Sterol regulation of scavenger receptor class B type I in macrophages

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Abstract **Scavenger receptor class B type I (SR-BI) is expressed in macrophages, but its role in sterol trafficking in these cells remains controversial. We examined the effect of sterol loading on SR-BI expression in human monocytes/macrophages, mouse peritoneal macrophages, and a cultured mouse macrophage cell line (J774 cells). Sterol loading using either acetylated LDL or 25-hydroxycholesterol resulted in a time- and concentration-dependent decrease in SR-BI protein and mRNA levels. Treatment of lipid-loaded J774 cells with cyclodextrin or HDL to promote cellular sterol efflux was associated with an increase in SR-BI expression. Studies were performed to determine if the sterol-associated downregulation of SR-BI in macrophages was mediated by either sterol regulatory element binding proteins (SREBPs) or the liver X receptor (LXR). Expression of constitutively active SREBPs failed to alter the expression of a luciferase reporter placed downstream of a 2,556 bp 5′ flanking sequence from the mouse SR-BI gene. Reduction in SR-BI expression was also seen in sterol-loaded peritoneal macrophages from mice expressing no LXRα and LXRβ. We conclude that SR-BI levels in macrophages are responsive to changes in intracellular sterol content and that these sterol-associated changes are not mediated by LXR and are unlikely to be mediated by an SREBP pathway.**—Yu, L., G. Cao, J. Repa, and H. Stangl. **Sterol regulation of scavenger receptor class B type I in macrophages.** ***J. Lipid Res.*** **2004.** 45: **889–899.**

Supplementary key words cholesterol • regulation • mouse scavenger receptor class B type I promoter

One of the earliest events in the formation of atherosclerotic plaque is the recruitment of macrophages into the arterial wall (1). Uptake of modified and oxidized LDL (ox-LDL) by scavenger receptors expressed in macrophages in the subendothelial space results in the formation of lipid-loaded foam cells (2, 3), which are the pathological hallmark of fatty streaks and atherosclerotic plaques (3).

Several proteins coordinate the uptake and removal of cholesterol from macrophages upon sterol loading. LDL receptor (LDLR) expression decreases to very low levels in cholesterol-loaded cells as a result of transcriptional regulation by sterol regulatory element binding proteins (SREBPs) (4). Unlike the LDLR, levels of the scavenger receptor class A and CD36, a member of the scavenger receptor class B family, both of which mediate the uptake of modified lipoproteins, remain high in sterol-loaded cells (5, 6). Oxidized lipids activate the nuclear receptor peroxisomal proliferator-activated receptor γ (PPARγ), resulting in increased expression of CD36 (6, 7). Lipid-laden macrophages also have increased levels of expression of two members of the ATP binding cassette (ABC) transporter family, ABCA1 (8, 9) and ABCG1 (10), which mediate the efflux of cholesterol from sterol-loaded cells (8–10). The genes encoding both of these ABC transporters are transcriptionally regulated by another nuclear receptor, liver X receptor (LXR) (9–11).

The scavenger receptor class B type I (SR-BI), a scavenger receptor that structurally resembles CD36 and participates in reverse cholesterol transport, is also expressed in macrophages (12–14). This cell surface receptor mediates the selective uptake of cholesterol and cholesterol esters (12) from lipoproteins to cells and the efflux of cholesterol from cells to lipoproteins (14, 15). Although SR-BI has previously been shown to be expressed in macrophages (13), its regulation in response to sterol loading remains controversial (16, 17). In this study, we examined

Abbreviations: ABC, ATP binding cassette; ac-LDL, acetylated LDL; BAC, bacterial artificial chromosome; FCS, fetal calf serum; 25-HC, 25-hydroxycholesterol; LDLR, LDL receptor; LXR, liver X receptor; M-CSF, macrophage colony-stimulating factor; M-SFM, macrophage serum-free medium; NCLPPS, newborn calf lipoprotein-deficient serum; ox-LDL, oxidized LDL; PPAR, peroxisomal proliferator-activated receptor; RAP, receptor-associated protein; SCAP, SREBP cleavage-activating protein; SF-1, steroidogenic factor 1; SR-BI, scavenger receptor class B type I; SREBP, sterol regulatory element binding protein.

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the effect of sterol loading and depletion on the expression of SR-BI in macrophages and consistently found that SR-BI protein was reduced to low levels with sterol loading in both mouse and human macrophages. This sterol regulation does not involve the two families of transcription factors that play an important role in the regulation of cholesterol homeostasis, SREBPs (4) and LXRs (9–11).

MATERIALS AND METHODS

Materials

J774 murine macrophages were obtained from the American Type Culture Collection. DMEM (low glucose), penicillin, and streptomycin sulfate were purchased from GIBCO BRL (Grand Island, NY). Fetal calf serum (FCS) and protease inhibitors (PMSF, leupeptin, pepstatin A, and aprotinin) were obtained from Sigma (St. Louis, MO), and a BCA protein assay kit was obtained from Pierce Chemical Co. (Rockford, IL). Cholesterol was purchased from Alltech (Deerfield, IL), 25-hydroxycholesterol (25-HC) from Steraloids (Wilton, NH), and hydroxypropyl β-cyclodextrin from Cyclodextrin Technologies Development, Inc. (Gainesville, FL). Human LDL (density 1.019–1.063 g/ml), human HDL (density 1.125–1.215 g/ml), newborn calf lipoprotein-deficient serum (NCLPPS; density >1.215 g/ml) (18, 19), and acetylated LDL (ac-LDL) were prepared as described previously (20). A rabbit polyclonal antipeptide antibody against the last 14 amino acids of murine SR-BI (Q820-6) was developed in our laboratory (21). Rabbit anti-bovine LDLR (638) (22) and anti-rat receptor-associated protein (RAP) polyclonal antibodies (692) (23) were kindly provided by Dr. Joachim Herz (University of Texas Southwestern Medical Center). A mouse anti-human CD36 antibody was obtained from Transduction Laboratories (Lexington, KY), and anti-Grp78 (BiP) was obtained from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada). Horseradish peroxidase-conjugated donkey anti-rabbit and sheep anti-mouse antibodies, the Enhanced Chemiluminescence Western Blotting Detection Kit, and [α-^{32}P]dCTP were purchased from Amersham Pharmacia (Piscataway, NJ). Plasmids were prepared using standard purification kits (Promega, Madison, WI). DNA sequencing was performed on an Applied Biosystems model 377 DNA sequencer (Foster City, CA).

Isolation of mouse peritoneal macrophages

Peritoneal macrophages were isolated from NIH Swiss Webster mice or *LXRα/β* knockout mice (9) (kindly provided by Dr. David J. Mangelsdorf) and their strain-matched controls. Mice were injected intraperitoneally with 2 ml of Brewer Thioglycollate Medium (DIFCO Laboratories, Detroit, MI), and 3 days later, resident peritoneal cells were harvested by lavage with PBS (24). Cells were washed with PBS, resuspended in medium A (low-glucose DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate) and plated at a density of 16 × 10^6 cells/100 mm dish. After 4 h, each dish was washed three times with DMEM to remove all nonadherent cells, after which the macrophage monolayers were incubated in medium A (day 0). On day 1, cells were incubated in medium B (low-glucose DMEM supplemented with 10% NCLPPS). On day 2, cells were switched to medium B supplemented with the indicated amount of ac-LDL or 25-HC/cholesterol. On day 3, cells were washed twice with ice-cold PBS and harvested.

