Specific Oxidized Phospholipids Inhibit Scavenger Receptor BI-mediated Selective Uptake of Cholesteryl Esters[*]

Mohammad Z. Ashraf[‡], **Niladri S. Kar**[‡], **Xi Chen**[§], **Jaewoo Choi**[§], **Robert G. Salomon**[§], **Maria Febbraio**[¶], **and Eugene A. Podrez**[‡1]

From the [‡]Department of Molecular Cardiology and the [¶]Department of Cell Biology, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio 44195 and the [§]Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106

We have recently demonstrated that specific oxidized phospholipids (oxPC$_{CD36}$) accumulate at sites of oxidative stress *in vivo* such as within atherosclerotic lesions, hyperlipidemic plasma, and plasma with low high-density lipoprotein levels. oxPC$_{CD36}$ serve as high affinity ligands for the scavenger receptor CD36, mediate uptake of oxidized low density lipoprotein by macrophages, and promote a pro-thrombotic state via platelet scavenger receptor CD36. We now report that oxPC$_{CD36}$ represent ligands for another member of the scavenger receptor class B, type I (SR-BI). oxPC$_{CD36}$ prevent binding to SR-BI of its physiological ligand, high density lipoprotein, because of the close proximity of the binding sites for these two ligands on SR-BI. Furthermore, oxPC$_{CD36}$ interfere with SR-BI-mediated selective uptake of cholesteryl esters in hepatocytes. Thus, oxidative stress and accumulation of specific oxidized phospholipids in plasma may have an inhibitory effect on reverse cholesterol transport.

Atherosclerosis is a chronic inflammatory disease in which macrophage accumulation of cholesterol and subsequent foam cell formation are critical events. Accumulation of cholesterol in macrophages is a result of failure to adequately adjust cellular cholesterol efflux in conditions of dramatically increased cholesterol acquisition. Efflux of excess of cholesterol from macrophages to HDL[2] and to free apolipoproteins and subsequent delivery to the liver for excretion, a process called reverse cholesterol transport, are critical for the maintenance of cholesterol balance. One of the key steps in reverse cholesterol transport is binding of HDL in the liver to hepatocyte SR-BI followed by selective uptake of cholesteryl esters (CE) from HDL. Multiple studies employing various murine models of atherosclerosis demonstrated that hepatic SR-BI is atheroprotective (1–4). Processes that interfere with SR-BI-mediated selective uptake of CE are of particular interest because they are potentially pro-atherogenic and may represent novel mechanisms contributing to the development of atherosclerosis.

SR-BI belongs to the evolutionarily conserved CD36 family of proteins, sharing 30% sequence identity with CD36 (5). SR-BI is an 82-kDa membrane glycoprotein containing a large extracellular domain and two transmembrane domains with short cytoplasmic amino- and carboxyl-terminal tails (6). Similar to CD36 it is a multifunctional protein; however, its major function is selective uptake of CE from HDL in steroidogenic tissues. SR-BI-mediated selective uptake of HDL CE is a two-step process. The first step involves lipoprotein binding to the extracellular domain of SR-BI, and the second step is the selective transfer of lipid from HDL to the plasma membrane. SR-BI shares with CD36 an affinity for a wide array of ligands, including native and modified lipoproteins, advanced glycation end products, and anionic phospholipids (7, 8).

Atherosclerosis is associated with oxidative stress and the generation of biologically active oxidized lipids. It has been previously demonstrated that these biologically active oxidized phospholipids initiate and modulate many of the cellular events attributed to the pathogenesis of atherosclerosis (9). We have previously described a novel family of oxidized choline glycerophospholipids (oxPC$_{CD36}$) that are formed during the oxidation of LDL by multiple distinct pathways and are present *in vivo* in human and animal atherosclerotic lesions and also accumulate in hyperlipidemic plasma and in plasma of subjects with low HDL levels (10–12). oxPC$_{CD36}$ serve as ligands for the scavenger receptor CD36, mediate highly specific cellular recognition and internalization of targets containing complex mixtures of biologically active oxidized lipids, thus directly induce foam cell formation via CD36 (13, 14). Based on the close protein similarity between CD36 and SR-BI, we hypothesized that oxPC$_{CD36}$ species would also serve as ligands for SR-BI and therefore could interfere with SR-BI-dependent processes.

In this study we demonstrate that oxPC$_{CD36}$ bind specifically to SR-BI and that binding of oxPC$_{CD36}$ prevents HDL association because of the close proximity of the binding sites for these two ligands on SR-BI. We then demonstrate that oxPC$_{CD36}$ is a potent inhibitor of SR-BI-mediated selective uptake of cho-

---

[1] To whom correspondence should be addressed: Cleveland Clinic, Lerner Research Institute, Dept. of Molecular Cardiology, ND-50, 9500 Euclid Ave., Cleveland, OH 44195. Tel.: 216-444-1019; Fax: 216-445-8204; E-mail: podreze@ccf.org.

[2] The abbreviations used are: HDL, high density lipoprotein; SR-BI, scavenger receptor class B, type I; oxPC, oxidized choline glycerophospholipid; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; LDL, low density lipoprotein; COE, cholesteryl oleate ether; PAPC, 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycero-3-phosphocholine; PLPC, -hexadecanoyl-2-octadec-9′,12′-dienoyl-*sn*-glycero-3-phosphocholine; POPC, 1-hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine; BSA, bovine serum albumin; KOOA, 5-keto-8-oxo-6-octenoic acid; CHO, Chinese hamster ovary; KDdiA, keto-6-octendioic acid; CE, cholesteryl ester; MPO, myeloperoxidase.

lesteryl esters in hepatocytes. Thus, our results suggest that specific oxidized phospholipids accumulated *in vivo* in oxidative stress may inhibit reverse cholesterol transport and contribute to the development of hypercholesterolemia and atherosclerosis.

EXPERIMENTAL PROCEDURES

Materials

Tissue culture media and additives were purchased from Invitrogen. Na^{125}I was supplied by ICN Biomedicals, Inc. (Costa Mesa, CA). [^{3}H]Cholesteryl oleate ether (COE) and 1,2-[^{3}H]dihexadecanoyl-*sn*-glycero-3-phosphocholine (DPPC) were from American Radiolabel Chemicals, Inc. (St. Louis, MO). 1-Hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycero-3-phosphocholine (PAPC), 1-hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycero-3-phosphocholine (POPC), 1-hexadecanoyl-2-octadec-9′,12′-dienoyl-*sn*-glycero-3-phosphocholine (PLPC), and DPPC were purchased from Avanti Polar Lipids (Alabaster, AL). The 9-keto-10-dodecendioic acid and 5-keto-6-octendioic acid esters of 2-lyso-PC (KDdiA-PC and KOdiA-PC, respectively) were purchased from Cayman, Inc. (Ann Arbor, MI). Anti-SR-BI blocking antibody was purchased from Novus Biologicals (Littleton, CO). All other reagents were obtained from Sigma unless otherwise specified.

