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

A Possible Mechanism of Endothelial Apoptosis

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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 athero-thrombotic 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

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 chromatogra-

phy 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′-TTGACAACCCCATCCAGA) 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$\pm$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.
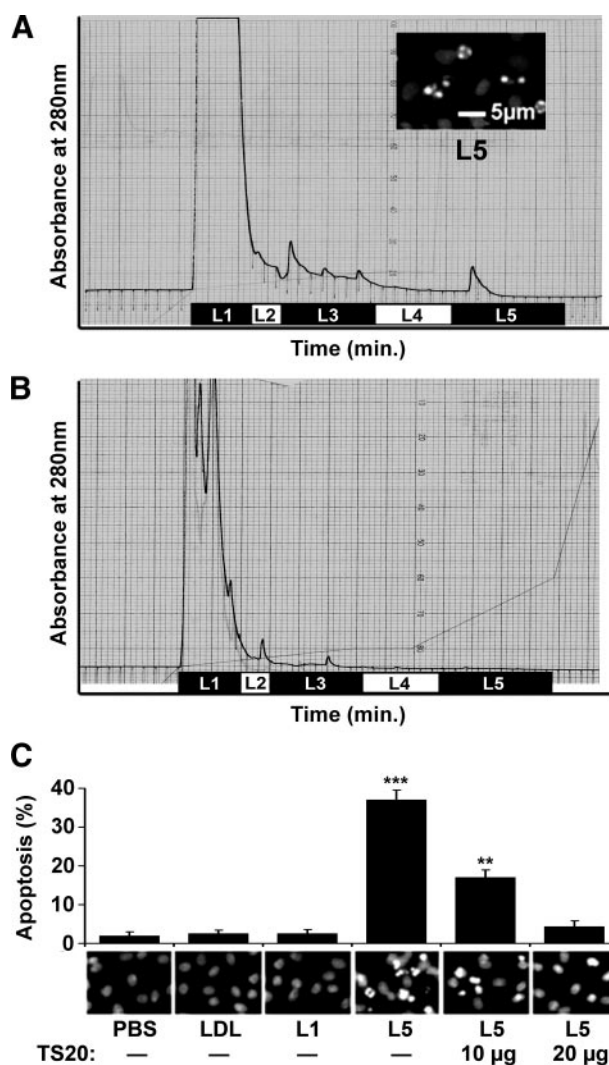

Figure 1. Presence of electronegative LDL subfractions in human hypercholesterolemic plasma and apoptotic activity of L5. A and B, Representative fast-protein liquid chromatography chromatograms of hypercholesterolemic LDL, yielding L5 (A), and normocholesterolemic LDL, where L5 is absent (B). C, BAECs 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
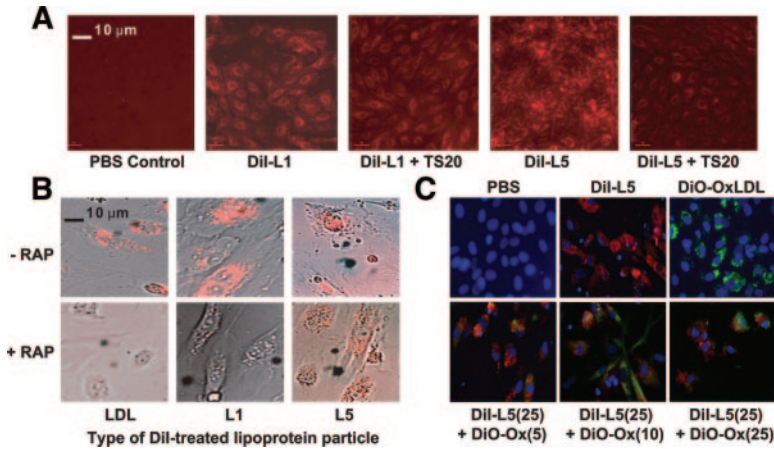
Figure 2. Internalization of L5 and oxLDL by LOX-1 and endocytosis of L1 by LDLR. A, Internalization of DiI-L1 and DiI-L5 was viewed in HUVECs with and without TS20 neutralization of LOX-1. B, Subconfluent BAECs were incubated with 50 μg/mL DiI-labeled L1 and L5, and particle internalization was monitored 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

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.

