Cholesterol-Lowering Drugs Inhibit Lectin-Like Oxidized Low-Density Lipoprotein-1 Receptor Function by Membrane Raft Disruption

Sara Matarazzo, Maria Chiara Quitadamo, Ruggiero Mango, Sarah Ciccone, Giuseppe Novelli, and Silvia Biocca

Department of Systems Medicine, Laboratory of Clinical Biochemistry (S.M., S.C., S.B.), Section of Cardiology (R.M.), Department of Biomedicine and Prevention, Section of Medical Genetics (M.C.Q., G.N.), and Center of Biostatistics and Bioinformatics (S.B., G.N.), School of Medicine, University of Tor Vergata, Rome, Italy; and National Agency for the Evaluation of Universities and Research, ANVUR, St. Peter Fatebenefratelli Hospital, Rome, Italy (G.N.)

Received March 23, 2012; accepted May 8, 2012

ABSTRACT

Lectin-like oxidized low-density lipoprotein (LOX-1), the primary receptor for oxidized low-density lipoprotein (ox-LDL) in endothelial cells, is up-regulated in atherosclerotic lesions. Statins are the principal therapeutic agents for cardiovascular diseases and are known to down-regulate LOX-1 expression. Whether the effect on the LOX-1 receptor is related to statin-mediated cholesterol-lowering activity is unknown. We investigate the requirement of cholesterol for LOX-1-mediated lipid particle internalization, trafficking, and processing and the role of statins as inhibitors of LOX-1 function. Disruption of cholesterol-rich membrane microdomains by acute exposure of cells to methyl-β-cyclodextrin or chronic exposure to different statins (lovastatin and atorvastatin) led to a spatial disorganization of LOX-1 in plasma membranes and a marked loss of specific LOX-1 function in terms of ox-LDL binding and internalization. Subcellular fractionation and immunocytochemical studies indicate that LOX-1 is naturally present in caveolae-enriched lipid rafts and, by cholesterol reduction, the amount of LOX-1 in this fraction is highly decreased (≥60%). In contrast, isoprenylation inhibition had no effect on the distribution and function of LOX-1 receptors. Furthermore, in primary cultures of atherosclerotic human aorta lesions, we confirm the presence of LOX-1 in caveolae-enriched lipid rafts and demonstrate that lovastatin treatment led to down-regulation of LOX-1 in lipid rafts and rescue of the ox-LDL-induced apoptotic phenotype. Taken together, our data reveal a previously unrecognized essential role of membrane cholesterol for LOX-1 receptor activity and suggest that statins protect vascular endothelium against the adverse effect of ox-LDL by disruption of membrane rafts and impairment of LOX-1 receptor function.

Introduction

Receptor-mediated endocytosis of oxidized low-density lipoprotein (ox-LDL) is the hallmark event in the pathogenesis of atherosclerosis. Elevated levels of ox-LDL are associated with macrophage differentiation in foam cells, apoptosis and necrosis of vascular endothelium, smooth muscle cell migration, and proliferation (Mitra et al., 2011) and are correlated to plaque instability in human coronary atherosclerotic lesions. Most of these effects are elicited by the lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), characterized as the primary receptor of ox-LDL in endothelial cells (Sawamura et al., 1997; Mehta et al., 2006). LOX-1 is a scavenger receptor overexpressed in atherosclerotic lesions and up-regulated during atherogenesis in atheroma-derived cells (Mehta et al., 2006; Vohra et al., 2006). LOX-1 activation triggers the oxidative stress response and has been shown to lead to plaque vulnerability and potential rupture, which is ultimately responsible for acute atherothrombotic vascular occlusion and tissue infarction. Moreover, LOX-1 is

ABBREVIATIONS: LDL, low-density lipoprotein; ox-LDL, oxidized low-density lipoprotein; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; MJCD, methyl-β-cyclodextrin; LDL-C, low-density lipoprotein-cholesterol; EC, endothelial cell; MAb, monoclonal antibody; IRβ, insulin receptor subunit β; HRP, horseradish peroxidase; FTL, farnesyl transferase inhibitor; ABT-100, (S)-6-[[2-(4-cyanophenyl)-1 (methyl)-1H-imidazol-5-yl]ethoxy]-4′-((trifluoromethoxy)-1′-biphenyl-3-carbonitrile; DMEM, Dulbecco’s modified Eagle’s medium; HEK, human embryonic kidney; E-64, trans-epoxysuccinic acid; Mes, 4-morpholineethanesulfonic acid; Dil, 1′,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Cav-1, caveolin-1.
up-regulated during myocardial ischemia reperfusion and appears to be associated with apoptosis, necrosis, and left ventricular functional deterioration (Li et al., 2003).