Methods

General Procedures—All buffers were passed over a column of Chelex-100 resin and supplemented with diethylenetriaminepentaacetic acid to remove any potential transition metal ions, which might catalyze phospholipid oxidation during incubations. Protein content was determined by the Markwell-modified Lowry protein assay with bovine serum albumin as standard (15). LDL and HDL were isolated from fresh plasma by sequential ultracentrifugation, and iodination with Na^{125}I was performed as described previously (16). Total synthesis of γ-keto-α,β-unsaturated aldehydic phospholipid, the 5-keto-8-oxo-6-octenoic acid esters of 2-lyso-PC (KOOA-PC), was performed as described earlier (14).

Lipoprotein Modification—LDL was modified utilizing the MPO-H$_2$O$_2$-NO$_2^-$ system by incubating LDL (0.2 mg of protein/ml) at 37 °C in 50 mM sodium phosphate (pH 7.4), 100 μM diethylenetriaminepentaacetic acid, 30 nM MPO, 100 μg/ml glucose, 20 ng/ml glucose oxidase, and 0.5 mM NaNO$_2$ for 8 h (16). Oxidation reactions were terminated by addition of 40 μM butylated hydroxytoluene and 300 nM catalase to the reaction mixture. LDL acetylation and oxidation of LDL by copper ions were performed as described previously (17).

Phospholipid Vesicle Preparation and Modification—Stock solutions (2 mg/ml) of small unilamellar vesicles consisting of PAPC, PLPC, or POPC were prepared in argon-sparged sodium phosphate buffer by extrusion (11 times) through a 0.1-μm polycarbonate filter using an Avanti Mini-Extruder Set (Avanti Polar Lipids) at 37 °C (14). For direct binding experiments, [^{3}H]DPPC (25 μCi/mg of phospholipids) was added to phospholipids (PAPC or PLPC) with equimolar amounts of specific synthetic oxidized phospholipids (oxPC$_{CD36}$). Phospholipid vesicles oxidized by reactive nitrogen species were prepared from PAPC or

PLPC vesicles (0.2 mg lipid/ml) by exposure to the MPO-H$_2$O$_2$-NO$_2^-$ system as described (16).

Cells—Hep G2 cells were from American Type Culture Collection (Manassas, VA). CHO cells lacking LDL receptor activity (ldlA7 cells) and a subcloned cell line expressing scavenger receptor class B type I (ldlA7-SR-BI) (a gift from Dr. M. Krieger, MIT, Boston) were cultured as described elsewhere (18). For generating the SR-BI overexpressing HEK-293T cell line, full-length human SR-BI was PCR-amplified from liver Marathon-Ready cDNA (BD Biosciences) and cloned into the pEF6V5-His vector (Invitrogen). HEK-293T cells were stably transfected with Lipofectamine 2000 (Invitrogen) using blasticidine as the selection marker. Clones overexpressing SR-BI were selected based on Western blot for SR-BI. Experiments were performed on confluent cell monolayer in Dulbecco's modified Eagle's medium/F-12 medium containing 10% fetal bovine serum, butylated hydroxytoluene (20 μM), diethylenetriaminepentaacetic acid (100 μM), and catalase (300 nM).

Selective Uptake of Cholesteryl Ester in Hepatocytes—Phospholipid liposomes containing [^{3}H]cholesteryl oleate ether (COE) were prepared as described elsewhere (19) and incubated with HDL in the presence of excess cholesteryl ester transfer protein and 0.5% BSA at 37 °C for 18 h. HDL was then re-isolated by ultracentrifugation and dialyzed. Specific activity of resulting [^{3}H]COE HDL was 4 cpm/ng HDL protein. Hep G2 cells were cultured for 18 h in the presence of 0.1 μM adrenocorticotropic hormone in Dulbecco's modified Eagle's/F-12 media containing 5% lipoprotein-deficient serum. Selective uptake of cholesteryl esters was determined as described before (19). Briefly, parallel experiments to assess HDL uptake were performed using [^{3}H]COE HDL and ^{125}I-HDL. After 5 h of incubation at 37 °C with indicated lipoproteins, the medium was removed, and the cells washed with warm PBS. Following a 30-min chase with 100 μg/ml unlabeled HDL, cells were lysed and cell-associated radioactivity was quantified. Degradation of ^{125}I-HDL was determined as described before (16), and total uptake of HDL protein was calculated as the sum of the ^{125}I associated with cells plus the noniodide-, nontrichloroacetic acid-precipitable ^{125}I in the medium. Selective uptake was calculated as the difference between total lipid uptake (^{3}H radioactivity) and the amount of lipid incorporation that could be accounted for by whole lipoprotein uptake as determined by the accumulated cellular ^{125}I.

