence of anti-LOX-1 antibody (lower) or IgG (upper). (d) Quantitative analysis of Dil-oxLDL accumulation in mesenteric artery in (c) ($n=8$, $^{**}P<0.005$). LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; oxLDL, oxidized low-density lipoprotein; SHR-SP, stroke-prone spontaneously hypertensive rats.

endothelial dysfunction/injury. Derangement of humoral factors, caused by enhanced activity of renin-angiotensin and sympathetic nervous systems, and increase in oxidative stress further make the pathophysiology of hypertension more complicated. Combination of high-fat diet

Fig. 7



Enhancement of permeability in mesenteric artery via oxLDL-LOX-1 pathway. (a) Effects of oxLDL on permeability of isolated mesenteric artery observed by Evans blue leakage. The vessels were pretreated with anti-LOX-1 antibody (lower) or IgG (upper). (b) Quantitative analysis of the vascular permeability of mesenteric artery in (a) ($n = 6$, $^*P < 0.05$). LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; oxLDL, oxidized low-density lipoprotein.

with salt loading to SHR-SP is a suitable and appropriate experimental model for the investigation of cellular and molecular mechanisms of the earliest process of atherosclerosis under hypertension and hyperlipidemia. Employing this model here, we demonstrated that LOX-1 mediates vascular lipid retention and augmentation of vascular permeability. high-fat diet induced characteristic localized lipid accumulation in mesenteric arteries of SHR-SP. This vascular lipid accumulation was associated with the augmented expression of LOX-1 and suppressed by the treatment of anti-LOX-1 antibody. These findings indicate that LOX-1 enhances oxLDL retention to the vessel wall. Conversely, oxLDL enhanced vascular permeability via LOX-1, leading to further lipid retention. Thus, LOX-1 might play a critical role in acute atherosis under hypertension. This vicious cycle under enhanced expression of LOX-1 may explain the molecular mechanisms of the response-to-retention model of atherosclerosis.

Anti-LOX-1 antibody, when administered *in vivo*, neither reduced blood pressure nor serum cholesterol concentration. LOX-1 expression is enhanced in SHR-SP and salt-loaded Dahl salt-sensitive hypertension rats [17], and reduction of blood pressure by either a calcium channel blocker or hydralazine effectively suppresses the expression of LOX-1 [24]. These findings suggest that LOX-1 system might be in the downstream cascade of hypertension. Enhanced expression of LOX-1 under hypertensive state is further likely to promote lipid deposition if hyperlipidemia coexists with hypertension. Indeed, hypertension and hypercholesterolemia have synergistic deleterious effects on coronary endothelial function in association with augmented expression of LOX-1 [25].

In hyperlipidemic rabbits, ApoB-containing LOX-1 ligands, presumably regarded as oxLDL, are accumulated in the plasma and atherosclerotic lesions [26]. Reflecting the elevated plasma levels of LOX-1 ligands, the expression of LOX-1 in endothelium of hyperlipidemic rabbits is augmented. This observation is explained by the experimental results that oxLDL itself induces the expression of LOX-1 in endothelial cells [19]. In the present investigation, the levels of LOX-1 ligands including oxLDL were increased by high-fat diet and reduced by vitamin E. Vascular lipid accumulation was also suppressed by vitamin E. From these findings, we speculate that enhanced expression of LOX-1 in the vasculature associated with elevated levels of LOX-1 ligands in the blood stream might be an important driving mechanism for atherogenesis. Indeed, direct blockade against LOX-1 significantly prevented lipid accumulation in arterial wall, demonstrating that the above hypothesis actually worked at least in the present hypertension model.

It is of note that anti-LOX-1 antibody reduced the enhanced vascular permeability in SHR-SP. Ex-vivo permeability analysis using Evans blue demonstrated that oxLDL augments vascular permeability via LOX-1 mediated pathway. Yamori *et al.* have suggested that vascular permeability might be an instigating mechanism preceding lipid deposition in vascular smooth muscle cells of SHR-SP. Thus, anti-LOX-1 antibody exerts its effects on lipid deposition both by suppression of vascular permeability in endothelium and by blocking of the uptake of lipids in smooth muscle cells.

Limitation of the study

Acute atherosis observed in SHR-SP is not precisely equal to atherosclerosis; rather, it is similar to vascular changes in human preeclampsia. The accumulation of macrophages was not observed in the present model even in the later stage, but smooth muscle cells incorporate lipids instead to transform into smooth muscle-derived foam cells. The present study focuses on elucidation of the role of LOX-1 in the process of lipid retention, and the issue of macrophage accumulation needs to wait for further study.

Cellular lipid uptake and foam cell formation are mediated by various pathway other than LOX-1, such as CD-36 and scavenger receptor-A. Previous investigations reported that compared with Apo E knockout mice those lacking SR-A or CD36 showed marked reduction in atherosclerotic lesion area [27,28]. In the present study, we focused on the role of LOX-1, and did not evaluate the involvement of these other pathways.

In summary, we have clarified that LOX-1 enhances vascular permeability and retention of lipids including oxLDL under the hypertensive state. Since oxLDL up-regulates expression of LOX-1, the vicious cycle composed of LOX-1, vascular permeability, and lipid retention might occur in the initial step of atherogenesis. Hence, the oxLDL-LOX-1 pathway may work in both endothelial dysfunction and the responses-to-lipid retention models.

Acknowledgements

The work was supported in part by the grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Ministry of Health, Labour and Welfare of Japan; the National Institute of Biomedical Innovation; Japan Science and Technology Agency; and the New Energy and Industrial Technology Development Organization.

There are no conflicts of interest.

