

# Regulation of SR-BI-mediated selective lipid uptake in Chinese hamster ovary-derived cells by protein kinase signaling pathways<sup>S</sup>

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**Abstract** Scavenger receptor, class B, type I (SR-BI) mediates binding and internalization of a variety of lipoprotein and nonlipoprotein ligands, including HDL. Studies in genetically engineered mice revealed that SR-BI plays an important role in HDL reverse cholesterol transport and protection against atherosclerosis. Understanding how SR-BI's function is regulated may reveal new approaches to therapeutic intervention in atherosclerosis and heart disease. We utilized a model cell system to explore pathways involved in SR-BI-mediated lipid uptake from and signaling in response to distinct lipoprotein ligands: the physiological ligand, HDL, and a model ligand, acetyl LDL (AcLDL). In Chinese hamster ovary-derived cells, murine SR-BI (mSR-BI) mediates lipid uptake via distinct pathways that are dependent on the lipoprotein ligand. Furthermore, HDL and AcLDL activate distinct signaling pathways. Finally, mSR-BI-mediated selective lipid uptake versus endocytic uptake are differentially regulated by protein kinase signaling pathways. The protein kinase C (PKC) activator PMA and the phosphatidylinositol 3-kinase inhibitor wortmannin increase the degree of mSR-BI-mediated selective lipid uptake, whereas a PKC inhibitor has the opposite effect. **Key words:** These data demonstrate that SR-BI's selective lipid uptake activity can be acutely regulated by intracellular signaling cascades, some of which can originate from HDL binding to murine SR-BI itself.—Zhang, Y., A. M. Ahmed, N. McFarlane, C. Capone, D. R. Boreham, R. Truant, S. A. Igdoura, and B. L. Trigatti. Regulation of SR-BI-mediated selective lipid uptake in Chinese hamster ovary-derived cells by protein kinase signaling pathways. *J. Lipid Res.* 2007. 48: 405–416.

**Supplementary key words** acetyl LDL • endocytosis • HDL • lipoprotein • phosphatidylinositol 3-kinase • protein kinase C • scavenger receptor, class B, type I

HDL plays an important role in protection against atherosclerosis, in part by mediating reverse cholesterol transport from macrophage foam cells in atherosclerotic plaque to the liver (1). Elimination of scavenger receptor,

class B, type I (SR-BI) expression in knock-out mice reduces hepatic HDL cholesterol clearance and biliary cholesterol secretion, and increases plasma levels of cholesterol associated with abnormally large, apolipoprotein E (apoE)-enriched HDL-like particles (as reviewed in Ref. 2). Thus, in mice, SR-BI plays an important role, both in hepatic HDL-cholesterol clearance, and in driving reverse cholesterol transport. Inactivation of SR-BI expression renders mice more susceptible to atherosclerosis induced by high-fat, high-cholesterol diets or disruption of either apoE or LDL receptor expression (as reviewed in Ref. 2). Mice deficient in both SR-BI and apoE develop severe occlusive coronary artery atherosclerosis and myocardial infarction and exhibit cardiac functional and conductance abnormalities prior to early death by 8 weeks of age (3). Hepatic SR-BI overexpression increases clearance of plasma lipoprotein cholesterol and biliary cholesterol secretion and decreases atherosclerosis (although decreased HDL cholesterol levels at high levels of overexpression may diminish atheroprotection) (as reviewed in Ref. 2). Elimination of SR-BI in bone marrow-derived cells, including macrophages, also results in increased atherosclerosis but without altering plasma lipoprotein cholesterol levels (4–6). Thus, both hepatic and macrophage SR-BI protect against atherosclerosis, and reverse cholesterol transport may be only one of multiple pathways involved.

SR-BI is a multiligand receptor that can bind to a variety of diverse ligands, including native lipoproteins (HDL,

Abbreviations: AcLDL, acetyl LDL; apo, apolipoprotein; CHO, Chinese hamster ovary; CTX-B, cholera toxin subunit B; DiI, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; ERK, extracellular signal-regulated kinase; KFM, potassium-free medium; MAPK, mitogen-activated protein kinase; MARCKS, myristoylated alanine-rich C-kinase substrate; mSR-BI, murine scavenger receptor, class A, type I; mSR-BI, murine scavenger receptor, class B, type I; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; SR-AI, scavenger receptor, class A, type I; SR-BI, scavenger receptor, class B, type I.

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LDL, VLDL, and chylomicrons) and modified lipoproteins (acetylated LDL, oxidized LDL, oxidized HDL, and acute-phase HDL containing serum amyloid  $\alpha$  instead of apo A-I) (as reviewed in Ref. 2). Mutational analyses have suggested that different amino acid residues of SR-BI are involved in its binding to distinct ligands (7–9). SR-BI mediates the bidirectional exchange of lipids between cells and bound lipoproteins, with the direction of net transfer (cellular uptake versus efflux) determined by the lipid concentration gradient (as reviewed in Ref. 10). Lipoprotein binding and lipid transfer appear to be separable activities of SR-BI, because small molecules (e.g., BLT-1 and related compounds) can block SR-BI-mediated lipid transfer without blocking lipoprotein binding (11).

SR-BI is heavily glycosylated, fatty acylated, and localized to lipid rafts and/or caveolae in a variety of cell types (as reviewed in Ref. 2). It undergoes internalization and recycling back to the cell surface. In polarized cells, cholesterol depletion induces SR-BI redistribution from the basal to apical membrane surface in a protein kinase A-dependent manner (12). In differentiating 3T3-L1 adipocytes and HepG2 human hepatoma cells, recruitment of SR-BI to the cell surface from internal sites is induced by insulin and/or serum and dependent on the phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B) signaling pathway (13, 14). Murine scavenger receptor, class B, type I (mSR-BI)-mediated HDL lipid uptake does not, however, appear to require endocytosis, because purified, reconstituted mSR-BI is active (15) and mSR-BI-mediated HDL lipid uptake in cells is not affected by inhibition of endocytosis (16, 17). Similarly, SR-BI-mediated sterol uptake by hepatocytes and trafficking to the bile canalicular (apical) space does not appear to be energy dependent (17, 18). Furthermore, the low selective uptake activities of SR-BII, a splice variant of SR-BI with a different carboxy-terminal cytoplasmic tail, and of a mutant form of SR-BI with an endocytic sequence inserted into its carboxy-terminal tail appear to be due to their increased endocytosis (19–21). On the other hand, mSR-BI does mediate the endocytosis and intracellular accumulation of a variety of other ligands, including serum amyloid  $\alpha$ , lipopolysaccharide, and apoptotic cells (22–26). SR-BI-mediated free cholesterol efflux from cells also appears to be dependent on the transient internalization and resecretion of HDL (27).

HDL binding to SR-BI leads to activation of diverse signaling pathways. These include ras-dependent activation of mitogen-activated protein kinases (MAPKs) (28, 29), activation of the PI3K/protein kinase B (Akt) pathway (30–33), and activation of protein kinase C (PKC) (28). HDL signaling through SR-BI mediates the activation of endothelial nitric oxide synthase in endothelial cells (30–32, 34, 35), endothelial cell migration (36), and the suppression of apoptosis in diverse cells (33, 37). The signaling events immediately following mSR-BI binding to its ligands, including HDL, remain unclear but may include transfer of signaling lipids (ceramides, sphingosine 1 phosphate, estradiol) and/or cholesterol either into or out of cells (30, 34, 38, 39). Alternatively, signaling may require the C-terminal cytoplasmic region of SR-BI and may in-

volve the binding of adaptor proteins (31, 32), possibly including PDZK1 (39, 40).