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).

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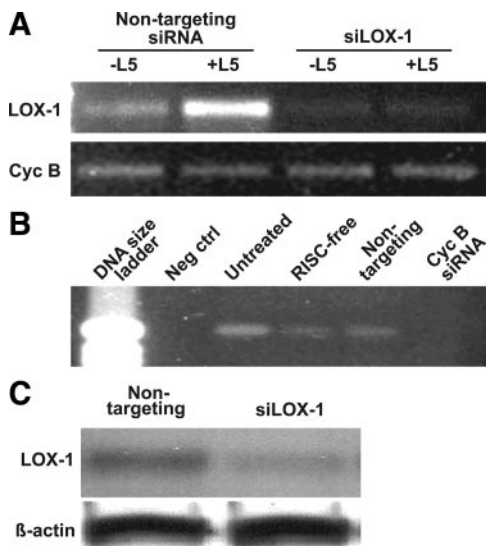
Comparison of oxLDL and L5 Activity in Modulating LOX-1 and FGF2

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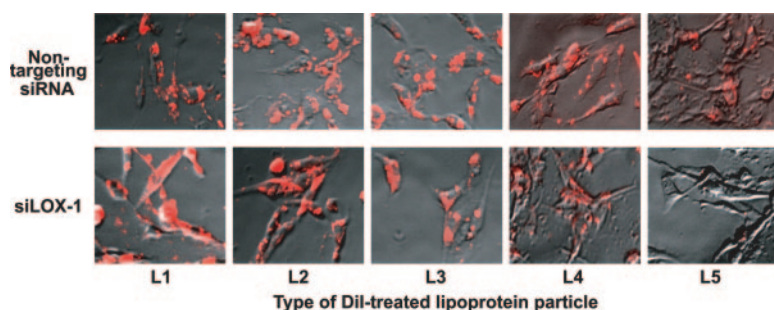
Figure 4. LDL subfractional binding preferences for LOX-1. BAECs transfected with nontargeting or specific LOX-1 siRNAs were incubated with charge-separated, DiI-labeled LDL fractions. Internalization was monitored by fluorescence microscopy.

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

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).

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).
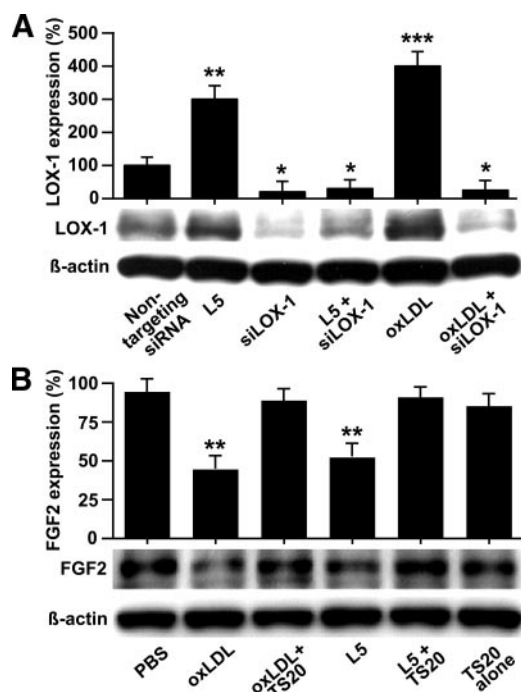
Discussion

