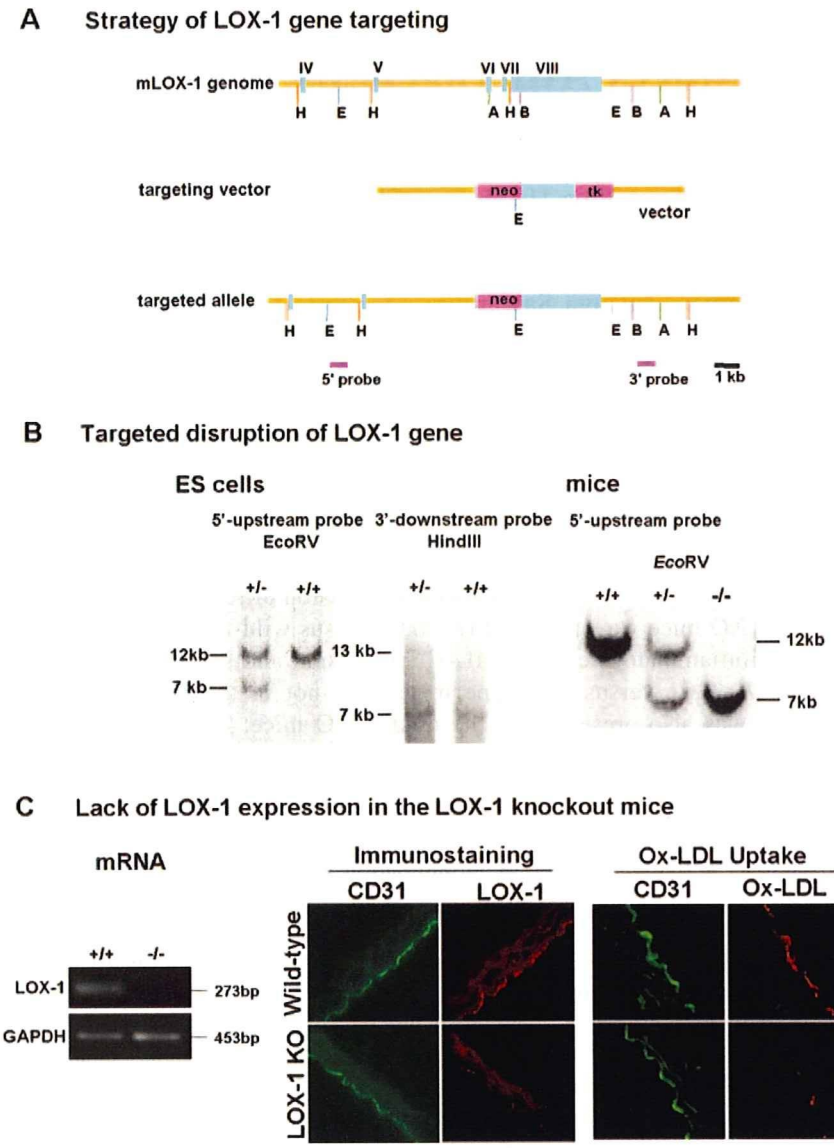

Figure 1. Strategy of LOX-1 gene targeting (A), targeted disruption of LOX-1 gene (B), and the absence of LOX-1 expression in the LOX-1 knockout mice (C). Note that ES cells (+/−) carrying disrupted LOX-1 gene show 7kb-band in the left panel and 13kb band in the middle panel. 12kb-band showing undisrupted LOX-1 gene disappears in the homozygous KO mice and has reduced signals on the heterozygous mice (B, right panel). The LOX-1 KO mice show no immunofluorescence signal for LOX-1 but do show signal for CD31. Uptake of oxLDL seen in the wild-type mice is not seen in the LOX-1 KO mice (C).

Materials and Methods

Generation of LOX-1 Gene Knockout Mice

Mouse LOX-1 gene was cloned by screening 129/SV mouse genomic DNA library made with Lambda FIXII by the use of mouse LOX-1 cDNA as a probe. The insert of a phage clone D was digested with SacI, and the resultant 3 fragments of the insert were subcloned into pBluescript SK(−) (Clone D3, D8, and D5). After sequencing and mapping with restriction enzymes, targeting vector was constructed according to the following strategy: (1) replacing a part of LOX-1 gene from the 6th to 8th exon with neomycin resistant gene (Figure 1A), (2) placing homologous part of LOX-1 gene at both sides of neomycin resistant gene (upstream: 3.8 kb of *ClaI*/*ApaI* digested fragment, downstream: 2 kb of *KpnI*/*Bam*HI fragment), (3) attaching thymidine kinase gene for negative selection at the downstream of the LOX-1 gene fragments (Figure 1A). The targeting vector was linearlized with *Xho*I digestion, and introduced by electroporation into 3×10^6 of RW-4 ES cells from 129/SvJ mice. Selection of ES clones was performed with 0.2 mg/mL G418,[23] and further screened with Southern blot analysis. Two homologously recombinated clones were obtained from 368 G418 resistant clones.

ES cells containing the disrupted gene were injected into blastocysts from C57BL/6 mice, and embryos were transferred to uteri of pseudopregnant mice. The chimeric mice were mated with C57BL/6 mice and resultant agouti mice were found to be carrying the disrupted LOX-1 gene. Offspring with the inactivated gene in germ line cells were backcrossed onto a C57BL/6 background 8 times. Homozygous LOX-1–deficient animals have no overt phenotype, and breed normally. The LOX-1 genotypes were verified by PCR analysis of genomic DNA extracted from tail with the primer pair for deleted portion of LOX-1 gene: 5′-GGCCAACCATGGCTTG GGAGAATGG-3′ and 5′-CAGCGAACACAGCTCCGTCTTG AAGG-3′; and for neomycin resistant gene: 5′-AGGATCTCGT CGTGACCCATGGCGA-3′ and 5′-GAGCGGCGATACCGTAA GCACGAGG-3′.

RT-PCR Analysis of LOX-1 Expression

Total RNA was extracted from the aortic lysate, and 1 μg was subjected to cDNA synthesis with Superscript II reverse transcriptase with random hexamer. Production was then subjected to PCR with LOX-1 primers (see above) producing 453 bp, and for GAPDH for internal standard: 5′-GACCACAGTCCATGACATGACATCA CT-3′ and 5′-TCCACCACCCTGTTGCTGTAG-3′ producing 273 bp. Thermal cycler condition was 94°C 40 sec, 60°C 1 minute, 72°C 1 minute repeated 35 times for LOX-1 and 25 times for GAPDH. Then, the PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide on UV transiluminator.

Immunofluorescence Staining

Expression of LOX-1 was analyzed in the cryothin sections of aorta with Cy3-labeled anti-mouse LOX-1 monoclonal antibody (1 μg/mL, JTX58). To confirm the presence of endothelium, immunostaining with biotinylated anti-CD31 (0.1 μg/mL) and avidin-FITC was performed and subjected to laser confocal microscopy.

Analysis of the Uptake of oxLDL

Thoracic aortas excised from mice were incubated for 12 hours at 37°C in DMEM/10% FCS containing 10 μg/mL DiI-labeled ox-LDL,[7] then washed with PBS 3 times and snap frozen. Cryothin sections were subjected to indirect immunofluorescence staining with biotinylated anti-CD31 and avidin-FITC to visualize endothelium and subjected to laser confocal microscopy.

Evaluation of Endothelial Nitric Oxide Synthase Activity

Aortic rings from wild-type male mice 10 weeks of age were used for vascular reactivity analysis.[24] Resting tension of the aortic ring was adjusted at 0.5 g. Contraction was induced with 5 μmol/L $PGF_{2\alpha}$. After stable contraction, endothelium-dependent vasorelaxation was induced with acetylcholine (Ach, 10^{-8} to 3×10^{-7} M). After washout, a second contraction was induced with $PGF_{2\alpha}$ in the presence of oxLDL 10 μg/mL. In some cases, aortic rings were preincubated with anti–LOX-1 antibody or nonspecific IgG (50 μg/mL) for 30 minutes.

Preparation of LDLR/LOX-1 Double Knockout Mice

Wild-type and homozygous LDLR KO mice were obtained from Jackson Laboratories (Bar Harbor, Maine). LDLR KO and LOX-1 KO mice were bred by brother-sister mating. First generation animals were all $LDLR^{+/-}$/$LOX\text{-}1^{+/-}$, which were cross-bred with either LDLR KO or LOX-1 KO. The genotype of second generation animals was examined by PCR, and LDLR KO/LOX-1 KO (double KO) mice were selected. They were housed in a room lit from 6:00 AM to 6:00 PM and kept at 21°C. All animal were given a high-cholesterol diet (4% cholesterol/10% cocoa butter) for 18 weeks from the age of 6 weeks. All experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Usage Committee.

Analysis of mRNA Expression by RT-PCR

Aortic specimens were derived from animals at 18 weeks. The mRNA isolation and RT-PCR amplification analysis of LOX-1 were performed as described previously.[13,14]

Analysis of Protein Expression

Standard methodologies[8–11,21] were used for determination of protein expression (Western blotting) and localization (immunohistochemistry). Relative (to β-actin) intensities of protein bands were analyzed. The intensity of immunohistochemistry stain was calculated by Image-Pro Plus program.

Superoxide Dismutase Activity

Mice liver homogenates were cleared by centrifugation at 15 000*g* for 30 minutes at 4°C, and supernatants were used for measurements of superoxide dismutase (SOD) activity as described previously.[25]

Analysis of Atherogenesis

After harvesting the animals, fatty deposits (index of atherosclerotic lesion formation) were quantitated as described previously.[26] Briefly, 5 to 7 mice from each group were euthanized and the aortas separated from surrounding tissues. After removal of the adventitial fat, aortas were opened longitudinally from the aorta arch to the iliac bifurcation, and fixed in 10% formalin for 24 hours. The aortas were then rinsed in 70% alcohol and stained with Sudan IV for 15 minutes. Aortas were then mounted and photographed with a camera connected to a dissection microscope. The images were analyzed by Image-Pro Plus (Media Cybernetics). The person performing the staining was blinded to the experimental protocol.

In the second method, entire aorta from the aortic arch above the aortic valves to the iliac bifurcation was stored in formalin and 5μ cross-sections were made at 5 predefined points (proximal ascending aorta, aortic arch, descending aorta, mid thoracic aorta, and abdominal aorta above the renal arteries). The sections were stained with H&E. In each case, the average value in each animal was used for measurement of intima thickness.

Measurement of Plasma Lipids by Nuclear Magnetic Resonance

The lipid components from 50 μL of plasma were isolated by the acetonitrile extraction followed by chloroform:methanol (2:1 volume ratio) extraction.[27] NMR spectra were acquired using a Bruker Avance spectrometer operating at 600.133 MHz for proton and equipped with a 5-mm triple resonance cryoprobe. Spectra were referenced to internal trimethylsilyl-2,2,–3,3-tetradeuteropropionic acid (TMSP). A total of 128 transients were collected for plasma extracts with a spectral width of 7183.91 Hz. The pulse width for proton was 8.10 μsec at a power level of 2.60 dβ.

Gradient-enhanced, phase sensitive ^{1}H-^{13}C HSQC spectra were acquired with a spectral width of 16.0221 ppm in the proton dimension and a width of 166.4892 ppm in the carbon dimension. For the plasma extracts, 104 transients were acquired with 1024 points in the time domain (t_2). A relaxation delay of 2.5 sec and 256 t_1 increments were used. The pulse width for proton was 11.00 μs at 2.40 dβ, whereas the pulse width for carbon was 16.00 μs at a power level of −1.40 dβ.

Spectra were processed using ACD/Labs 1D NMR Manager. All spectra were zero filled to 131 072 points followed by multiplication by a 0.3-Hz line broadening factor. Spectra were then Fourier transformed, phased using the "simple" method, and baseline corrected using the "Sp Averaging" method with a box half width of 61 and noise factor of 3 and autoreferenced to TMSP at 0.00 ppm. The final size in the t_2 dimension was 1024, whereas the final size in the t_1 dimension was 512. A squared sine bell apodization function and forward linear prediction applied before transformation along the t_1 dimension. Integration was done using 2D NMR Manager. Proton and HSQC were also acquired for lipid standards to identify individual lipids in the 2D HSQC spectra.

Statistical Analysis

Data are expressed as mean±SEM. Between-group difference was evaluated by unpaired *t* test. All other data were analyzed by a 2-way analysis of variance with a Bonferroni post-hoc test. A $P<0.05$ was considered significant.

Results

Characterization of LOX-1 Knockout Mice

The homologous recombination between targeting vector and mouse LOX-1 gene in ES cells and germ line transmission of the targeted allele are shown in Figure 1B. Lack of LOX-1 in the LOX-1 KO mice was confirmed by RT-PCR analysis and immunostaining of aortic tissues (Figure 1C). LOX-1 was not detected in the LOX-1 KO mice aorta, although the presence of endothelium was confirmed by simultaneous staining of CD31. LOX-1 was clearly present in the wild-type mice. Further, the uptake of oxLDL in endothelium was undetectable in LOX-1 KO mice, but clearly evident in wild-type mice (Figure 1C, right).

