SR-BI Mediates Cholesterol Efflux via Its Interactions with Lipid-Bound ApoE. Structural Mutations in SR-BI Diminish Cholesterol Efflux†

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ABSTRACT: Apolipoprotein E (apoE) and the lipoprotein receptor SR-BI play critical roles in lipid and lipoprotein metabolism. We have examined the cholesterol efflux from wild-type (WT) and mutant forms of SR-BI expressed in ldlA-7 cells using reconstituted discoidal particles consisting of apoE, 1-palmitoyl-2-oleoyl-L-phospatidylcholine (POPC), and cholesterol (C) as acceptors. POPC/C−apoE particles generated using apoE2, apoE3, apoE4, or carboxy-terminally truncated forms apoE4-165, apoE4-202, apoE4-229, and apoE4-259 caused similar (20−25%) cholesterol efflux from WT SR-BI. Cholesterol efflux mediated by POPC/C−apoE was not enhanced in the presence of lipid-free apoE. The rate of cholesterol efflux mediated by particles containing the WT or carboxy-terminally truncated forms of apoE was decreased to approximately 30% of the WT control with the Q402R/Q418R mutant SR-BI form that is unable to bind native HDL normally but binds LDL. The rate of cholesterol efflux was further decreased to approximately 7% of the WT control with another SR-BI mutant (M158R) that binds neither HDL nor LDL. The level of binding of POPC/C−apoE particles (150 μg/mL) to SR-BI mutant forms Q402R/Q418R and M158R was 70 and 8% of the WT control, respectively. SR-BI-dependent binding of lipid-free apoE to cells was undetectable, and cholesterol efflux was less than 0.5%. The findings establish that only lipid-bound apoE promotes SR-BI-mediated cholesterol efflux and that the amino-terminal region of residues 1−165 of apoE is sufficient for both receptor binding and cholesterol efflux. The SR-BI−apoE interactions may contribute to overall cholesterol homeostasis in cells and tissues that express SR-BI and apoE.

Cells and tissues achieve cholesterol homeostasis by both endogenous synthesis and uptake and efflux of cholesterol. These processes are mediated by apolipoproteins and different lipoprotein particles. The scavenger receptor SR-BI[1] is a multiligand receptor that binds HDL, LDL, VLDL, and chylomicrons, and is thought to play an important role in cholesterol homeostasis (*1*−*5*). SR-BI binds to HDL and reconstituted HDL (rHDL), at least in part by its interactions with apoA-I (*1*, *2*, *6*, *7*). Upon binding to HDL, SR-BI mediates selective uptake of both cholesteryl esters and other lipids from HDL to cells (*1*, *2*, *8*, *9*), bidirectional movement of unesterified cholesterol (*10*−*12*), and net efflux of excess cholesterol (*10*, *12*−*14*).

The biological significance of SR-BI in lipid homeostasis was studied in transgenic and knockout animal models. SR-BI overexpression in the liver of mice accelerated the clearance of the HDL and decreased the level of HDL cholesterol and apoA-I in a dose-dependent manner, irrespective of the diet (*15*−*17*). Deficiency of SR-BI in mice resulted in an increased total plasma cholesterol level, smaller stores of cholesteryl esters in steroidogenic tissues, and a decreased rate of cholesterol secretion without alterations in bile acid secretion, bile acid pool size, or fecal bile acid excretion (*18*−*20*). An important observation of these studies was that the knockout mice had abnormal large lipoprotein particles in the HDL2 and LDL size range that were enriched in apolipoprotein E (apoE) (*18*).

ApoE, which has three common isoforms (apoE2, apoE3, and apoE4) in the general population (*21*, *22*), is one of the most important proteins of the cholesterol transport system (*23*, *24*). ApoE promotes receptor-mediated clearance of lipoprotein remnants from the circulation, and contributes to lipid homeostasis (*23*, *25*). ApoE is also involved in

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[1] Abbreviations: ABCA1, ATP-binding cassette transporter; apoA-I, apolipoprotein A-I; apoE, apolipoprotein E; C, cholesterol; HDL, high-density lipoprotein; ldlA-7 cells, LDL receptor-deficient Chinese hamster ovary cells; ldlA[mSR-BI] cells, mSR-BI-expressing ldlA-7 cells; ldlA[Q402R/Q418R], mSR-BI[Q402R/Q418R]-expressing ldlA-7 cells; ldlA[M158R], mSR-BI[M158R]-expressing ldlA-7 cells; LDL, low-density lipoprotein; mSR-BI, murine SR-BI; POPC, 1-palmitoyl-2-oleoyl-L-phosphatidylcholine; POPC/C−apoE, reconstituted particles consisting of POPC, cholesterol, and apoE; rHDL, reconstituted HDL particles; SD, standard deviation; SR-BI, scavenger receptor class B type I; VLDL, very low-density lipoprotein; WT, wild-type.

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cholesterol efflux processes (*26*, *27*). In vitro and in vivo studies have shown that mutations in apoE that weaken binding of apoE-containing lipoproteins to the LDL receptor are associated with high plasma cholesterol levels and cause premature atherosclerosis in humans and experimental animals (*23*, *24*, *28*). Lipoprotein-bound apoE is a ligand for the LDL receptor as well as other receptors in vitro, and these interactions may lead to unidirectional delivery of cholesterol to cells (*25*, *29*, *30*). Furthermore, lipid-free apoE or minimally lipidated apoE, designated γLpE, may promote efflux of phospholipid and cholesterol from cells by the action of the ATP-binding cassette transporter (ABCA1) (*26*, *27*, *31*, *32*).

We have shown recently that POPC/C−apoE particles containing full-length and truncated apoE forms bind with similar affinities (K_d = 35−45 μg of protein/mL) to cells expressing SR-BI, and that the receptor binding domain of apoE is in the amino-terminal region of residues 1−165 (*33*). Other studies showed that binding of POPC/C−apoE particles to cells promotes selective cholesteryl ester uptake (*8*). The study presented here was designed to correlate receptor binding data with the ability of the WT and mutant apoE forms to promote efflux from cells expressing the WT or mutant SR-BI. We have found that lipid-bound apoE in the form of POPC/C−apoE particles containing WT and various truncated apoE forms promotes efficiently SR-BI-dependent cholesterol efflux. In contrast, lipid-free apoE does not exhibit detectable levels of SR-BI-dependent binding and does not promote SR-BI-dependent efflux. Cholesterol efflux promoted by POPC/C−apoE particles was nearly abolished in a SR-BI mutant that does not bind to HDL or LDL. Cholesterol efflux was also reduced to 30−35% of the WT control in a mutant that does not bind normally to HDL but binds to LDL. Competition experiments indicated that HDL and LDL compete efficiently and poorly, respectively, for the binding of POPC/C−apoE particles to the receptor. Comparison of the current findings with previously published work on apoA-I indicates similarities as well as differences in the interaction of the POPC/C−apoE particles and POPC/C−apoA-I particles with the WT and the mutant receptors (*13*, *33*). The ability of apoE to contribute both to cholesterol delivery and to cholesterol efflux via its interactions with SR-BI, as well as its other interactions with the LDL receptor and the ABCA1 transporter, apparently contributes to cell and tissue cholesterol homeostasis and may account for some of its atheroprotective properties (*34*, *35*).

