The hydrophobic tunnel present in LOX-1 is essential for oxidized LDL recognition and binding

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Abstract **Lectin-like oxidized LDL (ox-LDL) receptor-1 (LOX-1) is a type-II transmembrane protein that belongs to the C-type lectin family of molecules. LOX-1 acts as a cell surface endocytosis receptor and mediates the recognition and internalization of ox-LDL by vascular endothelial cells. Internalization of ox-LDL by LOX-1 results in a number of pro-atherogenic cellular responses implicated in the development and progression of atherosclerosis. In an effort to elucidate the functional domains responsible for the binding of ox-LDL to the receptor, a series of site-directed mutants were designed using computer modeling and X-ray crystallography to study the functional role of the hydrophobic tunnel present in the LOX-1 receptor. The isoleucine residue (I^{149}) sitting at the gate of the channel was replaced by phenylalanine, tyrosine, or glutamic acid to occlude the channel opening and restrict the docking of ligands to test its functional role in the binding of ox-LDL. The synthesis, intracellular processing, and cellular distribution of all mutants were identical to those of wild type, whereas there was a marked decrease in the ability of the mutants to bind ox-LDL. These studies suggest that the central hydrophobic tunnel that extends through the entire LOX-1 molecule is a key functional domain of the receptor and is critical for the recognition of modified LDL.**—Francone, O. L., M. Tu, L. J. Royer, J. Zhu, K. Stevens, J. J. Oleynek, Z. Lin, L. Shelley, T. Sand, Y. Luo, and C. D. Kane. **The hydrophobic tunnel present in LOX-1 is essential for oxidized LDL recognition and binding.** ***J. Lipid Res.*** **2009.** 50: **546–555.**

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Lectin-like oxidized LDL receptor-1 (LOX-1) is a member of the class E scavenger receptor family, a structurally diverse group of cell surface receptors of the innate immune system that recognize and internalize oxidized LDL (ox-LDL) in endothelial cells of large arteries (1). More recent studies have indicated that LOX-1 is expressed in other cells types, including macrophages (2), vascular smooth-muscle cells (3), and platelets (4). Its expression is not constitutive, but rather, markedly induced by proinflammatory, oxidative, and mechanical stimuli (5, 6), which leads to the activation of endothelial cells, transformation of smooth-muscle cells, and accumulation of lipids in macrophages, resulting in cellular injury and the development of atherosclerosis. Studies in animal models have provided further evidence in support of a role for LOX-1 in atherosclerosis. Overexpression of LOX-1 in mice leads to the formation of atheroma-like lesion areas (7). Conversely, its deletion sustains endothelial function and confers protection in the development of atherosclerosis in association with decreased inflammatory and pro-oxidant markers (8). Finally, human genetic studies strengthen the role of this receptor in cardiovascular disease (9–11).

LOX-1 is a disulfide-linked homodimeric type II transmembrane protein with a short 34-residue cytoplasmic tail, a single transmembrane domain, and an extracellular region consisting of an 80-residue domain predicted to be a coil followed by a 130-residue C-terminal C-type lectin-like domain (CTLD) responsible for ox-LDL recognition (12–14). Homodimers are formed via an interchain disulfide bond between Cys^{140} residues (15) that promotes noncovalent interactions leading to multimerization and ox-LDL binding facilitated by the neck region (16) and receptor density at the plasma membrane (17).

The structural domains within LOX-1 involved in the substrate recognition and binding remain unknown. Site-directed mutagenesis and X-ray crystallography (18, 19) have provided important clues as to how this receptor might interact with ox-LDL. Site-directed mutagenesis demonstrated that the conserved positively charged residues R^{208}, R^{209}, H^{226}, R^{229}, and R^{231} and the uncharged hydrophilic residues Q^{193}, S^{198}, S^{199}, and N^{210} are involved in ligand binding (17, 18), suggesting that ligand recogni-

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Abbreviations: CHO, Chinese hamster ovary; CTLD, C-terminal C-type lectin-like domain; LOX-1, lectin-like oxidized LDL receptor-1; MSD, Meso Scale Discovery; ox-HDL, oxidized HDL; TBARS, thiobarbituric acid-reactive substances.

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tion is dependent on electrostatic interaction and tridimensional conformation, which involves hydrophobic residues (14, 19). Truncation at the utmost C-terminal domain (13) and mutations of basic amino acid residues located between the third and fourth cysteine of the CTLD confirmed that the binding of ox-LDL requires the interaction between basic arginine residues and negatively charged domains within ox-LDL.

The crystal structure of LOX-1 has confirmed the findings from mutagenesis studies and provided new and important insights into the domains involved in the recognition of ox-LDL (18, 19). As expected from the sequence, the CTLD monomer is composed of two antiparallel B-sheets flanked by two α helices stabilized by three conserved intrachain disulfide bonds. The disulfide bridge between Cys^{140} present in monomers, together with the conserved W^{150} side chain, are key to the LOX-1 structure, and function by contributing to the formation of a dimer and favoring proper folding. The extracellular CTLD domain forms a heart-shaped homodimer with a ridge of six basic arginine residues extending diagonally across the apolar top of molecule. These residues, often referred to as the basic spine, play a key biological role in the binding of ox-LDL, as demonstrated by site-directed mutagenesis.

Interestingly, the X-ray structure of the LOX-1 dimer also reveals the presence of a central hydrophobic tunnel that extends through the entire molecule. This tunnel, first discovered by Park, Adsit, and Boyington (18), is ~7–8 Å in diameter and ~380 A^3 in volume. Interestingly, the volume and architecture of this channel are sufficient to accommodate the components of LDL, such as cholesterol, long-chain FAs, or possibly a nonpolar peptide. Hydrogen bonds to the main-chain residues F^{158}, D^{147}, A^{194}, and Y^{197}, and to side-chain residues I^{149} and Y^{197} determine the conformation of the central channel and restrict its opening to 4 Å. Although the topology of the channel suggests a prominent role in substrate binding and recognition, no study to date has addressed the significance and biological relevance of the portal to the binding of ox-LDL. Therefore, we attempted to gain insight into this key structural domain of LOX-1 protein, replacing I^{149} with structurally distinct amino acids to assess the functional significance of the hydrophobic tunnel in the binding of ox-LDL.