Isolation of human monocytes/macrophages

Human monocytes were prepared by a modification of the method of Johnson, Mei, and Cohn (25, 26). Briefly, 500 ml of human fasted blood was collected in a 600 ml bag (Baxter Biotech Fenwal Division, Deerfield, IL) containing 7.5 ml of a sterile solution of 0.25 M disodium EDTA, pH 7.5, and centrifuged at 600 *g* for 7 min at room temperature. The plasma layer was removed. The buffy coat was collected, recentrifuged, and resuspended in 40 ml of plasma. Twenty milliliter aliquots of the cell suspension were layered on top of 15 ml of Lymphocyte Separation Medium (ICN Biomedicals, Inc., Aurora, OH) in 50 ml tubes. The tubes were centrifuged at 400 *g* for 30–40 min at room temperature. Mononuclear cells were collected from the interphase, washed three times with RPMI 1640 medium, and resuspended in 40 ml of macrophage serum-free medium (M-SFM) (GIBCO BRL). Two-milliliter aliquots were transferred to 60 mm plastic Petri dishes. After 2 h (day 0), the nonadherent cells were removed by washing cells three times with RPMI 1640 medium. Adherent cells were incubated in 3 ml of M-SFM. After 24 h (day 1), M-SFM was changed to medium C [M-SFM supplemented with 50 ng/ml human recombinant macrophage colony-stimulating factor (M-CSF; R&D Systems, Minneapolis, MN)]. Cells were kept for up to 6 days in M-SFM with M-CSF. For the lipid-loading study, cells were incubated on day 4 with the indicated amount of ac-LDL or 25-HC/cholesterol. On day 6, cells were washed twice with ice-cold PBS and harvested.

Cell culture, lipid loading, and immunoblot analysis

J774 cells were plated on day 0 (3 × 10^6 cells/100 mm dish) and cultured in 8% CO_2 at 37°C in medium A. On day 1, cells

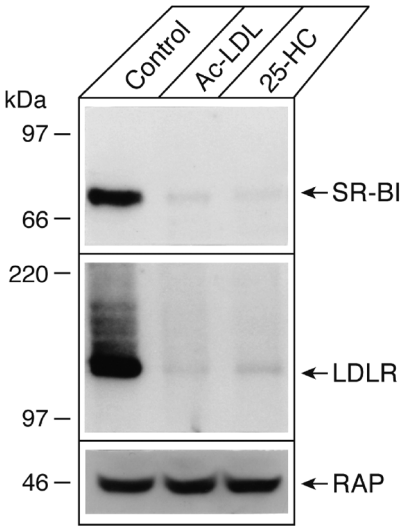

Fig. 1. Immunoblot analysis of scavenger receptor class B type I (SR-BI) and LDL receptor (LDLR) in murine peritoneal macrophages after incubation with acetylated LDL (ac-LDL) or 25-hydroxycholesterol (25-HC). Thioglycollate-stimulated mouse peritoneal macrophages were collected from NIH Swiss Webster mice as described in Materials and Methods. Cells were kept on day 0 in serum-containing medium (medium A) for 24 h and then incubated in lipoprotein-free serum medium (medium B) for another 24 h. On day 2, cells were treated with either ac-LDL (100 μg/ml) or 25-HC (1 μg/ml) in medium B. On day 3, cells were harvested and 50 μg of cell lysates was size-fractionated and immunoblotted with antibodies to SR-BI, LDLR, and receptor-associated protein (RAP). The blots were then incubated with horseradish peroxidase-conjugated donkey anti-rabbit antibody and developed using the Enhanced Chemiluminescence detection system. The filters were exposed to Kodak X-Omat Blue XB-1 film at room temperature for 30 s (SR-BI), 10 s (LDLR), or 2 s (RAP). Note the concurrent down-regulation of SR-BI and LDLR. This experiment was repeated once, and identical results were obtained.

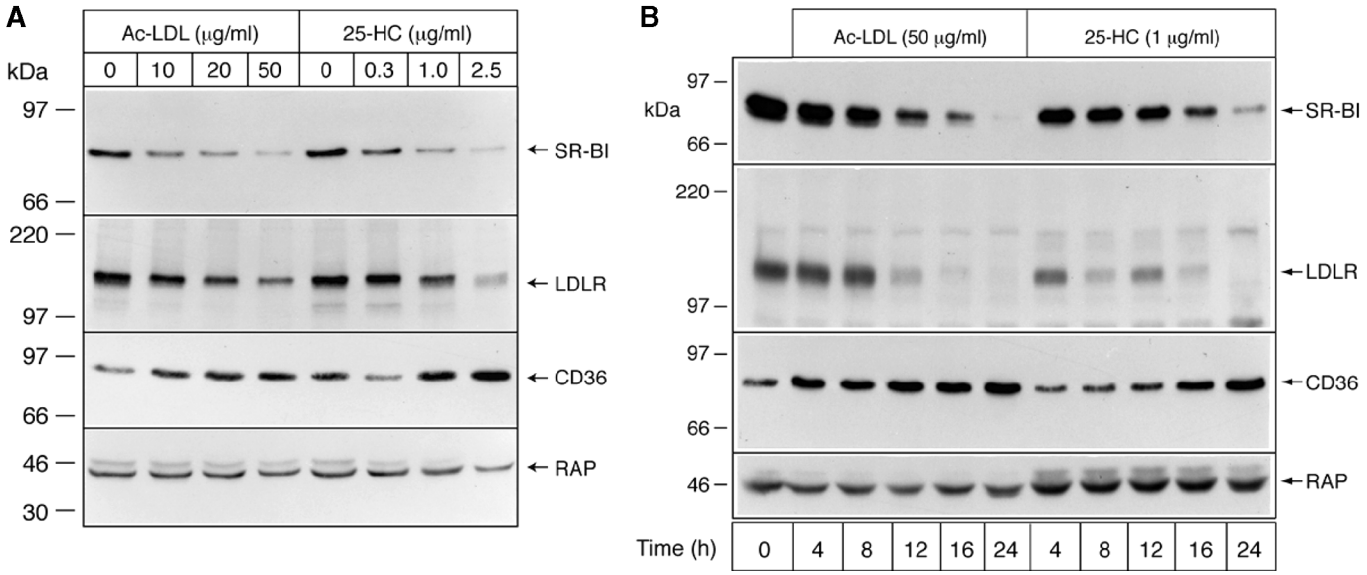

Fig. 2. Immunoblot analysis of SR-BI and LDLR in J774 cells. A: The murine macrophage cell line J774 was plated at a density of 3×10^6/100 mm dish and treated as described in Materials and Methods with either ac-LDL or 25-HC at the indicated concentrations for 24 h. Cells were collected and cell lysates were processed for immunoblotting as described in Fig. 1. The filters were also immunoblotted with a mouse monoclonal antibody to CD36. The filters were exposed to the film at room temperature for 15 s (SR-BI), 10 s (LDLR), 30 s (CD36), or 2 s (RAP). B: J774 cells were treated with either 50 μg/ml ac-LDL or 1 μg/ml 25-HC for the indicated times. The cell lysates were prepared and immunoblotted as described in A. Again, a downregulation of SR-BI and LDLR with dose and time is seen, which is in contrast to the upregulation of CD36. This experiment was performed six times, and similar results were achieved.