LOX-1 is encoded by a single gene, ORL1, located on human chromosome 12p12.3–p13.2 (Aoyama et al., 1999). Genetic association studies have identified different single nucleotide polymorphisms within the ORL1 gene, which play a role in cardiovascular disease susceptibility. A newly identified LOX-1 spliced isoform, named LOXIN, which lacks exon 5, is deficient in ox-LDL binding activity and is protective against LOX-1 induced apoptosis (Mango et al., 2005).

LOXIN isoform interacts with LOX-1 receptors, inhibiting its function through the formation of nonfunctional hetero-oligomers (Biocca et al., 2008). LOX-1 is a type II membrane glycoprotein with an extracellular C-type lectin-like ligand-binding domain (Sawamura et al., 1997), which forms a disulfide-linked heart-shaped homodimer. The C-type lectin-like ligand-binding domain possesses a basic spine structure across its ligand recognition surface known to play a role in the recognition of ox-LDL. LOX-1 dimers assemble in larger functional oligomers through noncovalent interactions (Ohki et al., 2005; Park et al., 2005; Biocca et al., 2008). More recently, other groups have reported that multimerization and cluster organization in plasma membrane are important requisites for LOX-1 activity (Cao et al., 2009; Ohki et al., 2011).

Several lines of evidence implicate cholesterol-enriched lipid microdomains, known as caveolae and lipid rafts, as essential docking sites for endocytosis of ligands, including ox-LDL, fatty acids, apoptotic cells, and also viruses. Despite the pathophysiological role of LOX-1-mediated ox-LDL endocytosis, details of the receptor-mediated lipid particle internalization, trafficking, and processing during atherosclerotic plaque formation are not yet understood. Scavenger receptors, such as CD36, CD209, and CD204 are localized in lipid rafts and use lipid rafts pathways for endocytosis (Lisanti et al., 1994; Zeng et al., 2003; Cambi et al., 2004; Kiyanagi et al., 2011). Although a clathrin-independent and dynamin-2-dependent pathway has been described and thought to be involved in LOX-1 endocytosis (Kashiwakura et al., 2004; Murphy et al., 2008), these studies have not investigated the lipid raft involvement. Caveolae and lipid rafts are specialized membrane domains, rich in cholesterol, sphingolipids, and glycosphospholipids and contain specific membrane proteins including glycosylphosphatidylinositol-anchored proteins, GTPases, and receptor-associated kinases (Parton and Simons, 2007). Cholesterol is a key component of caveolae and raft structure and is important in modulating the fluidity of plasma membranes and regulating their function.

Given the critical role of LOX-1 in atherogenesis, we wanted to investigate the requirement of cholesterol and the role of cholesterol-lowering drugs in LOX-1-mediated ox-LDL entry in human endothelial cells. To deplete cholesterol in plasma membranes, we used methyl-β-cyclodextrin (MβCD), which specifically extracts cholesterol from the plasma membranes (Ilangumaran and Hoessli, 1998) and two statins, lovastatin and atorvastatin, that inhibit HMG-CoA reductase, a rate-limiting enzyme in cholesterol biosynthesis (Wang et al., 2008). For their effect on lowering circulating total and low-density lipoprotein-cholesterol (LDL-C), statins are largely used in the clinic in the treatment of patients with cardiovascular diseases. We have focused our interest on the role of statins in the reduction of the membrane cholesterol level and disruption of lipid rafts in endothelial cells and the consequences on LOX-1 expression, membrane distribution, and function.

Here we report that LOX-1 is predominantly localized in caveolae/lipid rafts in the cell plasma membranes and its function is regulated by membrane cholesterol. A decrease in plasma membrane cholesterol by statin treatment leads to down-regulation of LOX-1 in lipid rafts, impairment of LOX-1-mediated ox-LDL internalization, and rescue of an ox-LDL-induced apoptotic phenotype in primary endothelial cells (ECs), suggesting that statins may protect vascular endothelium against the adverse effects of ox-LDL by disruption of LOX-1 receptor function.

**Materials and Methods**

**DNA Constructs.** For the expression in mammalian cells, human LOX-1 was subcloned into pEF/V5-His vectors (Invitrogen, Inchninnan, Paisley, UK), as described previously (Biocca et al., 2008).

