Scavenger receptor BI facilitates the metabolism of VLDL lipoproteins in vivo

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Abstract Scavenger receptor class B type I (SR-BI) functions as an HDL receptor that promotes the selective uptake of cholesteryl esters (CEs). The physiological role of SR-BI in VLDL metabolism, however, is largely unknown. SR-BI deficiency resulted in elevated VLDL cholesterol levels, both on chow diet and upon challenge with high-cholesterol diets. To specifically elucidate the role of SR-BI in VLDL metabolism, the plasma clearance and hepatic uptake of ^{125}I-β-VLDL were studied in SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice. At 20 min after injection, 66 ± 2% of the injected dose was taken up by the liver in SR-BI$^{+/+}$ mice, as compared with only 22 ± 4% (*P* = 0.0007) in SR-BI$^{-/-}$ mice. In vitro studies established that the B_{max} of ^{125}I-β-VLDL binding was reduced from 469 ± 30 ng/mg in SR-BI$^{+/+}$ hepatocytes to 305 ± 20 ng/mg (*P* = 0.01) in SR-BI$^{-/-}$ hepatocytes. Both in vivo and in vitro, limited to no selective uptake of CEs from β-VLDL was found. Interestingly, HDL effectively competed for the association of β-VLDL in the presence as well as in the absence of SR-BI, indicating a second common recognition site. In conclusion, SR-BI plays an important physiological role in the metabolism of VLDL (remnants).—Van Eck, M., M. Hoekstra, R. Out, I. S. T. Bos, J. K. Kruijt, R. B. Hildebrand, and T. J. C. Van Berkel. **Scavenger receptor BI facilitates the metabolism of VLDL lipoproteins in vivo.** ***J. Lipid Res.*** **2008.** 49: **136–146.**

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The scavenger receptor class B type I (SR-BI) is a 57 kDa cell surface receptor that mediates the selective uptake of cholesteryl esters (CEs) from HDLs (1–3). During this process, CEs from the core of the HDL particle are delivered into cells without degradation of the protein moiety. SR-BI is highly expressed in liver and steroidogenic tissues, plays a key role in HDL cholesterol metabolism, and regulates the supply of cholesterol to steroidogenic tissues (2, 4, 5). Several lines of evidence indicate an anti-atherogenic role for SR-BI in atherogenesis. Huszar et al. showed that LDL receptor-deficient (LDLR$^{-/-}$) mice with an attenuated expression of SR-BI are more susceptible to atherosclerotic lesion development (6). Furthermore, disruption of SR-BI in wild-type (7) as well as in LDLR$^{-/-}$ mice (8) results in a highly increased susceptibility to atherosclerotic lesion development. When cross-bred onto the apolipoprotein E knockout (apoE$^{-/-}$) background, SR-BI deficiency leads to severe cardiac dysfunction and premature death (9, 10). Hepatic overexpression of SR-BI, on the other hand, protects against the development of atherosclerosis (11–13).

The anti-atherogenic function of SR-BI is largely attributed to its role in the uptake of HDL CE by the liver. However, in addition to its role in controlling HDL cholesterol levels, SR-BI has also been implicated in the binding of a wide array of other ligands, including anionic phospholipids (14), advanced glycation end-products (15), apoptotic cells (16), and native and modified LDLs (2, 17–19). Distinct binding sites on SR-BI mediate the binding of this wide variety of ligands. In vitro studies have demonstrated that HDL competes for the binding of LDL to SR-BI, whereas LDL only poorly inhibits the binding of HDL (2, 17–19). Furthermore, SR-BI mutagenesis studies provided support for the proposal that the interaction of SR-BI with HDL differs from that with LDL (17).

Interestingly, SR-BI transgenics display reduced VLDL and LDL cholesterol levels (12, 20), whereas disruption of the SR-BI gene in apoE$^{-/-}$ mice results in an increase in circulating VLDL and LDL levels (9, 10). In addition, adenoviral overexpression of SR-BI in liver reduces VLDL and LDL levels in C57Bl/6 mice (11, 21) and reverses fibrate-induced hypercholesterolemia in apoE$^{-/-}$ mice (22). The anti-atherogenic effect of SR-BI could therefore also be caused in part by promoting the clearance of atherogenic apoB-containing lipoproteins. On the other hand, Webb et al. have recently shown that adenoviral overexpression of SR-BI in human apoB transgenic mice (23) and apoE$^{-/-}$ mice (24) only minimally affected LDL cholesterol levels. Furthermore, in the apoE$^{-/-}$ mice, no effect on VLDL cholesterol, the major cholesterol-

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transporting lipoprotein in these mice, was observed. The atherogenic form of VLDL that accumulates in animals on a high-cholesterol diet is β-migrating VLDL (β-VLDL), the ultimate remnant of LPL-mediated lipolysis of VLDL triglycerides (25, 26). In the present study, we utilized radiolabeled β-VLDL to exclude a possible limiting role of LPL activity in the clearance of VLDL. Direct evidence for a role of SR-BI in β-VLDL metabolism in vivo was obtained by performing serum decay and liver uptake studies in SR-BI-deficient mice and wild-type littermates. In addition, the effect of SR-BI deficiency on the metabolism of β-VLDL was determined in vitro using primary hepatocytes isolated from SR-BI-deficient and wild-type mice.

EXPERIMENTAL PROCEDURES

Mice

SR-BI knock-out mice were kindly provided by Dr. M. Krieger. In these mice, the entire coding region of the first exon, constituting the N-terminal cytoplasmic domain and a portion of the N-terminal transmembrane domain, were deleted, leading to a functionally null allele (27). Heterozygous SR-BI knock-out mice were crossed to generate wild-type (SR-BI$^{+/+}$), heterozygous mutant (SR-BI$^{+/-}$), and homozygous mutant (SR-BI$^{-/-}$) progeny. The presence of the targeted and/or wild-type SR-BI alleles was determined by PCR amplification of DNA extracted from tail biopsies. The primers 5′-GAT-GGG-ACA-TGG-GAC-ACG-AAG-CCA-TTC-T-3′ and 5′-TCT-GTC-TCC-GTC-TCC-TTC-AGG-TCC-TGA-3′ were used to detect the presence of the targeted and the wild-type SR-BI allele simultaneously. PCR resulted in a 1.0 kb band and a 1.5 kb amplification product diagnostic of the wild-type and the targeted allele, respectively. Mice were maintained on sterilized regular chow, containing 4.3% (w/w) fat and no added cholesterol (RM3; Special Diet Services, Witham, UK), or when indicated, a semi-synthetic Western-type diet, containing 15% (w/w) fat and 0.25% (w/w) cholesterol (Diet W; Abdiets, Woerden, The Netherlands) or a high-cholesterol/cholate diet, containing 15% (w/w) fat, 1% cholesterol, and 0.5% cholate (Diet N; Abdiets). Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with national laws and conducted in conformity with the Public Health Service Policy. All experimental protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

Cholesterol analyses

After an overnight fasting period, approximately 100 μl of blood was drawn by tail bleeding. The concentrations of free cholesterol in serum were determined by enzymatic colorimetric assays with 0.025 U/ml cholesterol oxidase (Sigma) and 0.065 U/ml peroxidase (Roche Diagnostics; Mannheim, Germany) in reaction buffer (1.0 KPi buffer, pH 7.7, containing 0.01 M phenol, 1 mM 4-amino-antipyrine, 1% polyoxyethylene-9-laurylether, and 7.5% methanol). Total cholesterol content was determined after addition of 15 μg/ml cholesteryl esterase (Roche Diagnostics). Absorbance was read at 490 nm. The distribution of cholesterol over the different lipoproteins in serum was determined by fractionation of 30 μl serum of each mouse using a Superose 6 column (3.2 × 300 mm; Smart-system, Pharmacia, Uppsala, Sweden). Total cholesterol content of the effluent was determined as above.

