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Cholesterol-lowering drugs inhibit LOX-1 receptor function by membrane raft disruption

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Running title: Statins inhibit LOX-1 receptor function

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247 words in Abstract

744 words in Introduction

1312 words in Discussion

Abbreviations: Ato, atorvastatin; Cav-1, caveolin-1; CTLD, C-type lectin-like domain; DC-SIGN, dentritic cell-specific intercellular grabbing non-integrin; DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethyllindocarbocyanine perchlorate; ECs, endothelial cells; FTI, farnesyl transferase inhibitor; HEK, human embryonic kidney; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IRβ, insulin receptor subunit β; LDL, low-density lipoprotein; LDL-C, low-density lipoprotein-cholesterol; Lov, lovastatin; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; Mab, monoclonal antibody; MβCD, methyl-β-cyclodextrin; ox-LDL, oxidized low-density lipoprotein.

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Abstract

Lectin-like oxidized low-density lipoprotein (LOX-1), the primary receptor for ox-LDL in endothelial cells, is up-regulated in atherosclerosis lesions. Statins are principal therapeutic agents for cardiovascular diseases and are known to down regulate LOX-1 expression. Whether the effect on LOX-1 receptor is related to statin-mediated cholesterol lowering activity is unknown. We seek to 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 (MβCD) 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 immunochemical 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 from 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 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.

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Introduction

Receptor-mediated endocytosis of ox-LDL (oxidized low-density lipoprotein) 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 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; Metha et al., 2006). LOX-1 is a scavenger receptor over-expressed in atherosclerotic lesions and up-regulated during atherogenesis in atheroma-derived cells (Metha et al., 2006; Vohra et al., 2006). LOX-1 activation triggers 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 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 12p13.2-13.1 (Aoyama et al., 1999). Genetic association studies have identified different SNPs within the ORL1 gene which play a role in cardiovascular diseases susceptibility. A new identified LOX-1 spliced isoform, which lacks exon 5, named LOXIN, 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 non-functional hetero-oligomers (Biocca et al., 2008). LOX-1 is a type II membrane glycoprotein with an extracellular C-type lectin-like ligand binding domain (CTLD) (Sawamura et al., 1997) which forms a disulfide-linked heart-shaped homodimer. The CTLD 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 non covalent interactions (Biocca et al., 2008; Ohki et al., 2005; Park et al., 2005). 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).

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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 pathophysiologic role of LOX-1-mediated ox-LDL endocytosis, a detailed understanding of the receptor-mediated lipid particle internalization, trafficking and processing during atherosclerotic plaque formation is not yet understood. Scavenger receptors, such as CD36, CD209 and CD204 are localized in lipid rafts and utilize 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 to be involved in LOX-1 endocytosis (Kashiwakura et al., 2004; Murphy et al., 2008), these studies have not investigated the lipid rafts involvement. Caveolae and lipid rafts are specialized membrane domains, rich in cholesterol, sphingolipids and glycerophospholipids and contain specific membrane proteins including GPI-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 thought 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 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a rate limiting enzyme in cholesterol biosynthesis (Wang et al., 2008). For their effect on lowering circulating total and LDL-cholesterol (LDL-C), statins are largely used in clinic in the treatment of patients with cardiovascular diseases. We have focused our interest on the role of statins in reducing 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

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mediated ox-LDL internalization and rescue of 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.

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Materials and Methods

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

Antibodies and reagents. Rat anti-LOX-1 (Biocca et al. 2008), Mab anti-V5 IgG (Invitrogen, Inchinnan, Paisley, UK), mouse anti-caveolin 1 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-insulin receptor β subunit (IRβ), BD Biosciences, Franklin Lakes, NJ, USA) and mouse anti-β-actin IgG (Affinity Bio Reagents, Golden, CO) were used as 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 (West Grove, PA). Filipin, MβCD and atorvastatin were purchased from Sigma-Aldrich (St.Louis, MO). Lovastatin (Alexis Biochemicals, San Diego, CA) was activated by NaOH addiction 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) ABT-100, (S)-6-[2-(4-cyanophenyl)-2-hydroxy-2-(1-methyl-1H-imidazol-5-yl)ethoxy]-4V-(trifluoromethoxy)-1,1V-iphenyl-3-carbonitrile) was provided by Abbott Laboratories (Abbott Park, IL). For in vitro studies, ABT-100 was dissolved in DMSO with dilutions made using DMEM plus 10% fetal bovine serum (FBS).