**Antibodies and Reagents.** Rat anti-LOX-1 (Biocca et al., 2008), MAb anti-V5 IgG (Invitrogen), mouse anti-caveolin-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-insulin receptor β subunit (IRβ), (BD, Franklin Lakes, NJ), and mouse anti-β-actin IgG (Affinity Bio Reagents, Golden, CO) were used as the primary antibodies. Secondary antibodies goat anti-rat IgG horseradish peroxidase (HRP), goat anti-mouse IgG HRP, donkey anti-rabbit IgG HRP, and Rhodamine Red X-conjugated AffiniPure donkey anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Filipin, MβCD, and atorvastatin were purchased from Sigma-Aldrich (St. Louis, MO). Lovastatin (Enzo Life Sciences, Farmingdale, NY) was activated by NaOH addition with a lovastatin/NaOH ratio (v/v) of 2:3, at 50°C and neutralized with HCl to pH 7. The farnesyl transferase inhibitor (FTI) (S)-6-[2-(4-cyanophenyl)-2-hydroxy-2-(1-methyl-1-phenylmethylsulfonyl-2-5-yl)ethoxy]-4′- (trifluoromethoxy)-1,1-biphenyl-3-carbonitrile (ABT-100) was provided by Abbott Laboratories (Abbott Park, IL). For in vitro studies, ABT-100 was dissolved in dimethyl sulfoxide with dilutions made using DMEM plus 10% fetal bovine serum.

**Cell Cultures and Transfection.** COS and HEK-293 cells were grown in DMEM (Biowest, Miami, FL) supplemented with 10% fetal bovine serum (Gibco; Invitrogen) and 100 U/ml penicillin-streptomycin (Euroclone, Devon, UK). COS cells were transiently transfected with JetPEI (Polyplus Transfection, Illkirch, France), following the manufacturer’s instructions, with a DNA/transfectant reagent ratio (w/v) of 1:2. For generation of stable clones, HEK-293 cells were transfected using SuperFect (QiAGEN, Hilden, Germany) with a DNA/lipid ratio (w/v) of 1.5. At least 30 clones (Zeocin)-resistant clones were isolated after 3 to 4 weeks. Of resistant clones, 20% were positive for LOX-1 expression. Details on the preparation of primary cultures of endothelial cells derived from aorta and infrarenal abdominal aortic aneurysm are in Supplemental Data.

**Purification of Caveolae-Enriched Membrane Fractions.** Caveolae-enriched membrane fractions were prepared by a detergent-free purification, as described previously (Song et al., 1996). Confluent 90-mm dishes of HEK-293 (clones 13 or 19) or transfected COS or primary endothelial cells were lysed in 500 mM sodium carbonate, pH 11, containing protease inhibitor cocktail set III (0.1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 0.5 μM aprotinin, 5 mM Bestatin, 1.5 μM trans-epoxysuccinic acid (E-64), 10 μM leupeptin, and 1 mM pepstatin A; (Calbiochem, La Jolla, CA) and 1 μM phenylmethylsulfonlfyl fluoride (Euroclone, Devon, UK), homogenized, and sonicated. A 5 to 45% discontinuous sucrose density gradient was formed in MBS (25 mM Mes, pH 6.5, and 0.15 M NaCl) and centrifuged at 39,000 rpm for 16 to 20 h in an SW-41 rotor (Beckman-Coulter, Fullerton, CA). Samples were fractionated in 1-ml aliquots from the top to the bottom. The protein concentration...
was measured in each fraction by the Bradford assay (Sigma-Aldrich). Proteins from each fraction were precipitated with 10% trichloroacetic acid and solubilized in SDS-polyacrylamide gel electrophoresis sample buffer.

**Western Blot Analysis.** Transected cells and human primary endothelial cells were lysed in ice-cold extraction buffer containing 10 mM Tris/HCl, pH 7.6, 100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, protease inhibitor cocktail set III, and phenylmethylsulfonyl fluoride and centrifuged for 20 min at 4°C at 15,000g. The supernatant fraction was analyzed by SDS-polyacrylamide gel electrophoresis in 10% acrylamide gels and transferred to polyvinylidene difluoride membranes (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) for 30 min at 15 V (Semi-Dry Transfer Cell; Bio-Rad Laboratories, Hercules, CA).

**Immunofluorescence Analysis and Surface Labeling Quantification.** Cell membrane immunofluorescence was performed as described previously (Cardinale et al., 2005) using MAb anti-V5 as the primary antibody and Rhodamine Red-X-conjugated AffiniPure donkey anti-mouse IgG as the secondary antibody. Samples were examined with a DMRA Leica fluorescence microscope, equipped with a charge-coupled device camera and with a confocal microscope (Nikon Instruments Spa, C1 on Eclipse TE2000; EZC1 software). A cyto-enzyme-linked immunosorbent assay for quantification of membrane-expressed proteins was performed as described previously (Biocca et al., 2008).

