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).

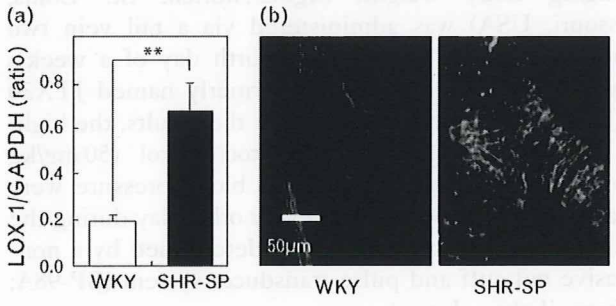
High-fat and salt diet-induced lipid deposition in SHR-SP

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

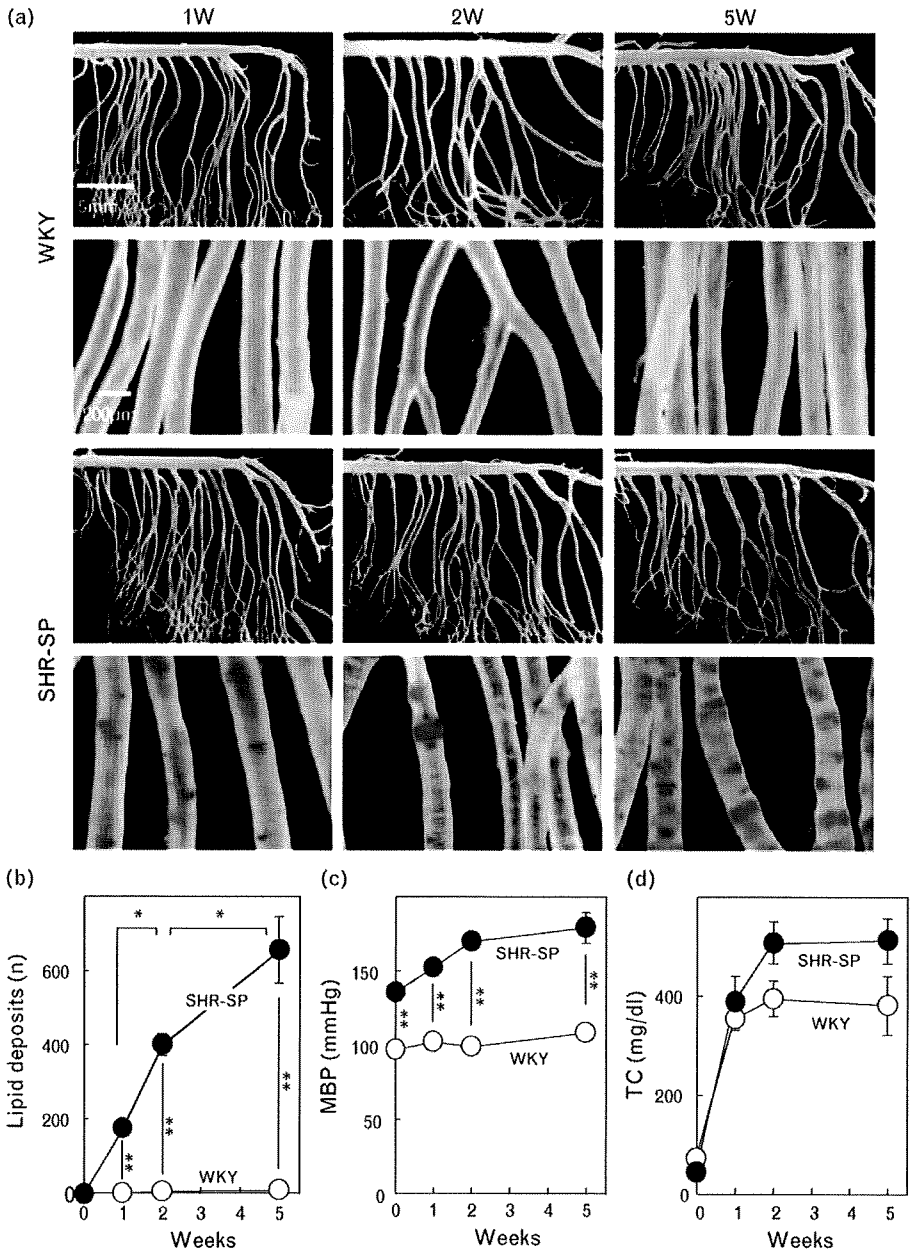
Next, we investigated spatial relationship between LOX-1 and oxLDL in the mesenteric arteries. OxLDL was accumulated in medial smooth muscle layer (Fig. 3a), and associated with LOX-1 expression (Fig. 3b). The expression of LOX-1 was observed in endothelium as well as smooth muscle cells (Fig. 3b-d). 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



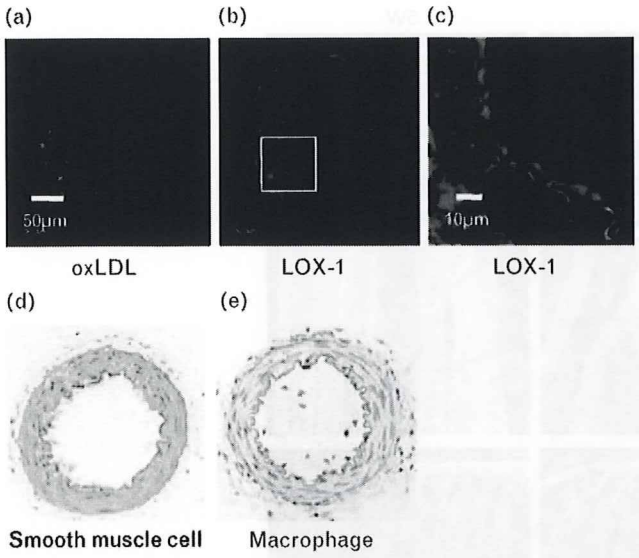
Lipid deposition in mesenteric artery of SHR-SP and WKY after high-fat (HF) diet and saline loading. (a) Oil Red O staining of lipid deposition in mesenteric artery of SHR-SP and WKY after 1, 2 and 5 weeks of HF diet and saline loading. Each upper panel shows the whole branches of mesenteric artery at 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).

