The Role of Human and Mouse Hepatic Scavenger Receptor Class B Type I (SR-BI) in the Selective Uptake of Low-Density Lipoprotein−Cholesteryl Esters[†]

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ABSTRACT: Low-density lipoprotein (LDL)−cholesteryl ester (CE) selective uptake has been demonstrated in nonhepatic cells overexpressing the scavenger receptor class B type I (SR-BI). The role of hepatic SR-BI toward LDL, the main carrier of plasma CE in humans, remains unclear. The aim of this study was to determine if SR-BI, expressed at its normal level, is implicated in LDL−CE selective uptake in human HepG2 hepatoma cells and mouse hepatic cells, to quantify its contribution and to determine if LDL−CE selective uptake is likely to occur in the presence of human HDL. First, antibody blocking experiments were conducted on normal HepG2 cells. SR-BI/BII antiserum inhibited ^{125}I-LDL and ^{125}I-HDL_3 binding (10 μg of protein/mL) by 45% ($p < 0.05$) and CE selective uptake by more than 85% ($p < 0.01$) for both ligands. Second, HepG2 cells were stably transfected with a eukaryotic vector expressing a 400-bp human SR-BI antisense cDNA fragment. Clone 17 (C17) has a 70% ($p < 0.01$) reduction in SR-BI expression. In this clone, ^{3}H-CE−LDL and ^{3}H-CE−HDL_3 association (10 μg of protein/mL) was 54 ± 6% and 45 ± 7% of control values, respectively, while ^{125}I-LDL and ^{125}I-HDL_3 protein association was 71 ± 3% and 58 ± 5% of controls, resulting in 46% and 55% ($p < 0.01$) decreases in LDL− and HDL_3−CE selective uptake. Normalizing CE selective uptake for SR-BI expression reveals that SR-BI is responsible for 68% and 74% of LDL− and HDL_3−CE selective uptake, respectively. Thus, both approaches show that, in HepG2 cells, SR-BI is responsible for 68−85% of CE selective uptake. Other pathways for selective uptake in HepG2 cells do not require CD36, as shown by anti-CD36 antibody blocking experiments, or class A scavenger receptors, as shown by the lack of competition by poly(inosinic acid). However, CD36 is a functional oxidized LDL receptor on HepG2 cells, as shown by antibody blocking experiments. Similar results for CE selective uptake were obtained with primary cultures of hepatic cells from normal (+/+), heterozygous (−/+), and homozygous (−/−) SR-BI knockout mice. Flow cytometry experiments show that SR-BI accounts for 75% of DiI−LDL uptake, the LDL receptor for 14%, and other pathways for 11%. CE selective uptake from LDL and HDL_3 is likely to occur in the liver, since unlabeled HDL (total and apoE-free HDL_3) and LDL, when added in physiological proportions, only partially competed for LDL− and HDL_3−CE selective uptake. In this setting, human hepatic SR-BI may be a crucial molecule in the turnover of both LDL− and HDL_3−cholesterol.

Lipoproteins are synthesized in the intestine and the liver. Once in the circulation, they may either take up cholesterol from cells (as free cholesterol) or deliver free or esterified cholesterol to cells. When the uptake of cholesteryl esters (CE)[1] occurs without a parallel uptake of the apolipoprotein, CE selective uptake is demonstrated. In the past, particular attention was given to high-density lipoprotein (HDL) derived CE (*1*, *2*) but not to low-density lipoprotein (LDL) derived CE even though LDL−CE selective uptake had been demonstrated in the perfused rat liver and human fibroblasts (*3*) and in human HepG2 hepatoma cell membranes (*4*). More recently, we have shown that mouse hepatocytes (*5*) and human HepG2 cells (*6*) perform the same task and that the selective uptake pathway is at least 5-fold better in bringing CE to the cell than the LDL receptor (LDLr) pathway (*5*, *7*). We then showed that selective uptake of LDL−CE is involved in HepG2 cell cholesterol homeostasis (*8*). Searching for the identity of the receptor involved, we found that the pathway for CE selective uptake in HepG2 cells can process CE from both native and modified lipoproteins and has a binding specificity similar to that of the class B

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[1] Abbreviations: AcLDL, acetylated low-density lipoprotein; apo, apolipoprotein; BSA, bovine serum albumin; CD36, cluster of differentiation 36; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; CHO, Chinese hamster ovary; CLA-1, CD36- and LIMPII-analogous-1; DiI, dioctadecyltetramethylindocarbocyanin; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HDL_3, high-density lipoprotein 3; LDL, low-density lipoprotein; LDLr, LDL receptor; M-BSA, maleylated bovine serum albumin; MEM, minimal essential medium; OxLDL, oxidized low-density lipoprotein; PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate-buffered saline; SR-BI, scavenger receptor class B type I; TBS, Tris-buffered saline; TCA, trichloroacetic acid.

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scavenger receptor family (*9*). The scavenger receptor class B type I (SR-BI) and the cluster of differentiation 36 (CD36) are two members of this family.

Rodent SR-BI was cloned by Krieger's group in 1994 as a binding receptor for LDL, oxidized LDL (OxLDL), acetylated LDL (AcLDL), and maleylated bovine serum albumin (M-BSA) (*10*). SR-BI was later shown to be a receptor for anionic phospholipids (*11*) and to mediate the selective uptake of HDL−CE (*12*). SR-BI mediates bidirectional flux of free cholesterol, i.e., selective uptake of free cholesterol and free cholesterol efflux to lipoprotein and nonlipoprotein acceptors [for a review, see Williams et al. (*13*)]. CD36- and LIMPII-analogous-1 (CLA-1) is the human orthologue of rodent SR-BI (*14*) and shows nearly identical binding properties (*15*, *16*). To simplify, human SR-BI will be used here when referring to CLA-1. SR-BI is expressed in steroidogenic organs and the liver in both rodents and humans (*17*, *18*) and in HepG2 cells (*14*). In vitro studies with SR-BI transfected cells demonstrated that SR-BI selectively takes up LDL−CE in COS (kidney) cells (*13*) and processes CE from LDL in Chinese hamster ovary (CHO) cells (*20*): the incoming cholesterol could down-regulate 3-hydroxy-3-methylglutaryl-CoA reductase (*21*). However, a recent study found that SR-BI could not transfer LDL−CE to transfected CHO cells (*22*) and challenged the role of SR-BI toward LDL in transfected cells overexpressing SR-BI. We found that LDL−CE from the selective uptake pathway is hydrolyzed in lysosomes in both primary cultures of mouse hepatocytes and HepG2 cells (*5*, *6*), while using cell imaging with HDL labeled with the fluorescent lipid DiI, HDL lipids were found in the extralysosomal compartment in CHO (*12*) and COS (*16*) cells. On the basis of these findings, it appears that SR-BI may not be involved in hepatic selective uptake of LDL−CE, unless this can be explained by tissue-specific differences in CE or lipoprotein metabolism.

Nevertheless, a number of in vivo studies in mice reveal that manipulation of SR-BI gene expression influences plasma LDL levels. Transgenic mice with a moderate (2-fold) increase in hepatic SR-BI have a reduction in both HDL− and LDL−cholesterol (*23*, *24*). Ueda and colleagues (*25*) have shown a reduction in plasma apolipoprotein (apo) B in mice overexpressing SR-BI. Moreover, a mouse strain with high (10-fold) SR-BI overexpression had virtually no plasma (V)LDL−cholesterol, the endogenous apoB level was decreased by 36%, and the clearance of human apoB was increased (*25*). Whether these effects on LDL catabolism were due to direct interaction of SR-BI and apoB-containing lipoproteins or other indirect effects in these mice was not ascertained. All of these studies rely on supraphysiological levels of SR-BI and, therefore, do not evaluate the role of SR-BI in normal mice. Thus, it appears critical to determine if hepatic SR-BI can process LDL−CE, especially in humans, where LDL are the main source of plasma CE.

Human CD36 (*26*) is expressed on HepG2 cell microvilli (*27*) and various cell types including endotheliocytes, adipocytes, and monocytes/macrophages. It has been implicated in a number of processes, such as intestinal cholesterol absorption, free fatty acid uptake in muscles and adipose tissue, and foam cell formation [for a review of CD36 roles, see Febbraio et al. (*28*)]. It was recently reported that CD36 binds very low density lipoprotein (VLDL), LDL, and HDL, reinforcing the similarity between members of the SR-B family (*29*). However, a recent study by Villiers et al. (*30*) has shown that LDL binding does not occur in COS cells expressing CD36. CD36 is able to mediate the association and degradation of OxLDL and AcLDL in a variety of cells (e.g., platelets, macrophages) (*31*, *32*). Comparative studies of CD36 and SR-BI functions revealed that CD36 mediates selective uptake of HDL−CE, albeit its >5-fold reduction in efficiency (*33*, *34*). CD36 participation in native lipoprotein metabolism is not firmly established.