**Ox-LDL Preparation, Labeling, and Fluorometric Assay.** Human LDL was prepared from fresh healthy normolipidemic plasma of volunteers by ultracentrifugation (Sattler et al., 1992), dialyzed in phosphate-buffered saline, and filtered (0.22-μm pore size). Oxidation was performed by incubating LDL (0.4 mg/ml) with 7.5 μM CuSO₄ in a CO₂ incubator at 37°C for 6 to 8 h, acquiring, at the end of the incubation period, the wavelength difference spectrum using LDL (without copper) as control. Oxidation was stopped by addition of 0.3 mM EDTA, and ox-LDL was dialyzed overnight in PBS containing 0.1 mM EDTA. Agarose gel electrophoresis of native and oxidized LDL was routinely performed in 0.8% (w/v) gel prepared in Tris-glycine (29 mM Tris base and 192 mM glycine), pH 8.3, and subjected to 100 V constant voltage for 60 min. Gels were stained with Oil Red O or Coomassie Blue R250. Relative electrophoretic mobility was calculated as the ratio between the migration distance with Oil Red O or Coomassie Blue R250 and that of native LDL and used as measure of oxidation. Ox-LDL was labeled with 1,1',3,3'-dioctadecyl-3,3',3',3'-tetrarnethylindocarbocyanine perchlorate (DiI-) (Invitrogen) as described previously (Stephan and Yurachek, 1993; Biocca et al., 2008). Dil-labeled ox-LDL was incubated in complete medium on ice for 1 h in the binding assay and at 37°C for different times in the uptake assay. Endothelial cells, treated or not with ox-LDL, were washed once with DMEM without serum and incubated with DMEM supplemented with lipid-depleted fetal calf serum (Biowest) for 1 h before the binding assay was performed. Quantitation of Dil-ox-LDL bound was assayed by Dil-extraction in isopropanol (Stephan and Yurachek, 1993), and fluorescence was determined in a spectrophuorometer (PerkinElmer Life and Analytical Sciences, Waltham, MA) with excitation and emission wavelengths set at 520 and 578 nm, respectively.

**Evaluation of Apoptosis.** Apoptotic cells were visualized by staining with an annexin V assay (Bossey-Weltzel and Green, 2000) (kit from BD) and with the blue fluorescent dye Hoechst 33342 (Sigma-Aldrich). Condensed and/or fragmented nuclei were counted as apoptotic nuclei.

**Statistical Data Analysis.** Data are reported as mean ± S.D. Comparison among groups was performed using one-way analysis of variance for parameters with gaussian distributions (after confirmation with histograms and the Kolmogorov-Smirnov test). p < 0.05 was considered statistically significant.

### Results

**Cholesterol Is Necessary for Spatial Organization of LOX-1 Surface Receptors.** To address the question of whether the membrane cholesterol level can alter the trafficking, distribution, and function of LOX-1 receptors, we used two compounds acting by different mechanisms. The polynene antibiotic filipin, a fluorescent drug that specifically binds to lipid rafts and nonraft membrane cholesterol (Rothberg et al., 1990) and MβCD. The latter specifically extracts cholesterol from the plasma membranes and therefore disrupts lipid rafts and caveolae (Ilangumaran and Hoessli, 1998). We first investigated the localization and physical interaction between LOX-1 receptors and cholesterol-rich membrane microdomains by comparing its membrane distribution with that of filipin. Double staining of nonfixed COS cells transiently transfected with human LOX-1-V5 and filipin is shown in Fig. 1A. We found many colocalization sites of the two fluorescence signals. In particular, LOX-1 accumulates in filipin-positive dots, which suggests that LOX-1 is preferentially associated with membrane-bound cholesterol. Then, we lowered the membrane cholesterol level and disrupted lipid rafts by MβCD and studied the trafficking and distribution of LOX-1 receptors. Whereas in the control (Fig. 1B, a and a’), most cells show an intense membrane fluorescence, the plasma membrane pool of LOX-1 receptors becomes more diffuse in MβCD-treated cells (Fig. 1B, b and b’).

![Fig. 1](image-url) Cholesterol level alters LOX-1 receptors surface organization. A, double staining of COS cells transiently transfected with LOX-1-V5 together with filipin. After a 30-min incubation with 100 μg/ml filipin, surface-expressed LOX-1 receptors were visualized with MAb anti-V5 for 1 h at 4°C. B, transfected COS cells were treated with 5 mM MβCD for 30 min in serum-free medium, surface stained with MAb anti-V5, and analyzed by confocal microscopy. Representative images of the membrane distribution of LOX-1 receptors in non-treated (a and a’) and in MβCD-treated cells (b and b’) are displayed. a’ and b’ report the LOX-1 red staining converted first to black and white and then inverted to white and black, using the channel mixer of Adobe Photoshop. Scale bar, 10 μm.
suggesting a perturbation of LOX-1 membrane localization. Taken together, these data indicate that LOX-1 is associated with cholesterol-rich domains and that plasma membrane cholesterol specifically regulates LOX-1 surface distribution.