MATERIALS AND METHODS

Lipoprotein and ligand preparation

Human LDL (1.019 to 1.063 g/ml) and HDL (1.063 to 1.21 g/ml) were isolated from the plasma of healthy subjects by sequential ultracentrifugation at 15°C. Ox-LDL and ox-HDL were purchased from Intracell, Frederik, MD. The degree of oxidation was determined by measuring the amount of thiobarbituric acid-reactive substances (TBARS). Values for TBARS in ox-LDL, ox-HDL, and native LDL were 17.0 nM, 5.2 nM, and 0.01 nM malonyldialdehyde/mg protein, respectively. Ox-LDL was biotinylated following the ECL protein biotinylation procedure (Amersham Biosciences, Piscataway, NJ) recommended by the manufacturer.

Cloning of human LOX-1 and generation of I^{149} mutants

A full-length human LOX-1 cDNA was cloned by RT-PCR using specific 5′ and 3′ terminal primers to the published LOX-1 cDNA sequence (accession number AB010710) and subsequently cloned into the *Bam*HI/*Not*I site of pcDNA5/FRT (Invitrogen, Carlsbad, CA). An additional expression vector was built to attach a V5-His epitope to the LOX-1 cDNA for detection by inserting the entire LOX-1 cDNA from the start codon to the 3′ end without the endogenous stop codon into pcDNA5/FRT/V5-His-Topo from Invitrogen.

Site-directed mutagenesis was conducted using the QuickChange Site-Directed Mutagenesis Kit (Strategene, La Jolla, CA) according to the manufacturer's protocol. Synthetic oligonucleotides 30–33 bases long carrying the mismatched bases were used to mutagenize nucleotides and replace isoleucine 149 (I^{149}) with phenylalanine (F), tyrosine (Y), or glutamic acid (E) using pcDNA5/FRT.hsLox-1 as a template. Full-length sequence confirmation of wild-type and mutants was done by dideoxynucleotide sequencing.

Transfection of wild-type and LOX-1 mutant cDNA into Chinese hamster ovary cells

Chinese hamster ovary (CHO) cells were maintained in DMEM/F12 medium (Gibco, Carlsbad, CA) with 10% FBS. pcDNA5 plasmids containing full-length wild-type or mutant human LOX-1 cDNAs were transfected into ChoFlp-in by using Lipofectamine according to the manufacturer's instructions. ChoFlp-in cells stably expressing human LOX-1 were maintained in DMEM/F12 with 10% FBS supplemented with 200 μg/ml of Hygomycin B (Invitrogen).

Immunofluorescent staining and confocal microscopy

Fluorescence microscopy of wild-type and I^{149} LOX-1 mutants was carried out in cells grown to 50% confluency in chamber culture slides (BD Biosciences, San Jose, CA) maintained in DMEM/F12 medium. The media was removed and cells were washed twice in PBS and then fixed for 5 min in 100% methanol, washed five times with PBS, and blocked for 20 min at room temperature in PBS containing 10% FBS. Blocking solution was removed, and anti-V5-FITC-conjugated antibody (Invitrogen) in PBS/10% FBS was added overnight at a 1:500 dilution in the dark at 4°C. Finally, cells were washed twice with PBS and observed using confocal microscopy.

Preparation and isolation of plasma membranes

The isolation of plasma membranes from wild-type and ChoFlp-in cells expressing LOX-1 was conducted according to the protocol described in the literature (20). Briefly, cells were grown until 70% confluency in DMEM/F12 medium containing 10% FBS, harvested, and homogenized in ice-cold HEPES medium containing 1 mM EDTA and protease inhibitors. Sucrose was added to a final concentration of 200 mM. The homogenate was centrifuged at 900 *g* for 5 min at 4°C, and the resulting supernatant was centrifuged at 110,000 *g* for 1 h at 4°C. The membrane pellets were washed with PBS containing 5 mM $CaCl_2$, and aliquots were kept frozen at −80°C. The concentration of the solution of solubilized membrane proteins was determined using a Bio-Rad protein assay kit.

Binding of wild-type and mutant LOX-1 to ox-LDL

Binding of ox-LDL to wild-type and mutant LOX-1 receptors was carried out using the Meso Scale Discovery (MSD) technology platform utilizing a Sector Imager 6000 (Meso Scale Discovery, Gaithersburg, MD). The MSD platform is essentially an immunoassay that utilizes electrochemiluminescence to measure binding.

