Figure 3

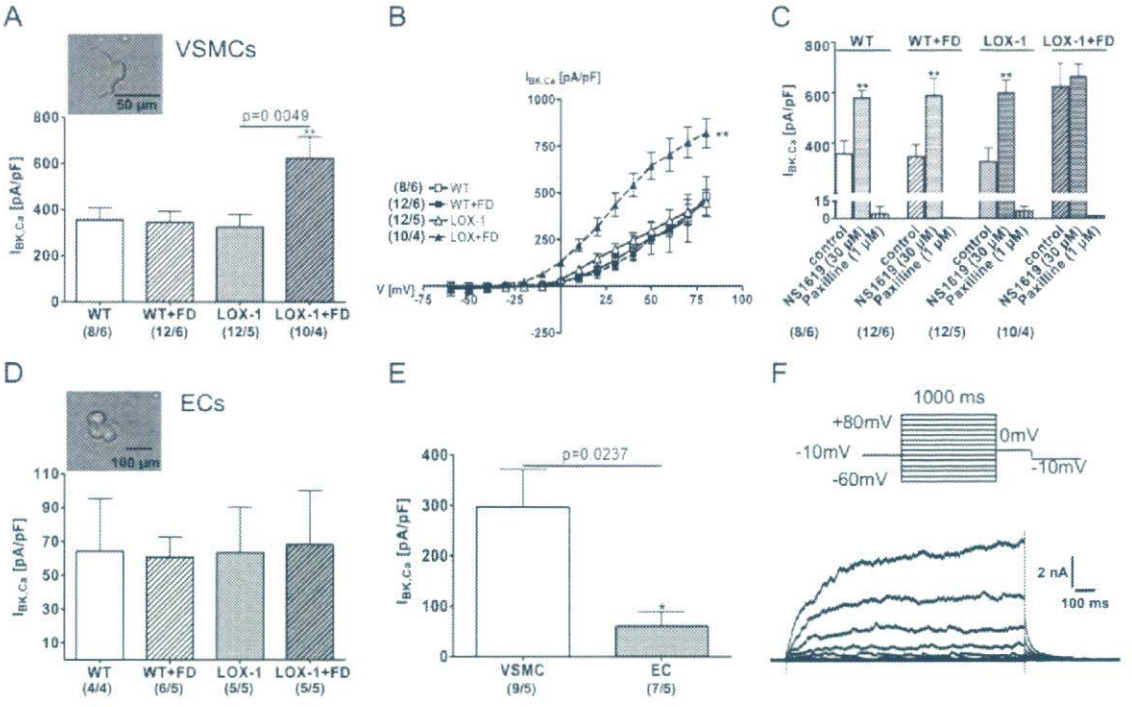


Figure 4

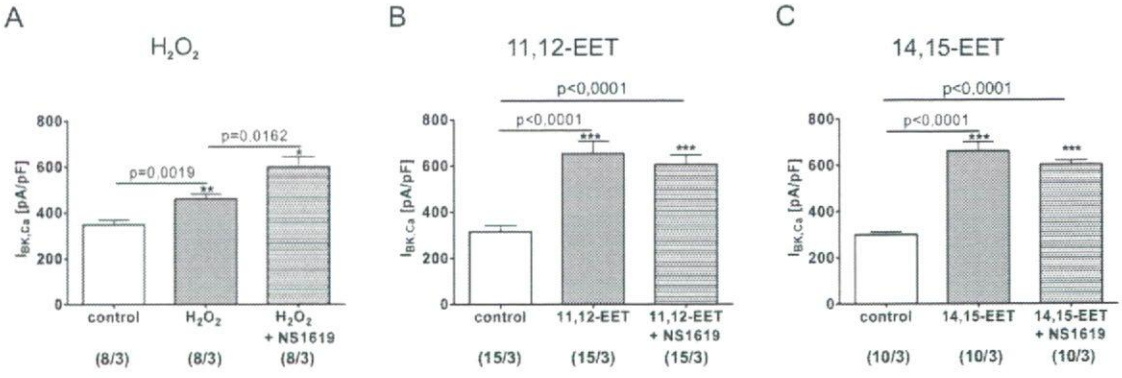
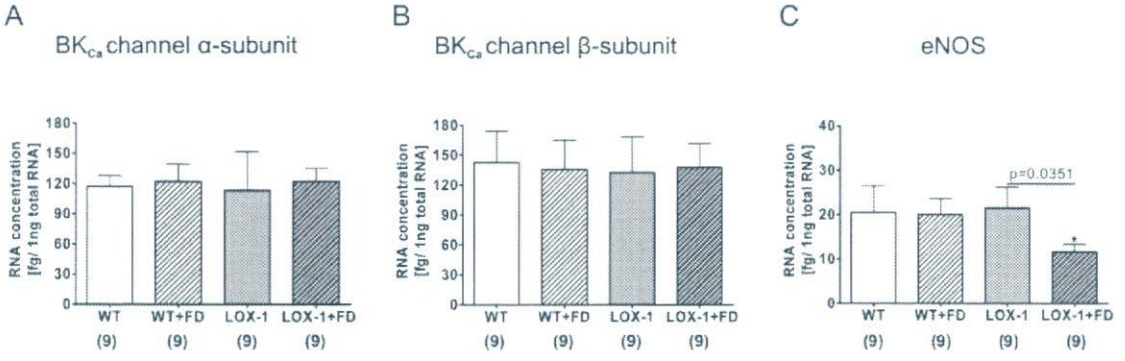


Figure 5



Mediation of Electronegative Low-Density Lipoprotein Signaling by LOX-1

A Possible Mechanism of Endothelial Apoptosis

Jonathan Lu, Jun-Hai Yang, Alan R. Burns, Hsin-Hung Chen, Daming Tang, Jeffrey P. Walterscheid, Shinichi Suzuki, Chao-Yuh Yang, Tatsuya Sawamura, Chu-Huang Chen

Abstract—The lectin-like oxidized LDL receptor LOX-1 mediates endothelial cell (EC) uptake of experimentally prepared copper-oxidized LDL (oxLDL). To confirm the atherogenic role of this receptor cloned against copper-oxLDL, we examined whether it mediates EC uptake of L5, an electronegative LDL abundant in dyslipidemic but not normolipidemic human plasma. Hypercholesterolemic (LDL-cholesterol, >160 mg/dL) human LDL was fractionated into L1–L5, increasingly electronegative, by ion-exchange chromatography. In cultured bovine aortic ECs (BAECs), L5 upregulated LOX-1 and induced apoptosis. Transfection of BAECs with LOX-1–specific small interfering RNAs (siLOX-1) minimized baseline LOX-1 production and restrained L5-induced LOX-1 upregulation. Internalization of labeled L1–L5 was monitored in BAECs and human umbilical venous ECs by fluorescence microscopy. LOX-1 knockdown with siLOX-1 impeded the endocytosis of L5 but not L1–L4. In contrast, blocking LDL receptor with RAP (LDL receptor–associated protein) stopped the internalization of L1–L4 but not L5. Although chemically different, L5 and oxLDL competed for EC entry through LOX-1. Via LOX-1, L5 signaling hampered Akt phosphorylation and suppressed EC expression of fibroblast growth factor-2 and Bcl-2. L5 also selectively inhibited Bcl-xL expression and endothelial nitric oxide synthase phosphorylation but increased synthesis of Bax, Bad, and tumor necrosis factor-α. Blocking Akt phosphorylation with wortmannin increased LOX-1 expression, suggesting a modulatory role of Akt in LOX-1 synthesis; L5 upregulated LOX-1 by dephosphorylating Akt. Because endothelial nitric oxide synthase and Bcl-2 activities are Akt-dependent, L5 impairs Akt-mediated growth and survival signals in vascular ECs by way of LOX-1. Thus, the L5/LOX-1 complex may play a critical role in atherogenesis and illuminate important targets for disease intervention. **(*Circ Res*. 2009;104:619-627.)**

Key Words: apoptosis ■ atherosclerosis ■ endothelium ■ lipoproteins ■ receptors

The lectin-like oxidized low-density lipoprotein (LDL) receptor LOX-1 (the type D scavenger receptor) was initially cloned from bovine aortic endothelial cells (BAECs) in 1997 by Sawamura et al via its ability to bind LDL oxidized ex vivo by copper (oxLDL).[1] Today, the atherothrombotic designation of LOX-1 is manifold, as has been reviewed.[2,3] LOX-1 is considered the major receptor for oxLDL in human and various animal vascular endothelial cells (ECs). In human atherosclerotic lesions, it is expressed in intimal smooth muscle cells and lipid-laden macrophages, as well as in plaque neovasculature.[4] LOX-1 plays a role in oxLDL-induced apoptosis of vascular smooth muscle cells and in the production of matrix metalloproteinases; hydroxymethylglutaryl-coenzyme A reductase inhibitors (statins) inhibit its expression in atheromas of Watanabe heritable hyperlipidemic rabbits.[5] In LOX-1 knockout mice, binding of oxLDL to aortic ECs was decreased and endothelium-dependent vasorelaxation was preserved after oxLDL treatment.[6]

The role of LOX-1 as an atherogenesis-related signaling transducer has yet to be confirmed, however, through naturally occurring modified LDL. Identifying such atherogenic particles is a priority in the sustained research initiative against the death and disability of cardiovascular disease. Small, dense LDL and the electronegatively charged subfraction of LDL in human blood are good suspects, and oxidative mechanisms are a key focus as their possible molecular modus operandi. The latter has been of interest since 1988, when Avogaro et al first characterized LDL(−) after its dichotomic separation by anion-exchange chromatography.[7] Whether lipid peroxidation is required for its formation remains in question; several routes independent of oxidation

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have been proposed.[8–10] Despite its minimal oxidation relative to oxLDL, LDL(−) can induce a spectrum of proinflammatory and cytotoxic responses in cultured vascular cells.[11,12] High concentrations of LDL(−) have been associated with major risk factors for cardiovascular disease, including hypercholesterolemia[13] and type 2 diabetes mellitus.[14]

Apparently more toxic still is L5, which is the most electronegative of the LDL subfractions separable by high-capacity ion-exchange chromatography according to charge that we first described in 2003.[11,15] L5 is present in hypercholesterolemic subjects and those with type 2 diabetes but not in healthy subjects with clinically normal lipid concentrations.[16] Distinct from L1–L4, it has marked potency in inducing EC apoptosis.[11]

In the present study, we extended our work with L5 to determine whether it signals through LOX-1. We examined that mechanism in coordination with the pathway of fibroblast growth factor (FGF)2, a potent antiapoptotic and prosurvival protein that functions by stimulating the phosphatidylinositol 3′-kinase (PI3K)-Akt axis.[17] We previously showed that L5 induces EC apoptosis in part by disrupting FGF2 autoregulation along the FGF2-PI3K-Akt loop.[18] Because FGF2 functions by activating downstream kinases and effectors (including Akt, Bcl-2, Bad, Bax, Bcl-xL, and endothelial nitric oxide synthase [eNOS]), we tested whether L5 dysregulates those FGF2-regulated targets through LOX-1. To better understand the atherogenic processes within hypercholesterolemic patients, we also examined how L5 upregulates LOX-1 to transduce its signaling.