Cell cultures and transfection. COS and HEK-293 cells were grown in DMEM medium (Biowest, Miami, FL, USA) supplemented with 10% fetal bovine serum (Gibco, Inchinnan, Paisley, UK) and 100U/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, HEH-293 cells were transfected using Superfect (Qiagen, Hilden, Germany) with a DNA/lipid ratio (w/v) of 1:5. At least 30 zeocin-resistant clones were isolated after 3–4 weeks. 20% of resistant clones were positive for LOX-1 expression. Details on the preparation of primary cultures of endothelial cells derived from aorta and infrarenal abdominal aortic aneurism are in Supplemental data.

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Purification of caveolae-enriched membrane fractions. Caveolae-enriched membrane fractions were prepared by a detergent-free purification, as described (Song et al., 1996). Two 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 proteases inhibitor cocktail set III (0.1 mM AEBSF hydrochloride, 0.5 μM aprotinin, 5 mM Bestatin, 1.5 μM E-64, 10 μM Leupeptin, 1 mM Pepstatin A) (Calbiochem, La Jolla, CA) and 1 μM phenylmethylsulfonyl fluoride (PMSF) (Euroclone, Devon, UK), homogenized and sonicated. A 5-45% discontinuous sucrose density gradient was formed in MBS (25 mM Mes, pH 6.5, 0.15 M NaCl) and centrifuged at 39,000 rpm for 16-20 h in an SW41 rotor (Beckman Instruments, Palo Alto, CA). Samples were fractionated in 1-ml aliquots from the top to the bottom. Protein concentration was measured in each fraction by Bradford assay (Sigma-Aldrich, St.Louis, MO). Proteins from each fraction were precipitated with 10% trichloroacetic acid (TCA) and solubilized in SDS-PAGE sample buffer.

Western blot analysis. Transfected cells and human primary endothelial cells were lysed in ice-cold extraction buffer (EB) containing 10mM Tris/HCl pH 7.6, 100 mM NaCl, 10mM ethylenediaminetetraacetic acid (EDTA), 0.5% Nonidet P40, 0.5% sodium deoxycholate, proteases inhibitor cocktail set III and PMSF, and centrifuged for 20 minutes at 4°C at 15,000xg. The supernatant fraction was analyzed by SDS-PAGE in 10% acrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Chalfont St. Giles, UK) for 30 minutes at 15 V (Semi-Dry Transfer cell, Biorad, Hercules, CA). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Sigma-Aldrich, St.Louis, MO).

Immunofluorescence analysis and surface labelling quantification. Cell membrane immunofluorescence was carried out as described (Cardinale et al., 2005) using Mab anti-V5 as primary antibody and Rhodamine Red-X-conjugated AffiniPure donkey anti-mouse IgG as secondary antibody. Samples were examined with a DMRA Leica fluorescence microscope, equipped with CCD camera and with a confocal microscope (Nikon Instruments Spa, C1 on Eclipse TE200; EZC1 software). CytoELISA assay for quantification of membrane expressed proteins was performed as previously described (Biocca et al., 2008).

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Ox-LDL preparation, labelling and fluorometric assay. Human LDL was prepared from fresh healthy normolipidemic plasma of volunteers by ultracentrifugation (Sattler et al., 1992), dialyzed in PBS and filtered (0,22 μm pore size). Oxidation was performed by incubating LDL (0,4mg/ml) with 7,5 μM CuSO4 in a CO_2 incubator at 37°C for 6-8 hours, acquiring, at the end of the incubation period, the wavelength difference spectrum using LDL (without copper) as control. Oxidation was stopped by adding 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 or Coomassie blue R250. Relative electrophoretic mobility (REM) was calculated as the ratio between the migration distance of ox-LDL and that of native LDL and used as measure of oxidation. Ox-LDL was labelled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyllindocarbocyanine perchlorate (DiI, Invitrogen, Inchinnan, Paisley, UK) as previously described (Stephan and Yurachek, 1993; Biocca et al., 2008). (DiI)-labelled ox-LDL was incubated in complete medium on ice for 1 hour in binding assay and at 37°C for different times in 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, Miami, FL) for 1h before performing the binding assay. Quantitation of Dil-ox-LDL bound was assayed by DiI extraction in isopropanol (Stephan and Yurachek, 1993) and fluorescence determined in a Perkin Elmer spectrofluorometer with excitation and emission wavelengths set at 520 and 578 nm, respectively.

Evaluation of Apoptosis. Apoptotic cells were visualized by staining with Annexin V assay (Bossy-Weltzel and Green, 2000) (Kit by BD Biosciences, Franklin Lakes, NJ) and with the blue fluorescent dye Hoechst 33342 (Sigma-Aldrich, St.Louis, MO). Condensed and/or fragmented nuclei were counted as apoptotic nuclei.