Impact of LOX-1 Deletion on Endothelium-dependent Vasorelaxation

Removal of endothelium did not affect $PGF_{2\alpha}$-induced constriction, but eliminated Ach-induced relaxation (data not

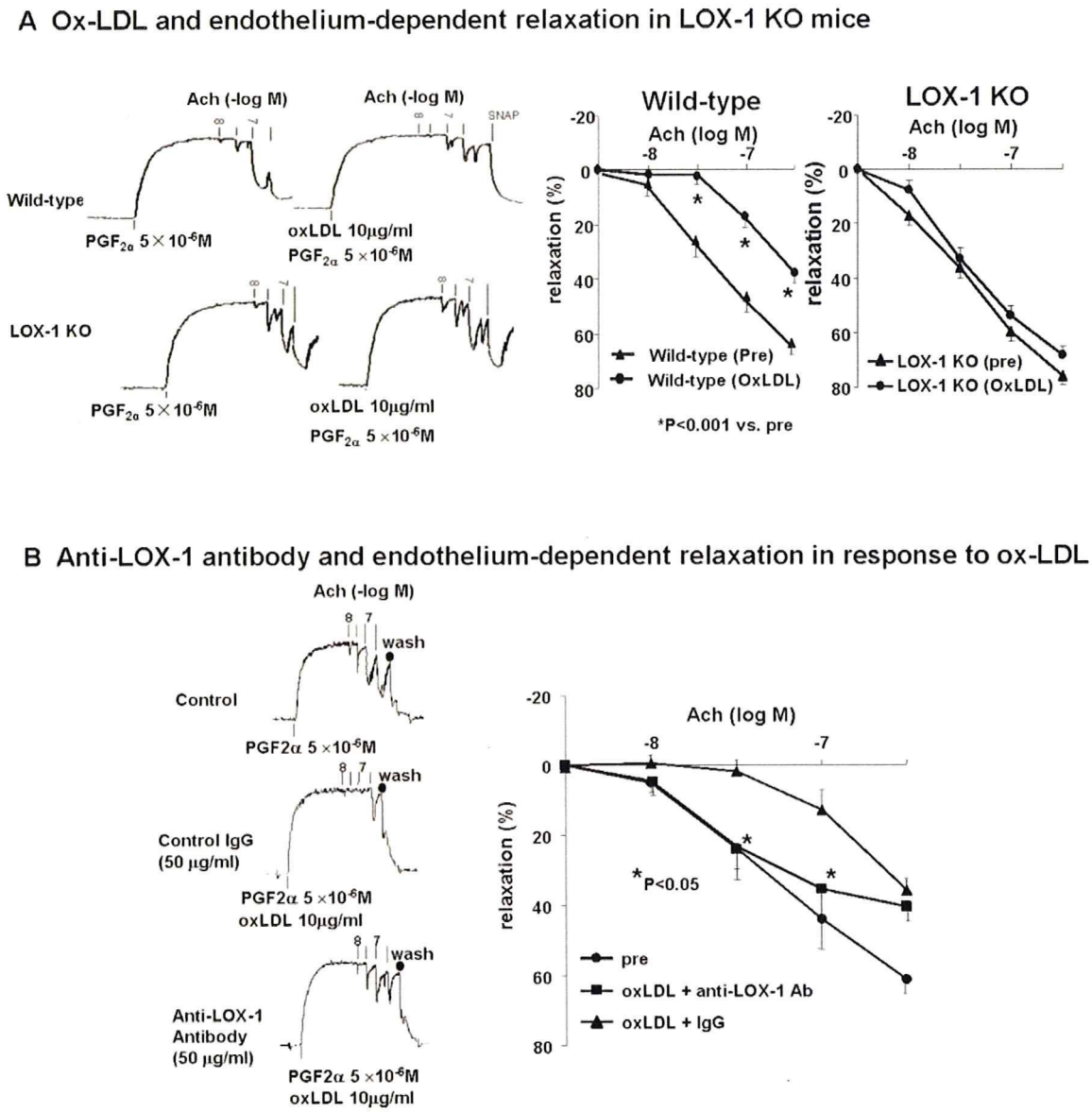

Figure 2. oxLDL and endothelium-dependent relaxation in the wild-type and LOX-1 KO mice. A, Loss of endothelium-dependent relaxation in response to oxLDL is absent in the LOX-1 KO mice. The NO donor SNAP induces relaxation of wild-type mice aortic rings treated with oxLDL. B, Loss of endothelium-dependent relaxation in response to oxLDL is abrogated by treatment of aortic rings by anti-LOX-1 antibody. The effects of anti–LOX-1 antibody are similar to those seen in the LOX-1 KO mice. Non-specific IgG was used as control for anti–LOX-1 antibody. The aortic rings were contracted with $PGF_{2\alpha}$ and endothelium-dependent relaxation assessed with exposure of precontracted rings to acetylcholine (Ach). Left panels show representative experiments and the right panels show summary (±SEM) data from multiple experiments.

shown). L-NAME, an inhibitor of endothelial nitric oxide synthase (eNOS), had the same effect as endothelium removal, indicating that NO released from endothelium mediates Ach-induced vasorelaxation in this system.

As shown in Figure 2A, basal relaxation in response to Ach was greater in aortic rings from LOX-1 KO mice (versus wild-type mice). Importantly, oxLDL treatment reduced Ach-induced relaxation in wild-type mice aortic rings, whereas SNAP, an NO donor, still induced relaxation. In contrast, Ach-induced relaxation was preserved in the LOX-1 KO mice aortic rings despite treatment with oxLDL. Next, we examined whether these changes in the LOX-1 KO mice are attributable to the deletion of LOX-1 by application of anti–LOX-1 antibody to wild-type mice aortic rings. As shown in Figure 2B, anti–LOX-1 antibody modulated the effects of oxLDL on Ach-induced relaxation, and the vasorelaxation became similar to that with LOX-1 ablation. The effects of oxLDL were abolished by the addition of SOD in the medium (data not shown), in accordance with the previous observation that the binding of oxLDL to LOX-1 induces the formation of ROS.[11]

High Cholesterol Diet and Increase in Plasma Lipids

Figure 3 (left) depicts the NMR proton spectra of lipids in plasma from different groups of mice. The HDL-cholesterol (0.77 to 0.83) was lower, and both LDL-cholesterol (region 0.84 to 0.88) and VLDL-cholesterol (0.88 to 0.92) higher in the double KO and LDLR KO mice ($P<0.01$ versus wild-type mice). There were no differences in LDL- and HDL-cholesterol peaks between the LDLR KO and double KO mice. Figure 3 (right) summarizes the data on the levels of HDL-, VLDL-, and LDL-cholesterol in different groups of mice.

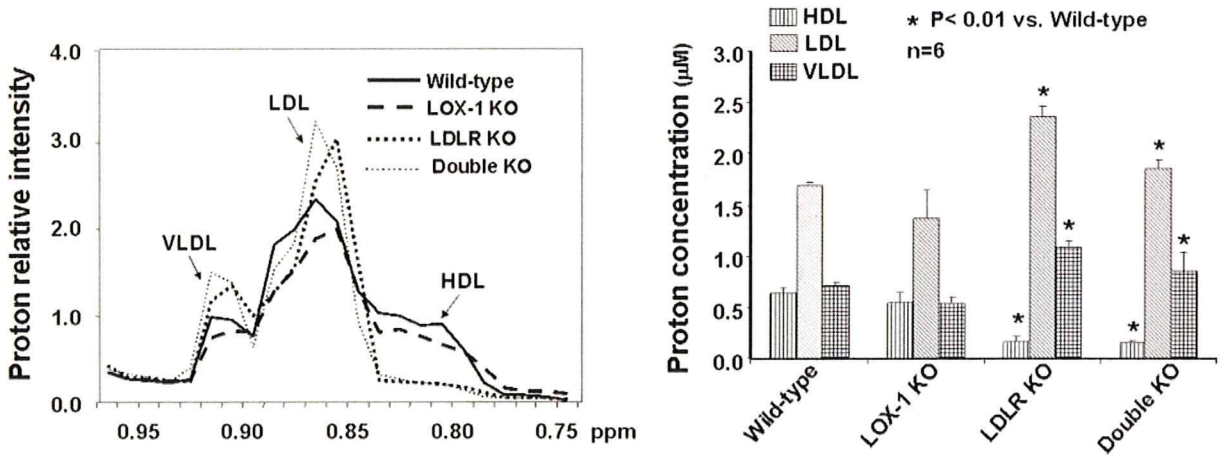

Figure 3. NMR spectra plot of the region around 0.77 to 0.94. The HDL-cholesterol levels are decreased, whereas LDL- and VLDL-cholesterol levels are higher in the double KO and LDLR KO mice (versus wild-type and LOX-1 KO mice; left panel). Bar graph on the right shows the summary (±SEM) data in plasma samples of different groups of mice.

Expression of LOX-1 and the Extent of Atherosclerosis

All wild-type mice exhibited modest LOX-1 expression. In keeping with previous data,[26] LDLR KO mice exhibited increased expression of LOX-1. The LOX-1 KO and double KO mice, as expected, did not express LOX-1 (Figure 4A).

There were small areas of sudanophilia in the wild-type mice; and the sudanophilic areas were much fewer in the LOX-1 KO mice ($P<0.01$ versus wild-type mice, Figure 4B). The LDLR KO mice showed extensive sudanophilia covering almost 60% to 80% of aortic surface; importantly, the sudanophilic areas were ≈50% less in the double KO mice. There was extensive intimal thickening in the LDLR KO mice with large areas of proliferation, which in several sections appeared to totally occlude the vascular lumen (see an example in Figure 4C). In contrast, the intimal thickening was much less in the double KO mice with relatively few occlusive lesions. The most advanced atherosclerotic lesion in a double KO mouse is shown in Figure 5C.

Mechanisms of Inhibition of Atherosclerosis

Endothelial continuity was studied by staining for von Willebrand factor. Figure 5 (left) shows endothelial disruption throughout the regions of plaque formation in the LDLR KO mice, particularly in areas with accumulation of macrophages and foam cells. On the other hand, LOX-1 deletion in the LDLR KO mice resulted in maintenance of endothelial continuity and few areas of macrophage accumulation. Figure 5 (right) shows marked reduction in eNOS expression in the LDLR KO mice and its restoration with LOX-1 deletion. Notably, LOX-1 KO mice had greater expression of eNOS ($P<0.01$ versus wild-type mice), complimenting the data on enhanced vasorelaxation in response to Ach (Figure 2A).

Atherosclerosis involves oxidative stress and inflammation.[3,28] In keeping with this concept, the expression of NF-κB was reduced in the LOX-1 KO mice (Figure 6A), indicating that LOX-1 deletion reduces basal expression of this transcription factor. The LDLR KO mice showed a marked increase in NF-κB expression ($P<0.01$ versus wild-type mice). Importantly, deletion of LOX-1 in the LDLR KO mice reduced NF-κB expression ($P<0.01$ versus LDLR KO mice).

Data on the expression of CD68, a general marker of macrophage infiltration,[29,30] are shown in Figure 6A (top right). LOX-1 KO mice exhibited much less CD68 expression ($P<0.01$ versus wild-type mice). There was a marked increase in CD68 expression in the LDLR KO mice ($P<0.01$ versus wild-type mice). In contrast, the LDLR KO mice with LOX-1 deletion exhibited much lower level of CD68 ($P<0.01$ versus LDLR KO mice).

Recent studies have shown reduced levels of the antiinflammatory cytokine IL-10 in atherosclerosis[31] and inhibition of atherogenesis in mice with IL-10 upregulation.[32] In this study, we found that the LDLR KO mice had reduced levels of IL-10 ($P<0.01$ versus wild-type mice). Importantly, deletion of LOX-1 significantly increased IL-10 expression in the wild-type and LDLR KO mice (Figure 6A, bottom left).

As a marker of antioxidant activity, we measured SOD activity in the mice liver. The LDLR KO mice were found to have less SOD activity (versus wild-type mice). Importantly, deletion of LOX-1 markedly increased SOD activity in the wild-type and LDLR KO mice, despite high-cholesterol diet. Importantly, extracellular SOD (ecSOD) expression was higher in the aortas of LDLR KO mice (versus wild-type mice), probably a response to increased oxidant stress. ecSOD expression increased further with LOX-1 deletion (Figure 6A, bottom right).

Next, we studied mitogen-activated protein kinase (MAPK) signaling in the presence of LOX-1 deletion. MAPK is a major signal in vascular inflammation.[33] As shown in Figure 6B, protein levels of both p38 and p44/22 MAPKs were not different in different groups of mice. Phosphorylated p38 MAPK levels was higher in the LDLR KO mice ($P<0.01$ versus wild-type mice), but not in the double KO mice. The levels of phosphorylated p42/44 MAPK were similar in all mice groups.