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes were purchased from New England Biolabs Inc. (Beverly, MA). Materials for polymerase chain reaction (PCR) were obtained from Perkin-Elmer Life Sciences Inc. (Boston, MA) and Promega (Madison, WI). Oligonucleotides for PCR, Max Efficiency DH5α competent cell, BJ 5183 electro competent cells, Ham's F-12 medium, Leibovitz's L-15 medium, DMEM, fetal bovine serum, trypsin/EDTA, penicillin/streptomycin, glutamine, and G418 sulfate were purchased from Invitrogen Corp. (Carlsbad, CA). Bactotryptone and bacto-yeast extract were obtained from VWR (Pittsburgh, PA). Other reagents (and sources) were as follows: sodium [^{125}I]iodide, [1,2-^{3}H]cholesterol (1 mCi/mL, specific activity of 40−60 Ci/mmol) from Perkin-Elmer Life Sciences Inc.; Iodo-Beads iodination reagent and D-salt dextran plastic desalting columns from Pierce (Rockford, IL); fatty acid-free bovine serum albumin, cholesterol, sodium cholate, and 1-palmitoyl-2-oleoyl-L-phosphatidylcholine (POPC) from Sigma Aldrich Corp. (St. Louis, MO); dialysis tubing from Spectrum Medical Industries, Inc. (Los Angeles, CA); Dc protein assay kit and protein assay dye reagent from Bio-Rad (Hercules, CA); and the bicinchoninic acid assay kit from Pierce. Dextran sulfate and epoxy-activated Sepharose 6B were purchased from Amersham Biosciences (Piscataway, NJ). All other reagents were purchased from Sigma Aldrich Corp., Bio-Rad, Amersham Biosciences, Fisher Scientific International Inc. (Suwanee, GA), or other standard commercial sources.

Plasmid and Recombinant Adenovirus Constructions. The construction of plasmids containing the WT or mutated human apoE gene has been described previously in detail (*36*). To generate the recombinant adenoviruses containing the WT and variant apoE forms, we used the Ad-Easy-1 system in which the pAdTrack-apoE vectors are used to electroporate BJ 5183 *Escherichia coli* cells along with the pAdEasy-1 helper vector, which contains the viral genome and the long terminal repeats of the adenovirus (*37*). Recombinant bacterial clones resistant to kanamycin were selected and characterized. The positive recombinant virus vectors expressing WT apoE and mutant apoE forms were propagated in DH5α competent cells and then linearized with PacI and used to infect 911 cells (*38*). Recombinant adenoviruses were subjected to three rounds of plaque purification using 911 cells. Following large-scale infection of HEK-293 cell cultures with the recombinant adenoviruses, the adenoviruses were purified by two consecutive $CsCl_2$ ultracentrifugation steps, dialyzed, and titrated as described previously (*36*, *39*). Usually, titers of approximately 5×10^{10} pfu/mL were obtained.

Production and Purification of ApoE Using the Adenovirus System. Human HTB13 cells (SW 1783, human astrocytoma), grown to 80% confluence in Leibovitz's L-15 medium containing 10% (v/v) fetal bovine serum (FBS) in roller bottles, were infected with adenoviruses expressing the WT or mutant apoE forms at a multiplicity of infection of 5. After they had been infected for 24 h, cells were washed twice with serum-free medium and preincubated in serum-free medium for 30 min, and then fresh serum-free medium was added. After 24 h, the medium was harvested and fresh serum-free medium was added to the cells. The harvests were repeated approximately eight times.

ApoE was purified from the culture medium of adenovirus-infected HTB-13 cells as described previously (*33*). The purification scheme involved dextran sulfate−Sepharose column fractionation (*33*).

Preparation of Discoidal Reconstituted ApoE−Phospholipid−Cholesterol Particles. Complexes comprising apolipoprotein, 1-palmitoyl-2-oleoyl-L-phosphatidylcholine (POPC), and cholesterol were prepared by the sodium cholate dialysis method (*40*), using POPC, cholesterol, apoE, and sodium cholate in a molar ratio of 100:10:1:100, as previously described (*13*, *41*). Apolipoprotein−lipid complexes were analyzed by native polyacrylamide gradient (4 to 15%) gel electrophoresis (Phast gel system, Amersham Biosciences).

Iodination of ApoE. ApoE was labeled with ^{125}I using Iodo-Beads (*42*) iodination reagent and Na^{125}I as described

previously (*33*). Each reaction used 1 mCi of ^{125}I, three beads, and 1 mg of apoE. The ^{125}I-labeled apoE was separated from the unincorporated Na^{125}I by gel filtration using dextran desalting columns. One microliter of each fraction was used for determination of the ^{125}I counts and 10 μL to measure the protein concentration. Specific activities of 650−1100 cpm/ng of protein were obtained.

Cell Cultures for [^{3}H]Cholesterol Efflux and Receptor Binding Assays. The ldlA-7 cell line is an LDL receptor-deficient Chinese hamster ovary (CHO) cell mutant which expresses very little SR-BI protein and displays very low HDL binding/selective uptake activity (*2*, *43*). The ldlA-7 cells were maintained in a monolayer culture in Ham's F-12 medium containing 5% fetal bovine serum, 100 units/mL penicillin, 100 mg/mL streptomycin, and 2 mM glutamine (medium A). The generation of cell lines expressing the WT and mutant forms of SR-BI designated as ldlA[mSR-BI], ldlA[Q402R/Q418R], and ldlA[M158R] was described previously (*2*, *10*, *44*). These cells were maintained as stock cultures in medium A supplemented with 500 μg/mL G418 (medium B). All incubations with cells were performed at 37 °C in a humidified 5% CO_2, 95% air incubator.

Flow Cytometric Analysis of SR-BI Cell Surface Expression. The levels of cell surface expression of SR-BI were determined by flow cytometry using the SR-BI-specific antibody KKB-1 (a generous gift from K. Kozarsky) (*10*). Cells were washed once with PBS and then incubated at 37 °C for 1 h in Ham's F-12 medium containing 0.5% (w/v) fatty acid-free bovine serum albumin (BSA), 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mM glutamine (medium C) containing KKB-1 antiserum (1:1000 dilution). The cells were then washed twice with PBS containing 0.5% fatty acid-free BSA and incubated at 37 °C for 1 h in buffer A (Ca^{2+}- and Mg^{2+}-free PBS, 0.5% fatty acid-free BSA, and 2 mM EDTA) containing FITC-conjugated goat anti-rabbit IgG (1:1000 dilution, Cappel, West Chester, PA). Finally, the cells were suspended by gentle pipetting in 5× volume of Ca^{2+}- and Mg^{2+}-free PBS containing 0.25% fatty acid-free BSA and 2 mM EDTA, pelleted at 500*g* for 2 min, resuspended in buffer A, and analyzed using a FACScan (BD Biosciences) flow cytometer (*10*, *44*, *45*). The ratio of the SR-BI cell surface expression of a mutant versus WT SR-BI form-expressing cell line was determined.

[^{3}H]Cholesterol Efflux from ldlA-7 Cells and Stably Transfected ldlA-7 Cells Expressing WT and Mutant Forms of mSR-BI. Efflux of [^{3}H]cholesterol from ldlA-7 cells and stably transfected ldlA-7 cells expressing WT and mutant forms of mSR-BI was assessed as described previously in detail (*13*). Cholesterol efflux from untransfected and transfected cells was also assessed in the presence and absence of the SR-BI blocking antibody KKB-1 (anti-mSR-BI KKB-1 serum at a 1:500 dilution) (*10*). The concentration of the cholesterol acceptors added to the cell cultures was 150 μg of protein/mL. Two complementary approaches were used to calculate the net SR-BI-dependent efflux. In the first approach, the rate of cholesterol efflux of the untransfected ldlA-7 cells was subtracted from the rate of cholesterol efflux of the SR-BI-expressing cells. In the second approach, the rate of cholesterol efflux in the presence of the SR-BI blocking antibody KKB-1 was subtracted from the rate of cholesterol efflux in the absence of the receptor-blocking antibody.