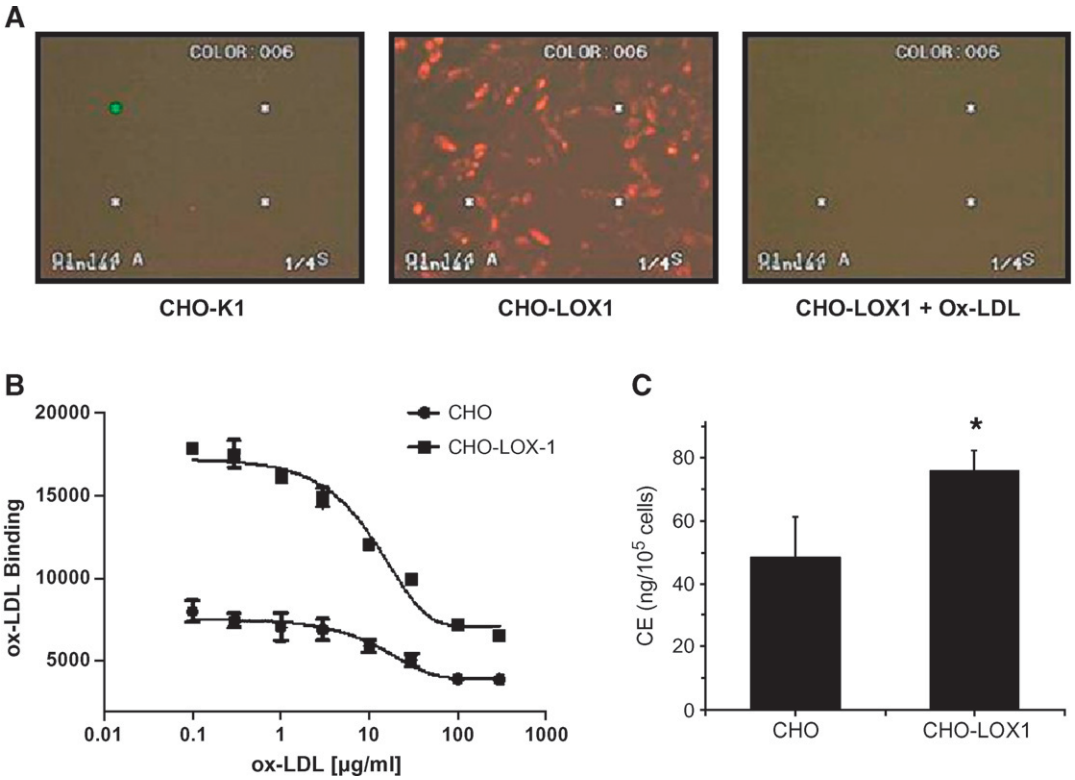


Fig. 1. Expression of human lectin-like oxidized LDL receptor-1 (LOX-1) by ChoFlp-in cells. A: Wild-type and LOX-1 ChoFlp-in-expressing cells were grown in DMEM/F12 as described in Materials and Methods. Fluorescent detection of wild-type and LOX-1 ChoFlp-in-expressing cells was accomplished by adding DiI-labeled oxidized LDL (ox-LDL) to cells for 1 h at room temperature. Cells expressing LOX-1 were incubated with a 50-fold excess of cold, unlabeled ox-LDL. B: Biotinylated ox-LDL binding to plasma membranes isolated from ChoFlp-in [Chinese hamster ovary (CHO)] or ChoFlp-in-LOX-1 (CHO-LOX-1) cells. Binding of ox-LDL was determined using the Meso Scale Discovery technology as described in Materials and Methods. C: Accumulation of cholesteryl esters in wild-type and LOX-1-expressing cells. Cells were grown to 80–90% confluency, as described in Materials and Methods. Cell monolayers were subsequently washed five times and resuspended in cold PBS. Lipids were extracted using chloroform-methanol (2:1). Cholesterol and cholesteryl esters were quantified in a gas chromatograph and expressed as $\mu g/10^5$ cells. Values are expressed as mean ± SD, n = 4. * $P < 0.05$.

Three micrograms of cell membrane (in 30 μl PBS) was added to each well of a 96-well, high-bind MSD plate (MSD L11XB-1) and incubated at room temperature with shaking for 1 h. Plates were then blocked for 1 h at room temperature with 50 μl PBS containing 3% BSA (essential FA-free; Sigma, St. Louis, MO) and 200 μg/ml of human LDL. After the blocking step, plates were washed twice with PBS. Binding buffer (Kreb's Ringer phosphate buffer plus 0.5% FA-free BSA) containing 30 μl/well of biotin-labeled human ox-LDL (10 μg/ml) and varied amounts of unlabeled ox-LDL (Intracel) were then added, and plates were incubated at 4°C, overnight. The next morning, the plates were washed five times with PBS, after which 30 μl/well of ruthenylated streptavidin (SA, MSD SULFO-TAG Streptavidin R32AD-1, and diluted 1:1,000 in MSD antibody diluent) was added to the wells for 1 h at room temperature. Plates were then washed five times with PBS. Read buffer (150 μl, MSD R92TC-3) was added to each well, and plates were read on a Sector Imager 6000. The Sector Imager 6000 applies an electric potential to each well, inducing emitted light (electrochemiluminescence) proportionate to the amount of ruthenylated streptavidin/biotin-labeled human ox-LDL/receptor complex.

1-Palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine, 1-*O*-hexadecyl-2-azelaoyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-(9′-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine, and 1-palmitoyl-2-(5′-oxo-valeroyl)-*sn*-glycero-3-phosphocholine were purchased from Avanti Polar Lipids (Alabaster, AL), dried under nitrogen, resuspended in chloroform-methanol-water (65:25:4), and further diluted in binding buffer containing biotin-labeled ox-LDL to achieve the desired final concentration. Their ability

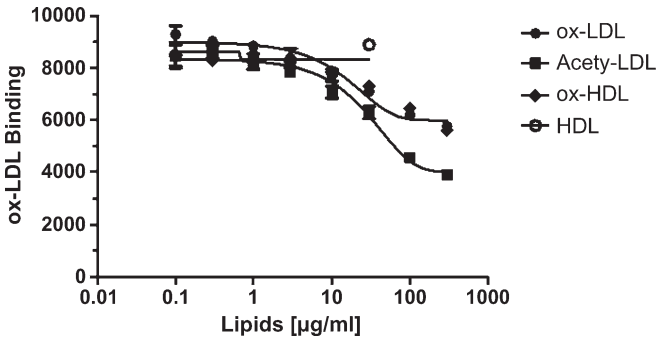


Fig. 2. Effects of an excess amount of HDL, ox-LDL, ox-HDL, and acetylated LDL on ox-LDL binding to LOX-1. Plasma membranes isolated from LOX-1-expressing cells were incubated with biotin-labeled ox-LDL in the presence or absence of excess amounts of HDL, ox-LDL, ox-HDL, and acetylated LDL, as described in Materials and Methods. Each data point represents the average of three independent experiments.