Discussion

Because endothelial dysfunction precedes morphological atherosclerotic changes, endothelial dysfunction has been hypothesized to be a key early lesion in atherogenesis.[4,34]

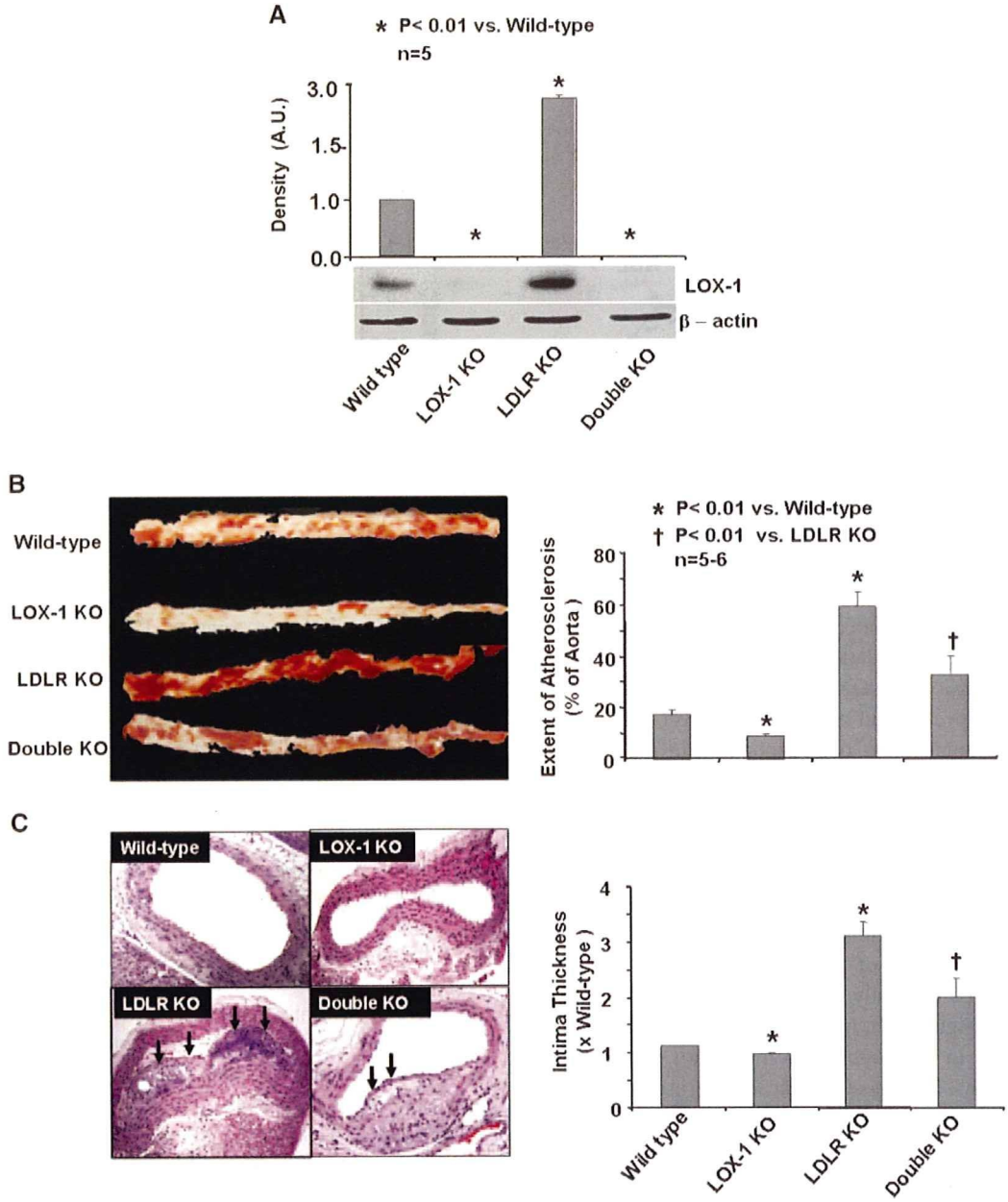

Figure 4. A, Expression of LOX-1 in various groups of mice. Note the absence of LOX-1 gene in the LOX-1 KO and double KO mice. B, Extent of atherosclerosis in different mice groups. LOX-1 KO mice have less atherosclerosis than wild-type mice, and LOX-1 ablation in the LDLR mice reduces atherosclerosis. Left panel shows representative aortas from each group and the bar graphs show summary (±SEM) data in each group. C, Intima thickness is significantly reduced in the LOX-1 KO mice (versus wild-type mice) and the double KO mice (versus LDLR KO mice). Left panel shows representative aortic sections. Arrows show regions of marked intimal thickening. Bar graphs show summary (±SEM) data in each group.

Among various insults to vascular endothelial cells, oxLDL has been postulated to be a major antigen.[34,35]

Ox-LDL is taken up in endothelial cells mostly via LOX-1.[7,8] LOX-1 activation in endothelial cells initiates a cascade of events that are intimately involved with atherogenesis.[11,12,21] The present study provides the missing link in this hypothesis, namely (1) endothelial dysfunction is mediated by LOX-1, and (2) LOX-1 is involved in the biology of atherosclerosis.

We show that Ach-mediated relaxation was greater at baseline in LOX-1 KO mice, probably reflecting the enhanced expression of eNOS in LOX-1 KO mice. Further, we demonstrate that the action of oxLDL is mediated, in large part, by LOX-1 activation. Pretreatment with anti-LOX-1 antibody protected the wild-type mice aortic rings from the adverse effects of oxLDL. Our data are supported by the recent description of the important role of LOX-1 in endothelial dysfunction in Apo-E KO mice.[36]

We also show that LOX-1 is causally involved in atherogenesis. We used LOX-1 KO mice backcrossed to C57BL/6 mice 8 times, and examined in 2 different conditions, namely, wild-type mice and LDLR KO mice with C57BL/6 genetic background all fed high cholesterol diet. Under these conditions, we observed a marked reduction in atherogenesis by LOX-1 ablation. SR-A and CD36 knockout mice all once were initially reported to reduce atherogen-

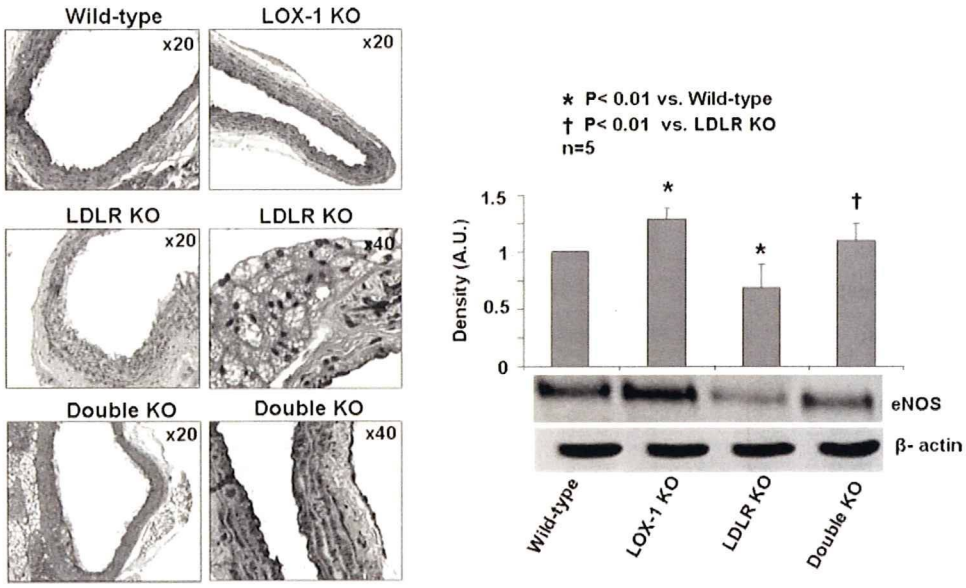

Figure 5. Left, Evidence of endothelial disruption throughout the areas of plaque formation in the LDLR KO mice, particularly in areas with accumulation of macrophages. On the other hand, double KO mice have maintenance of endothelial continuity. Right, There is marked reduction in eNOS expression in the LDLR KO mice and its restoration with LOX-1 deletion. LOX-1 KO mice have higher expression of eNOS (versus wild-type mice), complimenting the data on enhanced vascular relaxation in response to Ach shown in Figure 2. Data (±SEM) from 5 mice in each group.

esis.[37] However, subsequent reports showed opposite results.[38] This has led to the controversy on the role of these receptors in atherogenesis. Results of our study are important because LOX-1 is mainly expressed on endothelial cells.

The wild-type mice in our study had modest LOX-1 expression as well as atherosclerosis—most likely a result of high levels of LDL-cholesterol in response to 4% cholesterol/10% cocoa butter diet (Figure 3). On the other hand, LOX-1 KO mice had no LOX-1 expression and minimal atherosclerosis despite the same lipid levels. In a previous study in Apo-E KO mice,[26] we showed that lowering of LDL-cholesterol with an HMG CoA reductase inhibitor lowers LOX-1 expression. The basal expression of LOX-1 may be responsible for the basal state of oxidant stress and inflammation. In keeping with this suggestion, LOX-1 deletion reduced the expression of NF-κB and CD 68 as well as phosphorylated p38-MAPK.

Nuclear translocation of p65 has been observed in human and experimental atherosclerosis.[39,40] The canonical pathway of NF-κB activation that involves p65 is activated in atherosclerosis and results in selective upregulation of major proinflammatory mediators of the disease.[41] These findings suggest that the increased expression of p65 in our study is indicative of its nuclear translocation, resulting in increased inflammatory stress.

Data on LOX-1 deletion in the LDLR KO mice are particularly noteworthy. The double KO mice had much less atherosclerosis than the LDLR KO mice; the latter showed a marked increase in NF-κB and CD68 expression and a dramatic reduction in SOD activity and IL-10 expression. Deletion of LOX-1 in the LDLR KO mice attenuated these abnormal signals related to oxidant stress and inflammation. Recent studies show that upregulation of the antiinflammatory cytokine IL-10 reduces atherogenesis in the LDLR KO mice,[32,42] and IL-10 deficiency increases atherosclerosis in Apo-E KO mice.[43] Likewise, enhancement of antioxidant mechanisms has salutary effect on atherogenesis.[44]

Atherogenesis in LDLR KO mice was associated with enhanced phophorylation of p38 MAPK, but not of p44/42 MAPK, suggesting activation of proinflammatory pathways.[33] A previous in vitro study showed that oxLDL-mediated activation of LOX-1 is associated with p38 MAPK activation, and pretreatment of cells with antisense to LOX-1 blocks p38 MAPK activation.[45] Others have shown that p44/42 MAPK phosphorylation is also mediated by LOX-1.[46] However, we did not observe any changes in p44/42 MAPK phosphorylation, and the difference in the 2 studies may be a reflection of study design. The present study suggests a link between LOX-1 activation, oxidant stress, and atherogenesis in vivo. It is of note that the deletion of LOX-1 was not associated with any significant alteration in the levels of LDL-cholesterol.

We also demonstrated that LOX-1 deletion resulted in maintenance of endothelial continuity and eNOS expression in the LDLR KO mice, further confirming the importance of endothelial dysfunction in atherogenesis.[34,36] We stained aortic sections for von Willebrand factor as a measurement for endothelial integrity and attributed LOX-1 deletion to the preservation of endothelial integrity. This phenomenon needs to be confirmed at multiple stages of atherosclerotic process and/or in the primary cultured endothelial cells. The preservation of endothelial integrity may be secondary to the reduction in atherosclerotic process.

Study limitations

The lipoprotein metabolism in mice is quite different from that in man because of the lack of cholesterol ester transfer protein, presence of apoB100 in chylomicron/remnant instead of apoB48, and relative importance of Apo-E for the uptake of VLDL/LDL in the liver. Therefore, the results in mice will need to be confirmed in animals with lipid metabolism more akin to man. Further, LOX-1 is a multi-ligand receptor, and lipoprotein recognized by LOX-1 in vivo may not be produced in vitro, although its presence has been demonstrated in vivo and to correlate with atherosclerotic disease entities.

In summary, our data suggest that LOX-1 expression is important in atherogenesis, and its deletion limits atherogenesis despite high cholesterol diet, at least in part, by preserving endothelial function and integrity. The LOX-1 molecule that bridges the two phenomena, ie, endothelial dysfunction and atherogenesis, should be a good target for prediction, prevention and treatment of atherosclerosis-related diseases.