Actual data on SR-BI and CD36 functions are mainly drawn from studies with extrahepatic cell models that express caveolin-1 and thus exhibit caveolae at their surface. Caveolin expression in hepatoma cells and murine hepatocytes is controversial: HepG2 cells either do not express caveolin-1 (*35*) or express caveolin-1 (*36*; D. Rhainds, personal data), while it is either present in low amounts (*37*, *38*) or absent (L. Brissette, personal data) in murine hepatocytes. Both class B scavenger receptors are localized in caveolae in cultured cells (*39*, *40*), and selective uptake of HDL−CE occurs into caveolae in some cells (*41*). Thus, it is possible that the SR-BI and/or CD36 pathways described in nonhepatic cells will differ from those in hepatic cells. This also justifies the investigation in the hepatic cell devoid of caveolin/caveolae.

The aim of this study was to demonstrate clearly the implication of SR-BI in the selective uptake of LDL−CE in two hepatic cell models, the HepG2 hepatoma cell and mouse hepatocyte, and to determine its quantitative importance in the selective uptake of LDL−CE compared to HDL−CE. We also wanted to determine if CE selective uptake occurs in HepG2 cells in the presence of physiological amounts of lipoprotein competitors. Two strategies were designed in HepG2 cells to obtain an inhibition of SR-BI expression/function. First, a blocking antibody approach was used to inhibit SR-BI function. Second, we used a molecular biology approach based on constitutive human SR-BI antisense RNA expression in HepG2 cells. A panel of HepG2 cell clones were obtained, from which clone 17 (C17) was retained for its 70% reduction in SR-BI expression. Both approaches have shown that SR-BI is responsible for the major part of CE selective uptake from HDL and LDL particles. Similar results were obtained with primary cultures of mouse hepatic cells prepared from normal mice or SR-BI knockout mice. We also show that CE selective uptake in HepG2 can occur when physiological amounts of lipoprotein competitors were present.

EXPERIMENTAL PROCEDURES

*Material*s. The human hepatoma cell line HepG2 was obtained from the American Type Culture Collection (Rockville, MD). Minimal essential medium (MEM), fetal bovine serum (FBS), trypsin, penicillin, streptomycin, and geneticin were purchased from Life Technologies (Burlington, Ontario, Canada). [1,2-^{3}H]Cholesteryl oleate (50 mCi/mmol) was from Amersham Pharmacia Biotech (Laval, Quebec, Canada), and [^{125}I]iodine (as sodium iodide, 100 mCi/mmol) was from ICN Canada (Montreal, Quebec, Canada). DiI-labeled LDL (1 mg/mL) were from Intracel (Rockville, MD). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Restriction and modification enzymes were all from Amersham Pharmacia Biotech (Laval, Quebec, Canada).

Anti-SR-BI and anti-SR-BI/BII (blocking) polyclonal antibodies were purchased from Novus Biologicals (Littleton, CO), FA6-152 anti-CD36 monoclonal antibody was from Immunotech (Marseille, France), while anti-CD36 and anti-LDL receptor rabbit polyclonal antibodies were from Research Diagnostics (Flanders, NJ). Enhanced chemiluminescence substrate and Complete protease inhibitor cocktail tablets were from Roche Diagnostics (Laval, Quebec, Canada). Goat anti-rabbit IgG coupled to horseradish peroxidase was from Chemicon (Temecula, CA).

Animals. Three heterozygous B6/129S-*Srb1*^{tm1Kri} breeding pairs were obtained from Jackson Labs (Bar Harbor, ME) in order to generate a colony. These have generated mice with a 1:2:0.46 ratio of normal (+/+), heterozygous (−/+), and homozygous (−/−) knockout mice as determined by the PCR method of Rigotti et al. (*42*). The animals were provided with a standard mouse chow diet and drinking water ad libitum and were submitted to a 14 h light/10 h dark cycle. This study was conducted according to protocols approved by the Animal Care and Use Committee of the Université du Québec à Montréal (No. 0901-424-0904). Six to eight week old male mice were used.

Preparation and Primary Cultures of Mouse Hepatic Cells. Hepatic cells were isolated from mouse liver as described by us in refs *5* and *43*. Briefly, the portal vein was cannulated with a 23-gauge plastic cannula. First, the liver was perfused with calcium-free Hank's balanced salt solution (HBSS), pH 7.4, pregassed with 95% O_2/5% CO_2, at a flow rate of 5 mL/min. Subsequently, the liver was perfused with a collagenase solution (25 mg of collagenase/100 mL of HBSS containing 5 mM calcium) for 7 min. Hepatic cells were gently released from the Glisson capsule. Isolated hepatic cells were washed twice with Williams' E medium containing 10% newborn calf serum and 0.5% gentamycin. Hepatic cells were isolated with similar yields from livers of control, heterozygous, and homozygous SR-BI knockout mice, with approximately 5×10^7 cells per liver. Viability of hepatic cells was assessed by trypan blue exclusion immediately after isolation and was >85%. Cells were plated in 12-well plastic dishes, precoated with collagen (3 μg/mL), at 0.8×10^6 viable cells per well in 1 mL of culture medium. After a 3 h adherence period, nonviable cells were removed from the cultures by careful washing. The serum-containing medium was then replaced by MEM. They rapidly formed monolayers under these conditions. Typically, per day, two liver perfusions were conducted on a normal mouse and either a (−/+) or a (−/−) SR-BI knockout mouse. This allowed us to establish the value of (−/+) and (−/−) mouse hepatic cell association as a percentage of the normal value. Primary cultures of hepatic cells were used in assays after an overnight culture in MEM.

HepG2 Cell Culture. HepG2 cells were grown in 75 cm^2 flasks containing 15 mL of Eagle's MEM supplemented with 10% FBS, penicillin (100 units/mL), streptomycin (100 μg/mL), and glutamine (4 mM). Five days prior to the binding, association, or protein degradation assays, 3.0×10^5 cells were seeded in 3.8 cm^2 culture dishes (12-well plates), while 1.5×10^5 cells were seeded in 1.8 cm^2 culture dishes (24-well plates) for the experiments involving SR-BI blocking antibodies. When uptake of DiI-labeled LDL had to be investigated, 7.3×10^5 cells were seeded in 9.4 cm^2 dishes (6-well plates). The medium was changed every 2 days. In all cases, the cells were used when they were 80−90% confluent.

Creation of a Eukaryotic Vector Expressing Human SR-BI Antisense RNA. A 0.4 kb fragment from the 5′ end of the CLA-1 (human SR-BI) cDNA, including the beginning of the coding sequence, was recovered by an *Eco*RI−*Xho*I complete digestion of the eukaryotic vector pCEXV-3 (a gift from Dr. Miguel Angel Vega, Hospital de la Princesa, Madrid, Spain) (*14*). This fragment was inserted head to tail in the eukaryotic expression vector pRc/CMV. HepG2 cells at 80% confluence were transfected with the vector expressing antisense RNA or with the empty vector by the classic calcium phosphate method (*44*). Cells were placed under selection with 500 μg/mL geneticin (G418) for 3−4 weeks. Cell foci (clones) were then isolated and propagated. The maintenance medium contained 300 μg/mL G418.

Human SR-BI and CD36 Immunoblotting. Total cell proteins from either normal HepG2 cells, vector-transfected cells, or antisense RNA expressing cells were extracted by 1.4% Triton X-100 solubilization (*45*). Proteins (25−50 μg) were separated on 10% reducing SDS−PAGE and immunoblotted on nitrocellulose with anti-mouse SR-BI polyclonal antibody (Novus Biologicals) at 1:4000 or with anti-human CD36 polyclonal antibody (Research Diagnostics) at 1:250 followed by enhanced chemiluminescence detection on Kodak Biomax ML film. Protein expression was measured by densitometric scanning and analyzed with ImageQuant 5.2 software (Molecular Dynamics, Sunnyvale, CA). Results are based on averages of at least five different gel runs from different protein extractions.