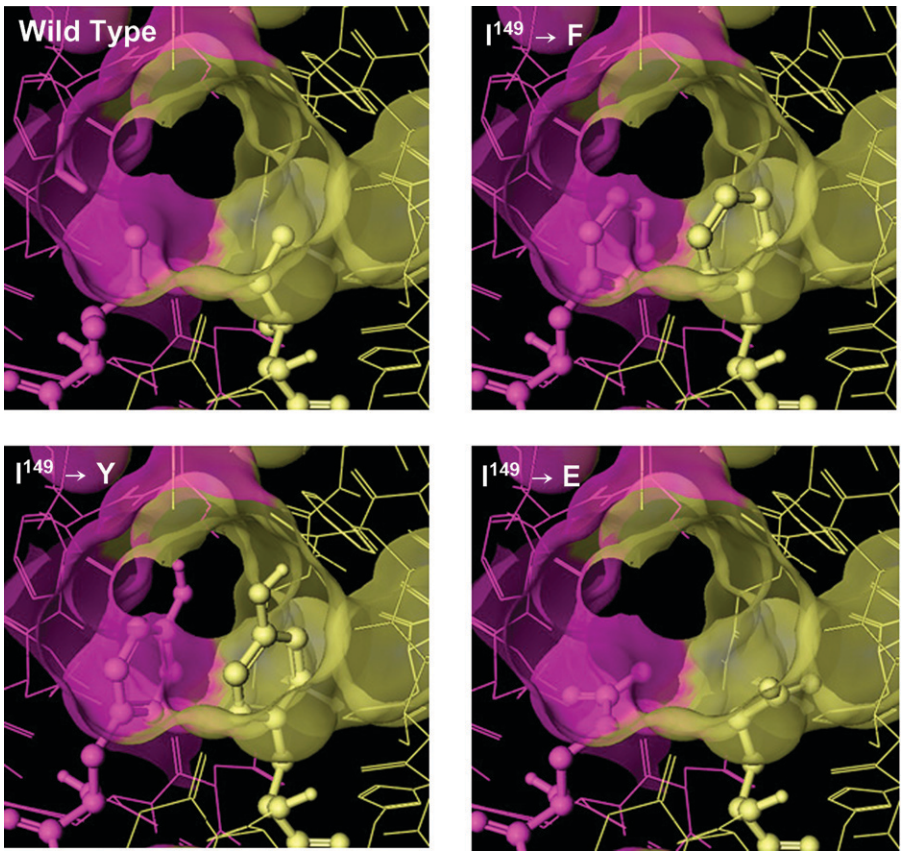


Fig. 3. Hydrophobic tunnel structure in wild-type and I^{149}-LOX-1 mutants. The protein structure of the native LOX-1 receptor was downloaded from Protein Data Bank (PDB ID, 1YPQ). The three mutant protein structures ($I^{149}F$, $I^{149}Y$, and $I^{149}E$) were built using the Maestro molecular modeling package. Chain A of the dimer is shown in magenta, and chain B is shown in yellow.

to block the binding of ox-LDL to LOX-1 was examined as described above.

Structure prediction and molecular modeling

The wild-type LOX-1 protein structure was downloaded from Protein Data Bank (PDB ID, 1YPQ). The structures of the mutant LOX-1 proteins ($I^{149}F$, $I^{149}Y$, and $I^{149}E$) were built using the Maestro molecular modeling package (Schrodinger, Inc., New York, NY). Nonpolar surface area and residue volume were obtained from the literature (21). The interactions between oxidized phospholipids and LOX-1 were modeled using Gold3.0 software (CCDC, Cambridge, United Kingdom). To prevent bias toward any particular binding site and binding mode, we defined the binding site as 20 Å around the dioxane molecule in the 1YPQ structure. The defined binding site was large enough to cover almost all of the protein surface. We also defined the number of genetic algorithm runs in GOLD as 1,000, because of the flexibility of the oxidized phospholipids.

RESULTS

Cloning and expression of wild-type human LOX-1

Expression of wild-type human LOX-1 was achieved by transfecting a full-length human LOX cDNA into ChoFlp-in cells as described in Materials and Methods. Cell surface expression of functional LOX-1 protein was demonstrated by the increased binding to labeled ox-LDL (**Fig. 1A**). Binding of labeled ox-LDL was displaced in the presence of a 50-fold excess of cold, unlabeled oxLDL (Fig. 1B). The increased binding of ox-LDL to cells expressing LOX-1 led to a 1.6-fold accumulation of cholesteryl esters when compared with control cells (75.9 ± 6.4 ng/10^5 cells and 48.4 ± 12.7 ng/10^5 cells, n = 4, $P < 0.005$, for LOX-1-transfected and wild-type cells, respectively; Fig. 1C). Cellular levels of unesterified choles-

TABLE 1. Changes in residue nonpolar surface area, volume, and energy introduced by site-directed mutagenesis of I^{149}

	Residue Properties	LOX-1 Energy	Nonpolar Surface Area	Volume
		Kcal/mol	A^2	A^3
I^{149}	Hydrophobic; nonpolar	−10,703	155	167
$I^{149} \rightarrow F$	Hydrophobic; nonpolar	−10,710	194	190
$I^{149} \rightarrow Y$	Hydrophobic; polar	−10,729	154	194
$I^{149} \rightarrow E$	Nonhydrophobic; polar	−10,817	69	138

LOX-1, lectin-like oxidized LDL receptor-1. LOX-1 energy calculations were made using Maestro software with optimized potential for liquid simultations force field. Nonpolar surface area and residue volume were obtained as described in Materials and Methods.

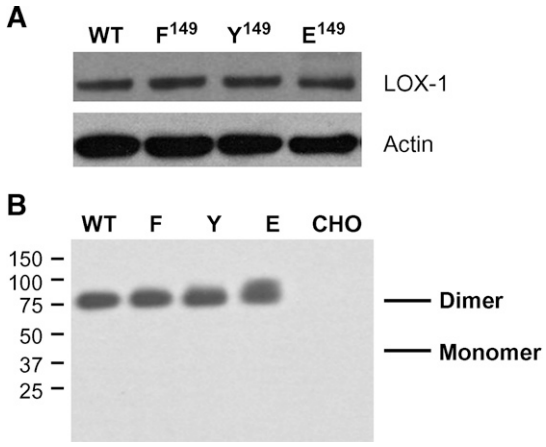


Fig. 4. Expression of wild-type and I^{149}-LOX-1 mutants. Cells expressing wild-type and mutant forms of LOX-1 were grown in DMEM/F12 tissue culture medium as described in Materials and Methods. A: Detection of wild-type and mutants forms of LOX-1. Cell lysates were fractionated by SDS-PAGE under reducing conditions and visualized by Western blotting using a rabbit anti-human LOX-1 or actin antibody. B: Detection of disulfite-linked dimeric forms of LOX-1. Cell lysates prepared from cells expressing wild-type and LOX-1 mutants were mixed with SDS sample buffer in the absence of β-mercaptoethanol and subjected to SDS-PAGE under nonreducing conditions. LOX-1 dimers were visualized by Western blot analysis using a rabbit anti-LOX-1 antibody. The molecular masses (in kDa) of protein standards are indicated.

terol were not significantly changed, consistent with the tight regulation of cholesterol levels in mammalian cells.