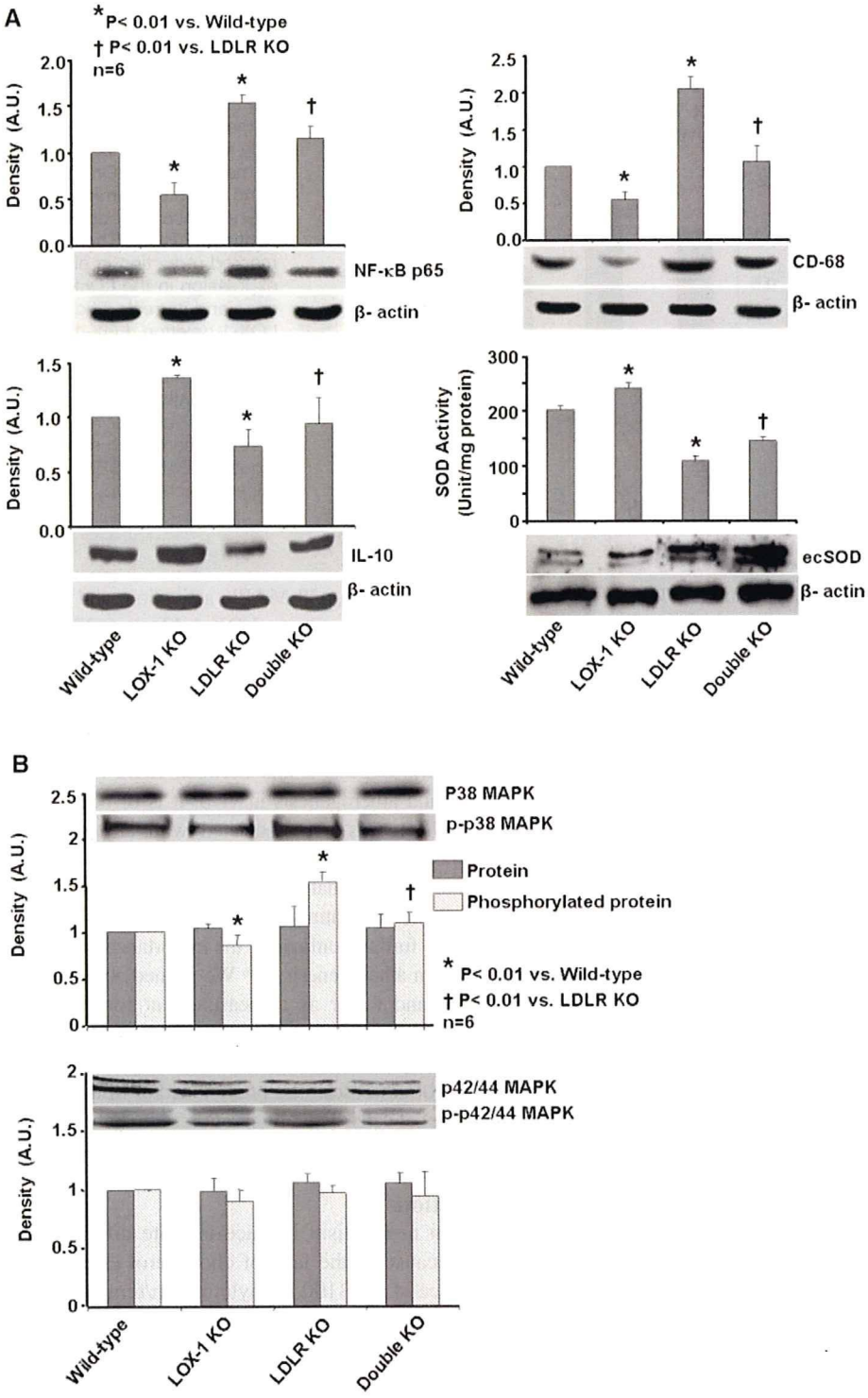

Figure 6. A, Expression of NF-κB and CD-68 in the aorta is reduced in the LOX-1 KO mice (versus wild-type mice) and the double KO mice (versus LDLR mice). IL-10 expression in the aorta and SOD activity in the liver is greater in the LOX-1 KO mice (versus wild-type mice) and the double KO mice (versus LDLR KO mice). ecSOD levels are higher in the aortas of LDLR KO mice and LOX-1 deletion further increases ecSOD expression. Bar graphs represent data (±SEM) from multiple experiments in each group. B, Expression of MAPK (both p38 and p44/42) protein is similar in all groups. p38 MAPK phosphorylation is reduced in the LOX-1 KO mice (versus wild-type mice) and in the double KO mice (versus LDLR mice). p44/42 MAPK phosphorylation is similar in all groups of mice. Bar graphs represent data (±SEM) from multiple experiments in each group.

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Disclosures

None.

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Modulation of ADP-Induced Platelet Activation by Aspirin and Pravastatin: Role of Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1, Nitric Oxide, Oxidative Stress, and Inside-Out Integrin Signaling

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ABSTRACT

Lectin-like oxidized low-density lipoprotein (LDL) receptor-1 (LOX-1), a receptor for oxidized-LDL, is up-regulated in activated endothelial cells, and it plays a role in atherothrombosis. However, its role in platelet aggregation is unclear. Both aspirin and HMG CoA reductase inhibitors (statins) reduce LOX-1 expression in endothelial cells. In this study, we investigated the effect of aspirin and pravastatin on LOX-1 expression on platelets. After ADP stimulation, mean fluorescence intensity of LOX-1 expression on platelets increased 1.5- to 2.0-fold. Blocking LOX-1 inhibited ADP-induced platelet aggregation in a concentration- and time-dependent manner. We also established that LOX-1 is important for ADP-stimulated inside-out activation of platelet $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ integrins (fibrinogen receptors). The specificity of this interaction was determined by arginine-glycine-aspartate-peptide inhibition. Furthermore, we found that LOX-1 inhibition of integrin activation is mediated by inhibition of protein kinase C activity. In other experiments, treatment with aspirin (1–10 mM) and pravastatin (1–5 μM) reduced platelet LOX-1 expression, with a synergistic effect of the combination of aspirin and pravastatin. Aspirin and pravastatin both reduced reactive oxygen species (ROS) released by activated platelets measured as malonyldialdehyde (MDA) release and nitrate/nitrite ratio. Aspirin and pravastatin also enhanced nitric oxide (NO) release measured as nitrite/nitrite + nitrate (NOx) ratio in platelet supernates. Small concentrations of aspirin and pravastatin had a synergistic effect on the inhibition of MDA release and enhancement of nitrite/NOx. Thus, LOX-1 is important for ADP-mediated platelet integrin activation, possibly through protein kinase C activation. Furthermore, aspirin and pravastatin inhibit LOX-1 expression on platelets in part by favorably affecting ROS and NO release from activated platelets.

Lectin-like oxidized LDL (ox-LDL) receptor-1 (LOX-1) is an ~50-kDa surface receptor initially found to be expressed on endothelial cells (Sawamura et al., 1997). It has been shown to bind to ox-LDL and to mediate endocytosis of ox-LDL by endothelial cells, causing endothelial activation and dysfunction (Moriwaki et al., 1998). The crystal structure of LOX-1 has been resolved, and it has a C-type lectin-like domain, neck domain, transmembrane domain, and a short 34-amino acid residue cytoplasmic domain (Ohki et al., 2005; Park et al., 2005). LOX-1 has been postulated to exert an important role in the pathogenesis of atherosclerosis. Numerous pathological effects of ox-LDL have been shown to be mediated by LOX-1, such as inflammation, oxidative stress, and apoptosis (Mehta et al., 2006). Mostly, the effects of LOX-1 in atherogenesis are mediated by LOX-1 expressed on endothelial cells. It is unclear whether LOX-1 expressed by other cell

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ABBREVIATIONS: LDL, low-density lipoprotein; ox-LDL, oxidized low-density lipoprotein; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; RGD, arginine-glycine-aspartate; PKC, protein kinase C; PRP, platelet-rich plasma; PPP, platelet-poor plasma; FACS, fluorescence-activated cell sorting; MDA, malonyldialdehyde; ROS, reactive oxygen species; NOx, nitrite + nitrate; MFI, mean fluorescence intensity; Ab, antibody.

types, such as immune cells or platelets, also contributes in disease progression, although inflammation and thrombosis are essential components of atherogenesis.

Platelets have also been shown to express small amounts of LOX-1 (Chen et al., 2001; Puccetti et al., 2005b). The HMG CoA reductase inhibitor atorvastatin was reported to decrease LOX-1 expression in hypercholesterolemic patients (Puccetti et al., 2005b). More recently, the role of oxidative stress in affecting platelet aggregation has been elucidated (Krötz et al., 2002, 2004). However, the role of LOX-1 in platelet aggregation is still largely unclear. It is tempting to speculate that LOX-1 plays a significant role in platelet aggregation, either indirectly through oxidative stress generated by ox-LDL or directly by influencing inside-out signaling of platelet integrin.

In this study, we report that the expression of LOX-1 on human platelets increases upon their exposure to ADP. We also report that blocking LOX-1 with a specific LOX-1 antibody inhibits ADP-induced platelet aggregation. Furthermore, we demonstrate that the inhibition of platelet aggregation occurs through interference with platelet binding to fibrinogen, which is mediated by platelet $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ integrins. We also show that treatment of platelets with aspirin or pravastatin reduces LOX-1 expression and that combination treatment of aspirin and pravastatin synergistically reduces LOX-1 expression.

Materials and Methods

Materials and Reagents. Monoclonal antibody against human LOX-1 (JTX92) was provided by Dr. T. Sawamura. This antibody is raised in mice with humanized Fc portion. Fluorescein isothiocyanate-conjugated secondary antibody was purchased from BD Biosciences (San Jose, CA). Monomeric fibrinogen conjugated with fluorescent Alexa Fluor 488 was purchased from Invitrogen (Carlsbad, CA). PepTag Assay for nonradioactive detection of protein kinase C (PKC) was obtained from Promega (Madison, WI). ADP and arginine-glycine-aspartate (RGD)-peptide were purchased from Sigma-Aldrich (St. Louis, MO).

Platelet Isolation. Platelet-rich plasma (PRP) was isolated from peripheral blood collected in 3.8% sodium citrate (nine parts of blood to one part of sodium citrate) from healthy donors. The blood was centrifuged at 200*g* for 15 min to obtain PRP. Platelet-poor plasma was isolated by centrifuging the plasma after PRP collection for another 10 min at 500*g* at room temperature. The protocol was approved by the Institutional Review Board, and informed consent was obtained from each subject.

Platelet Aggregation Study. Platelets were counted by flow cytometry (FACStar; BD Biosciences). Platelet count in PRP was adjusted at 300,000/mm^3 by adding PPP to PRP. PRP then was incubated at 37°C for 5 min before treatment. In the first set of experiments, before 10 μM ADP stimulation, platelets were incubated for 1, 5, and 10 min at room temperature with JTX92 at concentrations of 1, 5, and 10 μg/ml. For the second set of experiments, platelets were pretreated with aspirin at 1, 5, or 10 mM or with pravastatin at 1, 5, or 10 μM for 5 min at room temperature. Then, 10 μM ADP was added. Platelet aggregation was measured by using a four-channel platelet aggregometer (Bio/Data Corp., Horsham, PA).

LOX-1 Expression. In ADP-stimulated platelets, the expression of LOX-1 was studied with the use of JTX92 and fluorescein isothiocyanate-conjugated secondary antibody. First, platelets were washed and resuspended in FACS buffer containing phosphate-buffered saline, 2% fetal calf serum, and 0.1% sodium azide. Then, primary antibody against LOX-1 was added, and samples were briefly vortexed and incubated at room temperature for 10 min. Thereafter, primary antibody was washed with FACS buffer, secondary antibody was added, and samples were incubated for another 10 min at room temperature. Then, unbound secondary antibodies were washed and resuspended in FACS buffer before flow cytometric analysis. The expression levels were confirmed by FACS analysis using FACSCalibur (BD Biosciences).

Fibrinogen Binding to Platelets. Platelets were treated with anti-LOX-1 antibody (1, 5, and 10 μg/ml) and incubated for 10 min at room temperature. This was followed by the addition of 10 μM ADP together with 200 μg/ml Alexa Fluor 488-human fibrinogen, and the sample was incubated at 37°C for 15 min. Alexa Fluor 488-fibrinogen binding to platelets was determined by flow cytometry. For the RGD inhibition experiment, platelets were isolated from PRP, and then they were gel-filtrated on a Sepharose 4B column (GE Healthcare, Piscataway, NJ). Finally, they were eluted with elution buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 0.35 mM bovine serum albumin, 3.3 mM NaH_2PO_4, and 4 mM HEPES, pH 7.4. Then, 1 mM RGD-peptide was added together with 200 μg/ml Alexa Fluor 488-conjugated human fibrinogen supplemented with 1 mM $CaCl_2$ (Basani et al., 2001). Thereafter, 10 μM ADP was added followed by incubation at 37°C for 15 min. Alexa Fluor 488-fibrinogen binding to platelets was determined by flow cytometry.

In other experiments, platelets were initially treated with 10 mM aspirin or with 10 μM pravastatin for 5 min at room temperature. Then, 10 μM ADP was added together with 200 μg/ml Alexa Fluor 488-human fibrinogen, and the sample was incubated at 37°C for 15 min (Heilmann et al., 1994). Alexa Fluor 488-fibrinogen binding to platelets was determined by flow cytometry using FACSCalibur.