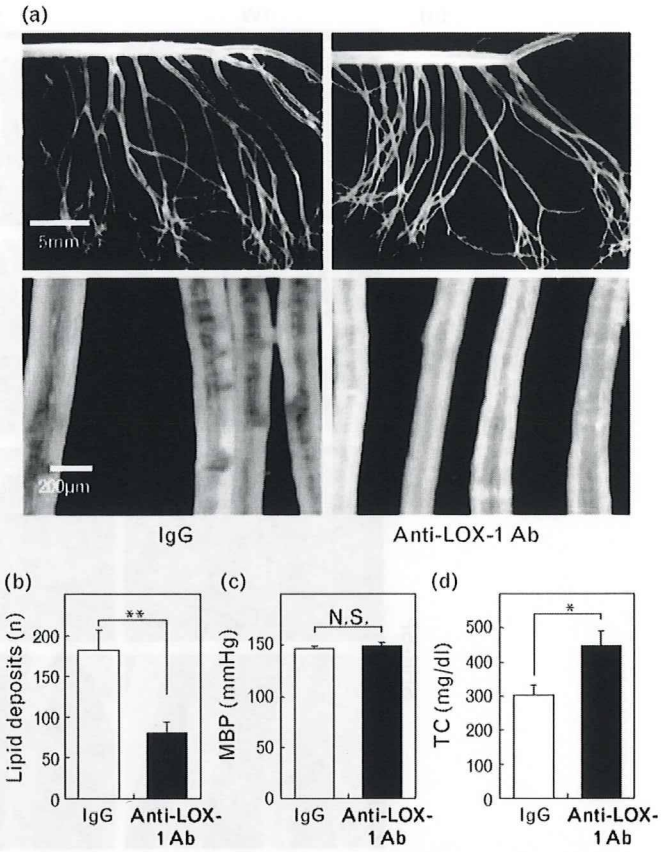
To clarify the significance of oxidative stress, the effects of vitamin E, a lipophilic antioxidant, were examined. As shown in Fig. 5a and b, vitamin E potently suppressed vascular lipid deposition. Concomitantly, the increase in serum levels of LOX-1 ligands induced by a high-fat diet

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).

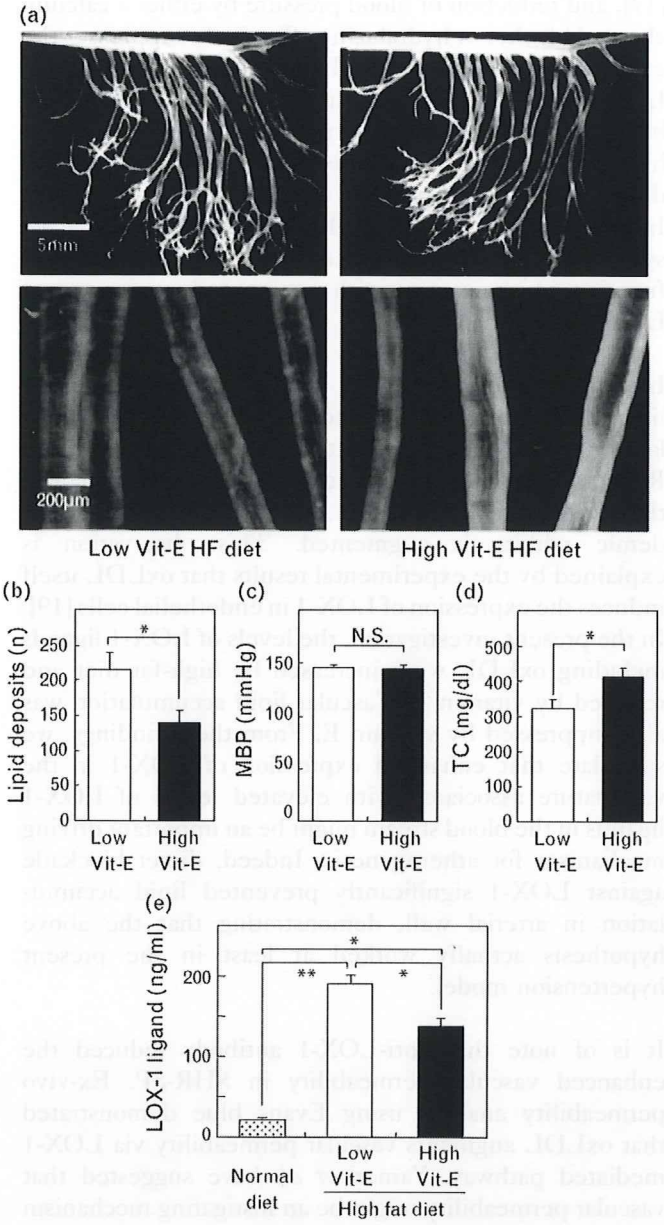
Enhancement of vascular permeability via LOX-1-mediated pathway

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

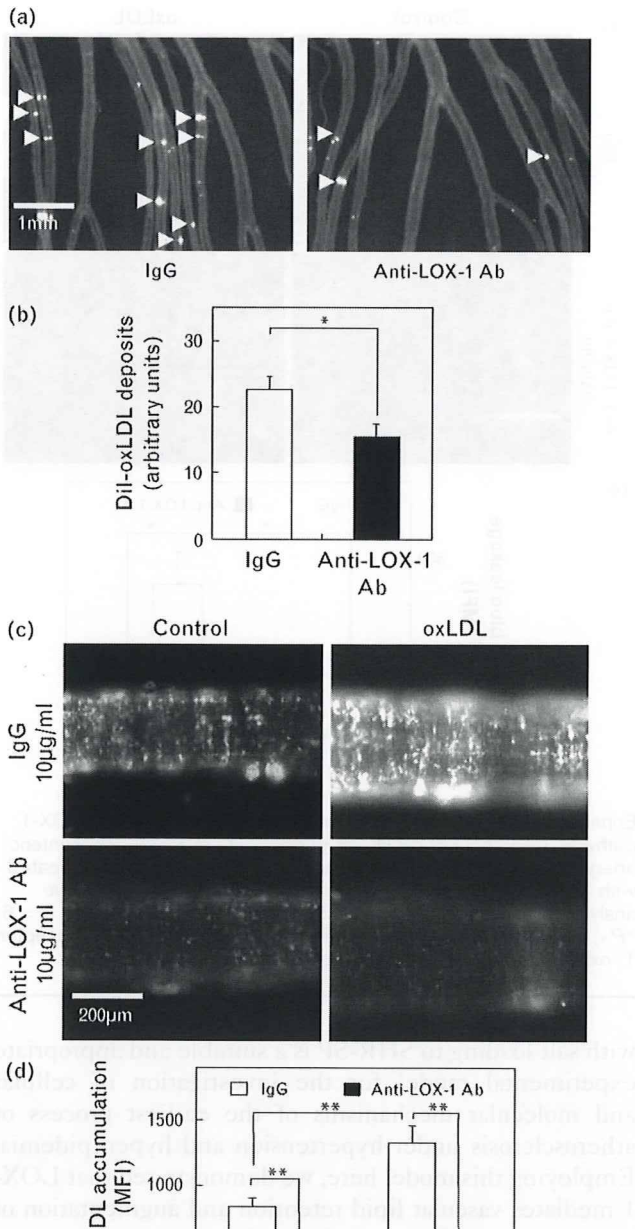
In the hypertensive state, mechanical stress induced by hemodynamic forces such as shear stress and stretch force is one of the most important factors contributing to

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.