A membrane binding assay was developed and optimized to assess the binding properties and substrate specificity of the human LOX-1 receptor. In agreement with the literature, incubation of ox-LDL with plasma membranes isolated from cells expressing LOX-1 demonstrated a dose-response relationship between human LOX-1 and ox-LDL. Binding of labeled ox-LDL was displaced by using increasing amounts of cold, unlabeled ox-LDL, acetylated-LDL, and ox-HDL (**Fig. 2**). In contrast, no inhibitory effect was observed using native HDL (Fig. 2) or native LDL (data not shown).

Computational properties of I^{149}-LOX-1 mutants

To assess the role that the hydrophobic tunnel plays in the recognition and binding of ox-LDL, we focused on I^{149}, which forms the portal to the channel, with its side chain pointing to the empty space in the center of the tunnel (**Fig. 3**). A series of single point mutations were designed using computational modeling to replace the isoleucine residue in position 149 with phenylalanine, tyrosine, or glutamic acid. These new residues were predicted to block the channel entrance because of their increased side-chain volume (F^{149} and Y^{149}) or polarity (E^{149}) without destabilizing the protein (**Table 1** and Fig. 3). The structures of the mutants ($I^{149}F$, $I^{149}Y$, and $I^{149}E$) were built using Maestro software, and the mutated residue side chains were optimized by Prime module. The final structures were checked by the Protein Report module in Maestro, and no steric clashes or improper torsions were found, suggesting that minimal, if any, disturbance of the overall tridimensional structure of the protein occurred, as supported by energy calculations in wild-type and mutants (Table 1).

Synthesis and intracellular processing of I^{149}-LOX-1 mutants

Stably transfected cells expressing either the wild-type or I^{149}-LOX-1 mutants were selected and grown to assess whether the expression and cellular trafficking of LOX-1 was impaired by the mutations. Cell lysates from cells expressing wild-type and I^{149}-LOX-1 mutants were subjected to PAGE, followed by immunoblotting to visualize either

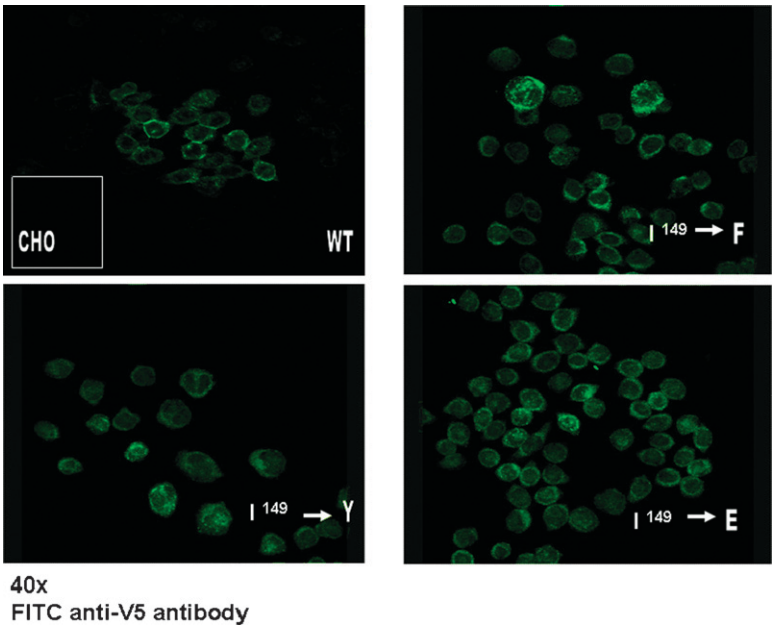


Fig. 5. Fluorescence microscopy of ChoFlp-in cells transfected with wild-type and I^{149}-LOX-1 mutants. Fluorescence microscopy of wild-type and I^{149}-LOX-1 mutants was carried out in cells grown to 50% confluency in chamber culture slides maintained in DMEM/F12 medium as described in Materials and Methods.

LOX-1 or actin. Actin was used as an internal standard to ensure equal protein loading. As shown in **Fig. 4A**, wild-type and mutant forms of LOX-1 were expressed at the same levels and have molecular weights similar to that of wild-type LOX-1. In addition, I^{149}-LOX-1 mutants maintain the ability to form dimers and retain the same molecular weight as wild-type LOX-1, as demonstrated using nondenaturing gradient gel electrophoresis (Fig. 4B).

To examine the cellular topology of wild-type and mutant LOX-1 proteins, a V5 tag was fused to the 3′ terminus of LOX-1 that greatly facilitated the visualization and detection of the LOX-1 protein without affecting the normal cellular distribution of the protein. The cellular distribution of I^{149}-LOX-1 mutants did not differ from that of wild-type LOX-1. Although no signal was observed in untransfected cells (insert, **Fig. 5**), cells expressing LOX-1 displayed a prominent fluorescence at the plasma membrane. These findings are consistent with the literature and the established role that this protein plays as a cell surface scavenger receptor. Taken together, the findings from our studies indicate that the I^{149}-LOX-1 mutants appear to be synthesized and topologically distributed in the same way as wild-type LOX-1. This suggests that these mutants share the same properties as wild-type LOX-1 and constitute an adequate model for conducting structure-function relationship studies.