Protein Kinase C Activity Assay. For determination of protein kinase C activity, platelets were pelleted and homogenized. Then, protein was passed through a DEAE cellulose column, and it was eluted as per protocol. After treatment with protease inhibitors, brightly colored protein kinase C peptide substrate was added, and the reaction was allowed to proceed at 30°C for 30 min. After adding 80% glycerol, samples were run on 0.8% agarose gel in 50 mM Tris-HCl, pH 8.0, until separation. Phosphorylated substrate migrates toward the cathode, and unphosphorylated substrate migrates toward the anode.

MDA and Nitrite/Nitrate Measurement. Malonyldialdehyde (MDA) concentration in the platelet supernates was measured spectrophotometrically, as an index of ROS generation, using an MDA kit (Oxis International, Inc., Foster City, CA).

End products of nitric oxide (NO) interactions, nitrite and total nitrite + nitrate (NOx), were analyzed using a chemiluminescence analyzer (model NOA 280i; Seivers, Boulder, CO). Nitrate concentration was calculated by subtracting nitrite concentration from total NOx concentration. The aqueous samples of the exiting media collected during the experiments were drawn using a gas-tight syringe (Hamilton, Reno, NV). A 250-μl volume was injected into the respective reducing solution in a radical purge vessel (Seivers). The reducing agent converts nitrite or total NOx from injected samples to NO. The nitrite reducing solution was 0.2 M KI and glacial acetic acid mixed in a 1:3 volumetric ratio. The nitrate reducing solution was a saturated solution of VCl_3 in HCl (stock solution of 0.8 g VCl_3 and 8 ml of HCl diluted to 100 ml in deionized water) at 95°C. The reducing solution was continuously bubbled with N_2 to purge NO from the solution and to transport NO into the chemiluminescence detector wherein it reacts with ozone and emits light in the infrared region. The concentrations were obtained by comparing them with $NaNO_2$ or $NaNO_3$ standard calibration curves. The nitrate/nitrite and nitrite/NOx ratio is referred to as the ROS level and the NO level, respectively (Kavdia et al., 2000; Kavdia, 2006).

Statistical Analysis. Results are shown as mean ± S.D. All data were analyzed by Fisher's exact test. $p < 0.05$ was accepted as

indicating statistical significance. All calculations were performed using the SPSS library (SPSS Inc., Chicago, IL).

Results

Up-Regulation of LOX-1 Expression on Platelets by ADP Stimulation. We confirmed that LOX-1 is expressed on human platelets by using JTX92 by flow cytometry (Fig. 1A). ADP stimulation significantly increased LOX-1 expression on platelets. Mean fluorescence intensity (MFI) of LOX-1 expression was found to increase 1.5- to 2.0-fold ($p < 0.01$) versus basal LOX-1 expression (Fig. 1B).

Engaging LOX-1 Receptor with Anti-LOX-1 Antibody Inhibits Platelet Aggregation. To determine whether LOX-1 has any role in platelet aggregation function, we used JTX92. This monoclonal antibody has been reported previously to block the LOX-1 effect (Li et al., 2003a,b). We found that pretreatment with anti-LOX-1 antibody blocked ADP-stimulated platelet aggregation in a concentration-dependent manner (Fig. 2A). Because JTX92 is an antibody against LOX-1 raised in mice but its Fc portion has been switched to human Fc (humanized), we used PPP as a control because it contains a considerable amount of human γ-globulin. Time course experiments were performed by preincubating PRP with 10 μg/ml anti-LOX-1 antibody for 1, 5, and 10 min. Incubation was not done longer than 10 min because antibody binding to receptors may induce internalization and may confound the data. The experiment revealed that inhibition of platelet aggregation occurs as early as 1 min into incubation of platelets with the antibody (Fig. 2B). These data suggest that LOX-1 plays an important role in ADP-mediated platelet aggregation.

Anti-LOX-1 Antibody Inhibits Integrin-Mediated Platelet Binding to Fibrinogen. ADP, through its ADP receptor, is known to activate platelet integrin by the inside-out integrin signaling pathway (Li et al., 2003c; Abrams, 2005). It is possible that LOX-1 receptor engagement by antibody activates intracellular signaling events that block the "inside-out" signaling pathway of integrin activation.

To identify more precisely the step(s) in platelet aggregation that is(are) inhibited by anti-LOX-1 antibody, we tested the binding of platelets to fibrinogen monomer conjugated with Alexa Fluor 488. Platelets were then analyzed by flow cytometry to determine their binding capacity to fibrinogen monomers. This assay enabled us to confirm that ADP stimulation increased binding of platelets to fibrinogen. Figure 3A shows the increase in fluorescently labeled fibrinogen binding to platelets after ADP stimulation. The level of binding to fibrinogen after ADP stimulation (open histogram) is 3-fold higher compared with the unstimulated level (solid histogram). This confirms that integrin adhesion to ligand containing a repetitive pattern of binding sites, such as fibrinogen, is stronger compared with adhesion to ligand with a single binding site, because FACS-based assay using soluble monomeric ligand to integrin, such as intercellular adhesion molecule-1 binding to leukocyte-integrin lymphocyte function-associated antigen 1, is known to be undetected by flow cytometry. These data, therefore, supported the importance of an increase in clustering as opposed to an increase in

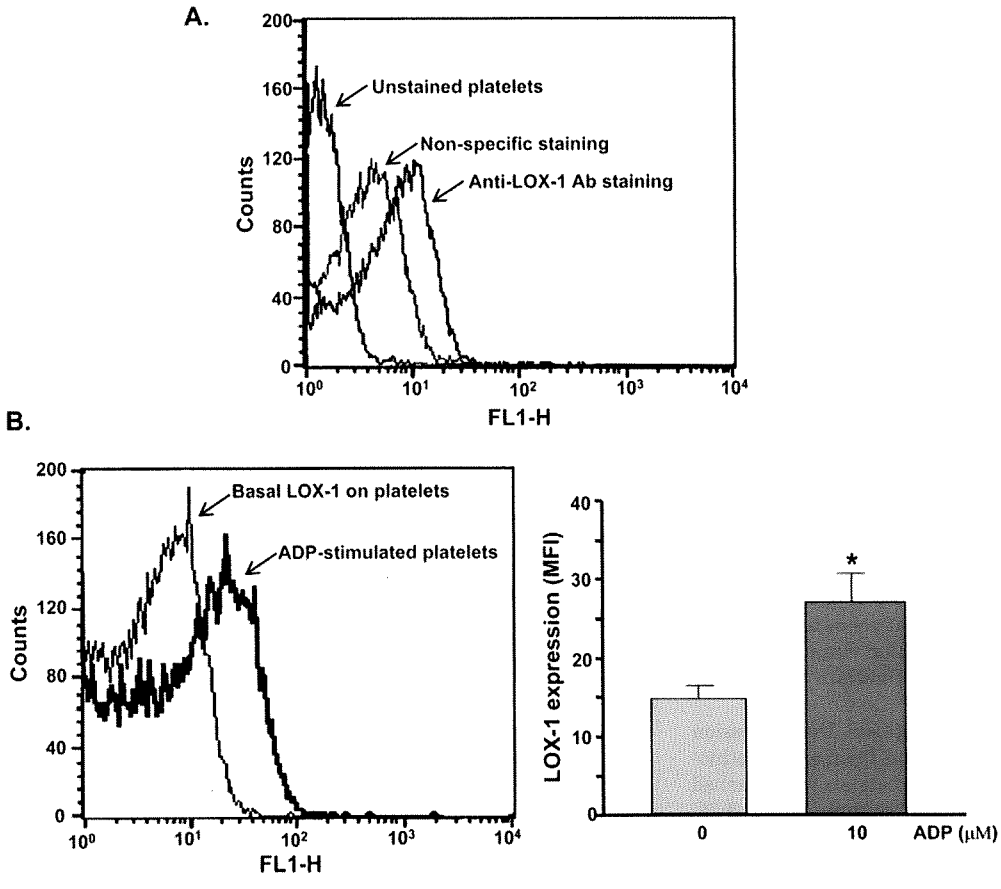


Fig. 1. FACS analysis of platelets showing the expression of LOX-1. A, representative histograms of unstained platelets, nonspecific binding, and platelets after treatment with anti-LOX-1 antibody (Ab) are shown. B, left, representative LOX-1 expression level without and with 10 μM ADP stimulation. Right, summary of data derived from FACS analysis. LOX-1 level is expressed as the MFI with data from three different healthy donors. *, $p < 0.01$ versus control (0 μM ADP).

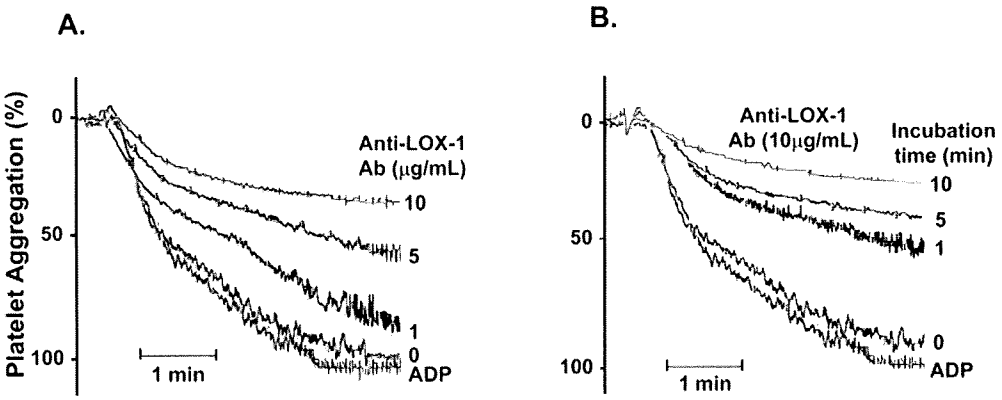


Fig. 2. Anti-LOX-1 Ab inhibits platelet aggregation. PRP was isolated from normal human volunteers. Platelet aggregation was measured by light transmission after stimulation with 10 μM ADP. Inhibition of platelet aggregation occurs after incubation of PRP with anti-LOX-1 antibody. The inhibition of platelet aggregation by LOX-1 antibody occurs in a dose- (A) and time-dependent manner (B).

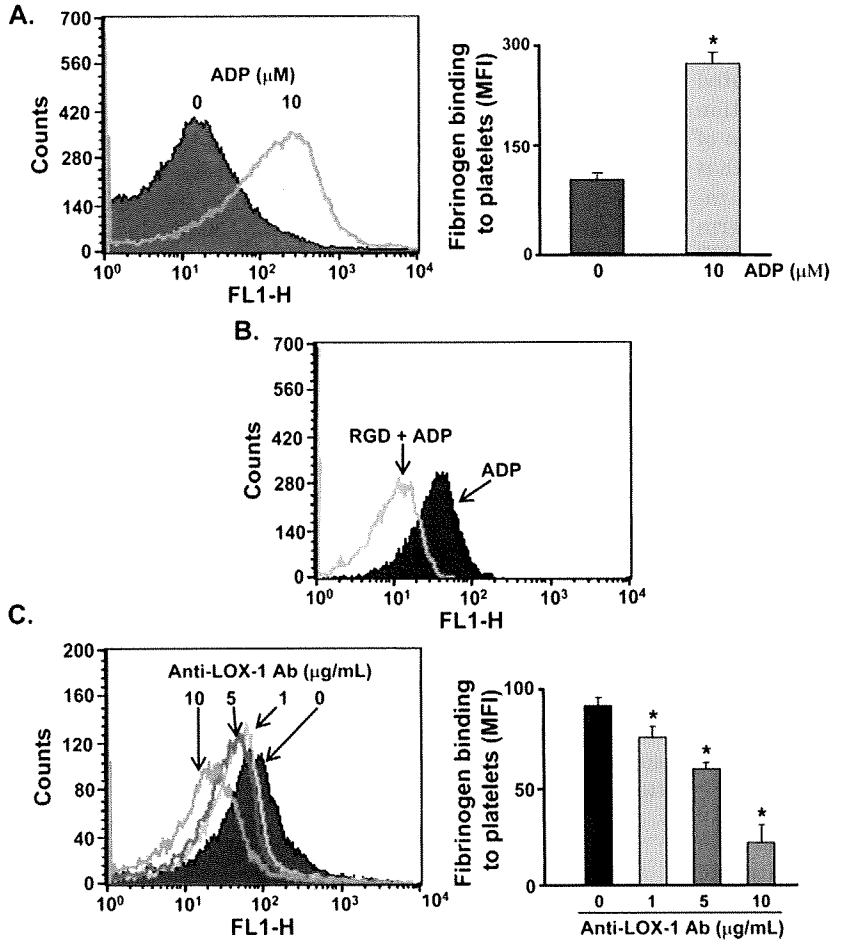


Fig. 3. Alexa Fluor 488-conjugated fibrinogen binding to platelets was tested by flow cytometry. A, increase in binding of Alexa Fluor 488-conjugated fibrinogen after 10 μM ADP stimulation compared with unstimulated platelets (*, $p < 0.01$). B, specificity of fibrinogen binding to platelet integrin is confirmed by the inhibition of 1 mM RGD peptide. C, left, inhibition of platelet integrin-mediated binding of platelets to fibrinogen by anti-LOX-1 antibody. Platelets were initially treated with anti-LOX-1 antibody and incubated for 10 min at room temperature with 10 μM ADP together with Alexa Fluor 488-conjugated fibrinogen and incubated for an additional 15 min. C, right, summary data from three separate experiments (*, $p < 0.01$ versus 0 μM anti-LOX-1 Ab).

affinity (conformational change) on integrin activity (Carman and Springer, 2003). To confirm that this interaction is specifically mediated by integrin, we used RGD-peptide as the inhibitor. RGD-peptide is known to act as a competitive inhibitor to integrin binding, because it is the 3-amino acid sequence of fibrinogen that binds to integrin. (Basani et al., 2001). Figure 3B shows RGD inhibition of fibrinogen-platelet interaction after ADP stimulation. Thus, we confirmed that platelet interaction to fibrinogen is specifically integrin-mediated.