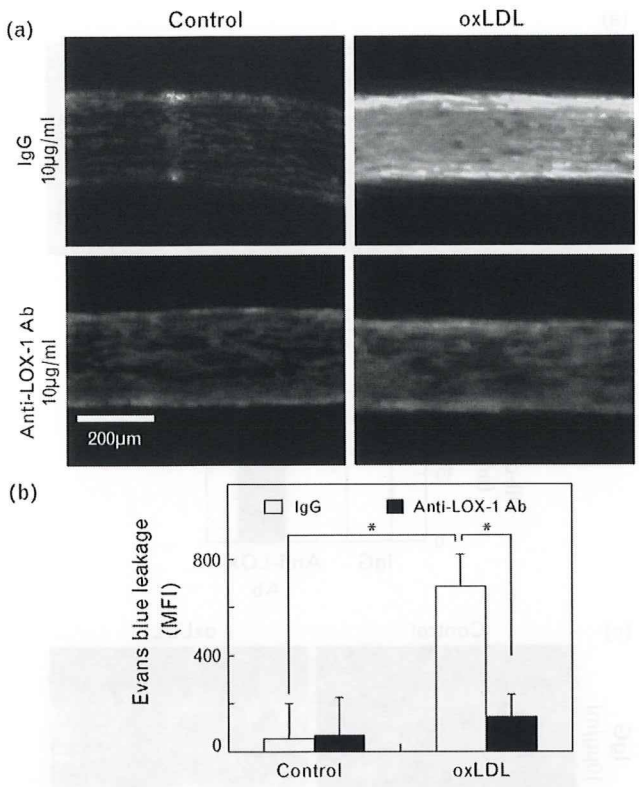
Fig. 6



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.

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There are no conflicts of interest.

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

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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

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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-β1,[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.

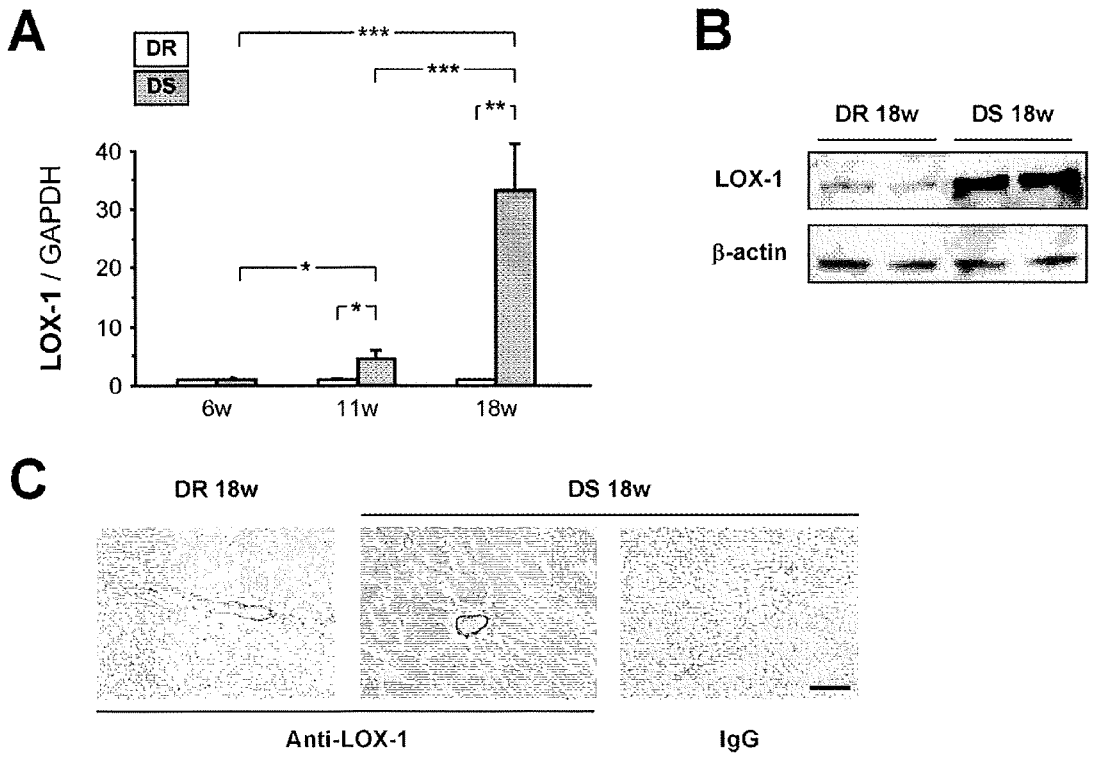

Figure 1. (A) Results of real-time RT-PCR. The amount of transcript for LOX-1 was normalized by that of GAPDH. The data shown are means ± SE. The mean value of DR rats at 6 weeks was set at 1.0. The number of animals in each group is shown in Table 1. *P<0.05, **P<0.01, ***P<0.005. (B) Whole cell lysates 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