Preparation, Modification, and Labeling of Lipoproteins. Human normolipidemic plasma (Royal Victoria Hospital, Montreal, Quebec, Canada) was supplemented with 0.01% ethylenediaminetetraacetate (EDTA), 0.02% sodium azide, 10 μM phenylmethanesulfonyl fluoride (PMSF), and 20 μM Trolox before the isolation of lipoproteins, which was achieved by ultracentrifugation as described in ref *46*. Human LDL ($d = 1.025-1.063$ g/mL), total HDL ($d = 1.063-1.21$ g/mL), and HDL_3 ($d = 1.125-1.21$ g/mL) were prepared as in ref *6*. LDL and HDL_3 contained no detectable amount of apoE as assessed by immunoblotting. Standardly oxidized LDL were prepared with 5 μM $CuSO_4$ as in ref *47* and characterized as in ref *9*. Oxidation was conducted after the radioiodination of proteins (^{125}I). LDL, OxLDL, and HDL_3 were iodinated by a modification (*48*) of the iodine monochloride method of McFarlane (*49*). One millicurie of sodium [^{125}I]iodide was used to iodinate 2.5 mg of LDL or HDL_3 in the presence of 30 nmol (10 nmol for HDL_3) of iodine monochloride in 0.5 M glycine−NaOH, pH 10. Free iodine was removed by gel filtration on Sephadex G-25 followed by dialysis in Tris-buffered saline (TBS). The specific radioactivity ranged from 100000 to 250000 cpm/μg of protein. LDL and HDL_3 were labeled with [^{3}H]cholesteryl oleate (CE) essentially as described by Roberts et al. (*50*). Thereafter, the labeled lipoproteins were reisolated by ultracentrifugation. The specific activity of lipoproteins labeled in CE ranged from 6800 to 11900 cpm/μg of protein. To ensure that [^{125}I]lipoproteins, ^{3}H-CE−lipoproteins, and their unlabeled counterpart did not behave differently one from another in their binding to HepG2 cells and adequately traced CE selective uptake, displacement curves of the binding of [^{125}I]lipoproteins by ^{3}H-CE−lipoproteins and

unlabeled lipoproteins were generated. Cells in 12-well plates were incubated for 2 h at 4 °C as for binding assays (see below) with a total ligand concentration (20 μg/mL) that was kept constant, but containing an increasing proportion of ^{3}H-CE-labeled or unlabeled competitor (from 100% to 5% [^{125}I]-lipoprotein, corresponding to 0−95% competitor). Experimental and theoretical displacement curves were compared, and no marked difference was evident ($n = 4$ for ^{3}H-CE-labeled and $n = 3$ for unlabeled competitors).

Lipoprotein Binding Assays. The cells were washed twice with 1 mL of phosphate-buffered saline (PBS). Cells were incubated for 2 h at 4 °C with 10 μg of protein/mL of [^{125}I]-lipoprotein (LDL or HDL_3) in a total volume of 250 μL containing 125 μL of MEM (2×) plus 4% bovine serum albumin (MEM−BSA) and 25 mM HEPES, pH 7.4 (total binding). Nonspecific binding was determined by the addition of 2 mg of protein/mL of unlabeled LDL or HDL_3. At the end of the incubation, the cells were washed twice with 1 mL of PBS plus 0.2% BSA (PBS−BSA) followed by two washes with 1 mL of PBS. The cells were then homogenized in 1.5 mL of 0.2 N NaOH. Radioactivity counts in the homogenates were obtained with a Cobra II counter (Canberra-Packard), and cell protein content was estimated. The specific binding was calculated by subtracting the nonspecific binding of [^{125}I]lipoprotein from the total binding.

Lipoprotein Cell Association and Degradation Assays. Cell association of ^{125}I-LDL and ^{125}I-HDL_3 (10 μg of protein/mL) was measured at 37 °C for 3 h in 12-well plates, as for the binding studies, but without HEPES. When needed, cells in 24-well plates were preincubated with SR-BI/BII antiserum (polyclonal Ab) or control nonimmune rabbit IgG at 600 μg/mL (1:20 dilution). At the end of the incubation, the dishes incubated with [^{125}I]lipoprotein were processed as for the binding studies. The results are expressed in micrograms of lipoprotein protein per milligram of cell protein. Association of ^{3}H-CE−lipoprotein (10 μg of protein/mL) was determined by in situ delipidation of cell monolayers with hexane/2-propanol, 3:2 (v/v), as in ref *6*. Associated ^{3}H-CE was quantitated by liquid scintillation counting (Wallack beta counter). To compare the association of lipoproteins labeled in protein (^{125}I) or in CE (^{3}H), the association data of ^{3}H-CE−lipoprotein were estimated as micrograms of protein per milligram of cell protein (apparent uptake). To achieve this, the specific activity of ^{3}H-CE−lipoprotein was expressed in counts per minute per microgram of lipoprotein protein. To assess degradation, trichloroacetic acid (TCA) was used at a final concentration of 12%, and degradation was estimated from the incubation medium as the non-iodine TCA-soluble fraction. ^{3}H-CE association due to selective uptake is calculated as the total ^{3}H-CE association minus the [^{125}I]-protein association and degradation. In some studies, cell-associated [^{125}I]protein radioactivity was sorted into surface-bound and internalized fractions. After the 3 h incubation and washes, cells were treated with 0.25% trypsin in PBS plus 1 mM EDTA for 15 min at 37 °C, gently detached from the wells, pelleted in microcentrifuge tubes, and washed once with PBS. Radioactivity in the two supernatants and the cell pellet (solubilized in 0.2 N NaOH) was counted and represents surface-bound and internalized lipoproteins, respectively. The efficiency of the trypsin protocol to sort bound/internalized lipoproteins was tested after binding assays at 4 °C and was found to detach 81 ± 7% of ^{125}I-LDL ($n = 3$) and 75 ± 6% of ^{125}I-HDL_3 ($n = 3$) bound to the cells.

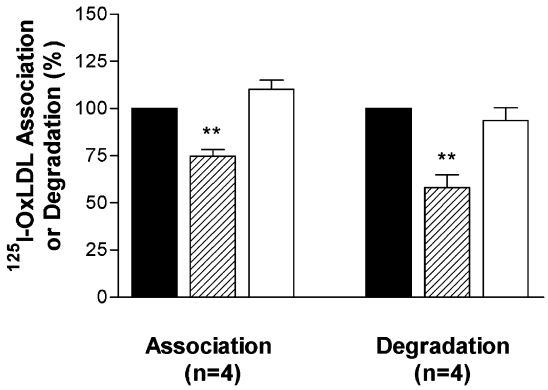

FIGURE 1: ^{125}I-OxLDL protein association and degradation in HepG2 cells in the presence of FA6-152 anti-human CD36 monoclonal antibody. After preincubation without antibody (closed bars) or with 20 μg/mL CD36 monoclonal antibody (hatched bars) or control nonimmune mouse IgG (open bars) for 20 min at 37 °C, radiolabeled ^{125}I-OxLDL (10 μg of protein/mL) were incubated with the cells for an additional 3 h at 37 °C in 12-well plates in duplicate. Nonspecific protein association/degradation was determined by the addition of unlabeled lipoprotein at 2 mg of protein/mL and subtracted from total values for each condition. Then, the HepG2 cell value (μg of protein/mg of cell protein) in each experiment was set as 100%. Results are shown as mean ± sem. * = statistically different (at least $p < 0.05$) from the value of HepG2 cells and HepG2 cells plus nonimmune IgG.

Flow Cytometry with DiI−LDL-Labeled Cells. For the analysis of DiI−LDL uptake, cells were seeded in 6-well plates. DiI-labeled LDL (5 μg of protein/mL) was incubated with cells for 90 min at 37 °C in a total volume of 500 μL containing 250 μL of MEM−BSA without HEPES. When needed, cells were incubated with SR-BI/BII antiserum (polyclonal Ab) or control nonimmune rabbit IgG at 300 μg/mL. Cells were then washed once with PBS−BSA and once with Ca- and Mg-free PBS and incubated for 30 min at 4 °C with PBS plus 2 mM EDTA to detach them from the plate and then treated for 5 min at 37 °C with 0.125% trypsin in PBS plus 1 mM EDTA to achieve complete dissociation of the cells. Cells were pelleted, washed once, and resuspended in Ca- and Mg-free PBS on ice for flow cytometry analysis (excitation 514 nm, emission 575 nm; FACS Vantage, Becton-Dickinson). Autofluorescence of unlabeled cells was determined and subtracted from total fluorescence. Mean fluorescence intensity was recorded and converted to percent values, with HepG2 cell fluorescence set as 100%.

Other Methods. Protein content was determined by the method of Lowry (*51*) with BSA as standard. Paired Student's *t* test or ANOVA-1 (with Tuckey's post-test) was used to obtain statistical comparison of the data. Differences were considered significant at $p < 0.05$.