Analysis of gene expression by real-time quantitative PCR

Total RNA was extracted from liver by the acid guanidium thiocyanate-phenol chloroform extraction method according to Chomczynski and Sacchi (28). cDNA was synthesized from 0.5–1 μg of total RNA using RevertAid™ M-MuLV Reverse Transcriptase according to the manufacturer's instructions. mRNA levels were quantitatively determined on an ABI Prism® 7700 Sequence Detection system (Applied Biosystems; Foster City, CA) using SYBR-green technology. PCR primers (**Table 1**) were designed using Primer Express 1.5 software with the manufacturer's default settings (Applied Biosystems). mRNA expression levels are indicated relative to the average of the housekeeping genes hypoxanthine phosphoribosyltransferase (HPRT), GAPDH, ribosomal protein 36B4, and 18Sr-RNA.

Immunoblot analysis

Liver homogenate was prepared by lysis in 50 mM Tris-HCl, 1% Triton-X100, 0.5% deoxycholate, 1% SDS containing 0.02 μg/ml leupeptin, 0.02 μg/ml aprotinin, and 0.02 μg/ml trypsin inhibitor. Cell debris was removed by centrifugation at 10,000 rpm for 10 min, and the protein concentration was determined according to Lowry et al. (29). Equal amounts of protein were separated on 7.5% SDS-PAGE gels and electrophoretically transferred to Protran nitrocellulose membrane (Schleicher and Schnell; Dassel, Germany). Immunolabeling was performed using either rabbit polyclonal α-SR-BI (ab3; Abcam, Cambridge, UK), rabbit polyclonal α-GAPDH (ab9485; Abcam), goat polyclonal α-LDL receptor-related protein type 1 (α-LRP-1) (sc16166; Santa Cruz Biotechnology, Santa Cruz, CA), or goat polyclonal α-LDLR (sc11824; Santa Cruz Biotechnology) as primary antibodies and horseradish peroxidase-conjugated goat-anti-rabbit IgG and donkey-anti-goat IgG (Jackson ImmunoResearch), respectively, as secondary antibodies. Finally, immunolabeling was detected by ECL (Amersham Bioscience, UK).

In vivo VLDL production

SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice were injected intravenously with 500 mg of Triton WR-1339 (Sigma) per kg body weight as a 15 g/dl solution in 0.9% NaCl after an overnight fast. Previous

TABLE 1. Primers for real-time PCR analysis

Gene	GenBank Accession Number	Forward Primer	Reverse Primer	Amplicon Size (bp)
SR-BI	NM016741	GGCTGCTGTTTGCTGCG	GCTGCTTGATGAGGGAGGG	63
LRP-1	NM008512	TGGGTCTCCCGAAATCTGTT	ACCACCGCATTCTTGAAGGA	95
LDLR	Z19521	CTGTGGGCTCCATAGGCTATCT	GCGGTCCAGGGTCATCTTC	68
HPRT	J00423	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAGC	91
GAPDH	NM008084	TCCATGACAACTTTGGCATTG	TCACGCCACAGCTTTCCA	103
36B4	NM007475	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG	85
18Sr-RNA	X00686	CCATTCGAACGTCTGCCC	GTCACCCGTGGTCACCATG	69

HPRT, hypoxanthine phosphoribosyltransferase; LDLR, LDL receptor; LRP-1, LDL receptor-related protein type 1; SR-BI, scavenger receptor class B type I.

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studies have shown that plasma VLDL clearance is virtually completely inhibited under these conditions (30). Blood samples (50 μl) were taken at 0, 1, 2, 3, and 4 h after Triton WR-1339 injection. Plasma triglycerides were analyzed enzymatically and were related to the body mass of the animals. The hepatic VLDL production rate was calculated from the slope of the curve and expressed as g/h/kg body weight.

Determination of lipolytic enzyme activity

To determine total body LPL and HL activity, blood was drawn from SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice after an overnight fast and at 20 min after an intravenous bolus injection of heparin (100 U/kg). The lipolytic activity of the postheparin plasma was measured by using a radiolabeled triolein emulsion, as described by Zechner (31). In brief, the substrate consisted of a radio-labeled triolein emulsion prepared by sonication of a mixture of 50 mCi glycerol-tri[9,10(n)-3 H]oleate, 4 mg of unlabeled glycerol trioleate, 0.1 mol/l Tris-HCl (pH 8.6), 0.1% Triton X-100, 2% BSA, and 2 ml of heat-inactivated human serum (a source of apoC-II, an LPL activator). Subsequently, 10 μl plasma was added to 0.2 ml of substrate and incubated for 30 min at 37°C. The reaction was stopped by addition of 3.25 ml of a mixture of chloroform-methanol-*n*-heptane (1:1.28:1.37, v/v/v) and 1 ml of 0.1 mol/l K_2CO_3-H_3BO_3 (pH 10.5). FFAs were extracted by vortexing this mixture for 15 s; phases were separated by centrifugation at 3,000 rpm at 4°C for 20 min, and 1 ml of the upper phase was counted for radioactivity. The lipolytic activity was calculated from the amount of FFAs released per milliliter per minute. The lipolytic activity was determined in the presence or absence of 1 mol/l NaCl to differentiate between LPL and HL activity. LPL activity was calculated as the portion of total lipase activity inhibited by 1 mol/l NaCl.

Lipoproteins

Human LDL (1.019 g/ml < d < 1.063 g/ml) and HDL (1.063 g/ml < d < 1.21 g/ml) was isolated from serum of healthy volunteers as described by Redgrave et al. (32).

β-VLDL was isolated from male Wistar rats (Charles River; Maastricht, The Netherlands) that were fed a diet containing 2% cholesterol, 5% olive oil, and 0.5% cholate (Hope Farms; Woerden, The Netherlands) for 2 weeks. After overnight fasting, blood was collected from the abdominal aorta and β-VLDL (d < 1.006 g/ml) was isolated using a discontinous KBr gradient, as described by Redgrave, Roberts, and West (32). The fraction of d < 1.006 g/ml was isolated and dialysed against PBS-1 mM EDTA. The β-VLDL consisted of 10 ± 0.7% protein (of which 42% was apoE, 25% apoB-100, 10% apoB-48, 10% apoC, 10% apoA-I, 2% apoA-II, and 1% apoA-IV) and 90 ± 7.3% lipids [of which 18% were phospholipids (PL), 16% triacylglycerols (TGs), 55% CEs, and 11% free cholesterol (FC)], and displayed β-mobility on agarose gels. The isolated β-VLDL was labeled with ^{125}I at pH 10.0 according to McFarlane (33), modified as described earlier (34), or labeled with cholesteryl [1α,2α (n)-^{3}H] oleate (Amersham) by exchange from donor particles as reported previously (35).