Statistical data analysis. Data are reported as mean ± standard deviation (SD). Comparison among groups was performed using one-way ANOVA for parameters with gaussian distributions (after

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confirmation with histograms and the Kolgomorov-Smirnov test). A p value <0.05 was considered statistically significant.

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Results

Cholesterol is necessary for spatial organization of LOX-1 surface receptors.

To address the question of whether membrane cholesterol level can alter the trafficking, distribution and function of LOX-1 receptors, we used two compounds acting by different mechanisms. The polyene antibiotic filipin, a fluorescent drug that specifically binds to lipid rafts and non-raft membrane cholesterol (Rothberg et al., 1990) and methyl-β-cyclodextrin (MβCD). The last 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 non-fixed COS cells transiently transfected with human LOX-1-V5 and filipin is shown in Figure 1A. We found many co-localization 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 membrane cholesterol level and disrupted lipid rafts by MβCD and studied the trafficking and distribution of LOX-1 receptors. While in control cells (Figure 1B, panels 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 (Figure 1B, panels b and b') suggesting a perturbation of LOX-1 membrane localization. Taken together these data indicate that LOX-1 is associated to 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 with Dil-labelled ox-

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LDL in serum-free medium. Representative confocal images of Dil-ox-LDL binding (1h at 4°C), and uptake (1h and 4h at 37°C) are shown in Figure 2A. DiI-ox-LDL efficiently binds to LOX-1 receptors in control cells (panels a and a'). Notably, MβCD caused a marked loss of specific ox-LDL binding making the typical membrane fluorescence more diffuse and less intense (panels b and b'). Moreover, the intracellular dots of endocyted ox-LDL after 1 hours incubation at 37°C (panels c and d) are markedly reduced both in size and number after MβCD treatment, indicating a strong inhibition of ox-LDL internalization (panels e and f). We also studied the intracellular uptake of ox-LDL at 37 °C for 4 h (panels e and f). In control cells most of the fluorescent ox-LDL is found inside the cells. By contrast, this intracellular pool is almost absent in MβCD treated COS cells where Dil-ox-LDL fluorescence remains at the level of the plasma membrane. Quantitation of bound DiI-ox-LDL was obtained by its extraction from stained cells with isopropanol and spectrofluorometric analysis. As it can be seen in Figure 2B, inhibition of ox-LDL binding is very severe, 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 non-treated cells (Figure 2B). Chronic exposure of cells with statins leads to a reduction of ox-LDL binding when compared to control cells. These data confirm that membrane cholesterol depletion in lipid rafts affects LOX-1 function. However, since statins inhibit the synthesis of isoprenoids and prevent isoprenylation of many proteins including Ras and Rho families of GTPase, exerting also cholesterol-independent or pleiotropic effects, 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 ABT100 before measuring Dil-ox-LDL binding. No effect was seen on ox-LDL binding, suggesting that cholesterol lowering rather than isoprenylation inhibition is responsible of LOX-1 function impairment.

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Whether reduction of plasma membrane cholesterol causes the marked decrease of 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 in Materials and Methods. The surface appearance of LOX-1 in COS transfected cells, treated or not with cholesterol lowering drugs is shown in Figure 2C. Interestingly, 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 Figure 1B) and is not related to variation on the number of exposed receptors. A decrease of surface LOX-1 receptors was only detected in cells treated for 48 hours at higher concentration of lovastatin. No significant difference of surface LOX-1 receptors were found with treatment of COS transfected cells with atorvastatin and the farnesyl transferase inhibitor ABT100.

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. Over 10 stably transfected clones were analyzed and compared for LOX-1 expression by Western blot and indirect immunofluorescence and the studies reported in this paper were carried out 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 46kDa in HEK transfected cells, is efficiently expressed and there is no change in the total cellular LOX-1 protein level immediately after the MβCD treatment (see Figure 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 (Figure 3A, fractions from 2 to 9). Interestingly, LOX-1 was mostly found