were switched to medium B. On day 2, cells were incubated in medium B supplemented with the indicated amount of ac-LDL or 25-HC/cholesterol (1:10). On day 3, the cells were washed twice with ice-cold PBS before harvesting. Cells were spun at 3,000 rpm for 5 min and then resuspended in lysis buffer [1% (v/v) Triton, 50 mM Tris, 2 mM $CaCl_2$, and 80 mM NaCl, pH 8] with protease inhibitors (0.5 mM PMSF, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, and 2 μg/ml aprotinin). After 15 min on ice, the cell lysate was centrifuged at 12,000 *g* for 10 min at 4°C. The supernatant was collected and protein concentration determined using the BCA kit. A total of 50 μg of cell lysate was size fractionated on a 6.5% SDS-polyacrylamide gel before transfer to Hybond-C Extra membranes (Amersham Pharmacia). The membranes were incubated with antibodies against SR-BI, LDLR, CD36, BiP, or RAP in PBS supplemented with 0.05% Tween 20, 5% powdered milk, and 5% newborn calf serum (all Sigma). Immunodetection was performed using the Enhanced Chemiluminescence Detection Kit according to the manufacturer's instructions. Filters were exposed to Kodak X-Omat Blue XB-1 films (Rochester, NY) at room temperature.

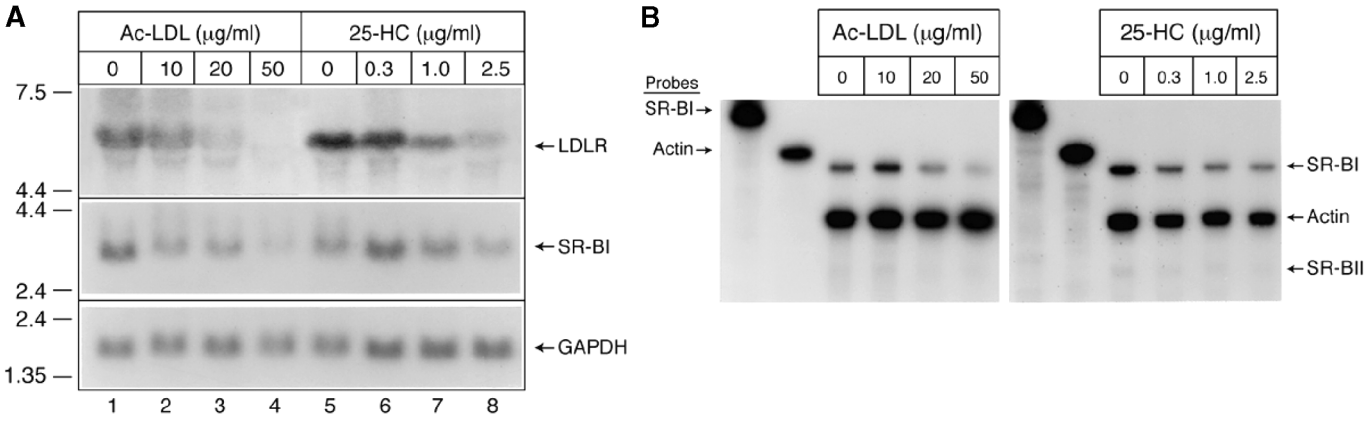

Fig. 3. Northern blot analysis (A) and RNase protection assay (B) of SR-BI and LDLR mRNAs in J774 cells incubated with ac-LDL or 25-HC. A: J774 cells were cultured and treated as described in Fig. 2. Total RNA was isolated from cultured cells, and 20 μg of total RNA was fractionated and transferred to the filter. The filter was then hybridized with α-^{32}P-labeled probes for SR-BI, LDLR (23), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 24) as a loading control. B: Ten micrograms of total RNA from each sample was analyzed by RNase protection assay. The purified protection fragments were fractionated on a 6% denaturing polyacrylamide gel. The protected fragments of SR-BI (307 bp), SR-BII (200 bp), and β-actin (used as an internal control) are indicated.

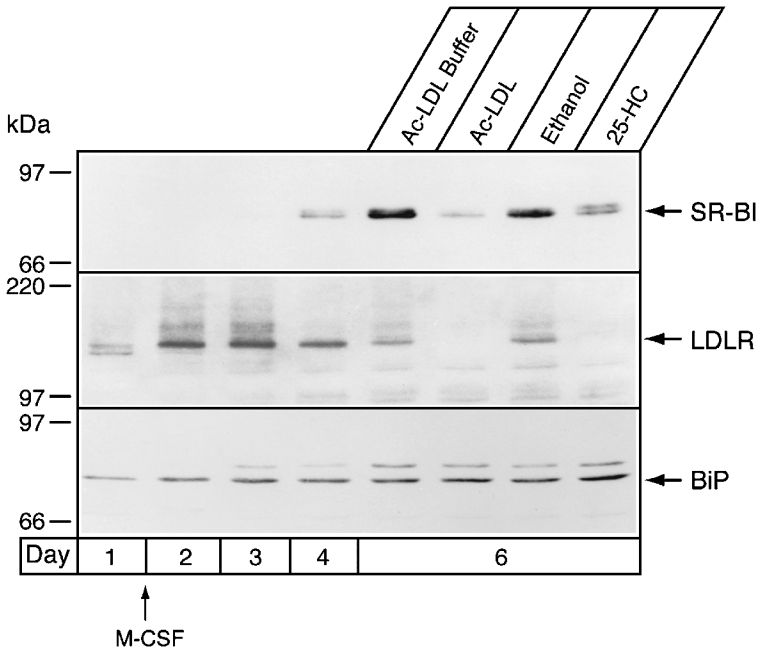

Fig. 4. Addition of ac-LDL and 25-HC to human macrophages results in reduced SR-BI levels. Human blood monocytes/macrophages were prepared as described in Materials and Methods and grown in medium C (macrophage serum-free medium) supplemented with 50 ng/ml human recombinant macrophage colony-stimulating factor (M-CSF). The cells were harvested at the indicated times, and cell lysates were prepared. On day 4, separate sets of cells were treated with either ac-LDL (100 μg/ml) or 25-HC (2.5 μg/ml) and cholesterol (10 μg/ml) or their appropriate controls ethanol and ac-LDL buffer. On day 6, cells were harvested. All cell lysates were immunoblotted with antibodies against SR-BI, LDLR, and Grp78 (BiP). BiP was used as a loading control for this experiment. The filters were exposed to the films at room temperature for 2 min (SR-BI), 2 min (LDLR), or 1 min (BiP). Note the increase in SR-BI with M-CSF treatment. The experiment was repeated once and gave similar results.