RESULTS

Demonstration of the Functional Presence of CD36 in HepG2 Cells. We have previously shown that HepG2 cells can selectively take LDL− and HDL_3−CE (*9*) by a pathway defined by the class B scavenger receptor ligands. Although CD36 had been detected earlier in HepG2 cells (*27*), its function was never assessed in this cell model. CD36 was first cloned as an OxLDL receptor (*31*). Thus, we conducted OxLDL association and degradation studies in HepG2 cells

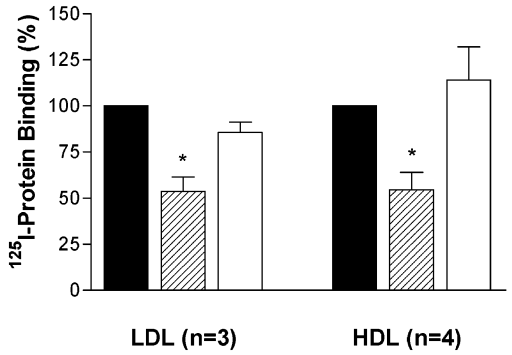

FIGURE 2: ^{125}I-LDL and ^{125}I-HDL$_3$ protein binding in HepG2 cells in the presence of SR-BI/BII antiserum (polyclonal antibody). After preincubation without antibody (closed bars) or with 600 μg/mL SR-BI polyclonal antibody (pAb) (hatched bars) or control nonimmune rabbit IgG (open bars) for 20 min at 4 °C, radiolabeled lipoproteins (10 μg of protein/mL) were incubated with the cells for 2 h at 4 °C in 24-well plates in duplicate. Nonspecific protein binding was determined by the addition of unlabeled lipoprotein at 2 mg of protein/mL and subtracted from total binding for each condition. Then, the HepG2 cell value (in μg of protein/mg of cell protein) in each experiment was set as 100%. Results are shown as mean ± sem. * = statistically different (at least $p < 0.05$) from the value of HepG2 cells and HepG2 cells plus nonimmune IgG.

in the presence of FA6-152 anti-human CD36 monoclonal antibody. Figure 1 reveals that CD36 is responsible for 25% of ^{125}I-OxLDL association but as much as 42% of ^{125}I-OxLDL degradation. Thus, CD36 is functionally present in HepG2 cells. Using the same antibody in ^{125}I-LDL binding and LDL−CE selective uptake experiments was without effect (data not shown), indicating that CD36 is not significantly involved in LDL binding or in LDL−CE selective uptake in HepG2 cells.

[^{125}I]Lipoprotein Binding and ^{3}H-CE Selective Uptake in the Presence of SR-BI/BII Blocking Antibody. Studies conducted in the presence of SR-BI/BII blocking antibody (*52*) during 2 h at 4 °C demonstrated that ^{125}I-LDL binding was reduced to 54% ($p < 0.05$), while ^{125}I-HDL$_3$ was reduced to 55% ($p < 0.05$) of the control values in the presence of nonimmune IgG (Figure 2). To define the importance of SR-BI in CE selective uptake, ^{3}H-CE association experiments were also undertaken in the presence of blocking antibody. As shown in Figure 3A, the antibody reduced the ^{125}I-LDL and ^{125}I-HDL$_3$ protein association by 66% ($p < 0.001$) and 58% ($p < 0.001$), respectively. The effect was stronger with ^{3}H-CE−LDL and ^{3}H-CE−HDL$_3$ association (Figure 3B), with 82% and 91% decreases, respectively ($p < 0.05$). Thus, antibody blockade studies show that SR-BI is responsible for 80% of total CE uptake in HepG2 cells. When CE association due to selective uptake is considered (Figure 3C), 87% of selective uptake is due to SR-BI in HepG2 cells. Trypsin treatment of cells incubated with ^{125}I-LDL and ^{125}I-HDL$_3$ for 3 h at 37 °C has shown that ^{125}I-LDL binding to the cell surface accounts for 17 ± 5% ($n = 5$) of the tracer association and ^{125}I-HDL$_3$ for 46 ± 6% ($n = 4$) of the tracer association. Correcting these values for the efficiency of the trypsin treatment on lipoprotein binding (see Experimental Procedures) shows that 21% of ^{125}I-LDL and 61% of ^{125}I-HDL$_3$ are bound at the end of 37 °C association assays. The reduction in lipoprotein binding due to SR-BI/BII blocking antibody could not account for the 66% decrease in ^{125}I-LDL protein association at 37 °C. The ^{125}I-LDL mass that

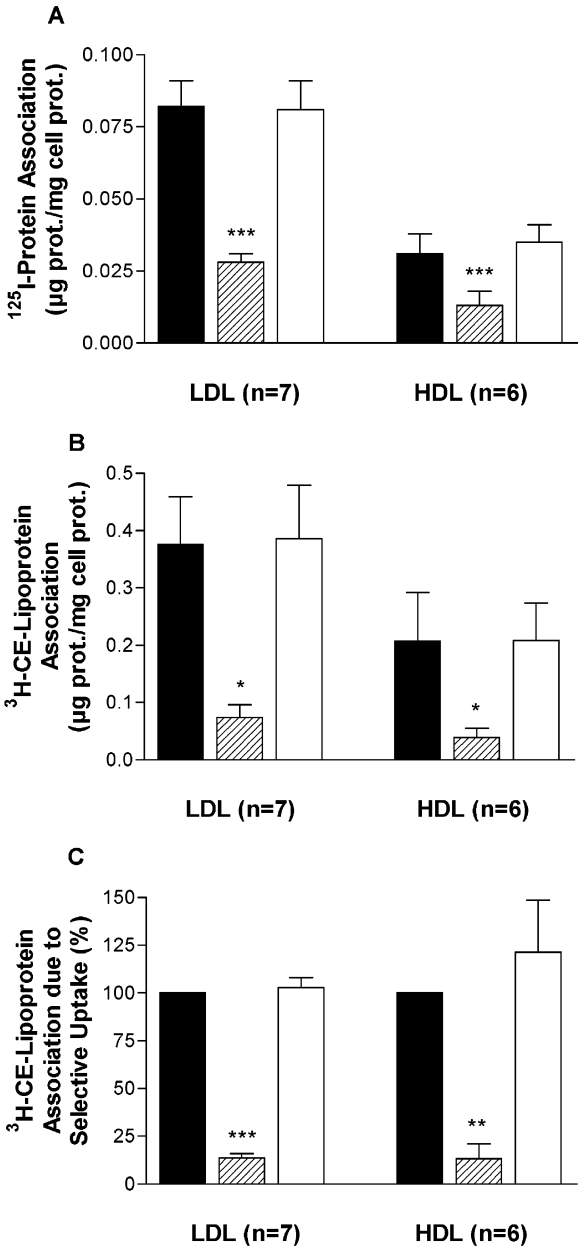

FIGURE 3: ^{125}I-LDL and ^{125}I-HDL$_3$ protein association (panel A), ^{3}H-CE−LDL and ^{3}H-CE−HDL$_3$ association (panel B), and ^{3}H-CE−lipoprotein association due to cholesteryl ester selective uptake (panel C) in HepG2 cells in the presence of SR-BI/BII antiserum (polyclonal antibody). After preincubation without antibody (closed bars) or with 600 μg/mL SR-BI pAb (hatched bars) or control nonimmune rabbit IgG (open bars) for 20 min at 37 °C, radiolabeled lipoproteins (10 μg of protein/mL) were incubated with the cells for an additional 3 h at 37 °C in 24-well plates in duplicate. Nonspecific protein association was determined by the addition of unlabeled lipoprotein at 2 mg of protein/mL and subtracted from total association for each condition. To determine the amount of CE association due to selective uptake, protein association was subtracted from CE association, both in μg of protein/mg of cell protein. Then, the HepG2 cell value in each experiment was set as 100%. Mean ± sem values for LDL−CE association due to selective uptake were 0.234 ± 0.076 (total), 0.046 ± 0.019 (with SR-BI pAb), and 0.305 ± 0.085 μg/mg of cell protein (with control IgG) and for HDL$_3$−CE association were 0.176 ± 0.078 (total), 0.025 ± 0.012 (with SR-BI pAb), and 0.174 ± 0.006 (with nonimmune IgG) μg/mg of cell protein. * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$, statistically different from the HepG2 cell value.

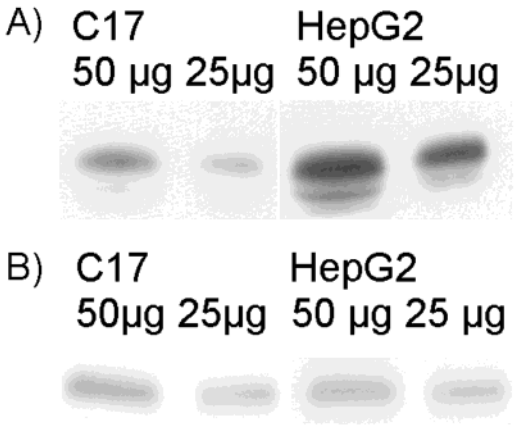

FIGURE 4: SR-BI (panel A) and CD36 (panel B) immunoblotting in the HepG2 cell and in the HepG2 cell clone 17 (C17) expressing constitutive SR-BI antisense RNA. Total cell proteins were extracted with 1.4% Triton X-100, and 25−50 μg of protein was separated on 10% reducing SDS−PAGE and immunoblotted with anti-mouse SR-BI polyclonal antibody (Novus Biologicals) at 1:4000 or anti-human CD36 polyclonal antibody (Research Diagnostics) at 1:250 followed by enhanced chemiluminescence detection. The blot is representative of five experiments. Differences in expression were quantified by densitometric scanning (Imagequant Software, Molecular Dynamics).

is lost in the presence of blocking antibody exceeds the binding of LDL to cells, while the ^{125}I-HDL$_3$ mass is equivalent to the binding of lipoproteins to cells. This suggests that, in HepG2 cells, SR-BI inhibition has some influence on LDL endocytosis but not on HDL endocytosis as previously suggested (*53)*.

