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Influence of the high density lipoprotein receptor SR-BI on reproductive and cardiovascular pathophysiology

BERNARDO TRIGATTI*, HELEN RAYBURN*, MARISA VIÑALS*, ANNE BRAUN*, HELENA MIETTINEN*, MARSHA PENMAN*, MIKI HERTZ*, MARK SCHRENZEL†, LUDWIG AMIGO‡, ATTILIO RIGOTTI*‡, AND MONTY KRIEGER*§

Departments of *Biology and †Comparative Medicine, Massachusetts Institute of Technology, Cambridge, MA 02139; and ‡Departamento de Gastroenterología, Facultad de Medicina, Pontificia Universidad Católica, Santiago, Chile

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ABSTRACT **The high density lipoprotein (HDL) receptor SR-BI (scavenger receptor class B type I) mediates the selective uptake of plasma HDL cholesterol by the liver and steroidogenic tissues. As a consequence, SR-BI can influence plasma HDL cholesterol levels, HDL structure, biliary cholesterol concentrations, and the uptake, storage, and utilization of cholesterol by steroid hormone-producing cells. Here we used homozygous null SR-BI knockout mice to show that SR-BI is required for maintaining normal biliary cholesterol levels, oocyte development, and female fertility. We also used SR-BI/apolipoprotein E double homozygous knockout mice to show that SR-BI can protect against early-onset atherosclerosis. Although the mechanisms underlying the effects of SR-BI loss on reproduction and atherosclerosis have not been established, potential causes include changes in (*i*) plasma lipoprotein levels and/or structure, (*ii*) cholesterol flux into or out of peripheral tissues (ovary, aortic wall), and (*iii*) reverse cholesterol transport, as indicated by the significant reduction of gallbladder bile cholesterol levels in SR-BI and SR-BI/apolipoprotein E double knockout mice relative to controls. If SR-BI has similar activities in humans, it may become an attractive target for therapeutic intervention in a variety of diseases.**

High density lipoprotein (HDL)-cholesterol levels are inversely proportional to the risk for atherosclerosis (1). This may be due partly to "reverse cholesterol transport" (RCT), in which HDL is proposed to remove excess cholesterol from cells, including those in the artery wall (2–7), and transport it, either indirectly or directly (8, 9), to the liver for biliary secretion. HDL also can deliver cholesterol directly to steroidogenic tissues (adrenal gland, testis, ovary) for storage in cytoplasmic cholesteryl ester droplets and for steroid hormone synthesis (10–12). Thus, HDL may influence a variety of endocrine functions, including reproduction. A key mechanism of receptor-mediated direct delivery of HDL cholesteryl esters to the liver and steroidogenic tissues is selective cholesterol uptake, in which only the cholesteryl esters of the HDL particles (not the apolipoproteins) are transferred efficiently to cells (8, 9).

The class B type I scavenger receptor, SR-BI, is a cell-surface HDL receptor that mediates selective lipid uptake (13–21; reviewed in refs. 22 and 23). It is most highly expressed in the liver and steroidogenic tissues, in which its activity is regulated by trophic hormones (13, 24–31). As a consequence, SR-BI is a key regulator of HDL cholesterol levels (17–21) and adrenal cholesterol stores (18). The finding that hepatic SR-BI overexpression leads to significant increases in biliary cholesterol content (17, 32) is consistent with gene-targeting studies (18, 19) that suggest an important role for SR-BI in RCT. In addition to HDL, SR-BI can bind other ligands, including lipoproteins [LDL, modified LDL, very low density lipoprotein (VLDL)] and apolipoproteins (33–37), and can mediate efflux of unesterified cholesterol from cells to HDL (38, 39).

Because inactivation of SR-BI is associated with both decreased RCT (18, 19) and increased plasma HDL cholesterol [albeit in abnormally large particles containing apolipoproteins A-I (apoA-I) and E (apoE) (18)], a key question has arisen: Do decreases in SR-BI expression inhibit or promote atherosclerosis? Here we addressed this question by studying crosses between apoE homozygous knockout (KO) mice, which on a chow diet spontaneously develop atherosclerosis at around 3 months of age (40–42), and SR-BI KO mice. The results clearly show that genetically suppressing SR-BI activity in apoE KO mice dramatically accelerates the onset of atherosclerosis. We also report that female mice deficient in SR-BI alone are infertile, apparently a result, in part, of abnormalities in the viability and developmental potential of their oocytes. Thus, genetic ablation of SR-BI has profound effects on both cardiovascular and reproductive pathophysiology in mice.