RNA hybridization

Total RNA was prepared from cultured cells using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX). For Northern blot analysis, 20 μg of total cellular RNA was size-fractionated on 1% (w/v) agarose and 2% (v/v) formaldehyde gel and transferred to BIOTRANS Nylon membranes (ICN, East Hills, NY). Filters were hybridized with ~1 × 10^6 cpm/ml of a 400 bp α-^{32}P-labeled probe generated from the mouse SR-BI cDNA or an α-^{32}P-

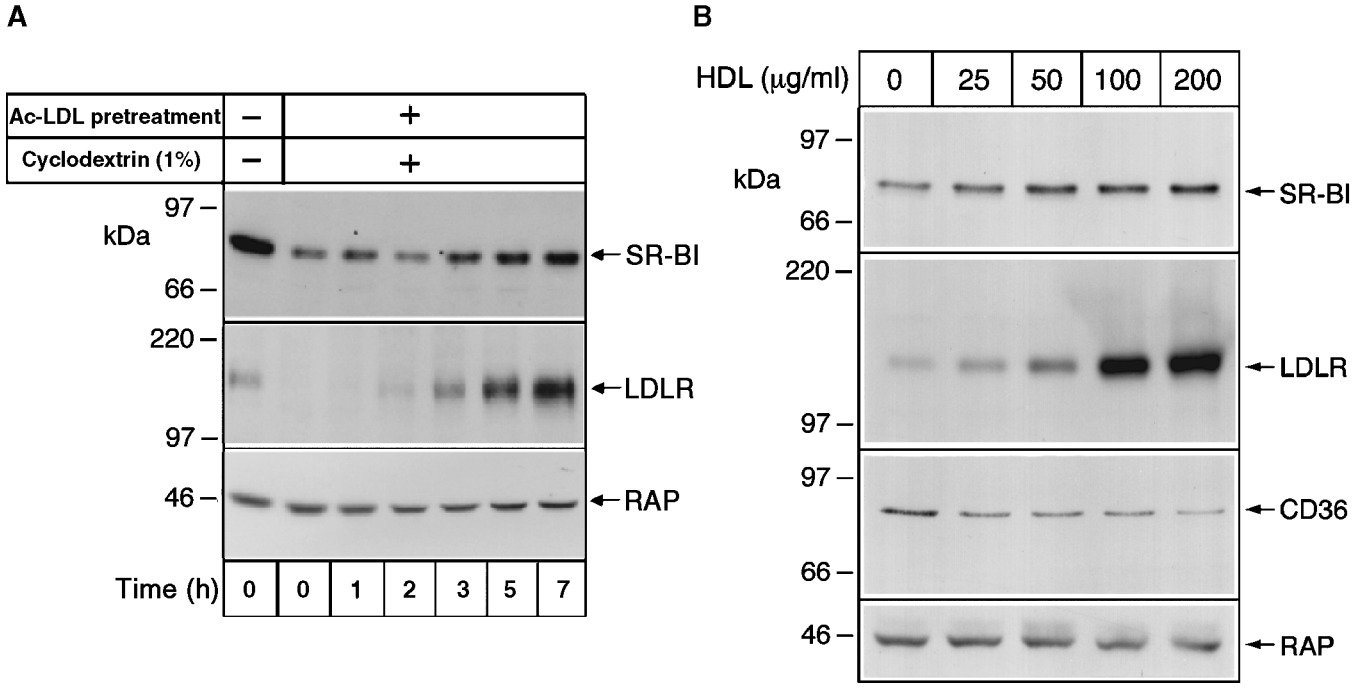

Fig. 5. Cholesterol depletion in J774 cells results in increased expression of SR-BI. A: Effects of cyclodextrin on SR-BI expression. J774 cells were plated on day 0 at a density of 6 × 10^6 cells/100 mm dish in medium A. On day 1, the medium was replaced with fresh medium A supplemented with 50 μg/ml ac-LDL. On day 2, the cells were washed twice with PBS and then grown in medium B supplemented with 50 μM compactin, 50 μM mevalonate, and 1% cyclodextrin. Cells were harvested at the indicated times and immunoblotted with antibodies to SR-BI, LDLR, and RAP as described in Fig. 1. The filters were exposed to film at room temperature for 15 s (SR-BI), 8 s (LDLR), or 2 s (RAP). B: Effects of HDL on SR-BI expression. J774 cells were plated at a density of 6 × 10^5 cells/100 mm dish in medium A on day 0. On day 1, cells were washed twice with PBS and then grown in medium B with the indicated amounts of HDL for 24 h. A total of 50 μg of cell lysates was used for immunoblot analysis against SR-BI, LDLR, CD36, and RAP as described in Fig. 2. The experiment was repeated twice, and similar results were obtained.

labeled cDNA probe for the mouse LDLR (27), rat glyceraldehydes-3-phosphate dehydrogenase (28), or mouse CD36 for 2 h at 65°C using Rapid-hyb™ buffer (Amersham Pharmacia). Afterward, filters were washed with 0.1× SSC/0.1% SDS (w/v) at 70°C for 60 min and exposed to Kodak X-Omat Blue XB-1 films with intensifying screens for 4–48 h at −70°C.

RNase protection assay

A 307 bp PCR fragment including the sequence encoding amino acids 397–499 of mouse SR-BI was amplified from murine hepatic total RNA by RT-PCR (Stratagene, La Jolla, CA). The oligonucleotides used were 5′-GGGCAAACAGGGAAGATCGAGCCA-3′ and 5′-ACCGTGCCCTTGGCAGCTGGTGAC-3′. The PCR product was subcloned into pGEM-T Easy vector (Promega). The cloned plasmid was then sequenced and linearized using *Nco*I. Afterward, an in vitro transcription reaction was performed in the presence of [α-^{32}P]CTP and SP6 polymerase (Promega) for 1 h at 37°C to generate the probe that included 192 bp of the vector sequence (total length of 499 bp) using the Riboprobe® in vitro transcription system (Promega). The DNA template was di-

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-2556 TAGCCTTGAT TGGCCTTGAA CTTCTGTTTC TTTGACCTCT TCCTTCCAAG TGCTGGGATC
                                    HSF
-2496 ACAGGGGTGC TCGCCATGCC TGTAAAGACC CCCATTTCCA ACAAATTGGA CACGAAGGCC
                                  ADR1
-2436 TTCTTTGATG AGTAGGCTTC AGGCTGAGAC TTGAGAGAGG AGGAGTCAGG CACTGAGGAT
-2376 CTAGAGAATT CCTTCATAAT AATCATGGCT GAGAAAACCC AGTCCCTCTG GTCTGAGATG
                                    dl/HSF
-2316 ACATCAGCTC CCTTTGGATA ACCTCCTGCA GACCAGATCT CTAGGATTTC GCTGCCTCAT
-2256 TCGGCCTCCT CCTAGGTCAC ATAGAATGGC CCAGTAGTGA CATCAGATAC CCCGGGGTTA
                                                          ADR1
-2196 ACACTCCTAA AGAATACGGG CCACTCAGGT TCCCGTTCAA ATCTAGAAGG TCTCCTCTTT
-2136 GGGACCACCT TTAGCAAACA TGTCCCTGAG CACCACGTAG TTTCTGGAGA GACTTTCAGC
                                                  HSF
-2076 CCCACTCCCC CCACCTCCCA TCTCCATCAT CAGGGGCACA GAGTGGGAGC AGGTTCAGAG
            ADR1                      STRE
-2016 GAACCTGGGC TGTGAGGACT CCCACCCCGA GTCCTGAGGA CCCTCTTCTG TAAGGTGAAG
                                ADR1
-1956 GTGTGAACTG CTCTTCTTAA AGCCAGTTGG TCGCTCATGA GCCTCTGAAC TTGGAACATG
                                c-Myb
-1896 CGCGGGTGTA ACACAGCCAA TGTTCCCAGG CCCCGCCCAC CCCTGCTCCT TTGTCTACTG
                                      Sp1
-1836 AACTTCTAGG CTCCTTTCTG TCTTTCTCCT GTGTCTACAC GTGTGCATGT TTGTGGGTGC
                     HSF     HSF
-1776 ACCTGAATGT GTTGTGTGAG ACAGAATATC TTACTAGGAA CCGGGTTAGA GAAGTCTGGC
-1716 TGGCCATCTG GCCCCAGGGA TCTTTCTTCC TGTCCCTGAC TCCCCTGCAC TGGAACTACC
  SF-1                  HSF
-1656 AGCCTAGAGT TTGTTTTTGT TTTGGACTTA CTTATTTTAT GTGTGCAATT GTCTTTCCTA
               SRY     SRY
-1596 CATGTCTGTC TGGGTACCAT GGGCATGCGT GGTATACTCA GAGGTCAGAG GAGGGGCATC
                                                        C/EBP
-1536 AGATCCCTTG GAACTGAAGT TAAGGATGGC TGTGAGCTGT CATGTGGGTT CTGGGGACTG
                                                      HSF
-1476 AACCCAGGTC CTCTGCAAGA GCAGCAAGTG CTCTTAACCA CTGAGCCATG TCTACCTCTG
-1416 AGCCATCTCT TCAGGCCTGG GTATCTCACC TGAACACGGA TGCTGGCGAC CAATATCAGG
                                deltaE
-1356 TGTCAAGCAC TCTACCAATA CGGTCCTTTG CACAGCTAAG TCTTTATTCC ATGTGATGGG
-1296 TGGAATCTGT CAGGGCTGGA AATATGACTT GGCTGTCTCT GGAGAACCTA CTCTGACTCA
                                                    HSF
-1236 ATATGAGCTA ACTCTGGGTC ATATTGCTGA AGGTGGTCTT CAAGAGCAGT CCTACTGGGT
-1176 TTTAACAGGA GCTGGGACTC GGTTTTAACA GGTGTGGGGC TGGGTTTTCT CAGCTCCGTA
                                              Sp1   dl/HSF
-1116 TCCACTTTTT GTGTCCTGAG TGGAATGCAG GCCTTGGCCT CACTGAGGAA GGAAGTCACC
                                  SF-1                          ADR1
-1056 CCATGCCTCA GGGTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TGGCTGTAAC