Inhibition of SR-BI Expression in HepG2 Cells by an Antisense RNA Strategy. The antibody strategy that we used to demonstrate the implication of SR-BI could be criticized, as an antibody to one receptor can impede the binding to a proximal receptor by steric hindrance. Furthermore, although this rabbit antiserum has been used by others (*52*, *54*), we cannot reject completely that the rabbit serum lipoproteins can partially compete with the labeled lipoproteins used in the assays. Thus, to demonstrate clearly the implication of human SR-BI in the hepatic metabolism of LDL−CE, we decided to attenuate its expression by a constitutive RNA antisense strategy. HepG2 cells were transfected with an expression vector (pRc/CMV) containing a 0.4 kb fragment of the 5′ end of the human SR-BI cDNA inserted head to tail. Cells resistant to G418 were obtained, and clones were propagated. From the 24 clones tested, seven did not show a reduction in SR-BI expression, nine exhibited a 20−30% inhibition, six a 30−50% inhibition, and two a >50% inhibition (data not shown). We chose to work with clone 17 (C17), which has a similar morphology and growth rate compared to HepG2 cells. Clones with a mild inhibition (20−30%) did not show a detectable fall in CE selective uptake, while clones with a moderate inhibition (30−50%) had a small reduction in LDL−CE uptake, an effect that did not reach statistical significance (data not shown). As shown in Figure 4 (panel A), C17 cells have a 70 ± 4% ($p < 0.01$) inhibition of SR-BI protein expression. Since there was virtually no difference in expression of SR-BI between HepG2 cells transfected with an empty vector and the parental cell line, HepG2 cell results are shown throughout this study. The 70% reduction in SR-BI expression did not modify either the CD36 expression level (Figure 4B) or the LDLr expression level on Western blots (data not shown).

LDL− and HDL$_3$−CE Selective Uptake in Cells with Attenuated SR-BI Expression. CE selective uptake experiments were performed in order to confirm that SR-BI has a role in both LDL− and HDL$_3$−CE selective uptake. HepG2 and C17 cells were incubated with 10 μg of protein/mL of radiolabeled lipoproteins for 3 h at 37 °C. As shown in Figure 5A, the 70% reduction in SR-BI expression in C17 resulted in a 29% ($p < 0.01$) decrease in ^{125}I-LDL−protein association, while ^{125}I-HDL$_3$−protein association was decreased by 42% ($p < 0.05$), corresponding to 41% and 60% decreases, assuming a 100% inhibition. SR-BI has been reported previously to mediate the selective uptake of CE by a mechanism that does not require lipoprotein particle endocytosis (*33*, *34*). Thus, if this was true in HepG2 cells with attenuated SR-BI, the decrease in protein association should not exceed LDL (21% of total association) and HDL$_3$ (61% of total association) binding to cells. Again, the significant reduction in cell association of both LDL and HDL$_3$ in cells with attenuated SR-BI shows that SR-BI may influence LDL particle endocytosis but not HDL endocytosis. ^{3}H-CE association experiments at 37 °C show that C17 cells have a 46% ($p < 0.05$) reduction in ^{3}H-CE−LDL association and a 55% ($p < 0.01$) reduction in ^{3}H-CE−HDL$_3$ association (Figure 5B). Since the ^{3}H-CE association for both lipoproteins (expressed as apparent protein association in micrograms of lipoprotein/mg of cell protein) exceeded the protein association, selective uptake was operational in our cells. By calculating the cell-associated ^{3}H-CE due to selective uptake and setting the HepG2 cell value to 100%, we show that the 70% reduction in SR-BI expression in C17 cells caused a 47% ($p < 0.01$) and 53% ($p < 0.001$) reduction in the ^{3}H-CE association due to selective uptake for LDL and HDL$_3$ (Figure 5C). If ^{3}H-CE selective uptake is normalized for SR-BI expression, we can estimate that SR-BI is responsible for 68% (LDL) and 74% (HDL$_3$) of all CE selective uptake in HepG2 cells.

General LDL Lipid Uptake in Cells with Attenuated SR-BI or LDLr Expression. To confirm our results concerning the SR-BI-mediated selective uptake of LDL−CE, we have used 5 μg of protein/mL of LDL labeled with the phospholipid tracer DiI-C18, an indocarbocyanin fluorescent molecule, in flow cytometry experiments. As shown in Figure 6, C17 cells had a 53% reduction ($p < 0.001$) in DiI−LDL uptake compared to normal HepG2 cells. It becomes evident that reducing SR-BI expression has an impact on the uptake of LDL−lipids in HepG2 cells. Moreover, the addition of anti-SR-BI/BII polyclonal antibody during the incubation with DiI-LDL further reduced the DiI−LDL uptake to 26 ± 3% ($p < 0.001$) for HepG2 cells and to 24 ± 2% ($p < 0.05$) for C17 cells, compared to cells incubated without antibody. Values for HepG2 cells and C17 cells in the presence of blocking antibody were not statistically different. Thus, SR-BI accounts for 75% of LDL−phospholipid uptake from LDL in HepG2 cells. We have previously produced a pool of HepG2 cells with an 85% inhibition of LDLr expression (*7*, *8*). As shown in Figure 6, the addition of anti-LDL receptor monoclonal antibody (IgG C7) and anti-SR-BI polyclonal antibody reduced DiI−LDL uptake by 89% in the LDLr-deficient (LDLr−) cells. The additive blockade of LDLr and SR-BI activities indicates that the LDLr is

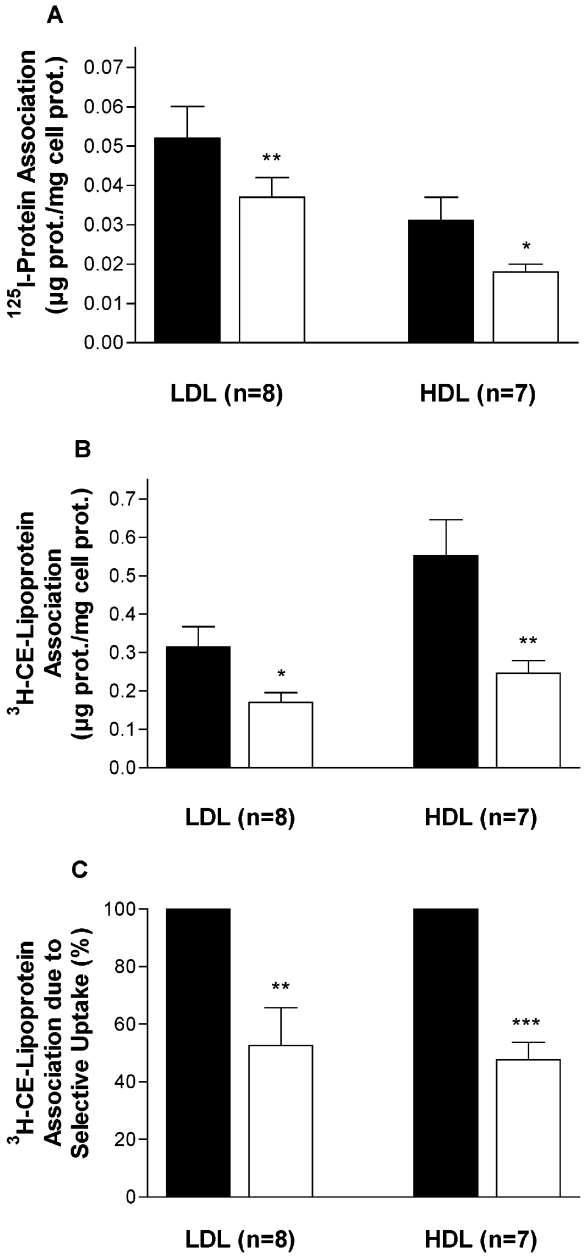

FIGURE 5: ^{125}I-LDL and ^{125}I-HDL$_3$ protein association (panel A), ^{3}H-CE−LDL and ^{3}H-CE−HDL$_3$ association (panel B), and ^{3}H-CE−lipoprotein association due to cholesteryl ester selective uptake (panel C) in HepG2 cells and HepG2 cells 70% deficient in SR-BI (C17). Radiolabeled lipoproteins (10 μg of protein/mL) were incubated with cells during 3 h at 37 °C in 12-well plates in triplicate. Nonspecific radioactive lipoprotein association was determined by the addition of unlabeled lipoprotein at 2 mg of protein/mL and subtracted from total association. ^{3}H-CE−lipoprotein association is calculated as apparent protein association since its specific activity is calculated as cpm/μg of lipoprotein protein. To determine the amount of CE association due to selective uptake, protein association was subtracted from CE association, both in μg of protein/mg of cell protein. Then, the HepG2 cell value in each experiment was set as 100%. Mean ± sem values for CE association due to selective uptake were 0.234 ± 0.034 (HepG2, closed bars) and 0.115 ± 0.029 (C17, open bars) μg/mg of cell protein and for HDL$_3$−CE association were 0.521 ± 0.094 (HepG2) and 0.229 ± 0.035 (C17) μg/mg of cell protein. * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$, statistically different from the HepG2 cell value.

responsible for ~14% of LDL−lipid uptake, while no more than 11% of LDL−lipid uptake occurs by a LDLr- and SR-BI-independent pathway.