Cloning and Purification of GST-SR-BI Ligand Binding Domain Fusion Protein—The sequence encoding full-length human SR-BI was amplified by PCR from liver Marathon-Ready cDNA and subcloned into the PCR-blunt vector (Invitrogen). A fragment containing a putative human HDL ligand-binding site, an extracellular amino-terminal domain of SR-BI (amino acids 144–205), was amplified by PCR with appropriate primers and cloned between BamHI and XbaI sites of the pGST-Parallel1 vector. The junction with GST was sequenced to confirm insertion of SR-BI-(144–205) in-frame. GST fusion protein expression in transformed *Escherichia coli* BL21(DE3) was induced by isopropyl 1-thio-β-D-galactopyranoside; cells were lysed, and fusion protein was isolated by affinity chromatography using glutathione-Sepharose 4B beads (GE Healthcare). The size, amount, and purity of the fusion protein
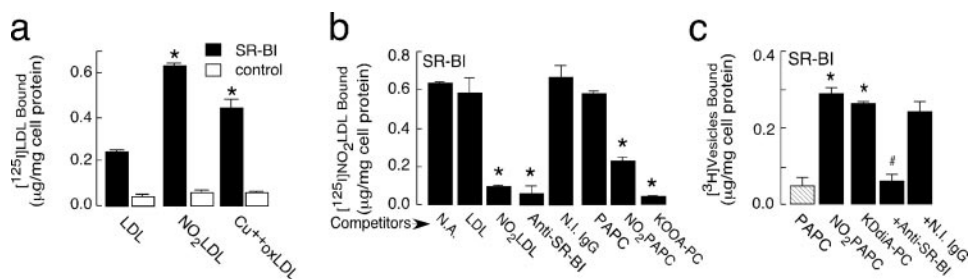
FIGURE 1. **Binding of NO$_2$-LDL and oxPC$_{CD36}$ to SR-BI-transfected cells.** *a,* ^{125}I-LDL or various modified ^{125}I-labeled lipoproteins (5 μg/ml) were incubated with SR-BI-transfected CHO cells (ldlA7-SR-BI) or control vector-transfected CHO cells (ldlA7). Cellular binding of lipoproteins was subsequently determined as described under "Experimental Procedures." (*Cu^{2+}oxLDL*, copper oxidized LDL). *, *p* < 0.001 for comparison *versus* LDL. *b,* ^{125}I-labeled NO$_2$-LDL was incubated with SR-BI-expressing cells either in the absence (*N.A.,* no addition) or presence of the indicated competitors. *Anti-SR-BI,* anti-SR-BI antibody; *N.I. IgG,* nonimmune IgG. *, *p* < 0.001 for comparison *versus* control (no addition). *c,* small unilamellar vesicles were generated containing tracer levels of [^{3}H]DPPC (25 μCi/mg vesicles) and consisting either of the unoxidized parent phospholipid alone (*PAPC*), phospholipid oxidized by MPO-H$_2$O$_2$- NO$_2^-$ system (*NO$_2$-PAPC*), or an equimolar mixture of parent phospholipid and the indicated synthetic oxidized phospholipids (*oxPC$_{CD36}$*). Cells were incubated with phospholipid vesicles, and cell-associated radioactivity was quantified. *, *p* < 0.001 for comparison *versus* PAPC; #, *p* < 0.001 for comparison *versus* KDdiA-PC and *versus* KDdiA-PC + nonimmune (*N.I.*) IgG.

were examined by SDS-PAGE. The molecular weight was found to be close to the predicted value, and purity was typically >95%.

Lipoprotein and Phospholipid Vesicle Binding Experiments—Cells were washed with serum-free medium, and the indicated amounts of ^{125}I-labeled lipoproteins were added in 250 μl of the appropriate media containing 10% fetal bovine serum. Lipoprotein binding to SR-BI expressing CHO cells (ldlA7-SR-BI) and control vector cells (ldlA7) was determined following 3 h of incubation at 37 °C with the indicated concentrations of native or modified forms of ^{125}I-labeled lipoproteins (17). In competition experiments, ^{125}I-labeled lipoproteins (5 μg/ml) were incubated with cells in the presence of the indicated concentrations of unlabeled competitor.

For phospholipid vesicle binding experiments, cells (0.25–0.3 mg of cell protein/well) were incubated with the corresponding radiolabeled phospholipid vesicles (10 μg/ml, 250 μl of Dulbecco's modified Eagle's medium) for 3 h at 37 °C in the presence of 40-fold excess of unlabeled POPC vesicles to block nonspecific binding. Unbound vesicles were then washed from cells with PBS/BSA; cells were solubilized with 0.1 M NaOH, and bound radioactivity was quantified.

GST-SR-BI binding experiments were performed by incubating GST or GST-SR-BI-(144–205) protein beads (1 μg of protein/tube) with the corresponding phospholipid vesicles containing the radioactive tracer (14) at 10 μg lipid/ml in PBS for 3 h at 25 °C with gentle shaking. Unbound vesicles were removed from the beads by repeated washing with PBS, and then bound radioactivity was quantified. HDL binding and competition assays were performed by incubating ^{125}I-HDL (5 μg/ml) in PBS with GST or GST-SR-BI-(144–205) beads (1 μg of protein/tube) for 3 h at 25 °C. In competition experiments, unlabeled competitors were added at 20-fold excess. Where indicated, the beads were preincubated for 15 min with anti-SR-BI blocking antibody. HDL binding parameters for HEK-293T cells overexpressing SR-BI and GST-SR-BI-(144–205) fusion protein were obtained from nonlinear regression analysis in Prism 4 software (GraphPad Software Inc., San Diego).

Statistics—Each experimental point represents the mean ± S.D. for triplicate determinations of a representative experiment performed at least three times. Statistical analyses were made using a paired Student's *t* test. For all hypotheses, the significance level was 0.05. When multiple comparisons were made, a Bonferoni correction to the significance criterion for each test was made.