 -996 ACGAAGCATT TAGAATTAAA GGCTTAAAGG CTCTGGCTGT GGTCTGAGAA CGAGCTAACT
                                           AML-1a     HSF
 -936 GGGCACTGGG CAGAGCAGGG AAAGACAGAG TCCCTACCAC CCTGCTGGTA TCCTGAGTGG
 -876 GGTTCATGGC CAAAGAACGC ATAACTGAGC TCAACTGAGC TGGTGGTGTC TCTGGGTGAT
         SF-1
 -816 AAGGAAGAGT CAGCAGAGAG CATAGCCGCC AGGCAGTCTG ATAAGCCCCT CGAACACATT
 -756 CCTGGATGGA GTCAGGGGCC CAGCGCGTGT CTGGGGCCTG GAGTTTTGAG TGGAGGGTAT

 -696 GCTTAAGGCC TCGAGGGACA CAGAATTCTG TACGAAGGGT GTGCAAAGGT CCCGGGAGAC
                                                  C/EBP
 -636 ATGGAATTCC GAGGGGACAG TGTGCAAAGG CCACGAGGGA CACAGAATGG AGGGTGTGCA
                            C/EBP        N-Myc
 -576 AAAGCCCAAA GGGATATGGA ATTCCGAGTG GGGGAAGTGC AAAGGGCCCC GAGGGACACG
 -516 GAATTTTGAG TGGAGGGCGT TCATGGCCCA GAGGCTGGCC CGGGCTGACT GCAACTGATT
                               SF-1
 -456 TTAATGCAGG GGAGCGGGAG GCATTCGCAG GAGTCCGGAA GAAAGAAGAG GTCGCAGGGG
                                                                  Sp1
 -396 CGGGGGTAGT GGATGCAGAC GGTGCCAGGG TCTTCTGCCC TCTGTAGAGG GCACATCGGT

 -336 TCCCACCTAG ACCAGCAACC ACCAGGAAAG CCCAGCAGCT CGGAGGGGCG GCGCCCAAAG
                                c-Rel
 -276 GAAGCCACGC CCACGCCTCA CCATCAGAGC ACCGCCCACT CCCCGCCTCT TCCCACCCCT
                                              p300      Sp1
 -216 CGCCGGAATC CCGCGCCGAA CTCGGGGGCG GGCTGCCCGG GCCATGGCGC ATAAAGCCTC
                                  Sp1              SF-1      TATA Box
                                                    ▶
 -156 TGGCCACCTG CAGGGCTACT GCTGCTCCGG CCACCGCCAG GCACACACCT TGCTGCTGAG
        E-Box                                              SF-1
  -96 GGAGTCTCGG CTTCTGTCAT CTCTGTGGCC TCCGTCACCT CTGTCTCCGT CTCCTTCAGG
  -36 TCCTGAGCCC CGAGAGCCCC TTCCGCGCAC GCGGACATG
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Fig. 6. Nucleotide sequence of the mouse SR-BI 5′ flanking region. Nucleotide position +1 is assigned to the A of the ATG start codon (boldface). The potential transcriptional start site is indicated by an arrow. A potential TATA box-like sequence is underlined twice. Potential sites for transcriptional factor binding were identified by TFSEARCH version 1.3** computer analysis software (Yutaka Akiyama, Kyoto University, Kyoto, Japan).

gested by incubating the reaction product with 1 U of RQ DNase (Promega) for 15 min at 37°C. The reaction was then diluted with RNase-free water to 50 μl, extracted once with 50 μl of phenol-chloroform (1:1), and then purified using a G-50 spin column (5′ to 3′, Inc., Boulder, CO). A HybSpeed kit from Ambion, Inc. (Austin, TX), was used for the RNase protection assay. A total of 1×10^5 cpm of probe was mixed with 10 μg of total RNA that was isolated from the cultured cells using RNA STAT-60 (Tel-Test, Inc.). The RNase protection assay was performed as recommended by the manufacturer. The purified protection fragments were size-fractionated on a 6% denaturing polyacrylamide gel. The gels were dried and exposed to Reflection™ NEF496 films (NEN-Dupont, Wilmington, DE).

Cloning of the mouse SR-BI promoter

A bacterial artificial chromosome (BAC) clone (BACM16) containing the murine SR-BI gene (29) was kindly provided by Drs. Yukihiko Ueda and Edward M. Rubin (University of California, Berkeley, CA). The 5′ flanking sequence of mouse SR-BI was sequenced using BACM16 as the template. A fragment that extended 2,556 bp upstream of the methionine start codon was amplified by long-range PCR (Takara LA Taq kit; Takara Shuzo Co., Ltd.) with the following two primers designed: 5′-ACGGCTAGCCTTGATTGGCCTTGAAC-3′ and 5′-ATAAGCTTGTCCGCGTGCGCGGAAGG-3′. The PCR fragment was cloned into pGL3-Basic luciferase vector (Promega) to generate a reporter construct called pmSRBI.1. The integrity of the upstream fragment was confirmed by sequencing. Constructs containing smaller fragments of 5′ flanking sequence of the murine SR-BI gene were also cloned into pGL3-Basic, including 1,578 bp (pmSRBI.2), 687 bp (pmSRBI.3), and 294 bp (pmSRBI.4) fragments.