Despite SR-BI's importance for HDL lipid uptake, reverse cholesterol transport, and protection against atherosclerosis in mice, little is known about how its activity is regulated at the posttranslational level. It has recently been demonstrated that SR-BI-dependent binding of phosphatidyl serine-containing liposomes and apoptotic cells leads to the activation of MAPK signaling cascades (p38 MAPK, extracellular signal-regulated kinase (ERK)1/2, and JNK), which feed back to regulate SR-BI-dependent engulfment (26). In contrast, little is known about the effects of signaling pathways stimulated by SR-BI's lipoprotein ligands on SR-BI-mediated lipid uptake.

Here we demonstrate in a model cell system that SR-BI-mediated lipid uptake from distinct lipoprotein ligands, acetyl LDL (AcLDL) and HDL, occurs via distinct endocytic and nonendocytic pathways. We also demonstrate that the physiological ligand, HDL, but not the model ligand, AcLDL, stimulates PKC activity in an SR-BI-dependent manner, whereas both ligands stimulate PI3K and MAPK pathways. HDL also stimulates the retention of SR-BI on the cell surface in a manner that is not dependent on PKC, PI3K, or ERK signaling. Finally, we demonstrate that PKC activation and PI3K inactivation lead to an increased ratio of uptake of lipids to uptake of protein components of lipoproteins, suggesting increased selective lipid uptake activity of SR-BI. These data support the idea that endocytosis may be a point at which SR-BI's activity is regulated and that PKC and PI3K may play important roles in that regulation.

## EXPERIMENTAL PROCEDURES

### Materials

Antibodies were obtained from the following suppliers. Rabbit anti-SR-BI antibody (NB400-101) and anti-phospho-myristoylated alanine-rich C-kinase substrate (MARCKS) (NB500-140) were from Novus Biologicals, Inc. (distributed by Cedarlane Laboratories Ltd, Hornby, Ontario, Canada). Phospho- and total Akt, ERK1/2, and p38 MAPK (generously provided by Karen Mossman) were from Cell Signaling Technologies, Inc. (Danvers, MA). Mouse-anti- $\beta$ -actin was from MP Biomedicals (Aurora, OH). Horseradish peroxidase-conjugated goat anti-rabbit antibody and streptavidin-agarose were from Amersham Biosciences (Piscataway, NJ). Horseradish peroxidase-conjugated donkey anti-mouse antibody was from Jackson ImmunoResearch (distributed by BioCan Scientific, Inc., Etobicoke, Ontario). The anti-SR-BI blocking antiserum was generously provided by Karen Kozarsky. Western Lighting Enhanced Chemiluminescence assay reagent was from PerkinElmer Life and Analytical Sciences (Boston, MA). Alexa 488 cholera toxin subunit B (CTX-B), alexa 488 protein-labeling reagent, alexa 594-transferrin, and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate [DiI (C18)] were from Invitrogen/Molecular Probes (Eugene, OR). The Pierce Biotechnology, Inc. bicinchoninic acid protein assay reagent and EZ-Link sulfo-NHS-biotin were from MJS Biolinx (Brockville, Ontario, Canada). BLT-1 (ID 5234221) was from ChemBridge Corp. (San Diego, CA). RO31-8220 (3-[1-[3-(amidinothio)propyl]-1H-indol-3-yl]3-(1-methyl-1H-indol-3-yl)-

maleimide) and wortmannin were from EMD Biosciences (La Jolla, CA), and PMA, cytochalasin D and colchicine were from Sigma Chemical Co. (St. Louis, MO).

### Preparation of DiI- or DiI/alexa 488-labeled lipoproteins

HDL and LDL were prepared from human plasma by KBr density gradient ultracentrifugation and labeled with DiI as previously described (41–43). DiI-AcLDL and unlabeled AcLDL were prepared by acetylation of either DiI-LDL or unlabeled LDL (44). DiI/alexa 488 double-labeled HDL was prepared by alexa 488 labeling of DiI-HDL. DiI/alexa 488 double-labeled AcLDL was prepared from DiI-LDL by alexa 488 labeling, followed by acetylation (44). Labeled lipoproteins were dialyzed against 0.9% NaCl and 1 mM EDTA, sterilized by 0.2  $\mu$ m filtration, and stored under N<sub>2</sub> gas at 4°C. Newborn calf lipoprotein-deficient serum was prepared by KBr density gradient centrifugation of newborn calf serum (45). Maleyl-BSA was prepared as described by Goldstein et al. (46).

### Cell lines and culture conditions

The following cell lines were kindly provided by Monty Krieger (Massachusetts Institute of Technology): The IdIA7 mutant of Chinese hamster ovary (CHO) cells, lacking functional LDL receptor (47); CHO-K1; IdIA7 cells overexpressing mouse SR-BI (IdIA[mSR-BI] cells) (43); and CHO cells overexpressing murine scavenger receptor class A, type I (CHO[mSR-AI] cells) (48). Cells were cultured in Ham's F12 medium containing 5% FBS (Hyclone), 2 mM L-glutamine, 50 U/ml penicillin, and 50  $\mu$ g/ml streptomycin (medium A). For experiments, cells were seeded in medium B (same as medium A except that 5% FBS was replaced with 3% newborn calf lipoprotein-deficient serum). CHO[mSR-AI] and IdIA[mSR-BI] cells were enriched by fluorescence-activated cell sorting after incubation with DiI-AcLDL or DiI-HDL, respectively (see below).

### Cell surface biotinylation, SDS-PAGE and immunoblotting

Cells, cultured for 2 days in medium B, were released from dishes by mild trypsinization, washed once in Ham's F12 medium, and suspended ( $2 \times 10^6$  cells/ml) in Ham's F12 medium without additions. Cells were incubated for 20 min at 37°C with rocking to recover. The cells were then treated as described for the indicated times in the absence or presence of 25  $\mu$ M monensin. The cells were chilled on ice, and 1 ml of ice-cold PBS was added. Cells were washed twice in 2 ml and suspended in 0.5 ml of ice-cold PBS (pH 8.0) containing 2 mM sulfo-NHS-biotin and incubated for 60 min at 4°C with rocking. Biotinylation was quenched by addition of ice-cold PBS containing 100 mM glycine, and cells were pelleted by centrifugation for 5 min at 4,000 rpm in a Spectramax microcentrifuge. The cell pellets were washed twice with PBS with 100 mM glycine, and cells were lysed in ice-cold 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 0.5% Triton X-100, 1 mM PMSF, 10  $\mu$ M leupeptin, and 10  $\mu$ g/ml aprotinin. Lysates were cleared by centrifugation at 14,000 rpm for 5 min, and biotinylated proteins in the cleared lysate were captured by centrifugation (as above) after incubation for 1 h at 4°C with streptavidin-agarose. The precipitates were washed sequentially in PBS for 30 s, PBS containing 0.5 M NaCl for 10 min with rocking, and PBS for 30 s with centrifugation (as above) after each wash. Biotinylated proteins in the pellets were released by boiling for 1 min in 10  $\mu$ l of a solution containing 10 mM EDTA and 95% formamide, followed by the addition of 40  $\mu$ l of SDS-PAGE sample buffer and further boiling for 3 min. Proteins were analyzed by SDS-PAGE and immunoblotting as described previously (49).