Materials and Methods

Cell Culture

Primary BAECs (Cambrex) were used after 3 or 4 passages and maintained in DMEM (Invitrogen) containing 10% FBS. Primary human umbilical venous ECs (HUVECs), maintained in EGM medium (Cambrex), were cultured from umbilical cords of newborn infants and used after the first passage.[19] The content of FBS was reduced to 5% during LDL treatments. The LOX-1–neutralizing antibody TS20 was provided by T.S. All procedures were approved by the Baylor College of Medicine Institutional Review Board.

LDL Isolation and oxLDL Preparation

To prevent contamination and experimental oxidation, 50 mU/mL aprotinin, 1% ampicillin/streptomycin, and 5 mmol/L EDTA were added to human plasma samples immediately after collection. LDL particles were isolated from hypercholesterolemic (LDL-cholesterol, >160 mg/dL) volunteers by sequential potassium bromide density centrifugation to remove chylomicrons, very-low-density lipoprotein, and intermediate-density lipoprotein fractions, yielding LDL at a final density of 1.019 to 1.063 g/mL.[20] OxLDL was prepared by incubating the dialyzed portion of L5-free LDL, obtained from healthy volunteers, with 5 μmol/L $CuSO_4$ at 37°C for 24 hours.[20]

Ion-Exchange Purification of L5

Whole LDL was equilibrated by dialysis in a column loaded with buffer A, comprising 20 mmol/L Tris HCl, pH 8.0, 0.5 mmol/L EDTA, and 0.01% NaN_3. Approximately 100 mg of LDL material was injected onto a UnoQ12 anion-exchange column (Bio-Rad) by using an LCC-500 programmable fast-protein liquid chromatography pump (Pharmacia) and eluted according to a multistep sodium chloride gradient as previously described.[15]

Apoptosis Measurements

Apoptosis was assessed by the Vybrant Apoptosis Assay kit (Molecular Probes), with visualization by a Zeiss Axiovert 200 fluorescence microscope and filters to capture digitally images based on Hoechst 33342, propidium iodide, and calcein AM staining of nuclear or apoptotic DNA.[11] Cytoplasmic histone-associated DNA fragmentation was examined by using the Cell Death Detection ELISA Assay (Roche) according to the protocol of the manufacturer.

LOX-1 Small Interfering RNA Transfection

Complementary antisense constructs of the bovine LOX-1 receptor were custom designed (Dharmacon) from GenBank accession no. NM_174132. The set, designed to disrupt the lectin-recognition domain of LOX-1, was composed of the following sequences: 5′-GAACCTGAATCTCCAAGAA, to clamp bases 366 to 384; 5′-GAAAGAGGCAGCAAACTAT, to complement bases 390 to 408; 5′-CCAGGTCTCTGATCTCATA, to bind bases 171 to 189; and 5′-AGAAGGAACTCAAAGAAAT, to target bases 284 to 302 of LOX-1 mRNA. For a positive control, silencing efficiency was determined by transfection with siCONTROL cyclophilin B small interfering (si)RNA. For a negative control, primary BAEC cultures were transfected with the siCONTROL nontargeting siRNA pool, which is a mixture of DNA constructs that produce siRNAs but do not bind to cellular mRNA. Primary BAECs were transfected in 6-well plates at 70% confluence. The transfection was executed according to the recommended protocol of 100 nmol/L siRNA mixed with Oligofectamine (Invitrogen). After transfection, the supernatant was replaced with 5% FBS DMEM, and cells were exposed to experimental treatments.

LDL Labeling With 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindocarbocyanine Perchlorate and 3,3′-Dioctadecyloxacarbocyanine Perchlorate

Lipophilic dyes 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and 3, 3′-dioctadecyloxacarbocyanine perchlorate (DiO) were purchased from Invitrogen. DiI was used to label LDL subfractions. In brief, DiI-LDL subfractions (L1–L5) were freshly prepared before use, as described by Pitas et al[21] L1–L5 were diluted to 1 mg/mL with PBS and incubated with 80 μmol/L DiI at 37°C overnight. The labeled L1–L5 were then purified by ultracentrifugation at a density of 1.063 g/mL and subsequently dialyzed against PBS-EDTA (0.5 mmol/L), with all steps protected from light. DiO was used to label oxLDL with the same procedure.

Fluorescence Monitoring of L1–L5 Internalization

The DiI-LDL subfractions were normalized by protein content and added to primary BAECs at a concentration of 50 μg/mL. Various amounts of DiO-oxLDL were also used for receptor binding analysis. Internalization was observed using a Zeiss Axiovert 200 fluorescence microscope to record the positions of DiI-LDL and DiO-oxLDL with respect to bright field images in overlay. To antagonize LDL binding to LDL receptors, cells were pretreated with a final concentration of 2 μmol/L human LDL receptor (LDLR)-associated protein (RAP) (EMD Biosciences).

Western Blot Analysis

To measure protein levels by Bradford assay, cells were solubilized in NET-N lysis buffer. Electrophoresis was conducted with 10% SDS-PAGE by using 10 μg of each cell lysate.[20] The separated proteins were transferred to nitrocellulose paper (Bio-Rad) and blocked with SuperBlock (Pierce). Monoclonal mouse anti–LOX-1 (provided by T.S.), polyclonal rabbit anti-FGF2, monoclonal mouse anti-Bcl-2, polyclonal goat anti–tumor necrosis factor (TNF)-α (R&D systems), polyclonal rabbit anti–phospho-Akt (Ser473), polyclonal rabbit anti-Akt, monoclonal rabbit anti-p-eNOS, monoclonal

rabbit anti-eNOS, monoclonal mouse anti-Bax, polyclonal rabbit anti-Bad and anti-Bcl-xL (Cell Signaling Technology), and monoclonal mouse anti–β-actin (Sigma-Aldrich) antibodies were used to probe membranes to evaluate relative quantities of specific proteins before and after treatments. Secondary anti-rabbit, -goat, and -mouse IgG antibodies were conjugated to horseradish peroxidase (Amersham Biosciences). Signals were recorded on Kodak films by using ECL Plus chemiluminescent reagents (Amersham Biosciences). The results were normalized to those of β-actin.

Semiquantitative RT-PCR

The mRNA-silencing efficiency of transfected siRNA was evaluated by semiquantitative RT-PCR (Invitrogen SuperScript III One-Step RT-PCR kit). Total RNA was isolated from BAECs by using the RNeasy Midi Kit (Qiagen). Bovine LOX-1 primers (forward: 5′-GTACCCTGCTGCTGTGA; reverse: 5′-TTGACAA CCCCATCCAGA) and bovine cyclophilin B primers (forward: 5′-GTACTTTGACCTGCGAA; reverse: 5′-GCCCTATTCCTTGGCAA) were designed to amplify the respective regions.

Statistical Analysis

The significance of differences was assessed by a paired Students *t* test with Bonferroni correction. Probability values of $P<0.05$ were considered significant. Results are expressed as mean values±SEM.

Results

L5 Isolation and Activity

Consistent with our previous report,[11] L5 was present in hypercholesterolemic (Figure 1A) but not normocholesterolemic (Figure 1B) human plasma. In BAECs incubated with 50 μg/mL each LDL preparation for 24 hours, neither LDL from healthy donors nor L1 from hypercholesterolemic subjects showed detectable apoptotic activity, whereas BAEC apoptosis was substantially greater after exposure to L5 (Figure 1A and 1C). The apoptotic activity of L5 was quenched, in a concentration-dependent manner, by the addition of 10 μg/mL TS20, a LOX-1–neutralizing antibody (Figure 1C).

Subfractionated LDL Internalization With Regard to LDL Receptor and LOX-1

We typically evaluate apoptotic activity in ECs after an overnight incubation with LDL subfractions. However, internalization of the LDL fractions should be an early event in the apoptosis cascade.[22] Therefore, we monitored intracellular fluorescence by deconvolution microscopy from the outset of treating HUVECs with 50 μg/mL DiI-L1 or DiI-L5. At 30 minutes, both DiI-L1 and DiI-L5 were visible intracellularly. Pretreatment with TS20 to neutralize LOX-1 activity blocked L5 endocytosis but had no effect on L1 (Figure 2A).

To examine how the chromatographically separated LDL particles were internalized with regard to LDLR, we used recombinant human RAP to selectively antagonize LDLR activity.[23] DiI-labeled L1 and L5 were applied to BAECs, and the cells were photographed after overnight incubation. In the negative control (−RAP), cells internalized L1 and L5 equally well. In contrast, RAP pretreatment (+RAP) obstructed the internalization of L1–L4 but did not prevent internalization of L5 (Figure 2B; L2–L4 not shown). Although L5 fits within the physical characteristics of LDL,[16] these results show that it is not a ligand for LDLR.

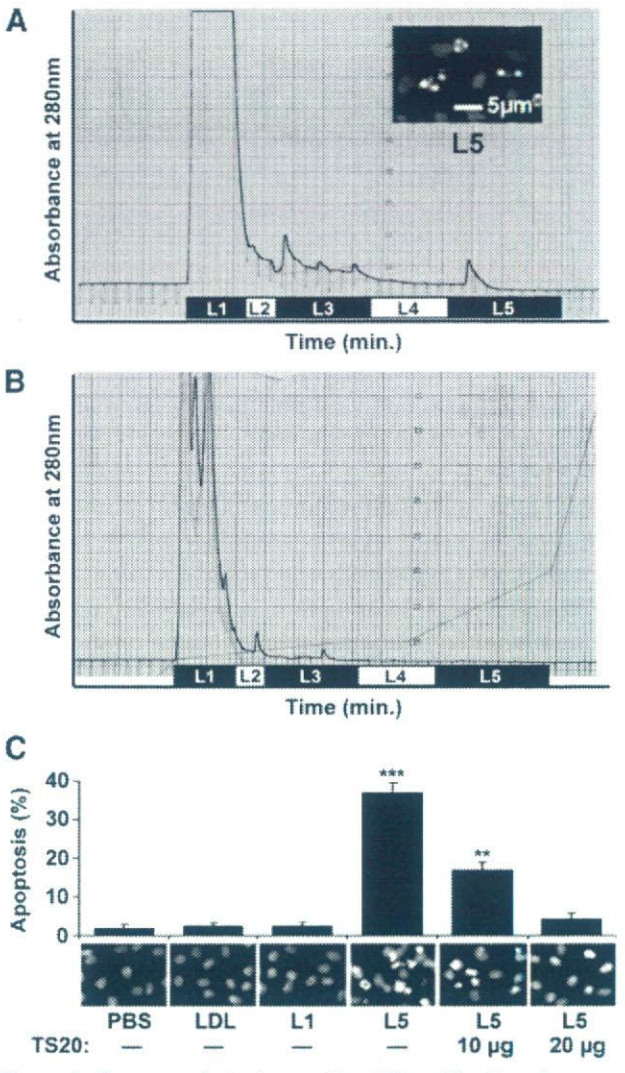

Figure 1. Presence of electronegative LDL subfractions in human hypercholesterolemic plasma and apoptotic activity of L5. A and B, Representative fast-protein liquid chromatography chromatograms of hypercholesterolemic LDL, yielding L5 (A), and normocholesterolemic LDL, where L5 is absent (B). C, BAECs exhibiting condensed, fragmented nuclei by epifluorescence microscopy were considered to be apoptotic. Percentage of cells undergoing apoptosis was evaluated in 6 samples. $^{**}P<0.01$, $^{***}P<0.001$ vs PBS.