MATERIALS AND METHODS

Animals. Mice (mixed C57BL/6 × 129 background) were housed and fed a normal chow diet as described (18). SR-BI$^{-/-}$ mice (18) and apoE$^{-/-}$ mice (The Jackson Laboratory; refs. 40 and 41) were mated and the double heterozygous offspring were intercrossed. The resulting SR-BI$^{+/-}$ApoE$^{-/-}$ offspring were mated to produce single apoE KO and double SR-BI/apoE KO animals. Genotypes were determined by PCR analysis (ref. 18; also see The Jackson Laboratory web site). Estrus cycles were followed by vaginal cytology (43) and external appearance (44). Superovulation was induced by i.p. injection of 5 each of pregnant mare's serum (Calbiochem) and human chorionic gonadotropin (Organon) as described (44). Pseudopregnancy was induced by mating (confirmed by detection of vaginal seminal plug) with vasectomized males (Taconic Farms) (44). Ovaries were harvested and prepared for sectioning as described below, and oocytes and preimplantation embryos were harvested as described (44) and cultured in KSOM medium with amino acids (Specialty Media, Lavallette, NJ).

Plasma and Bile Analysis. Blood was collected in a heparinized syringe by cardiac puncture from mice fasted over-

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Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; SR-BI, scavenger receptor class B type I; apo, apolipoprotein; KO, homozygous knockout; RCT, reverse cholesterol transport.

§To whom reprint requests should be addressed at: Biology Department, Room 68-483, Massachusetts Institute of Technology, Cambridge, MA 02139. E-mail: krieger@mit.edu.

night. Plasma was subjected to FPLC analysis (18), either immediately after isolation or after storage at 4°C. Total cholesterol was assayed as described (18). Cholesterol from non-apoB-containing lipoproteins was determined either by using the EZ HDL kit (Sigma, based on an antibody that blocks detection of cholesterol in non-HDL lipoproteins, and validated by us by using human or mouse lipoproteins, not shown) or after precipitation with magnesium/dextran sulfate (Sigma; refs. 40 and 45). Plasma (0.4 μl) and FPLC fractions or pools were analyzed by SDS/PAGE (18) or agarose gel electrophoresis (46) and immunoblotting (47, 48) with chemiluminescence detection as described previously (18), by using primary anti-apolipoprotein antibodies (Sigma, or gifts from J. Herz and H. Hobbs, University of Texas Southwestern Medical Center) and corresponding horseradish peroxidase-coupled secondary antibodies (Jackson ImmunoResearch or Amersham). The Attophos chemifluorescence kit (Amersham) and an alkaline phosphatase-coupled goat anti-rabbit secondary antibody (gift from D. Housman, Massachusetts Institute of Technology) were used with a Storm Fluorimager (Molecular Dynamics) for quantitative analysis. Plasma progesterone concentrations were determined by radioimmunoassay (Diagnostic Products, Los Angeles). Cholesterol was extracted from gallbladder bile and assayed as described (49).

Histology and Immunofluorescence Microscopy. Mice anesthetized with 2.5% avertin were perfused through the left ventricle with 20 ml of ice-cold PBS containing 5 mM EDTA. Hearts were collected directly, or the mice were perfused (5 ml) with paraformaldehyde and the hearts were collected and treated as described (50). Hearts and ovaries were frozen in Tissue Tek OCT (Sakura, Torrance, CA). Serial cross-sections [10-μm thickness through aortic sinuses (41, 51, 52), 5 μm for ovaries, Reichert–Jung cryostat)] were stained with oil red O and Mayer's hematoxylin (53). Images were captured for morphometric analysis by using a computer-assisted microscopy imaging system, and lesion size was quantified as the sum of the cross-sectional areas of each oil red O-staining atherosclerotic plaque in a section (51) by using NIH IMAGE software. Immunohistochemistry with a monoclonal anti-α smooth-muscle actin antibody (Sigma; gift from R. Hynes, Massachusetts Institute of Technology) was performed as described (24). Cumulus/oocyte complexes, isolated from the oviducts of superovulated females as described (44), or denuded oocytes (zona pellucida removed as in ref. 44) were immunostained with polyclonal rabbit anti-murine SR-BI antibodies (ref. 13 or a gift from K. Kozarsky, Smith Kline Beecham Pharmaceuticals) and Cy3-labeled donkey anti-rabbit secondary antibodies (gift from R. Rosenberg, Massachusetts Institute of Technology) as described (14, 54).