### Treatment of cells and analysis of lipoprotein lipid and protein uptake

Cells ( $5.0 \times 10^4$ ) were seeded in 35 mm dishes and cultured for 2 days in medium B. Cells were washed twice with Ham's F12 without additives. Potassium depletion was performed by incubation of cells at 37°C for 5 min in 1 ml of 0.5 $\times$  strength Ham's F12, followed by a 1 h incubation at 37°C in either potassium-free medium (KFM) containing 140 mM NaCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5.5 mM glucose, 0.1% BSA, and 20 mM HEPES (pH 7.4) or control medium (KFM containing 10 mM KCl) (50). For exposure to hypertonic sucrose, cytochalasin D, or colchicine, cells were incubated for 1 h at 37°C with 1 ml of Ham's F12 containing 0.5% BSA without additions (control medium) or with 0.4 M sucrose (hypertonic medium) (51) or 10  $\mu$ M of either cytochalasin D or colchicine. Treatment with PMA, RO31-8220, and/or wortmannin was performed similarly but for 10 min. Labeled lipoproteins (to 5  $\mu$ g/ml) with or without competitors (unlabeled HDL, AcLDL, or maleyl-BSA, each to 200  $\mu$ g/ml) were added, and incubations were continued for 2 h at 37°C. Cells were washed twice with ice-cold PBS containing 0.5% BSA, washed twice with ice-cold PBS, released from dishes by mild trypsinization, suspended in ice-cold PBS containing 0.1% BSA, and analyzed by flow cytometry (see below) (52). Alternatively, cells were allowed to remain on glass-bottomed dishes (Biopix, Beaver Falls, PA) and analyzed by live cell fluorescence microscopy (see below).

### Flow cytometry

Cells suspended in PBS containing 0.1% BSA were kept on ice until analysis. Cells were analyzed by flow cytometry using either a FACScan II (BD Biosciences) or a Beckman Coulter Epics XL. Excitation was at 488 nm, and emission was at  $515 \pm 10$  nm (FACScanII) or  $525 \pm 10$  nm (Beckman Coulter Epics XL) for alexa 488 and  $575 \pm 10$  nm for DiI. The geometric mean fluorescence value of the population of cells was taken as a measure of the level of uptake for each sample. In some instances, a 20-fold excess of either maleyl-BSA [competitor for both SR-BI and scavenger receptor, class A, type I (SR-AI)] or unlabeled lipoprotein was included. For those samples, specific uptake was determined as the difference between total uptake (in the absence of competitor) and nonspecific uptake (in the presence of competitor). We saw no differences when we used either excess maleyl-BSA or excess unlabeled lipoproteins (AcLDL or HDL) as competitors (data not shown). In all cases, untransfected IdIA7 cells were included as controls. The ratio of the specific uptake of lipoprotein-lipid (DiI label) to protein (alexa 488 label) was taken as a measure of the degree of selective lipid uptake.

### Live-cell fluorescence microscopy

Epifluorescence microscopy was performed at 37°C on a Nikon TE200 inverted fluorescence microscope with a 175 W xenon arc lamp (Sutter Instruments) light source, using a Nikon 63 $\times$  plan apochromat (NA 1.3) oil immersion objective and a heated stage. Texas Red filter sets (Semrock Technologies) were used for imaging of DiI. The images were captured using a Hamamatsu ORCA ER digital camera and SimplePCI 5.2 software (C-imaging).

## RESULTS

### Murine SR-BI mediates lipid uptake from HDL and AcLDL via distinct pathways

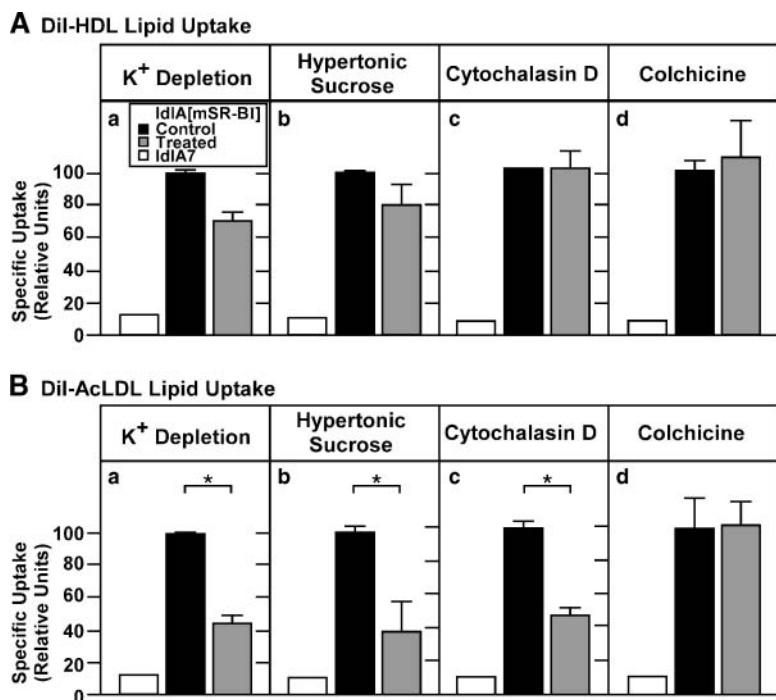
We tested the effects of inhibiting endocytosis on mSR-BI-mediated lipid uptake from different lipoprotein



ligands. We chose the physiological ligand, HDL, as well as the model ligand, AcLDL, because their binding to mSR-BI involves distinct residues in the extracellular region of the receptor (7, 8). We used *IdIA*[mSR-BI] cells, which lack a functional LDL receptor and do not express high levels of other scavenger receptors, allowing us to analyze mSR-BI activity in the absence of other pathways of lipoprotein uptake. Cells were either treated with hypertonic (0.4 M) sucrose or depleted of intracellular potassium, which interferes with clathrin-dependent endocytosis by preventing the interaction of clathrin with adaptor proteins (53) and/or reducing the formation of clathrin-coated pits on the cell surface (50, 54). Hypertonic shock and potassium depletion also reduce clathrin-independent endocytosis, although the mechanisms involved are unclear (51). We also tested cytochalasin D, which disrupts actin polymerization (55, 56), and colchicine, which depolymerizes microtubules (56, 57). As reported previously (16), neither hypertonic sucrose nor potassium depletion significantly decreased DiI-HDL lipid uptake by mSR-BI (Fig. 1A, panels a and b). In control experiments (Fig. 2A), both treatments did significantly reduce (by 80–90%) cell association of DiI-AcLDL by CHO[murine scavenger receptor, class A, type I (mSR-AI)] cells. These cells overexpress mSR-AI, known to mediate clathrin-dependent endocytosis of AcLDL (58). Both treatments also inhibited transferrin uptake by *IdIA*[mSR-BI] cells (data not shown), in agreement with previously reported findings (16). In contrast, only potassium depletion significantly reduced the uptake of CTX-B (Fig. 2B), which binds the ganglioside GM1 and is internalized in a GM1-dependent pathway that involves lipid rafts/caveolae but not clathrin (59, 60). Similarly, treatment of *IdIA*[mSR-BI] cells with cytochalasin D did not affect DiI-HDL uptake (Fig. 1A,