RESULTS

oxPC$_{CD36}$ Represent Ligands for SR-BI—SR-BI is closely related to scavenger receptor CD36 and shares with CD36 several ligands, including oxLDL (13, 20, 21). We hypothesized that oxLDL could bind to SR-BI via specific oxidized phospholipids (oxPC$_{CD36}$) that were previously shown to be ligands for CD36. LDL oxidized by reactive nitrogen species generated by the MPO-H$_2$O$_2$-NO$_2$-system (NO$_2$-LDL) is a carrier of oxPC$_{CD36}$ and represents a biologically relevant and well characterized model of oxidized LDL that selectively binds to CD36 (13, 14). First, we tested whether NO$_2$-LDL was recognized by SR-BI and whether oxPC$_{CD36}$ present in NO$_2$-LDL serve as ligands for SR-BI. ^{125}I-Labeled NO$_2$-LDL binding to SR-BI-overexpressing CHO cells was significantly higher than control cells (Fig. 1*a*). Binding of ^{125}I-acLDL (a specific ligand for the scavenger receptor class A) was low (data not shown). Native LDL and LDL oxidized by alternative methods (*e.g.* Cu^{2+}) also bound to SR-BI-overexpressing cells in agreement with previous reports (22, 23). The specificity of SR-BI-mediated recognition of ^{125}I-labeled NO$_2$-LDL was verified by competition experiments. Excess amounts of unlabeled NO$_2$-LDL effectively competed with ^{125}I-labeled NO$_2$-LDL for binding to SR-BI-overexpressing cells (Fig. 1*b*). In contrast, a 40-fold molar excess of native LDL had no significant effect (Fig. 1*b*). An anti-SR-BI blocking antibody significantly inhibited binding of ^{125}I-labeled NO$_2$-LDL to SR-BI-overexpressing cells, whereas an isotype-matched nonimmune antibody had no effect (Fig. 1*b*). These findings confirmed that LDL modified by the MPO-H$_2$O$_2$-NO$_2^-$ system was recognized specifically by SR-BI. We then tested whether SR-BI recognized NO$_2$-LDL via its oxidized phospholipid moiety. Small unilamellar vesicles comprised of synthetic homogeneous phosphatidylcholine molecular species were oxidized by the MPO-H$_2$O$_2$-NO$_2^-$ system (NO$_2$-PAPC) and then tested for their ability to compete for ^{125}I-labeled NO$_2$-LDL binding to SR-BI-overexpressing cells. NO$_2$-PAPC significantly inhibited binding of ^{125}I-labeled NO$_2$-LDL (Fig. 1*b*) indicating that free (not bound covalently to protein) oxidized phospholipid, in NO$_2$-LDL, served as at least one of the ligands for SR-BI. A similar pattern of recognition was demonstrated by our group previously for CD36 (11, 14). Because CD36 and SR-BI belong to the same family of proteins and share evolutionarily conserved domains, these results suggest that CD36 and SR-BI recognize NO$_2$-LDL via similar
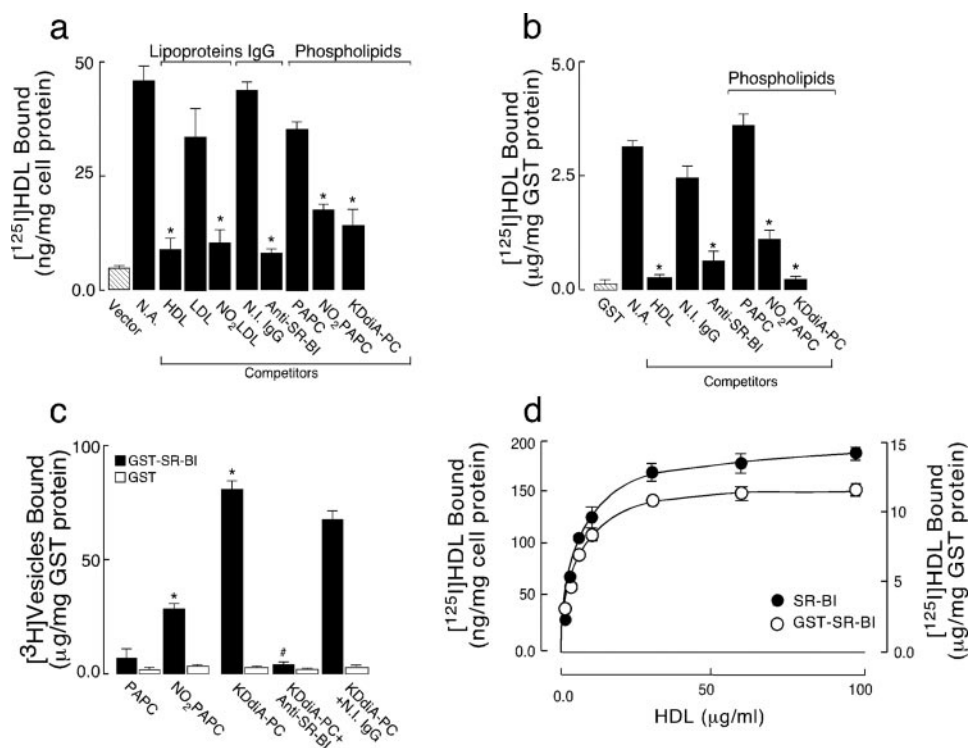
FIGURE 2. **NO$_2$-LDL and oxPC$_{CD36}$ compete for the binding of HDL to SR-BI.** *a,* ^{125}I-HDL (5 μg/ml) was incubated with ldlA7-SR-BI cells or vector control cells, and bound ^{125}I-HDL was quantified. The concentrations of competitors used were 200 μg of protein/ml for lipoproteins, 40 μg of lipid/ml for vesicles, and 20 μg/ml for antibody. *Anti-SR-BI,* anti-SR-BI antibody; *N.I. IgG,* nonimmune IgG. *, $p < 0.001$ for comparison *versus* control (*N.A.,* no addition). *b,* ^{125}I-HDL (5 μg/ml) was incubated with GST-SR-BI-(144–205) protein beads, either in the absence (no addition) or presence of 20-fold excess unlabeled indicated competitors in PBS/BSA. Beads were repeatedly washed with PBS to remove unbound lipoproteins, and bound radioactivity was then quantified. *, $p < 0.001$ for comparison *versus* control (no addition). *c,* GST-SR-BI-(144–205) protein beads were incubated with small unilamellar vesicles consisting of PAPC, NO$_2$-PAPC, or the indicated oxPC$_{CD36}$ with tracer levels of [^{3}H]DPPC (25 μCi/mg vesicles) (10 μg/ml), washed repeatedly with PBS, and assayed for bead-associated radioactivity. *, $p < 0.001$ for comparison *versus* PAPC. #, $p < 0.001$ for comparison *versus* KDdiA-PC. *d,* concentration dependence of ^{125}I-HDL binding to SR-BI-overexpressing HEK-293T cell and GST-SR-BI-(144–205) protein. Indicated concentrations of ^{125}I-HDL were incubated with either cells or GST-SR-BI-(144–205) beads at 25 °C for 3 h and then washed, and bound radioactivity was quantified. Specific ^{125}I-HDL binding was determined by subtracting background binding to control vector-transfected cells or GST beads alone, respectively.

oxPC$_{CD36}$ vesicles further demonstrating specificity. These data demonstrate that SR-BI, similar to CD36, recognizes oxPC$_{CD36}$.

oxPC$_{CD36}$ Interfere with HDL Binding to SR-BI Because of Overlap of Binding Sites—We next tested whether NO$_2$-LDL or oxPC$_{CD36}$ interfered with SR-BI-mediated HDL binding. Binding of HDL to SR-BI-expressing cells was significantly higher than vector-transfected cells as anticipated (Fig. 2*a*). Excess unlabeled HDL and anti-SR-BI blocking antibody, but not isotype-matched nonimmune antibody, inhibited binding to the SR-BI-transfected cells down to the level of binding to vector-transfected cells, demonstrating specificity. Forty-fold molar excess of unlabeled NO$_2$-LDL competed with ^{125}I-HDL for binding to SR-BI (Fig. 2*a*). In contrast, native LDL failed to significantly block the binding of HDL to SR-BI-transfected cells. NO$_2$-PAPC significantly inhibited binding of ^{125}I-HDL, in agreement with the results in Fig. 1, *b* and *c*. At the same time, native unoxidized PAPC was found to be a weak competitor (Fig. 2*a*). Finally, small unilamellar vesicles containing individual synthetic oxPC$_{CD36}$ (data for KDdiA-PC are shown) were potent inhibitors of ^{125}I-HDL binding to

mechanisms. We next examined individual synthetic phospholipids previously identified by our group as specific endogenous ligands for CD36 (oxPC$_{CD36}$) (14), such as KOOA-PC (the 5,8-dioxooct-6-enoic acid) and KODA-PC (9,12-dioxododec-10-enoic acid ester of 2-lyso-PC), for their ability to compete for the binding to SR-BI. As shown in Fig. 1*b*, oxPC$_{CD36}$ (data for KOOA-PC are shown), but not their unoxidized phospholipid precursors (data for PAPC are shown), inhibited binding of ^{125}I-labeled NO$_2$-LDL to SR-BI-expressing cells.