Statistical Analysis. Data were analyzed by using either a two-tailed, unpaired Student's *t* test (total or EZ HDL cholesterol from plasma, bile, or FPLC fractions, and progesterone and apoA-I levels) or an unpaired, nonparametric Kruskall–Wallis test (atherosclerotic plaque lesion sizes) (STATVIEW and Microsoft EXCEL). Values are presented as means ± SD.

RESULTS AND DISCUSSION

Reproductive Pathophysiology. Homozygous SR-BI KO males exhibit normal fertility (18). In contrast, homozygous KO females are infertile. In a 2-month pairing of either homozygous KO or heterozygous females with homozygous SR-BI KO males (n = 8 for each), heterozygous females produced 19 litters and 82 healthy offspring, whereas the homozygous females produced no healthy offspring. Although two pups from two homozygous SR-BI KO females were born, they died soon after.

There were no obvious gross morphological abnormalities in SR-BI KO ovaries (not shown). Histochemical analysis of

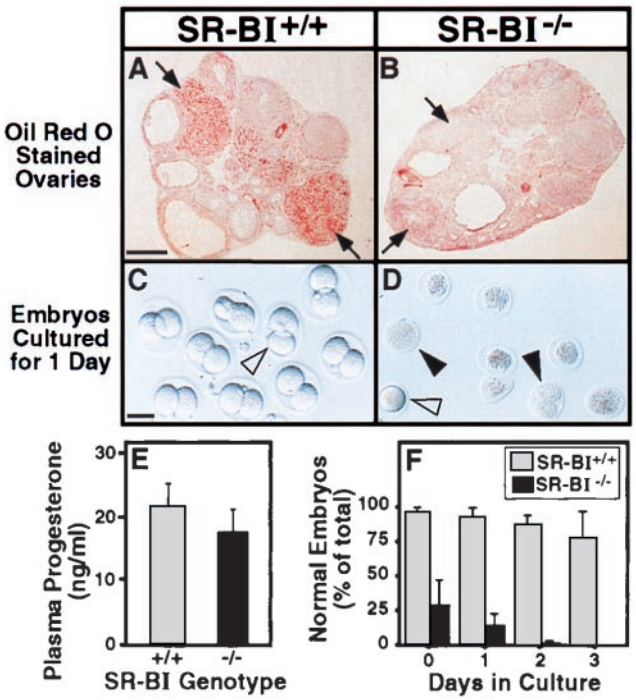

FIG. 1. *In vivo* ovarian lipid accumulation in and *in vitro* development of preimplantation embryos from wild-type and SR-BI KO mice. Six-week-old female mice were superovulated and were mated to males of the other genotype (i.e., SR-BI$^{+/+}$ females mated to SR-BI$^{-/-}$ males and *vice versa*) to generate embryos with heterozygous mutant genotypes. Ovaries and preimplantation embryos were harvested the next morning (day 0). (*A* and *B*) Typical oil red O staining of lipids in ovaries from SR-BI$^{+/+}$ (*A*) or SR-BI$^{-/-}$ (*B*) animals. The arrows indicate corpora lutea. (Bar = 450 μm.) (*C* and *D*) Phase-contrast microscopy of preimplantation embryos (cultured for 1 day) from SR-BI$^{+/+}$ (*C*) or SR-BI$^{-/-}$ (*D*) females mated to males of the opposite genotype. Similar results were observed when SR-BI$^{-/-}$ males were mated to SR-BI$^{-/-}$ females. Open arrowheads indicate morphologically normal, one- or two-cell embryos; solid arrowheads indicate embryos with abnormal, nonrefractive morphology. (Bar = 100 μm.) (*E*) Plasma progesterone concentrations from pseudopregnant females (6 days postmating, ages 6–10 weeks, weight = 19–25 g, n = 8; P = 0.08). (*F*) Percentage of preimplantation embryos from SR-BI$^{+/+}$ (open bars) or SR-BI$^{-/-}$ (solid bars) females with normal morphology during 3 days of culture. The values represent the averages from five animals of each genotype. Total number of embryos: SR-BI$^{+/+}$, 131; SR-BI$^{-/-}$, 167.