Membrane Cholesterol Depletion Inhibits LOX-1-Mediated ox-LDL Binding and Internalization. To study whether the altered membrane distribution of LOX-1 receptors is accompanied by impairment of ox-LDL binding and internalization, we transfected COS cells with LOX-1-V5 and 24 h after transfection, we treated cells with MβCD and incubated them with Dil-labeled ox-LDL in serum-free medium. Representative confocal images of Dil-ox-LDL binding (1 h at 4°C) and uptake (1 and 4 h at 37°C) are shown in Fig. 2A. Dil-ox-LDL efficiently binds to LOX-1 receptors in control cells (Fig. 2A, a and a′). Of note, MβCD caused a marked loss of specific ox-LDL binding, making the typical membrane fluorescence more diffuse and less intense (Fig. 2A, b and b′). Moreover, the intracellular dots of endocytosed ox-LDL after a 1-h incubation at 37°C (Fig. 2A, c and d) are markedly reduced both in size and number after MβCD treatment, indicating strong inhibition of ox-LDL internalization. We also studied the intracellular uptake of ox-LDL at 37°C for 4 h (Fig. 2A, e and f). In control cells, most of the fluorescent ox-LDL is found inside the cells. In contrast, this intracellular pool is almost absent in MβCD-treated COS cells, in which Dil-ox-LDL fluorescence remains at the level of the plasma membrane. Quantitation of bound Dil-ox-LDL was obtained by its extraction from stained cells with isopropanol and spectrofluorometric analysis. As can be seen in Fig. 2B, inhibition of ox-LDL binding is very strong, reaching 47 ± 5% reduction of LOX-1 binding after treatment with MβCD.

Lipid rafts are also disrupted by statins, drugs that are largely used in clinics for their activity in lowering circulating cholesterol. We treated LOX-1-V5-COS transfected cells with lovastatin and atorvastatin, and quantified the fluorescent Dil-ox-LDL binding in treated and nontreated cells (Fig. 2B). Chronic exposure of cells to statins leads to a reduction of ox-LDL binding compared with that in control cells. These data confirm that membrane cholesterol depletion in lipid rafts affects LOX-1 function. However, because statins inhibit the synthesis of isoprenoids and prevent isoprenylation of many proteins including Ras and Rho families of GTPase, exerting cholesterol-independent or pleiotropic effects also, they may impair the function of cell surface receptors that interact with signaling molecules. To examine the role of isoprenylation on LOX-1 function, transfected COS cells were pretreated with the farnesyl transferase inhibitor ABT-100 before Dil-ox-LDL binding was measured. No effect was seen on ox-LDL binding, suggesting that cholesterol lowering rather than isoprenylation inhibition is responsible for LOX-1 function impairment.

Whether reduction of plasma membrane cholesterol causes the marked decrease in LOX-1 activity by modulating the
number of exposed receptors was studied by using a surface labeling quantification assay (Biocca et al., 2008), as described under Materials and Methods. The surface appearance of LOX-1 in COS transfected cells, treated or not with cholesterol-lowering drugs is shown in Fig. 2C. Of interest, there is no statistically significant difference in the amount of exposed LOX-1 receptors immediately after MβCD, lovastatin, and atorvastatin were washed off the cells, indicating that the effect of cholesterol reduction at the level of plasma membranes is mostly on LOX-1-mediated ox-LDL binding activity and on its distribution (as shown in Fig. 1B) and is not related to a variation in the number of exposed receptors. A decrease in surface LOX-1 receptors was only detected in cells treated for 48 h at higher concentration of lovastatin. No significant difference in surface LOX-1 receptors was found with treatment of COS transfected cells with atorvastatin and the farnesyl transferase inhibitor ABT-100.

**Cholesterol-Lowering Drugs Disrupt LOX-1 Distribution in Caveolae/Lipid Rafts.** To better understand how membrane cholesterol regulates LOX-1 functional state, we studied the subcellular distribution of LOX-1 receptors in plasma membranes by fractionation of cellular membranes and purification of cholesterol-rich lipid rafts. We generated HEK-293 cell lines stably transfected with human LOX-1-V5. More than 10 stably transfected clones were analyzed and compared for LOX-1 expression by Western blot and indirect immunofluorescence, and the studies reported in this article were performed with two representative expressing clones (13 and 19). First, we quantified the total cellular LOX-1 content in HEK-293 cells incubated or not with MβCD by Western blot. LOX-1 protein runs at 46 kDa in HEK-293 transfected cells and is efficiently expressed, and there is no change in the total cellular LOX-1 protein level immediately after the MβCD treatment (Fig. 3A, INPUT). We then isolated caveolin-rich domains by a detergent-free procedure and sucrose gradient flotation centrifugation (Song et al., 1996). An aliquot of each fraction was subjected to immunoblot analysis (Fig. 3A, fractions from 2 to 9). Of interest, LOX-1 was mostly found in fractions 5. This fraction is composed of lipid rafts, as confirmed by the presence of caveolin-1, and we have calculated that LOX-1, by using this fractionation scheme, is purified approximately 200-fold relative to total cell lysate. Strikingly, MβCD treatment led to a marked depletion of LOX-1 from caveolin-enriched membranes, which was superimposable to that of caveolin itself, as seen in the blot visualized with anti-Cav-1 antibodies. It is worth noting that the amount of LOX-1 and caveolin-1 not clearly present in lipid rafts is now detected as associated with nonraft membrane fractions (fraction 9) and, in a lower amount, in fractions 10 to 12 (not shown). To evaluate the specificity of our data, we determined the expression of another plasma membrane protein, IRβ, which is located in raft and nonraft membranes, under the same experimental conditions (Winter et al., 2012). As shown in Fig. 3A, changes in cellular cholesterol did not influence the insulin receptor expression and localization in lipid rafts. The fold change in LOX-1, caveolin-1, and IRβ bands in fraction 5 in control and MβCD-treated cells was evaluated by densitometric quantification (Fig. 3B).