SR-BI promoter activity assay

HEK-293 cells were grown in medium A to a density of 1×10^5 cells per well in 12-well plates at 37°C under 8% CO_2. On day 1, cells were cotransfected with 50 ng of the luciferase reporter constructs, 20 ng of pCMV-β-galactosidase plasmid, and 50 ng of a plasmid expressing constitutively active SREBPs using the MBS kit (Stratagene). The pcDNA3 plasmid (Invitrogen) was used to adjust the total DNA to 1 μg per well. The mixed DNA was diluted to a final volume of 45 μl with water, followed by the addition of 5 μl of solution I and 50 μl of solution II supplied in the kit, and the mixture was incubated at room temperature for 15 min. The medium in each well was removed and replaced with 1 ml of 6% MBS DMEM low-glucose medium. A total of 100 μl of the resuspended DNA mixture was added to each well. After 3 h of incubation at 35°C under 3% CO_2, cells were gently washed twice with PBS and then cultured at 37°C under 8% CO_2 in medium B supplemented with 1 μg/ml 25-HC and 10 μg/ml cholesterol. After 24 h of incubation, cells were washed twice with PBS and harvested in 1× Reporter Lysis Buffer (Promega). Cell lysates were centrifuged at 10,000 *g* for 2 min at 4°C. Supernatants were assayed for luciferase and β-galactosidase activities in a Luminometer (MGM Instruments, Inc., Hamden, CT) using the Luciferase Assay System (Promega) and the Luminescent β-Galactosidase Reporter System 3 (Clontech Laboratories, Inc., Palo Alto, CA), respectively.

RESULTS

Reduction of SR-BI protein by ac-LDL and 25-HC in mouse peritoneal macrophages and a cultured mouse macrophage cell line (J774)

To evaluate the effect of cholesterol loading on SR-BI in macrophages, we incubated mouse peritoneal macrophages with ac-LDL or 25-HC. The protein levels of SR-BI and LDLR both decreased dramatically in peritoneal macrophages after 24 h of incubation with ac-LDL (100 μg/ml) or 25-HC (1 μg/ml) (**Fig. 1**). The amount of RAP did not change with treatment, indicating specific downregulation of SR-BI and LDLR. Similar dose-dependent reductions in SR-BI and LDLR were seen in the cultured mouse macrophage J774 cell line (**Fig. 2A**). In contrast to SR-BI and LDLR, the levels of CD36 increased after the addition of either ac-LDL or 25-HC (Fig. 2A), although SR-BI and CD36 are both members of the scavenger receptor class B family. The reduction in SR-BI was both dose dependent (Fig. 2A) and time dependent (Fig. 2B), with the amount of immunodetectable SR-BI decreasing by at least 50% within 12 h after the addition of either compound (Fig. 2B).

Reduction of SR-BI mRNA in J774 cells

To determine if the reduction of SR-BI in J774 cells was attributable to a decrease in the level of SR-BI mRNA, Northern blot analysis was performed. The levels of SR-BI

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Mouse    CCCACGCCTCACCATCAGAGCACCGCCCACTCCCCGCCTCTTCCCACCCCTCGCCGGAAT
Rat      CCCACGCCTCACCATCAGAGCACCGCCCACTCCCCGCCTTTTCCCACCCCTAGCCGGAAT
Human    CCCCGACCCCGCC--CCGGGC-CCGCTCAGGCCCCGCCCCTGCC-GCCGGAATCCTGAAG
         ***   ** * **  * * ** **** **  *******  * **  **     ** ***

Mouse    CCCGCGCCGAACTCGGGGGCGG----GCTGCCCGGGCCATGGCGCATAAAGCCTCTGGCC
Rat      CCCGCGCCGAACACGGGGGCGG----GCTGCCCGGGCCATGGCGCATAAAACCTCTGGCC
Human    CCCAAGGCTG-CCCGGGGGCGGTCCGGCGGCGCCGGCGATGGGGCATAAAACCACTGGCC
         ***  * *   * *********    ** ** * *** **** ******* ** ******

                                                  ↱
Mouse    ACCTGCAGGGCTACTGCTGCTCCGGCCACCGCCAGGCACACACCTTGCTGCTGAGGGAGT
Rat      ACCTGCAGGGCTACTGCTGCTCTGGCCACTGTCAGGCACACACCTTGCTGCT--------
Human    ACCTGCCGGGCTGCTCCTGCGTGCGCTGCCGTCCCGGATCCACCGTGCCTCTGCGGCCTG
         ****** ***** ** ****    **  * * *  * *  **** ***  **

Mouse    CTCGGCTTCTGTCATCTCTGTGGCCTCCGTCACCTCTGTCTCCGTCTCCT-TCAGGTCCT
Rat      --CGGTTTCTGTCATCTCTGTGGCCTCCGTCGCCTCTGTCGCCGTCCCCT-TCAGGTCCT
Human    CGTGCCCGGAGTCCCCGCCTGTGTCGTCTCTGTCGCCGTCCCCGTCTCCTGCCAGGCGCG
            *      ***  * *    * *  *     * * *** ***** ***  ****  *

Mouse    GAGCCCCGAGAGCCCCTT-------CCGCGCACGCGGAC
Rat      GAGCCCAGCGACTCTCGG-------CCGCGCACGCGGAC
Human    GAGCCCTGCGAGCCGCGGGTGGGCCCCAGGCGCGCAGAC
         ****** * **  * *          **  ** *** ***
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Fig. 7. Comparison of the mouse, rat, and human 5′ flanking regions using the CLUSTAL W Multiple Sequence Alignment Program (version 1.8, June 1999). The rat and human SR-BI transcription start sites are underlined (boldface) [(based on refs. (34) and (33), respectively]. A potential mouse SR-BI transcription start site is deduced from the comparison and indicated by an arrow.

and LDLR mRNA decreased dramatically with sterol loading (**Fig. 3A**), which suggests an effect either at the transcriptional level or on mRNA stability. Because the SR-BI gene is alternatively spliced at its 3′ end (30), we examined the relative effect of cholesterol loading on the expression of the two transcripts, SR-BI and SR-BII, by performing an RNase protection assay (Fig. 3B) (31). The level of SR-BI transcript declined with sterol treatment, as did SR-BII mRNA, which was barely detectable.

Reduction of SR-BI protein by ac-LDL and 25-HC in human macrophages

Fresh monocytes harvested from normolipidemic humans were cultured in serum-free medium for 24 h before the addition of human M-CSF to stimulate differentiation. Cells were incubated for the indicated times with human M-CSF to assess SR-BI protein levels, which increased with differentiation (**Fig. 4**). On day 6, a prominent upregulation of SR-BI was seen, whereas a downregulation of the LDLR occurred after an initial increase with M-CSF. On day 4, separate sets of M-CSF-treated cells were incubated with 100 μg/ml ac-LDL or 2.5 μg/ml 25-HC plus 10 μg/ml cholesterol, with the appropriate controls for ethanol or ac-LDL buffer. A significant reduction of SR-BI and LDLR protein was seen after 48 h of addition of either ac-LDL or 25-HC (Fig. 4). Thus, sterol loading also decreased the levels of SR-BI protein in human macrophages.

Cholesterol depletion increased SR-BI protein levels in macrophages

Next, we wanted to assess if cholesterol depletion increases SR-BI levels. Cyclodextrin, an agent that removes sterols from the cellular membranes of intact cells (32), was used to deplete lipid-loaded J774 cells of cholesterol. The amount of immunodetectable LDLR and SR-BI in cholesterol-loaded J774 cells increased with cyclodextrin treatment, although the magnitude of the change was greater for the LDLR than for SR-BI (**Fig. 5A**), indicating a regulation of SR-BI by the intracellular cholesterol content of macrophages.