References

1 Franklin SS, Pio JR, Wong ND, Larson MG, Leip EP, Vasan RS, Levy D. Predictors of new-onset diastolic and systolic hypertension: the Framingham Heart Study. *Circulation* 2005; **111**:1121–1127.
2 Yamori Y, Hamashima Y, Horie R, Handa H, Sato M. Pathogenesis of acute arterial fat deposition in spontaneously hypertensive rats. *Jpn Circ J* 1975; **39**:601–609.
3 Yamori Y, Horie R, Sato M, Fukase M. Hypertension as an important factor for cerebrovascular atherogenesis in rats. *Stroke* 1976; **7**:120–125.
4 Ross R. Atherosclerosis: an inflammatory disease. *N Engl J Med* 1999; **340**:115–126.
5 Williams KJ, Tabas I. The response-to-retention hypothesis of early atherogenesis. *Arterioscler Thromb Vasc Biol* 1995; **15**:551–561.
6 Tabas I, Williams KJ, Boren J. Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. *Circulation* 2007; **116**:1832–1844.
7 De Wolf F, Brosens I, Robertson WB. Ultrastructure of uteroplacental arteries. *Contrib Gynecol Obstet* 1982; **9**:86–99.
8 Pijnenborg R, Vercruysse L, Hanssens M. The uterine spiral arteries in human pregnancy: facts and controversies. *Placenta* 2006; **27**:939–958.
9 Sawamura T, Kume N, Aoyama T, Moriwaki H, Hoshikawa H, Aiba Y, *et al.* An endothelial receptor for oxidized low-density lipoprotein. *Nature* 1997; **386**:73–77.
10 Chen J, Mehta JL, Haider N, Zhang X, Narula J, Li D. Role of caspases in Ox-LDL-induced apoptotic cascade in human coronary artery endothelial cells. *Circ Res* 2004; **94**:370–376.
11 Cominacini L, Pasini AF, Garbin U, Davoli A, Tosetti ML, Campagnola M, *et al.* Oxidized low density lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells induces the activation of NF-kappaB through an increased production of intracellular reactive oxygen species. *J Biol Chem* 2000; **275**:12633–12638.
12 Shin HK, Kim YK, Kim KY, Lee JH, Hong KW. Remnant lipoprotein particles induce apoptosis in endothelial cells by NAD(P)H oxidase-mediated production of superoxide and cytokines via lectin-like oxidized low-density lipoprotein receptor-1 activation: prevention by cilostazol. *Circulation* 2004; **109**:1022–1028.
13 Ma FX, Zhou B, Chen Z, Ren Q, Lu SH, Sawamura T, Han ZC. Oxidized low density lipoprotein impairs endothelial progenitor cells by regulation of endothelial nitric oxide synthase. *J Lipid Res* 2006; **47**:1227–1237.
14 Aoyama T, Fujiwara H, Masaki T, Sawamura T. Induction of lectin-like oxidized LDL receptor by oxidized LDL and lysophosphatidylcholine in cultured endothelial cells. *J Mol Cell Cardiol* 1999; **31**:2101–2114.
15 Li D, Saldeen T, Romeo F, Mehta JL. Oxidized LDL upregulates angiotensin II type 1 receptor expression in cultured human coronary artery endothelial cells: the potential role of transcription factor NF-kappaB. *Circulation* 2000; **102**:1970–1976.
16 Chen H, Li D, Sawamura T, Inoue K, Mehta JL. Upregulation of LOX-1 expression in aorta of hypercholesterolemic rabbits: modulation by losartan. *Biochem Biophys Res Commun* 2000; **276**:1100–1104.
17 Nagase M, Hirose S, Sawamura T, Masaki T, Fujita T. Enhanced expression of endothelial oxidized low-density lipoprotein receptor (LOX-1) in hypertensive rats. *Biochem Biophys Res Commun* 1997; **237**:496–498.
18 Chen J, Li D, Schaefer R, Mehta JL. Cross-talk between dyslipidemia and renin-angiotensin system and the role of LOX-1 and MAPK in atherogenesis studies with the combined use of rosuvastatin and candesartan. *Atherosclerosis* 2006; **184**:295–301.
19 Chen M, Kakutani M, Minami M, Kataoka H, Kume N, Narumiya S, *et al.* Increased expression of lectin-like oxidized low density lipoprotein receptor-1 in initial atherosclerotic lesions of Watanabe heritable hyperlipidemic rabbits. *Arterioscler Thromb Vasc Biol* 2000; **20**:1107–1115.
20 Kataoka H, Kume N, Miyamoto S, Minami M, Moriwaki H, Murase T, *et al.* Expression of lectin-like oxidized low-density lipoprotein receptor-1 in human atherosclerotic lesions. *Circulation* 1999; **99**:3110–3117.
21 Sankaralingam S, Xu Y, Sawamura T, Davidge ST. Increased lectin-like oxidized low-density lipoprotein receptor-1 expression in the maternal vasculature of women with preeclampsia: role for peroxynitrite. *Hypertension* 2009; **53**:270–277.
22 Kakutani M, Masaki T, Sawamura T. A platelet-endothelium interaction mediated by lectin-like oxidized low-density lipoprotein receptor-1. *Proc Natl Acad Sci U S A* 2000; **97**:360–364.
23 Sato Y, Nishimichi N, Nakano A, Takikawa K, Inoue N, Matsuda H, Sawamura T. Determination of LOX-1-ligand activity in mouse plasma with a chicken monoclonal antibody for ApoB. *Atherosclerosis* 2008; **200**:303–309.
24 Nagase M, Kaname S, Nagase T, Wang G, Ando K, Sawamura T, Fujita T. Expression of LOX-1, an oxidized low-density lipoprotein receptor, in experimental hypertensive glomerulosclerosis. *J Am Soc Nephrol* 2000; **11**:1826–1836.
25 Rodriguez-Porcel M, Lerman LO, Herrmann J, Sawamura T, Napoli C, Lerman A. Hypercholesterolemia and hypertension have synergistic deleterious effects on coronary endothelial function. *Arterioscler Thromb Vasc Biol* 2003; **23**:885–891.
26 Kakutani M, Ueda M, Naruko T, Masaki T, Sawamura T. Accumulation of LOX-1 ligand in plasma and atherosclerotic lesions of Watanabe heritable hyperlipidemic rabbits: identification by a novel enzyme immunoassay. *Biochem Biophys Res Commun* 2001; **282**:180–185.
27 Suzuki H, Kurihara Y, Takeya M, Kamada N, Kataoka M, Jishage K, *et al.* A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature* 1997; **386**:292–296.
28 Podrez EA, Febbraio M, Sheibani N, Schmitt D, Silverstein RL, Hajjar DP, *et al.* Macrophage scavenger receptor CD36 is the major receptor for LDL modified by monocyte-generated reactive nitrogen species. *J Clin Invest* 2000; **105**:1095–1108.

Advance Publication by J-STAGE

Circulation Journal
Official Journal of the Japanese Circulation Society
http://www.j-circ.or.jp

Left Ventricular Expression of Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1 in Failing Rat Hearts

Tomohide Takaya, MA*,†,‡‡; Hiromichi Wada, MD*,‡‡; Tatsuya Morimoto, MD*; Yoichi Sunagawa, MA*,††; Teruhisa Kawamura, MD*; Rieko Takanabe-Mori, MA*; Akira Shimatsu, MD**; Yoshiko Fujita, PhD‡; Yuko Sato, PhD‡; Masatoshi Fujita, MD††; Takeshi Kimura, MD†; Tatsuya Sawamura, MD‡; Koji Hasegawa, MD*

Background: Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is a multiple ligand receptor induced by oxidative stress. However, its role in chronic heart failure remains unknown.

Methods and Results: The left ventricular (LV) expression of LOX-1 was examined in a salt-sensitive Dahl rat model of hypertension. Compared with controls, LOX-1 mRNA levels increased by 4.7-fold in the LV with hypertrophy, and by 32-fold in the LV with decreased systolic function. LV LOX-1 mRNA levels strongly correlated with the decrease in LV ejection fraction (EF) (r=−0.772), and with increases in the LV mRNA levels of B-type natriuretic peptide (r=0.814), monocyte chemoattractant protein-1 (r=0.943), transforming growth factor-β_1 (r=0.936), and a macrophage marker, F4/80 (r=0.560). Serum levels of soluble LOX-1 were significantly elevated in patients with LV systolic dysfunction and hypertrophy, and significantly correlated with the decrease in EF (r=−0.495).

Conclusions: Marked increase in the LV expression of LOX-1 in failing hearts may contribute to increased serum levels, and might be involved in chronic inflammation during the development of heart failure.