To verify whether chronic inhibition of cholesterol biosynthesis by statins also resulted in perturbation of LOX-1 distribution in caveolae/lipid rafts, we used HEK-293 cells stably expressing LOX-1 and treated cells with lovastatin or atorvastatin for 3 days. We then isolated lipid rafts by flotation centrifugation and analyzed the presence of LOX-1, caveolin-1, and IRβ in each fraction of the gradient. A representative blot of the gradient derived from lovastatin-treated cells and the analysis of the band intensity in fraction 5 is shown in Fig. 4, A and B, respectively. It is worth mentioning that lovastatin, as MβCD, does not change the total LOX-1 expression (Fig. 4A, INPUT). Lovastatin treatment leads to a marked decrease in LOX-1 protein (lovastatin 38 ± 6% versus control 100%) and caveolin-1 (lovastatin 50 ± 3% versus control 100%) in lipid rafts (fraction 5). In these cells the amount of LOX-1 not present in fraction 5 is detected mostly in fraction 12. No significant difference in the intensity of the band was detected for the IRβ subunit. As shown in Fig. 4C, a comparable decrease in the intensity of LOX-1 band in fraction 5 was obtained by incubating cells with 2 μM atorvastatin. Of importance, the farnesyl transferase inhibitor ABT-100 does not lead to a reduction in LOX-1 in lipid rafts (Fig. 4C).

**Lovastatin Acts on LOX-1 Function in Human Endothelial Primary Cultures from Aortic Aneurysm.** It is well established that ox-LDL treatment of vascular endothelial cells results in apoptosis and necrosis and that most of these effects are mediated by an increase in expression and activation of LOX-1 receptors (Chen et al., 2002). To further

---

**Fig. 3.** Detergent-free purification of caveolin-rich domains by sucrose gradient. A, distribution and localization of LOX-1 receptors on membranes derived from HEK-293 cells stably transfected with LOX-1-V5 (clone 19). Lysates of untreated cells or cells treated with 5 mM MβCD at 37°C for 30 min were subjected to sucrose gradient centrifugation after homogenization in a buffer containing sodium carbonate (see under Materials and Methods) and analyzed by Western blot. Total protein extract (5%, 15 μg) (INPUT) was also loaded as positive internal control of electrophoretic mobility. Immunoblot analysis was carried out with anti-V5 (LOX-1), anti-caveolin 1 (Cav-1), and anti-IRβ antibodies. Fractions 4 to 6 were designated as caveolin-enriched lipid rafts indicated by the marker protein caveolin-1. B, histogram shows the densitometric measurements performed to compare the intensity of LOX-1, caveolin-1, and IRβ in fraction 5 derived from control or treated cells. The data represent the average ± S.D. of three separate experiments.
explore the significance of the effect of cholesterol modulation and raft disruption by statins on LOX-1 receptor activity, we have used human endothelial primary cells isolated from atherosclerotic lesions of human aorta (infraaortic abdominal aortic aneurysm), as a model system. ECs were isolated as described in the Supplemental Data and characterized by fluorescence-activated cell sorter analysis using different markers (Supplemental Fig. 1). We incubated primary EC cells with ox-LDL or ox-LDL plus lovastatin for 24 h and analyzed the binding of fluorescent ox-LDL, as described under Materials and Methods (Fig. 5A). In control cells, binding is very low because of the low endogenous LOX-1 level of expression. As expected, upon induction of LOX-1 with ox-LDL incubation, many cells show a much higher fluorescent signal (Fig. 5A, center panel). Lovastatin treatment results in a marked reduction of positive cells (right panel, ox-LDL + Lov). Because most of the Dil-ox-LDL-positive cells do not thrive and many of these cells exhibit cell shrinkage, which is a feature of apoptosis, we measured the apoptotic effects after different treatments, performing annexin V membrane staining and Hoechst 33342 nuclear staining performed on control or treated cells. The data represent the average ± S.D. of three separate experiments. C, Western blot analysis for LOX-1 present in fraction 5 of sucrose gradients derived from HEK-293 (clone 19) treated or not with 2 μM lovastatin (Lov), 2 μM atorvastatin (Ato), or 0.1 μM PTI (ABT-100) for 48 h.