The SR-BI-dependent cholesterol efflux from cells expressing the mutant forms of SR-BI was corrected for the different level of mutant receptor expression in cells relative to the WT receptor expression.

Radioreceptor Competition Binding Assay. On day 0, ldlA-7 and ldlA[mSR-BI] cells were plated at concentrations of 7.5 × 10^4 cells/well in 24-well dishes in medium A for ldlA-7 or medium B for the other cells (*10*, *33*). On day 2, the monolayers were washed twice with Ham's F-12 medium and then re-fed with 0.4 mL of a medium C/25 mM Hepes/KOH (pH 7.4) mixture containing 15 μg of protein/mL of [^{125}I]POPC/C−apoE4 particles and unlabeled competitor protein (POPC/C−apoE4, HDL, and LDL) in the concentration range of 0.1−300 μg protein/mL. SDS−PAGE analysis showed that the HDL used for competition contains traces of apoE. After a 1.5 h incubation at 37 °C, the cells were washed twice at 4 °C with buffer B [50 mM Tris-HCl (pH 7.4) and 0.15 M NaCl] containing 2 mg/mL fatty acid-free BSA, followed by one rapid wash with buffer B alone. The cells were then solubilized with 0.1 M NaOH (500 μL in each well). Aliquots of 200 μL were used for radioactivity determinations, and aliquots of 20 μL were used for determination of the protein concentration using the Bradford protein assay. The level of binding obtained in the absence of competitor was set to 100%. The net competition of binding of POPC/C−apoE4 particles was calculated as the difference in the curves obtained in the ldlA[mSR-BI] cell minus those obtained in the control ldlA-7 cells for each experimental point.

Immunoreceptor Binding Assay Using ELISA. On day 0, ldlA[mSR-BI], ldlA[Q402R/Q418R], and ldlA[M158R] cells were plated at concentrations of 4.5−5 × 10^4 cells/well in 24-well dishes in medium A for ldlA-7 or medium B for the other cells (*10*, *33*). On day 2, the monolayers were washed twice with Ham's F-12 medium and then re-fed with 0.4 mL of a medium C/25 mM Hepes/KOH (pH 7.4) mixture containing POPC/C−apoE4 particles at a concentration of 150 μg/mL or lipid-free apoE at the indicated concentrations. After a 1.5 h incubation at 37 °C, the cells were washed twice at 4 °C with buffer B containing 2 mg/mL fatty acid-free BSA, followed by one rapid wash with buffer B alone. The cells were then solubilized in lysis buffer [300 μL in each well of Ca^{2+}- and Mg^{2+}-free PBS, 1% Triton X-100, and 1% protease inhibitor cocktail (Sigma Aldrich Corp.)] and incubated, with shaking, at 4 °C for 30 min. The cell lysates were collected by scraping the wells and were clarified by centrifugation at 14 000 rpm in a microfuge for 10 min. Aliquots of 200 μL were used for the immunoreceptor assay using ELISA, and aliquots of 20 μL were used for determination of the protein concentration using the bicinchoninic acid assay.

For receptor association experiments, the Maxisorb 96-well plates were coated with mouse anti-human apoE monoclonal antibody (1 mg/mL) (Ottawa Heart Institute) diluted 1:100 in PBS at 100 μL/well and stored at 37 °C for 1 h. The coating solution was aspirated, and the wells were washed three times with washing buffer (0.05% Tween 20 in PBS) at 300 μL/well. Blocking buffer (10% nonfat dry milk in washing buffer) was then added (300 μL/well). The plates were incubated at 25 °C for 1 h and washed three times with washing buffer. Cell lysates (200 μL containing ∼25 μg of protein) were added in each well, and then

incubated at 37 °C for 1 h. To obtain a standard curve, POPC/C−apoE particles containing different amounts of apoE (10, 25, 50, 75, and 100 ng), mixed with 25 μg of the lysate of cells (ldlA-7 or ldlA-7 expressing the WT or mutant SR-BI forms) that were not treated with POPC/C−apoE particles or any lipoprotein, were adjusted to a total volume of 200 μL and were added to different wells. After the plates were washed with washing buffer three times, the secondary antibody (goat polyclonal anti-human apoE, horseradish peroxidase-conjugated, 0.5 mg/mL) (Biodesign) diluted 1:500 in blocking buffer was added (100 μL/well). The plates were incubated at 37 °C for 45 min. After the plates were washed with washing buffer three times, an aliquot of 100 μL of *o*-phenylenediamine dihydrochloride substrate (0.4 mg/mL *o*-phenylenediamine dihydrochloride, 0.4 mg/mL urea hydrogen peroxide, and 0.05 M phosphate-citrate buffer) was added to each well. After incubation for 30 min at room temperature, the reaction was terminated by adding 50 μL of 2 N H_2SO_4 per well. The absorbance was measured at 490 nm using a microtiter plate reader. The binding (cell association) values were expressed as nanograms of apoE associated with the cells per milligram of total cell protein. The level of specific binding was obtained by subtracting the level of binding of the untransfected cells (ldlA-7) from the level of binding of receptor-expressing cell lines ldlA-[mSR-BI], ldlA[Q402R/Q418R], and ldlA[M158R] (*10*, *33*). The level of specific binding to cells expressing the mutant forms of SR-BI was corrected for the different level of mutant receptor expression in cells relative to the WT receptor expression. The correction factor was the level of expression of WT SR-BI divided by the levels of expression of the mutant SR-BI, determined by flow cytometry. Receptor binding to the untransfected and transfected cells was also assessed in the presence and absence of the SR-BI blocking antibody KKB-1 (anti-mSR-BI KKB-1 serum at a 1:500 dilution) as described previously (*10*).

RESULTS

Cholesterol Efflux Mediated by Wild-Type SR-BI Using Full-Length ApoE: Effects of Carboxy-Terminal ApoE Deletions on Cholesterol Efflux. We have shown that lipid-bound apoE is a ligand for the SR-BI, and that the amino-terminal 1−165 domain of apoE suffices for receptor binding (*33*). To examine the effects of the carboxy-terminal apoE deletions on the ability of the mutant apoE to interact functionally with SR-BI, we prepared discoidal rHDL particles (designated POPC/C−apoE) containing the WT or the mutant apoE forms along with phospholipid and cholesterol at a defined POPC:C:apoE ratio of 100:10:1, as described previously (*33*). These preparations were incubated at 37 °C with control ldlA-7 cells that express almost no SR-BI (*2*) or ldlA-7 cell lines that express high levels of wild-type mSR-BI (designated ldlA[mSR-BI]) (*2*, *3*, *10*). The efflux of [^{3}H]cholesterol from cells prelabeled with this sterol was assessed as a function of time in the presence of POPC/C−apoE at a fixed concentration of 150 μg of protein/mL (4.4 μM POPC/C−apoE). The net rate of SR-BI-dependent cholesterol efflux was determined by comparing the rate of cholesterol efflux from the SR-BI-expressing and control cells and by comparing the rate of cholesterol efflux from the SR-BI-expressing cells in the presence or absence of an SR-BI blocking antibody, as described in Experimental Procedures and Figure 1. Both approaches gave identical results. Figure 1A shows the time course of efflux of [^{3}H]-cholesterol from control ldlA-7 cells and ldlA[mSR-BI] cells. The differences between the rates measured in the transfected (ldlA[mSR-BI]) and untransfected control (ldlA-7) cells represent the SR-BI-dependent efflux. Figure 1B shows the time course of efflux of [^{3}H]cholesterol from ldlA-7 cells and ldlA[mSR-BI] cells in the presence and absence of the SR-BI blocking antibody KKB-1. The difference between the rate of cholesterol efflux in the absence of antibodies minus the rate of cholesterol efflux in the presence of antibodies represents the rate of SR-BI-dependent efflux. To simplify the analysis of the cholesterol efflux, we tabulated the percent of cholesterol efflux after incubation for 4 h at 37 °C. Figure 1C shows the percent of [^{3}H]cholesterol efflux values from ldlA-7 cells and ldlA[mSR-BI] cells in the presence and absence of SR-BI blocking antibody KKB-1 after incubation for 4 h at 37 °C. The data establish that cholesterol efflux from the ldlA-7 cells is the same in the absence or presence of the SR-BI blocking antibody, whereas cholesterol efflux from ldlA[mSR-BI] cells is much greater in the absence of the antibodies. The cholesterol efflux value from the SR-BI-expressing cells in the presence of the antibodies is equal to the cholesterol efflux value observed in ldlA-7 cells in either the absence or presence of the SR-BI blocking antibodies. The data of Figure 1C show that the net SR-BI-dependent cholesterol efflux (hatched bars) from ldlA-7 cells is undetectable, whereas the net SR-BI-dependent cholesterol efflux from ldlA[mSR-BI] cells is 22% of the total cellular cholesterol. Figure 1D (black bars) shows the percent of SR-BI-dependent [^{3}H]cholesterol efflux values after incubation for 4 h at 37 °C for the cell lines expressing the WT SR-BI to POPC/C−apoE particles containing WT and truncated (carboxy-terminal residue indicated) apoE forms. Results similar to those shown in panels C and D of Figure 1 were obtained using the cholesterol efflux values determined after incubation for 2 h (data not shown). Cholesterol efflux to cell lines expressing two mutant receptors (white and hatched bars) is discussed later.