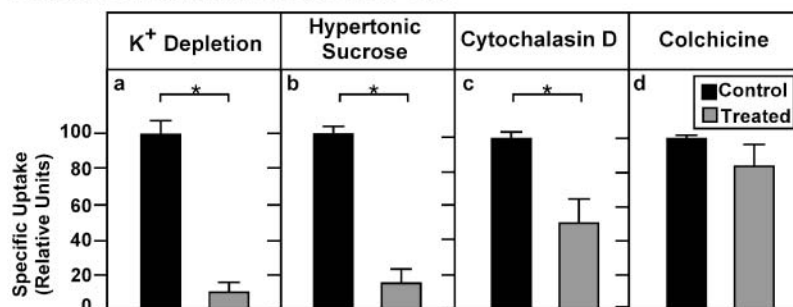
panel c) whereas SR-AI-mediated uptake of DiI-AcLDL was reduced by ~50% (Fig. 2A, panel c). Surprisingly, when DiI-AcLDL was used instead of DiI-HDL, DiI uptake by *IdIA*[mSR-BI] cells was significantly reduced by 50–70% by potassium depletion, exposure to hypertonic sucrose, or treatment with cytochalasin D (Fig. 1B, panels a–c). Lipid uptake by control, untransfected *IdIA7* cells was only 10–14% of that by untreated *IdIA*[mSR-BI] cells (Fig. 1). Therefore, potassium depletion, hypertonic shock, and treatment with cytochalasin D inhibited SR-BI-dependent lipid uptake from DiI-AcLDL. Colchicine did not affect DiI uptake from either HDL or AcLDL by mSR-BI in *IdIA*[mSR-BI] cells (Fig. 1A, B, panel d) or by SR-AI in control CHO[SR-AI] cells (Fig. 2). Colchicine did, however, disrupt the trafficking of DiI-containing vesicles in both CHO[mSR-AI] (Fig. 2C, panels a and c, and Supplementary video files a and c) and *IdIA*[mSR-BI] cells incubated with DiI-AcLDL (Fig. 2C, panels b and d, and Supplementary video files b and d). Similar results were obtained for *IdIA*[mSR-BI] cells incubated with DiI-HDL (data not shown). These data confirm that endocytosis is not required for HDL lipid uptake mediated by murine SR-BI. However, the data demonstrate that the same receptor mediates lipid uptake from AcLDL via an apparently endocytic process that is actin dependent and microtubule independent.

We next tested whether cell surface mSR-BI was internalized during lipid uptake from either HDL or AcLDL. We used cell surface biotinylation as a probe for the amount of mSR-BI remaining on the cell surface after incubation, for various times, in the absence or the presence of either HDL or AcLDL. Monensin was included, to block the recycling of SR-BI (12, 61, 62), allowing us to monitor the internalization step in isolation (63). In the

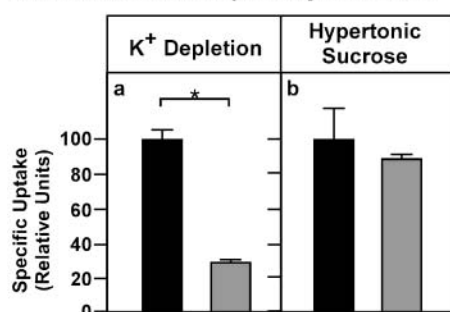


**Fig. 1.** Effects of potassium depletion, hypertonic sucrose, cytochalasin D, or colchicine on murine scavenger receptor, class B, type I (mSR-BI)-mediated 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) uptake from either HDL or acetyl LDL (AcLDL). Cells overexpressing mSR-BI were either depleted of intracellular potassium, incubated in medium containing 0.4 M sucrose or treated with 10  $\mu$ M of either cytochalasin D or colchicine to disrupt actin filaments or microtubules, as indicated (gray bars). Untreated control *IdIA*[mSR-BI] (black bars) and untransfected *IdIA7* cells (white bars) were included in each case. After treatment, cells were incubated with 5  $\mu$ g/ml of either (A) DiI-HDL or (B) DiI-AcLDL for 2 h at 37°C in the absence or presence of 200  $\mu$ g/ml of maleyl-BSA (a general competitor for mSR-BI-mediated lipoprotein lipid uptake). Specific uptake of DiI was measured by flow cytometry as the difference in the mean fluorescence of the population of cells incubated without and with maleyl-BSA (equivalent results were obtained when unlabeled lipoproteins were used instead of maleyl-BSA). Values were normalized to control untreated cells (black bars, set at 100% for ease of comparison), and data are expressed as the mean  $\pm$  SD of triplicates. \**P* < 0.05 for comparisons between untreated and treated *IdIA*[mSR-BI] cells by Student's *t*-test.

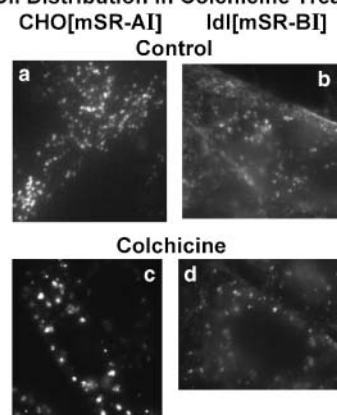
### A DiI-AcLDL Uptake by CHO[mSR-AI] Cells