Key Words: Heart failure; Hypertension; Inflammation; LOX-1; Receptors

The lectin-like oxidized low-density lipoprotein (oxLDL) receptor-1 (LOX-1) was originally identified as an endothelial receptor for oxLDL.[1] LOX-1 expression in vascular cells is relatively low in the normal state, but can be induced by various stimuli such as oxLDL,[2] tumor necrosis factor-α (TNF-α),[3] transforming growth factor-β_1 (TGF-β_1),[4] interleukin-1β (IL-1β),[5] angiotensin II,[6,7] and endothelin-1 (ET-1)[8] in vitro. LOX-1 upregulation is involved in oxLDL-induced apoptosis through the intracellular production of reactive oxygen species.[9,10] Endothelial expression of LOX-1 in vivo is increased in hypertension (HT),[11] diabetes mellitus (DM),[12] hyperlipidemia,[13] hypercholesterolemia,[14] and atherosclerosis.[15] OxLDL-induced LOX-1 regulates the expression of monocyte chemoattractant protein-1 (MCP-1), a cytokine that mediates macrophage infiltration,[16] and is considered to be involved in the pathogenesis of atherosclerosis at an early stage.[17] The membrane proximal extracellular domain of LOX-1 can be proteolytically cleaved and released as soluble forms.[18] Levels of soluble LOX-1 (sLOX-1) in sera are increased in acute coronary syndrome,[19] type 2 DM,[20] and obesity.[21]

LOX-1 expression in cultured cardiomyocytes is also very low in the basal state, and can be induced by norepinephrine and ET-1, neurohormonal factors that are activated in heart failure (HF).[22] The cardiac LOX-1 pathway is activated by oxidative stress in vitro and by ischemia–reperfusion injury in vivo.[23] Although the activation of LOX-1 induces apoptosis in cardiomyocytes, the administration of anti-LOX-1 antibody is able to suppress their apoptosis in vitro[22] and reduces the extent of myocardial infarction (MI) in vivo.[23] Left ventricular (LV) expression of LOX-1 is also increased in salt-sensitive Dahl (DS) rats with hypertensive HF compared with control normotensive salt-resistant Dahl (DR) rats.[24] The administration of eplerenone, an aldosterone blocker, reduces LOX-1 activation and recovers the cardiac function of DS rats.[24] In the present study, we examined the

Received July 2, 2009; revised manuscript received December 22, 2009; accepted December 24, 2009; released online February 27, 2010 Time for primary review: 48 days

*Division of Translational Research, **Clinical Research Institute, Kyoto Medical Center, National Hospital Organization, †Department of Cardiovascular Medicine, ††Department of Human Health Sciences, Graduate School of Medicine, Kyoto University, Kyoto and ‡Department of Vascular Physiology, National Cardiovascular Center Research Institute, Suita, Japan

‡‡The first two authors contributed equally to the work presented here.

Mailing address: Koji Hasegawa, MD, Division of Translational Research, Clinical Research Institute, Kyoto Medical Center, National Hospital Organization, 1-1 Mukaihata-cho, Fukakusa, Fushimi-ku, Kyoto 612-8555, Japan. E-mail: koj@kuhp.kyoto-u.ac.jp

ISSN-1346-9843 doi:10.1253/circj.CJ-09-0488

Table 1. Morphometric and Hemodynamic Parameters of Dahl Rats

	6 weeks		11 weeks		18 weeks	
	DR	DS	DR	DS	DR	DS
N	5	5	5	5	4	4
BW (g)	207±5	178±4*	374±13	327±8*,†	479±9	333±11*,†
LVW/BW	2.38±0.02	2.40±0.02	2.19±0.12	2.85±0.12*,†	1.84±0.06	3.16±0.16*,†
SBP (mmHg)	107±2	113±3	130±6	191±8*,†	126±3	213±10*,†
DBP (mmHg)	64±8	69±6	105±6	141±5*,†	96±2	149±7*,†
Heart rate (beats/min)	449±11	405±25	417±20	404±13	376±13	354±33
LVESD (mm)	3.21±0.13	2.80±0.31	4.15±0.25	3.15±0.26†	5.26±0.18	5.47±0.81†,‡
LVEDD (mm)	7.49±0.22	7.01±0.42	8.63±0.28	7.61±0.21*	9.37±0.15	8.27±0.74
LVPWT (mm)	0.93±0.07	0.98±0.10	1.31±0.12	1.73±0.07*,†	1.25±0.17	2.03±0.18*,†
EF (%)	91.9±1.2	93.6±1.2	88.2±2.3	92.8±1.1	82.2±1.3	71.2±5.6†,‡
Plasma BNP (pg/ml)	113±13	98±1	120±18	137±18	108±8	303±97†

Data are means±SE. *P<0.05 vs corresponding DR; †P<0.05 vs DS at 6 weeks; ‡P<0.05 vs DS at 11 weeks.
DR, salt-resistant Dahl; DS, salt-sensitive Dahl; BW, body weight; LVW, left ventricular (LV) weight; SBP, systolic blood pressure; DBP, diastolic blood pressure; LVESD, LV end-systolic dimension; LVEDD, LV end-diastolic dimension; LVPWT, LV posterior wall thickness; EF, ejection fraction; BNP, B-type natriuretic peptide.

correlation between LV expression of LOX-1 and progression of HF using Dahl rats. Furthermore, we found that serum sLOX-1 levels are increased in patients with chronic HF and LV hypertrophy (LVH).

Methods

Dahl Rats

Male Dahl rats were fed a low-salt diet (0.3% NaCl) until the age of 6 weeks, after which, to induce HT, they were fed a high-salt diet (8% NaCl). All animal experiments conformed with the Guide for the Care and Use of Laboratory Animals by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, and the protocol was approved by the Ethics Committee of the Graduate School of Medicine, Kyoto University.

Physiological Analysis

Blood pressure (BP) was measured in the Dahl rats by the tail-cuff method. Cardiac functions were noninvasively evaluated by echocardiography, as previously described.[25] In brief, images were recorded using a 10- to 12-MHz phased-array transducer (model 21380A with HP SONOS 5500 imaging system; Agilent Technologies). LV end-diastolic and end-systolic dimensions (LVEDD and LVESD) were measured with M-mode tracings from the short-axis view of the LV at the papillary muscle level. All measurements were performed in a blinded fashion according to the guidelines of the American Society for Echocardiology and averaged over 3 consecutive cardiac cycles. After physiological studies, surviving rats were euthanased, and their hearts were removed.

Measurement of Plasma B-Type Natriuretic Peptide (BNP)

Blood samples were obtained from surviving rats for measurement of plasma BNP concentrations using a radioimmunoassay kit (Peninsula Lab).

Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNAs from the LVs were isolated, reverse transcribed, and subjected to quantitative real-time RT-PCR as previously described.[26] Primer sequences of LOX-1,[27] BNP,[28] MCP-1,[29] TGF-β_1,[30] IL-1β,[31] F4/80,[32] and GAPDH[26] have been described previously.

Western Blotting

Whole cell lysates from rat LVs were prepared and subjected to Western blotting as described previously,[33] using mouse monoclonal anti-LOX-1[1] and mouse monoclonal anti-β-actin (Sigma) antibodies. Protein amounts were semi-automatically quantified by using Image J software (National Institutes of Health).

Histological Analysis

The excised hearts were cut into 2 transverse slices at the mid-level of the papillary muscles. The specimens were fixed in 10% formalin, embedded in paraffin, sliced into 4-μm-thick sections, and stained using mouse monoclonal anti-LOX-1 antibody.[1]

Human Subjects

A cross-sectional study was carried out during a specified period between July and September 2007. Patients with chronic congestive HF and LVH (CHF-LVH) and apparently healthy subjects with normal cardiac function without LVH (controls) were recruited in the Outpatient Department of Cardiovascular Disease of Kyoto Medical Center. CHF was defined according to the ACC/AHA Guideline.[34] LVH was defined as LV mass index (LVMI) >116 g/m^2 in men and >104 g/m^2 in women on echocardiographic examination. Chronic HF was defined as the patient being in a stable New York Heart Association functional class for at least 3 months. Most of the controls attended for further examination of risk factors after periodical health checkup. The echocardiographic criteria for CHF-LVH were defined as the presence of LVH, ejection fraction (EF) <60%, and LVEDD >50 mm, and those for controls were LVMI <100 g/m^2, EF >60%, and LVEDD <50 mm. Exclusion criteria were: (1) infection or illness with pyrexia; (2) recent (<3 month) acute coronary syndrome, MI, or stroke; (3) chronic, systemic illness, including renal failure, hepatic impairment, cancer, and inflammatory connective tissue disease; inflammatory bowel disease. BP was measured twice with an automatic electronic sphygmomanometer (BP-103i II; Nippon Colin, Komaki, Japan).