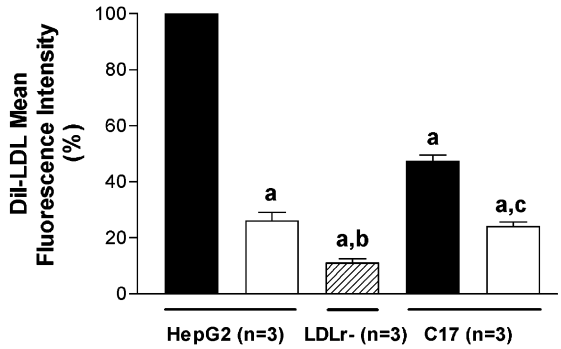

FIGURE 6: DiI−LDL uptake in HepG2 cells, HepG2 cells 70% deficient in SR-BI (C17), and HepG2 cells 85% deficient in LDL receptor (LDLr−). DiI−LDL were incubated (5 μg of protein/mL) with cells for 90 min at 37 °C in 6-well plates with (open bars) or without (closed bars) SR-BI pAb at 300 μg/mL or with LDLr mAb at 40 μg/mL (hatched bar). Cells were then washed and incubated for 30 min at 4 °C with PBS plus 2 mM EDTA to detach them from the plate and treated for 5 min at 37 °C with 12 μg/mL trypsin to achieve complete dissociation of the cells. Cells were pelleted, washed once with PBS, and resuspended in PBS for flow cytometry analysis (excitation 514 nm, emission 575 nm; FACS Vantage, Becton-Dickinson). Autofluorescence of unlabeled cells was determined and subtracted from total fluorescence. Mean fluorescence intensity was recorded and converted to percent values, with HepG2 cell fluorescence set as 100%. Key: (a) statistically different ($p < 0.001$) from the HepG2 cell value; (b) statistically different from all of the other values (at least $p < 0.05$); (c) statistically different ($p < 0.001$) from the C17 cell value in the absence of antibody.

Competition of LDL− and HDL$_3$−CE Association by LDL and HDL on HepG2 Cells. Both LDL and HDL$_3$ appear to be equally processed by the retroendocytic pathway in HepG2 cells, and both lipoproteins were affected by the reduction in SR-BI expression by either antibody blockade or antisense RNA expression. In the human context, where plasma LDL−cholesterol is more than twice the HDL−cholesterol (120 vs 50 mg/dL), it is important to determine if LDL−CE selective uptake can occur in the presence of HDL particles. Data on human SR-BI shows that LDL is as efficient as HDL to compete for HDL binding to cells (*15*), while LDL partially competes for HDL−CE association (*16*). However, murine SR-BI does not allow displacement of HDL by LDL particles (*12*). On HepG2 cells, unlabeled HDL$_3$ competes for 76% of ^{3}H-CE−LDL association to HepG2 cells, while LDL reduced its own association by 93% (Figure 7A). As expected, HDL$_3$ were not able to displace the LDLr-dependent ^{3}H-CE−LDL association, which accounts for less than 24% of total LDL−CE association (Figure 7A and ref *9*). Figure 7B shows that both LDL and HDL$_3$ competed for 71% of ^{3}H-CE−HDL$_3$ association to HepG2 cells. However, LDL were the most efficient competitors when compared on a per particle basis (Figure 7B, right panel), since the maximal effect was reached at a 5-fold lower particle concentration. When maximal inhibitory effect on ^{3}H-CE association is considered, reciprocal competition between LDL and HDL$_3$ was observed. Thus, human SR-BI may be different from murine SR-BI regarding its simultaneous interaction with LDL and HDL$_3$ particles.

Since the total plasma HDL−protein concentration is two times higher than the LDL−protein concentration in the blood,[2] we conducted competition assays with 40 μg of protein/mL of both radiolabeled lipoproteins (in order to allow high- and moderate-affinity binding) in the presence of 20 μg of protein/mL of LDL or 80 μg of protein/mL of

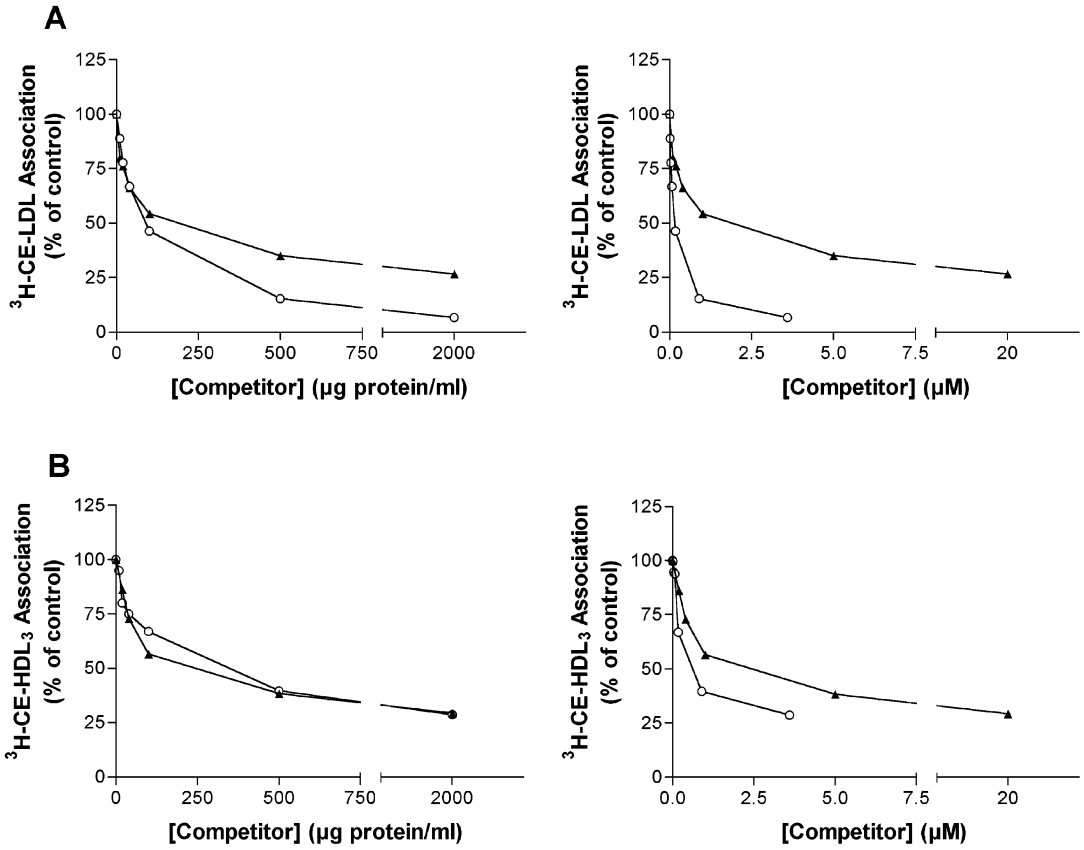

FIGURE 7: Competition curves of ^{3}H-CE−lipoprotein association in the presence of unlabeled competitors LDL and HDL$_3$. ^{3}H-CE−LDL (panel A) and ^{3}H-CE−HDL$_3$ (panel B) at 40 μg of protein/mL were incubated with cells during 3 h at 37 °C in 12-well plates in duplicate after a 20 min preincubation with unlabeled competitors at 0−2 mg/mL LDL (0−3.6 μM, open circles) and HDL$_3$ (0−20 μM, black triangles). ^{3}H-CE−lipoprotein association is calculated as apparent protein association since its specific activity is calculated as cpm/μg of lipoprotein protein. Then, the value without competitor in each experiment was set as 100%. Shown here are curves from one experiment conducted in duplicate, representative of three experiments that gave essentially identical results. The same data were plotted against competitor concentration in μg of protein/mL (left panels) or in μmol of particle/L (right panels) assuming 550 kDa protein/mol of LDL and 100 kDa protein/mol of HDL.

either total HDL (d = 1.063−1.21 g/mL) or HDL$_3$ (d = 1.125−1.21 g/mL). Then, CE association due to selective uptake was calculated. In these experiments, HDL$_3$ particle concentration was kept at ∼11 times the LDL particle concentration. Figure 8 shows that total HDL and apoE-free HDL$_3$ are equivalent in their capacity to compete for LDL−CE selective uptake, which was reduced by 45% ($p < 0.05$). LDL competed for 27% ($p < 0.05$) of HDL$_3$−CE selective uptake. These results imply that CE selective uptake from both LDL and HDL$_3$ can occur simultaneously, since neither ligand could abolish the CE selective uptake of the heterologous lipoprotein. There was no difference between the maximum effect of LDL as a competitor for HDL$_3$ or for the two types of HDL as competitors for LDL. On the basis of the lower LDL particle concentration needed to reach a similar effect, LDL is a more efficient competitor for selective uptake than HDL.