DiO-oxLDL was used as a positive control and a competitive ligand of L5 to LOX-1. To avoid massive apoptosis, BAECs were treated with 25 μg/mL of either DiI-L5 or DiO-oxLDL in initial experiments. At 24 hours, both could be easily detected inside their respective cells (Figure 2C). In cells loaded with 25 μg/mL DiI-L5, addition of DiO-oxLDL exhibited a progressive increase of colocalization of both particles in a concentration-dependent manner. When DiO-oxLDL was increased to 25 μg/mL, the combined toxicity of L5 and oxLDL resulted in marked apoptosis. Because oxLDL is also internalized by LOX-1,[1] the results suggest that L5 and oxLDL compete for EC entry through this particular receptor (Figure 2C).

Characterization of LOX-1–Specific siRNAs

Before using siRNA in experiments to support our hypothesis, we tested its activity in our model system. Using a

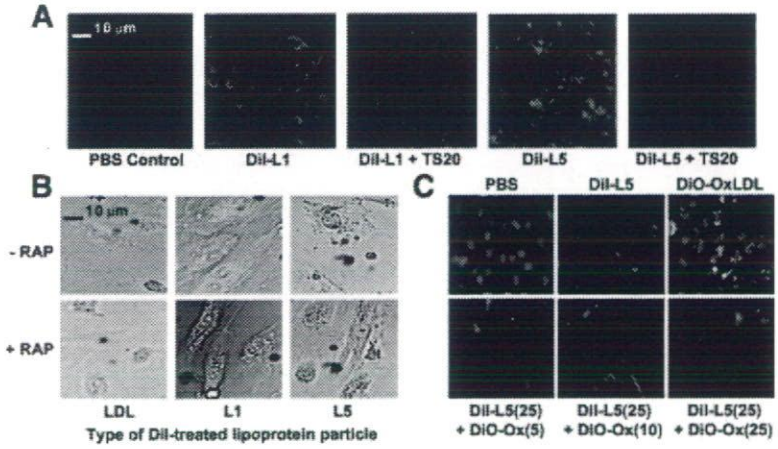

Figure 2. Internalization of L5 and oxLDL by LOX-1 and endocytosis of L1 by LDLR. A, Internalization of DiI-L1 and DiI-L5 was viewed in HUVECs with and without TS20 neutralization of LOX-1. B, Subconfluent BAECs were incubated with 50 μg/mL DiI-labeled L1 and L5, and particle internalization was monitored after 24 hours by fluorescence microscopy in cells with and without RAP peptide antagonism of LDLR. C, Internalization of DiI-L5 and DiO-oxLDL, alone (25 μg/mL) or in combinations at concentrations indicated, was monitored in BAECs at 24 hours.

combination of four custom-made bovine LOX-1–specific constructs (siLOX-1), we were able to suppress basal and L5-stimulated LOX-1 mRNA in BAECs, whereas the nontargeting, siRNA negative controls had no effect (Figure 3A). To determine transfection efficiency and specificity, we used cyclophilin B siRNAs and RT-PCR to measure cyclophilin B in BAECs. Two siRNA controls, without specific mRNA targets, were used to observe the effects of siRNA production. With the RNA-induced silencing complex (RISC)-free and nontargeting template sets, there was some inhibition of cyclophilin B mRNA, likely attributable to the transfection process itself. However, the cyclophilin B–positive control potently deleted the mRNA to a level below the detection by RT-PCR (Figure 3B). The effectiveness of siRNA treatment was confirmed by Western blot analysis, which showed a significant reduction of LOX-1 expression compared with control (Figure 3C).

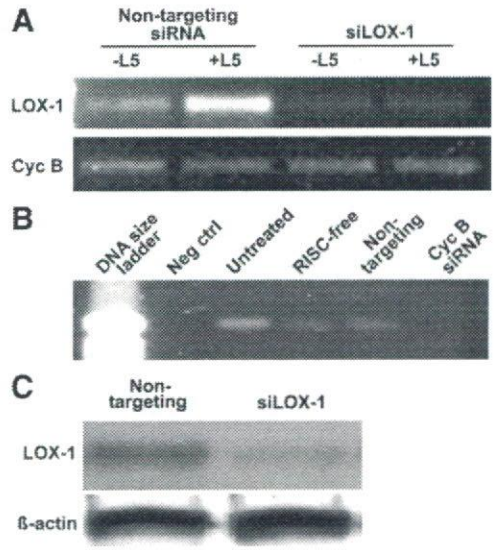

Figure 3. Characterization of LOX-1 siRNA silencing. A, Results of RT-PCR showing relative levels of LOX-1 mRNA in BAECs treated with L5 and siLOX-1. Cyclophilin B (Cyc B) was amplified as an internal control. B, RT-PCR controls using cyclophilin B to determine transfection efficiency and specificity. The negative control is a reaction without the addition of a template. The untreated lane represents cells without transfection or empty liposomal treatment. RISC-free corresponds to cells treated with constructs that prevent RISC assembly. The nontargeting lane is a set of specific siRNAs developed not to bind to any product of the human genome. The cyclophilin B control is a set of siRNAs that target cyclophilin B mRNA. C, Western blot of LOX-1 protein before and after siLOX-1 treatment. As an internal control, β-actin was referenced for protein normalization.

Subfractionated LDL Internalization With Regard to LOX-1

In experiments parallel to our studies with LDLR, we incubated BAECs with 50 μg/mL DiI-labeled L1–L5 and used siLOX-1 to specifically block its cellular expression. All the LDL subfractions were equally internalized among cells transfected with a pool of nontargeting siRNAs (Figure 4, top row). In LOX-1–silenced BAECs, all but DiI-L5 were internalized (Figure 4, bottom row). These findings indicate that L5 internalization requires LOX-1 expression, whereas the less electronegative LDL subfractions rely on the LDL receptor.

Comparison of oxLDL and L5 Activity in Modulating LOX-1 and FGF2

Much of the present literature on LDL oxidation is based on results that use the experimentally prepared oxLDL for eliciting cellular responses.[24] Copper-oxLDL is well characterized, and more easily obtained than pathologically derived L5, so we used it as a positive control for our assays. We treated BAECs with 50 μg/mL L5 or oxLDL and determined the amount of LOX-1 protein by Western blot. Both L5 and oxLDL enhanced LOX-1 expression to a similar extent (Figure 5A). BAECs transfected with siLOX-1 maintained a significantly reduced baseline LOX-1 and easily resisted the stimulatory effects of L5 and oxLDL.

Because L5 causes apoptosis, we hypothesized that LOX-1 activation inhibits the expression of FGF2, a crucial factor for EC growth and survival. We measured FGF2 protein in L5- or oxLDL-treated BAECs with or without TS20 inactivation of LOX-1. L5 and oxLDL were equally effective in reducing FGF2 protein production, yet this activity was prevented by TS20 (Figure 5B). Therefore, LOX-1 is an inducible receptor for oxLDL and L5, which downregulates FGF2 expression when activated.

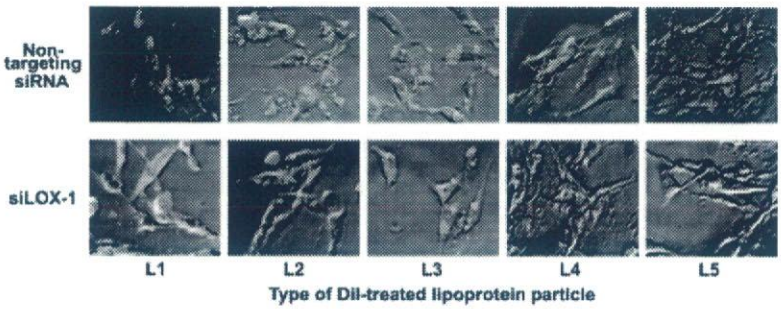

Figure 4. LDL subfractional binding preferences for LOX-1. BAECs transfected with nontargeting or specific LOX-1 siRNAs were incubated with charge-separated, DiI-labeled LDL fractions. Internalization was monitored by fluorescence microscopy.

Role of LOX-1 in L5 Signaling and Mechanism of L5-Induced LOX-1 Expression

The signals directed by FGF2 work through the PI3K/Akt pathway, ultimately to raise Bcl-2 levels and discourage apoptosis while promoting EC growth.[17] Because of the importance of Akt and Bcl-2 in maintaining EC health, we examined at which points L5 and LOX-1 disrupt this survival loop. BAECs were treated with 50 μg/mL L5 for 24 hours, and the lysates were assayed for FGF2, phosphorylated Akt, and Bcl-2 by Western blot analysis. Cells treated with L5 displayed a significant reduction of FGF2 protein, whereas cells transfected with siLOX-1 were free of such L5 effects (Figure 6A). Cells treated with 20 ng/mL FGF2 showed a significant increase of phosphorylated Akt relative to total Akt protein, whereas cells treated with L5 were unable to maintain a basal level (Figure 6B). However, treatment with siLOX-1 prevented a further reduction in phosphorylated Akt when challenged with L5. In the final step, L5 restrained Bcl-2 expression, but the addition of FGF2 stimulated Bcl-2 expression above basal levels (Figure 6C). Cells transfected with siLOX-1 maintained a steady amount of Bcl-2, even when stimulated with L5.

L5 signaling was accompanied by augmented LOX-1 production. Because LOX-1 was upregulated during Akt dephosphorylation in L5-exposed cells (Figure 5), we tested whether baseline LOX-1 expression is negatively modulated by Akt. The findings that hindering Akt activation with PI3K inhibitor wortmannin increased LOX-1 expression in a concentration-dependent manner verified the hypothesis (Figure 6D). Thus, by dephosphorylating Akt (Figure 6D), L5 activated LOX-1 expression that is normally suppressed by Akt.

To further examine the role of LOX-1 in L5 signaling, we performed additional siRNA transfection studies. In nontransfected BAECs, treatment with 50 μg/mL L5 for 24 hours resulted in apoptotic nuclear changes as expected, whereas cells that had been transfected with siLOX-1 were highly resistant to the toxic effects of L5 (Figure 7A and 7B). Transfection with siLOX-1 or nontargeting siRNA alone did not affect cell survival. The apoptotic effect of L5 was accomplished by its ability to selectively downregulate prosurvival proteins (Bcl-2, Bcl-xL, eNOS, p-eNOS) (Figures 6C and 7C) while concomitantly upregulating proapoptotic factors (Bax, Bad, TNF-α) (Figure 7C).

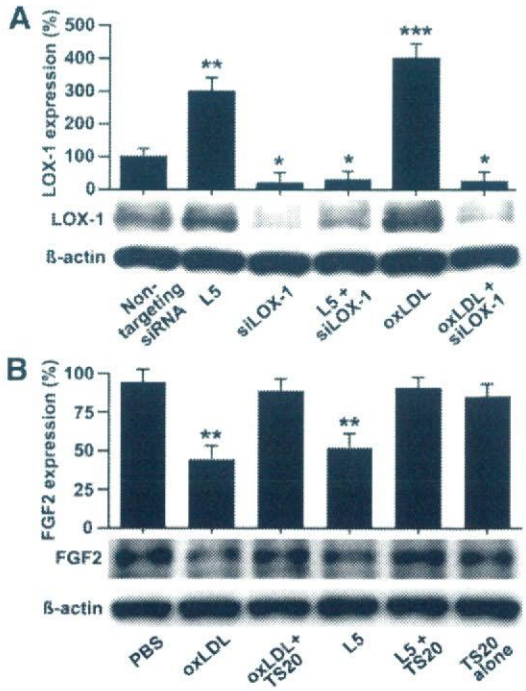

Figure 5. Comparison of oxLDL and L5 in modulation of LOX-1 and FGF2. A, Western blot of BAECs treated with 50 μg/mL L5 or oxLDL, in combinations with siLOX-1 knockdown. The bar graph shows LOX-1 intensity normalized to β-actin. B, Western blot of BAECs treated with 50 μg/mL L5 or oxLDL, in combinations with TS20. The bar graph relates FGF2 intensity to β-actin. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs nontargeting control or IgG control (n=3).