A similar increase in LDLR and SR-BI protein levels was seen when J774 cells were treated with increasing amounts of HDL for 24 h, which serves as a cholesterol acceptor (Fig. 5B), although the relative change in SR-BI levels was not as prominent as that with cyclodextrin treatment. The level of CD36 decreased under the same conditions, indicating a differential regulation of the two members of the scavenger receptor class B family in response to changes in the cholesterol status of the macrophages.

SREBPs do not transactivate proximal SR-BI promoter

To explore the role of SREBPs, an important cholesterol sensor in cells, in the regulation of SR-BI, we cloned and sequenced a 2,556 bp fragment from the 5′ flanking sequence of the mouse SR-BI gene (**Fig. 6**) and placed it upstream of a luciferase reporter gene (pmSRBI.1). The transcription start site of the murine SR-BI gene has not been defined, but comparison of the sequence with that of human (33) and rat (34) predicts that it is very likely located as indicated by the arrow in **Fig. 7**. Plasmid pmSRBI.1 was cotransfected into HEK-293 cells with expression plasmids encoding the N-terminal domains of human SREBP-1a (pTK-SREBP-1a460) (35), SREBP-1c (pTK-SREBP-1c436) (35), and SREBP-2 (pTK-SREBP2-461; kindly provided by Hitoshi Shimano, University of Texas Southwestern Medical Center) or with a mutant form of hamster SREBP cleavage-activating protein (SCAP) [pCMV-SCAP (D443N)] (36). A luciferase reporter construct (pSRE-Luc) with three tandem copies of the sterol regulatory element from the LDLR promoter placed downstream of a luciferase reporter gene was used as a positive control (36). Expression of the nuclear form of SREBP-1a or SREBP-2 or the mutant SCAP resulted in an increased luciferase activity in HEK-293 cells transfected with plasmid pSRE-Luc but had no effect on the luciferase expression in cells transfected with plasmid pmSRBI.1 (**Fig. 8A**).

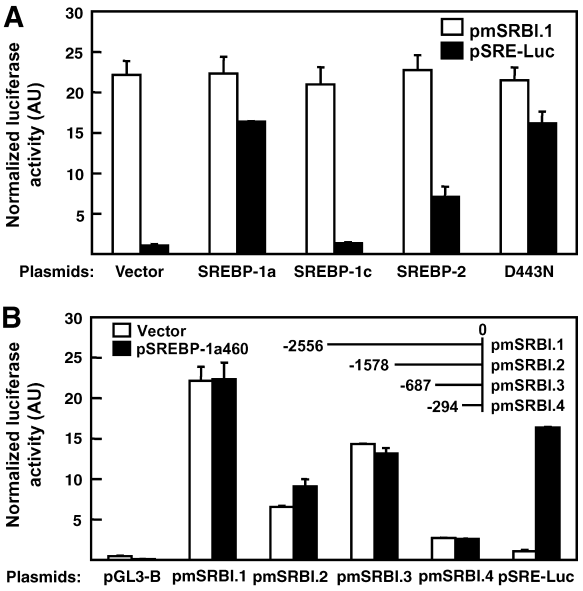

Fig. 8. Constitutively active sterol regulatory element binding protein-1a (SREBP1a) has no effect on mouse SR-BI promoter activity. A: HEK-293 cells were grown in medium A to a density of 1×10^5 cells per well in 12-well plates at 37°C. The next day, the cells were transfected using the MBS kit (Stratagene) with plasmids pmSRBI.1 (open bars) or pSRE-Luc (closed bars) (36) and cotransfected with plasmids expressing either constitutively active SREBPs or mutant SREBP cleavage-activating protein (SCAP) (D443N): pTK-vector (35), pTK-SREBP1a460 (35), pTK-SREBP1c436 (35), pTK-SREBP2-461, and pCMV-SCAP (D443N) (36), as described in Materials and Methods. B: HEK-293 cells transfected with the indicated luciferase constructs were cotransfected with either pTK-vector (open bars) or pTK-SREBP1a460 (closed bars). Plasmid pSRE-Luc with a luciferase gene placed downstream of a synthetic SRE-1 *cis* element was used as a positive control in this experiment. After incubation for 24 h, cells were harvested and luciferase activity was measured and normalized to β-galactosidase activity. Each value represents the average of triplicate incubations ± SD. This experiment was repeated four times and produced similar results. AU, arbitrary units.

To determine if a negative regulatory element was responsible for the absence of regulation of pmSRBI.1 expression by mature SREBP-1a, we generated a series of deletion constructs (pmSR-BI.2, pmSR-BI.3, and pmSR-BI.4) (Fig. 8B). Coexpression of pTK-SREBP-1a460 had no effect on luciferase activity in cells transfected with any construct (Fig. 8B). Again, pSRE-Luc was used as a positive control. To assess if endogenous SREBPs have any effect on the activity of the 2.556 kb mouse SR-BI proximal promoter, HEK-293 cells and mouse Raw 264.7 macrophage cells were transiently transfected with plasmid pmSRBI.1 and grown in either medium B (sterol minus medium) or medium B with either 50 μg/ml ac-LDL or 1 μg/ml 25-HC and 10 μg/ml cholesterol (sterol plus medium) for 24 h before luciferase assay. There was no difference observed in luciferase activities between cells grown in sterol minus and sterol plus media (data not shown). Thus, we found no evidence that SREBPs transactivate the transcription of murine SR-BI.

LXRα and LXRβ are not required for sterol-associated reduction in SR-BI protein expression in macrophages

To determine if sterol regulation of SR-BI is mediated by LXR in macrophages, peritoneal macrophages obtained from wild-type and *Lxrα/β*$^{-/-}$ mice (9) were treated with 100 μg/ml ac-LDL or 1 μg/ml 25-HC for 18 h as described in Materials and Methods, and SR-BI and LDLR protein levels were estimated by Western blot analysis. The treatment of ac-LDL and 25-HC resulted in a similar reduction in the levels of SR-BI and LDLR proteins in peritoneal macrophages of both genotypes (**Fig. 9**). Similar changes were seen at the mRNA level (data not shown). These data demonstrate that LXRα and LXRβ are not required for the sterol-mediated regulation of SR-BI in mouse macrophages.

DISCUSSION

In this study, we show that SR-BI levels in human and mouse macrophages are responsive to the sterol content of cells. SR-BI levels decreased when human and murine macrophages were cultured in media containing ac-LDL or 25-HC, and SR-BI levels increased with sterol depletion by cyclodextrin or HDL. SR-BI mRNA and protein levels were downregulated with cholesterol loading in cultured mouse macrophages. LXR was not required for the sterol-associated regulation of SR-BI in macrophages, as shown by the reduction in SR-BI protein in sterol-treated peritoneal macrophages from *Lxrα/β*$^{-/-}$ mice. We were unable to demonstrate transactivation of a reporter gene placed downstream of an ~2.5 kb 5′ flanking sequence from the start codon of the murine SR-BI gene. Additional studies will be required to determine the molecular mechanism responsible for the sterol regulation of SR-BI in macrophages.