### B Alexa-488 CTX-B Uptake by CHO Cells



### C DiI Distribution in Colchicine Treated Cells



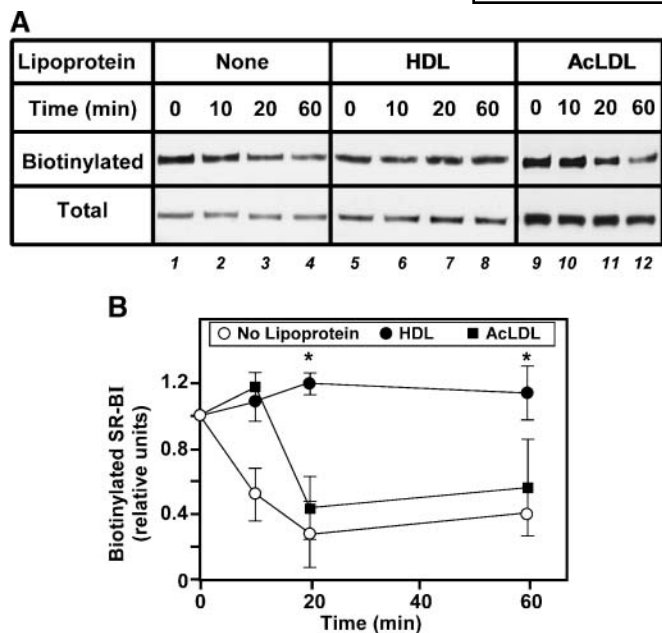
**Fig. 2.** Effect of potassium depletion, hypertonic sucrose or disruption of actin filaments or microtubules on murine scavenger receptor, class A, type I (mSR-AI)-mediated DiI-AcLDL uptake or on uptake of cholera toxin subunit B (CTX-B). Chinese hamster ovary (CHO) cells expressing mSR-AI (a known endocytic receptor for AcLDL) (A) or untransfected CHO cells (B) were either depleted of intracellular potassium, incubated in medium containing 0.4 M sucrose, or treated with 10  $\mu$ M of either cytochalasin D or colchicine (as indicated, gray bars) prior to incubation with either (A) 5  $\mu$ g/ml DiI-AcLDL (without or with 200  $\mu$ M maleyl BSA as a specific competitor) or (B) alexa 488-labeled CTX-B for 2 h at 37°C. Control cells (black bars) were treated in parallel. Specific uptake of DiI-AcLDL was measured by flow cytometry as the difference between the geometric mean fluorescence of the population incubated with DiI-AcLDL in the absence and presence of the specific competitor maleyl-BSA. Total uptake of alexa 488 CTX-B was determined by flow cytometry using 488 nm excitation and emission between 505–525 nm (FL1 channel). Mean fluorescence values were determined for each population. Values were normalized to those of control and untreated cells. Data are expressed as mean  $\pm$  SD of triplicate determinations. C: Cells, cultured on glass-bottomed dishes, were treated without or with 10  $\mu$ M colchicine and labeled with DiI-AcLDL as described in Experimental Procedures. Cells were imaged by live-cell fluorescence microscopy. Single frames are shown and correspond to supplementary video files a–d.

absence of lipoproteins, and in the presence of monensin, there was a time-dependent decrease in cell surface mSR-BI over 60 min (Fig. 3A, top left panel; quantified in Fig. 3B), which was blocked if cells were incubated at 4°C throughout (not shown). Total mSR-BI levels in cell lysates did not change under any of the conditions (Fig. 3A, bottom panels). When cells were treated with monensin in the presence of AcLDL, cell surface mSR-BI decreased following a similar time course (Fig. 3A, top right panel; quantified in Fig. 3B). In contrast, HDL blocked the time-dependent, monensin-triggered decrease in cell surface mSR-BI (Fig. 3A, middle panel; quantified in Fig. 3B). Thus, HDL, but not AcLDL, appears to stabilize mSR-BI on the cell surface, possibly by preventing its internalization. These data are consistent with the idea that mSR-BI-mediated lipid uptake from HDL and AcLDL occur via

distinct pathways. mSR-BI-mediated uptake of lipids from AcLDL has the characteristics of an endocytic pathway, whereas mSR-BI-mediated HDL lipid uptake appears to be independent of endocytosis.

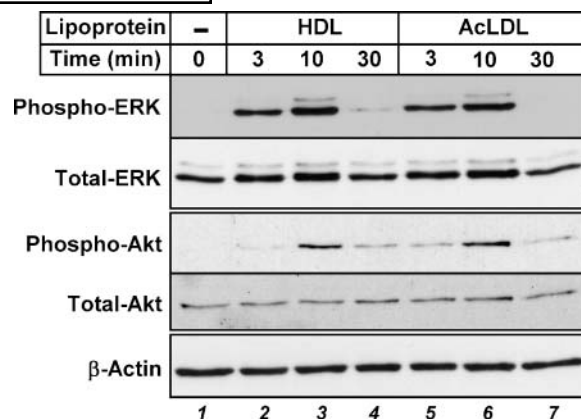
#### HDL, but not AcLDL, stimulates PKC activity

We reasoned that HDL and AcLDL interactions with SR-BI may also induce different downstream signaling pathways. We measured the time dependence of Akt and ERK phosphorylation after cells were incubated with either HDL or AcLDL (Fig. 4). Both HDL and AcLDL rapidly and transiently stimulated phosphorylation of ERK (Fig. 4, upper panel), and Akt (Fig. 4, middle panel). Phosphorylation of both ERK and Akt peaked at  $\sim$ 10 min and declined by 30 min. No changes were observed in total ERK or Akt protein levels. We also measured the abilities of HDL



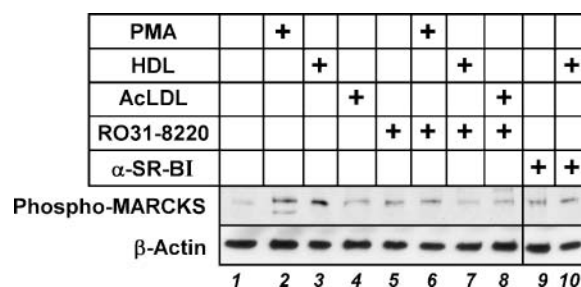
**Fig. 3.** Effect of HDL and AcLDL on scavenger receptor, class B, type I (SR-BI) internalization. A: Cells overexpressing mouse SR-BI were treated with monensin in either the absence of lipoproteins (lanes 1–4), the presence of HDL (lanes 5–8), or the presence of AcLDL (lanes 9–12) as indicated. At 0, 10, 20, and 60 min, cell surface proteins were biotinylated at 4°C. Cells were then washed to remove excess biotinylation reagent and lysed in TBS containing 0.1% Triton X-100 at 4°C. Biotinylated proteins were captured with streptavidin-agarose and analyzed by SDS-PAGE and immunoblotting for SR-BI (top panel, Biotinylated). Equal volumes of starting lysate (equivalent to 10% of the amount subjected to streptavidin pull-down) were also analyzed for SR-BI (bottom panel of A, Total). B: Bands from three independent experiments were quantified using a Kodak Image Station 440CF. The data are expressed as the relative band intensity, with values at time 0 set to 1. Data are the mean  $\pm$  SD of triplicate experiments. \* $P < 0.05$  for the 20 and 60 min values for HDL treatment compared with either AcLDL or no lipoprotein (Student's *t*-test).  $P > 0.05$  for all other comparisons between treatments.

and AcLDL to stimulate PKC activity, by monitoring the phosphorylation of MARCKS, using a phospho-MARCKS-specific antibody. Cells were treated for 3 min with either no addition (unstimulated control), PMA, HDL, or AcLDL in the absence or the presence of RO31-8220 (Fig. 5, lanes 1–6, 9, and 10). The phorbol ester, PMA, is a potent activator of a variety of PKC isoforms (64, 65), and serves as a positive control for PKC activation. RO31-8220 is a potent PKC inhibitor (64). As expected, treatment of cells with PMA induced MARCKS phosphorylation (Fig. 5, lane 2; compare with lane 1), and this was blocked by RO31-8220 (Fig. 5, lanes 5 and 6). HDL likewise stimulated MARCKS phosphorylation (Fig. 5, lane 3) in a manner that was also blocked by RO31-8220 (Fig. 5, lane 7). In contrast, AcLDL did not stimulate MARCKS phosphorylation above the background (Fig. 5, lanes 4 and 8; compare with lane 1). HDL-dependent MARCKS phosphorylation was blocked by an anti-SR-BI blocking antiserum (Fig. 5, lanes 9 and 10), demonstrating that binding to mSR-BI was required for HDL-dependent PKC activation in *Id1A*[mSR-BI] cells.