The study protocol was approved by the Institutional Ethics Committee of Kyoto Medical Center.

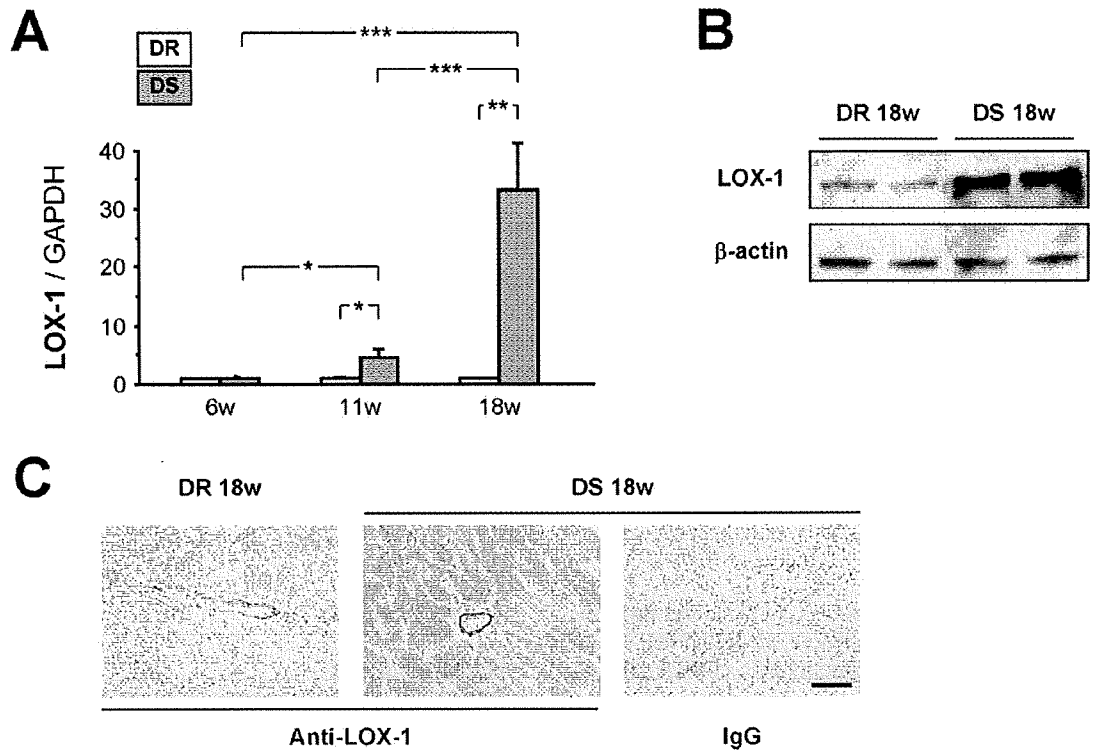


Figure 1. (A) Results of real-time RT-PCR. The amount of transcript for LOX-1 was normalized by that of GAPDH. The data shown are means ± SE. The mean value of DR rats at 6 weeks was set at 1.0. The number of animals in each group is shown in Table 1. *P<0.05, **P<0.01, ***P<0.005. (B) Whole cell lysates from the LV of Dahl rats at 18 weeks were subjected to Western blotting with anti-LOX-1 and anti-β-actin antibodies. (C) Representative photographs of LOX-1-stained sections of LV myocardium from Dahl rats at 18 weeks. Scale bar=20 μm. DR, salt-resistant Dahl rat; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; LV, left ventricle; RT-PCR, reverse transcriptase polymerase chain reaction; w, weeks.

Measurement of sLOX-1

Patients' blood samples were taken from the antecubital vein in the morning after a 12-h fast. Blood was immediately centrifuged and the serum obtained was divided into aliquots. Serum sLOX-1 concentrations were measured by ELISA. The analyses were performed by an investigator who was unaware of the source of each sample.

Statistical Analysis

Results are presented as means ± SE. Statistical comparisons were performed using ANOVA with Scheffe's test. Linear regression analysis with Pearson's coefficients was performed to investigate correlations. The Mann-Whitney U test was used for comparisons of human sLOX-1. P<0.05 was taken to indicate significance.

Results

Development of HF in Dahl Rats

Cardiac function of the Dahl rats was assessed before and after (at 11 and 18 weeks) they were fed a high-salt diet from the age of 6 weeks. As shown in Table 1, BP was significantly higher than in the DS compared with the DR rats at 11 and 18 weeks. Accordingly, DS rats exhibited LVH: increased LV weight-to-body weight ratio (LVW/BW) and LV posterior wall thickness (LVPWT) compared with DR rats at 11 and 18 weeks. The LVEF of DS rats was preserved at 11 weeks but significantly reduced at 18 weeks. These data demonstrate that DS rats showed progressive LVH at 11 weeks, followed by systolic dysfunction at 18 weeks. The LVW/BW ratio was significantly higher in the DS (5.14±0.30) than in the DR (3.71±0.04) rats at 18 weeks. The increased lung weights and plasma BNP levels in the DS compared with the DR rats suggest that the LV end-diastolic pressure increased at 18 weeks. LV dilatation would subsequently occur after 18 weeks in the DS rats. However, LV dilatation was not observed in this series of experiments, because DS rats rapidly die after 18 weeks and the time period of LV dilatation is very short.

LV Expression of LOX-1 in Dahl Rats

Real-time RT-PCR analysis indicated that LV mRNA levels of LOX-1 in the DS rats progressively increased at 11 and 18 weeks, while those in the DR rats did not change (Figure 1A). LOX-1 expression revealed 4.7- and 32-fold increases in the DS compared with the DR rats at 11 and 18 weeks, respectively. Compatible with the mRNA levels, the amount of LOX-1 protein in the LV was greater in the DS rats than in the DR rats at 18 weeks (Figure 1B). DS rats showed a 5.8± 3.3-fold increase in the levels of LOX-1 protein compared with the DR rats at 18 weeks. Sections of LV from these rats at 18 weeks were stained using anti-LOX-1 antibody (Figure 1C). LOX-1 immunoreactivity was observed in vessel walls and very faintly in the cardiomyocytes of DR rats. However in the DS rats, LOX-1 was strongly and clearly detected in cardiomyocytes as well as vessel walls. These