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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, that 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 are now detected associated with non-raft membrane fractions (see fraction 9) and, in a lower amount, in fractions 10-12 (not shown). In order to evaluate the specificity of our data, we determined the expression of another plasma membrane protein under the same experimental conditions, the insulin receptor β-subunit (IRβ), which is located in raft and non-raft membranes (Winter et al. 2012). As shown in Figure 3A, changes in cellular cholesterol did not influence the insulin receptor expression and localization in lipid rafts. The fold change of LOX-1, caveolin-1 and IRβ bands in fraction 5 in control and MβCD treated cells was evaluated by densitometric quantification (Figure 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 Figure 4A and Figure 4B respectively. It is worth mentioning that lovastatin, as MβCD, does not change the total LOX-1 expression (see INPUT). Lovastatin treatment leads to a marked decrease of LOX-1 protein (Lov: 38±6% vs Ctrl: 100%) and caveolin-1 (Lov: 50±3% vs Ctrl: 100%) in lipid rafts (fraction 5). In these cells the amount of LOX-1 not present in fraction 5 is detected mostly in fractions 12. No significant difference in the intensity of the band was detected for the IRβ subunit. As shown in Figure 4C, a comparable decrease in the intensity of LOX-1 band in

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fraction 5 was obtained by incubating cells with 2μM atorvastatin. Importantly, the farnesyl transferase inhibitor ABT100 does not lead to reduction of LOX-1 in lipid rafts (Figure 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 of expression and activation of LOX-1 receptors (Chen et al., 2002). To further 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 (infrarenal abdominal aortic aneurysm), as a model system. ECs were isolated as described in Supplemental Data and characterized by FACS analysis using different markers (Supplemental Figure 1). We incubated primary EC cells with ox-LDL or ox-LDL plus lovastatin for 24h and analyzed the binding of fluorescent ox-LDL, as described in Materials and Methods (Figure 5A). In control cells, binding is very low due to 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 (Figure 5A, central panel). Lovastatin treatment results in a marked reduction of positive cells (panel ox-LDL + Lov). Since most of 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 following different treatments, carrying out Annexin V membrane staining and Hoechst 33342 nuclear staining (Figure 5B). The percentage of apoptotic cells significantly increased when ECs were exposed to ox-LDL (ox-LDL treatment: 47% vs Ctrl: 5%, on the basis of Annexin V assay ($p<0,01$)). In contrast, lovastatin co-incubation resulted in rescue of ox-LDL induced phenotype (ox-LDL + Lov treatment: 16% vs ox-LDL: 47% ($p<0,01$)). Indeed, the percentage of apoptotic cells in this population (16±2 %) approaches to that of cells treated with lovastatin alone (10±3 %).

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Ox-LDL-dependent induction of endogenous LOX-1 receptors was also studied by Western blot with anti-LOX-1 polyclonal antiserum (Figure 5C). As it can be seen, LOX-1 band is induced in human primary endothelial cells. The 48kDa LOX-1 band is very intense. However, its intensity markedly decreases in lysates derived from cells simultaneously incubated with ox-LDL and lovastatin (57%±5 reduction). As expected, LOX-1 in lysates derived from non-treated cells or derived from cells incubated with lovastatin 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 plus lovastatin, 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 hour at 37 °C (Figure 6A). In the absence of ox-LDL, LOX-1 receptors are distributed in caveolae/lipid rafts (fraction 4 and 5) and non-lipid rafts membranes (fractions 8 and 9). Interestingly, upon incubation with ox-LDL, 100% of LOX-1 receptor molecules are found in fraction 4 and 5, indicating that active receptors are localized in caveolae/lipid rafts membranes.

Figure 6B shows gradients derived from primary EC cells obtained from aortic aneurysm. In non-treated control cells, LOX-1 is distributed in rafts and non-rafts 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 caveolae/lipid rafts fraction (see fractions 4-6). Treatment of EC cells with 2µM lovastatin does not change LOX-1 distribution, but significantly lower the intensity of both LOX-1 and caveolin-1 bands. We then compared (by densitometric quantification) the two proteins in ox-LDL and in ox-LDL plus lovastatin EC treated cells. As observed in transfected COS cells, in the presence of the ligand ox-LDL, LOX-1 is mostly concentrated in lipid rafts (52%±3). In primary cells, however, 38%±6 of LOX-1 is also found in fractions 11 and 12. Since over 45% of ox-LDL treated cells undergo apoptosis (Figure 5B), LOX-1 detected at the bottom of the gradient may belong to apoptotic bodies. In contrast, in the gradient derived from cells that have been co-incubated with

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lovastatin and ox-LDL, LOX-1 is not concentrated in fractions 4 and 5 but has a distribution very similar to that seen in control cells and is present in all membranous fractions. As shown above, these cells show a substantial rescue of ox-LDL induced apoptotic phenotype.

Finally, it is worth noting that caveolin-1 is barely detectable in lovastatin treated endothelial cells. From a densitometric analysis of band intensity from different experiments, gradients derived from lovastatin-treated EC cells present a reduction of ≥50% of caveolin-1 band in fractions 4-6, a value which is comparable to that observed in HEK-293 LOX-1 expressing cells (-55%) incubated with lovastatin (see Figure 4).