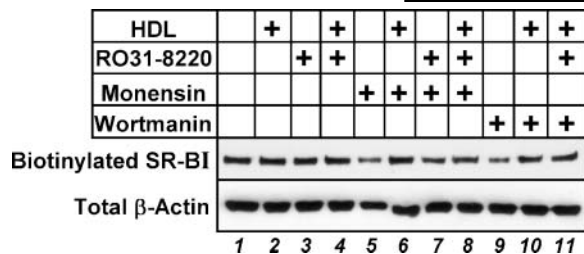
**Fig. 4.** Both HDL and AcLDL stimulate phosphorylation of extracellular signal-regulated kinase (ERK) and Akt. mSR-BI-overexpressing (*Id1A*[mSR-BI]) cells were cultured in medium containing 3% lipoprotein-deficient serum. On the day of the experiment, cells were washed, serum starved in Ham's-F12 medium containing 0.5% BSA for 2 h, and either harvested (0 time) or incubated in the same medium containing either HDL or AcLDL (each at 100  $\mu$ g/ml) for 3, 10, or 30 min prior to harvest. After incubations, cells were rapidly chilled to 4°C and washed, lysed, and analyzed by SDS-PAGE and immunoblotting for either phospho-ERK (top panel) or phospho-Akt (middle panel). Data from a single representative experiment are shown.

To determine whether either the PI3K/Akt or the PKC signaling pathways are involved in HDL-dependent retention of mSR-BI at the cell surface, we treated cells with monensin and HDL either in the absence or the presence of the PI3K inhibitor (wortmannin) (66) or RO31-8220 (Fig. 6). Neither wortmannin nor RO31-8220 disrupted HDL's ability to stimulate the cell surface retention of mSR-BI, indicating that neither the PI3K/Akt nor the PKC pathways were required. Wortmannin treatment did, how-



**Fig. 5.** HDL, but not AcLDL, stimulates phosphorylation of the protein kinase C (PKC) substrate myristoylated alanine-rich C-kinase substrate (MARCKS). mSR-BI-overexpressing (*Id1A*[mSR-BI]) cells, prepared as described in the legend to Fig. 4, were given serum-free Hams F12 medium containing either no additions (lane 1), 0.5  $\mu$ M PMA, HDL, or AcLDL (each at 50  $\mu$ g/ml), 5  $\mu$ M RO31-8220, a 1:1000 dilution of an anti-SR-BI blocking antiserum, or combinations as indicated. Cells were incubated for 3 min, at which time they were rapidly chilled on ice, then washed, lysed, and analyzed by SDS-PAGE and immunoblotting for either phospho-MARCKS or  $\beta$ -actin. The data are representative of multiple experiments. For the experiment shown, lanes 9 and 10 were from a different portion of the same gel.



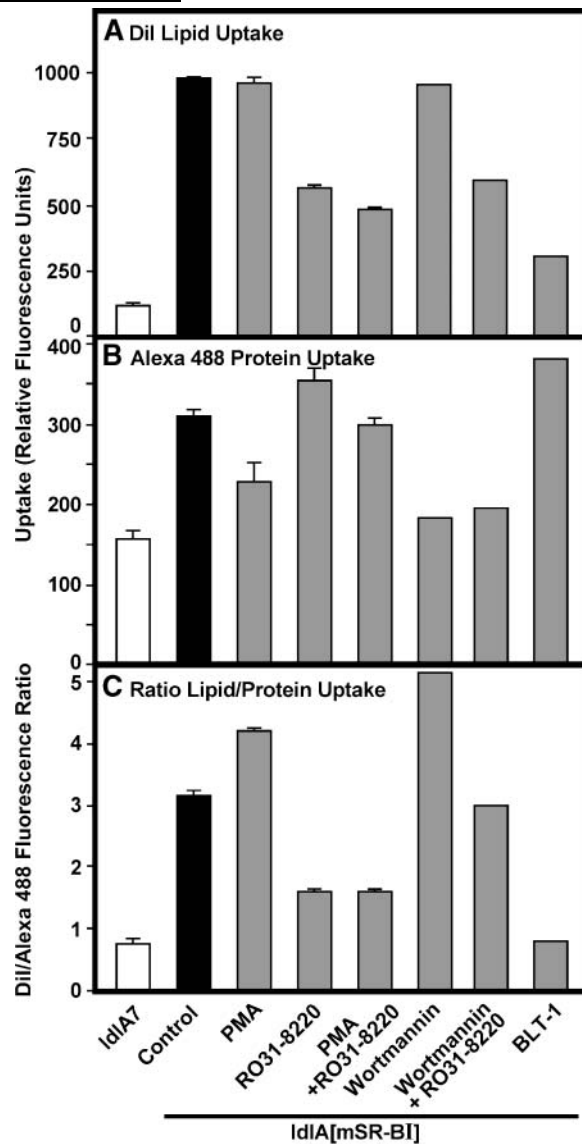


**Fig. 6.** HDL-mediated stabilization of SR-BI on the cell surface is not sensitive to the PKC inhibitor RO31-8220. mSR-BI-overexpressing (IdIA[mSR-BI]) cells were cultured in the absence of lipoproteins, washed, and released from culture dishes. Cells were treated either without (control, no additions, lane 1) or with (individually or in combination as indicated) HDL (100  $\mu$ g/ml), the PKC inhibitor RO31-8220 (5  $\mu$ M), monensin (25  $\mu$ M), and/or the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (1  $\mu$ M). After the incubation, cells were rapidly chilled to 4°C and biotinylated at 4°C. Cells were then washed and lysed at 4°C, and biotinylated proteins were captured with streptavidin-agarose and analyzed by SDS-PAGE and immunoblotting for SR-BI (top panel, Biotinylated SR-BI). Lysates of cells were also run on a separate gel and immunoblotted using an antibody against  $\beta$ -actin (lower panel).

ever, reduce cell surface (biotinylated) mSR-BI in otherwise untreated cells (Fig. 6, lane 9; compared with lane 1), in agreement with its reported effects in 3T3-L1 adipocytes and HepG2 human hepatoma cells (13, 14). HDL blocked the wortmannin-induced decrease in cell surface SR-BI (Fig. 6, lanes 9 and 10), similar to its ability to block the monensin-induced decrease in cell surface SR-BI (Fig. 3 and Fig. 6; lanes 5 and 6). RO31-8220 did not alter the amount of cell surface SR-BI (Fig. 6). Thus, HDL-stimulated retention of SR-BI on the cell surface does not appear to require either PKC or PI3K signaling. Similarly, neither ERK nor PI3K signaling was sufficient, because both were activated by AcLDL (Fig. 4) under conditions that did not lead to retention of mSR-BI on the cell surface (Fig. 6).