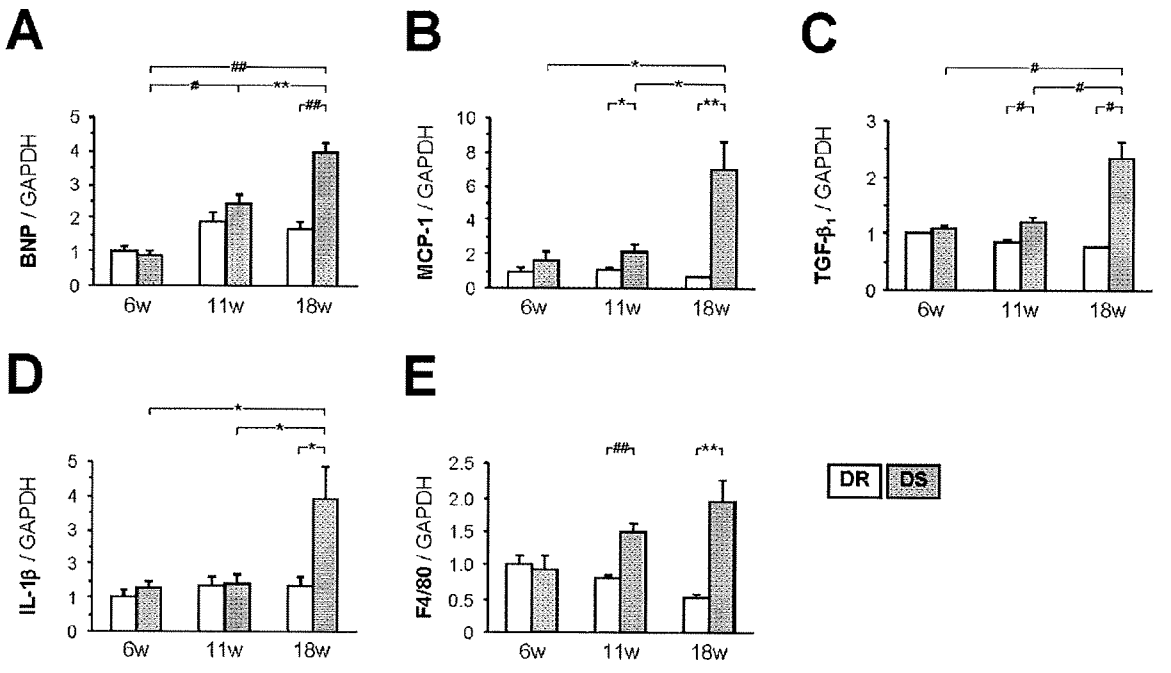

Figure 2. Results of real-time reverse transcriptase polymerase chain reaction. The amount of each of the transcripts for BNP (A), MCP-1 (B), TGF-β_1 (C), IL-1β (D), and F4/80 (E) was normalized by that of GAPDH. Data are means ± SE. The mean values of DR rats at 6 weeks were set at 1.0. The number of 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).

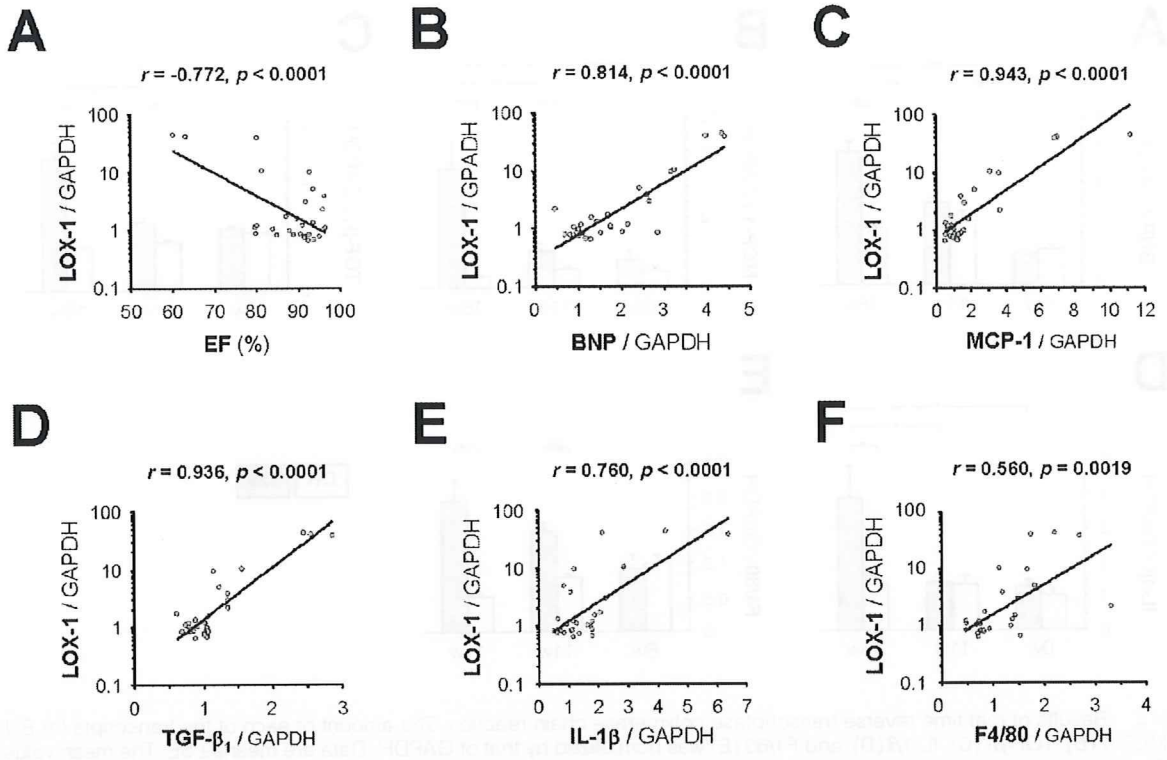

Figure 3. Correlations between the LV mRNA levels of LOX-1 and the parameters of heart failure. Abbreviations see in Tables 1 and 2.

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.

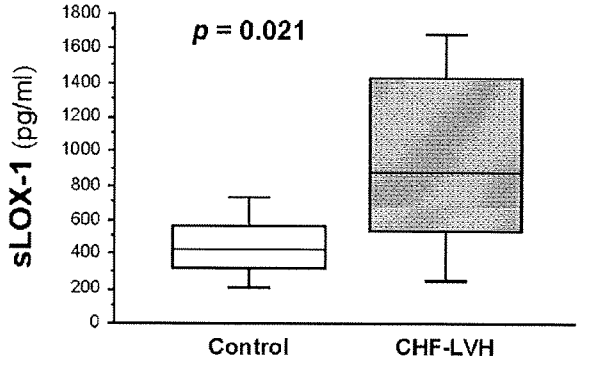

Figure 4. Serum levels of soluble LOX-1 (sLOX-1) in chronic heart failure patients with left ventricular hypertrophy (CHF-LVH, n=7) and those without organic cardiac diseases (control, n=11). LOX-1, lectin-like oxidized low-density lipoprotein receptor-1.

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

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

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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

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[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.

of monocyte chemoattractant protein 1 (MCP-1) and adhesion molecules *(11–13)*. In addition to oxidized LDL, LOX-1 binds various ligands, e.g., apoptotic cells, activated platelets, leukocytes, and C-reactive protein *(14–17)*. Accumulating evidence suggests that LOX-1 is involved in endothelial dysfunction, inflammation, atherogenesis, myocardial infarction, and intimal thickening after balloon catheter injury *(16, 18–23)*.

Recently, we developed a system to measure the biological activity of apolipoprotein B (ApoB)-containing lipoprotein based on binding to LOX-1 *(24)*. The activity of LOX-1 ligand containing ApoB (LAB) might reflect atherogenicity of LDL better than measurements of oxidized lipids, oxidized LDL, and LDL. In addition, recent reports have shown that the serum concentrations of soluble LOX-1 (sLOX-1), which is released from the cell surface by proteolysis of LOX-1, might be a useful biomarker for the diagnosis of acute coronary syndrome *(25, 26)*. Accordingly, we hypothesized that the product of LAB and sLOX-1, here designated "LOX index," might be an even better marker reflecting the interaction of atherogenic lipoproteins and their receptors.