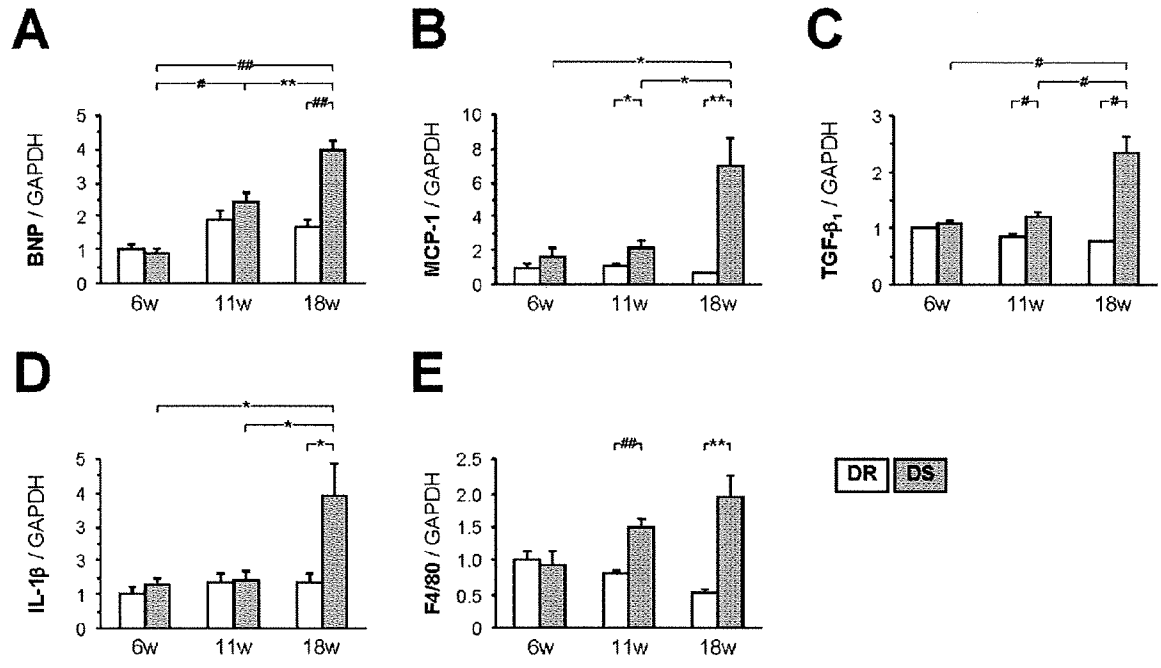


Figure 2. Results of real-time reverse transcriptase polymerase chain reaction. The amount of each of the transcripts for BNP (A), MCP-1 (B), TGF-β_1 (C), IL-1β (D), and F4/80 (E) was normalized by that of GAPDH. Data are means ± SE. The mean values of DR rats at 6 weeks were set at 1.0. The number of animals in each group is described in Table 1. *P<0.05, **P<0.01, #P<0.005, ##P<0.001. BNP, B-type natriuretic peptide; DR, salt-resistant Dahl rat; DS, salt-sensitive Dahl rat; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; TGF, transforming growth factor; w, weeks.

Table 2. Correlation Between LOX-1 mRNA and Parameters of Heart Failure

	vs LOX-1/GAPDH	
	R	P value
LVW/BW	0.620	0.0004
SBP	0.748	<0.0001
DBP	0.604	0.0007
Heart rate	−0.426	0.0268
LVESD	0.555	0.0022
LVEDD	0.172	0.3808
LVPWT	0.638	0.0002
EF	−0.772	<0.0001
Plasma BNP	0.744	<0.0001
BNP/GAPDH	0.814	<0.0001
MCP-1/GAPDH	0.943	<0.0001
TGF-β_1/GAPDH	0.936	<0.0001
IL-1β/GAPDH	0.760	<0.0001
F4/80/GAPDH	0.560	0.0019

Correlations between LV mRNA levels of LOX-1 and parameters of heart failure for all 28 rats shown in Table 1.
GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MCP, monocyte chemoattractant protein; TGF, transforming growth factor; IL, interleukin. Other abbreviations see in Table 1.

results clearly indicate that the expression of LOX-1 in LV cardiomyocytes was upregulated during the development of LVH and HF in DS rats.

LV Expression of Cytokines Involved in HF

Levels of the mRNA of BNP, MCP-1, TGF-β_1, IL-1β, and F4/80 in the LV were also quantified by real-time RT-PCR (Figure 2). Those of BNP, which reflect the extent of LV wall stress, were increased in the DS rats during the development of HF and were significantly higher than those of the DR rats at 18 weeks (Figure 2A). Those of MCP-1 in the DS rats showed 2.1- and 10.2-fold increases at 11 and 18 weeks, respectively, compared with the DR rats (Figure 2B). Those of TGF-β_1 (Figure 2C) and IL-1β (Figure 2D) showed 3.1- and 2.9-fold increases, respectively in the DS compared with the DR rats, at 18 weeks. Compatible with the increased expression of these cytokines in the DS rats, the LV mRNA level of F4/80, a marker of macrophages, showed 1.9- and 3.7-fold increases at 11 and 18 weeks, respectively, in the DS compared with the DR rats (Figure 2E).

Correlation Between LOX-1 Expression and Parameters of HF

As shown in Table 2, LV mRNA levels of LOX-1 positively correlated with the levels of HT (systolic and diastolic BP) and LVH (LVW/BW and LVPWT). LOX-1 expression was also associated with deterioration of systolic function (increase in LVESD and decrease in EF, Figure 3A). In addition, LOX-1 strongly indicated positive correlations with the plasma and mRNA levels of BNP (Figure 3B). Thus, LOX-1 expression was closely associated with the extent of HF in Dahl rats. Importantly, the LV mRNA levels of LOX-1 were most closely correlated with those of MCP-1 (Figure 3C). The levels also strongly correlated with those of TGF-β_1 (Figure 3D) and IL-1β (Figure 3E). In addition to these cytokines, the LV mRNA levels of LOX-1 significantly correlated with those of F4/80 (Figure 3F).

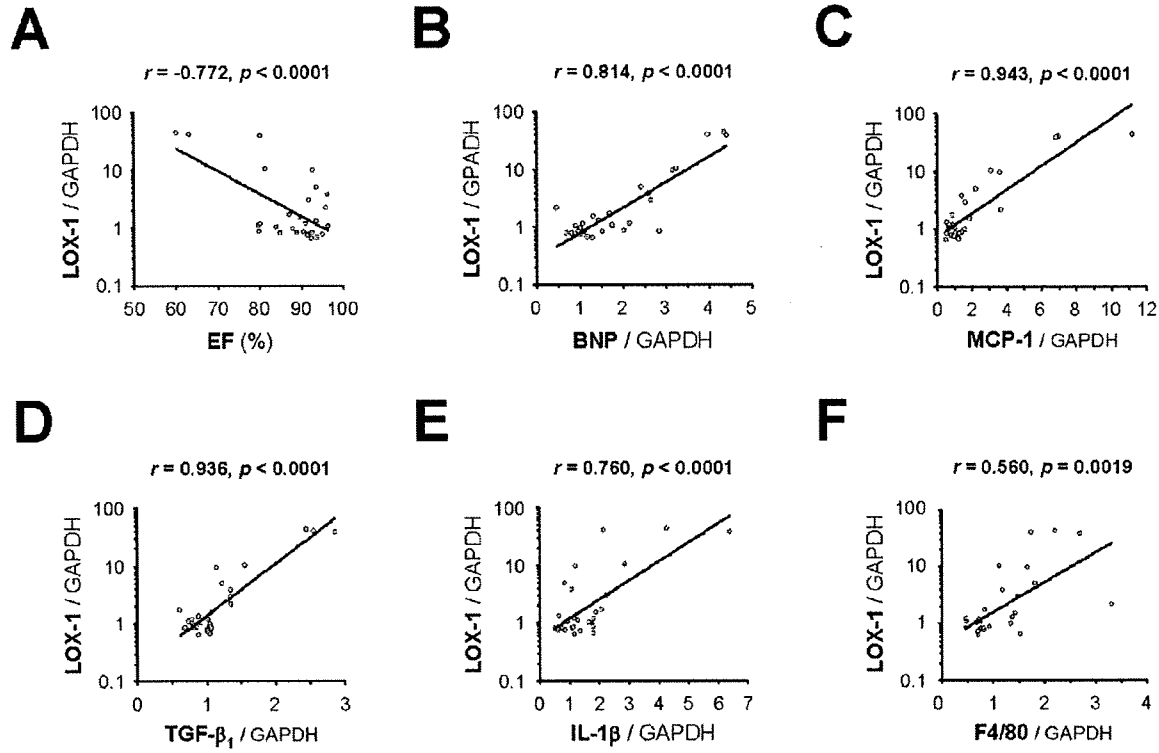


Figure 3. Correlations between the LV mRNA levels of LOX-1 and the parameters of heart failure. Abbreviations see in Tables 1 and 2.