#### Effects of PMA, RO31-8220, and wortmannin on SR-BI activity

To test the effects of PKC and PI3K signaling pathways on the efficiency of selective lipid uptake mediated by mSR-BI, IdIA[mSR-BI] cells were treated with either PMA, RO31-8220, or wortmannin, and the uptake of both lipid and protein components of HDL were measured using HDL that was doubly labeled with DiI (lipid) and alexa 488 (protein). **Figure 7** shows the levels of cell association of lipid (DiI fluorescence; panel A), protein (alexa 488 fluorescence; panel B), and the ratio of lipid/protein (DiI/alexa 488 fluorescence; panel C). When IdIA[mSR-BI] cells were incubated with DiI/alexa 488-doubly labeled HDL, they accumulated both DiI and alexa 488 labels in a time-dependent manner (not shown) and became enriched in DiI, versus alexa 488. The ratio of DiI/alexa 488 associated with cells after 2 h at 37°C was  $\sim$ 6:1, whereas the starting ratio in the doubly labeled HDL was  $\sim$ 1:1, indicative of efficient selective lipid uptake. Treatment of cells with PMA did not significantly affect the cell asso-



**Fig. 7.** mSR-BI-mediated selective uptake of HDL lipid is sensitive to activators and inhibitors of PKC and PI3K signaling. mSR-BI-overexpressing (IdIA[mSR-BI]) cells (black or gray bars) or control untransfected IdIA7 cells (white bars) were cultured in the absence of lipoproteins, washed, and treated for 10 min either without or with 0.5  $\mu$ M PMA, 5  $\mu$ M RO31-8220, 1  $\mu$ M wortmannin, or 0.3  $\mu$ M BLT-1 either singly or in combination as indicated below panel C. After 10 min, 5  $\mu$ g/ml of DiI/alexa 488-doubly labeled HDL was added and cells were incubated for a further 2 h at 37°C. After 2 h, cells were washed, released from dishes, and analyzed by flow cytometry for DiI fluorescence (A), alexa 488 fluorescence (B), or the ratio of DiI/alexa 488 fluorescence (C). Untreated cells, white or black bars; treated cells, gray bars. Data are the mean  $\pm$  SD of triplicate determinations, with the exception of data corresponding to wortmannin, wortmannin + RO31-8220, and BLT-1 treated IdIA[mSR-BI] cells, which are from a single determination. All of the data are representative of multiple independent experiments.

ciation of HDL lipid (Fig. 7, panel A), but cell association of protein (Fig. 7, panel B) was substantially reduced. Thus, PMA treatment increased the degree of selective lipid uptake (lipid/protein ratio) (Fig. 7, panel C). RO31-8220-treated cells took up substantially less HDL lipid

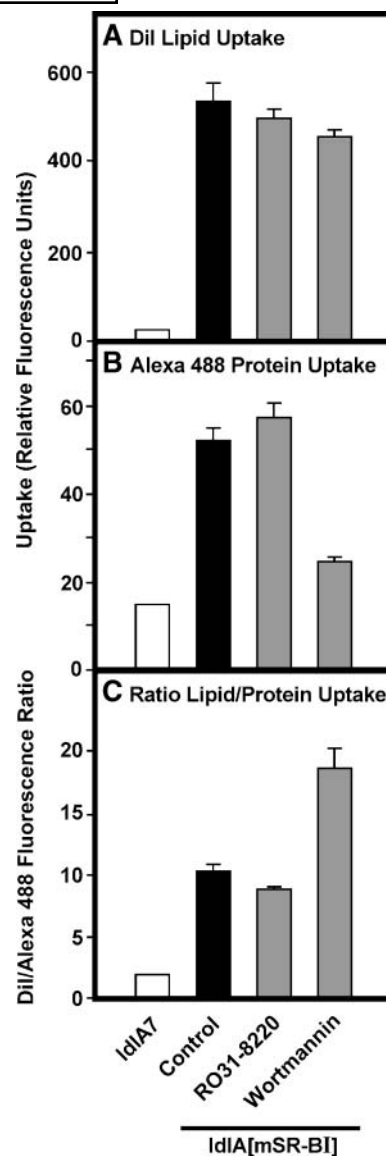
but slightly more HDL protein than controls. Therefore, RO31-8220 reduced the degree of selective lipid uptake in both the absence and the presence of PMA. The effect of the PKC inhibitor was similar to that of the small molecule BLT-1, which selectively inhibits lipid uptake while enhancing HDL binding to mSR-BI (11). In contrast, wortmannin did not alter the level of cell association of HDL lipid but did decrease the cell association of HDL protein, resulting in an increased degree of selective lipid uptake (lipid/protein ratio) (Fig. 7, panel C). Therefore, the mSR-BI-mediated selective lipid uptake appears to be stimulated by PKC and decreased by PI3K signaling in ldlA[mSR-BI] cells.

We also tested the effect of RO31-8220 and wortmannin on the uptake of lipid and protein from DiI/alexa 488-doubly labeled AcLDL in ldlA[mSR-BI] cells. As for doubly labeled HDL, cells accumulated both DiI and alexa 488 labels in a time-dependent manner (not shown) but only became enriched DiI versus alexa 488 labels by ~2-fold. The ratio of DiI/alexa 488 associated with cells after incubation with doubly labeled AcLDL was ~10:1 (Fig. 8, panels A, B, and C), whereas the starting ratio in the DiI/alexa-488-doubly labeled AcLDL was ~5:1 (not shown). This is consistent with endocytosis playing a more prominent role in mSR-BI-mediated lipid uptake from AcLDL than from HDL. Treatment of cells with RO31-8220 did not alter cell association of DiI or alexa 488 from doubly labeled AcLDL. In contrast, treatment of cells with wortmannin decreased cell association of alexa 488 (Fig. 8, panel B) without affecting cell association of DiI (Fig. 8, panel A), resulting in an ~4-fold increase in the degree of selective lipid uptake of AcLDL lipid (Fig. 8, panel C). Therefore, wortmannin appears to block mSR-BI's endocytic activity and to increase the efficiency of mSR-BI-mediated selective lipid uptake, regardless of the lipoprotein ligand.

## DISCUSSION

We have shown that mSR-BI-mediated lipid uptake from AcLDL and HDL occurs via distinct pathways. mSR-BI-mediated lipid uptake from AcLDL has the characteristics of an endocytic process, which include a low level of selectivity for the lipid versus the protein component of the lipoprotein particle; sensitivity to potassium depletion and to exposure to hypertonic media; and inhibition by the actin-depolymerizing agent, cytochalasin D. These results are consistent with findings that SR-BI mediates the internalization/intracellular accumulation of a variety of other ligands, including advanced glycation end-product-modified BSA, lipopolysaccharide, serum amyloid  $\alpha$ , liposomes containing phosphatidyl serine (PS-liposomes), and apoptotic cells (22–24, 26, 67, 68).