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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 rafts analysis techniques. First, LOX-1 receptors co-localize 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 which contains caveolin-1 and that we define caveolae/lipid rafts, in all cell types that we have 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 caveolae. Importantly, disruption of caveolae/lipid rafts by acute treatment of 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 demonstrate 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, 2009). As shown here, also LOX-1 receptors change their distribution and lose their physiological location when cells

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are treated with cholesterol-depleting drugs. However, interestingly, disruption of caveolae/lipid rafts and mislocalization of LOX-1 receptors do not lead to reduction of 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 evidences from our laboratory and from others have recently suggested that multimerization and cluster formation is 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 of LOX-1 function (Biocca et al., 2008; Biocca et al., 2009). Here we indicate cholesterol-enriched microdomains (caveolae/lipid rafts) as the sites of multimerization. Whether ox-LDL can engage multiple interactions with several LOX-1 dimers and these interactions strengthen the binding affinity in vivo, as it was demonstrated in vitro (Ohki et al., 2011), is under study. In support to this hypothesis, it is worth mentioning that intact lipid rafts are essential for HIV-1 virus entry by scavenger receptors (Cambi et al., 2004; Carter et al., 2002; Waheed and Freed, 2009). For DC-SIGN, a dendritic cell specific C-type lectin receptor, a direct correlation between the distribution of receptors in microdomains, rather than randomly distributed, and their capacity to bind and internalize virus-sized ligand-coated particles in dendritic cells has been described (Cambi et al., 2004). Alternative non-mutually exclusive mechanisms to explain the effects of cholesterol lowering drugs on LOX-1 activity can be proposed and require further studies: i) a direct specific interaction of cholesterol with LOX-1 receptor; ii) an indirect mechanism on membrane physical properties, which may affect other molecules involved in LOX-1-mediated ox-LDL endocytosis.

Statins, as potent cholesterol-lowering drugs, are principal therapy for more than 25 million people at risk for cardiovascular diseases worldwide. They are largely used to lower total and LDL cholesterol and are beneficial in primary and secondary prevention of cardiovascular diseases. Lowering circulating cholesterol is thought to be the principal beneficial effect of statins. However,

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they can also exert cholesterol-independent responses, due to the inhibition of the synthesis of isoprenoids, which are important lipid attachments for post-translational modifications of many proteins, such as Ras, Rho, Rac and nuclear lamina (Wang et al., 2008). Notably, treatment of cells with the farnesyl transferase inhibitor ABT100 had no effects on LOX-1 membrane distribution and its activity. We describe a different pleiotropic effect of statins: reduction of membrane-associated cholesterol which influences the density of membrane rafts and disrupts LOX-1 cluster distribution in plasma membranes. This new mechanism of LOX-1 inhibition may explain the beneficial effects of lovastatin incubation in ox-LDL-treated primary endothelial cells derived from aortic aneurysm and suggests that statins protect vascular endothelium by inhibiting LOX-1-mediated entry of ox-LDL. Indeed, in EC cells we show that lovastatin treatment results either to a marked reduction of ox-LDL binding, similar to that shown in statin-treated LOX-1-COS transfected cells and to a consequent substantial rescue of ox-LDL induced apoptotic phenotype. Although other studies have reported the effects of statins on decreasing LOX-1 expression in animal and cellular models (Li et al., 2001; Li et al., 2002; Hofnagel et al., 2006; Dje N'Guessan et al., 2009), none of these reports have focused on the relationship between the potent membrane cholesterol lowering effect and LOX-1-mediated ox-LDL binding and internalization activity. It is worth mentioning, however, that in vivo and ex-vivo studies have recently shown that statin-mediated membrane raft depletion and membrane cholesterol reorganization influence immune cell function, such as natural killer cell citotoxicity and foam cell formation and accumulation in atherosclerotic lesions (Hillyard et al., 2007; Hofnagel et al., 2007; Hillyard et al., 2004; Salvary et al., 2012). In clinical use, the discrimination of the circulating LDL-cholesterol lowering effect from other pleiotropic effects of statins may be more evident in the early phase of treatment, since only 10% reduction of LDL-C level is detectable after 24 hours and at least 6-7 days are necessary to lower it significantly (Corsini et al., 2007). Interestingly, in a recent trial, an early window of protection by statin without LDL-C lowering effects has been described. A pretreatment of 12 hours with 40mg of atorvastatin before percutaneous coronary intervention improved clinical outcomes in patients with acute coronary syndromes, indicating pleiotropic properties of statins (Patti et al., 2007).