Discussion

LOX-1 is highly expressed in vivo in large human arteries, particularly at the predilection sites of atherosclerosis.[1,25] One of the prototypical endothelial scavenger receptors that bind to negatively charged molecules, LOX-1 has emerged as the principal receptor that mediates oxLDL uptake in the vascular wall in both mammals and humans.[1,25,26] Thus, it may be the most important scavenger receptor in the development of atherothrombotic disease, given that high susceptibility to lipid peroxidation is believed to be a key characteristic of atherogenic LDL.[24] In LOX-1 studies, the high conservation among species of its lectin-like domain at the long, extracellular C terminus[1] facilitates the use of human oxLDL in animal EC models.[27] Our present studies demonstrated that LOX-1 also mediates the actions of pathophysiologically derived human

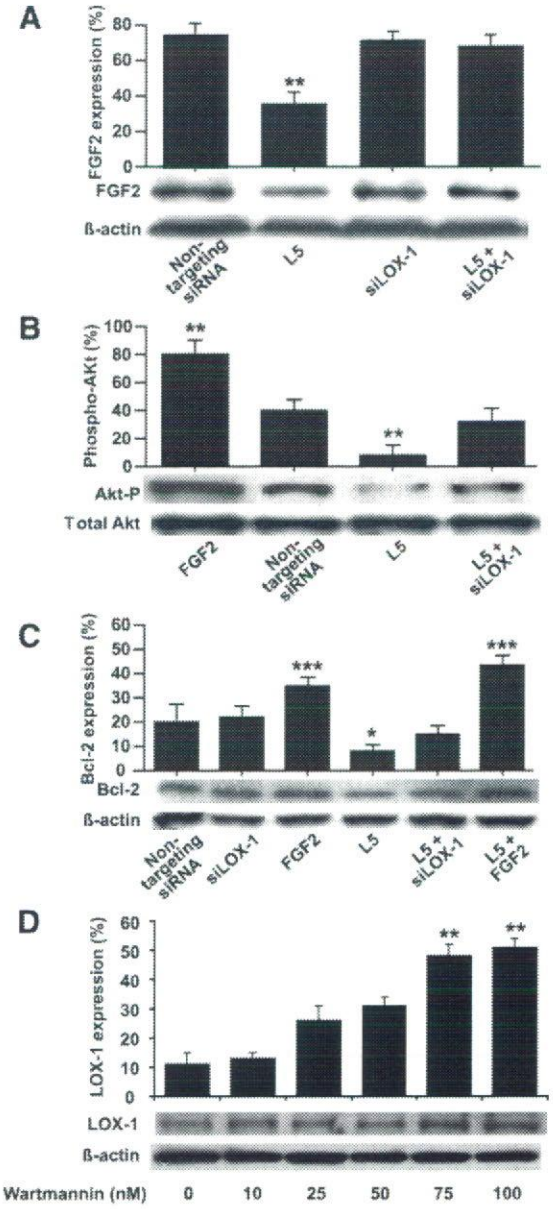

Figure 6. Role of LOX-1 in L5 signaling and mechanism of L5-induced LOX-1 expression. A, FGF2 protein concentrations were assayed by Western blot analysis 24 hours after BAECs were treated with 50 μg/mL L5, with or without siLOX-1 blockade. B, The degree of Akt phosphorylation was measured by normalization to total Akt in BAECs treated with 20 ng/mL FGF2 or 50 μg/mL L5, with or without siLOX-1 transfection. C, Bcl-2 protein levels were assayed by Western blot analysis and normalized to β-actin. BAECs had been treated with 20 ng/mL FGF2, 50 μg/mL L5, or a combination of L5 and FGF2, with or without siLOX-1 transfection. D, LOX-1 expression in BAECs was analyzed by Western blot, in the absence or presence of the PI3K inhibitor wortmannin in graded concentrations. *$P<0.05$, **$P<0.01$ vs nontargeting siRNA control; ***$P<0.001$ vs L5 alone (n=3).

electronegative LDL, perhaps a native atherogenic lipoprotein species.

Our experiments were performed using 5 LDL subfractions, L1 to L5. L5, the most electronegative subfraction, accounts for a smaller proportion (1% to 3%)[11,15] of LDL than does the dichotomically separated LDL(−).[7,9] L5 decreases intracellular FGF2 production in vascular ECs, thus contributing to apoptosis and impaired angiogenic capacity.[11] In addition, it induces monocyte–EC adhesion,[15] a step that precedes the subendothelial deposition of inflammatory cells and modified lipids in atherogenesis. Unlike its normal LDL counterpart, LDL(−) also induces a spectrum of atherogenic responses and production inflammatory chemokines from ECs.[28–31]

In this study, LOX-1 protein levels were increased by 3-fold when the cells were exposed to L5. Exposing ECs to oxLDL yielded a similar effect. Baseline protein expression of LOX-1 was greatly suppressed in BAECs transfected with siLOX-1. Receptor knockdown also completely prevented its upregulation by L5 or oxLDL. A growing body of studies shows that a variety of agents, including oxLDL, TNF-α, angiotensin II, and endothelin 1, affect LOX-1 at both the mRNA and protein levels.[32–34]

DiI-labeled LDL subfractions L1–L5 could be visualized inside normal BAECs after 24 hours of incubation. Labeling with DiI, a lipophilic fluorescence probe with excitation and emission wavelengths of 520 and 578 nm,[35] preserves the functional properties of LDL, including its affinity to LDLR and subsequent interactions with cells.[36] That LOX-1 knockdown prevented the internalization of L5 exclusively indicates that L5 was the only subfraction endocytosed by LOX-1, whereas the other subfractions were internalized by other receptors. Neutralizing LOX-1 with TS20 was equally effective in blocking L5 entry into the cells. L4 entry was minimally obstructed in LOX-1–silenced cells, suggesting that a small portion of L4 particles may exhibit L5-like properties, as explained above.

RAP, known for its potent antagonistic effect against members of the LDLR family,[37] blocked the entry of L1–L3 into BAECs but had no effect on L5 internalization. Again, there was a partial effect on L4. In combination, these findings indicate that L5 is an extreme and pure form of LDL(−) that is endocytosed specifically by LOX-1, not by the normal LDL receptor. The more electropositive subfractions L1 to L3 are internalized by LDLR but not LOX-1. Most L4 particles enter the ECs through LDLR, with a small portion entering the cells through LOX-1 owing to possible amalgamation.

Because our original observation was made at 24 hours after the cells had been treated with L5, the rest of our assays were completed at 24 hours for consistency and comparability. However, the initial internalization of these particles may occur much sooner. In a series of experiments using HUVECs, we found that the uptake of L1 and L5 may be complete as early as 30 minutes. Neutralizing LOX-1 with TS20 inhibited the entry of L5 but not L1. This further confirms the mediator role of LOX-1 in L5 internalization in both human and bovine ECs.

LOX-1 mediates oxLDL-induced EC apoptosis.[38] Here, neutralizing LOX-1 with TS20 attenuated the apoptotic effect of L5. The role of LOX-1 was confirmed by gene silencing of this receptor by a LOX-1–specific siRNA cocktail. The efficiency of siRNA transfection can be influenced by factors including siRNA design, transfection reagents, and RISC

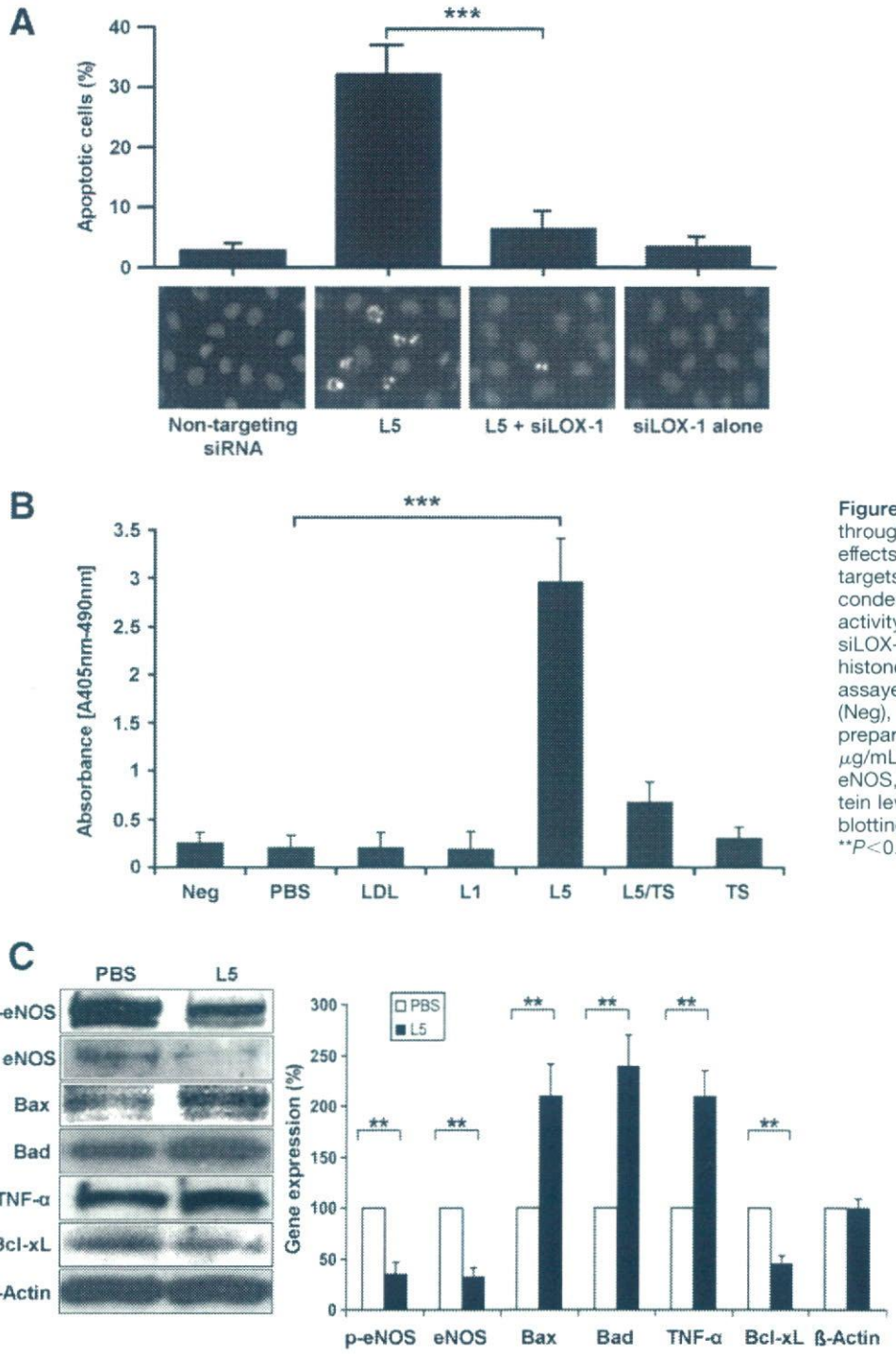

Figure 7. L5 induces EC apoptosis through LOX-1 and exerts opposite effects on prosurvival vs proapoptotic targets. A, Fluorescence microscopy of condensed nuclear staining shows the activity of L5 in BAECs with and without siLOX-1 transfection. B, Cytoplasmic histone-associated DNA fragmentation assayed in BAECs treated with nothing (Neg), PBS, LDL (50 μg/mL for all LDL preparation), L1, L5, L5 with TS20 (10 μg/mL), or TS20 alone. C, p-eNOS, eNOS, Bax, Bad, TNF-α, and Bcl-xL protein levels were evaluated by Western blotting and normalized to β-actin. $^{**}P<0.01$, $^{***}P<0.001$ (n=3).

formation.[39] The positive control cyclophilin B siRNA demonstrated near-complete silencing, and the designed LOX-1 siRNA demonstrated 80% silencing efficiency, indicating a sufficient delivery of siRNA. To test whether L5 and oxLDL compete for the same receptor, we challenged BAECs with DiI-L5 and DiO-oxLDL. The results indicated that L5 and oxLDL are both internalized by LOX-1 in a competitive manner.