In keeping with this hypothesis, treatment with anti-LOX-1 antibody decreased binding of fibrinogen monomer to platelets in a concentration-dependent manner (Fig. 3C). In this experiment, we first treated PRP with anti-LOX-1 antibody for 10 min at room temperature and then with ADP together with Alexa Fluor 488-conjugated fibrinogen at 37°C for 15 min. Even with a short incubation time, we could identify inhibition of fibrinogen binding to platelets. This confirms that LOX-1 is important in ADP-mediated inside-out signaling of platelet integrins.

Anti-LOX-1 Antibody Treatment Inhibits Protein Kinase C Activation. The finding that anti-LOX-1 antibody can block integrin-mediated platelet binding to fibrinogen suggests that LOX-1 engagement with antibody may inter-

fere with intracellular signaling of inside-out activation of integrin. Protein kinase C has been reported to mediate this pathway (Abrams, 2005). One of the responses of platelets to most agonists is the activation of phospholipase C. Phospholipase Cβ is primarily responsible for the rapid burst of phosphoinositide hydrolysis that occurs during platelet activation by agonists. Activated phospholipase C hydrolyzes phosphatidylinositol-4,5-bisphosphate to diacylglycerol and inositol-1,4,5-trisphosphate. Diacylglycerol activates protein kinase C, and it contributes to protein phosphorylation. Inositol-1,4,5-trisphosphate binds to receptors in the dense tubular system and the cell membrane, and it allows an influx of Ca^{2+} into the cytoplasm (Abrams, 2005; Han et al., 2006; Yacoub et al., 2006)

We studied the activity of protein kinase C to phosphorylate its substrate. Treatment with LOX-1 antibody indeed inhibited protein kinase C activity (Fig. 4). Phosphorylated protein kinase C, which represents the activated form, travels toward the cathode (up), whereas the unphosphorylated protein migrates toward the anode (down). The inhibition seems to readily take place with the lowest concentration of antibody used, i.e., 0.1 μg/ml. In essence, we established that LOX-1 antibody inhibits activation of protein kinase C intracellular signaling, which is important for platelet integrin activation. As a control, we included the well-known PKC inhibitor staurosporine to show that protein kinase C activation by ADP is specific.

Inhibition of Platelet Aggregation by Aspirin and Pravastatin. In other experiments, we studied platelet inhibitory effects of aspirin and pravastatin on platelet aggregation and the role of LOX-1 in this process.

As shown in Fig. 5A, aspirin inhibited ADP-induced platelet aggregation in a concentration-dependent manner. Pravastatin, in contrast, did not induce significant inhibition of platelet aggregation. The combination of aspirin and pravastatin showed a modest synergistic effect. With 1 mM aspirin and 1 μM pravastatin, inhibition of platelet aggregation was 17 $\pm$ 4% (versus 22 $\pm$ 6% with aspirin alone; $p < 0.05$).

LOX-1 Down-Regulation by Aspirin and Pravastatin. As mentioned earlier, LOX-1 is expressed on human platelets, and ADP stimulation significantly increases LOX-1 expression (Fig. 1B). Upon treatment with 1 to 10 mM aspirin, LOX-1 expression was found to be reduced in a concentration-dependent manner ($p < 0.05$) (Fig. 5B). Likewise, 1 to 10 μM pravastatin treatment reduced LOX-1 expression in a concentration-dependent manner ($p < 0.05$). Combination of 1 mM aspirin and 1 μM pravastatin significantly reduced LOX-1 expression level ($p < 0.05$ versus aspirin or pravastatin alone).

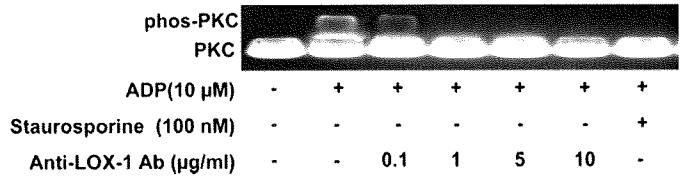


Fig. 4. Protein kinase C activity is inhibited by anti-LOX-1 antibody treatment. Phosphorylated protein kinase C substrate migrates up, and unphosphorylated protein kinase C substrate migrates down. The first lane (left-most) represents untreated platelets, the second lane represents ADP stimulation, the third through sixth lanes represent 10 μM ADP stimulation in the presence of anti-LOX-1 antibody (0.1, 1, 5, and 10 μg/ml, respectively). The seventh lane represents ADP stimulation in the presence of 100 nM staurosporine, a protein kinase C inhibitor, as a positive control. This figure is representative of three separate experiments.

Treatment with Aspirin and Pravastatin Reduces Oxidative Stress. Because LOX-1 has been widely implicated as a receptor for ox-LDL, which is up-regulated in the presence of oxidative stress (Mehta et al., 2006), we measured the level of oxidative stress in ADP-treated platelets. Using MDA as a measurement of oxidative stress, we found that ADP increased MDA concentration, and aspirin and/or pravastatin treatment reduced MDA levels in the PRP (Fig. 6A). Furthermore, the combination of small amounts of aspirin (1 mM) and pravastatin (1 μM) synergistically reduced MDA levels ($p < 0.01$ versus aspirin alone or pravastatin alone).

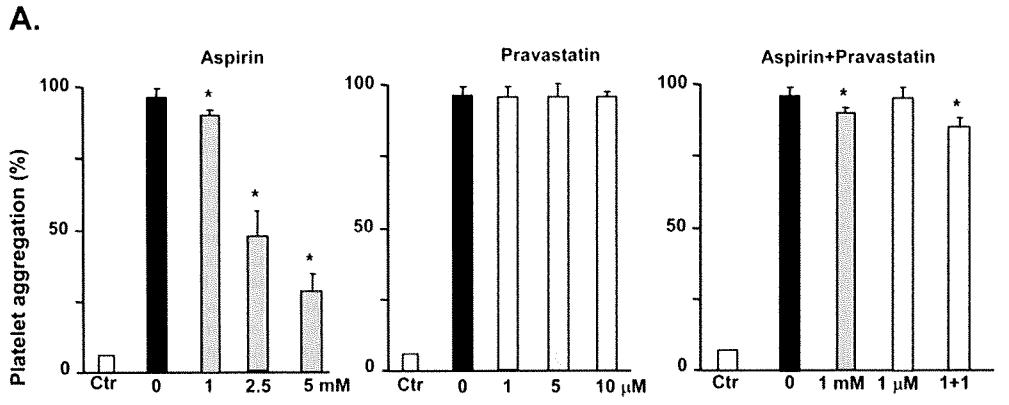
NO reacts readily with molecular oxygen and O_2^--derived free radicals. The oxidation of NO at physiological pH by O_2 leads to nitrite formation via intermediates nitrogen dioxide and nitrous anhydride. With superoxide, NO forms peroxynitrite that leads to formation of nitrate as the main end product. Thus, in two different treatments, if total NOx concentration is constant, increase in nitrate-to-nitrite ratio can be interpreted by increased ROS, whereas decrease in nitrate-to-nitrite ratio can be interpreted by increased NO levels and decreased ROS (Kavdia et al., 2000; Kavdia, 2006). Treatment with aspirin and pravastatin, the nitrate-to-nitrite ratio decreased with increase in respective dose compared with control (Fig. 6B). This indicated that both these agents inhibited ROS and increased NO levels. This trend is also supported by the increase in nitrite-to-total NOx ratio, as seen in Fig. 6C, with increase in drug dose, because a reduction in ROS level increases the nitrite formation rates through metabolism with molecular oxygen.

Aspirin, but Not Pravastatin, Inhibits Integrin-Mediated Binding of Platelets to Fibrinogen. As shown in Fig. 7, aspirin treatment reduced the binding capacity of fibrinogen to platelets, whereas pravastatin had a small effect. These data demonstrate that aspirin-mediated reduction in LOX-1 expression is associated with reduction in ROS generation and platelet aggregation. However, pravastatin-mediated decrease in LOX-1 is only associated with reduction in ROS, but only minimal inhibition of platelet aggregation, suggesting separate mechanisms linking platelet aggregation and ROS reduction.

Discussion

We have demonstrated that blocking LOX-1 inhibits platelet aggregation and that this phenomenon is mediated by inhibition of protein kinase C activity.

LOX-1, a receptor for oxidized-LDL, was initially reported to be mainly expressed in endothelial cells (Sawamura et al., 1997). It is present in human atherosclerotic lesions (Kataoka et al., 1999). Its role in the pathogenesis of atherosclerosis has been mainly attributed to endothelial dysfunction (Mehta et al., 2006). Some studies have shown the presence of LOX-1 on human platelets. The expression of LOX-1 in endothelial cells has been shown to mediate its interaction with platelets (Kakutani et al., 2000). Because platelets are also important players in atherogenesis (Gawaz et al., 2005), we postulated that LOX-1 may influence platelet function (Chen et al., 2001; Puccetti et al., 2005b). In the current



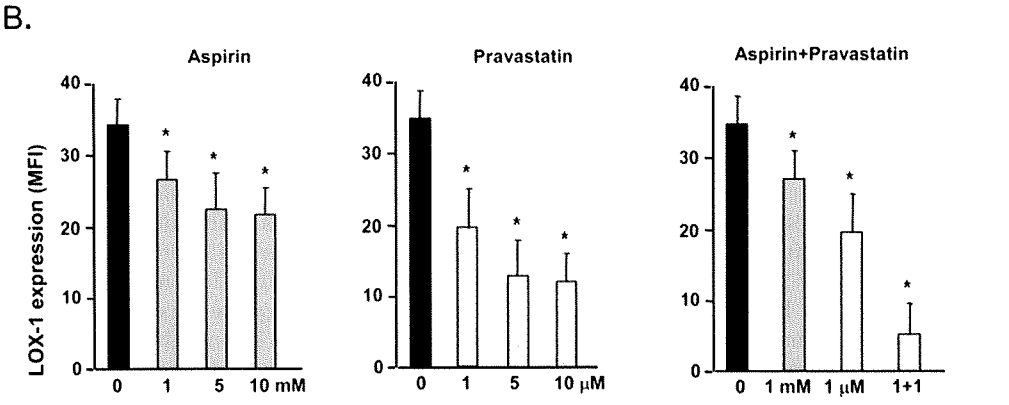
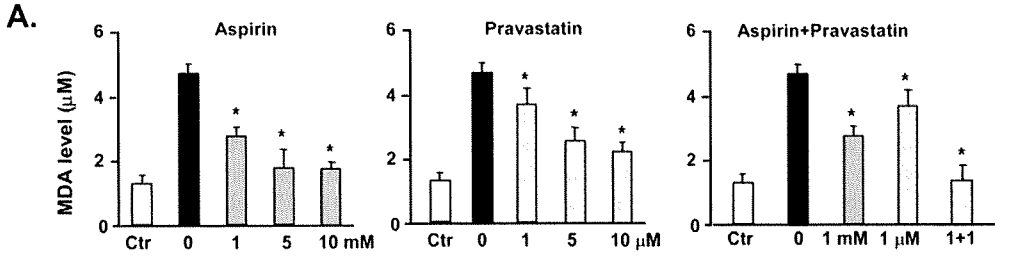
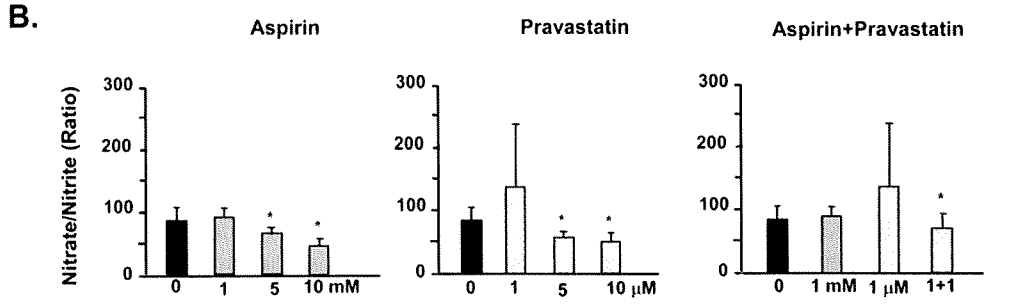


Fig. 5. Inhibition of platelet aggregation and LOX-1 expression by aspirin and pravastatin. A, aspirin inhibits platelet aggregation in a dose-dependent manner (left), but pravastatin does not have a significant inhibitory effect (middle). The combination of both aspirin and pravastatin (each in lowest concentration) inhibits platelet aggregation (right). B, LOX-1 expression from FACS analysis demonstrated as graph bars. Platelets collected from PRP are stimulated with ADP in the presence of aspirin, pravastatin, and their combination. LOX-1 level is expressed as the MFI with data from three different healthy donors. Aspirin (left), pravastatin (middle), and combination of both (right) reduce the expression level of LOX-1. *, $p < 0.05$ versus control.