Table 3. Clinical and Echocardiographic Measurements of Human Subjects

	Controls	CHF-LVH patients	P value
n (M/F)	11 (7/4)	7 (4/3)	
Age (years)	64.8±4.4	67.7±3.8	NS
BMI (kg/m²)	24.0±1.3	22.9±1.5	NS
SBP (mmHg)	125.8±5.3	113.4±3.7	NS
DBP (mmHg)	74.5±4.2	61.9±3.4	0.077
Heart rate (beats/min)	71.8±4.2	76.8±5.0	NS
LVEDD (mm)	42.1±1.0	64.1±4.7	<0.001
EF (%)	71.5±1.9	37.5±2.9	<0.001
LV mass (g)	115.1±6.5	247.8±33.9	<0.001
LVMI (g/m²)	71.7±4.9	157.3±26.2	<0.001
Diabetes mellitus, n (%)	0 (0)	4 (57)	
History of hypertension, n (%)	0 (0)	4 (57)	
Etiology of CHF, n (%)			
Ischemic	0 (0)	4 (57)	
Non-ischemic	0 (0)	3 (43)	
Idiopathic dilated cardiomyopathy	0 (0)	3 (43)	
NYHA functional class, n (%)			
I	11 (100)	0 (0)	
II	0 (0)	6 (86)	
III	0 (0)	1 (14)	
IV	0 (0)	0 (0)	

Data are means±SE.
Controls, no organic cardiac diseases; CHF-LVH, chronic heart failure with LV hypertrophy; NS, not significant; NYHA, New York Heart Association. Other abbreviations see in Table 1.

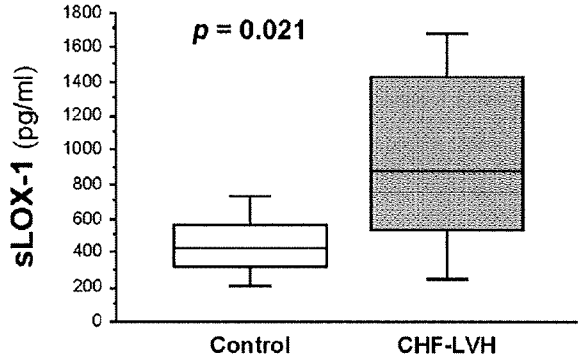


Figure 4. Serum levels of soluble LOX-1 (sLOX-1) in chronic heart failure patients with left ventricular hypertrophy (CHF-LVH, n=7) and those without organic cardiac diseases (control, n=11). LOX-1, lectin-like oxidized low-density lipoprotein receptor-1.

Serum Levels of sLOX-1 in Chronic HF Patients

The clinical and echocardiographic measurements in the patients with CHF-LVH and the apparently healthy subjects with normal cardiac function (control) are shown in Table 3. LVEDD, LV mass, and LVMI were significantly larger and EF significantly lower in the CHF-LVH patients than in the control group. However, there were no significant differences in age, body mass index, BP, and heart rate between the 2 groups. Interestingly, serum levels of sLOX-1 were significantly increased in the CHF-LVH group compared with the controls (Figure 4). In simple regression analysis, there was a weak, but non-significant correlation between serum sLOX-1 levels and LVMI (r=0.437, P=0.07). However, there was a significant negative correlation between serum sLOX-1 levels and EF (r=−0.495, P=0.037). Since previous reports have shown that sLOX-1 levels are increased in patients with DM and those with HT, we compared the sLOX-1 levels in CHF-LVH patients with and without DM or HT. There was no significant difference in the sLOX-1 levels of CHF-LVH patients with and without DM or HT (DM 892±316 pg/ml vs non-DM 1,023±311 pg/ml, P=0.8; HT 845±275 pg/ml vs non-HT 1,086±366 pg/ml, P=0.6). To evaluate whether the etiology of chronic HF affects sLOX-1 levels, we compared sLOX-1 levels in patients with CHF-LVH caused by ischemic heart disease (IHD) with those in patients with CHF-LVH caused by dilated cardiomyopathy (DCM). There was no significant difference (P=0.5): IHD, 1,083±348 pg/ml; DCM, 769±178 pg/ml.

Discussion

In the present study, we showed that levels of mRNA and LOX-1 protein were markedly upregulated in the LV of DS rats with HF, which was compatible the results of a previous report.[24] We have found that LV mRNA levels of LOX-1 closely correlated with decreased EF and increases in the plasma and mRNA levels of BNP. These findings suggest that LV expression of LOX-1 serves as a novel biomarker of HF in hypertensive heart disease. We have also shown that the serum levels of sLOX-1 were significantly increased in chronic HF patients with LVH and that they correlated with the decrease in EF. Thus, a marked increase in the LV expression of LOX-1 in the failing heart may significantly contribute to increased serum levels of sLOX-1. However, the origin of increased serum sLOX-1 levels during hypertensive heart disease should be examined in further studies, because HT enhances LOX-1 expression not only by the heart, but also by the vascular endothelium.[11]

LV mRNA levels of LOX-1 showed a very strong positive correlation with those of MCP-1, an important chemotactic factor for macrophages. LOX-1 expression also closely correlated with those of TGF-β_1 and IL-1β, proinflammatory cytokines produced by macrophages. Furthermore, LV expression of LOX-1 positively correlated with that of F4/80, a maker of macrophages, suggesting that increased LOX-1 expression is involved in macrophage infiltration and inflammation. In the heart, MCP-1 expression and the number of interstitial macrophages in the LV are significantly increased in models of hypertensive heart disease with HF[31] and of post-MI HF.[35] The number of macrophages in the LV myocardium shows nearly a 4-fold increase in DS compared with DR rats at 11 and 18 weeks.[31] Our results for the LV mRNA levels of F4/80, a marker of macrophages, are compatible with those of the previous report.

MCP-1 is considered to be downstream of LOX-1 because the antisense to LOX-1 inhibits MCP-1 expression in endothelial cells.[17] Inhibition of MCP-1 in a mouse MI model reduced macrophage infiltration and the levels of cytokines such as TGF-β_1 in the heart.[35] It has also been reported that anti-LOX-1 antibody reduces IL-1β expression in vascular cells.[36] Our results indicated that LOX-1 upregulation in the LV of DS rats compared with DR rats was most prominent at the stage of systolic HF. Furthermore, LV expression of LOX-1 showed a close relationship with that of inflammatory cytokines, as well as MCP-1 and F4/80, which are markers of increased macrophage infiltration. These findings suggest that LOX-1-induced MCP-1 enhances macrophage infiltration, and that the migrating macrophages then produce proinflammatory cytokines in the heart. TGF-β_1 and IL-1β are well-known inducers of LOX-1,[4,5] so it is possible that increased LOX-1, macrophage infiltration, and the release of inflammatory cytokines may form a feed-back loop that progresses to fibrosis and apoptosis during the progression of HF. At present, it is unknown whether activation of LV LOX-1 is a cause or result of HF. However, our results, together with those of previous reports, suggest that upregulation of LOX-1 in HF is a very important key event, leading to inflammation of the heart. The development of a specific antagonist is awaited to clarify the precise role of LOX-1, and to investigate the therapeutic potential of the antagonist for chronic HF.

Acknowledgments

We thank Mika Kiriyama, Noboru Chiba, Shuichi Ura, Akira Yamada, and Yuko Iida for their technical assistance and Akemi Wada for secretarial assistance. This work was supported by grants in-aid for scientific research awarded to K. Hasegawa from Ministry of Education, Culture, Sports, Science and Technology of Japan and from Research on Publicly Essential Drugs and Medical Devices, The Japan Health Sciences Foundation, and awarded to H. Wada from Suzuken Memorial Foundation, Japan Research Foundation for Clinical Pharmacology, and the Smoking Research Foundation.