In agreement with our previous findings,[11,20] both L5 and oxLDL reduced the protein level of prosurvival FGF2×40% to 50%. Because of the mediator role of LOX-1 in L5-induced EC apoptosis, it is not surprising to find that L5-induced FGF2 downregulation could be prevented by blockade or knockdown of this receptor. One of the early effectors of FGF2 is Akt, whose activation through phosphorylation by PI3K signaling is stimulated by exogenous or autocrine production of FGF2.[40] Opposite to its stimulatory effect on FGF2 transcription through the FGF2-PI3K-Akt autoregulatory loop,[18] Akt was found to retain LOX-1 expression to a minimum at the baseline. By means of dephosphorylating Akt, L5 lifts this suppression and augments LOX-1 production.

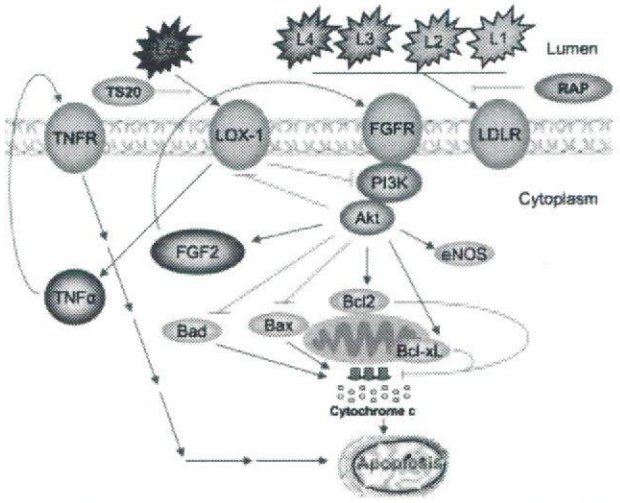

Figure 8. Schematic summary of L5 signaling through LOX-1 in vascular endothelial cells. L5 signals and is internalized through LOX-1, whereas L1–L4 are endocytosed via LDLR. Green arrows indicate stimulation; blue arrows indicate direction of transportation; red arrowheads indicate release from mitochondria; red lines with end bar indicate inhibition. RAP, LDLR inhibitor; TS20, LOX-1–neutralizing antibody.

The antiapoptotic protein Bcl-2 represents a relatively downstream effector of FGF2.[11] The opposing stimulatory effects of FGF2 and inhibitory effects of L5 on Akt phosphorylation and Bcl-2 expression support the hypothesis that L5 induces EC apoptosis by inhibiting FGF2-dependent survival mechanisms. The preservation of Akt phosphorylation and Bcl-2 expression in LOX-1 knockdown cells indicates that L5 interrupts the normal FGF2–Akt–Bcl-2 signaling by means of activating LOX-1. Additionally, L5 suppressed Bcl-xL, eNOS, and p-eNOS while upregulating Bax, Bad, and TNF-α. Taken together, by way of LOX-1, L5 selectively suppresses prosurvival mechanisms but concomitantly activates proapoptotic effectors to force the cells into apoptosis (Figure 8).

Ectopic expression of LOX-1 facilitates the removal of plasma oxLDL and attenuates atherosclerosis development in apolipoprotein E–deficient mice.[41] Our finding that the naturally occurring L5 signals through LOX-1 suggests a significant role of the L5-LOX-1 axis in atherogenesis.

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Disclosures

None.

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Oxidized LDL binding to LOX-1 enhances MCP-1 expression in cultured human articular chondrocytes

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Summary

Objective: It has been suggested that oxidized low-density lipoprotein (ox-LDL) has some roles in progression of osteoarthritis. The purpose of this study is to investigate whether ox-LDL binding to lectin-like ox-LDL receptor 1 (LOX-1) enhances monocyte chemoattractant protein 1 (MCP-1) expression in cultured human articular chondrocytes (HACs).

Method: The time course and dose response of MCP-1 mRNA expression and MCP-1 protein release into medium following ox-LDL stimulation were investigated using quantitative Real time PCR (delta–delta Ct method) and enzyme-linked immunosorbent assay (ELISA), respectively. To examine the receptor specificity of ox-LDL action, HACs were preincubated with anti-human LOX-1 monoclonal antibody (TS92).

Results: A time-course study revealed that MCP-1 mRNA expression increased 5.09 ± 0.86 fold 12 h after ox-LDL stimulation compared to time-0. ox-LDL stimulation increased MCP-1 protein level in conditioned medium in a time-dependent manner. Increased MCP-1 level was evident 6 h after stimulation, reaching 830 ± 91 pg/ml at 24 h (33 ± 8 pg/ml at time-0). Dose responses of MCP-1 expression were also evident in mRNA and protein levels. Pretreatment with TS92 markedly suppressed these stimulating effects of ox-LDL, although that with non-specific IgG did not. Native LDL did not affect MCP-1 expression.

Conclusion: Our results suggest that ox-LDL enhances MCP-1 expression in HACs and supports the hypothesis that ox-LDL is involved in cartilage degeneration.

Key words: Oxidized LDL, MCP-1, Chondrocytes, LOX-1, Cartilage degeneration.

Introduction

Chemokines were originally identified by their chemotactic activity for inflammatory cells[1,2]. Besides the implicated involvement of the chemokine/chemokine receptor system in the pathogenesis of inflammatory joint diseases[3–5], accumulating evidence has indicated involvement of the system in cartilage degeneration in osteoarthritis[5–9], that is chondrocytes produce the chemokines[7] and express their receptors[8], whose interaction induces matrix metalloproteinase (MMP)-1, 3, 13 and *N*-acetyl-β-D-glucosaminidase in chondrocytes, inhibits proteoglycan synthesis by chondrocytes and enhances proteoglycan release from cartilage[8,9]. These findings strongly suggest that this system plays a key role in the cartilage degradation, possibly acting in an autocrine/paracrine manner.

Recently, it has been shown that oxidized low-density lipoprotein (ox-LDL) uptake through lectin-like ox-LDL receptor 1 (LOX-1) expressed on vascular endothelial cells is involved in endothelial activation and dysfunction in atherogenesis[10–12]. Interestingly, expression of LOX-1 and association with ox-LDL in chondrocytes were noted in zymosan-induced arthritis rats[13]. We recently demonstrated *in vitro* that ox-LDL binding to LOX-1 increases production of intracellular reactive oxygen species (ROS), resulting in activation of nuclear factor-kappaB (NF-κB)[14]. However, functional consequences caused by the ox-LDL-induced NF-κB activation have not been investigated.

It is known that ox-LDL increases monocyte chemoattractant protein 1 (MCP-1) expression in macrophages[15] and endothelial cells[16], and that ox-LDL-induced MCP-1 expression in endothelial cells plays an important role in monocyte transmigration into the subendothelial space[16,17]. The purpose of this study was to investigate whether ox-LDL binding to LOX-1 increases MCP-1 expression in cultured human articular chondrocytes (HACs).

Materials and methods

HAC CULTURES

HAC culture was performed using commercially available cryopreserved human normal chondrocytes according to the manufacturer's instruction (NHAC-kn, Cambrex Corp., East Rutherford, NJ, USA). After the cells had been thawed in a 37°C water bath, resuspended chondrocytes were seeded at a density of $1 \times 10^4/cm^2$ in growth culture medium (CGM BulletKit,

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Cambrex Corp., East Rutherford, NJ, USA) and incubated at 37°C in a humidified 5% CO_2 incubator. Chondrocytes cultured in the media were expanded in two passages. We first confirmed that the cells used maintained properties of chondrocytes after the cell expansion. Reverse transcription-polymerase chain reaction (RT-PCR) and Real time PCR showed that the cultured HACs without any stimulation constitutively expressed mRNA of both type II collagen and aggrecan gene (data not shown). After reaching 70% confluence in monolayers, cells were cultured in the serum-free culture medium for 12–24 h and then stimulated with various agents in the serum-free medium.

PREPARATION OF NATIVE LDL (n-LDL) and ox-LDL

Human LDL (density 1.019–1.063) was isolated from fresh plasma by ultracentrifugation as described previously[10]. LDL was oxidized at a protein concentration of 3 mg/ml by exposure to 7.5 μM $CuSO_4$ for 20 h at 37°C. Oxidation was monitored by measuring the amount of thiobarbituric acid-reactive substances (10.7 nmol/mg protein) produced, and the increased mobility on agarose gel electrophoresis, due to increased negative charge, was compared with that of n-LDL (relative electrophoretic mobility was 3.25)[10].

PREPARATION OF ANTI-HUMAN LOX-1 MONOCLONAL ANTIBODY

Briefly, lysate of cells expressing human LOX-1 was immunized to the zenomouse[18], and hybridoma producing anti-human LOX-1 monoclonal antibody (TS92, which was called JTX92 in the past) was obtained according to the conventional method to prepare monoclonal antibody. TS92 was purified using protein A from serum-free medium of the hybridoma. The purity of the antibody was verified by SDS-PAGE. Specificity and blocking ability of the antibody against human LOX-1 were confirmed by western blot and suppression of DiI-labeled ox-LDL uptake by LOX-1, respectively (data not shown). This monoclonal antibody has been used in some experimental studies to block ox-LDL binding to human LOX-1 and to show presence of human LOX-1 in immunohistochemistry[11,12,19–21].