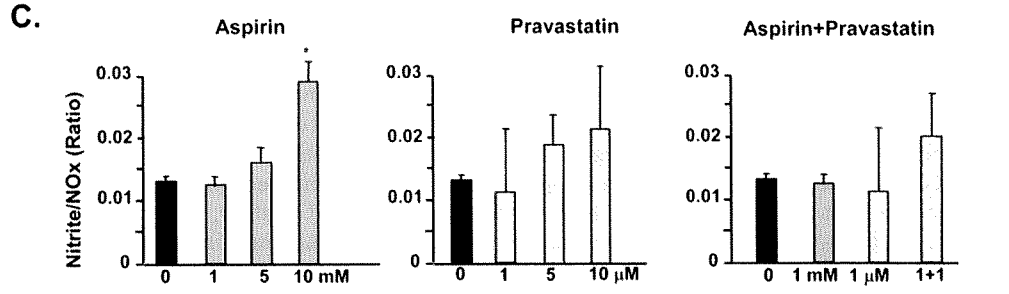


Fig. 6. A, dose-dependent decrease in nitrate-to-nitrite ratio and MDA levels following platelet treatment with aspirin, pravastatin, and their combination compared with control, indicating the inhibition of ROS and increase in NO levels by both aspirin and pravastatin. B, dose-dependent decrease in the nitrite/nitrate ratio. C, dose-dependent increase in nitrite/NOx ratio, which supports the results of the inhibition of ROS and increase in NO levels. *, $p < 0.05$ versus control.

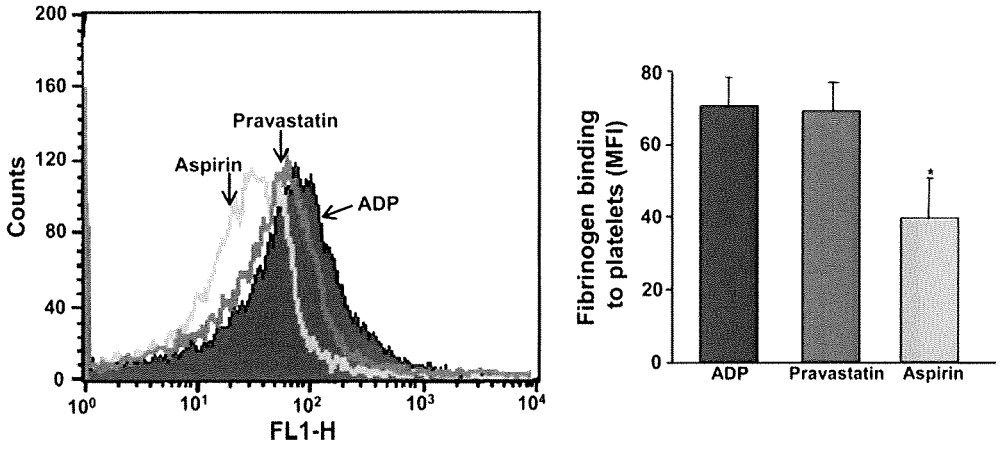


Fig. 7. Alexa Fluor 488-conjugated fibrinogen binding to platelets was examined by flow cytometry. Platelets were initially treated with 10 mM aspirin or 10 μM pravastatin, and then 10 μM ADP was added together with 200 μM Alexa Fluor 488-fibrinogen, and the mixture was incubated at 37°C for 15 min. Left, inhibition with aspirin of Alexa Fluor 488-conjugated fibrinogen to 10 μM ADP-stimulated platelets; pravastatin has a minimal effect in a representative experiment. Right, bar graphs of MFI from three independent experiments. *, $p < 0.05$ versus ADP alone.

study, we addressed the question of whether LOX-1 on platelets plays a role in platelet aggregation.

LOX-1 is known to mediate binding to ox-LDL, which itself is a product of oxidative stress. The role of oxidative stress in thrombosis has become appreciated recently (Krötz et al., 2004). ROS released by endothelial cells and immune cells (neutrophils, monocytes, and macrophages) and by platelets may play an important role in thrombus formation. Here, we provide another possible contribution of LOX-1 in modifying platelet aggregation. LOX-1 is an important component of integrin-mediated platelet binding to fibrinogen, which is the ultimate step in thrombosis.

ADP stimulation activates platelet integrins, mainly $\alpha_{IIb}\beta_3$ and to a lesser extent $\alpha_2\beta_1$, through a process called inside-out signaling (Li et al., 2003c; Abrams, 2005). This signaling pathway, common in all other integrins, induces conformational changes of these important adhesion molecules (Takagi et al., 2002; Vinogradova et al., 2002). In the present study, we show that LOX-1 affects the inside-out signaling of platelet integrins by inhibiting protein kinase C activation. To confirm the specificity of this reaction, we used RGD-peptide to inhibit integrin binding to fibrinogen (Basani et al., 2001; Xiong et al., 2002), and we were able to show similar response. Protein kinase C has been reported to be involved in inside-out signaling (Abrams, 2005; Han et al., 2006; Yacoub et al., 2006). It is likely that antibody engagement of LOX-1 on platelet triggers other signaling events that interfere with this process. The downstream signaling pathway of LOX-1 is still largely unknown. However, it is clear that antibody engagement may induce clustering of LOX-1 receptors and activate its downstream signaling cascades, or that it may block them (Li et al., 2003a,b). Further studies are warranted to clarify this issue.

We also show that aspirin and pravastatin reduce LOX-1 expression. This finding correlates with reduction of ROS generation and enhanced NO release. It is noteworthy that aspirin and pravastatin had synergistic effects on these measurements. Because oxidative stress stimulates LOX-1 expression (Mehta et al., 2006) and LOX-1 expression itself induces oxidative stress, it is conceivable that the reduction in LOX-1 expression by aspirin and pravastatin is either the basis and/or a consequence of reduction in oxidative stress.

In a previous study in endothelial cells (Mehta et al., 2004), aspirin in a dose- and time-dependent manner was shown to reduce ox-LDL-mediated LOX-1 expression. The effect of aspirin was thought to be the effect of salicylate moiety, because treatment of endothelial cells with salicylate, but not indomethacin, resulted in the suppression of LOX-1 expression, an effect similar to that of aspirin. It is noteworthy that both aspirin and its component salicylate decreased superoxide anion generation. Other studies in endothelial cells have shown that the HMG CoA reductase inhibitors simvastatin and atorvastatin reduce LOX-1 expression, up-regulate protein kinase B activity, and reduce ^{125}I-ox-LDL uptake in endothelial cells (Mehta et al., 2001). This was thought to result in an increase in endothelial nitric-oxide synthase expression and activity.

It is possible that inhibition of platelet aggregation by aspirin reflects oxidative stress reduction. Oxidative stress has clearly been shown to enhance platelet aggregation and thrombosis (Krötz et al., 2004). Platelets produce ROS because they have NAD(P)H-oxidase activity (Seno et al., 2001; Krötz et al., 2002). ROS affect thrombosis by increasing platelet recruitment into growing thrombus (Krötz et al., 2002). It is noteworthy that we did not see inhibition of platelet aggregation by pravastatin, even though ROS was significantly reduced. However, this does not necessarily mean that pravastatin does not have antithrombotic inhibition. It is possible that by reducing ROS level, pravastatin reduces platelet recruitment into the growing thrombus. In support of this hypothesis are the observations from our laboratory that another HMG CoA reductase inhibitor, atorvastatin, reduced the weight of thrombus in the rat aorta exposed to oxidative stress (Gaddam et al., 2002). Furthermore, there is evidence that regulation of integrins may be affected by redox condition owing to the modifications of its disulfide bonds that may affect its affinity through conformational changes (Lahav et al., 2002; Walsh et al., 2004). Again, we observed that aspirin, but not pravastatin, by reducing ROS, inhibited integrin-mediated binding of platelet to fibrinogen. It is still unclear whether the redox state that can affect integrin disulfide bonds can be replicated in our fibrinogen binding assay, or for that matter, whether the redox state that alters disulfide bonds occurs in vivo. It is conceivable that ROS produced during oxidative stress may increase platelet LOX-1 expression. Clues in favor of this assumption

are emerging from preliminary observations on the effects of direct ROS generation by the Fe(III)-ascorbate reaction (Ciuffi et al., 1999) on thrombus formation in carotid arteries of wild-type and LOX-1-deficient mice.

In hypercholesterolemic patients, atorvastatin has been reported to reduce LOX-1 expression on platelets (Puccetti et al., 2005b). The level of expression of LOX-1 was measured by flow cytometry with a different clone of monoclonal antibody (JM90) than the antibody we used in this study. Both monoclonal antibodies are humanized, but most probably they recognize different epitopes of LOX-1 (Puccetti et al., 2005b). Even though the data of Puccetti et al. (2005b) were presented as percentage of LOX-1-positive platelets rather than MFI, this supports our findings of LOX-1 expression on platelets. This study and other studies show that platelet activation with thrombin, as opposed to ADP, also up-regulates expression levels of LOX-1 on the surface of platelets (Bruni et al., 2005; Puccetti et al., 2005a,b). Moreover, statins reduce the expression levels of P-selectin (Bruni et al., 2005; Puccetti et al., 2005a). This finding has been suggested to explain the antiplatelet effect of statins. Interestingly, in agreement with our study is the report that the effect of statins on down-regulation of LOX-1 on platelets occurs much earlier than the reduction in serum LDL-cholesterol level. This suggests that the antiplatelet effects of statins are independent of serum cholesterol effects. It is not clear whether HMG-CoA reductase is fully functional in platelets. In our system, this confounding factor was excluded. It is unlikely that the effect of pravastatin seen in the present study could have been mediated by the HMG-CoA inhibitory effect, because the LOX-1 down-regulation effect was observed almost immediately.

In conclusion, we propose that platelet LOX-1 plays an important role in ADP-induced platelet integrin binding to fibrinogen via interaction with protein kinase C and that LOX-1 thereby participates in inside-out signaling of platelet integrins (Fig. 8). Our study also shows that aspirin and pravastatin can reduce LOX-1 expression level associated with reduction in ROS production by platelets. These data suggest the importance of LOX-1 in platelet aggregation. Furthermore, they elucidate its role in the mechanism of antiplatelet effect of combination of aspirin and pravastatin. More importantly, we also demonstrate the synergistic effect of aspirin and pravastatin as antiplatelet agents.

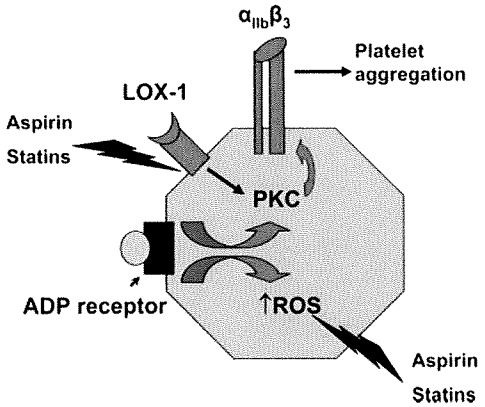


Fig. 8. ADP stimulation through ADP receptor activates inside-out signaling pathway of platelet integrins, mainly $\alpha_{IIb}\beta_3$, which involves PKC. LOX-1 blocking with anti-LOX-1 monoclonal antibody inhibits integrin activation by inhibiting PKC activation. Aspirin and pravastatin reduce both expression of LOX-1 and ROS.