References

1. Sawamura T, Kume N, Aoyama T, Moriwaki H, Hoshikawa H, Aiba Y, et al. Masaki, An endothelial receptor for oxidized low-density lipoprotein. *Nature* 1997; **386:** 73–77.
2. Mehta JL, Li DY. Identification and autoregulation of receptor for ox-LDL in cultured human coronary artery endothelial cells. *Biochem Biophys Res Commun* 1998; **248:** 511–514.
3. Kume N, Murase T, Moriwaki H, Aoyama T, Sawamura T, Masaki

T, et al. Inducible expression of lectin-like oxidized LDL receptor-1 in vascular endothelial cells. *Circ Res* 1998; **83:** 322–327.
4. Minami M, Kume N, Kataoka H, Morimoto M, Hayashida K, Sawamura T, et al. Transforming growth factor-β1 increases the expression of lectin-like oxidized low-density lipoprotein receptor-1. *Biochem Biophys Res Commun* 2000; **272:** 357–361.
5. Hofnagel O, Luechtenborg B, Stolle K, Lorkowski S, Eschert H, Plenz G, et al. Proinflammatory cytokines regulate LOX-1 expression in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2004; **24:** 1789–1795.
6. Li DY, Zhang YC, Philips MI, Sawamura T, Mehta JL. Upregulation of endothelial receptor for oxidized low-density lipoprotein (LOX-1) in cultured human coronary artery endothelial cells by angiotensin II type 1 receptor activation. *Circ Res* 1999; **84:** 1043–1049.
7. Morawietz H, Rueckschloss U, Niemann B, Duerrschmidt N, Galle J, Hakim K, et al. Angiotensin II induces LOX-1, the human endothelial receptor for oxidized low-density lipoprotein. *Circulation* 1999; **100:** 899–902.
8. Morawietz H, Duerrschmidt N, Niemann B, Galle J, Sawamura T, Holtz J. Induction of the oxLDL receptor LOX-1 by endothelin-1 in human endothelial cells. *Biochem Biophys Res Commun* 2001; **284:** 961–965.
9. Li D, Mehta JL. Upregulation of endothelial receptor for oxidized LDL (LOX-1) by oxidized LDL and implications in apoptosis of human coronary artery endothelial cells: Evidence from use of antisense LOX-1 mRNA and chemical inhibitors. *Arterioscler Thromb Vasc Biol* 2000; **20:** 1116–1122.
10. Kataoka H, Kume N, Miyamoto S, Minami M, Morimoto M, Hayashida K, et al. Oxidized LDL modulates bax/bcl-2 through the lectinlike ox-LDL receptor-1 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2001; **21:** 955–960.
11. Nagase M, Hirose S, Sawamura T, Masaki T, Fujita T. Enhanced expression of endothelial oxidized low-density lipoprotein receptor (LOX-1) in hypertensive rats. *Biochem Biophys Res Commun* 1997; **237:** 496–498.
12. Chen M, Nagase M, Fujita T, Narumiya S, Masaki T, Sawamura T. Diabetes enhances lectin-like oxidized LDL receptor-1 (LOX-1) expression in the vascular endothelium: Possible role of LOX-1 ligand and AGE. *Biochem Biophys Res Commun* 2001; **287:** 962–968.
13. Chen M, Kakutani M, Minami M, Kataoka H, Kume N, Narumiya S, et al. Increased expression of lectin-like oxidized low density lipoprotein receptor-1 in initial atherosclerotic lesions of Watanabe heritable hyperlipidemic rabbits. *Arterioscler Thromb Vasc Biol* 2000; **20:** 1107–1115.
14. Chen H, Li D, Sawamura T, Inoue K, Mehta JL. Upregulation of LOX-1 expression in aorta of hypercholesterolemic rabbits: Modulation by losartan. *Biochem Biophys Res Commun* 2000; **276:** 1100–1104.
15. Kataoka H, Kume N, Miyamoto S, Minami M, Moriwaki H, Murase T, et al. Expression of lectinlike oxidized low-density lipoprotein receptor-1 in human atherosclerotic lesions. *Circulation* 1999; **99:** 3110–3117.
16. Li D, Mehta JL. Antisense to LOX-1 inhibits oxidized LDL-mediated upregulation of monocyte chemoattractant protein-1 and monocyte adhesion to human coronary artery endothelial cells. *Circulation* 2000; **101:** 2889–2895.
17. Hamakawa Y, Omori N, Ouchida M, Nagase M, Sato K, Nagano I, et al. Severity dependent up-regulations of LOX-1 and MCP-1 in early sclerotic changes of common carotid arteries in spontaneously hypertensive rats. *Neurol Res* 2004; **26:** 767–773.
18. Murase T, Kume N, Kataoka H, Minami M, Sawamura T, Masaki T, et al. Identification of soluble forms of lectin-like oxidized LDL receptor-1. *Arterioscler Thromb Vasc Biol* 2000; **20:** 715–720.
19. Hayashida K, Kume N, Murase T, Minami M, Nakagawa D, Inada T, et al. Serum soluble lectin-like oxidized low-density lipoprotein receptor-1 levels are elevated in acute coronary syndrome: A novel marker for early diagnosis. *Circulation* 2005; **112:** 812–818.
20. Tan KC, Shiu SW, Wong Y, Leng L, Bucala R. Soluble lectin-like oxidized low density lipoprotein receptor-1 in type 2 diabetes mellitus. *J Lipid Res* 2008; **49:** 1438–1444.
21. Brinkley TE, Kume N, Mitsuoka H, Phares DA, Hagberg JM. Elevated soluble lectin-like oxidized LDL receptor-1 (sLOX-1) levels in obese postmenopausal women. *Obesity* 2008; **16:** 1454–1456.
22. Iwai-Kanai E, Hasegawa K, Sawamura T, Fujita M, Yanazume T, Toyokuni S, et al. Activation of lectin-like oxidized low-density lipoprotein receptor-1 induces apoptosis in cultured neonatal rat cardiac myocytes. *Circulation* 2001; **104:** 2948–2954.
23. Kataoka K, Hasegawa K, Sawamura T, Fujita M, Yanazume T, Iwai-Kanai E, et al. LOX-1 pathway affects the extent of myocardial ischemia-reperfusion injury. *Biochem Biophys Res Commun* 2003; **300:** 656–600.
24. Kobayashi N, Yoshida K, Nakano S, Ohno T, Honda T, Tsubokou Y, et al. Cardioprotective mechanisms of eplerenone on cardiac performance and remodeling in failing rat hearts. *Hypertension* 2006; **47:** 671–679.
25. Morimoto T, Sunagawa Y, Kawamura T, Takaya T, Wada H, Nagasawa A, et al. The dietary compound curcumin inhibits p300 histone acetyltransferase activity and prevents heart failure in rats. *J Clin Invest* 2008; **118:** 868–878.
26. Takaya T, Ono K, Kawamura T, Takanabe R, Kaichi S, Morimoto T, et al. MicroRNA-1 and microRNA-133 in spontaneous myocardial differentiation of mouse embryonic stem cells. *Circ J* 2009; **73:** 1492–1497.
27. Hinagata J, Kakutani M, Fujii T, Naruko T, Inoue N, Fujita Y, et al. Oxidized LDL receptor LOX-1 is involved in neointimal hyperplasia after balloon arterial injury in a rat model. *Cardiovasc Res* 2006; **69:** 263–271.
28. Iwanaga Y, Kihara Y, Takenaka H, Kita T. Down-regulation of cardiac apelin system in hypertrophied and failing hearts: Possible role of angiotensin II-angiotensin type 1 receptor system. *J Mol Cell Cardiol* 2006; **41:** 798–806.
29. Hevener AH, Olefsky JM, Reichart D, Nguyen MTA, Bandyopadyhay G, Leung HY, et al. Macrophage PPARγ is required for normal skeletal muscle and hepatic insulin sensitivity and full antidiabetic effects of thiazolidinediones. *J Clin Invest* 2007; **117:** 1658–1669.
30. Khanna AK, Plummer MS, Hilton G, Pieper GM, Ledbetter S. Anti-transforming growth factor antibody at low but not high doses limits cyclosporine-mediated nephrotoxicity without altering rat cardiac allograft survival: Potential of therapeutic applications. *Circulation* 2004; **110:** 3822–3829.
31. Shioi T, Matsumori A, Kihara Y, Inoko M, Ono K, Iwanaga Y, et al. Increased expression of interleukin-1β and monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 in the hypertrophied and failing heart with pressure overload. *Circ Res* 1997; **81:** 664–671.
32. Rahman MM, Kukita A, Kukita T, Shobuike T, Nakamura T, Kohashi O. Two histone deacetylase inhibitors, trichostatin A and sodium butyrate, suppress differentiation into osteoclasts but not into macrophages. *Blood* 2003; **101:** 3451–3459.
33. Morimoto T, Fujita M, Kawamura T, Sunagawa Y, Takaya T, Wada H, et al. Myocardial regulation of p300 and p53 by doxorubicin involves ubiquitin pathways. *Circ J* 2008; **72:** 1506–1511.
34. Hunt SA, Abraham WT, Chin MH, Feldman AM, Francis GS, Ganiats TG, et al. ACC/AHA 2005 Guideline Update for the Diagnosis and Management of Chronic Heart Failure in the Adult: A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Update the 2001 Guidelines for the Evaluation and Management of Heart Failure): Developed in Collaboration With the American College of Chest Physicians and the International Society for Heart and Lung Transplantation: Endorsed by the Heart Rhythm Society. *Circulation* 2005; **112:** e154–e235.
35. Hayashidani S, Tsutsui H, Shiomi T, Ikeuchi M, Matsusaka H, Suematsu N, et al. Anti-monocyte chemoattractant protein-1 gene therapy attenuates left ventricular remodeling and failure after experimental myocardial infaction. *Circulation* 2003; **108:** 2134–2140.
36. Shin HK, Kim YK, Kim KY, Lee JH, Whan K. Remnant lipoprotein particles induce apoptosis in endothelial cells by NAD(P)H oxidase-mediated production of superoxide and cytokines via lectin-like oxidized low-density lipoprotein receptor-1 activation: Prevention by cilostazol. *Circulation* 2004; **109:** 1022–1028.