Fig. 5. Lovastatin effects on human primary endothelial cells. A, EC cells treated or not with ox-LDL (100 μg/ml) or ox-LDL plus 2 μM lovastatin (Lov) were incubated with 10 μg/ml Dil-ox-LDL for 1 h at 4°C. Fluorescence of Dil-ox-LDL was detectable only in ox-LDL-treated EC cells. Scale bar, 10 μm. B, apoptosis assay by annexin V membrane staining and Hoechst 33342 nuclear staining performed on control EC cells, ox-LDL, ox-LDL plusLovastatin, andLovastatin-treated cells. The data represent the average ± S.D. of three separate experiments. **, p < 0.01. C, Western blot analysis for LOX-1 on EC cells treated or not with ox-LDL, ox-LDL plus lovastatin, and lovastatin alone. The gel is representative of five separate experiments. Ctrl, control.

Fig. 4. Statins disrupt LOX-1 membrane distribution in caveolin-rich lipid rafts. A, HEK-293 stably expressing LOX-1-V5 (clone 19) were treated or not with 2 μM lovastatin (Lov) for 3 days. Lysates of untreated and treated cells were subjected to sucrose centrifugation to isolate caveolin-enriched lipid rafts and immunoblotted with anti-V5 (LOX-1), anti-caveolin (Cav-1), and anti-IRβ antibodies (IRβ). Total protein extract (5%, 15 μg) (INPUT) was also loaded as a positive internal control of electrophoretic mobility. B, densitometric measurements of LOX-1, Cav-1, and IRβ subunit bands in fraction 5 derived from control or treated cells. The data represent the average ± S.D. of three separate experiments. C, Western blot analysis for LOX-1 present in fraction 5 of sucrose gradients derived from HEK-293 (clone 19) treated or not with 2 μM lovastatin (Lov), 2 μM atorvastatin (Ato), or 0.1 μM PTI (ABT-100) for 48 h. Ox-LDL-dependent induction of endogenous LOX-1 receptors was also studied by Western blot with anti-LOX-1 polyclonal antiserum (Fig. 5C). As can be seen, the LOX-1 band is induced in human primary endothelial cells. The 48-kDa LOX-1 band is very intense. However, its intensity markedly decreases in lysates derived from cells simultaneously incubated with ox-LDL andLovastatin (57 ± 5% reduction). As expected, LOX-1 in lysates derived from nontreated cells or derived from cells incubated withLovastatin alone is under the detection threshold.

To study the intracellular distribution of endogenous LOX-1 in primary endothelial cells incubated with ox-LDL or ox-LDL plusLovastatin, we isolated lipid rafts by flotation centrifugation. We first analyzed LOX-1 distribution in transiently transfected COS cells incubated or not with 100 μg of ox-LDL for 1 h at 37°C (Fig. 6A). In the absence of ox-LDL, LOX-1 receptors are distributed in caveolae/lipid raft (fractions 4 and 5) and nonlipid raft membranes (fractions 8 and 9). Of interest, upon incubation with ox-LDL, 100% of LOX-1 receptor molecules are found in fractions 4 and 5, indicating that active receptors are localized in caveolae/lipid raft membranes.

Figure 6B shows gradients derived from primary EC cells obtained from an aortic aneurysm. In nontreated control cells, LOX-1 is distributed in raft and nonraft membranous fractions. A small, but significant, amount of LOX-1 was also found in the final fractions 11 and 12 at the bottom of the tube. As expected, caveolin-1 is almost entirely found in the caveola/lipid raft fraction (fractions 4–6). Treatment of EC


### Discussion

Here we demonstrate that LOX-1 is distributed within lipid rafts in the plasma membranes and that its distribution in cholesterol-enriched microdomains is an absolute requirement for its capacity to bind and internalize ox-LDL. Our observation that LOX-1 resides within lipid rafts is based on several well-established raft analysis techniques. First, LOX-1 receptors colocalize with filipin, a fluorescent marker of membrane cholesterol. Second, cholesterol sequestration by MβCD leads to mislocalization of LOX-1 receptors in a more diffuse distribution in the plasma membrane. Third, by biochemical fractionation of cell membranes, we show that functional LOX-1 is found almost entirely in a fraction containing caveolin-1 and that we define caveolae/lipid rafts in all cell types we analyzed including human primary endothelial cells. A more detailed morphological analysis at high resolution is necessary, however, to establish whether LOX-1 associates exclusively with caveolaee. Of importance, disruption of caveolae/lipid rafts by acute treatment with MβCD or chronic incubation of cells with lovastatin or atorvastatin results in a marked reduction of LOX-1-mediated ox-LDL binding and uptake.