To demonstrate that oxPC$_{CD36}$ directly bind to SR-BI, small unilamellar vesicles containing PLPC or PAPC as a carrier lipid, synthetic oxPC$_{CD36}$ species, and tracer levels of [^{3}H]DPPC were made and binding assays were performed as described previously (14). Vesicles comprised of PAPC (or PLPC, data not shown) alone failed to demonstrate SR-BI-specific binding (Fig. 1*c*). However, the NO$_2$-PAPC and NO$_2$-PLPC vesicles bound to SR-BI transfected cells at significantly greater levels than control cells (Fig. 1*c* and data not shown). Vesicles containing any of the individual synthetic oxPC$_{CD36}$ (data for KDdiA-PC are shown) readily bound to SR-BI-expressing cells (Fig. 1*c*). Addition of a blocking anti-SR-BI antibody, but not isotype-matched control antibody, inhibited binding of the

SR-BI. Collectively, these results show that LDL and phospholipids vesicles upon oxidation acquire the ability to block HDL binding to SR-BI because of oxPC$_{CD36}$.

SR-BI is a multiligand receptor, one potential mechanism for the observed inhibition is that the binding sites for HDL and oxPC$_{CD36}$ on SR-BI overlap. To test this hypothesis an extracellular amino-terminal domain of human SR-BI (amino acids 144–205) containing the putative HDL ligand-binding site was expressed as a GST fusion protein, isolated and purified by affinity chromatography on glutathione-Sepharose 4B beads, and used in binding and competition studies. The selection of this SR-BI-(144–205) peptide was based on separate studies of lipoprotein binding domains in CD36, a close relative of SR-BI that can also bind HDL (24, 25). Binding of ^{125}I-HDL to GST-SR-BI-(144–205) was significantly higher than control GST beads (Fig. 2*b*). Binding was specific because excess unlabeled HDL significantly inhibited binding of ^{125}I-HDL. Importantly, the anti-SR-BI blocking antibody inhibited HDL binding to GST-SR-BI-(144–205) protein to the same extent as in SR-BI-expressing cells (Fig. 2*b*), suggesting that GST-SR-BI-(144–205) contains the true binding site for HDL. Other properties of the ^{125}I-HDL-binding site on GST-SR-BI-(144–205) mirrored

those of the full-length SR-BI expressed on cells. Specifically, NO_2-PAPC and vesicles containing individual synthetic $oxPC_{CD36}$ were potent inhibitors of ^{125}I-HDL binding (results for KDdiA-PC are shown), whereas vesicles consisting of native unoxidized PAPC or PLPC were weak competitors (Fig. 2b, data for PAPC are shown). Direct binding experiments demonstrated that small unilamellar vesicles comprised of PAPC or PLPC alone failed to bind significantly to GST-SR-BI-(144–205) (Fig. 1c and data not shown), whereas NO_2-PAPC and vesicles containing any of the individual synthetic $oxPC_{CD36}$ (data for KDdiA-PC are shown) were found to bind to GST-SR-BI-(144–205) at significantly greater levels than control GST (Fig. 2c). Addition of the anti-SR-BI antibody, but not isotype-matched control antibody, blocked binding of the vesicles containing $oxPC_{CD36}$. Comparison of SR-BI-overexpressing cells and purified GST-SR-BI-(144–205) fusion protein showed similar saturable HDL binding curves (Fig. 2d). The values for half-maximal concentration for HDL binding were similar, 5.6 ± 0.5 μg/ml for cells overexpressing SR-BI and 4.6 ± 0.1 μg/ml for the GST fusion protein. Together these results suggest that the ligand-binding site of SR-BI lies in the region between amino acids 144 and 205 and that the binding sites for HDL and $oxPC_{CD36}$ on SR-BI overlap.

$oxPC_{CD36}$ Bind to Hepatocyte SR-BI and Interfere with SR-BI-mediated Selective Cholesteryl Uptake—Selective uptake of HDL cholesteryl esters in hepatocytes is a major physiological function of SR-BI and is a critical part of the reverse cholesterol transport pathway in the body. Because $oxPC_{CD36}$ accumulate in plasma under conditions of increased oxidative stress and dyslipoproteinemia, and are associated with atherosclerosis development (10, 11, 14), we tested whether $oxPC_{CD36}$ can affect SR-BI-mediated selective uptake of cholesteryl esters in hepatocytes. We first used NO_2-LDL to test whether hepatocytes recognize this mildly modified form of oxidized LDL via SR-BI and whether $oxPC_{CD36}$ present in NO_2-LDL serve as ligands for hepatocyte SR-BI. Hep G2 cells bound significant amounts of ^{125}I-labeled NO_2-LDL (Fig. 3a). SR-BI was the major receptor on Hep G2 cells for ^{125}I-labeled NO_2-LDL because an anti-SR-BI blocking antibody but not isotype-matched nonimmune antibody significantly inhibited binding. Excess amounts of unlabeled HDL but not native LDL effectively competed with ^{125}I-labeled NO_2-LDL for binding to Hep G2 cells (Fig. 3a). In agreement with the data obtained using SR-BI-overexpressing cells, binding was significantly inhibited by $oxPC_{CD36}$ but not by native unoxidized phospholipids. These findings demonstrated that LDL modified by the MPO-H_2O_2-NO_2^- system was recognized specifically by SR-BI on hepatocytes via its oxidized phospholipid moiety.