Figure 1D also shows that POPC/C−apoE particles containing the three naturally occurring apoE isoforms (apoE2, apoE3, and apoE4) have statistically similar cholesterol efflux capacity from cells expressing the WT SR-BI. In addition, POPC/C−apoE particles containing carboxy-terminal apoE4 mutants extending from amino acid 1 to amino acid 259, 229, 202, or 165 promote cholesterol efflux from cells expressing the WT receptor as efficiently as the full-length apoE forms. Control experiments showed that cholesterol efflux mediated by POPC/C particles that do not contain apoE from cells expressing the WT SR-BI was around 2% of total cholesterol. The data indicate that the domain of apoE responsible for cholesterol efflux is within the amino-terminal region of residues 1−165.

Effect of Mutations in SR-BI on Receptor Binding and Cholesterol Efflux. To explore further the interactions of POPC/C−apoE particles with SR-BI, we used two cell lines expressing mutant forms of SR-BI. The first cell line expresses a double mutant form (Q402R/Q418R). This mutant binds to and mediates lipid transfer from LDL, but does not exhibit detectable high-affinity binding and lipid transfer to HDL (*44*). The rate of cholesterol efflux from

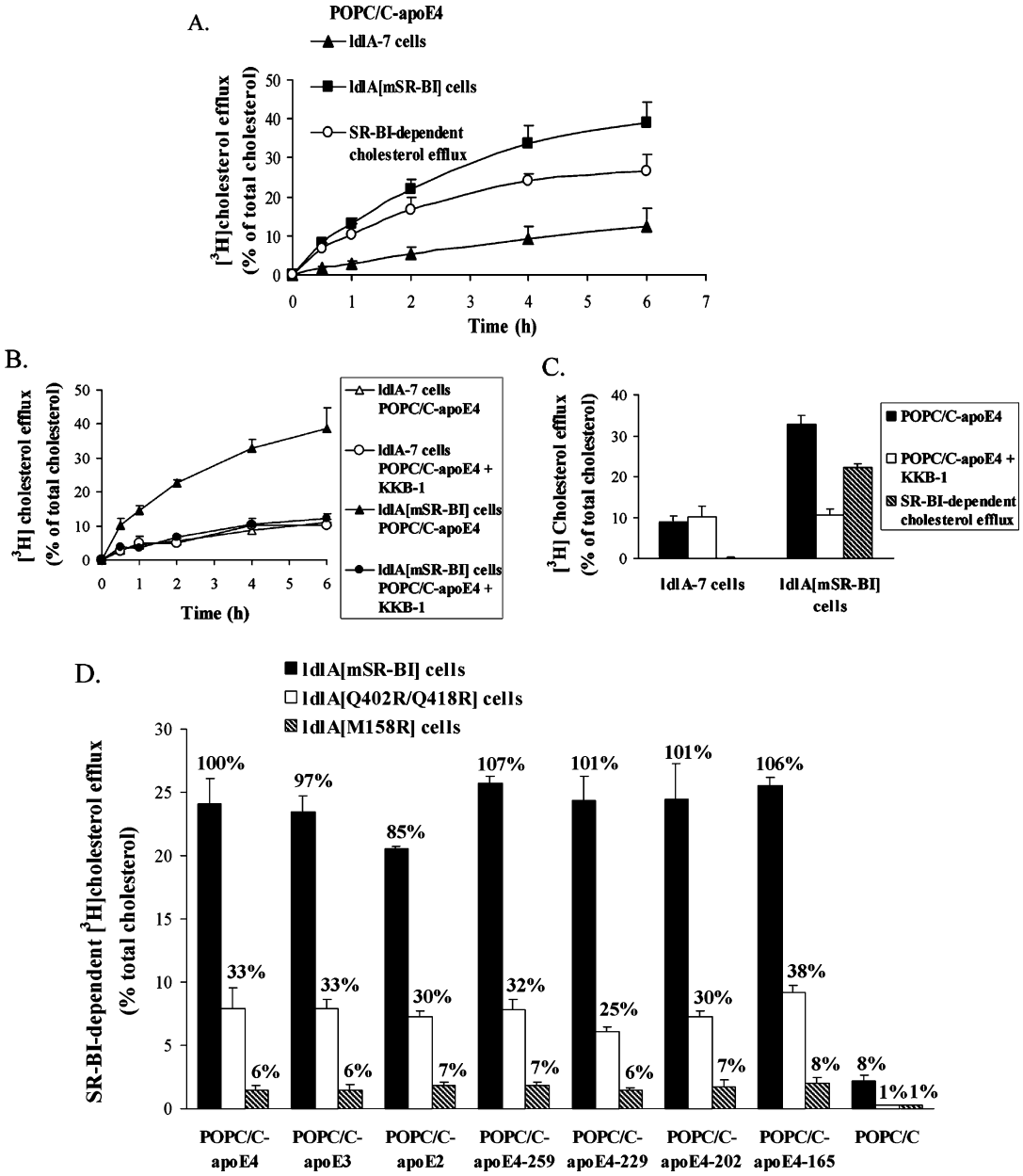

FIGURE 1: (A−D) SR-BI-dependent cholesterol efflux from cells expressing the WT SR-BI or two mutant forms of SR-BI (Q402R/Q418R and M158R), using POPC/C−apoE particles as cholesterol acceptors. Cells expressing the WT or mutant forms of SR-BI and the control untransfected cells were plated at a density of 200 000 cells/well in six-well dishes and labeled with [^{3}H]cholesterol for 48 h. The medium was then aspirated and replaced with fresh Ham's F-12 medium containing 150 μg of POPC/C−apoE/mL. Cholesterol efflux was assessed after incubation at 37 °C for up to 6 h. The percent of [^{3}H]cholesterol efflux was determined as the amount of radioactivity released in the medium divided by the total radioactivity present in the culture medium and the cell lysate. Panel A shows the time course of cholesterol efflux from control ldlA-7 cells and ldlA[mSR-BI] cells using POPC/C−apoE4 as the cholesterol acceptor. Values are the means ± SD from three independent experiments performed in duplicate. Panel B shows the time course of cholesterol efflux from control ldlA-7 cells and ldlA[mSR-BI] cells using POPC/C−apoE4 as the cholesterol acceptor in the absence or presence of the SR-BI blocking antibody KKB-1. Two independent experiments were performed in duplicate for each cell line. Panel C shows the percent values of cholesterol efflux from control ldlA-7 cells and ldlA[mSR-BI] cells in the absence or presence of the SR-BI blocking antibody KKB-1 after a 4 h incubation at 37 °C using POPC/C−apoE4 particles as cholesterol acceptors. Two independent experiments were performed in duplicate for each cell line. Panel D shows the percent values of net SR-BI-dependent cholesterol efflux after incubation for 4 h at 37 °C from cells expressing the WT and the two mutant SR-BI using POPC/C−apoE particles containing the WT or truncated apoE forms as cholesterol acceptors (150 μg of protein/mL) (rate of cholesterol efflux). The net rate of SR-BI-dependent [^{3}H]cholesterol efflux was calculated as the difference in the rate of cholesterol efflux between cells expressing the WT or mutant forms of SR-BI and the control untransfected cells. The SR-BI-dependent cholesterol efflux from cells expressing the mutant forms of SR-BI was corrected for the different level of mutant receptor expression in cells relative to the level of WT receptor expression. Values are the means ± SD from three independent experiments performed in duplicate.

cells expressing this mutant to POPC/C−apoE containing WT or mutant forms of apoE was reduced to 25−38% of the WT control (Figure 1D). The level of binding of POPC/C−apoE4 particles at concentrations of 150 μg/mL to this mutant receptor was 70% of the WT control (Figure 2). When corrected for binding efficiency, the rate of SR-BI-mediated cholesterol efflux from this mutant receptor to POPC/C−apoE4 was 47% of the WT control. The second mutant (M158R) does not bind to and does not mediate lipid transfer from either HDL or LDL (*44*). The rate of cholesterol

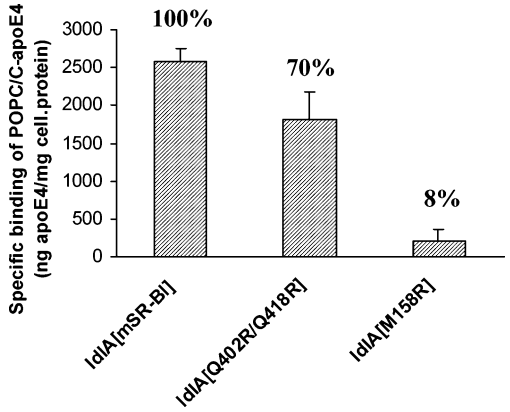