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Lectin-like oxidized LDL receptor-1 as extracellular chaperone receptor: Its versatile functions and human diseases

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Abstract

Well-known coronary risk factors such as hyperlipidemia, hypertension, smoking, and diabetes are reported to induce the oxidative stress. Under the oxidative stress, low-density lipoprotein (LDL) is oxidatively modified in the vasculature, and formed oxidized LDL induces endothelial dysfunction, expression of adhesion molecules and apoptosis of vascular smooth muscle cells. It has become evident that these cellular responses induced by oxidized LDL are mediated by lectin-like oxidized LDL receptor-1 (LOX-1). LOX-1 was originally identified from cultured aortic endothelial cells as a receptor for oxidized LDL; however, recent investigations revealed that LOX-1 has diverse roles in the host-defense system and inflammatory responses, and it is involved in the pathogenesis of various diseases such as atherosclerosis-based cardiovascular diseases and septic shock. Beside oxidized LDL, LOX-1 recognizes multiple ligands including apoptotic cells, platelets, advanced glycation end products, bacteria, and heat shock proteins (HSPs). The HSPs function as a chaperone to affect protein folding of newly synthesized or denatured proteins. There are accumulating evidences that the HSPs released into the extracellular space have potent biological activities and it may work as a kind of cytokines. It is demonstrated that LOX-1 works as a receptor for HSP70, since it has high affinity for HSP70. The interaction of LOX-1 with HSP70 is involved in the cross-presentation of antigen. Given the potent and wide variety of biological activities, more understanding their interaction provides potential therapeutic strategy for various human diseases.

Keywords: Oxidative stress; Oxidized LDL; Heat shock protein

1. Identification of LOX-1 and its versatile functions

According to the response to injury theory, the endothelial injury associated with dysfunction is a very first step of atherosclerosis. Possible causes of endothelial dysfunction leading to atherosclerosis include hypertension, diabetes, oxidative stress, hemodynamic forces such as high shear stress, some pathogens, or oxidized LDL. Clinical investigations have demonstrated that well-known coronary risk factors, including diabetes, hyperlipidemia, hypertension, obesity and smoking, are associated with oxidative stress. Under the oxidative stress, LDL particles, which were trapped in the vessel wall, are oxidatively modified. Formed oxidized LDL is extensively accumulated into macrophages, resulting in the formation of foam cells. The treatment of endothelial cells with oxidized LDL induces pro-inflammatory responses. Oxidized LDL induces the production of wide variety of inflammatory cytokines or chemokines by vascular cells. Oxidized LDL induces the adhesion of blood cells to endothelium and vascular inflammation, which are essential in pathogenesis of atherosclerosis-based cardiovascular diseases. Therefore, the identification of signaling pathway induced by oxidized LDL and the clarification of detailed mechanisms is important for the understanding the pathogenesis of atherosclerosis-based cardiovascular diseases. In 1997, the lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) was cloned from a DNA library of bovine aortic endothelial cells employing expression cloning as the receptor for

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oxidized LDL on endothelial cells [1]. It is now evident that the atherogenic properties of oxidized LDL are mediated mainly via LOX-1. The activation of LOX-1 by oxidized LDL induces the up-regulation of monocyte chemotatic factor (MCP)-1, intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1, and the release of reactive oxygen species via NADPH oxidase [2,3]. LOX-1 expression is up-regulated in various pathological conditions affecting vasculature including hypertension, diabetes, hyperlipidemia and atherosclerosis [4,5].

LOX-1 is a type II membrane glycoprotein with a molecular weight of 50 kDa, as shown in Fig. 1. LOX-1 exists as a disulfide-linked homodimer on the cell surface and the C-type lectin-like domain acts as an oxidized LDL binding domain [6]. A single homodimer of LOX-1 displays a lower affinity for oxidized LDL; however, chemical cross linking experiments demonstrate that LOX-1 assembles as a three homodimeric hexamer at the cell surface, and the clustering of LOX-1 on the cell surface results in the specific high affinity binding to oxidized LDL as shown in Fig. 2 [7].

To investigate the role of LOX-1 in endothelial dysfunction and atherogenesis *in vivo*, we generated mice overexpressing LOX-1 (LOXtg) in C57BL/6 and apolipoproteinE-null mice (apoEKO) backgrounds [8]. In the LOXtg mice, the expression of the transgene was prominent in coronary vessels and cardiomyocytes. The immunohistochemical analysis of LOXtg/apoEKO mice revealed that both oxidized LDL and 8-hydroxy-deoxyguanosine accumulated in the coronary arteries. Thus, LOX-1 expression in the coronary arteries was closely associated with the oxidative stress in the LOXtg/apoEKO mice. Furthermore, the expression of ICAM-1 and VCAM-1, as well as the number of macrophages around blood vessels, were significantly increased in LOXtg/apoEKO mice compared to control littermates. Furthermore, the LOXtg/apoEKO mice displayed accelerated coronary atherosclerosis. Interestingly, the lipid deposition was frequently associated with atherosclerotic plaques in the LOXtg/apoEKO mice, as shown in Fig. 3. Compared to the apoEKO mice, the LOXtg/apoEKO mice showed an approximately 10-fold increase in the atherosclerotic plaque area after they were maintained 3-weeks on a high-fat diet. Thus, LOX-1 plays a critical role in the pathogenesis of atherosclerosis-based cardiovascular diseases.

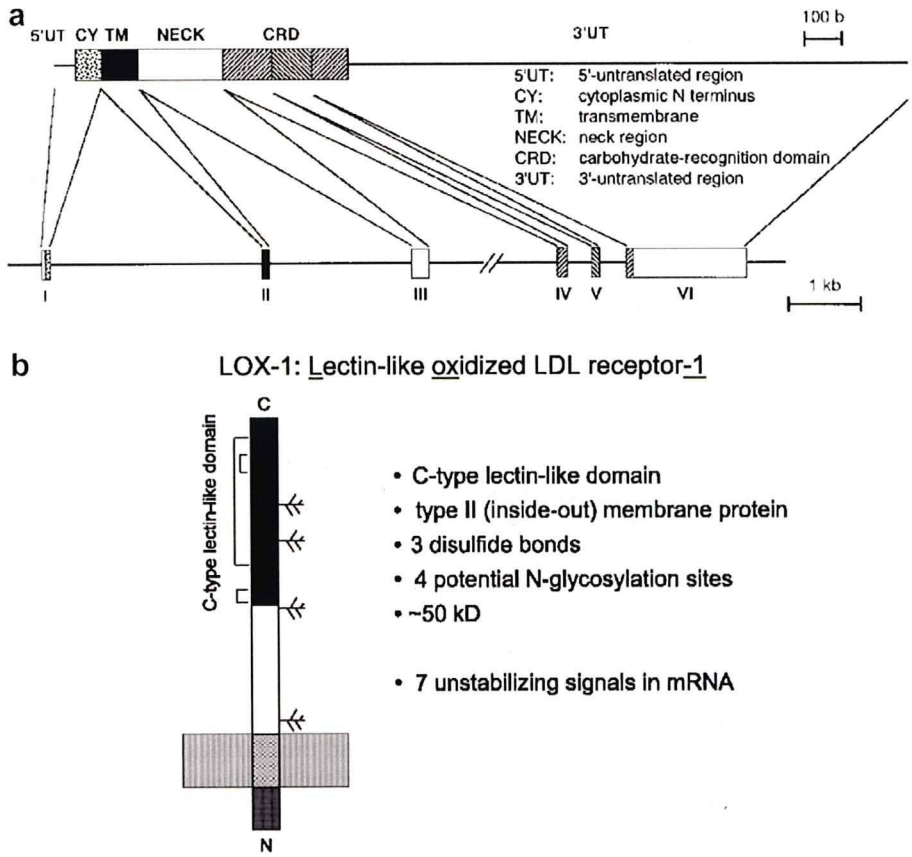

Fig. 1. (a) Structural organization of the human LOX-1 gene and mRNA. The scheme indicates the domain structure of the LOX-1 protein encoded by LOX-1 mRNA. The translated region is indicated by boxes: dotted box, cytoplasmic region; black box, transmembrane domain; open box, neck region; and striped box, CRD domain. The lower scheme indicates the exon–intron organization of the human LOX-1 gene. Exons are indicated by boxes numbered I–VI; introns, and 5′- and 3′-flanking sequences are indicated by lines. (b) Schema indicating the structure of the LOX-1 protein. Cited from Ref. [6].

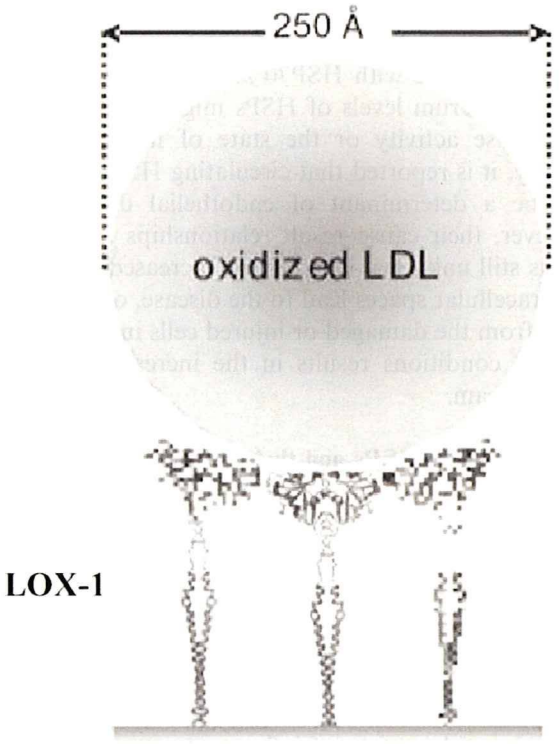

Fig. 2. The schema showing the binding of oxidized LDL to LOX-1. Cited from Ref. [7].

LOX-1 is highly homologous in the lectin-like domain of NKR-P1, which is essential for the activation of natural killer cells and is a member of the natural killer cell receptor family discovered in this group of C-type lectin-like receptors. This observation suggests the possibility that LOX-1 could recognize various ligands other than oxidized LDL, and that it plays some role in the host-defense system. In fact, recent progress in research on LOX-1 reveals that this hypothesis is true. LOX-1 recognizes not only oxidized LDL but also acidic phospholipids, apoptotic cells, damaged cells, platelets, bacteria, and advanced glycation end products (AGEs). Thus, besides playing a significant role in atherogenesis, LOX-1 is an important member in the host-defense system and is involved in inflammatory responses [9,10].

2. LOX-1 and inflammatory responses and host-defense system

The significance of LOX-1 in the inflammatory responses was demonstrated by animal models of septic shock. In a rat model of endotoxemia, injection of a high dose of endotoxin into rats induced leucopenia and death of the animals [11]. Pretreatment of anti-LOX-1 antibody reduced the degree of leucopenia and rescued the animals, whereas control IgG had no effect. Furthermore, in a model of endotoxin-induced uveitis, anti-LOX-1 antibody significantly suppressed leukocytes infiltration and protein exudation. In situ videomicroscopic analysis revealed that this LOX-1 blocking antibody reduced the number of rolling leukocytes and increased the velocity of rolling. These findings suggest that LOX-1 function as a vascular tethering ligand. Thus, LOX-1 is a key molecule in the inflammatory process as an adhesion molecule for leukocyte recruitment.

Furthermore, it has become evident that LOX-1 is critical for host-defense system and tumor immunity via the

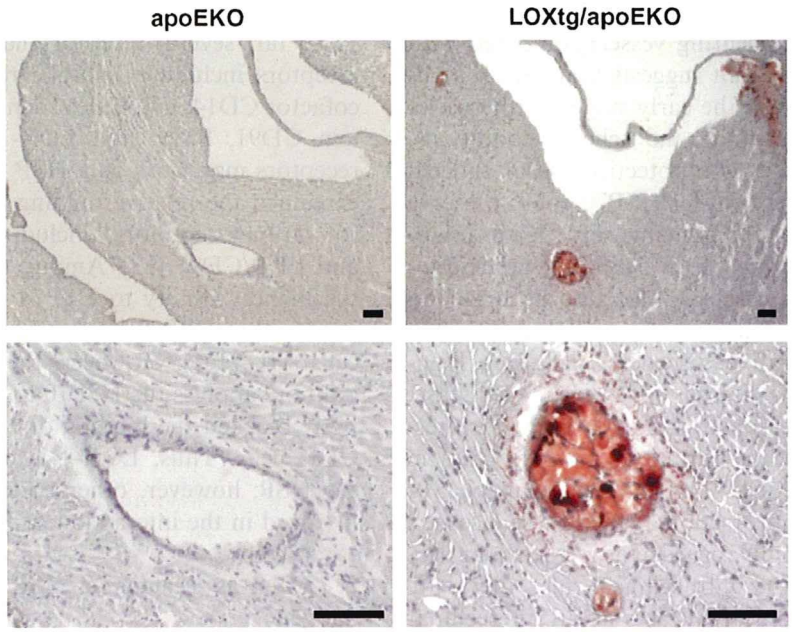

Fig. 3. Association of lipid deposition with atherosclerotic lesion. This figure is an oil-red O staining of the heart section of the apoE knockout mice and LOX-1 transgenic/apoE knockout mice who were fed a high-fat diet for 3 weeks. Small coronary arteries of the LOX-1 transgenic/apoE knockout mice show atherosclerotic changes with lipid deposition. Scale bars = 100 μm. Cited from Ref. [8].