VLDL serum decay and liver uptake

The serum decay and liver uptake of β-VLDL was determined in SR-BI$^{-/-}$ mice and wild-type SR-BI$^{+/+}$ littermates as described previously (36, 37). Briefly, mice were anesthetized by subcutaneous injection of ketamine (60 mg/kg; Eurovet), fentanyl citrate, and fluanisone (1.26 mg/kg and 2 mg/kg, respectively; Janssen). The abdomen was opened and 10 μg ^{125}I-β-VLDL or ^{3}H-β-VLDL was injected into the vena cava inferior. At the indicated times, blood samples (50 μl) were drawn from the vena cava inferior, and liver lobules were tied off, excised, and weighed. Serum samples and liver lobules were counted for radioactivity. The total amount of radioactivity in serum was determined with the equation: serum volume (ml) = [0.0219 × body weight (g)] +2.66. At the indicated times after injection, the distribution of ^{125}I over the different lipoproteins in serum was analyzed by fractionation of 30 μl serum of each mouse using a Superose 6 column (3.2 × 30 mm, Smart-system; Pharmacia) and determination of the radioactivity in the effluent.

In vitro association and degradation studies with primary hepatocyes

Hepatocytes were isolated from SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice by linear perfusion at 37°C according to the method of Seglen (38), as previously described (39). Briefly, the liver was perfused with a 0.05% (w/v) collagenase type IV (Sigma) in a buffer of 67 mM NaCl, 6.7 mM KCl, 4.8 mM $CaCl_2$, 67 mM Hepes, and 2% BSA for 10 min at a flow rate of 14 ml/min. Subsequently, hepatocytes were isolated by gently mincing the liver in Hanks' buffer containing 0.3% BSA, filtered through nylon gauze, and centrifuged three times for 10 min at 50 *g*. The cell pellets consisted of pure (>99%) hepatocytes as judged by light microscopy, and the viability of the cells was >90% as determined by trypan blue exclusion. Using the isolated hepatocytes, β-VLDL binding and association/degradation studies were carried out with the indicated amounts of ^{125}I-β-VLDL or ^{3}H-CE-β-VLDL and the indicated amounts of competitor for 3 h at 4°C or 37°C, respectively, in a circulating lab-shaker at 150 rpm. For incubations at 37°C, the air was saturated with carbogen (95% O_2, 5% CO_2) every 30 min. Aspecific binding/association was determined by performing incubations in the presence of a 10-fold excess of unlabeled β-VLDL. After incubation, the cells were washed three times with washing buffer (50 mM Tris-HCl, 0.9% NaCl, 1 mM EDTA, 5 mM $CaCl_2$, and 0.2% BSA, pH 7.4) followed by two washing steps without BSA. To discriminate between cell association and cellular uptake, cells were incubated with 2 mg/ml proteinase K for 2 h at 0°C to break down the membrane-associated β-VLDL. Finally, the cells were lysed in 0.1 M NaOH, and the radioactivity was determined. Cell protein was measured by the method of Lowry et al. (29). Degradation of ^{125}I-β-VLDL was determined by precipitation with the trichloroacetic acid method as described by Henriksen, Mahoney, and Steinberg (39). To study the effect of chondroitin sulfate proteoglycans on β-VLDL association, parenchymal liver cells were preincubated with 0.24 U/ml chondroitin lyase ABC for 40 min at 37°C prior to the incubation with β-VLDL. GST-RAP, a fusion protein of glutathion S-transferase (GST) and the 39 kDa receptor-associated protein (RAP), was produced using *Escherichia coli* (DH5α) that were transformed with a plasmid (pGEX) encoding GST-RAP (generous gift of Dr. J. Herz, Dallas, TX) (40). The potency of the isolated GST-RAP to displace trypsin-activated ^{125}I-α_2-macroglobulin binding from its receptor was essentially equal to values described in the literature (IC_{50}, ~1 nM).

Statistical analyses

Statistically significant differences among the means of the different populations were tested using Student's *t*-test (Graphpad Instat software, San Diego, CA).

RESULTS

SR-BI deficiency results in an increase in serum VLDL cholesterol levels

On chow diet, containing 4.3% fat and no added cholesterol, HDL transports the majority of cholesterol in

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wild-type mice. As previously reported, SR-BI deficiency results in a dramatic increase in HDL cholesterol levels. Interestingly, under these conditions VLDL cholesterol levels were 45.9 ± 8.8 μg/ml in SR-BI-deficient mice as compared with only 6.1 ± 2.3 μg/ml in wild-type mice ($P = 0.017$) (**Fig. 1**). Challenging of SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice with a Western-type diet containing 15% fat and 0.25% cholesterol increased VLDL cholesterol levels to 242 ± 32.5 μg/ml and 453 ± 72 μg/ml ($P = 0.019$), respectively. Feeding a more severe high-cholesterol/cholate diet containing 15% fat, 1% cholesterol, and 0.5% cholate resulted in an increase of VLDL cholesterol to 871 ± 255 μg/ml in SR-BI$^{+/+}$ mice. Also under these conditions, VLDL cholesterol levels were significantly higher in SR-BI$^{-/-}$ mice as compared with SR-BI$^{+/+}$ mice (2,075 ± 464 μg/ml; $P = 0.041$) (Fig. 1).

The observed increase in VLDL levels could be a consequence of increased VLDL production by the liver or of a prolonged residence time owing to impaired receptor-mediated removal or impaired lipolysis.

SR-BI deficiency does not affect VLDL synthesis, hepatic LDL receptor and LRP expression, or lipolysis

Established receptor systems for the removal of VLDL (remnants) from the circulation are the LDLR and LRP-1. Therefore, we first investigated the effect of SR-BI deficiency on the hepatic expression of the LDLR and LRP-1 using real-time PCR and Western blotting (**Fig. 2**). No effect of SR-BI deficiency was observed on the mRNA expression of the LDLR or LRP-1, whereas on the protein level, a slight increase was observed.

To assess the effect of SR-BI deficiency on VLDL production by the liver, SR-BI-deficient mice and wild-type littermates were injected with Triton WR-1339. Triton WR-1339 blocks the lipase system, resulting in a virtually complete inhibition of VLDL clearance (30). As indicated in **Fig. 3A**, VLDL triglyceride production was 3.6 ± 1.1 g/h/kg in SR-BI$^{-/-}$ mice as compared with 5.2 ± 0.6 g/h/kg in SR-BI$^{+/+}$ mice, suggesting that the increased VLDL levels were not the result of increased VLDL production. LPL and HL activities were determined in postheparin plasma at 20 min after injection (Fig. 3B). The activity of both LPL and HL tended to be slightly higher in the absence of SR-BI, but this did not attain statistical significance. LPL activity in SR-BI-deficient mice was 63 ± 6 nmol FFA/ml/min (n = 5) as compared with 45 ± 15 nmol FFA/ml/min (n = 3) in wild-type controls. HL activity was 44 ± 5 nmol FFA/ml/min in SR-BI-deficient mice, as compared with 35 ± 3 nmol FFA/ml/min in wild-type animals.