LDL− and HDL$_3$−CE Selective Uptake by Primary Cultures of Hepatic Cells from Normal and SR-BI Knockout Mice. To define the importance of mouse hepatic SR-BI in LDL− and HDL$_3$−CE selective uptake, association assays were conducted with primary cultures of hepatic cells obtained from normal (+/+), heterozygous (−/+), and homozygous (−/−) SR-BI knockout mice. This mouse model provided us with cells that are 50% or 100% deficient in SR-BI and gave the opportunity to determine if CE selective uptake is correlated to SR-BI expression in hepatic cells. Figure 9 shows that (−/+) hepatic cells lost 45% of their ability to selectively take up CE from LDL and HDL$_3$, while the (−/−) hepatic cells lost 87%. We conclude that in mouse hepatic cells CE selective uptake is proportional to SR-BI level, that SR-BI is responsible for the major part of this pathway, and that there is no significant backup mechanism for CE selective uptake when SR-BI is absent in murine hepatic cells.

DISCUSSION

In this study, we have been successful in reducing the expression and/or activity of human SR-BI by two different approaches in a completely autologous system, including HepG2 human hepatoma cells, human antisense RNA, and assays with defined human lipoproteins, where SR-BI levels are not manipulated by the culture conditions. The first approach took advantage of a commercial polyclonal antibody directed against the extracellular domain of SR-BI, which is identical in SR-BII (*55*). Albeit its low titer, this antibody has been reported, while this study was conducted,

[2] These were calculated as follows: LDL contain 20−22% protein and 45% total cholesterol. Total HDL contain 50% protein and 20% total cholesterol. From the total cholesterol/protein ratio and the human plasma LDL− and HDL−cholesterol (100 vs 50 mg/dL) values, there is 2.1 times more HDL−protein than LDL−protein.

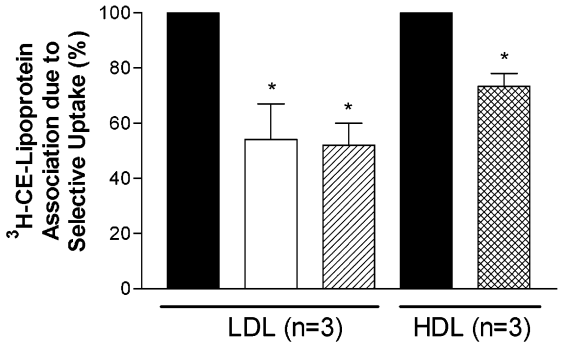

FIGURE 8: ^{3}H-CE−lipoprotein association due to cholesteryl ester selective uptake in the presence of physiological amounts of lipoprotein competitors. ^{3}H-CE−lipoproteins (40 μg of protein/mL) were incubated with cells during 3 h at 37 °C in 12-well plates in triplicate after a 20 min preincubation without (closed bars) or with unlabeled lipoprotein competitor at 80 μg of protein/mL of HDL_3 (open bar) or 80 μg/mL total HDL (hatched bar) or 20 μg of protein/mL of LDL (cross-hatched bar). Nonspecific radioactive lipoprotein association was determined by the addition of unlabeled lipoprotein at 2 mg of protein/mL and subtracted from total association. ^{3}H-CE−lipoprotein association is calculated as apparent protein association since its specific activity is calculated as cpm/μg of lipoprotein protein. To determine the amount of CE association due to selective uptake, protein association was subtracted from CE association, both in μg of protein/mg of cell protein. Then, the value without competitor in each experiment was set as 100%. Mean ± sem values for CE association due to selective uptake were 0.522 ± 0.082 (LDL) and 0.233 ± 0.062 (HDL_3) μg/mg of cell protein. * = $p < 0.05$, statistically different from the value without competitor.

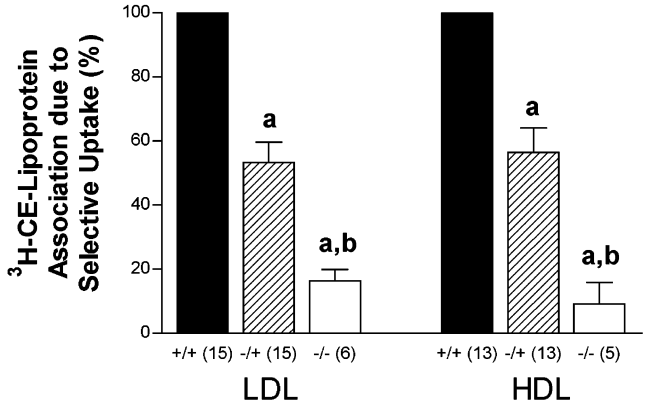

FIGURE 9: ^{3}H-CE−lipoprotein association due to cholesteryl ester selective uptake in primary cultures of hepatic cells from control (+/+), SR-BI heterozygous (−/+), and SR-BI null (−/−) mice. SR-BI knockout mice were bred and genotyped as described in Experimental Procedures. Primary cultures of mouse hepatic cells were obtained by in situ perfusion with collagenase as described in Experimental Procedures. Radiolabeled lipoproteins (20 μg of protein/mL) were incubated with cells during 3 h at 37 °C in 12-well plates in triplicate. Nonspecific radioctive lipoprotein association was determined by the addition of unlabeled lipoprotein at 2 mg of protein/mL and subtracted from total association. ^{3}H-CE−lipoprotein association is calculated as apparent protein association since its specific activity is calculated as cpm/μg of lipoprotein protein. To determine the amount of CE association due to selective uptake, protein association was subtracted from CE association, both in μg of protein/mg of cell protein. Then, the control mouse (+/+) value in each experiment was set as 100%. Results are shown as mean ± sem of the number of mice indicated in parentheses. CE association due to selective uptake was 0.962 ± 0.149 μg of protein/mg of cell protein for LDL and 0.699 ± 0.199 μg of protein/mg of cell protein for HDL_3 (mean ± sem) in control (+/+) mice. Key: (a) statistically different ($p < 0.001$) from the control mice value; (b) statistically different ($p < 0.001$) from the heterozygous (−/+) mice value.

to inhibit SR-BI-mediated HDL−CE selective uptake at similar concentrations (*52*). Our results show that this antibody is also a useful tool to block LDL−CE selective uptake and LDL/HDL binding. Second, a constitutive antisense RNA strategy was designed on the basis of a human SR-BI cDNA fragment that includes the beginning of the coding sequence. Antisense RNA from the 5′ end are believed to be more efficient (*56*). A 70% inhibition of human SR-BI expression was obtained, as measured by repeated immunoblotting during the course of these experiments. Our results show for the first time that human SR-BI is implicated not only in a well-known HDL−CE selective uptake process but also in LDL−CE selective uptake in hepatocyte-derived cells. Both approaches show that SR-BI is responsible for the majority of CE selective uptake in a human liver cell model. In our hands, SR-BI mediates 68−87% of LDL−CE selective uptake and 74−87% of HDL_3−CE selective uptake, depending on the approach considered. A similar conclusion was reached with the study of primary cultures of hepatic cells from normal and SR-BI knockout mice. Selective uptake by SR-BI is not limited to lipoprotein−CE: phospholipids and triglycerides can also be taken up from lipoprotein particles (*57*, *58*). Our results with the phospholipid tracer DiI show that LDL−lipid uptake in HepG2 cells is mediated by SR-BI (∼75%) and by the LDLr as a minor (∼14%) contributor. These results confirm our previous reports with LDLr-deficient cells (*7*, *8*) showing that selective uptake by SR-BI brings more lipids to HepG2 cells than the LDLr in vitro. Thus, this study is the first to quantify the contribution of SR-BI in HDL_3−CE and LDL−CE selective uptake in both human and mouse hepatic cell systems and to demonstrate that hepatic SR-BI expressed at normal levels can process LDL−CE. In vivo studies with SR-BI transgenic mice have demonstrated that SR-BI overexpression increases (V)LDL−cholesterol catabolism more than apoB-100 catabolism (*25*). These studies imply that hepatic SR-BI has direct and/or indirect effects on LDL particle catabolism. Our study conducted on human HepG2 and mouse hepatic cells shows that SR-BI is responsible for the bulk of LDL−CE selective uptake, similarly to HDL_3−CE selective uptake. Thus, hepatic SR-BI can have direct effects on LDL−lipids.