FIGURE 2: Binding of POPC/C−apoE particles to confluent monolayers of ldlA-7 cells expressing the WT SR-BI and the mutants Q402R/Q418R and M158R SR-BI. Cells in 24-well plates were washed and incubated with 150 μg of POPC/C−apoE4/mL. The total level of binding to ldlA-7 cells expressing the WT or the mutant receptors and the total level of binding to untransfected ldlA-7 cells were obtained as described in Experimental Procedures. The specific binding shown in this figure was assessed by subtracting the values of binding to the ldlA-7 cells from the corresponding values of binding to the ldlA-7 cells expressing the WT or each of the two mutant receptors. The level of specific binding to cells expressing the mutant forms of SR-BI was corrected for the different level of mutant receptor expression in cells relative to the WT receptor expression. Four independent experiments were performed in duplicate for each cell line.

efflux from cells expressing this mutant to POPC/C−apoE particles containing WT or mutant forms of apoE was reduced to 6−8% of the WT control (Figure 1D). Binding studies showed that the level of SR-BI-dependent binding of POPC-C/C−apoE4 to cells expressing this mutant receptor was 8% of the WT control (Figure 2), indicating that the diminished rate of cholesterol efflux was correlated with the diminished level of binding to the receptor. These findings suggest that structural mutations in SR-BI affect the physical and functional interactions between SR-BI and lipid-bound apoE and affect SR-BI-dependent cholesterol efflux.

Control experiments showed that cholesterol efflux mediated by POPC/C particles that do not contain apoE from cells expressing the two mutant SR-BI forms was around 0.3% of total cholesterol.

Table 1 compares the binding and cholesterol efflux properties of different ligands to WT and mutant SR-BI forms.

Specificity of Binding of POPC/C−ApoE Particles for SR-BI. SR-BI is a multiligand receptor that binds both HDL and rHDL particles that contain apoA-I and LDL that has apoB as its only apolipoprotein (*1*, *13*). The specificity of binding of the apoE-containing POPC/C particles in relation to the specificities of HDL and LDL particles was examined with competition experiments. It was found that excess unlabeled POPC/C−apoE4 as well as excess unlabeled HDL competed strongly and with similar efficiency for the binding of 15 μg of [^{125}I]POPC/C−apoE4/mL to ldlA[mSR-BI], but excess unlabeled LDL competed poorly (Figure 3). Previous studies also showed that HDL and POPC/C−apoA-I compete similarly for the binding of POPC/C−apoA-I to SR-BI (*7*). The findings indicate that HDL and POPC/C−apoE may share common elements that contribute to their recognition by SR-BI.

Lipid-Free ApoE Is Not a Ligand for SR-BI and Does Not Promote SR-BI-Dependent Cholesterol Efflux. Previous reports indicated that lipid-free apoE competes for the binding of POPC−apoA-I to human adrenal cells (NCI-H295R) and promotes uptake of cholesteryl esters (*46*). To test the ability of lipid-free apoE to bind to and promote cholesterol efflux from cells overexpressing SR-BI, we performed cholesterol efflux studies in control ldlA-7 and ldlA[mSR-BI] cells (*2*, *3*, *10*) that express high levels of WT mSR-BI using the WT and mutant apoE forms described in Figure 1 as cholesterol acceptors. Figure 4A shows the time course of efflux of [^{3}H]cholesterol from control ldlA-7 cells and ldlA[mSR-BI] cells at a fixed lipid-free apoE4 concentration (150 μg of protein/mL, 4.4 μM). The differences between the rates measured in the transfected (ldlA[mSR-BI]) and untransfected control (ldlA-7) cells represent SR-BI-dependent efflux. Figure 4B shows the percent of SR-BI-dependent [^{3}H]cholesterol efflux values from cells after incubation for 4 h at 37 °C with WT and truncated lipid-free apoE forms in comparison with the values of the SR-BI-dependent cholesterol efflux promoted by POPC/C−apoE4. The analysis shown in panels A and B of Figure 4 establishes that there was virtually no SR-BI-dependent efflux to lipid-free apoE4 (<0.5%) in contrast to the 24% efflux from the same cells to POPC/C−apoE particles (Figure 1A,D).

To assess whether lipid-free apoE exerts any effect in the presence of lipid-bound apoE, we performed cholesterol efflux studies using either a mixture of 150 μg of protein/mL each of lipid-free apoE and POPC/C−apoE or a mixture of 150 μg of protein/mL each of lipid-free apoE and HDL. This analysis presented in panels A and B of Figure 5 showed that the rate of cholesterol efflux to POPC/C−apoE or to HDL in either control or SR-BI-expressing cells was not affected by the presence of lipid-free apoE.

To interpret the cholesterol efflux data of panels A and B of Figure 4, we performed receptor binding studies in ldlA-[mSR-BI] cells and control ldlA-7 cells. To obtain the level of SR-BI-dependent binding of apoE, we subtracted the level of binding to the ldlA-7 cells from the level of binding to the ldlA[mSR-BI] cells for the different apoE concentrations used to determine the binding curves. This analysis showed a very high level of nonspecific binding of apoE to cells, but failed to detect any SR-BI-dependent binding (Figure 6).