Binding of ox-LDL to wild-type and I^{149}-LOX-1 mutants

The biological significance of the hydrophobic tunnel present in LOX-1 was tested by introducing amino acids with larger side chains (phenylalanine and tyrosine) or by increasing the polarity (glutamic acid) to perturb the entrance to the tunnel. The effect on binding to ox-LDL was determined by measuring the interaction of biotin-labeled ox-LDL with enriched plasma membrane preparations expressing either wild-type or I^{149}-LOX-1 mutant forms. Complete loss of binding activity was obtained by disruption of the basic spine ($R^{231}N$) or induction of dimer disarrangement ($W^{150}A$), in agreement with previous studies (19) and confirming the functionality of the LOX-1 protein (data not shown). All three human LOX-1 mutants ($I^{149}F$, $I^{149}Y$, $I^{149}E$) were detected by Western blotting (Fig. 4A) and located correctly at the cell surface (Fig. 5), but the ligand binding ability varied among mutants. Whereas $I^{149}Y$ retained approximately 50% of wild-type binding activity, the $I^{149}E$ and $I^{149}F$ mutants showed a marked reduction of ligand binding and retained less than 20% of the wild-type binding activity (**Fig. 6A**). Interestingly, the remaining ox-LDL binding activity in all mutants was displaced when the incubation was performed in the presence of unlabeled ox-LDL (Fig. 6B).

To better understand the role that the hydrophobic tunnel plays in the recognition of putative ligands, we examined the ability of five commercially available oxidized phospholipids (see **Table 2**) to displace the binding of ox-LDL to the receptor. In addition, we modeled the interaction of these oxidized phospholipids with wild-type and I^{149}-LOX-1 mutant proteins. Binding of ox-LDL to LOX-1 was impacted differently by the phospholipids tested (see

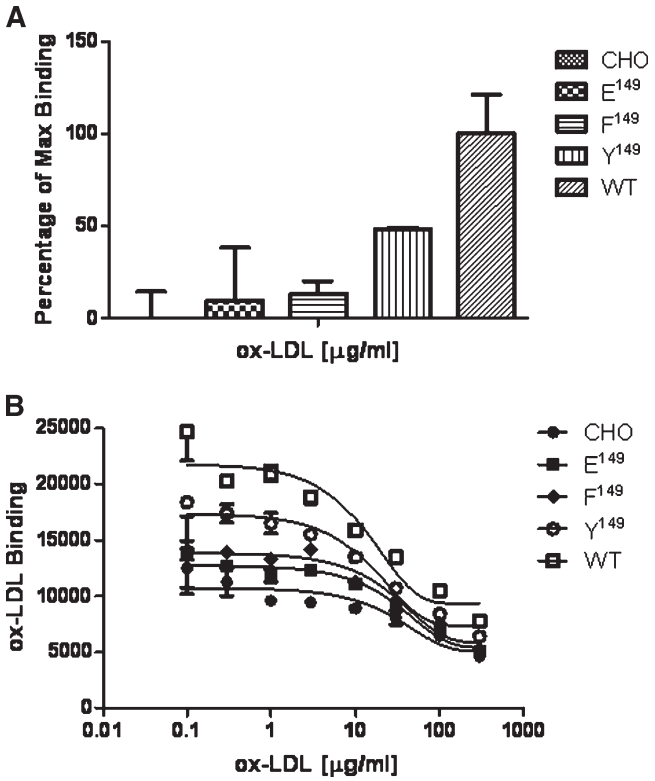
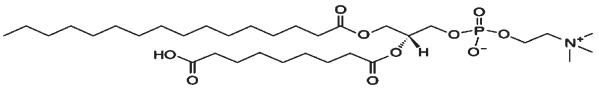
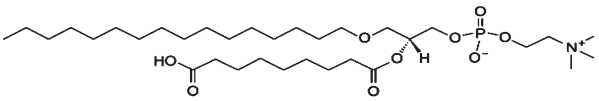
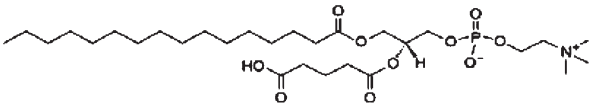
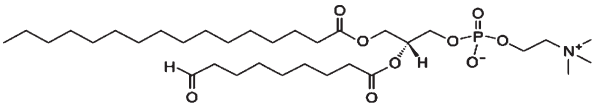
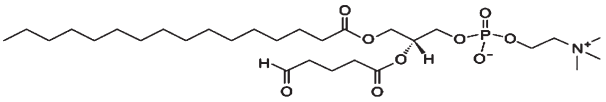


Fig. 6. Binding of ox-LDL to wild-type and I^{149}-LOX-1 mutants. Binding of biotin-labeled ox-LDL to wild-type or I^{149}-LOX-1 mutants was determined as described in Materials and Methods. A: Binding of ox-LDL to I^{149}-LOX-1 mutants expressed as a percentage of the maximum binding achieved with wild-type LOX-1. B: Plasma membranes isolated from wild-type ChoFlp-in and ChoFlp-in cells expressing wild-type and I^{149}-LOX-1 mutants were incubated with biotin-labeled ox-LDL in the presence of increasing amounts of unlabeled ox-LDL. The values represent the mean ± standard deviation of four separate experiments.

Table 2, and **Fig. 7A**). Although compounds 1, 2, and 5 displaced the binding of ox-LDL to LOX-1 with an IC_{50} of 17, 23, and 54 μM, respectively (see Table 2), shorter oxidized *sn*-2 FAs were markedly diminished in their ability to compete for the binding of ox-LDL. These findings are consistent with the molecular modeling experiments that predicted that oxidized phospholipids can act as ligands and mediate, at least in part, the binding of ox-LDL to the receptor. The insertion of the long aliphatic FA chain of the oxidized phospholipids into the hydrophobic tunnel in the LOX-1 dimer interface is favored by the hydrophobic residues I-149A (chain A), F-158A, A-194A, I-149B (chain B), L-157B, and F-158B (Fig. 7B, C) in the LOX-1 dimer interface. These residues form an excellent hydrophobic environment for the insertion of the long aliphatic FA chain of the oxidized phospholipids into the tunnel (Fig. 7B, C) and displacement of ox-LDL. The positively charged trimethylammonium group on the phosphocholine head group interacts with D-189B, whereas the acidic carboxylic group of the oxidized *sn*-2 FA moiety is more likely interacting with the K-171A. The model predicts, in agreement with the binding data ob-