We next investigated the effect of $oxPC_{CD36}$ on the binding of ^{125}I-HDL to Hep G2 cells. Hep G2 cells bound significant amounts of ^{125}I-HDL, as anticipated. Excess of unlabeled HDL and anti-SR-BI blocking antibody, but not isotype-matched nonimmune antibody, inhibited ^{125}I-HDL binding to Hep G2 cells demonstrating specificity of the binding. Importantly, excess of all competitors containing $oxPC_{CD36}$, including unlabeled NO_2-LDL, NO_2-PAPC and vesicles containing individual synthetic $oxPC_{CD36}$ significantly inhibited binding of ^{125}I-HDL to SR-BI (Fig. 3b), in agreement with the results in Fig. 2. In
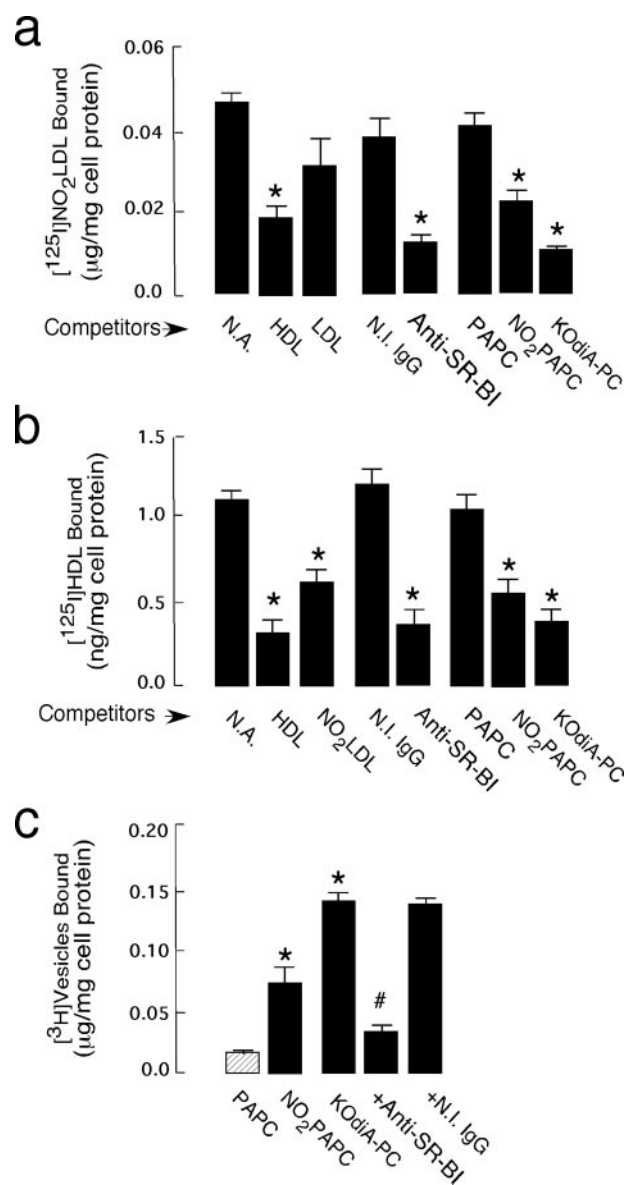

FIGURE 3. **Binding of NO_2-LDL and $oxPC_{CD36}$ to Hep G2 cells.** *a*, ^{125}I-NO_2-LDL (5 μg/ml) was incubated with Hep G2 cells either in the absence (*N.A.*, no addition) or presence of the indicated competitors at 4 °C for 3 h in the appropriate media. Cellular binding of lipoproteins was subsequently determined as described under "Experimental Procedures." *Anti-SR-BI*, anti-SR-BI antibody; *N.I. IgG*, nonimmune IgG. *, $p < 0.001$ for comparison *versus* control (no addition). *b*, ^{125}I-HDL (5 μg/ml) was incubated with Hep G2 cells either in the absence (no addition) or presence of the indicated competitors. *, $p < 0.001$ for comparison *versus* control (no addition). *c*, small unilamellar vesicles were generated as described in Fig. 1, and Hep G2 cells were incubated with phospholipid vesicles (10 μg/mg of cell protein), following 3 h of incubation at 4 °C and washed, and cell-associated radioactivity was quantified. *, $p < 0.001$ for comparison *versus* PAPC. #, $p < 0.001$ for comparison *versus* KDdiA-PC and *versus* KDdiA-PC + nonimmune IgG.

contrast, native PAPC or PLPC (data for PAPC are shown) had no significant effect on the binding (Fig. 3b).

To demonstrate that $oxPC_{CD36}$ directly bind to SR-BI on hepatocytes, small unilamellar vesicles containing PLPC or PAPC as a carrier lipid, synthetic $oxPC_{CD36}$ species, and tracer levels of $[^3H]$DPPC were made, and binding assays were performed as in Fig. 1c. Vesicles comprised of PAPC (or PLPC, data not shown) alone failed to demonstrate significant binding to Hep G2 cells (Fig. 3c). NO_2-PAPC vesicles bound to SR-BI-
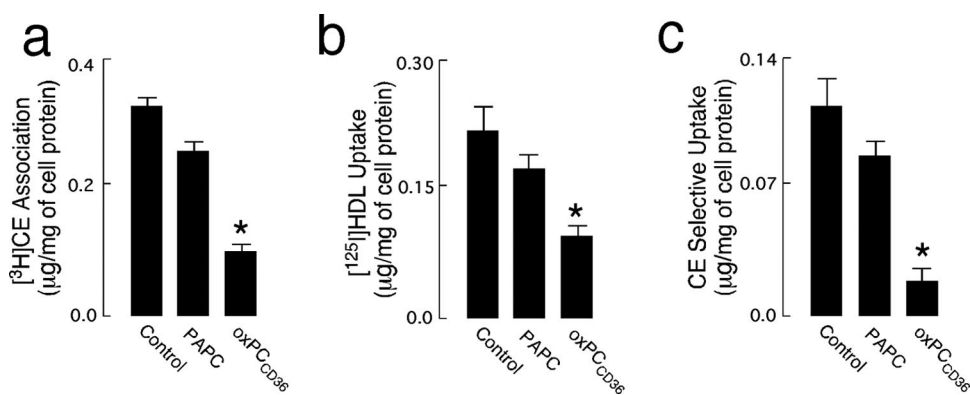
a

b

c

FIGURE 4. **oxPC$_{CD36}$ interfere with SR-BI-mediated selective cholesteryl ester uptake.** Hep G2 cells were incubated with either [^{3}H]COE HDL (*a*) or ^{125}I-HDL (*b*) at a concentration 10 μg/ml for 5 h 37 °C, with indicated additions, and then cells were washed with warm PBS and chased for another 30 min in the presence of 100 μg/ml unlabeled HDL. *a,* cells were lysed; cell-associated radioactivity was quantified and [^{3}H]COE HDL association determined. *b,* ^{125}I-HDL uptake was determined as described under "Experimental Procedures." *c,* selective uptake was calculated from data in *a* and *b* as described under "Experimental Procedures." *, $p < 0.001$ for comparison *versus* control.

transfected cells at significantly greater levels than PAPC vesicles (Fig. 3*c*). Vesicles containing individual synthetic oxPC$_{CD36}$ (data for KOdiA-PC are shown) readily bound to Hep G2 cells (Fig. 3*c*). Blocking anti-SR-BI antibody, but not isotype-matched control antibody, inhibited binding of the oxPC$_{CD36}$ vesicles to hepatocytes, demonstrating that SR-BI is the major receptor for oxPC$_{CD36}$ on hepatocytes.