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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 cardiovascular system has been recently evidenced. 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 over-expression 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 particle both in vitro and in vivo. The result that statins reduce caveolin-1 abundance in endothelial cells have been reported (Pelat et al., 2003; Plenz et al., 2004) and may act in synergy to 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 caveolae/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 intervention.

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Authorship Contributions

Participated in research design: Biocca, Mango, Novelli.

Conducted experiments: Matarazzo, Quitadamo, Ciccone, Biocca.

Contributed new reagents or analytic tools: Mango, Novelli.

Performed data analysis: Matarazzo, Quitadamo.

Wrote or contributed to the writing of the manuscript: Biocca.

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Footnotes

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Figures Legends

Figure 1 ***Cholesterol level alters LOX-1 receptors surface organization.***

(A) Double staining of COS cells transiently transfected with LOX-1-V5 together with filipin. After 30 min incubation with 100 µg/ml of 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 5mM 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 (panels a and a') and in MβCD treated cells (panels b and b') are displayed. Panels 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.

Figure 2 ***Effect of cholesterol lowering drugs on LOX-1 mediated ox-LDL binding and uptake.***

(A) COS cells transiently transfected with LOX-1-V5 were incubated or not with 5mM MβCD in serum-free medium at 37 °C for 30 min, washed and then incubated with 10 µg/ml of DiI-ox-LDL on ice for 1 h (binding), 1h or 4 h at 37 °C (uptake). Panels a'- f' report the Dil-ox-LDL 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. B) Cells were treated or not with MβCD or with 2µM or 4µM lovastatin, 2µM atorvastatin or 0,1µM FTI (ABT100) for the times indicated. Histograms show quantification of Dil-ox-LDL binding measuring fluorescence by spectrofluorimeter. (C) Surface receptors were measured by cytoELISA assay by using Mab anti-V5. The data represent the average ± standard deviation of four separate experiments. A p value <0.05 was considered to be statistically significant (* $p< 0.05$; ** $p<0.01$); the ns label indicates no significant difference.

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Figure 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 5mM MβCD at 37 °C for 30 min were subjected to sucrose gradient centrifugation after homogenization in a buffer containing sodium carbonate (see in Materials and Methods) and analyzed by Western blot. 5% of total protein extract (15µg) (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-insulin receptor β subunit (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 subunit in fraction 5 derived from control or treated cells. The data represent the average ± standard deviation (SD) of 3 separate experiments.

Figure 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 2µM lovastatin for 3 days. Lysates of untreated and treated cells were subjected to sucrose gradient centrifugation to isolate caveolin-enriched lipid rafts and immunoblotted with anti-V5 (LOX-1), anti-caveolin (Cav-1) and anti- IRβ antibodies (IRβ). 5% of total protein extract (15µg) (INPUT) was also loaded as 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 ± standard deviation (SD) of 3 separate experiments. (C) Western blot analysis for LOX-1 present in fraction 5 of sucrose gradients derived from HEK-293#19 treated or not with 2µM lovastatin, 2µM atorvastatin or 0,1µM FTI (ABT100) for 48 hours.

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Figure 5 ***Lovastatin effects on human primary endothelial cells.***

(A) EC cells treated or not with ox-LDL (100 µg/ml) or ox-LDL plus 2µM lovastatin were incubated with 10 µg/ml of DiI-ox-LDL for 1h at 4°C. Fluorescence of DiI-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 plus lovastatin and lovastatin-treated cells. The data represent the average ± standard deviation (SD) of 3 separate experiments. Asterisks represent p values (** $p<0.01$). (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 5 separate experiments.

Figure 6 ***Effect of ox-LDL and lovastatin on LOX-1 membrane distribution in COS and human endothelial primary cells derived from aortic aneurisms.***

(A) COS cells transiently transfected with LOX-1-V5 were incubated or not with 100 µg/ml ox-LDL for 1 h at 37°C. Lysates of untreated and treated cells were subjected to sucrose gradient centrifugation and immunoblotted with Mab anti-V5. The experiment was repeated 3 times. (B) Distribution and localization of LOX-1 receptors on membranes from EC cells derived from aortic aneurisms. Lysates of untreated and treated cells with 2µM lovastatin, ox-LDL (100 µg/ml) and ox-LDL plus lovastatin were subjected to sucrose gradient centrifugation to isolate caveolin-enriched lipid rafts and immunoblotted with specific antibodies directed against LOX-1 and caveolin-1. All collected fractions are shown, except for fraction 1, which does not contain proteins. The data are representative of 5 separate experiments.