To verify further the lack of SR-BI-dependent binding, we performed receptor binding studies in the untransfected and transfected cells in the presence of SR-BI receptor-blocking antibodies (*10*, *33*). This analysis showed that the binding of lipid-free apoE was similar in the ldlA-7 control cells and the ldlA[mSR-BI] cells, thus confirming the lack of SR-BI-dependent binding (Figure 7A). Control experiments showed that when POPC/C−apoE particles were used as the ligand, the receptor-blocking antibodies did not affect the binding to ldlA-7 cells, but showed inhibition of binding to ldlA[mSR-BI] (Figure 7B). These findings confirm the data of Figures 1A−D and 2 that established unequivocally that only the lipid-bound apoE is a high-affinity ligand for SR-BI and promotes SR-BI-dependent cholesterol efflux.

Table 1: Binding and Cholesterol Efflux Properties of Different Ligands to WT and Mutant SR-BI Forms

	cholesterol efflux				SR-BI binding			
	LDL	HDL	POPC/C−apoA-I [a]	POPC/C−apoE	LDL	HDL	POPC/C−apoA-I [a]	POPC/C−apoE
WT SR-BI	+	+	100%	100%	+	+	+	+
SR-BI[Q402R/Q418R]	+	−	24%[b]	47%[b]	+	−	+ (nonproductive)	+ (nonproductive)
SR-BI[M158R]	−	−	∼71%[b]	∼75%[b]	−	−	weak (productive)	very weak (productive?)

[a] Data from ref *13*. [b] Cholesterol efflux corrected for binding efficiency.

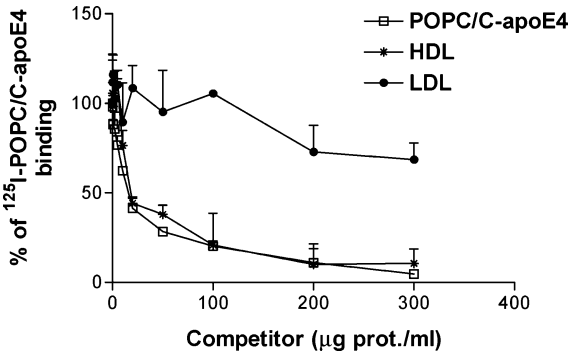

FIGURE 3: Competition of binding of [^{125}I]POPC/C−apoE by excess of HDL, LDL, and unlabeled POPC/C−apoE. On day 2 after plating, ldlA-7 cells and ldlA[mSR-BI] cells were incubated with 15 μg of ^{125}I-labeled POPC/C−apoE4/mL and 0.1−300 μg of unlabeled POPC/C−apoE4, HDL, or LDL/mL as explained in Experimental Procedures. The level of binding obtained in the absence of competitor was set to 100%. The specific activity of ^{125}I-labeled POPC/C−apoE4 was in the range of 650−1100 cpm/ng. Competition experiments were performed in ldlA[mSR-BI] cells and control untransfected ldlA-7 cells. The net competition of binding of POPC/C−apoE4 particles was calculated as the difference in the curves obtained in the ldlA[mSR-BI] cell minus those obtained in the control ldlA-7 cells for each experimental point. Values are the means ± SD from two independent experiments performed in duplicate.

DISCUSSION

This study has focused on the functional interactions between apoE and SR-BI that promote cholesterol efflux. ApoE plays an important role in cholesterol homeostasis in the circulation (*47*) and the brain (*36*, *48*). Lipid-bound apoE is a ligand for the LDL receptor and other receptors in vitro (*25*, *29*, *30*, *36*) and promotes the clearance of the apoE-containing lipoproteins from the circulation (*23*, *24*, *49*). Mutations in apoE that prevent its interactions with the LDL receptor are associated with dyslipidemia and premature atherosclerosis (*23*, *24*, *28*). With one exception (*50*), low-level expression of apoE by either bone marrow transplantation, retroviral gene transfer, helper-dependent adenovirus gene transfer, or ectopic expression in the adrenals protected mice from atherosclerosis (*51*−*56*). All these data reinforce the atheroprotective properties of apoE (*34*, *35*). These properties may be related to the ability of lipid-bound apoE to clear cholesterol via the LDL receptor (*25*, *49*), as well as the ability of lipid-free apoE to promote ABCA1-dependent cholesterol efflux (*31*, *32*). Finally, this study and previous studies have established that lipid-bound apoE is a ligand for SR-BI that promotes lipid efflux (*33*) and also is capable of promoting selective uptake of cholesteryl esters (*8*) (Figure 8).

Specificity of the Interactions of ApoE with SR-BI. This study establishes that lipid-bound apoE in the form of POPC/C−apoE particles, but not lipid-free apoE, promotes SR-BI-dependent cholesterol efflux. This interaction is specific and can be blocked by receptor-blocking antibody and by specific mutations in SR-BI. On the other hand, the specificity of apoE binding to SR-BI and promotion of SR-BI-dependent cholesterol efflux were not affected by carboxy-terminal deletions of apoE that eliminate a portion of or the entire region of residues 166−299. This indicates that the amino-terminal domain of apoE-containing amino acids 1−165 is sufficient for functional interactions with SR-BI. The observed specificity of POPC/C−apoE for SR-BI most likely is determined by its conformation on discoidal particles and may differ when apoE is bound to other naturally occurring lipoprotein particles.

Cross-competition experiments showed that excess unlabeled POPC/C−apoE or HDL competed efficiently for the binding of [^{125}I]POPC/C−apoE, while LDL competed poorly. Previous studies also showed that excess unlabeled HDL, POPC/C-apoE, and LDL competed efficiently, less efficiently, and relatively poorly, respectively, for the binding of [^{125}I]HDL (*33*). The nonreciprocal cross-competition between POPC/C−apoE and HDL and the different effect of SR-BI mutations on the binding properties of the different ligands (*44*) suggest that the binding sites of lipid-bound apoE and apoA-I may share common elements but have different affinities and are not equivalent. SR-BI exhibits complex binding properties suggesting the existence of multiple classes of binding sites or different modes of binding of the individual ligands (*2*, *10*, *44*). In this study, the competition and SR-BI mutagenesis experiments also suggest that the HDL and POPC/C−apoE binding sites may differ from the LDL binding site (*33*, *44*).

The specificity of POPC/C−apoE particles for WT and mutant SR-BI forms observed in this study has some similarities, as well as differences, with the specificity of POPC/C−apoA-I particles for WT and mutant SR-BI forms (*13*). The apoA-I studies showed that the affinity of binding of POPC/C−apoA-I particles to WT SR-BI and the Q402R/Q418R and M158R mutants was approximately 4.4, 10, and 48 μg of protein/mL, respectively, and the relative values of cholesterol efflux promoted by the WT or mutant receptors were 100, 12, and 32%, respectively. When the cholesterol efflux values were corrected for binding, the relative values were 100, 24, and 71%, respectively, indicating that cholesterol efflux did not correlate directly to the binding affinity of the ligand. On the basis of these and other data, we propose that efficient SR-BI-mediated cholesterol efflux to lipoproteins may require not only direct binding of the lipoprotein to the receptor (*10*) but also the formation of a "productive complex" (*13*) in which both the lipoprotein and the receptor should be precisely aligned, have the capacity to undergo appropriate conformational changes, or both (*13*, *57*).