RT-PCR FOR LOX-1 mRNA

Total RNA (1 μg) extracted from cultured HACs using Isogen (Nippon Gene, Tokyo, Japan) was reverse transcribed using the OneStep RT-PCR kit (Qiagen Japan, Tokyo, Japan). Reverse transcribed material (1.5 μL) was amplified with Taq DNA polymerase (Bex, Tokyo, Japan) using primer pairs specific to human LOX-1 (sense primer, 5′-GGGGTACCCACCTACAT TATGCAGC-3′; antisense primer, 5′-CCGCTCGAGCGGCCTGGTTG CAAGCCTATAATC-3′). The LOX-1 PCR products were 834 bp long, respectively. For PCR amplification of LOX-1, 30 cycles of 94°C for 45 s, 53°C for 45 s, and 72°C for 60 s were used. In the same experiments, bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified with equal efficiency as a relative internal reference. A primer pair for human GAPDH was used (sense primer, 5′-CTGCCGTCTAGAAAAACC-3′; antisense primer, 5′-CCAAATTCGTTGTCATACC-3′). The PCR product was 200 bp long. For GAPDH PCR amplification, 30 cycles of 94°C for 45 s, 53°C for 45 s, and 72°C for 60 s were used. The amplified samples were visualized on 1.5% agarose gels using ethidium bromide.

RNA EXTRACTION AND REAL TIME PCR ANALYSIS

Cells were seeded at 1×10^5 cells/well in a 24-well plate and were allowed to grow to 70% confluence. All experiments were set up in triplicate per same lot, and two different lots were used in this analysis.

Cell pellets were resuspended in 350 μl of RNeasy lysis buffer from the RNeasy kit (Qiagen Inc., Valencia, CA, USA) and homogenized through Qiashredder columns (Qiagen). RNA extraction was performed through RNeasy columns according to manufacturer's instructions. Extracted RNA was eluted with 30 μl of diethylpyrocarbonate (DEPC) water. Single strand cDNA was prepared from total RNA using random primer under standard conditions with the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The cDNA from each sample was diluted and used for quantification of *MCP-1* and *β-actin* expression. Quantitative Real time PCR with total cDNA was performed using Perfect Real time Premix Ex-Taq™ (TAKARA BIO, Inc., Shiga, Japan) and following primer/probe sets (listed 5′ to 3′ in the order of forward primer, probe, reverse primer): *β-actin*, GGTCATCACCATTGGCAATG, CGGTTCCGCTGCCCTGAGGC, CCA CAGGACTCCATGCCC; *MCP-1*, AGTGTCCCAAAGAAGCTGTGA, TCAA GACCATTGTGGCCAAGGAGAT, CCTGAACCCACTTCTGCTTG. The primer sets were designed to span exons to distinguish cDNA from genomic DNA products. Probes were dual labeled with FAM and TAMRA (all primers and probes were purchased from Sigma Genosys, Tokyo, Japan). The PCR amplifications were performed with the 7100 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) at 95°C for 5 min followed by 35 cycles of 95°C, 30 s; 60°C, 30 s; and 72°C, 30 s. Quantification of gene expression was based on the cycle threshold (Ct) value for each sample. Delta Ct was calculated as (gene of interest Ct) – (*β-actin* Ct) using Sequence detector (Applied Biosystems) and Microsoft Excel (Microsoft corp., Redmond, WA, USA). The relative quantity of MCP-1 mRNA expressions was calculated by delta–delta Ct calculation as $2^{-((\text{treated sample delta Ct})-(\text{control sample delta Ct}))}$. The amplification efficiencies of the target and the endogenous reference were confirmed to be approximately equal by observing that the plot of cDNA dilution vs delta Ct is close to zero (<0.05). All experiments included negative controls consisting of no cDNA for each primer pair.

MCP-1 PROTEIN QUANTIFICATION

Chondrocytes were seeded at a density of 2×10^5/ml in 12-well culture plates and cultured for 5 days, reaching 70% confluence in monolayers. All experiments were set up in duplicate per same lot, and five different lots were used in this analysis.

After incubation of the chondrocytes under the indicated conditions, the conditioned medium was collected and centrifuged at $14{,}000 \times g$ for 5 min. The MCP-1 protein level (pg/ml) was measured in the supernatant using a human MCP-1 enzyme-linked immunosorbent assay (ELISA) kit (sandwich method, Human MCP-1 Biotrak ELISA system, GE Healthcare UK Ltd., Buckinghamshire, UK) according to the manufacturer's instruction. The absorbance at 450 nm was measured within 10 min of addition of a reaction-stopping reagent using a microplate reader (TEACAN SPECTRA Micro-Plate Reader 539-67021, MTX Lab System Inc., Vienna, VA, USA).

STATISTICAL ANALYSIS

Results are presented as means ± standard deviation (SD). Analyses of variance, Scheffe's tests, and un-paired Student's *t* tests were used for statistical assessments. A level of $P < 0.05$ was considered statistically significant.

Results

LOX-1 mRNA EXPRESSION IN CULTURED HACs

Previous studies had shown that LOX-1, one of the receptors for oxidized LDL, is expressed in cultured rat[22] and bovine[14] articular chondrocytes. To confirm LOX-1 expression in cultured HACs, changes in LOX-1 mRNA expression by ox-LDL and interleukin 1β (IL-1β) stimulation were investigated by RT-PCR. A time-course study revealed that basal level of LOX-1 mRNA expressed constitutively and that by addition of 0.1 ng/ml IL-1β and 50 μg/ml ox-LDL it reached a peak after 6 and 12 h, respectively (data not shown).

INCREASE IN MCP-1 mRNA EXPRESSION IN CULTURED HACs BY ox-LDL

After the HACs were washed with serum-free medium three times, they were stimulated with 50 pg/ml IL-1β, 50 μg/ml ox-LDL, or 50 μg/ml n-LDL, and the time course of MCP-1 mRNA expression was investigated by Real time PCR. To examine the receptor specificity of ox-LDL action, HACs were pretreated with 40 μg/ml anti-human LOX-1 monoclonal antibody (TS92) for 30 min and then stimulated with 50 μg/ml ox-LDL. We tried some concentrations of TS92 in preliminary experiments to block LOX-1 and noted that 40 μg/ml TS92 provided consistent results in complete suppression of ox-LDL-induced MCP-1 expression. ox-LDL increased MCP-1 mRNA expression in HACs. Twelve hours after stimulation MCP-1 mRNA expression reached a peak and the mean increase in MCP-1 mRNA expression was estimated to be 5.09 ± 0.86 fold ($n = 3$) greater than time-0. Pretreatment of HACs with 40 μg/ml TS92 for 30 min significantly suppressed the increase in MCP-1 mRNA expression induced by ox-LDL, although that with non-specific IgG (human IgG, Equitech-Bio, Inc., Kerrville, TX, USA) did not [Fig. 1(A)].

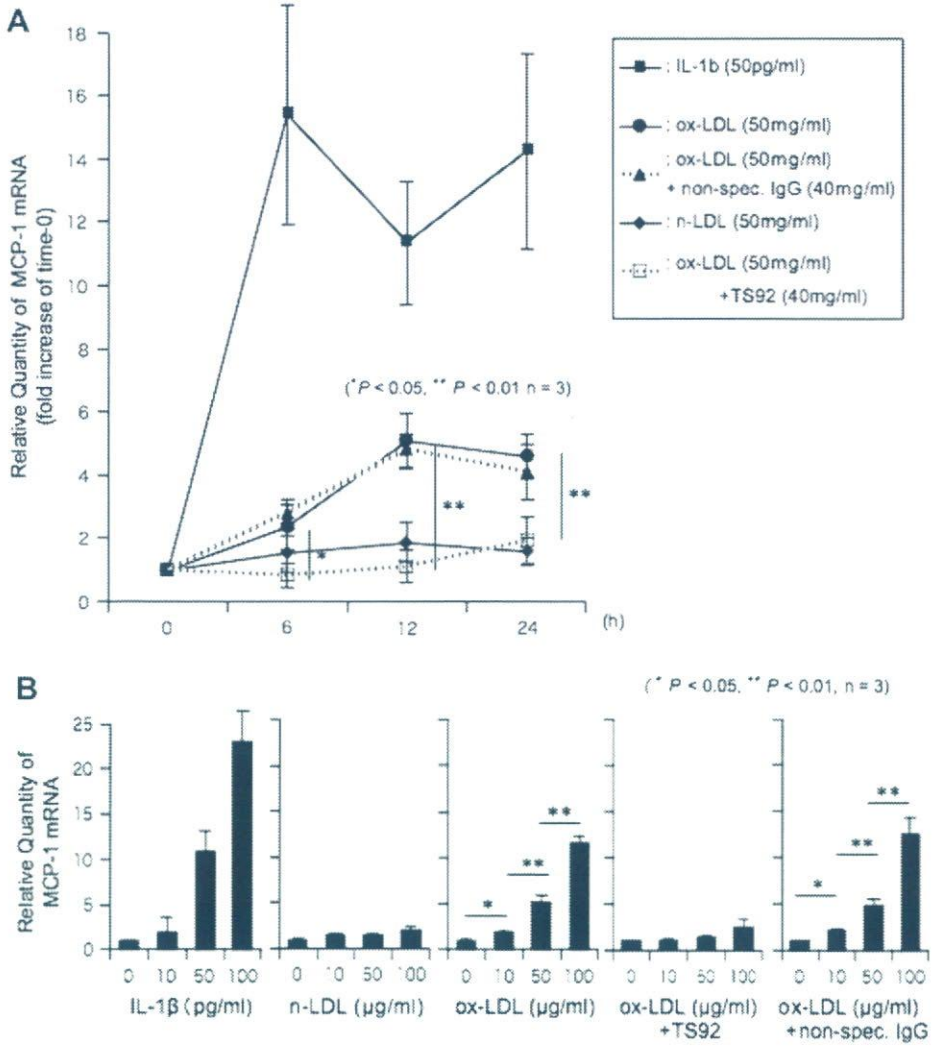

Fig. 1. Time and dose effects of ox-LDL on MCP-1 mRNA expression. HACs were incubated with 50 g/ml IL-1β, 50 μg/ml ox-LDL, or 50 μg/ml n-LDL, and MCP-1 mRNA expression was investigated by Real time PCR at the indicated times (A). HACs were incubated with the indicated concentration of IL-1β, n-LDL or ox-LDL for 12 h, and MCP-1 mRNA expression was investigated by Real time PCR (B). Cells preincubated with 40 μg/ml anti-human LOX-1 mAb (TS92) or non-specific IgG for 30 min were also stimulated with ox-LDL. Relative quantity of MCP-1 mRNA was calculated by the delta–delta Ct method. Error bars indicate SDs ($n = 3$).

The dose dependency of ox-LDL stimulation for MCP-1 mRNA expression was then investigated. HACs were incubated with IL-1β (0, 10, 50, and 100 pg/ml), n-LDL (0, 10, 50, and 100 μg/ml) or ox-LDL (0, 10, 50, and 100 μg/ml) for 12 h, and MCP-1 mRNA expression was investigated by Real time PCR. HACs were pretreated with 40 μg/ml TS92 for 30 min and then stimulated with ox-LDL (0, 10, 50, and 100 μg/ml). IL-1β and ox-LDL increased MCP-1 mRNA expression in a dose-dependent manner. n-LDL did not significantly affect MCP-1 mRNA expression. The mean increase in MCP-1 mRNA expression was estimated to be 1.91 ± 0.32, 5.19 ± 0.67 and 11.6 ± 0.72 fold greater than control for cells, when stimulated with 10, 50 and 100 μg/ml ox-LDL, respectively. Pretreatment of HACs with TS92 significantly suppressed the increase [Fig. 1(B)].