Levels of SR-BI tend to be inversely related to sterol content in cell types other than macrophages, including steroidogenic cells and hepatocytes (21, 37). SR-BI levels are increased in lipid-depleted adrenocortical cells of apolipoprotein A-I knockout mice (38) and of the hypolipidemic estrogen-treated rat (21, 39). Conversely, SR-BI levels are reduced in sterol-enriched hepatocytes of estrogen-treated rats (21) and in hepatic parenchymal cells of rats fed a high-cholesterol diet (40). SR-BI levels are low in human monocytes (14) but increase upon differentiation into macrophages (Fig. 4). We and others consistently found that upon treatment with modified LDL, SR-BI levels are reduced (17) (Figs. 1–3). However, Hirano et al. (16) reported that both ox-LDL and ac-LDL increased the levels of immunodetectable SR-BI and SR-BI mRNA in freshly isolated human monocytes/macrophages. A possi-

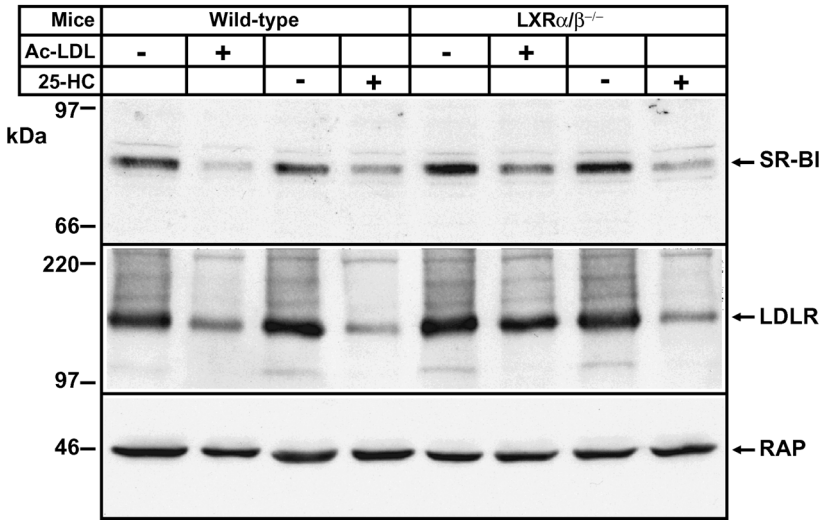

Fig. 9. Sterol-associated reduction in SR-BI levels is maintained in peritoneal macrophages from mice expressing no liver X receptor-α (LXRα) and LXRβ. Thioglycollate-stimulated peritoneal macrophages were harvested on day 0 as described in Materials and Methods from wild-type and *Lxrα/β*$^{-/-}$ mice (9). Cells were cultured and treated with 100 μg/ml ac-LDL and 1 μg/ml 25-HC, and SR-BI, LDLR, and RAP proteins were immunoblotted as described in Fig. 1.

ble explanation for this discrepancy may be the differences in the state of differentiation of the monocytes/macrophages used in the different studies. SR-BI expression increases with the differentiation of human monocytes to macrophages (41, 42) (Fig. 4). The cells used in the experiment reported by Hirano et al. (16) were cultured for only 3 days before lipid loading, and the medium was not supplemented with M-CSF; therefore, the cells may not have been fully differentiated.

The major regulatory proteins responsible for sterol-mediated changes in many of the genes involved in lipid metabolism are SREBPs (4). Previously, Lopez and McLean (34) reported that a constitutively active form of human SREBP-1a binds in a sequence-specific manner to the 5′ flanking region of rat SR-BI and transactivates a reporter gene placed downstream of an ~2.2 kb rat SR-BI 5′ flanking sequence. We failed to find any regulatory effects of SREBP-1a or SREBP-2 on the proximal mouse SR-BI promoter in either HEK-293 cells or HTB-9 cells (data not shown), the same cell line used in the study of Lopez and McLean (34). Although we cannot completely rule out a possible role of SREBPs in the sterol-associated regulation of SR-BI in macrophages without using macrophages from macrophage-specific SREBP pathway-deficient mice, which are not available at present, SR-BI mRNA and protein levels in the livers of mice expressing constitutively active forms of SREBP-1a and SREBP-2 are either similar or reduced (data not shown), which argues against these transcription factors directly regulating the SR-BI gene.

Another major class of transcription factors that regulate genes involved in cellular responses to lipids is the orphan nuclear receptor (43). LXRα and LXRβ orchestrate the regulation of numerous genes involved in cholesterol trafficking and metabolism in macrophages, including two members of the ABC transporter family, ABCA1 (9, 11) and ABCG1 (10). We found no evidence that these transcription factors participate in the regulation of SR-BI by sterols in macrophages (Fig. 9), which is consistent with a previous report that LXR agonists do not affect the expression of SR-BI in small intestine (9).

The orphan nuclear receptor steroidogenic factor 1 (SF-1) stimulates SR-BI promoter activity in vitro (33) and is required for the expression of SR-BI in steroidogenic tissues during murine embryogenesis (44). We used RT-PCR to determine if SF-1 is expressed in murine J774 macrophages and found no evidence for the presence of SF-1 mRNA in these cells (L. Yu and H. H. Hobbs, unpublished observation).

Finally, ligands of PPARα and PPARγ upregulate SR-BI expression in human monocytes/macrophages in the atherosclerotic lesion of apolipoprotein E-null mice (42). Therefore, it is unlikely that PPARs would downregulate SR-BI in our macrophage model after PPAR activation by sterol loading. CD36, which is increased in macrophages by the treatment of oxidized lipid via PPARγ (6, 7), is upregulated with 25-HC or ac-LDL treatment, whereas SR-BI is downregulated, suggesting the differential regulation of the two members of the scavenger receptor class B family. Moreover, PPARα and PPARγ exert their effect on other cholesterol-regulated genes, such as ABCA1, through the enhanced expression of LXRs (7, 45), which we have shown is not required for the sterol regulation of SR-BI. Thus, we failed to find evidence that any of the well-characterized pathways for sterol regulation contribute to the sterol-mediated repression of SR-BI in macrophages.

The levels of SR-BI mRNA were decreased dramatically with sterol loading (Fig. 3A, B), which suggests an effect either on the transcription rate or on mRNA stability. The regulation of SR-BI expression can also occur at the post-translational level in a tissue-specific manner, as shown in *pdzk1* knockout mice, in which hepatic SR-BI protein levels were reduced dramatically but SR-BI mRNA abundance remained unchanged (46). Mardones et al. (47) also showed that fibrates reduced SR-BI protein levels in the liver without changing its mRNA levels. We have shown in this study that the dramatic sterol-mediated reduction of SR-BI protein was associated with a downregulation of SR-BI mRNA, suggesting a different regulatory mechanism.

SR-BI has been detected by immunohistochemistry in atherosclerotic lesions from humans and mice (14, 16, 42). Based on our findings, the expression of SR-BI should be very low in foam cells. If SR-BI levels were increased in foam cells, cholesterol would be expected to be caught in a futile cycle, being transported between foam cells and lipoproteins, as has been observed in Raw macrophages expressing high levels of both recombinant SR-BI and ABCA1 (48). If macrophage SR-BI played a significant role in lipid trafficking in the atherosclerotic lesion, we would predict, based on the results of these studies, that it is at a very early stage of lesion development, when it is acting as an uptake receptor to deliver cholesteryl esters from cholesterol-laden particles in the circulation.

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