Thus, the observed increase in VLDL levels in SR-BI-deficient mice is not a result of increased VLDL production, impaired lipolysis, or reduced clearance via the LDLR or LRP-1.

Reduced serum decay and liver uptake of β-VLDL in absence of SR-BI

To study the direct role of SR-BI in VLDL metabolism in vivo, the kinetics of serum decay and liver uptake of

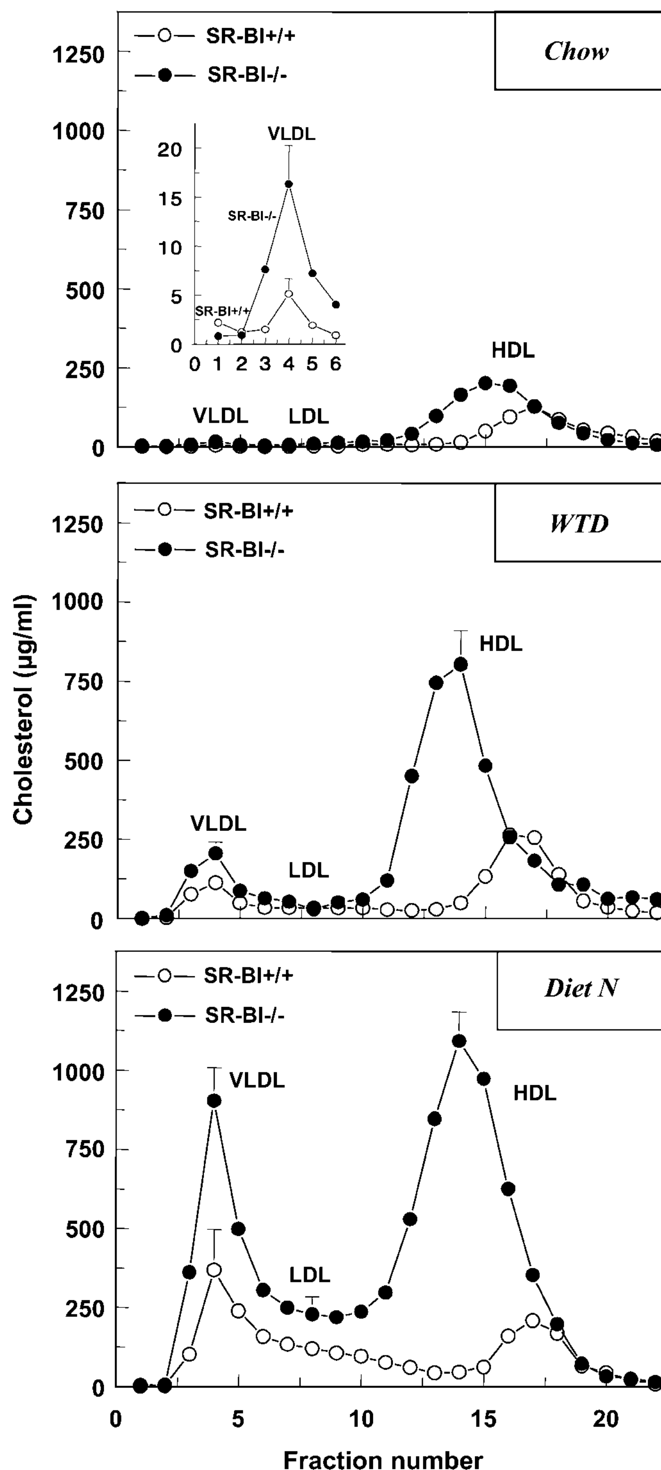

Fig. 1. Effect of scavenger receptor class B type I (SR-BI) deficiency on plasma cholesterol distribution. Blood samples were drawn after an overnight fast while feeding regular chow diet, 8 weeks Western-type diet (WTD), or 8 weeks high-cholesterol/cholate diet (Diet N). Sera from individual mice were loaded onto a Superose 6 column, and fractions were collected. Fractions 3 to 7 represent VLDL; fractions 8 to 14, LDL; and fractions 15 to 19, HDL, respectively. The distribution of cholesterol over the different lipoproteins in SR-BI$^{+/+}$ (open circles) and SR-BI$^{-/-}$ (closed circles) animals is shown. Values represent the mean ± SEM of six mice. SEMs are shown only for fractions containing the top of the VLDL, LDL, and HDL peaks.

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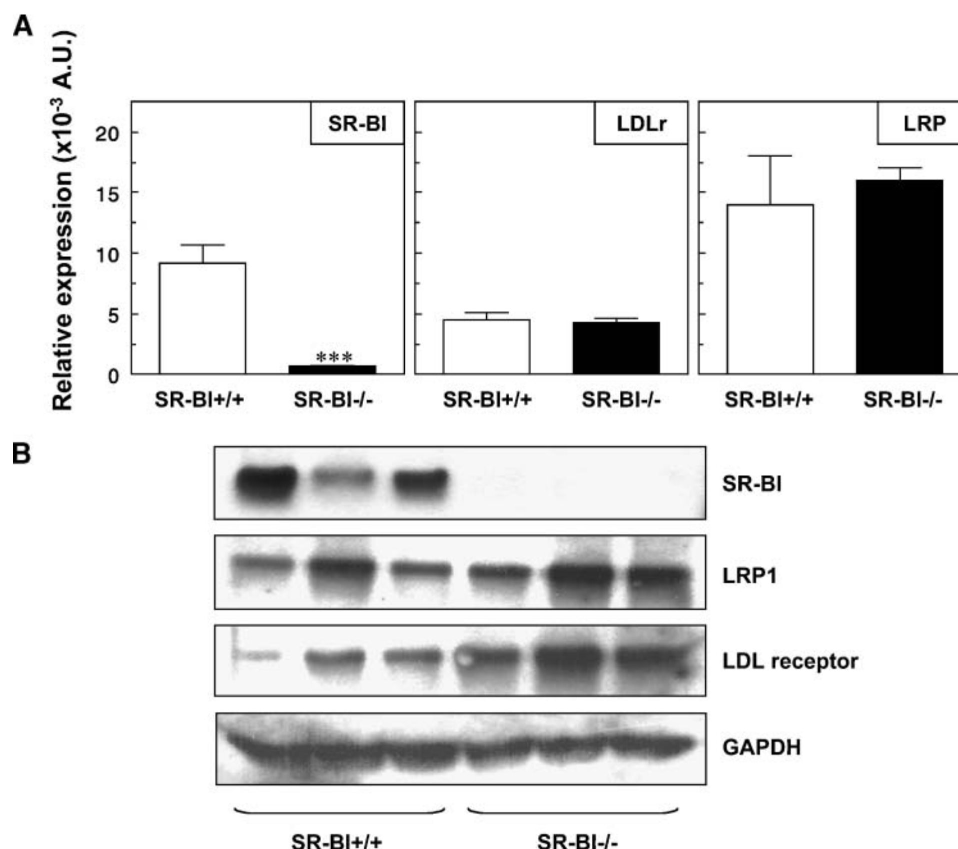