In human blood, HDL particles are more abundant than LDL particles. Our results show that, in HepG2 cells, LDL−CE selective uptake can occur in the presence of physiological proportions of bulk HDL or HDL_3 competitors and vice versa. HDL and LDL particles, albeit the >11-fold higher particle concentration for HDL, had roughly the same effect on CE selective uptake. This may be related to the higher lipid content and particle mean diameter of LDL compared to HDL. It has been reported that small LDL (LDL-III) are better substrates for selective uptake in vitro (*3*), but the detailed interaction between SR-BI and LDL subclasses is still unknown. Thus, LDL−CE selective uptake is likely to exist in the liver in species where there are important amounts of LDL−cholesterol, and both LDL− and HDL_3−CE will occur at a lower rate than in in vitro assays with isolated ligands. Human LDL are CE-rich particles (∼40% w/w, 1300 molecules) that can donate higher amounts of lipids to cells than HDL, albeit the lower LDL particle concentration (as in this study where 20 μg of protein/mL is ∼36 nM for LDL

particles and ~200 nM for HDL_3 particles) and lower fractional uptake of CE from LDL particles compared to HDL_3 particles (*19*). The characteristics of LDL particles after the CE selective uptake process, especially their pro- or anti-atherogenic behavior, will have to be defined in future experiments. However, our previously published results have shown that LDL incubation with HepG2 cells can reduce significantly the CE content of these particles (*6*). While this depletion of LDL−lipids appears to be desirable at first sight (a reduction in LDL−cholesterol), the interaction of lipid-depleted LDL with the LDLr has not been defined. Are lipid-depleted LDL similar to small dense LDL in having a reduced binding to the LDLr (*59*)? Reduced interaction of LDL with the LDLr impacts negatively on the proper control of LDL particle concentration via the uptake of whole LDL particles. Alternatively, the lipid-depleted LDL may be a competent ligand for whole particle clearance by the LDLr as suggested by the increased clearance of apoB100 in SR-BI transgenic mice (*25*). From the compelling evidence in mice, SR-BI is an anti-atherogenic molecule. SR-BI gene disruption leads to the formation of abnormal HDL particles and increased coronary atherosclerosis (*60*). Conversely, mild hepatic SR-BI overexpression reduces atherosclerosis, while high overexpression, accompanied by a severe decrease in anti-atherogenic HDL, worsens the atherogenic apoB hemizygous phenotype (*61*). Adenovirus-mediated SR-BI hepatic expression also attenuates atherosclerosis in LDLr knockout mice fed a Western diet (*62*). In human, LDL−cholesterol clearance by SR-BI may represent an additional mechanism to eliminate CE transferred from HDL particles by cholesteryl ester transfer protein (CETP). Understanding the changes induced by SR-BI in lipoprotein structure, especially LDL, will be crucial for the proper evaluation of human SR-BI as part of anti-atherosclerotic strategies.

Our results indicate that LDLr- and SR-BI-independent LDL−lipid uptake represents one-tenth of total lipid uptake and likely does not involve CD36. However, our study establishes clearly that CD36 leads to the degradation of OxLDL particles in HepG2 cells. Our results are in agreement with experiments by Villiers et al. (*30*). They demonstrated that CD36 transfected in COS cells does not interact with LDL, while it is a bona fide OxLDL receptor. We also show that more than half of the OxLDL degradation is due to another receptor (Figure 1). On the basis of the recent study by Gillotte-Taylor and colleagues (*63*), it is likely that SR-BI is another OxLDL receptor in HepG2 cells. They have shown that SR-BI transfected in CHO cells leads to the degradation of OxLDL but not to selective uptake of OxLDL lipids. The efficient uptake and degradation of OxLDL by SR-BI in vivo, while not yet demonstrated, could be an additional anti-atherogenic role for the receptor. Looking for the identity of the receptor involved in the SR-BI-independent LDL−CE selective uptake activity, we investigated if the class A scavenger receptor ligand, poly(inosinic acid) [poly-(I)], had an effect on LDL and HDL_3 association in HepG2 cells and found none, excluding a role for this class of receptors (D. Rhainds, personal data). SR-BI-independent pathways for the LDL−CE selective uptake have been reported: one implicating lipoprotein lipase and proteoglycans in CHO cells and in muscles in vivo (*22*) and the other implicating apoE and a RAP-sensitive α_2-macroglobulin receptor, likely the LDLr-related protein (LRP), in adrenocortical cells (*64*). Recent findings on human adipocytes clearly show that the LRP is able to mediate the selective uptake of HDL−CE (*65*). In HepG2 cells, such a pathway would involve chondroitin sulfate proteoglycans and their extracellular apoE blanket (*66*). In our study, HDL_3 were used to avoid the direct interaction of apoE from total HDL with the LRP or the LDLr. However, interaction of LDL and HDL_3 particles and the consecutive enrichment in apoE may lead to selective uptake by LRP. The role of LRP in the SR-BI-independent CE selective uptake, which represents $\leq$30% of CE selective uptake in HepG2 cells, is under investigation.

In SR-BI low expressor (C17) cells and HepG2 cells treated with SR-BI/BII blocking antibody, ^{125}I-LDL association and ^{125}I-HDL_3 association were significantly decreased. This is in agreement with previous studies where SR-BI expression is correlated with HDL binding to cells (*33*, *34*). In this study, the reduction in HDL protein association due to SR-BI blockade/inhibition was equivalent to the bound fraction in association assays (61% of total association, as seen by trypsin treatment of cells), while the reduction in LDL protein association exceeded the bound fraction in association assays (21% of total association), suggesting that SR-BI may influence LDL, but not HDL_3, endocytosis. Indeed, a previous study showed that SR-BI transfected cells had an increase in LDL protein degradation (*15*). A direct effect of SR-BI on LDL endocytosis or an indirect effect, on LDLr-mediated endocytosis, may be present. An important study by Silver et al. (*53*) has demonstrated that SR-BI in murine hepatocytes is an endocytic receptor that trafficks in the endosomal recycling compartment (ERC) and is recycled to the basolateral (sinusoidal) pole. HDL−lipids are directed toward the apical pole (biliary canaliculus) for secretion, while CE-depleted particles are resecreted basolaterally. This study also shows that SR-BI does not lead to degradation of HDL−apolipoproteins, which appears to depend on another hepatic receptor (*67*). While the first steps of lipoprotein CE selective uptake in murine hepatocytes appear to involve binding to SR-BI and internalization of ligand−receptor complexes, the precise mechanism of CE transfer to cells and/or membranes is still elusive. Transfer of CE may involve both hemifusion between lipoprotein and membrane phospholipids or a hydrophobic channel for neutral lipids such as CE and TG created by the extracellular domain of SR-BI (*68*). Accordingly, purified SR-BI per se mediates CE incorporation into membranes (*69*). In cells such as adrenocortical cells and ovarian granulosa cells (*70*, *71*), SR-BI is located in microvillar channels. These are closely juxtaposed microvilli where both LDL and HDL can be trapped at the cell surface (*72*). It has been proposed that lipoproteins in microvillar channels are substrates for CE selective uptake by SR-BI (*71*). Both hepatic cell lines (*73*) and hepatocytes (*74*, *75*) exhibit microvilli at their sinusoidal pole, while isolated hepatocytes are covered with numerous microvilli (*76*). HepG2 cells also have morphologically visible microvilli in electron microscopy images (*27*). However, to our knowledge, there is no report on these microvilli forming a microvillar channel compartment in hepatocytes. Thus, we cannot exclude that such a compartment participates in CE selective uptake in HepG2 cells. Additional studies are required to distinguish between the implication of human SR-BI in retroendocytosis of lipopro-

tein particles and cell (surface microvillar) selective uptake in hepatocytes.

HepG2 cells will be an excellent system to study the sorting of lipids from LDL and HDL by human SR-BI, since they are polarized cells; i.e., they form a closed biliary vacuole, where secretion and reuptake of lipids occur (*77*). Another interesting feature of HepG2 cells is the absence of caveolae. Our results show that both HDL_3 and LDL selective uptake transfers nonnegligible amounts of CE to HepG2 cells in the absence of caveolin expression. Thus, SR-BI membrane domain localization and organization in hepatocytes need additional investigation. Overall, our results show that SR-BI is responsible for the bulk of CE selective uptake from the most abundant human plasma lipoproteins, LDL and HDL, in the hepatic cell model, HepG2 cells, and in mouse hepatic cells. Selective uptake of both LDL− and HDL−CE is likely to occur simultaneously in the liver.

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