In contrast, HDL stimulates the retention of mSR-BI on the cell surface in an apparently PKC-, PI3K-, and ERK-independent manner and SR-BI-mediated HDL lipid uptake does not involve endocytosis [our data and the data of Nieland et al. (16) and Harder et al. (17)]. The mechanisms by which HDL and AcLDL trigger different lipid



**Fig. 8.** The degree of mSR-BI-mediated selective lipid uptake from AcLDL is increased by the PI3K inhibitor wortmannin. mSR-BI-overexpressing (ldlA[mSR-BI]) cells were cultured in the absence of lipoproteins, washed, and treated for 10 min either without (Control, black bars) or with 5  $\mu$ M RO31-8220, or 1  $\mu$ M wortmannin (gray bars) as indicated below panel C. After 10 min, 5  $\mu$ g/ml of DiI/alexa 488-doubly labeled AcLDL was added and cells were incubated for a further 2 h at 37°C. After 2 h, cells were washed, released from dishes, and analyzed by flow cytometry for DiI fluorescence (A), alexa 488 fluorescence (B), or the ratio of DiI/alexa 488 fluorescence (C). Untransfected ldlA7 cells were included as a control (white bars). Samples are mean  $\pm$  SD of triplicate determinations from a single representative experiment.

uptake pathways remain to be discovered. It is currently unclear whether SR-BI's oligomerization state (69, 70) and/or localization in lipid rafts/caveolae (49) determine endocytic versus nonendocytic pathways, or are influenced by binding to distinct ligands. Nevertheless, our data suggest that mSR-BI endocytosis versus retention on the cell surface may be a step at which SR-BI's selective uptake activity can be regulated. This is consistent with recent



studies that suggest that the lower selective uptake activity of SR-BII, a splice variant of SR-BI, which has a different C-terminal cytoplasmic tail, appears to be the result of increased endocytosis and that the introduction of an endocytic motif into the carboxy-terminal cytoplasmic tail of SR-BI led to its increased endocytosis and decreased selective lipid uptake activity (20).


Our results demonstrate that mSR-BI-mediated selective lipid uptake, in CHO-derived IdIA7 cells overexpressing the receptor, is acutely regulated by protein kinase signaling pathways. PMA increases and RO31-8220 decreases the degree of SR-BI-mediated selective lipid uptake. In contrast, RO31-8220 did not affect the mSR-BI-mediated endocytic uptake of AcLDL lipid. This suggests that in these cells, a phorbol ester-activated and RO31-8220-sensitive signaling pathway activates SR-BI-mediated selective uptake and inhibits SR-BI-mediated endocytosis. PMA and other phorbol esters are potent activators and RO31-8220 is a potent inhibitor of PKC, although both also similarly affect other kinases, including p90Rsk, Msk1, and the p70S6 kinase (64, 65). The nature of the signaling pathway, controlling SR-BI activity in these cells, is not yet clear. The finding that HDL activates PKC activity (as measured by MARCKS phosphorylation) in an SR-BI-dependent manner suggests the involvement of one or more PKC isoforms and raises the possibility that SR-BI's selective uptake activity may be subject to positive feedback regulation by HDL.

PI3K also appears to stimulate mSR-BI-mediated endocytic uptake and to suppress selective lipid uptake, because wortmannin reduced the cell association of lipoprotein particles (alexa 488 protein label) while increasing the efficiency of selective lipid uptake for both HDL and AcLDL. Our findings differ somewhat from the reported wortmannin-dependent reduction in HDL selective lipid uptake in 3T3-L1 adipocytes and HepG2 cells (13, 14). It is not clear whether this is due to differences in the cell type and/or experimental protocols used. In particular, we demonstrate that in non-serum-starved cells, wortmannin treatment reduced the level of cell surface SR-BI, and that this was blocked by HDL (Fig. 6). In contrast, in serum-starved HepG2 cells, wortmannin blocked HDL-dependent recruitment of SR-BI to the cell surface (14). On the other hand, our findings are consistent with reports that wortmannin inhibits hepatocyte uptake of HDL particles (14) and the transport of HDL particles from the basolateral to the apical membrane of polarized hepatocytes (71). Our findings are also consistent with the report that apoptotic cells and phosphatidyl serine-containing liposomes, both of which are ligands for SR-BI (72, 73), were able to stimulate SR-BI-dependent phosphorylation of Akt, and the MAPKs ERK, p38, and JNK in rat Sertoli cells (26). Moreover, pharmacological blockade of ERK and p38 MAPK activity resulted in reduced SR-BI-dependent phagocytosis of apoptotic cells and PS-liposomes (26). We have demonstrated that mSR-BI-mediated AcLDL lipid uptake is sensitive to cytochalasin D-mediated actin depolymerization, and that cell association of both AcLDL and HDL protein, which includes binding and internalization of

lipoprotein particles, is reduced by treatment of cells with wortmannin. Likewise, Osada, Shiratsuchi, and Nakanishi (26) demonstrated that SR-BI-dependent Sertoli cell engulfment of apoptotic cells was blocked by actin depolymerization and by wortmannin, further supporting the conclusion that AcLDL lipid uptake mediated by mSR-BI in IdIA7 cells involves an endocytic process. It is currently not clear whether AcLDL lipid uptake mediated by mSR-BI is also inhibited by pharmacological blockade of ERK and/or p38 MAPK activity, in a manner analogous to SR-BI-mediated phagocytosis (26).

HDL, but not AcLDL, activated PKC signaling in an mSR-BI-dependent manner in the transfected IdIA[mSR-BI] cells. In contrast, both HDL and AcLDL activated the PI3K and MAPK pathways. To our knowledge, this is the first demonstration that different mSR-BI ligands activate different signaling pathways in the same cells. How the engagement of mSR-BI by its ligands is coupled to signaling is not clear. Early events downstream of ligand binding, which may affect signaling, could include the transfer of cholesterol either into or out of signaling domains such as caveolae/lipid rafts in the plasma membrane (39, 74–76). Other research implicates the transfer of bioactive lipids (e.g., estradiol, ceramides, sphingosine 1 phosphate) from bound lipoproteins into cells (30, 34, 38, 77). Alternatively, SR-BI could bind one or more signaling proteins directly and/or adaptor proteins that may couple it to signaling proteins. In support of this, an antibody against the C-terminal cytoplasmic region of SR-BI could block HDL-stimulated endothelial nitric oxide synthase activation in isolated endothelial cell membranes (31). Furthermore, a mutant form of SR-BI lacking the C-terminal cytoplasmic region, which was still able to mediate HDL lipid uptake, was not able to mediate HDL-stimulated proliferation and survival of MCF-7 cells in culture (33). These studies suggest that the C-terminal cytoplasmic domain of SR-BI may play a role in at least some signaling pathways. It has recently been proposed that SR-BI's C-terminal PDZ-K1 binding sequence may participate in HDL-dependent signaling in endothelial cells (39). Whether PDZ-K1 itself participates in SR-BI-dependent signaling is not clear.

In conclusion, we have provided evidence that SR-BI's selective uptake and endocytic activities can be regulated by the PKC and PI3K signaling pathways, at least in CHO-derived IdIA[mSR-BI] cells. PKC activation and PI3K inhibition increase the efficiency of mSR-BI-mediated selective lipid uptake, whereas PKC inhibition and PI3K activation reduce its efficiency. This suggests the possibility that SR-BI's selective uptake and endocytic activities may also be modulated by protein kinase signaling in other cell types, such as hepatocytes, steroidogenic cells, macrophages, and/or endothelial cells. These findings may have important implications for SR-BI's *in vivo* functions, including reverse cholesterol transport and HDL-dependent protection against atherosclerosis. The ability to modulate SR-BI activity in both hepatocytes and other cell types (including macrophages) in atherosclerotic plaques by pharmacological activation or inhibition of protein kinase signaling path-

ways could be a therapeutic approach to modulating the development of atherosclerosis and heart disease. 

## NOTE ADDED IN PROOF

Kimura et al. (2006) *J. Biol. Chem.* **281**: 37457–37467 have reported evidence that PDZK1 is involved in SR-BI-mediated HDL signaling in endothelial cells.

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