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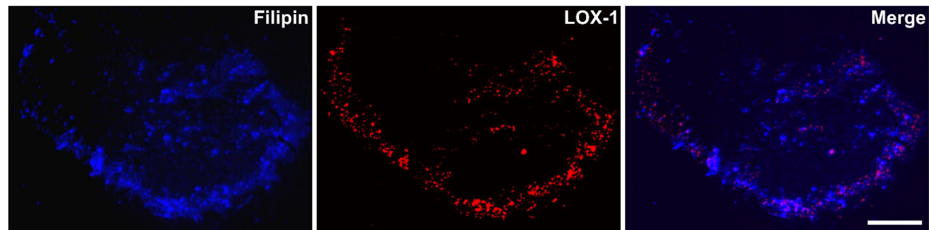
Figure 1



B

Ctrl

MβCD

a a′ b b′

Figure 2

A

Ctrl

MβCD

1h 4°C

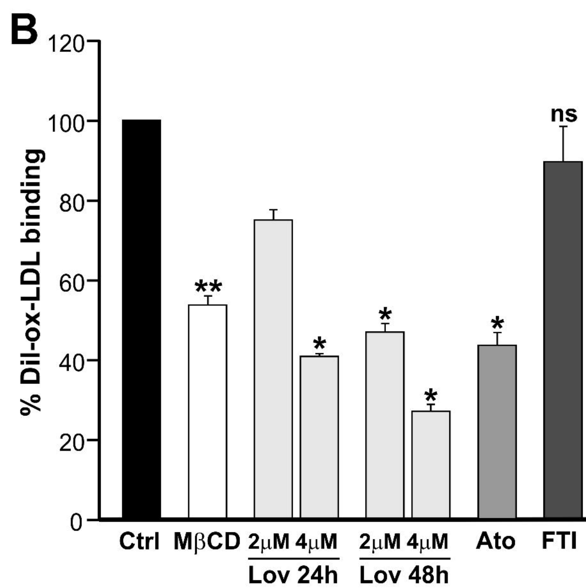
1h 37°C

4h 37°C

a a′ b b′

c c′ d d′

e e′ f f′



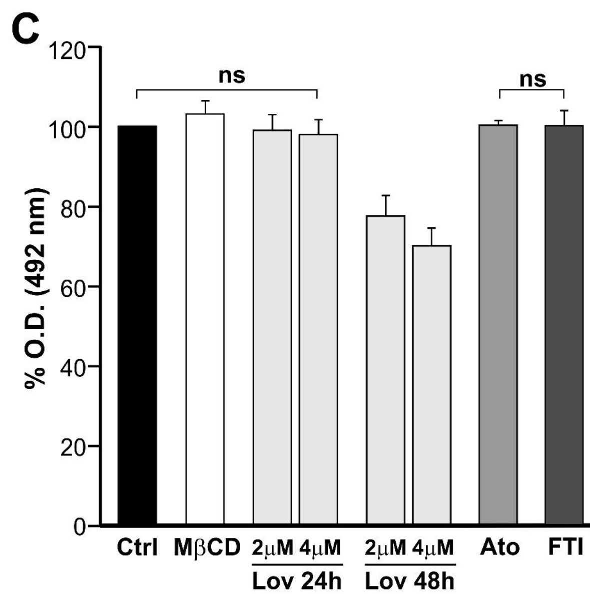


Figure 3

A

INPUT

Top

Bottom

1 2 3 4 5 6 7 8 9

MβCD

kDa

48 –

34 –

LOX-1

−

+

kDa

24 –

Cav-1

−

+

90 –

IRβ

−

+

Lipid rafts

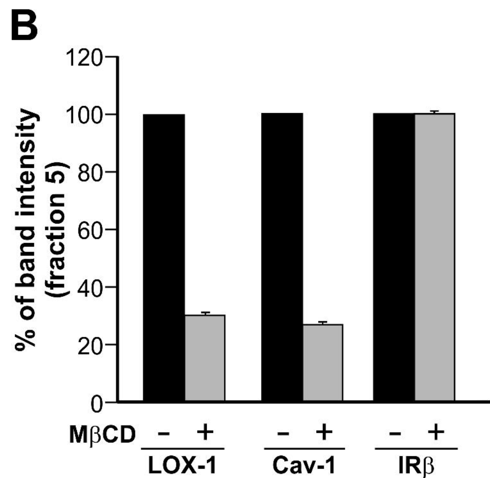


Figure 4

A

INPUT Top Bottom