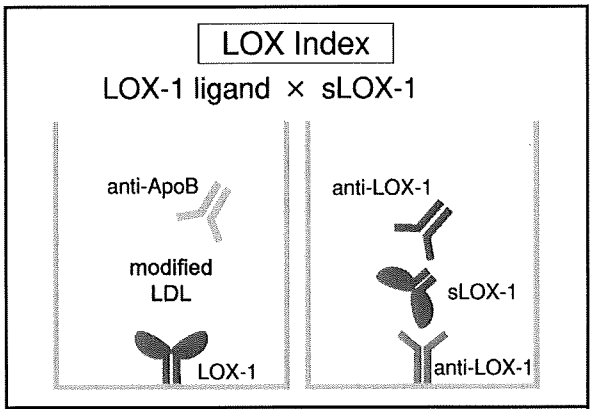

Fig. 1. Scheme of the detection systems for LAB and sLOX-1 to determine LOX index.

Materials and Methods

STUDY POPULATION

The Suita Study is a population-based cohort study in an urban area performed by the National Cardiovascular Center, the details of which have been reported *(2, 27, 28)*. Briefly, in 1989, 6485 men and women, aged 30–79 years, were enrolled as study participants randomly selected from the community of Suita City. They underwent medical examinations every 2 years. In these participants, we set the baseline of the present study as the medical examination held between April 1994 and February 1995, since at that time serum samples were collected and stored at −80 °C. During this 10-month time period, 2437 participants were followed until December 31, 2007. Of these, 142 participants were excluded or the following reasons: history of CHD or stroke (n = 94), lost to follow-up (n = 17), and other reasons such as missing data (n = 31). Data from the remaining 2295 participants (1094 men and 1201 women) were included in the analysis. Informed consent was obtained from all participants. This study was approved by the institutional review board at the National Cardiovascular Center.

BASELINE MEDICAL EXAMINATION

A baseline survey included questionnaires, anthropometric measurements, and blood sample testing after overnight fasting (at least 10 h). Height and weight were measured in light clothing, and body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Blood pressure of participants in a sitting position after at least 5 min of rest was measured 3 times by well-trained physicians, using a standard mercury sphygmomanometer *(27)*. The average of the second and third measurement was used in the analysis. Hypertension was defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, and/or the use of antihypertensive agents. Serum total cholesterol (TC), HDL cholesterol, and fasting serum glucose were analyzed with an automated analyzer at the laboratory of the National Cardiovascular Center. Non-HDL cholesterol was calculated by subtracting HDL cholesterol from TC. Diabetes was defined as serum glucose concentrations ≥7.0 mmol/L (126 mg/dL) in fasting or ≥11.1 mmol/L (200 mg/dL) in nonfasting samples and/or current use of medications for diabetes. Well-trained health nurses obtained information on the smoking, alcohol drinking, and medical histories of the participants.

MEASUREMENT OF LAB

A schematic presentation of the detection system for sLOX-1 and LAB is shown in Fig. 1.

We immobilized recombinant human LOX-1(61–273) (0.25 μg/well) on 384-well plates (Greiner 384 Plate High Bind 781061) by incubating overnight at 4 °C in 50 μL PBS *(9, 24)*. After 3 washes with PBS, we blocked the plates with 80 μL of 3% BSA in HEPES buffer (10 mol/L HEPES, 150 mmol/L NaCl, pH 7.0). After 3 washes with PBS, the plates were incubated for 2 h at room temperature with 40 μL standard oxidized LDL or samples. We prepared samples by 20-fold dilution of serum with EDTA-BSA-HEPES buffer [2 mmol/L EDTA, 5% BSA/HEPES buffer (10 mmol/L HEPES, 150 mmol/L NaCl, pH 7.0)] and standards by dilution of oxidized LDL with EDTA-BSA-HEPES buffer. After 3 washes with PBS, the plates were incu-

bated for 1 h at room temperature with 83 μg/L chicken monoclonal anti-ApoB antibody (HUC20) in EDTA-BSA-HEPES buffer *(24)*. After 3 washes with PBS, the plates were incubated for 1 h at room temperature with peroxidase-conjugated donkey antichicken IgY (AP194P; Chemicon) diluted 6000 times with EDTA-BSA-HEPES buffer. After 5 washes with PBS, we added the substrate solution containing 3,3′5,5′-tetramethylbenzidine (TMB solution; Bio-Rad) to the plates and incubated them for 30 min at room temperature. The reaction was terminated with 2 mol/L sulfuric acid. We determined peroxidase activity by measuring absorbance at 450 nm; the functional sensitivity of the measurement was 7.8 μg/L, and the range of the measurement was 7.8–500 μg/L oxidized LDL. Imprecision (CV) was 7.5% intraassay and 12.5% interassay at 50 μg/L (n = 10).

MEASUREMENT OF sLOX-1

We immobilized antihuman LOX-1 antibody (TS92, 0.25 μg/well) on 384-well plates (Corning 384 Plate High Bind 3700) by incubating overnight at 4 °C in 50 μL PBS *(9)*. After 3 washes with PBS, the plates were blocked with 20% ImmunoBlock (DS Pharma). After 3 washes with PBS, the plates were incubated with 40 μL standard oxidized LDL or samples for 2 h at room temperature. We prepared samples by 4-fold dilution of the serum with 1% BSA/PBS containing 0.04% Tween20 and 2 mmol/L EDTA and also by dilution of recombinant extracellular LOX-1(61–273) with the same buffer. After 3 washes with PBS, the plates were incubated with 0.16 μg/mL chicken monoclonal antihuman LOX-1 antibody (HUC5–40) in PBS containing 0.04% Tween20 and 2 mmol/L EDTA for 1 h at room temperature *(29)*. After 3 washes with PBS, the plates were incubated with the peroxidase-conjugated donkey antichicken IgY diluted 5000 times. After 5 washes with PBS, the substrate solution containing TMB solution was added to the plates and incubated for 30 min at room temperature. The reaction was terminated with 2 mol/L sulfuric acid. We determined peroxidase activity by measuring absorbance at 450 nm; the functional sensitivity of the measurement was 15.6 ng/L and the range of the measurement of sLOX-1 was 15.6–2500 ng/L. Imprecision (CV) was 8.5% intraassay and 14.7% interassay at 150 ng/L (n = 10).

ENDPOINT DETERMINATION

The method of endpoint determination for the Suita Study has been reported *(2, 27, 28)*. The endpoints of the current follow-up study were (1) date of first CHD or stroke event; (2) date of death; (3) date of leaving Suita city; and (4) December 31, 2007.