Fig. 2. Effect of SR-BI deficiency on mRNA and protein expression of SR-BI, LDL receptor (LDLR), and LDL receptor-related protein type 1 (LRP-1) in liver. A: mRNA levels of the indicated genes in livers of SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice were quantified using real-time PCR with SYBR-green detection. Values represent the mean ± SEM of six mice. Statistically significant difference of ***$P < 0.001$ as compared with SR-BI$^{+/+}$ mice. B: Protein expression of SR-BI, LDLR, and LRP-1 in livers of SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice. Hepatic lysates were separated on 7.5% SDS-PAGE and immunoblotted with α-SR-BI, α-LRP-1, and α-LDLR. α-GAPDH was used for analysis of equal loading.

β-VLDL were determined in SR-BI-deficient mice and wild-type littermates. β-VLDL is a VLDL remnant that is enriched in apoE (42% of total apoprotein content) and cholesterol esters and depleted in triglycerides (18% PL, 16% TG, 55% CE, and 11% FC). In comparison, endogenous VLDL from SR-BI$^{-/-}$ mice on chow comprised 13% PL, 73% TG, 7.8% CE, and 6.1% FC, and VLDL from SR-BI$^{+/+}$ mice comprised 15% PL, 79% TG, 3.7% CE, and 2.5% FC. HDL, the most abundant circulating particle, comprised 56% PL, 2.7% TG, 26% CE, and 16% FC in SR-BI$^{-/-}$ mice and 69% PL, 0.8% TG, 29% CE, and 1.3% FC in SR-BI$^{+/+}$ mice. As previously shown by Rigotti et al., HDL in SR-BI$^{-/-}$ mice is enriched in apoE (9). Because β-VLDL is the ultimate VLDL remnant, it allows investigation of the role of SR-BI in VLDL metabolism independent of any factors, such as lipolysis, involved in the remodeling of the VLDL particle.

At 30 min after injection, 71 ± 2% of the injected trace amount of ^{125}I-β-VLDL was cleared from the circulation in SR-BI$^{+/+}$ mice, as compared with only 38 ± 4% (P = 0.002) in SR-BI$^{-/-}$ mice (**Fig. 4A**). Upon iodination of β-VLDL, all apolipoproteins on the particle that can rapidly exchange with HDL in the circulation are iodinated, including apoC (41). To exclude that the decreased clearance of β-VLDL in the SR-BI-deficient mice was a direct effect of the high HDL cholesterol levels, serum was fractionated at 0, 2, and 30 min after injection and the label distribution over the different lipoproteins in the circulation was determined (Fig. 4B). At 2 min after injection, only 16 ± 3% of the injected dose was still associated with the VLDL fraction in wild-type mice, as compared with 40 ± 4% (P = 0.0096) in SR-BI-deficient animals. No difference in the exchange of label with HDL was observed at this time point (17 ± 4% in wild-type mice as compared with 18 ± 3% in SR-BI-deficient animals). At 30 min after injection, 18 ± 1% of the injection dose was still present in the VLDL fraction in SR-BI-deficient mice, as compared with only 3.3 ± 0.4% in wild-type animals. Clearance of HDL from the circulation is very slow. Therefore, at this time point, 17 ± 2% of injected label was still in the HDL fraction of wild-type mice. In SR-BI-deficient mice, the label exchange to HDL increased to 34 ± 7% of the injected dose owing to the prolonged circulation of ^{125}I-β-VLDL.

In both wild-type and SR-BI-deficient mice, the maximum association value of ^{125}I-β-VLDL to the liver was reached at 20 min after injection. At this time point, 66 ± 2% of the injected dose of ^{125}I-β-VLDL was taken up by

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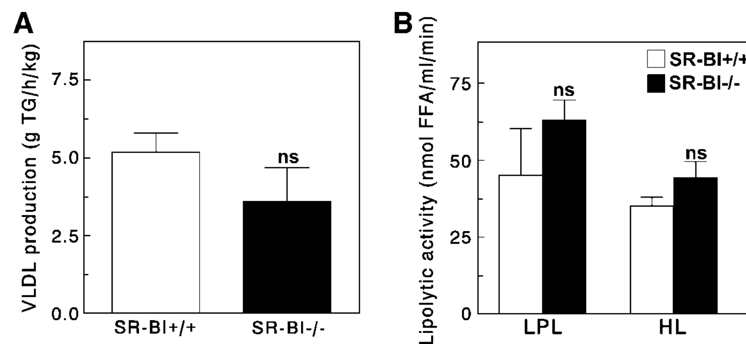

Fig. 3. Effect of SR-BI deficiency on VLDL synthesis and lipolytic activity. A: The hepatic VLDL production rate was determined by injection of SR-BI$^{+/+}$ (open bar) and SR-BI$^{-/-}$ (closed bar) mice with Triton WR-1339 (500 mg/kg). Serum triglycerides were determined prior to injection and at 1, 2, 3, and 4 h after injection. The hepatic VLDL production rate was calculated from the slope of the curve. B: Lipoprotein lipase (LPL) and hepatic lipase (HL) activity of postheparin plasma of SR-BI$^{+/+}$ (open bars) and SR-BI$^{-/-}$ (closed bars) mice was determined using a substrate-based assay. No statistically significant differences were observed (ns). Values represent mean ± SEM.

the liver in wild-type mice, as compared with only 22 ± 4% ($P = 0.0007$) in SR-BI-deficient mice, indicating that SR-BI expression in the liver facilitated the removal of ^{125}I-β-VLDL. SR-BI mediates the selective uptake of CEs from HDL. To investigate the importance of SR-BI for the selective uptake of CEs from β-VLDL in vivo, serum decay and hepatic uptake of β-VLDL labeled with ^{3}H-cholesteryl oleate was determined. In wild-type mice, the serum decay of ^{3}H-CE-β-VLDL was slightly slower as compared with ^{125}I-β-VLDL, probably as a result of exchange of the iodinated apolipoproteins. The liver uptake values were similar for both ^{3}H-CE-β-VLDL and ^{125}I-β-VLDL (68 ± 4% and 66 ± 2%, respectively, at 20 min after injection), indicating that no selective uptake of CEs occurs from β-VLDL in vivo. In the absence of SR-BI, however, the association of ^{3}H-CE-β-VLDL to the liver is even lower as compared with ^{125}I-β-VLDL (10 ± 2% and 22 ± 4%, respectively, at 20 min after injection). Thus, SR-BI does play an important role in the hepatic removal of β-VLDL, albeit without selective uptake of its CEs.