The finding that LOX-1 function is highly inhibited by treatment with cholesterol-depleting drugs highlights, for the first time, the critical importance of cholesterol in receptor activity and demonstrates a new role of statins as inhibitors of LOX-1 activity. The plasma membrane cholesterol is an essential determinant of membrane fluidity by modulating the structure of the phospholipid bilayer. Formation and maintenance of lipid rafts and caveolae are strictly dependent on cholesterol. These specialized cholesterol-rich subdomains, highly abundant in endothelial cells, regulate various signal transduction pathways and are characterized by the presence of the caveolin protein family (Li et al., 2005). Treatment with statins reduces the amount of cholesterol in these sites, increases membrane fluidity, and induces a redistribution of caveolin-1 and other resident membrane proteins in the endoplasmic reticulum and plasma membrane. Many important membrane properties are affected, including cell endocytosis, permeability, and transport functions (Goonasekara et al., 2010). As shown here, LOX-1 receptors also change their distribution and lose their physiological location when cells are treated with cholesterol-depleting drugs. However, interestingly, disruption of caveolae/lipid rafts and mislocalization of LOX-1 receptors do not lead to a reduction in surface exposed LOX-1 receptors. Notwithstanding their presence, it appears that their affinity for ox-LDL decreases dramatically, suggesting that the assembly of LOX-1 in multimers in specific membrane microdomains is a crucial requirement for ox-LDL binding and internalization. Thus, when LOX-1 is randomly distributed in the membrane, it is unable to bind ox-LDL. Its clustered distribution is essential to enhance the interaction efficiency as well as the internalization of LOX-1-ox-LDL complexes. Several pieces of evidence from our laboratory and from others have recently suggested that multimerization and cluster formation are necessary for LOX-1 activity (Biocca et al., 2008; Cao et al., 2009; Ohki et al., 2011). Hetero-oligomerization with LOX-1 mutant isoforms such as LOXIN or K167N LOX-1 leads to a very severe reduction in LOX-1 function (Biocca et al., 2008, 2009). Here we indicate cholesterol-enriched mi-
Cholesterol may be more evident in the early phase of treatment, because only a 10% reduction in the LDL-C level is detectable after 24 h and at least 6 to 7 days are necessary to lower it significantly (Corsini et al., 2007). Of interest, in a recent trial, an early window of protection by statin without LDL-C-lowering effects has been described. A pretreatment of 12 h with 40 mg of atorvastatin before percutaneous coronary intervention improved clinical outcomes in patients with acute coronary syndromes, indicating the pleiotropic properties of statins (Patti et al., 2007).

Finally, we found a marked down-regulation of caveolin-1 in lovastatin-treated human primary endothelial cells. The physiological role of caveolin-1 and caveolae in the cardiovascular system has been seen recently. Caveolin-1 is important for the biogenesis of caveolae and is also involved in cholesterol trafficking to and from plasma membrane. In fact, caveolin-1 directly binds cholesterol with high affinity, which can explain the high concentration of cholesterol in caveolae (Li et al., 2005). In particular, endothelial-specific overexpression of Cav-1 enhances the progression of atherosclerosis and loss of caveolae through Cav-1 gene deletion is protective against atherosclerosis (Frank et al., 2008; Fernández-Hernando et al., 2010). Cav-1(−/−) mice show defects in the aortic uptake of LDL particles both in vitro and in vivo. The result is that statins reduce caveolin-1 abundance in endothelial cells (Pelat et al., 2003; Plenz et al., 2004) and may act in synergy with the inhibitory effect on LOX-1 receptor activity. Whether caveolin-1 directly interacts with LOX-1 receptors and this interaction modulates the internalization of ox-LDL remains to be determined.

The delineation of the caveola/raft-mediated pathway for endocytosis of ox-LDL through LOX-1 receptors provides the basis of future studies and supports a novel effect of statins on membrane raft function that may be relevant for other membrane receptors in cardiovascular diseases, paving the way for new therapeutic interventions.

Acknowledgments

We thank Prof. Arnaldo Ippoliti for providing surgery explants, Dr. Massimo Sanchez for his assistance with fluorescence-activated cell sorter analysis, Prof. Federica Sanguinulo and Prof. Nadia Canu for critical reading of the manuscript, and Prof. Fabrizio Lorenzi for helpful suggestions. We are also very grateful to Graziano Bonelli for his expert graphical assistance.

Authorship Contributions

Participated in research design: Mango, Novelli, and Biocca.
Conducted experiments: Matarazzo, Quitadamo, Ciccone, and Biocca.

Contributed new reagents or analytic tools: Mango and Novelli.
Performed data analysis: Matarazzo, Quitadamo, and Biocca.
Wrote or contributed to the writing of the manuscript: Biocca.

References