The first step in the survey for CHD and stroke involved checking the health status of all participants by repeated clinical visits every 2 years and yearly questionnaires sent by mail or conducted by telephone. In the second step, in-hospital medical records of participants who were suspected of having CHD or stroke were reviewed by registered hospital physicians or research physicians who were blinded to the baseline information. To complete the surveillance for fatal CHD and stroke, we conducted a systematic search for death certificates. The criteria for stroke were defined according to US National Survey of Stroke criteria *(30)*. Classification of patients into stroke subtypes (ischemic stroke, intracerebral hemorrhage, and subarachnoid hemorrhage) was based on examination of computed tomography, magnetic resonance imaging, or autopsy. Definite and probable myocardial infarction (MI) were defined according to the criteria of the MONICA (Monitoring Trends and Determinants of Cardiovascular Disease) project *(31)*. The criteria for a diagnosis of CHD included first-ever MI, coronary artery bypass surgery, or angioplasty. Sudden deaths of unknown origin that occurred within 24 h from onset were classified as CHD in the present study. We also defined cardiovascular disease (CVD) as a composite outcome of CHD or stroke.

STATISTICAL ANALYSIS

In addition to sLOX and LAB, we calculated the LOX index by multiplying serum concentrations of sLOX-1 by those of LAB. We set the cutoff points of sLOX, LAB, and LOX index according to the quartile ranges. Statistical methods of data analysis included ANOVA for assessing mean differences between groups and χ^2 tests for proportions. Multivariable analysis combining patients of both sexes was performed because there was no interaction between sex and LOX index (or LAB, sLOX) on the incidence of CHD or stroke. We calculated the multivariable-adjusted hazard ratios (HRs) of sLOX, LAB, and LOX index for CHD or stroke using a proportional hazards regression model after adjusting for sex, age, hypertension, diabetes, use of lipid-lowering agent, BMI, and current smoking and alcohol drinking (model 1). Further adjustment for non-HDLC was also performed (model 2). All CIs were estimated at the 95% level, and significance was set at $P < 0.05$. We used the SAS Statistical Package (release version 8.2, SAS Institute) for all the analyses.

Results

LAB and sLOX-1 concentrations at baseline [mean (SD)] were 516.1 (17.1) μg/L and 1060.1 (8.6) ng/L in men and 782.3 (23.7) μg/L and 797.8 (0.2) ng/L in women. The mean baseline serum TC was 181.5 (1.3) mg/dL [4.70 (0.034) mmol/L] in men and 224.5 (2.0) mg/dL [5.81 (0.052) mmol/L] in women in this population. Table 1

Table 1. Sex-specific means and prevalence of risk factors according to LAB or sLOX-1 quartiles at the baseline survey.[a]

	Mean LAB, μg/L (range)					Mean sLOX1, ng/L (range)				
	Q1, 217 (21–349)	Q2, 496 (350–644)	Q3, 870 (649–1128)	Q4, 1931 (1133–6379)	*P* (trend)	Q1, 558 (85–754)	Q2, 925 (755–1085)	Q3, 1289 (1084–1534)	Q4, 2367 (1540–9874)	*P* (trend)
Men										
n	261	257	288	288		229	251	284	330	
Age, years	61 (12)	60 (12)	60 (12)	59 (12)	0.41	62 (12)	61 (13)	60 (13)	59 (12)	0.01
TC										
mg/dL	186 (30)	195 (30)	192 (33)	203 (30)	<0.001	195 (33)	194 (29)	193 (34)	196 (32)	0.65
mmol/L	4.82 (0.78)	5.05 (0.78)	4.97 (0.85)	5.26 (0.78)		5.05 (0.85)	5.02 (0.75)	5.00 (0.88)	5.08 (0.83)	
HDL cholesterol										
mg/dL	54 (14)	56 (14)	54 (15)	53 (12)	0.06	54 (14)	55 (15)	55 (14)	53 (13)	0.33
mmol/L	1.40 (0.36)	1.45 (0.36)	1.40 (0.39)	1.37 (0.31)		1.40 (0.36)	1.42 (0.39)	1.42 (0.36)	1.37 (0.34)	
Non-HDL cholesterol										
mg/dL	132 (31)	139 (32)	139 (34)	151 (32)	<0.001	141 (33)	139 (30)	138 (34)	142 (34)	0.35
mmol/L	3.42 (0.80)	3.60 (0.83)	3.60 (0.88)	3.91 (0.83)		3.65 (0.85)	3.60 (0.78)	3.57 (0.88)	3.68 (0.88)	
Hypertension, %[b]	35	37	37	39	0.96	34	37	37	39	0.99
Lipid-lowering agent use, %	4	4	3	5	0.53	5	4	4	4	0.88
Diabetes, %[c]	7	6	5	6	0.79	6	6	7	5	0.62
Current smoking, %	41	44	43	39	0.71	32	37	45	48	<0.001
Current alcohol drinking, %[d]	69	68	71	67	0.75	70	73	65	67	0.21
Women										
n	312	317	286	286		344	323	290	244	
Age, years	57 (12)	59 (12)	57 (12)	57 (12)	0.05	58 (12)	57 (12)	57 (13)	58 (13)	0.71
TC										
mg/dL	200 (32)	204 (33)	207 (32)	216 (37)	<0.001	208 (33)	207 (35)	203 (34)	207 (35)	0.19
mmol/L	5.18 (0.83)	5.28 (0.85)	5.36 (0.83)	5.59 (0.96)		5.39 (0.85)	5.36 (0.91)	5.26 (0.88)	5.36 (0.91)	
HDL cholesterol										
mg/dL	62 (14)	62 (14)	61 (13)	59 (13)	0.06	62 (14)	62 (13)	60 (13)	60 (13)	0.04
mmol/L	1.61 (0.36)	1.61 (0.36)	1.58 (0.34)	1.53 (0.34)		1.61 (0.36)	1.61 (0.34)	1.55 (0.34)	1.55 (0.34)	
Non-HDL cholesterol										
mg/dL	138 (32)	142 (33)	146 (34)	156 (39)	<0.001	146 (34)	145 (36)	143 (35)	148 (36)	0.48
mmol/L	3.57 (0.83)	3.68 (0.85)	3.78 (0.88)	4.04 (1.01)		3.78 (0.88)	3.76 (0.93)	3.70 (0.91)	3.83 (0.93)	
Hypertension, %	33	27	30	32	0.84	29	30	30	34	0.42
Lipid-lowering agent use, %	8	5	7	6	0.27	8	5	7	7	0.62
Diabetes, %	3	3	3	5	0.26	3	3	4	4	0.72
Current smoking, %	7	12	10	10	0.31	6	8	11	16	<0.001
Current alcohol drinking, %	25	28	26	24	0.84	25	27	23	28	0.52

[a] Data are mean (SD) unless noted otherwise.
[b] Hypertension was defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, or use of antihypertensive agents.
[c] Diabetes was defined as fasting serum glucose ≥7.0 mmol/L (126 mg/dL), use of antidiabetic agents, or both.
[d] Alcohol drinking was defined as consuming at least 1 drink per week.

shows the baseline characteristics of the participants in each LAB or sLOX-1 quartile. In both sexes, there were significant differences in the mean concentrations for TC and non-HDL cholesterol according to LAB quartile, with higher concentrations in the higher LAB quartiles. Conversely, serum TC and non-HDLC were not found to be associated with sLOX-1 quartiles. In women, HDL cholesterol was lower in the higher LOX-1 quartiles. The prevalence of smoking was higher in the upper sLOX-1 quartiles but was not associated with LAB. There were no significant differences across quartiles in the prevalence of hypertension and diabetes.