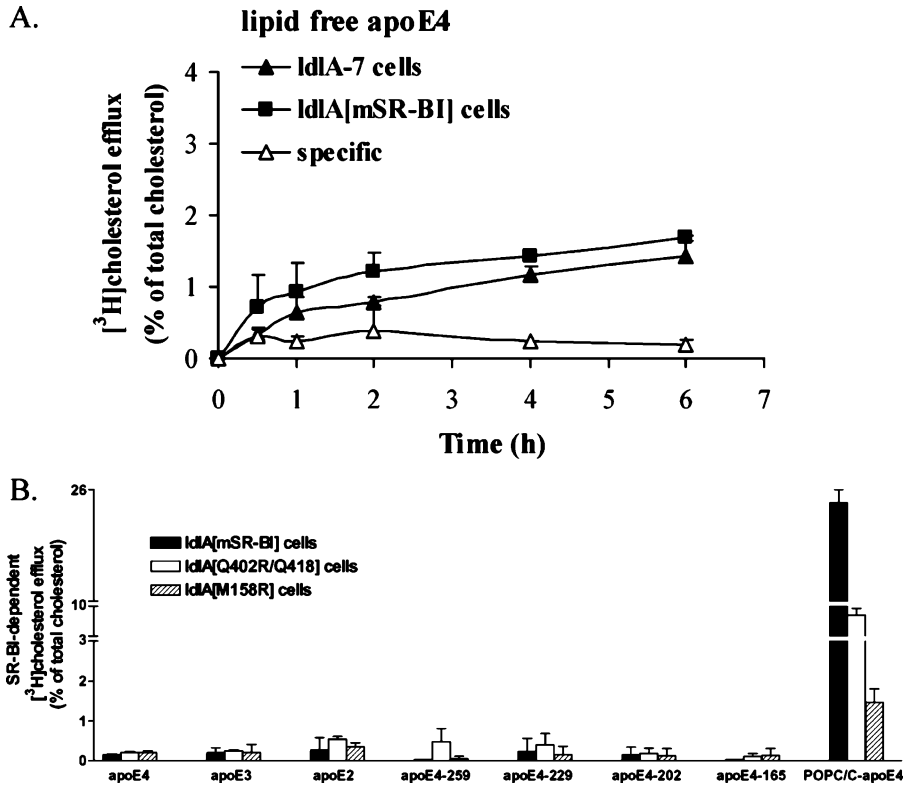

FIGURE 4: (A and B) SR-BI-dependent cholesterol efflux from cells expressing the WT SR-BI (ldlA[mSR-BI]) or two mutant forms of SR-BI (Q402R/Q418R and M158R), using lipid-free apoE as the cholesterol acceptor. (A) Control ldlA-7 cells and the ldlA[mSR-BI] cells were labeled with [^{3}H]cholesterol, and the time course of cholesterol efflux was determined following incubation with 150 μg of apoE4/mL as described in the legend of Figure 1. Values are the means ± SD from three independent experiments performed in duplicate. Panel B shows the percent values of net SR-BI-dependent cholesterol efflux after a 4 h incubation at 37 °C from cells expressing the WT and the two mutant forms of SR-BI using the WT or the truncated lipid-free apoE forms as cholesterol acceptors, in comparison with the percent values of net SR-BI-dependent cholesterol efflux promoted by POPC/C−apoE4. The SR-BI-dependent cholesterol efflux from cells expressing the mutant forms of SR-BI was corrected for the different level of mutant receptor expression in cells relative to the WT receptor expression. Values are the means ± SD from three independent experiments performed in duplicate.

Thus, the binding of POPC/C−apoA-I and POPC/C−apoE4 to SR-BI[Q402R/Q418R] appears to be nonproductive, since the rate of cholesterol efflux after correction for POPC/C−apoA-I and POPC/C−apoE4 binding is 24 and 47%, respectively (Table 1). On the other hand, the binding of POPC/C−apoA-I to SR-BI[M158R] is relatively productive. As shown in Table 1, cholesterol efflux after correction for POPC/C−apoA-I binding is 71% of the WT control (*13*). Similarly, cholesterol efflux after correction for binding of POPC/C−apoE4 to SR-BI[M158R] is approximately 75% of the WT control (Table 1). Thus, despite the very low affinity of this ligand for the mutant receptor, the binding appears to be relatively productive. However, given the diminished values of both receptor binding (8%) and cholesterol efflux (6%), it is also possible that the very low values of cholesterol efflux may simply reflect the absence of functional interactions between POPC/C−apoE4 and SR-BI[M158R].

Lipid-Free ApoE Is Not a Ligand for SR-BI and Does Not Promote SR-BI-Dependent Cholesterol Efflux. Previous studies using competition experiments indicated that lipid-free apoE may bind directly to SR-BI, and this interaction was reported to be associated with an increased level of CE uptake from HDL as well as VLDL (*46*). Earlier studies, prior to the discovery of SR-BI, also had suggested that apoE bound to the cell membrane may facilitate cholesteryl ester uptake from HDL (*58*, *59*). However, in our study, we could not detect direct binding of lipid-free apoE to SR-BI or evidence of inhibition of binding with receptor-blocking antibody. Another study has also shown that in macrophages overexpressing SR-BI, the net SR-BI-mediated cholesterol efflux to lipid-free apoE was approximately 0.5% (*60*). Thus, it appears that lipid-free apoE, prepared as described in Experimental Procedures, is not a ligand for SR-BI. Competition experiments also showed that lipid-free apoE competes inefficiently for the binding of HDL to SR-BI-expressing cells (*61*).

Consistent with the binding data (Figures 6 and 7), cholesterol efflux experiments also showed that lipid-free apoE does not promote SR-BI-dependent cholesterol efflux. In this study, the rate of SR-BI-dependent POPC/C−apoE-mediated cholesterol efflux was 48-fold greater compared to that of lipid-free apoE. Furthermore, when lipid-free apoE was mixed with lipid-bound apoE or HDL, it neither enhanced nor inhibited cholesterol efflux to either POPC/C−apoE or HDL from SR-BI-expressing or control cells. Thus, the previously reported effect of lipid-free apoE in the stimulation of cholesteryl ester uptake from HDL3 or VLDL (*46*) might have been due to SR-BI-independent mechanisms.

Physiological Significance of ApoE−SR-BI Interactions. ApoE is remarkably structurally (*62*, *63*) and functionally similar to apoA-I (*47*). Recent studies have shown that lipid-free apoA-I interacts functionally with ABCA1 and promotes

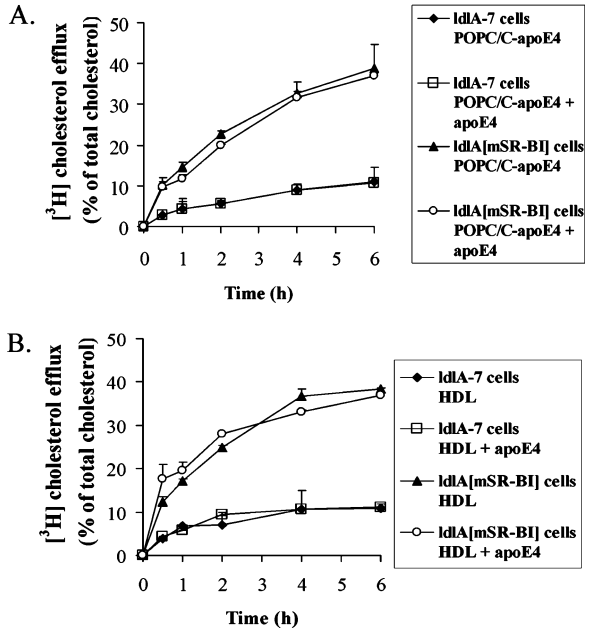

FIGURE 5: (A and B) Cholesterol efflux from control ldlA-7 cells and ldlA[mSR-BI] cells, using a mixture of lipid-free apoE and POPC/C−apoE particles or a mixture of lipid-free apoE and HDL as cholesterol acceptors. Cells expressing the WT form of SR-BI and the control untransfected cells were labeled with [^{3}H]cholesterol for 48 h as described in the legend of Figure 1 and incubated with 150 μg of lipid-free apoE4/mL for 1 h, followed by addition of 150 μg POPC/C−apoE4/mL (A) or 150 μg of HDL/mL (B). Cholesterol efflux was determined after incubation at 37 °C for up to 6 h. The percent of [^{3}H]cholesterol efflux from ldlA-7 cells and ldlA[mSR-BI] cells was determined as described in the legend of Figure 1. Values are the means ± SD from two independent experiments performed in duplicate.