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
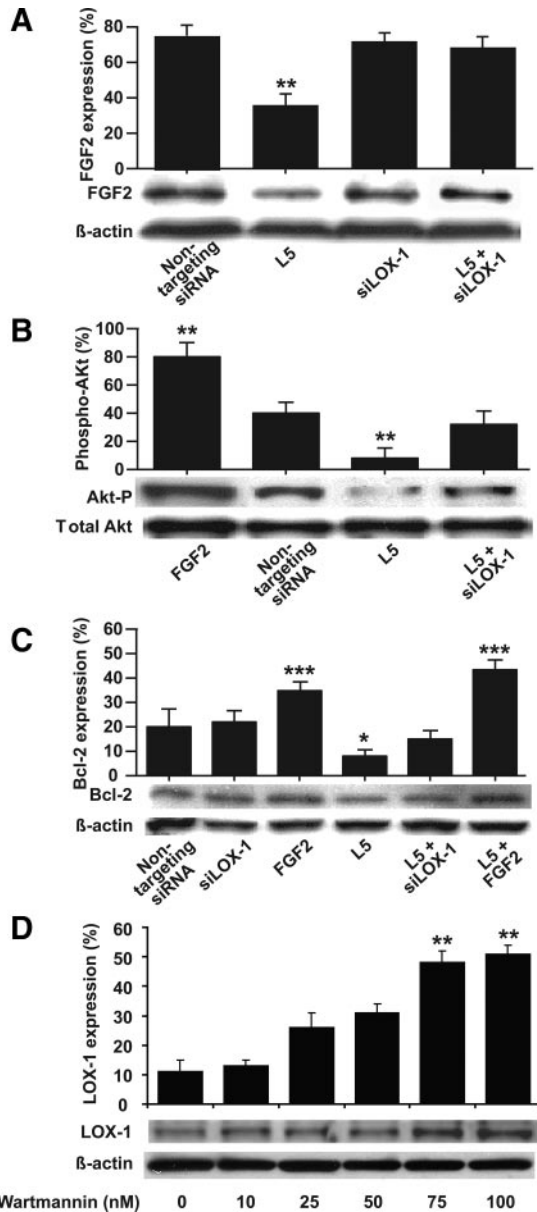
Figure 6. Role of LOX-1 in L5 signaling and mechanism of L5-induced LOX-1 expression. A, FGF2 protein concentrations were assayed by Western blot analysis 24 hours after BAECs were treated with 50 μg/mL L5, with or without siLOX-1 blockade. B, The degree of Akt phosphorylation was measured by normalization 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
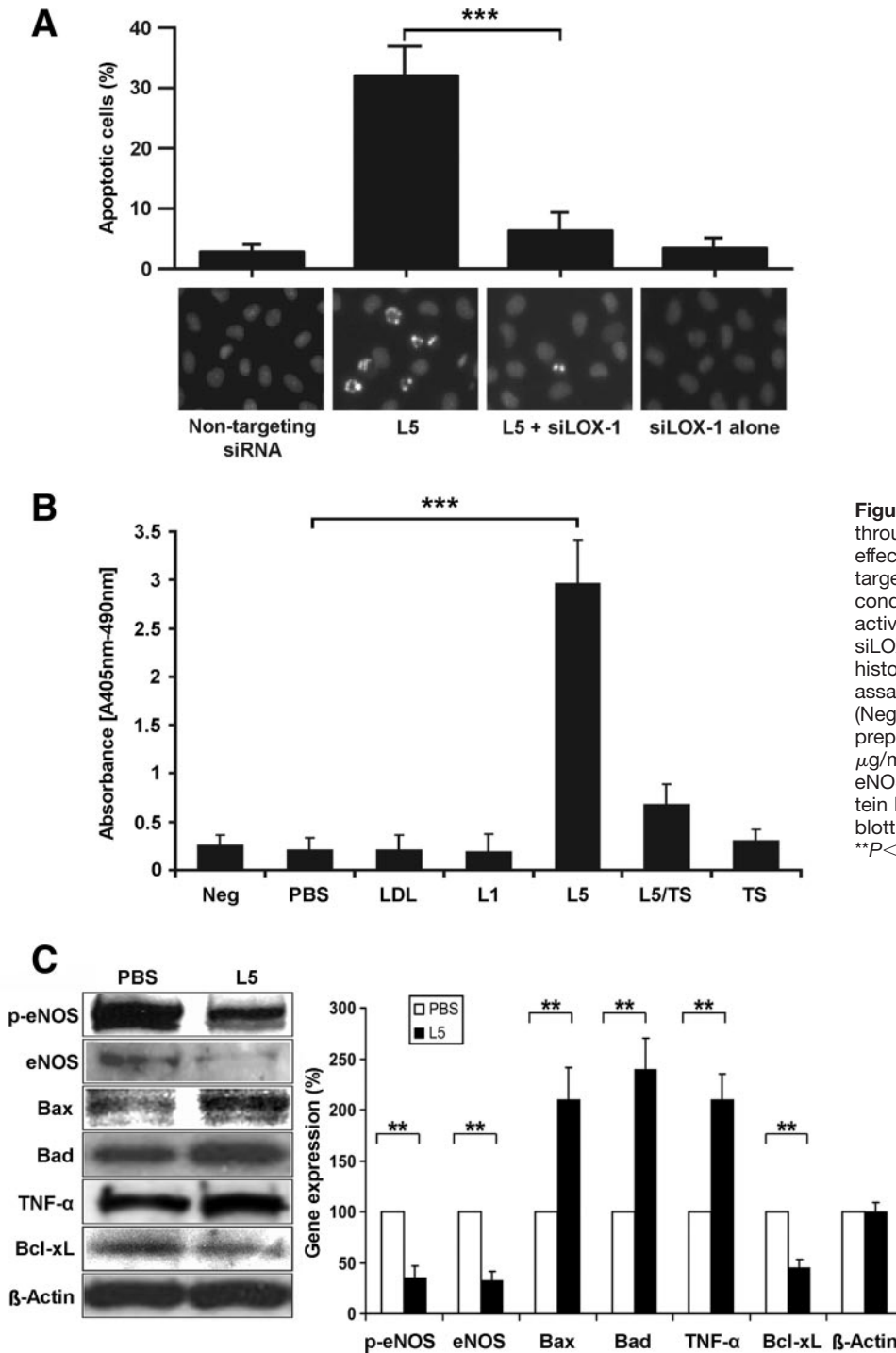
Figure 7. L5 induces EC apoptosis through LOX-1 and exerts opposite effects on prosurvival vs proapoptotic targets. A, Fluorescence microscopy of condensed nuclear staining