Reduced association of β-VLDL to primary hepatocytes from SR-BI-deficient mice

To analyze the precise role of SR-BI in the recognition of β-VLDL, in vitro studies were performed using freshly isolated hepatocytes from SR-BI-deficient mice and wild-type littermates. Incubation for 3 h at 4°C revealed a B_{max} of ^{125}I-β-VLDL binding to hepatocytes isolated from SR-BI$^{+/+}$ animals of 469 ± 30 ng/mg cell protein (**Fig. 5A**). The B_{max} of ^{125}I-β-VLDL binding to hepatocytes isolated from SR-BI$^{-/-}$ animals, however, was only 305 ± 20 ng/mg ($P = 0.01$). ^{125}I-β-VLDL association at 37°C was ~1.6-fold lower in SR-BI$^{-/-}$ hepatocytes as compared with SR-BI$^{+/+}$ hepatocytes (Fig. 5B). No specific accumulation of degradation products of ^{125}I-β-VLDL in the medium was observed for both types of hepatocytes. ^{125}I-β-VLDL association at 37°C was significantly higher as compared with ^{125}I-β-VLDL binding at 4°C. The amount of β-VLDL uptake as compared with membrane association was determined by degradation of the β-VLDL associated to the outer membrane using proteinase K for 2 h at 4°C. For both wild-type and SR-BI knockout hepatocytes, the majority of the associated label appeared to be actually taken up by the cells (71 ± 4% for wild-type hepatocytes and 76 ± 1% for SR-BI-deficient cells).

To determine whether SR-BI mediates selective uptake of CE from β-VLDL in vitro, hepatocytes from SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice were incubated with β-VLDL labeled with ^{3}H-cholesteryl oleate (^{3}H-CE). To allow direct assessment of selective lipid uptake, ^{3}H-CE cell association was expressed as the amount of apparent β-VLDL protein uptake by the cells, as introduced by Pittman et al. (42). Incubation of the wild-type hepatocytes with increasing concentrations of ^{3}H-CE-β-VLDL resulted in the association of increasing amounts of ^{3}H label that exceeded the ^{125}I-β-VLDL association only ~1.1-fold, indicating only very limited selective uptake of CEs from β-VLDL. As observed for ^{125}I-β-VLDL, the apparent uptake of ^{3}H-CE-β-VLDL by SR-BI$^{-/-}$ hepatocytes was 2.1-fold lower as compared with the uptake by wild-type hepatocytes (Fig. 5B). The association of ^{3}H label, however, did not exceed the ^{125}I-β-VLDL association in the absence of SR-BI.

To further characterize the nature of the recognition sites for β-VLDL on isolated hepatocytes in the presence and absence of SR-BI, competition studies were performed. Hereto, 10 μg/ml ^{125}I-β-VLDL and ^{3}H-CE-β-VLDL was coincubated with 100 μg/ml β-VLDL, LDL, or HDL or 10 μg/ml GST-RAP, an effective inhibitor of LRP-1. Both ^{125}I-β-VLDL and ^{3}H-CE-β-VLDL association were inhibited effectively by unlabeled β-VLDL to ~10–20% of the association in the absence of the competitor in both SR-BI$^{+/+}$ and SR-BI$^{-/-}$ hepatocytes (**Fig. 6**). Using LDL as a competitor reduced ^{125}I-β-VLDL association by only ~30%, while virtually no effect was observed on ^{3}H-CE-β-VLDL association. This is probably the result of the loss of the LDLRs during the hepatocyte isolation procedure. Inhibition of LRP-1 association with GST-RAP reduced β-VLDL association, with only ~20% in SR-BI$^{+/+}$

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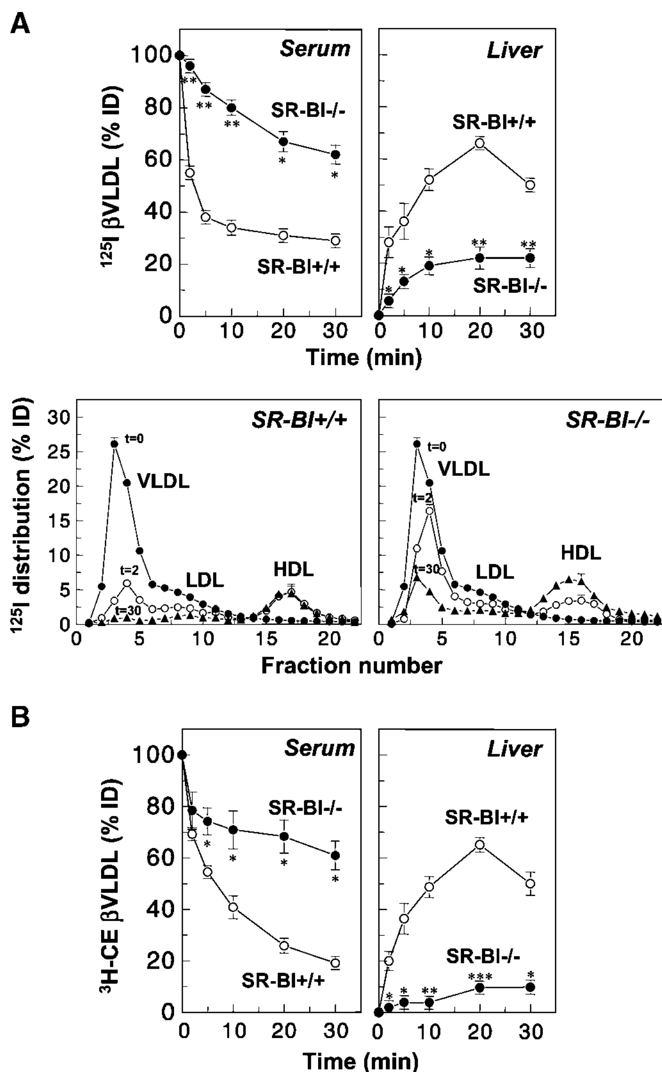

Fig. 4. Effect of SR-BI deficiency on serum decay and liver uptake of β-VLDL. A tracer amount of 10 μg ^{125}I-β-VLDL (A) or ^{3}H-CE-β-VLDL (B) was injected into anesthetized SR-BI$^{+/+}$ (open circles) and SR-BI$^{-/-}$ (closed circles) mice and the serum decay and liver uptake were followed over time. At 0, 2, and 30 min after injection of ^{125}I-β-VLDL into SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice, serum was fractionated using a Superose 6 column, and the label distribution over the different lipoproteins in the circulation was determined. Fractions 3 to 7 represent VLDL; fractions 8 to 14, LDL; and fractions 15 to 19, HDL, respectively. Values represent the mean ± SEM of three mice. Statistically significant difference of $*P < 0.01$, $**P < 0.001$, and $**P < 0.0001$ compared with SR-BI$^{+/+}$ mice.

hepatocytes. In the absence of SR-BI, inhibition of LRP-1 by GST-RAP appeared slightly more effective (~40% inhibition). No significant effect of degradation of chondroitin sulfate proteoglycans on the association of β-VLDL to hepatocytes in the absence or presence of SR-BI was observed. In contrast, HDL competed effectively for β-VLDL association and reduced ^{125}I-β-VLDL association to ~45% of the association in the absence of competitor in both SR-BI$^{+/+}$ and SR-BI$^{-/-}$ hepatocytes. In the absence of SR-BI, HDL is thus still an effective competitor for β-VLDL association. Interestingly, HDL competition for the association of ^{3}H-CE-β-VLDL (~70–80% reduction) was even more effective than that for ^{125}I-β-VLDL (~50–60% reduction).