TABLE 2. Chemical name, molecular structure, and inhibition of ox-LDL binding to LOX-1 by oxidized phospholipids

Compound ID	Chemical name	Molecular structure	IC_{50} (μM)
1	1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine		17 ± 14
2	1-O-hexadecyl-2-azelaoyl-sn-glycero-3-phosphocholine		23 ± 11
3	1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine		>>400
4	1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine		157 ± 36
5	1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine		54 ± 25

Plasma membranes isolated from ChoFlp-in cells expressing LOX-1 were incubated with biotin-labeled ox-LDL in the presence of increasing amounts of oxidized phospholipids, as described in Materials and Methods. IC_{50} values are expressed as mean ± SD, n = 4.

tained experimentally, that the distance between the carboxylic acid group and the trimethylammonium group is key in facilitating the hydrophobic interaction with the L-157A and L-175A. The substitution of phenylalanine, tyrosine, or glutamic acid for isoleucine 149 does not favor the insertion and docking of the FA aliphatic chain to the hydrophobic tunnel present in the LOX-1 receptor. These findings are consistent with restricted ligand binding activity observed experimentally (Fig. 6).

DISCUSSION

LOX-1 is increasingly linked to atherosclerosis plaque formation. Recent information gathered from animal models, as well as genetic studies, continues to support the hypothesis that the oxidative modification of LDL and subsequent binding and internalization of oxidized lipoproteins by scavenger receptors like LOX-1 trigger a series of mechanisms leading to endothelial dysfunction and foam cell formation, key steps in the initiation and progression of atherosclerosis. These findings have prompted significant activity within the lipoprotein field aimed at gaining insight into the mechanisms, structural domains, and structure-function relationships that determine the interaction between modified lipoproteins and scavenger receptors like LOX-1. LOX-1 is a type II integral membrane glycoprotein with a short N-terminal cytoplasmic domain, a single transmembrane domain, a short neck or stalk region, and an extracellular C-type lectin-like domain. Structural characterization and mutagenesis of LOX-1 have provided important clues into the domains necessary for the recognition of ox-LDL. The formation of a heart-shaped homodimer with a ridge of six basic arginine residues extending diagonally across the apolar top of the molecule appears to be of critical importance to the recognition and binding of ox-LDL.

In this study, we provide functional evidence for an additional domain and conformational feature within the LOX-1 receptor that is of fundamental importance to the recognition and binding of ox-LDL, i.e., the hydrophobic tunnel. We examined its role by creating mutant forms of LOX-1 using the information available from the X-crystal structure and computer modeling. The binding properties of wild-type and LOX-1 receptor mutants were measured using biotin-labeled ox-LDL and plasma membranes iso-

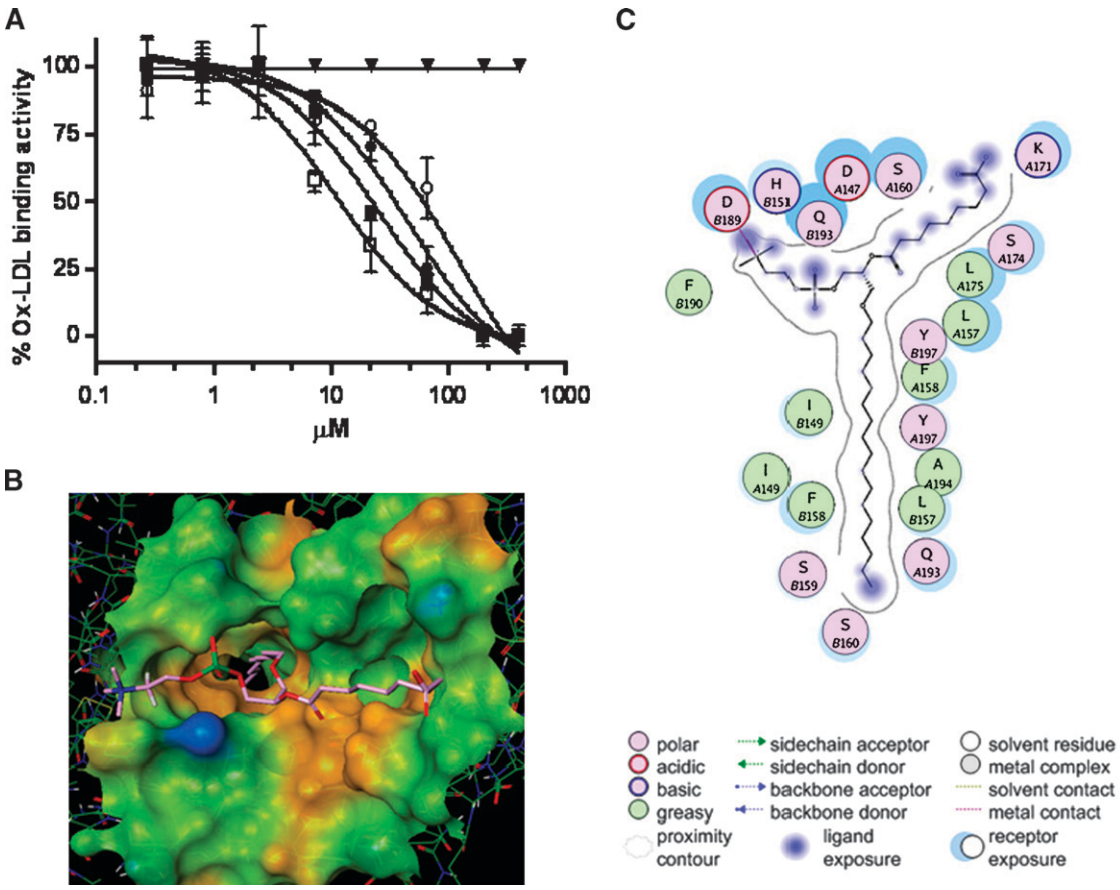


Fig. 7. Computer modeling and interaction of oxidized phospholipids with LOX-1. A: Inhibition of biotin-labeled ox-LDL binding to LOX-1 by oxidized phospholipids. Binding of oxidized phospholipids to LOX-1 was determined as described in Materials and Methods. Data are shown as mean ± standard deviation of four separate experiments. Open squares: compound 1; closed squares: compound 2; closed circles: compound 5; open circles: compound 4; closed triangles: compound 5. B: Computer modeling results of compound 1 in hydrophobic channel. The protein surface is colored based on hydrophobicity. Orange: hydrophobic; green: polar; blue: acidic. C: Two-dimensional ligand-protein interaction map. The interactions between oxidized phospholipids and LOX-1 were modeled using Gold3.0 software.