shows the activity of L5 in BAECs with and without siLOX-1 transfection. B, Cytoplasmic histone-associated DNA fragmentation assayed 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.
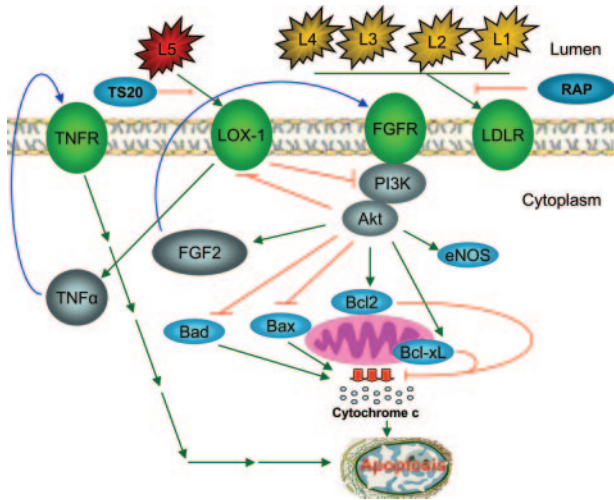
Figure 8. Schematic summary of L5 signaling through LOX-1 in vascular endothelial cells. L5 signals and is internalized through LOX-1, whereas L1–L4 are endocytosed via LDLR. Green arrows indicate stimulation; blue arrows indicate direction of transportation; red arrowheads indicate release from mitochondria; 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.

Disclosures
None.

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