During the mean follow-up period of 11 years, there were 68 incident cases of CHD and 91 cases of stroke, including 60 cases of ischemic stroke. The number of incident cases and multivariable-adjusted HRs for CVD, stroke, ischemic stroke, and CHD stratified by LAB and sLOX-1 are shown in Table 2. The HRs for stroke, ischemic stroke, and CHD were highest in the highest LAB quartile, and except for CHD, the trends in HRs across quartiles were statistically significant. The HR for CVD was highest in the top quartile of LAB, and the trend across quartiles was statistically significant. For sLOX-1, however, the across-quartile trends in HRs did not reach statistical significance.

The number of incident cases and multivariable-adjusted HRs for CVD, stroke, ischemic stroke, and CHD stratified by LOX index (LAB × sLOX) are shown in Table 3. The HR for ischemic stroke was constantly high from the second to the highest quartile: 3.39 (95% CI 1.34–8.53), 3.15 (1.22–8.13), and 3.23 (1.24–8.37), respectively. Furthermore, the HR of the highest quartile of LOX index was 2.09 (1.00–4.35) for CHD. In the highest LOX index quartile, the incidence of CVD was approximately 2-fold that in the lowest LOX index quartile, and the associated HR was 1.83 (1.03–2.96).

After additional adjustment for HDL cholesterol or the exclusion of sudden cardiac death from CHD, the results of all the analyses listed above remained the same (data not shown).

Discussion

In the present study, we followed 1094 men and 1201 women for a mean period of 11 years to investigate the impact of LAB, sLOX-1, and LOX index (LAB × sLOX) on the incidence of CVD. We found LAB and LOX index to be significantly associated with the incidence of CVD and CHD, especially ischemic stroke. This investigation is the first cohort study on the relationship between CVD and LOX index–related or oxidized LDL–related parameters in a general population.

Instead of oxidized LDL, here we measured the serum concentrations of LAB. Researchers have applied several methods to measure circulating concentrations of oxidized LDL, including measurement of oxidation-dependent epitopes in the ApoB moiety of LDL. These methods, however, which evaluate the amount of oxidized moiety on LDL, do not necessarily reflect biological activity. In contrast, the present assay system using recombinant LOX-1 and anti-ApoB antibody has the ability to evaluate the biological activity of the atherogenic lipoproteins (Fig. 1). On the other hand, circulating sLOX-1 concentrations might reflect the expression levels of LOX-1, the target site of the atherogenic lipoproteins in vascular wall. Therefore, LOX index (LAB × sLOX-1) could represent ligand (LAB)–receptor (LOX-1) interaction leading to vascular dysfunction. The present results confirmed LOX index as a predictor of the incidence of CVD. This suggests that LOX-1 may be important in the pathogenesis of CVD, and indicates that the evaluation of LOX-1–mediated signaling may serve as a potential tool for risk stratification.

It is well known that increased blood pressure, smoking, diabetes, and atrial fibrillation are major risk factors for stroke, especially for ischemic stroke *(32)*. In contrast with these risk factors, we found either no relationship or a weakly positive one between TC or LDL cholesterol and ischemic stroke in several cohort studies performed in the Japanese population *(32–34)*. A large metaanalysis of individual data from 61 prospective studies performed mainly in Western populations also showed no association between TC or non-HDL cholesterol and stroke mortality *(4)*. We recently reported that we found no association between LDL cholesterol or non-HDL cholesterol concentrations and the incidence of ischemic stroke in this cohort using another baseline survey, shortly before that of the present study *(2)*. In contrast, the present investigation demonstrated that the LOX index is a predictor of not only CHD but also ischemic stroke. A strong association of LOX-1 with ischemic stroke in experimental models has been reported. For example, expression of LOX-1 and MCP-1 is increased in the early stage of atherosclerotic changes of common carotid arteries in spontaneously hypertensive rats *(35)*. Schwarz et al. *(36)* reported that LOX-1 expression was induced >10-fold at ischemic core sites during experimental stroke. Furthermore, we found that LOX-1 contributed to the formation of arterial thrombus (unpublished data). Thus, activation of LOX-1 might facilitate the pathophysiological conditions leading to stroke. In the present study, the risk for ischemic stroke was significantly increased from the second to the fourth quartile of LOX index, which suggests a protective role

Table 2. Age- and multivariable-adjusted odds ratios (95% CIs) for the incidence of cardiovascular disease and its subtypes according to LAB and sLOX-1 quartiles.