DISCUSSION

The risk of developing coronary artery disease is directly related to VLDL/LDL cholesterol levels and inversely associated with the concentration of HDL. In this study, we show that in addition to its established function in HDL metabolism, SR-BI plays an important role in the metabolism of VLDL remnants.

VLDL cholesterol levels were increased in SR-BI-deficient mice on chow diet as well as while feeding a Western-type diet or a more severe high-cholesterol/cholate diet. The observed increase in VLDL cholesterol levels in the absence of SR-BI was not caused by an increased VLDL secretion rate or decreased expression of the LDLR or LRP-1, suggesting a direct involvement of SR-BI in the catabolism of VLDL. Interestingly, the postprandial triglyceride response after an intragastric fat load is higher in the absence of SR-BI, indicating that chylomicron metabolism also is altered in SR-BI-deficient mice (37). Furthermore, a common single nucleotide polymorphism in exon 8 ($C^{1050} \rightarrow T$) of CLA-1, the human homolog of SR-BI, was associated with lower LDL cholesterol levels in women, whereas several studies on common polymorphisms of CLA-1 have shown that some SR-BI variants interfere with the metabolism of apoB lipoproteins in humans, although the effects vary with age and gender (43–46). In addition, Pérez-Martínez et al. have shown that subjects carrying the exon 1 (G→A) single nucleotide polymorphism display increased LDL cholesterol levels on a saturated fatty acid-rich diet (47). Thus, genetic variation at the CLA-1 gene locus is also associated with alterations in the metabolism of apoB-containing lipoproteins in humans.

In vitro studies have shown that in addition to HDL, SR-BI binds apoB-containing lipoproteins (2, 17–19). It is thus possible that SR-BI directly facilitates the clearance of VLDL. Therefore, we subsequently assessed the role of SR-BI in the metabolism of VLDL by analyzing the clearance from the circulation and hepatic uptake of ^{125}I-β-VLDL. β-VLDL is the atherogenic form of VLDL that accumulates in animals on a high-cholesterol diet, in hyperlipidemic animal models, and in patients with type III hyperlipoproteinemia (25, 26). It is a VLDL remnant that is relatively enriched in CEs and depleted in triglycerides (41). The removal of both ^{125}I-β-VLDL and ^{3}H-CE-β-VLDL from the circulation was largely reduced in the absence of SR-BI. In agreement, Ueda et al. have shown that the clearance of ^{125}I-LDL was increased in transgenic mice overexpressing SR-BI (48). We now show that the reduced clearance of β-VLDL from the circulation was a direct effect of an impaired uptake of β-VLDL by the liver as a result of SR-BI deficiency. No differences were observed between the liver association of ^{125}I-β-VLDL and ^{3}H-CE-β-VLDL in either the SR-BI knockout or the wild-type mice. In contrast, for HDL, we have shown that ^{3}H-

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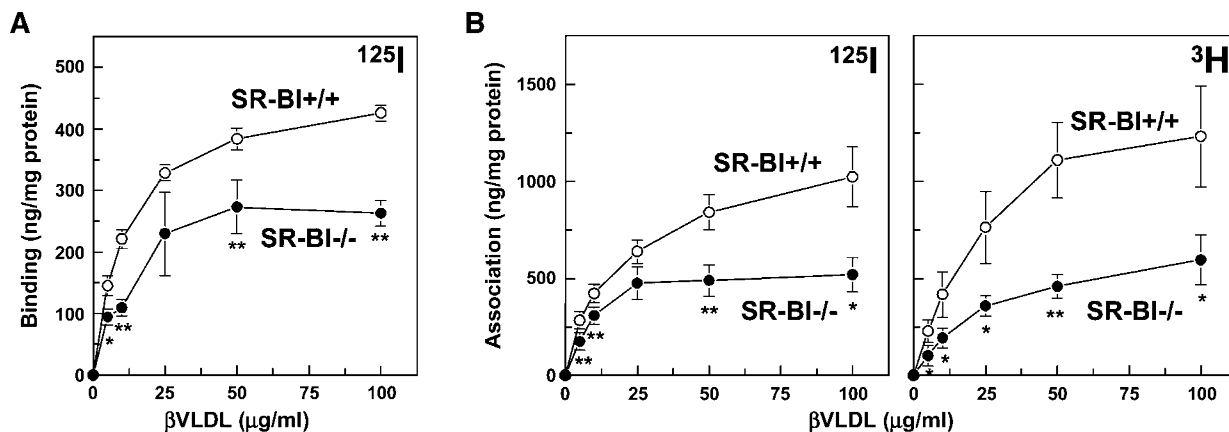

Fig. 5. Effect of SR-BI deficiency on β-VLDL association to primary hepatocytes. Freshly isolated hepatocytes from SR-BI$^{+/+}$ (open circles) and SR-BI$^{-/-}$ (closed circles) mice were incubated for 3 h at 4°C (A) or 37°C (B) with the indicated amounts of ^{125}I-β-VLDL or ^{3}H-CE-β-VLDL in DMEM/2% BSA. Values represent the specific binding (A) or association (B) as nanogram of ligand per milligram of cell protein. Values represent mean ± SEM of three independent experiments performed in triplicate. Statistically significant difference of *$P < 0.05$ and **$P < 0.01$ as compared with SR-BI$^{+/+}$ mice.