lated from CHO cells expressing wild-type and mutant LOX-1 proteins. Taken together, the findings from our studies demonstrate that changes in the overall architecture and opening of the hydrophobic tunnel markedly influence the recognition of ox-LDL, as demonstrated by site-directed mutagenesis. Replacing isoleucine 149 with either tyrosine or phenylalanine augmented the volume of the side chains pointing to the tunnel open space, resulting in the narrowing of the opening to 2.8 Å ($I^{149}Y$) or 4 Å ($I^{149}F$) when compared with 7 Å for wild-type LOX-1. Binding of ox-LDL is greatly diminished in these mutants. Similarly, an increase in the polarity at the channel's gate resulting from replacing I^{149} with glutamic acid virtually blocked the binding to ox-LDL. Substitutions made at I^{149} did not alter the synthesis or processing of the LOX-1 protein. More importantly, energy calculations indicate that these mutations do not have consequences such as steric clashes, improper torsions, and destabilization of the protein.

How these mutations impair the binding and recognition of ox-LDL is unclear. It is believed that during the oxidative modification of LDL, multiple pathways contribute to the formation of a variety of oxidized molecules, including oxidized phospholipids (22), which can act as ligands to the receptor and can mediate, at least in part, the binding of these modified lipoproteins to scavenger receptors. This hypothesis has been studied and confirmed for CD36, another scavenger receptor involved in the binding and uptake of ox-LDL by macrophages (23). Several lines of evidence suggest that oxidized phospholipids might also mediate the interaction, at least in part, of ox-LDL with the LOX-1 receptor. X-ray crystallography data and computer modeling predict that the aliphatic chain at the *sn*-1 position of the phospholipid molecule anchors in the tunnel and that mutations at the I^{149} significantly hamper its docking. Experiments using I^{149} mutants together with computer modeling confirm that the geometry of the channel is critical to the docking of the *sn*-1 FA and ox-LDL binding. The structural motifs in oxidized phospholipids also appear to be important in supporting the binding of ox-LDL to LOX-1. From the molecular modeling results, it appears that the existence

of an *sn*-2 carboxylic acid group and its distance to the positively charged trimethylammonium group are critical to the strength of the binding of oxidized phospholipids to LOX-1. If the distance is too short, for example as in compound 3, the negatively charged carboxylic acid group will be positioned in a hydrophobic environment (L-175A, L-157A) and will no longer achieve the optimal interaction with the basic residue K-171A, resulting in the loss of binding to LOX-1. When the carboxylic acid group is changed to a neutral aldehyde (compound 4), the distance to the trimethylammonium group remains the same but the acid-base interaction with K-171A no longer exists. As a consequence, compound 4 has a marked decrease in binding affinity to LOX-1. On the other hand, when the acid group on the oxidized phospholipid is replaced by a neutral aldehyde, the distance to the trimethylammonium group is reduced (compound 5) but its binding activity is retained. A neutral aldehyde group in a hydrophobic environment (L-157A, L-175A) will receive much less energy penalty than the acid group present in compound 3. The modeling results are confirmed by our experimentally measured IC_{50}. The importance of oxidized phospholipids as putative ligands to LOX-1 is supported by our findings using the E06 monoclonal antibody. The binding of ox-LDL to LOX-1 is blocked by the E06 monoclonal antibody that specifically recognizes the phosphocholine headgroup of phosphocholine-containing oxidized phospholipids, such as that found in 1-palmitoyl-2-(5′-oxo-valeroyl)-*sn*-glycero-3-phosphocholine (data not shown), further supporting the hypothesis that oxidized phospholipids participate in the interaction between ox-LDL and LOX-1.

Conceptually, it is plausible to envision that the recognition of a modified lipoprotein by LOX-1 requires the cooperation of various domains in the receptor in addition to the hydrophobic tunnel. The anchoring of the *sn*-1 FA to the tunnel and subsequent interaction between the positively charged trimethylammonium group on the phosphocholine head group, and the acidic carboxylic group of the oxidized *sn*-2 FA moiety, might represent the initial steps in the recognition of modified lipoproteins by LOX-1. Additional oxidized lipids and modified protein epitopes within the lipoproteins might also participate in the binding to the receptor. Subsequent interactions with the cluster of basic residues and the hydrophobic patches on the surface of the receptor could lead to the stabilization and docking of oxidized lipoproteins to LOX-1. Our studies suggest that protein modification within the apolipoprotein B molecule in LDL might not play a major role in binding, inasmuch as approximately 80% of ox-LDL binding to LOX-1 is abolished by the occlusion of the channel opening. In addition, ox-HDL also interferes with the binding of ox-LDL to the LOX-1 receptor. The implication of our findings in other structurally diverse LOX-1 ligands, such as apoptotic bodies, activated platelets, aged cells, and both Gram-positive and Gram-negative bacteria, remains unknown. Additional studies will be required for understanding the various domains involved and the sequence of events leading to the recognition, binding, and internalization of modified lipoproteins and other structurally distinct ligands by the LOX-1 receptor. The present study suggests that changes in the geometry of the hydrophobic tunnel could be a possible strategy to develop selective receptor antagonists and abrogate the ox-LDL-induced oxidative damage and endothelial dysfunction observed in atherosclerosis.

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