INCREASES IN MCP-1 PROTEIN LEVEL INDUCED BY ox-LDL IN CONDITIONED MEDIUM

To examine whether ox-LDL stimulates MCP-1 production by HACs, MCP-1 levels in conditioned medium were determined by ELISA. First, the time-dependent effects of ox-LDL on MCP-1 production were observed in HACs incubated with 50 pg/ml IL-1β, 50 μg/ml ox-LDL, or 50 μg/ml n-LDL. IL-1β stimulation increased MCP-1 production by chondrocytes, reaching a mean concentration 1803 ± 62 pg/ml 24 h after stimulation. ox-LDL stimulation also increased MCP-1 level in conditioned medium in a time-dependent manner. Increased MCP-1 level was evident 6 h after stimulation with ox-LDL, reaching 830 ± 71 pg/ml at 24 h. Pretreatment of HACs with TS92 significantly suppressed the increase in MCP-1 protein level by ox-LDL stimulation although that with non-specific IgG did not [Fig. 2(A)].

Next, the dose dependence of MCP-1 production was investigated after 24 h of stimulation with IL-1β (10, 50, and 100 pg/ml), n-LDL (10, 50, and 100 μg/ml) or ox-LDL (10, 50, and 100 μg/ml). IL-1β and ox-LDL dose-dependently increased MCP-1 protein level. The mean increase in MCP-1 level was estimated to be 7.5 ± 0.92, 11.5 ± 2.22 and 10.4 ± 1.75 fold greater than control with stimulation with 10, 50 and 100 μg/ml ox-LDL, respectively. Pretreatment

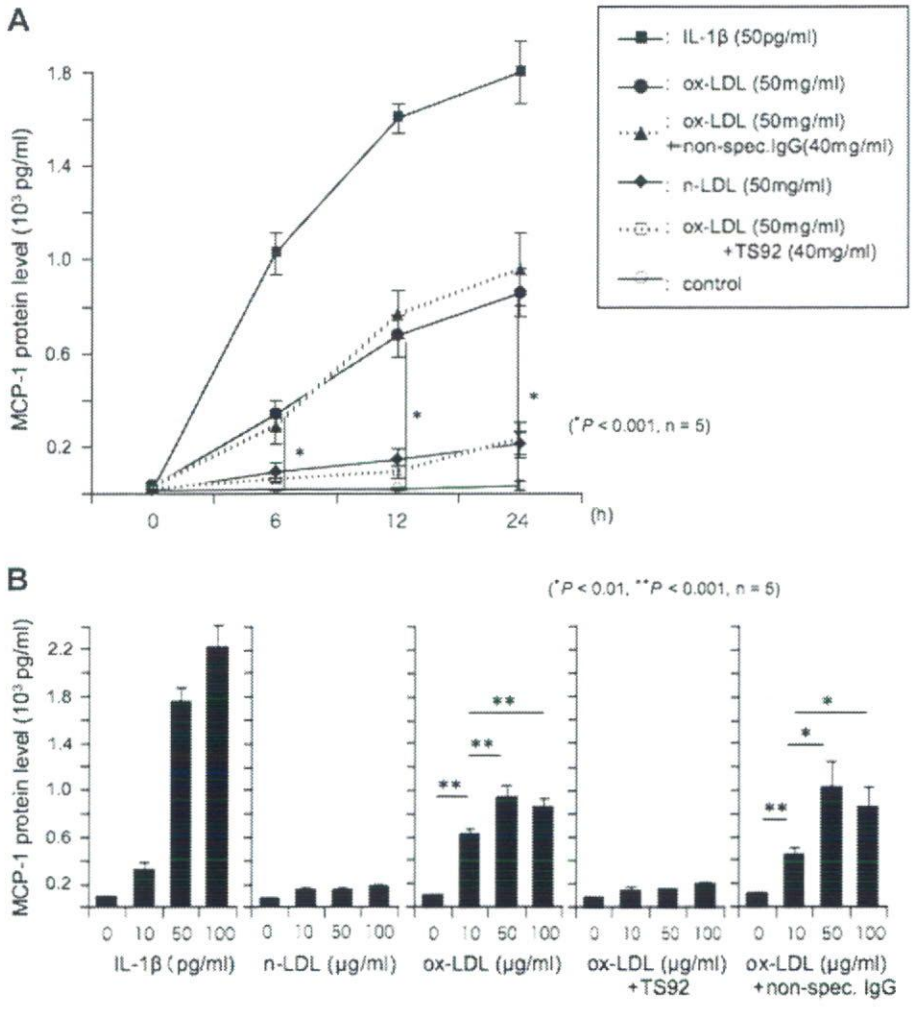

Fig. 2. Time and dose effects of ox-LDL on MCP-1 protein level. MCP-1 protein level was determined by ELISA of conditioned medium. Time dependent increases in MCP-1 level were observed when cells were incubated with 50 pg/ml IL-1β or 50 μg/ml ox-LDL (A). HACs were incubated with the indicated concentration of IL-1β, n-LDL or ox-LDL for 24 h (B). Cells preincubated with anti-human LOX-1 mAb (TS92) or non-specific IgG for 30 min were also stimulated with ox-LDL. Error bars indicate SDs ($n = 5$).

of HACs with TS92 significantly suppressed the increase in MCP-1 level by ox-LDL stimulation [Fig. 2(B)].

Discussion

Our data indicated that ox-LDL binding to LOX-1 enhanced MCP-1 expression in HACs. Increased MCP-1 mRNA expression and protein level in conditioned medium were evident 6 h after stimulation with ox-LDL and continued after 24 h, suggesting that MCP-1 upregulation by ox-LDL may have both a primary effect due to ox-LDL binding to LOX-1 and a secondary effect through enhancement of LOX-1 expression due to its ligand ox-LDL[11,12,14,22].

In the dose–response experiments, MCP-1 level reached a peak with ox-LDL stimulation at 50 μg/ml, while at 100 μg/ml of ox-LDL, the stimulation of MCP-1 level showed a decreasing trend, although expression of MCP-1 mRNA showed an significant increasing trend at both ox-LDL doses. This could be explained by a cytotoxic effect of high-dose ox-LDL. Nakagawa *et al.* reported that ox-LDL reduced chondrocyte viability through suppression of the PI3 kinase/Akt pathway[22]. We previously showed that ox-LDL reduces glycosaminoglycan synthesis with the decrease in cell viability[23]. Reduction in MCP-1 level with high-dose ox-LDL may therefore be attributable to the reduced chondrocyte viability.

In a previous *in vitro* study, we demonstrated ox-LDL-induced ROS results in the NF-κB activation in chondrocytes[14]. It is likely that one of functional consequences of the ox-LDL-induced NF-κB activation in chondrocytes is enhancement of MCP-1 expression, because the NF-κB is known as a nuclear transcription factor for chemokine expression[24–26]. Activation of mitogen-activated protein kinase (MAPK) may be another candidate of the signaling pathway involved in the ox-LDL-mediated MCP-1 expression. Actually, it has been demonstrated that activation of MAPK may play a critical role in signal transduction in ox-LDL-mediated MCP-1 expression in endothelial cells[16]. Interestingly, Pulai *et al.* have recently shown that fibronectin-fragment stimulation results in activation of MAPK in chondrocytes, which further trigger NF-κB activation, regulating MCP-1 expression[27].

In conclusion, we indicated in this study that ox-LDL enhances MCP-1 expression in cultured HACs. ox-LDL may play a significant role in progression of cartilage degeneration in osteoarthritis.

Conflict of interest

The authors declare they have no conflict of interest in connection with this paper.

Acknowledgments

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Plasma Tetrahydrobiopterin/Dihydrobiopterin Ratio
— A Possible Marker of Endothelial Dysfunction —

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Background: Although endothelium-dependent vasodilatation has been used as a marker of endothelial dysfunction (ED), there have been no reliable plasma markers for ED. Oxidative stress, which is a major determinant of ED, oxidizes tetrahydrobiopterin (BH4), an essential cofactor of endothelial type nitric oxide synthase (eNOS), and resulted in the relative deficiency of BH4.
Method and Results: In 163 patients with cardiovascular disorders, the plasma levels of BH4 and 7, 8–dihydrobiopterin (BH2) by high performance liquid chromatography were measured and compared with the flow-mediated (FMD) vasodilatory response of the brachial artery, which was measured by ultrasonography. The effects of atorvastatin on plasma pteridine levels and FMD were examined in patients with multiple coronary risk factors. There was a positive relationship between FMD and plasma BH4 levels and a negative relationship between FMD and plasma BH2 levels. Subsequently, a strong positive relationship between FMD and the BH4/BH2 ratio ($r=0.585$, $P<0.0001$) was found. Although we did not find any significant relationship between pteridine levels and individual traditional risk factors, the BH4/BH2 ratio in patients with more than 2 risk factors showed significant reductions compared with that in those without risk factors. Statin treatment improved FMD in association with an increase in the plasma BH4/BH2 ratio.
Conclusions: Plasma pteridine levels were associated with endothelial dysfunction in cardiovascular disorders.

Key Words: Endothelial dysfunction; FMD; Plasma pteridine level; Statin

Endothelial dysfunction (ED) plays a critical role in the initiation and progression of atherosclerosis[1,2] and is associated with risk factors for coronary artery diseases, including smoking, hypertension, hyperlipidemia, diabetes mellitus and obesity[3–7] Furthermore, endothelial function was demonstrated to serve as a predictor of cardiovascular events[8,9] Therefore, the evaluation of endothelial function is important to determine the therapeutic strategy for atherosclerotic diseases.

Clinically, endothelial function has mostly been evaluated by the extent of endothelium-dependent relaxation (EDR), which is almost exclusively mediated by nitric oxide (NO). Particularly, flow-mediated vasodilatation (FMD) induced by reactive hyperemia following the release of a forearm-occluding cuff is an established method for assessing endothelial function[10] By using this technique, the relationships between coronary risk factors and the ED have been assessed in many clinical studies, and the close linkage has been reported between endothelial function of the brachial artery and that of the coronary arteries[11] Now it is well recognized that the extent of ED depends on the burden of coronary risk factors[12]

There have been, however, no reliable plasma markers found for ED in humans. When EDR is used as a standard representing endothelial function, only limited numbers of clinical studies have shown a correlation between a given plasma marker and EDR[13,14] Oxidative stress has been shown as an important factor leading to ED. Oxidative stress oxidizes tetrahydrobiopterin (BH4), an essential cofactor for endothelial type NO synthase (eNOS), to its oxidative form 7, 8–dihydrobiopterin (BH2) in vascular tissue, particularly in the endothelium[15,16] The resultant relative deficiency of BH4 causes the uncoupling of the L-arginine-NO pathway (uncoupling of eNOS), which is at least partly involved in the ED in various vascular disorders[16,17]

In certain pathological conditions such as renal failure, changes in plasma BH4 and BH2 have been reported[18] As oxidative stress is the major factor damaging endothelial

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function and BH4 is a molecular target of reactive oxygen species (ROS);[15] we hypothesized that plasma levels of pteridine such as BH4 and BH2 might reflect endothelial function. In the present study, we measured plasma pteridine levels in patients with cardiovascular disorders, and examined the relationship between FMD in the brachial artery and plasma pteridine levels. We then examined the effects of HMG-CoA reductase inhibitors (statin) on plasma pteridine levels, which were demonstrated to improve the impaired EDR.