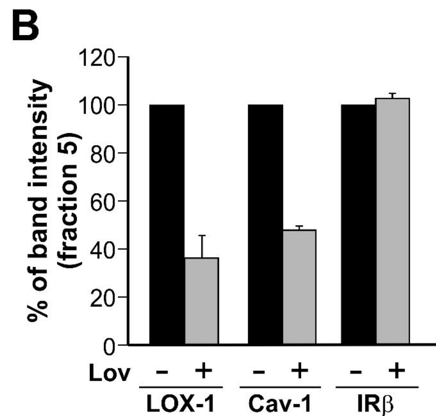
kDa 1 2 3 4 5 6 7 8 9 10 11 12 Lov

LOX-1 48 – 48 – +

Cav-1 24 – 24 +

IRβ 90 – 90 +

Lipid rafts



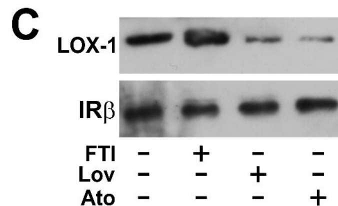
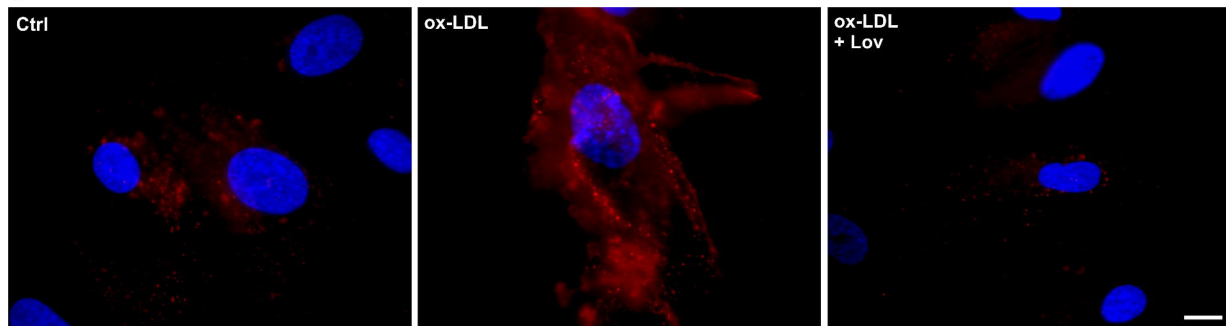
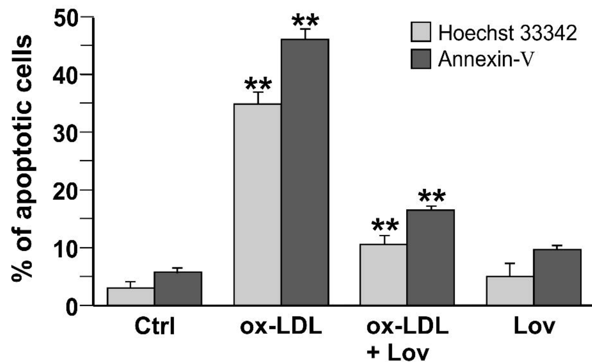


Figure 5





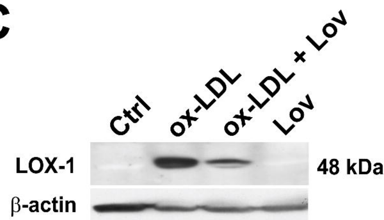
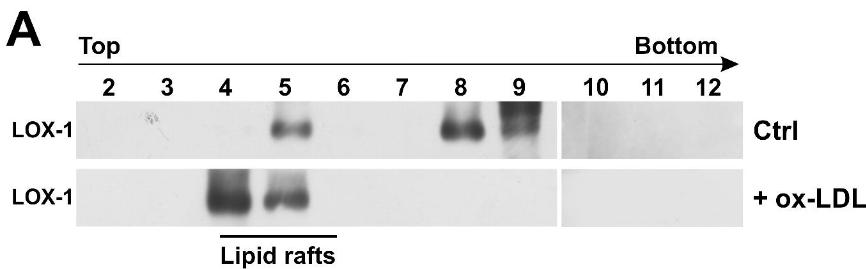


Figure 6



B
Top
Bottom
2 3 4 5 6 7 8 9 10 11 12
LOX-1
Cav-1
Ctrl
LOX-1
Cav-1
+ Lov
LOX-1
Cav-1
+ ox-LDL
LOX-1
Cav-1
+ ox-LDL
+ Lov
Lipid rafts