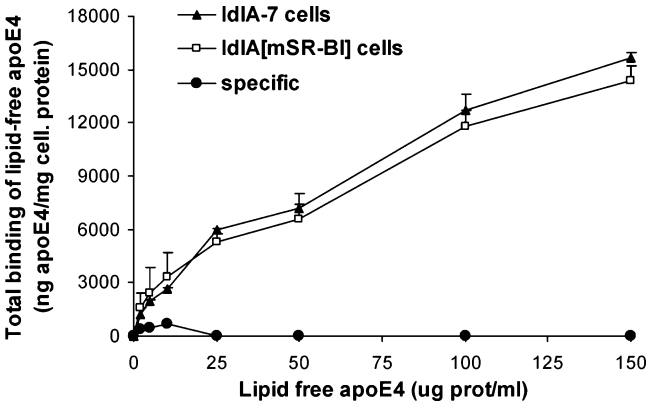

FIGURE 6: Concentration-dependent binding of lipid-free apoE4 to confluent monolayers of ldlA[mSR-BI] and control ldlA-7 cells. ldlA-7 cells and ldlA[mSR-BI] cells in 24-well plates were washed and incubated with various concentrations of lipid-free apoE4. The total level of binding to ldlA-7 cells expressing the WT SR-BI and the total level of binding to untransfected ldlA-7 cells were obtained as described in Experimental Procedures.

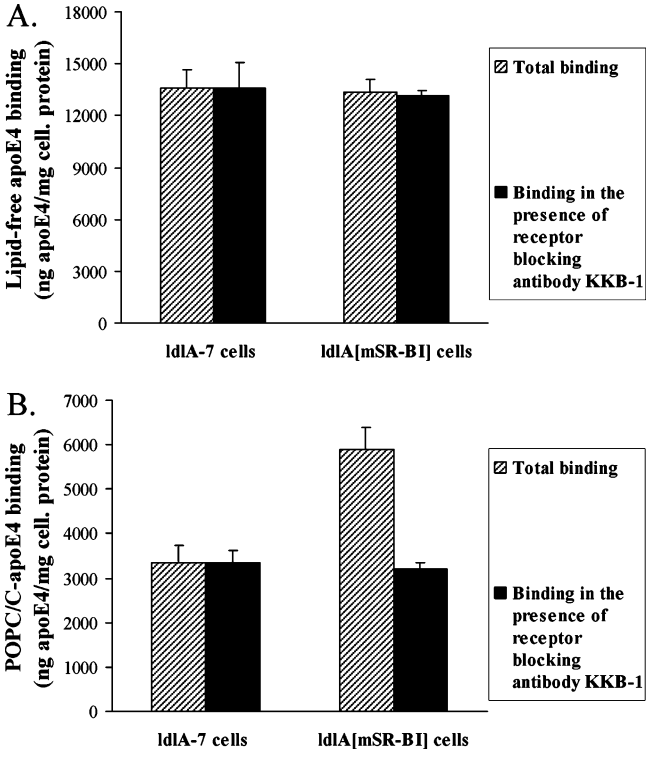

FIGURE 7: (A and B) SR-BI-dependent binding of lipid-free apoE and POPC/C−apoE4 complexes to confluent monolayers of control ldlA-7 cells and ldlA[mSR-BI]. ldlA-7 cells and ldlA[mSR-BI] cells in 24-well plates were washed and incubated with 150 μg/mL lipid-free apoE4 or POPC/C−apoE4. The level of binding of lipid-free apoE4 (A) and POPC/C−apoE4 (B) to control ldlA-7 cells and ldlA[mSR-BI] cells in the absence or presence of SR-BI-blocking monoclonal antibodies KKB-1 was obtained as described in Experimental Procedures. Note that the total binding as well as the binding in the presence of receptor-blocking monoclonal antibodies of lipid-free apoE4 between the ldlA-7 and ldlA[mSR-BI] cells is the same (A). Also note that the enhanced total binding of POPC−apoE to the SR-BI-expressing cells is blocked by receptor-blocking monoclonal antibodies (B). Two independent experiments were performed in duplicate for each cell line.

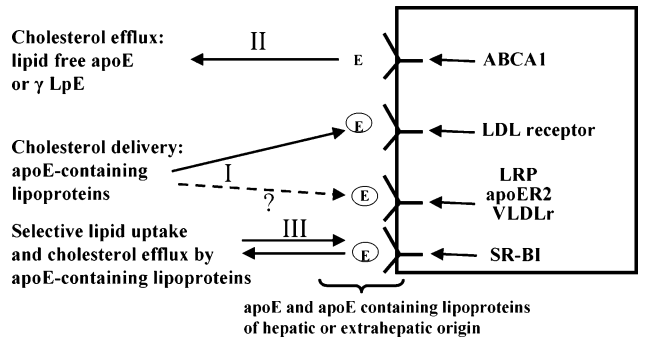

FIGURE 8: Contribution of apoE, apoE-containing lipoproteins, LDL receptor family members, ABCA1, and SR-BI in cellular cholesterol homeostasis. VLDLr represents very low-density lipoprotein receptor. apoER2 represents apoE receptor 2. LRP represents LDL receptor-related protein.

efflux of phospholipid and cholesterol and thus promotes biogenesis of HDL (*64*). In addition, apoA-I bound to HDL or rHDL promotes SR-BI-mediated lipid uptake and cholesterol efflux (*1*, *6*, *13*). In vivo studies using transgenic and knockout animals demonstrated that SR-BI controls the structure and composition of plasma HDL, the cholesterol contents of HDL, the adrenal gland, the ovaries, and the bile (*16*−*20*), and helps protect against atherosclerosis in mice (*65*−*67*). SR-BI deficiency also causes accumulation of large lipoprotein particles in the HDL2 and LDL size range that are enriched in apoE (*18*), indicating that SR-BI−apoE interactions may affect the structure and atheroprotective functions of HDL and apoE. The unique ability of SR-BI either to provide cholesteryl esters and other lipids to cells or to remove free cholesterol from cells via its interactions with HDL and apoE-containing lipoproteins may explain its

importance in the overall lipid homeostasis and its atheroprotective properties (*47*). In addition to its role in either the pathogenesis of or protection from cardiovascular disease, apoE, which is the only medium-size apolipoprotein in the brain (*48*), has been implicated in Alzheimer's disease (*48*, *68*, *69*). Figure 8 illustrates the interactions of apoE with SR-BI, the ABCA1 lipid transporter, and apoE-recognizing receptors that control lipid efflux and uptake and contribute to the overall lipid homeostasis in cells and tissues, including the brain. It is possible that in addition to potential interactions of apoE with amyloid peptide β (Aβ) and other brain proteins, disturbances in the lipid homeostatic mechanism may contribute to neurodegeneration observed in Alzheimer's disease (*48*).

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