Methods

Subjects

From patients admitted to Kobe University Hospital from March 2006 to April 2008, 163 patients with cardiovascular disorders from which informed consent was obtained were used in the present study. Patients with acute coronary syndrome, those with heart failure, and those with an infectious disease were excluded from the study. In addition, patients with a percentage left ventricular ejection fraction of <40% were not included in the study. A clinical history and physical examination (blood pressure (BP), height, body weight, body mass index (BMI), waist circumference) were undertaken prior to the study. The left ventricular function was assessed by either transthoracic echocardiography or left ventricular angiography. Cardiovascular risk factors and medications were fully documented. An additional 46 patients with more than 2 coronary risk factors were used to examine the effects of treatment with atorvastatin (10 mg/day) for 3 months on plasma pteridine levels as well as the brachial endothelial function. Those 46 patients were randomly divided into the statin treatment group and the control group. Among the 23 patients enrolled in the control group (who did not receive statin treatment), 2 were dropped from the study protocol and 21 were re-examined 3 months later.

Coronary risk factors examined in the present study patients included hypertension, hypercholesterolemia, diabetes mellitus and cigarette smoking. Hypertension was defined as a systolic and/or diastolic pressure of ≥140 and/or 90 mmHg, respectively, or if the patient was being treated with anti-hypertensive drugs. Hypercholesterolemia was defined as serum cholesterol levels of ≥220 mg/dl or if the patient was under treatment. Diabetes mellitus was diagnosed by the presence of fasting plasma glucose (FPG) (≥126 mg/dl) or if the patient was being treated for diabetes mellitus. The Brinkman Index was obtained by taking the number of cigarettes smoked/day times the number of years smoking occurred.

All patients provided written informed consent, and the study was approved by the Institutional Review Board of the Kobe University School of Medicine.

Study Design

All medications including vasoactive drugs and statins, and smoking were stopped at least 12 h prior to the measurement of vasodilatory response. Blood samples were taken in the morning before the measurement of the vasodilatory response. In an additional group, re-examinations of both the brachial vasodilatory response and the blood sample measurements were conducted 3 months after the first examinations. To assess endothelial function in the brachial artery, FMD was measured by high-resolution ultrasonography in the early morning after overnight fasting.[19] The diameter of the brachial artery was measured from B-mode ultrasound images using a 7.5-MHz linear array transducer (SSH-140A; Toshiba Medical Co, Tokyo, Japan). The brachial artery was scanned over a longitudinal section 3–5 cm above the antecubital fossa. When a satisfactory transducer position was found, the surface of the skin was marked, and the arm remained in the same position throughout the study. After baseline measurement of the diameter and the flow velocity in the brachial artery was conducted, a BP cuff placed around the forearm was inflated with a pressure of 220–250 mmHg, and released 5 min later. Fifteen minutes later, the second resting scan was recorded. A sublingual nitroglycerin spray (0.3 mg) was then administered, and the brachial artery was imaged 4 min later. Measurements were taken from the anterior to the posterior interface between the media and adventitia ("m" line) at the end diastole. The diameters at 4 cardiac cycles were analyzed for each scan, and measurements for the reactive hyperemia were taken 45–60 s after the cuff deflation to measure the peak diameter. Responses of the vessel diameter to the reactive hyperemia and nitroglycerin were expressed as the percentage increase to the baseline value of the diameter, and defined as FMD (%) and nitroglycerin-mediated vasodilation (NMD) (%), respectively. All scans were analyzed by the same experienced observers, who were blinded to the identity and the clinical and biochemical data of the subjects. As to the reproducibility of this ultrasound examination, the coefficient of variation for repeated measurements of FMD was 5.60±1.39% and the interobserver correlation coefficient of FMD was 0.985.

Biochemical Analysis

Blood for biochemical analysis was obtained from fasting venous samples. For measurements of plasma levels of pteridines (BH4 and BH2), we minimized the oxidation during the assay by adding 1,4-Dithioerythritol in blood sampling tubes, which has been revealed to inhibit oxidation of BH4 very effectively.[20] Plasma levels of BH4 and BH2 were measured directly by high performance liquid chromatography (HPLC) with the electrochemical detection method, as previously described.[21,22]

Total cholesterol (TC), triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), FPG and insulin, hemoglobin (Hb) A_{1c}, and high-sensitive C-reactive protein (CRP) were measured by using a standard assay. Insulin resistance was evaluated by a homeostasis model assessment of insulin resistance (HOMA-IR). The HOMA-IR value was calculated by using a formula: IR = fasting serum insulin (μU/ml) × FPG (mgl/dl)/405.

Statistical Analysis

Statistical analysis was conducted with a commercially available software package (Stat view ver. 5.0; SAS Institute Inc, Cary, NC, USA). The Spearman correlation coefficient analysis was used to assess associations between FMD and measured parameters. The significance of the difference between group means was analyzed by one-way ANOVA followed by a post-hoc test (PRISM 4.0, GraphPad). After 3 months, the differences between the statin group and the control group were tested by non-parametric analysis (Mann–Whitney U-test). We used a Student's paired t- or Wilcoxon-signed rank test to compare the value before and after each treatment, and the effects of statins were analyzed by using one-way ANOVA with a post-hoc test. The relationships between FMD and the risk factors, and each medi-

Table 1. Baseline Characteristics of Patients

No. of patients	163
Characteristics	
Age (years)	66.2±11.2
Gender (M/F)	91/72
Brinkman index	395.6±550.8
BMI (kg/m^2)	23.7±3.6
Waist circumference (cm)	85.1±10.8
SBP (mmHg)	125.2±20.3
DBP (mmHg)	66.8±11.3
Blood chemistry	
TC (mg/dl)	200.0±43.6
TG (mg/dl)	138.3±68.4
HDL-C (mg/dl)	53.7±18.1
LDL-C (mg/dl)	122.7±34.1
HbA_{1c} (%)	5.9±1.1
FPG (mg/dl)	106.1±27.5
Underlining diseases (%)	
Coronary artery disease	65.6
Arrhythmia	16.6
Valvular disease	6.1
Hypertension	11.7
Medications (%)	
β-blockers	37.4
Ca-blockers	35.0
ACEI	16.6
ARB	43.6
Nitrates	27.0
Oral antidiabetics and insulin	30.7
Statins	33.7
Aspirins	51.5
Diuretics	27.0
Risk factors (%)	
Diabetes mellitus	38.7
Hypertension	68.7
Hyperlipidemia	69.9
Smoking	46.6

Data are mean±SEM.
BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; HbA_{1c}, hemoglobin A_{1c}; FPG, fasting plasma glucose; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin type II receptor blocker.

cation, were assessed using multiple stepwise regression analysis. The effects of statin treatment on the ΔFMD and ΔBH4/BH2 ratio were also assessed by multiple stepwise regression analysis. Results were expressed as the mean± SEM and a P value of <0.05 was considered significant.

Results

The clinical characteristics of the study population are summarized in **Table 1**. Approximately 66% of patients were those with coronary artery disease. Nineteen patients were those with hypertension, 27 with arrhythmia, and 10 with valvular disease having preserved ventricular function. When patients were sub-grouped according to the number of coronary risk factors, most of the patients with coronary artery disease had more than 2 risk factors, whereas only a limited numbers of patients without coronary artery disease had more than 2 risk factors.

We then assessed the relationship between FMD and plasma pteridine levels. There was a positive relationship between FMD and plasma BH4 levels (**Figure 1A**, P< 0.0001, r=0.319) and a negative relationship between FMD and plasma BH2 levels (**Figure 1B**, P<0.0001, r=–0.439). Subsequently, we demonstrated a strong positive relationship between FMD and the BH4/BH2 ratio (**Figure 1C**, P< 0.0001, r=0.585). There were no correlations between NMD and plasma pteridine levels (data not shown). **Table 2** shows the basal clinical characteristics divided by the numbers of risk factors. When we examined the relationship between the plasma BH4/BH2 ratio and the total numbers of coronary risk factors, the BH4/BH2 ratio in patients with more

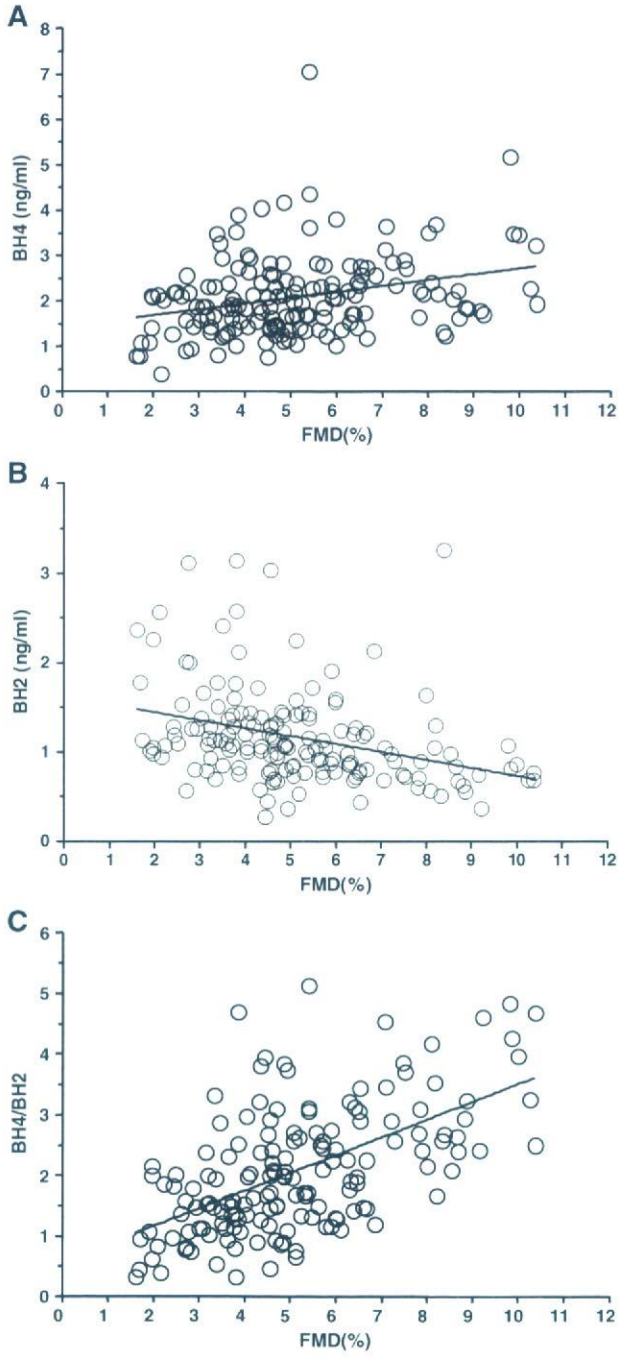

Figure 1. Relationship between flow-mediated vasodilatation (FMD) and plasma tetrahydrobiopterin (BH4) levels (**A**), plasma 7,8-dihydrobiopterin (BH2) levels (**B**), and the plasma BH4/BH2 ratio (**C**), respectively. There was a positive relationship between FMD and plasma BH4 levels (P<0.0001, r=0.319) and a negative relationship between FMD and plasma BH2 levels (P<0.0001, r=–0.439). Subsequently, a strong positive relationship between FMD and the plasma BH4/BH2 ratio was found (P<0.0001, r=0.585).