Finally, we tested the effect of ligands containing oxPC$_{CD36}$ on SR-BI-mediated [^{3}H]cholesteryl ester association, ^{125}I-HDL uptake, and selective cholesteryl ester uptake using small unilamellar phospholipid vesicles. Native PAPC had a mild effect on cholesteryl ester association, ^{125}I-HDL uptake, and selective uptake of HDL cholesteryl esters in Hep G2 cells (Fig. 4, *a–c*). At the same time, vesicles containing oxPC$_{CD36}$ significantly inhibited all tested SR-BI-mediated processes (Fig. 4, *a–c*). Importantly, oxPC$_{CD36}$ not only significantly reduced holoparticle uptake of HDL as evidenced by decreased uptake of ^{125}I-HDL (Fig. 4*b*), it also almost completely inhibited hepatocyte selective uptake of cholesteryl esters (Fig. 4*c*). This result strongly suggests that oxPC$_{CD36}$ also interferes with the second step of SR-BI-mediated selective uptake, i.e. lipid transfer from HDL to the cell membrane. An earlier report suggested that reverse cholesterol transport may be proportional to cell surface expression of SR-BI (26). No change in surface expression of SR-BI was observed in Hep G2 cells pretreated with either HDL or with HDL plus oxPC$_{CD36}$ for the periods of time used in this experiment (data not shown). Taken together, these results support our hypothesis that ligands for SR-BI that contain oxPC$_{CD36}$ compete for the binding of HDL to SR-BI and disrupt SR-BI-mediated cholesteryl ester selective uptake from HDL.

DISCUSSION

Pro-atherosclerotic lipid abnormalities are associated with enhanced oxidative stress and the generation of biologically active oxidized lipids. Oxidized phospholipids accumulate at sites of oxidative stress, including plasma and in the vessel wall (10–12). They are involved in all stages of atherosclerosis and can mediate many atherogenic processes from the earliest entry of monocytes into the vessel wall to thrombus formation (9–11,

14, 27). In this study we show that a recently described family of specific oxidized phospholipids represents a ligand for the scavenger receptor BI and interferes with the major physiological function of SR-BI, namely selective uptake of cholesteryl esters from HDL.

We demonstrated the following: (i) oxPC$_{CD36}$ are recognized by SR-BI when presented in various forms. (ii) oxPC$_{CD36}$ interfered with HDL binding to SR-BI because of an overlap of binding sites for oxPC$_{CD36}$ and HDL on SR-BI. (iii) oxPC$_{CD36}$ prevented SR-BI-mediated selective uptake of cholesteryl esters from HDL in hepatocytes. These observations suggest a novel pro-atherogenic activity of specific oxidized phospholipids: an inhibition of reverse cholesterol transport.

SR-BI belongs to the CD36 family of proteins that is well known for multiligand specificity. SR-BI and CD36 share significant sequence homology and ligands, including HDL, oxidized LDL, and anionic phospholipids (28–30). Oxidized LDL had been shown to interact with SR-BI (20, 21, 23), although the molecular mechanism of this interaction has not been elucidated. We previously reported that a structurally conserved family of oxidized choline glycerophospholipids with an oxidatively truncated *sn*-2 acyl group incorporating a terminal γ-hydroxy (or oxo)-α,β-unsaturated carbonyl serves as high-affinity ligands for macrophage CD36, mediating recognition of various oxidized forms of LDL (11, 14). oxPC$_{CD36}$ are present *in vivo* in human and animal atherosclerotic lesions and accumulate in significant amounts in plasma in dyslipoproteinemia or in normolipidemia associated with low HDL levels (10, 11). oxPC$_{CD36}$ mediate macrophage foam cell formation and promote platelet hyper-reactivity via scavenger receptor CD36 (10, 11). Based on the close similarity between CD36 and SR-BI, we hypothesized that oxPC$_{CD36}$ could serve as ligands for SR-BI as well. Using direct binding and competition assays, we demonstrated that SR-BI binds oxidized LDL in the same fashion as CD36, via recognition of oxPC$_{CD36}$ formed during oxidative modification of LDL.

SR-BI is intimately involved in HDL metabolism *in vivo* (30, 31). Based on ^{125}I-labeled NO$_2$-LDL and oxPC$_{CD36}$ binding studies, we hypothesized that oxPC$_{CD36}$ could interfere with the ability of SR-BI to mediate cholesterol transport to and from HDL. Our competition experiments using ^{125}I-HDL as a ligand and various competitors containing oxPC$_{CD36}$ demonstrated that oxPC$_{CD36}$ significantly reduced HDL binding to SR-BI, and thus affect HDL-mediated processes. To elucidate whether the observed inhibition is because of spatial hindrance or more complex cellular events, we performed competition experiments using the extracellular amino-terminal domain of human SR-BI (amino acids 144–205) containing the putative HDL ligand-binding site. Importantly, the K_d value of the binding of HDL to this protein was very similar to that determined

for the full-length SR-BI expressed on cells (Fig. 2, *a*, *b*, and *d*). Moreover, an anti-SR-BI blocking antibody inhibited HDL binding to GST-SR-BI-(144–205) peptide, strongly suggesting that it contains the true binding site for HDL. NO$_2$-PAPC and vesicles containing synthetic oxPC$_{CD36}$ bound specifically to GST-SR-BI-(144–205) with a K_d value similar to that for HDL (Fig. 2*c* and data not shown). Others have reported that a M158R mutant does not bind either HDL or LDL (32) in agreement with our data. Taken together, these results suggest that the ligand-binding sites for both HDL and oxPC$_{CD36}$ on SR-BI lie in the region of amino acids 144–205 and that the binding sites for HDL and oxPC$_{CD36}$ on SR-BI overlap, thus providing the molecular basis for the observed competition between the two ligands on cells.