Clinical Chemistry 56:4
000–000 (2010)

LOX Index, a Novel Predictive Biochemical Marker for Coronary Heart Disease and Stroke

Nobutaka Inoue,[1] Tomonori Okamura,[2] Yoshihiro Kokubo,[2] Yoshiko Fujita,[1] Yuko Sato,[1] Mamoru Nakanishi,[3] Kazuki Yanagida,[3] Akemi Kakino,[1] Shin Iwamoto,[1] Makoto Watanabe,[2] Sayoko Ogura,[1] Kazunori Otsui,[1] Haruo Matsuda,[4] Kagehiro Uchida,[3] Ryo Yoshimoto,[1] and Tatsuya Sawamura[1]*

BACKGROUND: Lectin-like oxidized LDL receptor 1 (LOX-1) is implicated in atherothrombotic diseases. Activation of LOX-1 in humans can be evaluated by use of the LOX index, obtained by multiplying the circulating concentration of LOX-1 ligands containing apolipoprotein B (LAB) times that of the soluble form of LOX-1 (sLOX-1) [LOX index = LAB × sLOX − 1]. This study aimed to establish the prognostic value of the LOX index for coronary heart disease (CHD) and stroke in a community-based cohort.

METHODS: An 11-year cohort study of 2437 residents age 30–79 years was performed in an urban area located in Japan. Of these, we included in the analysis 1094 men and 1201 women without history of stroke and CHD. We measured LAB and sLOX-1 using ELISAs with recombinant LOX-1 and monoclonal anti–apolipoprotein B antibody and with 2 monoclonal antibodies against LOX-1, respectively.

RESULTS: During the follow-up period, there were 68 incident cases of CHD and 91 cases of stroke (with 60 ischemic strokes). Compared with the bottom quartile, the hazard ratio (HR) of the top quartile of LOX index was 1.74 (95% CI 0.92–3.30) for stroke and 2.09 (1.00–4.35) for CHD after adjusting for sex, age, body mass index, drinking, smoking, hypertension, diabetes, non-HDL cholesterol, and use of lipid-lowering agents. Compared with the bottom quartile of LOX index, the fully adjusted HRs for ischemic stroke were consistently high from the second to the top quartile: 3.39 (95% CI 1.34–8.53), 3.15 (1.22–8.13) and 3.23 (1.24–8.37), respectively.

CONCLUSIONS: Higher LOX index values were associated with an increased risk of CHD. Low LOX index values may be protective against ischemic stroke.

Therapeutic interventions for dyslipidemia such as hypercholesterolemia have proven their effectiveness for the primary as well as secondary prevention of coronary heart disease (CHD).[5] It is also well known that the risk for CHD is significantly associated with high serum concentrations of LDL cholesterol or low concentrations of HDL cholesterol in both Japanese *(1, 2)* and Western populations *(3)*. In contrast, dyslipidemia has much weaker relationship to stroke than CHD *(4)*. Although the pathogenesis of ischemic stroke and CHD is based largely on atherosclerotic changes of arteries, there is still much unresolved discrepancy.

Oxidized LDL induces a wide variety of cellular responses, such as induction of the expression of adhesion molecules and proinflammatory cytokines, which enhance progression of atherothrombotic cardiovascular diseases. Using antibodies against oxidation-dependent epitopes of LDL, cross-sectional studies reported association of oxidized LDL concentrations with ischemic heart disease, and a cohort study reported association of oxidized LDL with metabolic syndrome *(5–8)*.

Lectin-like oxidized LDL receptor 1 (LOX-1) is the receptor for oxidized LDL identified in endothelial cells *(9, 10)*. Activation of LOX-1 in endothelial cells induces various changes relevant to endothelial dysfunction, e.g., superoxide generation, reduction in the release of nitric oxide, and induction of the expression

[1] Department of Vascular Physiology and [2] Department of Preventive Cardiology, National Cardiovascular Center, Osaka, Japan; [3] Biomarker Science Co. Ltd., Osaka, Japan; [4] Laboratory of Immunobiology, Department of Molecular and Applied Biosciences, Graduate School of Biosphere Science, Hiroshima University, Hiroshima, Japan.
* Address correspondence to this author at: Department of Vascular Physiology, National Cardiovascular Center, 5-7-1, Fujishirodai, Suita, Osaka, 565-8565 Japan. Fax +81-6-6872-7485; e-mail t-sawamura@umin.ac.jp.

Received November 15, 2009; accepted December 18, 2009.
Previously published online at DOI: 10.1373/clinchem.2009.140707

[5] Nonstandard abbreviations: CHD, coronary heart disease; LOX-1, lectin-like oxidized LDL receptor 1; MCP-1, monocyte chemoattractant protein 1; ApoB, apolipoprotein B; LAB, LOX-1 ligand containing ApoB; sLOX-1, soluble LOX-1; TC, total cholesterol; MI, myocardial infarction; MONICA, Monitoring Trends and Determinants of Cardiovascular Disease; CVD, cardiovascular disease; HR, hazard ratio; BMI, body mass index.