CE-HDL liver association is 5-fold higher as compared with ^{125}I-HDL association in wild-type mice, whereas the same low level of association was found in SR-BI-deficient mice (49). Thus, SR-BI mediates the selective uptake of CEs by the liver from HDL, but not β-VLDL. ApoA-I is important for the binding and selective uptake of CE from HDL by SR-BI, although it is not solely responsible for inducing selective uptake (50–52). In addition to apoA-I, apoE also binds to SR-BI. ApoE is a 34 kDa arginine-rich protein that is an important constituent of VLDL and chylomicron remnants and that plays an important role in lipoprotein metabolism. It serves as a high-affinity ligand for several receptor systems in the liver, including the LDLR, LRP-1, and proteoglycans (53). ApoE deficiency in mice leads to impaired catabolism of remnant lipoproteins and an increased susceptibility to atherosclerosis (54, 55). As compared with normal VLDL, β-VLDL is considerably enriched in apoE (41). Using reconstituted HDL particles, Thuahnai et al. have shown that particles containing apoE bind as well to SR-BI as particles containing apoA-I (56). However, the apoA-I-containing particles were better in inducing CE selective uptake. Other studies have confirmed that apoE is a ligand for SR-BI (57–59) and that the N-terminal region of apoE is involved in the binding to SR-BI (57). Interestingly, lipid-free apoE enhances the CE transfer from lipoproteins, whereas apoE associated to lipids does not modulate the binding or selective uptake of CE from lipoproteins (59). On the other hand, Thuahnai et al. showed that free apolipoproteins, including apoE, only partly compete with the binding of HDL to SR-BI, whereas lipidated apolipoproteins compete fully (57). The importance of apoE for SR-BI recognition in vivo was further illustrated by Arai et al., who demonstrated that the selective uptake of CE from HDL was decreased in apoE-deficient mice (60). Webb, de Beer, and Van der Westhuyzen showed that adenoviral overexpression of SR-BI in apoE-deficient mice resulted in only a modest depletion of LDL cholesterol levels, whereas no effect on VLDL cholesterol levels was observed (24). These studies might indicate that apoE on β-VLDL is important for recognition by SR-BI. However, disruption of the SR-BI gene in apoE$^{-/-}$ mice does result in an increase in circulating VLDL and LDL levels (9,10). Furthermore, β-VLDL isolated from apoE-deficient mice did associate to Chinese hamster ovary cells transfected with SR-BI, albeit without resulting in selective uptake of CE from the apoE-

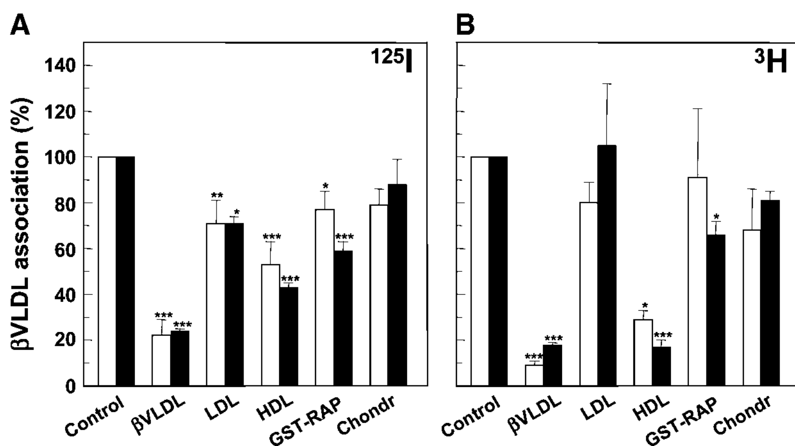

Fig. 6. Effect of different competitors on the association of ^{125}I- (A) and ^{3}H-CE-labeled (B) β-VLDL to primary SR-BI$^{+/+}$ and SR-BI$^{-/-}$ hepatocytes. Freshly isolated hepatocytes from SR-BI$^{+/+}$ (open bars) and SR-BI$^{-/-}$ (closed bars) mice were incubated for 3 h at 37°C with 10 μg/ml ^{125}I-β-VLDL or ^{3}H-CE-β-VLDL in DMEM/2% BSA in the presence of 100 μg/ml unlabeled β-VLDL, LDL, or HDL, 10 μg/ml GST-RAP, or after degradation of chondrotin sulfate proteoglycans. Values represent the association as nanogram of ligand per milligram of cell protein. Values represent mean ± SEM of two independent experiments performed in triplicate. Statistically significant difference of *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ as compared with SR-BI$^{+/+}$ mice.

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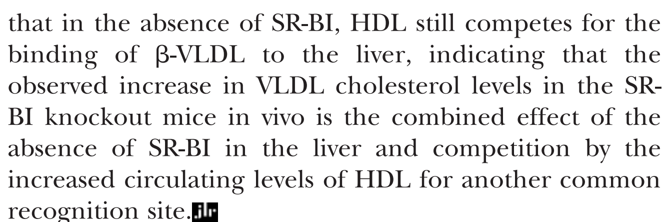
deficient β-VLDL (24). In the current study, we show that both the binding and the association of apoE-containing β-VLDL to primary hepatocytes is reduced in the absence of SR-BI. A substantial amount of β-VLDL is taken up by the cells in both the absence and the presence of SR-BI. Interestingly, SR-BI deficiency results in a reduced cellular uptake of β-VLDL, suggesting that SR-BI mediates the uptake of β-VLDL. Historically, SR-BI has been considered a nonendocytotic receptor, mainly because chloroquine and monensin inhibitors of clathrin-coated pit endocytosis did not affect CE uptake, in contrast to apoA-I uptake (61, 62). More recently, however several studies have also indicated a so-called retro-endocytosis pathway, which describes the holo-particle uptake of HDL followed by resecretion of CE-poor HDL (63–65). It might thus be possible that SR-BI mediates the holo-particle uptake of β-VLDL. Previous studies have indicated both SR-BI-dependent and SR-BI-independent (23, 66, 67) mechanisms for selective uptake of CEs from LDL. However, in agreement with the results obtained in vivo, limited to no selective uptake of CE from β-VLDL by isolated hepatocytes was observed.

Uptake of VLDL and chylomicron remnants by the liver is described by the so-called sequestration-capture model (68). According to this model, initial rapid clearance of remnants by the liver begins with apoE-mediated sequestration within the space of Disse by proteoglycans. Subsequently, the remnants undergo further processing in the space of Disse by hepatic and lipoprotein lipases, which may also serve as ligands mediating remnant uptake. Finally, the remnants are endocytosed via the LDLR or LRP-1. By performing competition studies with LDL and GST-RAP, we demonstrated that the importance of the LDLR and LRP-1 for the association of β-VLDL to isolated hepatocytes is limited. HDL, however, competed effectively for the association of β-VLDL in both the absence and the presence of SR-BI. These data suggest the existence of another common recognition site for β-VLDL and HDL, in addition to SR-BI. Although HDL selective uptake is completely abolished in SR-BI-deficient mice, no effect of SR-BI deficiency on the association of ^{125}I-HDL was observed, also indicating another recognition site for HDL. Martinez et al. identified the β chain of ATP synthase, a principal protein complex of the mitochondrial inner membrane, as a high-affinity receptor for HDL that is expressed on the plasma membrane of hepatocytes (69). Interestingly, Beisiegel et al. (70) and Mahley et al. (71) described almost two decades ago that the β chain of ATP synthase binds apoE-containing lipoproteins with high affinity. It is highly interesting to speculate that in addition to SR-BI, as we demonstrate in this study, the β chain of ATP synthase is the recognition site responsible for displacement of β-VLDL association to hepatocytes by HDL.

In the present study, we have provided evidence that SR-BI, in addition to its established role in HDL metabolism, significantly contributes to the metabolism of β-VLDL in mice, as evidenced by the reduced association of β-VLDL to primary hepatocytes and the accumulation of VLDL in vivo in mice lacking SR-BI. Our in vitro studies also show



that in the absence of SR-BI, HDL still competes for the binding of β-VLDL to the liver, indicating that the observed increase in VLDL cholesterol levels in the SR-BI knockout mice in vivo is the combined effect of the absence of SR-BI in the liver and competition by the increased circulating levels of HDL for another common recognition site.

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