	Mean LAB, μg/L (range)					Mean sLOX1, ng/L (range)				
	Q1, 217 (21–349)	Q2, 496 (350–644)	Q3, 870 (649–1128)	Q4, 1931 (1133–6379)	*P* (trend)	Q1, 558 (85–754)	Q2, 925 (755–1085)	Q3, 1289 (1084–1534)	Q4, 2367 (1540–9874)	*P* (trend)
Person-years	6447	6343	6323	6156		6376	6446	6173	6276	
Stroke										
Cases, n	16	27	22	26		21	20	25	25	
Age-adjusted	1	1.60 (0.86–2.99)	1.44 (0.75–2.75)	1.86 (0.99–3.50)	0.09	1	0.93 (0.50–1.72)	1.18 (0.66–2.12)	1.17 (0.65–2.11)	0.45
Multivariable-adjusted, model 1[a]	1	1.68 (0.90–3.15)	1.49 (0.77–2.89)	2.07 (1.09–3.91)	0.05	1	0.89 (0.48–1.65)	1.16 (0.64–2.09	1.12 (0.61–2.03)	0.52
Multivariable-adjusted, model 2	1	1.69 (0.90–3.17)	1.50 (0.77–2.90)	2.09 (1.10–3.98)	0.05	1	0.89 (0.48–1.66)	1.17 (0.64–2.10)	1.12 (0.61–2.04)	0.52
Ischemic stroke										
Cases, n	9	18	13	20		12	17	13	18	
Age-adjusted	1	1.99 (0.89–4.44)	1.51 (0.64–3.56)	2.62 (1.18–5.79)	0.04	1	1.37 (0.65–2.87)	1.02 (0.46–2.25)	1.34 (0.64–2.81)	0.63
Multivariable-adjusted, model 1	1	2.12 (0.94–4.77)	1.63 (0.68–3.90)	3.18 (1.41–7.12)	0.01	1	1.38 (0.65–2.94)	1.02 (0.46–2.25)	1.29 (0.60–2.74)	0.72
Multivariable-adjusted, model 2	1	2.10 (0.93–4.74)	1.62 (0.67–3.88)	3.11 (1.37–7.04)	0.15	1	1.38 (0.65–2.94)	1.03 (0.46–2.29)	1.29 (0.60–2.74)	0.71
CHD										
Cases, n	12	18	15	23		12	17	23	16	
Age-adjusted	1	1.46 (0.69–3.07)	1.35 (0.63–2.90)	2.02 (1.00–4.08)	0.06	1	1.47 (0.69–3.16)	2.12 (1.03–4.37)	1.44 (0.66–3.12)	0.24
Multivariable-adjusted, model 1	1	1.54 (0.73–3.26)	1.44 (0.66–3.16)	2.18 (1.06–4.44)	0.04	1	1.53 (0.71–3.30)	1.97 (0.95–4.08)	1.42 (0.64–3.07)	0.31
Multivariable-adjusted, model 2	1	1.42 (0.67–3.02)	1.35 (0.62–2.96)	1.82 (0.88–3.76)	0.13	1	1.66 (0.77–3.58)	2.13 (1.02–4.42)	1.47 (0.67–3.21)	0.27
Cardiovascular disease										
Cases, n	28	45	37	49		33	37	48	41	
Age-adjusted	1	1.52 (0.94–2.45)	1.38 (0.84–2.26)	1.89 (1.18–3.02)	0.02	1	1.13 (0.70–1.81)	1.50 (0.96–2.35)	1.26 (0.79–2.02)	0.18
Multivariable-adjusted, model 1	1	1.60 (0.99–2.59)	1.44 (0.87–2.38)	2.05 (1.28–3.29)	0.01	1	1.11 (0.69–1.80)	1.43 (0.91–2.24)	1.21 (0.75–1.94)	0.27
Multivariable-adjusted, model 2	1	1.56 (0.96–2.52)	1.41 (0.85–2.32)	1.91 (1.18–3.08)	0.02	1	1.13 (0.70–1.83)	1.48 (0.94–2.33)	1.21 (0.76–1.95)	0.26

[a] Model 1 adjusted for age, sex, BMI, smoking, drinking, hypertension, diabetes, and use of lipid-lowering agents; model 2 as model 1 with the addition of non-HDL cholesterol.

Table 3. Age- and multivariable-adjusted odds ratios (95% CIs) for the incidence of cardiovascular disease and its subtypes according to LOX index ($\times 10^6$) quartiles.

	Mean LOX index (range)				
	Q1, 0.21 (0.017–0.363)	Q2, 0.52 (0.364–0.7040)	Q3, 0.97 (0.7043–1.314)	Q4, 2.64 (1.315–44.22)	*P* (trend)
Person-years	6416	6396	6314	6144	
Stroke					
Cases, n	17	24	25	25	
Age-adjusted	1	1.49 (0.80–2.80)	1.50 (0.80–2.79)	1.68 (0.90–3.14)	0.12
Multivariable-adjusted, model 1[a]	1	1.44 (0.76–2.71)	1.59 (0.85–2.99)	1.74 (0.92–3.28)	0.08
Multivariable-adjusted, model 2	1	1.44 (0.76–2.71)	1.60 (0.85–3.00)	1.74 (0.92–3.30)	0.09
Ischemic stroke					
Cases, n	6	20	17	17	
Age-adjusted	1	3.54 (1.41–8.87)	2.80 (1.10–7.14)	3.03 (1.18–7.74)	0.07
Multivariable-adjusted, model 1	1	3.40 (1.35–8.56)	3.22 (1.25–8.29)	3.31 (1.28–8.56)	0.03
Multivariable-adjusted, model 2	1	3.39 (1.34–8.53)	3.15 (1.22–8.13)	3.23 (1.24–8.37)	0.04
CHD					
Cases, n	12	19	12	25	
Age-adjusted	1	1.81 (0.85–3.81)	1.07 (0.47–2.44)	2.40 (1.17–4.93)	0.05
Multivariable-adjusted, model 1	1	1.70 (0.80–3.64)	1.04 (0.45–2.40)	2.37 (1.15–4.90)	0.05
Multivariable-adjusted, model 2	1	1.67 (0.78–3.59)	1.02 (0.44–2.35)	2.09 (1.00–4.35)	0.11
Cardiovascular disease					
Cases, n	29	43	37	50	
Age-adjusted	1	1.58 (0.98–2.55)	1.31 (0.80–2.15)	1.92 (1.20–3.07)	0.02
Multivariable-adjusted, model 1	1	1.49 (0.92–2.42)	1.35 (0.82–2.23)	1.95 (1.21–3.13)	0.01
Multivariable-adjusted, model 2	1	1.48 (0.91–2.41)	1.31 (0.80–2.17)	1.83 (1.13–2.96)	0.03

[a] Model 1 adjusted for age, sex, BMI, smoking, drinking, hypertension, diabetes, and use of lipid-lowering agents; model 2 as model 1 with the addition of non-HDL cholesterol.

against ischemic stroke. Additional epidemiologic studies to establish clinical cut points of LOX index are warranted.

The positive relationship between LOX index and ischemic stroke bridged, for the first time, the missing link between stroke and cholesterol-related parameters. In those with high LOX index, breaking the interaction between LAB and LOX-1 might be effective in preventing stroke. The most straightforward approach would be to apply a LOX-1 antagonist, which is yet to be developed. Although most of the randomized controlled trials failed to find a beneficial effect of antioxidants for the prevention of CVD *(21)*, we may reduce LAB concentration per se by statin therapy, thereby increasing LDL receptor expression, leading to an increase in the turnover of LDL and a decrease in the chance of LDL modification *(37)*. Actually, in the present study, serum concentrations of LAB showed a significant association with TC and non-HDLC. In addition, a positive relationship between smoking and LOX-1 suggests the possibility of smoking cessation to reduce sLOX-1 concentration by decreasing the expression level of LOX-1.

The present study has some limitations. First, a recent report from the Hisayama study showed a positive relationship between LDL cholesterol and atherothrombotic infarction, which accounts for one fourth of all ischemic stroke *(38)*. Therefore, the relation between LOX index and each subtype of ischemic stroke would be worth analyzing; however, the relatively small sample size of the current study precludes such analysis. Second, the participants in the present investigation were all Japanese; therefore, the study should be repeated in other ethnic populations. Because the incidence of stroke in Japan is much greater than in other countries *(39)*, some Japanese-specific factors might be affecting the present findings. Finally, since the number of cardiovascular events was not sufficiently large to enable a sex-specific analysis, especially in women, we did not perform such analysis.