Our results suggest that under conditions of increased oxidative stress, accumulation of specific oxidized phospholipids may promote atherosclerosis not only by inducing uptake of modified lipoproteins by macrophages via CD36, but can also interfere with reverse cholesterol transport by preventing SR-BI-mediated selective cholesteryl ester uptake in hepatocytes.

REFERENCES

1. Arai, T., Wang, N., Bezouevski, M., Welch, C., and Tall, A. R. (1999) *J. Biol. Chem.* **274,** 2366–2371
2. Kozarsky, K. F., Donahee, M. H., Glick, J. M., Krieger, M., and Rader, D. J. (2000) *Arterioscler. Thromb. Vasc. Biol.* **20,** 721–727
3. Ueda, Y., Gong, E., Royer, L., Cooper, P. N., Francone, O. L., and Rubin, E. M. (2000) *J. Biol. Chem.* **275,** 20368–20373
4. Huszar, D., Varban, M. L., Rinninger, F., Feeley, R., Arai, T., Fairchild-Huntress, V., Donovan, M. J., and Tall, A. R. (2000) *Arterioscler. Thromb. Vasc. Biol.* **20,** 1068–1073
5. Acton, S. L., Scherer, P. E., Lodish, H. F., and Krieger, M. (1994) *J. Biol. Chem.* **269,** 21003–21009
6. Krieger, M. (1999) *Annu. Rev. Biochem.* **68,** 523–558
7. Acton, S., Rigotti, A., Landschulz, K. T., Xu, S., Hobbs, H. H., and Krieger, M. (1996) *Science* **271,** 518–520
8. Ohgami, N., Nagai, R., Miyazaki, A., Ikemoto, M., Arai, H., Horiuchi, S., and Nakayama, H. (2001) *J. Biol. Chem.* **276,** 13348–13355
9. Berliner, J. A., and Watson, A. D. (2005) *N. Engl. J. Med.* **353,** 9–11
10. Podrez, E. A., Byzova, T. V., Febbraio, M., Salomon, R. G., Ma, Y., Valiyaveettil, M., Poliakov, E., Sun, M., Finton, P. J., Curtis, B. R., Chen, J., Zhang, R., Silverstein, R. L., and Hazen, S. L. (2007) *Nat. Med.* **13,** 1086–1095
11. Podrez, E. A., Poliakov, E., Shen, Z., Zhang, R., Deng, Y., Sun, M., Finton, P. J., Shan, L., Febbraio, M., Hajjar, D. P., Silverstein, R. L., Hoff, H. F., Salomon, R. G., and Hazen, S. L. (2002) *J. Biol. Chem.* **277,** 38517–38523
12. Hoff, H. F., O'Neil, J., Wu, Z., Hoppe, G., and Salomon, R. L. (2003) *Arterioscler. Thromb. Vasc. Biol.* **23,** 275–282
13. Podrez, E. A., Febbraio, M., Sheibani, N., Schmitt, D., Silverstein, R. L., Hajjar, D. P., Cohen, P. A., Frazier, W. A., Hoff, H. F., and Hazen, S. L. (2000) *J. Clin. Investig.* **105,** 1095–1108
14. Podrez, E. A., Poliakov, E., Shen, Z., Zhang, R., Deng, Y., Sun, M., Finton, P. J., Shan, L., Gugiu, B., Fox, P. L., Hoff, H. F., Salomon, R. G., and Hazen, S. L. (2002) *J. Biol. Chem.* **277,** 38503–38516
15. Markwell, M. A., Haas, S. M., Bieber, L. L., and Tolbert, N. E. (1978) *Anal. Biochem.* **87,** 206–210
16. Podrez, E. A., Schmitt, D., Hoff, H. F., and Hazen, S. L. (1999) *J. Clin. Investig.* **103,** 1547–1560
17. Goldstein, J. L., Ho, Y. K., Basu, S. K., and Brown, M. S. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 333–337
18. Ashkenas, J., Penman, M., Vasile, E., Acton, S., Freeman, M., and Krieger, M. (1993) *J. Lipid Res.* **34,** 983–1000
19. Greene, D. J., Skeggs, J. W., and Morton, R. E. (2001) *J. Biol. Chem.* **276,** 4804–4811
20. Gillotte-Taylor, K., Boullier, A., Witztum, J. L., Steinberg, D., and Quehenberger, O. (2001) *J. Lipid Res.* **42,** 1474–1482
21. Han, J., Nicholson, A. C., Zhou, X., Feng, J., Gotto, A. M., Jr., and Hajjar, D. P. (2001) *J. Biol. Chem.* **276,** 16567–16572
22. Rigotti, A., Acton, S. L., and Krieger, M. (1995) *J. Biol. Chem.* **270,** 16221–16224
23. Nishikawa, K., Arai, H., and Inoue, K. (1990) *J. Biol. Chem.* **265,** 5226–5231
24. Calvo, D., Gomez-Coronado, D., Suarez, Y., Lasuncion, M. A., and Vega, M. A. (1998) *J. Lipid Res.* **39,** 777–788
25. Gu, X., Trigatti, B., Xu, S., Acton, S., Babitt, J., and Krieger, M. (1998) *J. Biol. Chem.* **273,** 26338–26348
26. Shetty, S., Eckhardt, E. R., Post, S. R., and van der Westhuyzen, D. R. (2006) *Arterioscler. Thromb. Vasc. Biol.* **26,** 2125–2131
27. Chang, M. K., Binder, C. J., Miller, Y. I., Subbanagounder, G., Silverman, G. J., Berliner, J. A., and Witztum, J. L. (2004) *J. Exp. Med.* **200,** 1359–1370
28. Jessup, W., Gelissen, I. C., Gaus, K., and Kritharides, L. (2006) *Curr. Opin. Lipidol.* **17,** 247–257
29. Connelly, M. A., and Williams, D. L. (2004) *Curr. Opin. Lipidol.* **15,** 287–295
30. Rigotti, A., Miettinen, H. E., and Krieger, M. (2003) *Endocr. Rev.* **24,** 357–387
31. Yancey, P. G., Bortnick, A. E., Kellner-Weibel, G., de la Llera-Moya, M., Phillips, M. C., and Rothblat, G. H. (2003) *Arterioscler. Thromb. Vasc. Biol.* **23,** 712–719
32. Gu, X., Kozarsky, K., and Krieger, M. (2000) *J. Biol. Chem.* **275,** 29993–30001