ovaries from superovulated females showed reduced oil red O staining of lipids in the ovarian corpora lutea of SR-BI KO relative to those of wild-type animals (Fig. 1 *A* and *B*). This suggests there was reduced cholesteryl ester storage, as observed previously in adrenal glands from SR-BI KO mice (18). This raised the possibility that there might have been insufficient amounts of cholesterol substrate in the corpora lutea to sustain adequate steroid hormone production for pregnancy. However, plasma progesterone levels between pseudopregnant control and KO females 6 days after mating, either without (Fig. 1*E*) or with superovulation (not shown), were not significantly different. Furthermore, several other murine homozygous knockout mutants (e.g., LCAT, ACAT, and apoA-I) exhibit similar lipid depletion in steroidogenic tissues (12, 55, 56) without apparent female infertility. Thus, normal lipid stores are not required for synthesis of adequate amounts of steroid hormones for female fertility.

Although KO females were infertile, they exhibited no obvious defects in their estrus cycles or numbers of oocytes ovulated, either during normal estrus (data not shown) or after superovulation [wild type (n = 4), 52 ± 5 oocytes; SR-BI KO (n = 3), 41 ± 8, P = 0.2]. Because the estrus cycle and

ovulation depend on estrogen (e.g., for follicular development and induction of luteinizing hormone receptors) and progesterone (e.g., for follicular rupture) (57), KO females apparently synthesize adequate levels of intra- and extraovarian steroids for at least some, if not all, ovarian functions.

Because the extent of ovulation by the KO mice appeared normal, we compared the viability and development of heterozygous (SR-BI$^{+/-}$) preimplantation (one-cell) embryos placed into culture the morning (day 0) after mating with males. Almost all embryos from wild-type females had normal morphologies (Fig. 1*C*) and developed into morulas or blastocysts after 3 days in culture (Fig. 1*F*; data not shown). In contrast, the majority of embryos from KO females at harvesting had an abnormal, nonrefractile morphology (Fig. 1*D*, solid arrowheads), reminiscent of that seen in embryos mechanically damaged during pronuclear injection (ref. 44; data not shown). The abnormal (presumably dead) embryos did not develop further (Fig. 1*F*, solid bars; data not shown). All of the other embryos from SR-BI KO females that appeared normal on day 0 eventually adopted the abnormal morphology and arrested (most at the one- or two-cell stages) in culture (Fig. 1 *D*, solid arrowheads, and *F*, solid bars). We also observed a similar abnormal morphology in oocytes from wild-type females that had been treated in culture with 50 μg/ml nystatin or filipin (not shown), cholesterol-binding drugs that can perturb membrane structure (58). Further work will be required to establish whether membrane abnormalities, perhaps a result of potential cholesterol deficiency, contribute to the SR-BI KO oocyte abnormalities.

The same abnormal morphology was seen in newly harvested, unfertilized oocytes from SR-BI KO ($n = 6$), but not wild-type ($n = 7$), superovulated females, although at a lower frequency (31 $\pm$ 22%) than in fertilized preimplantation embryos (69 $\pm$ 19%, $P = 0.02$). Therefore, some of the oocyte abnormalities apparently are fertilization- and cell division-independent. Using immunostaining with anti-SR-BI antibodies, we did not detect a signal for SR-BI in wild-type oocytes, either denuded (zona pellucida removed) or in cumulus complexes, above the background seen in oocytes from KO animals (not shown), suggesting that after ovulation, murine oocytes do not express high levels of SR-BI (also see ref. 29). In contrast, substantial expression of SR-BI was detected in the expanded cumulus cells surrounding ovulated oocytes from wild-type, but not SR-BI KO, mice (not shown). These cells are derived from follicular granulosa cells and are believed to play a key role in oocyte development (57). SR-BI expression has been reported to be induced in follicular granulosa cells soon after a luteinizing pulse of human chorionic gonadotropin (27–30).

Infertility in SR-BI KO females may be due to inadequate delivery of HDL-cholesterol for membrane synthesis or steroidogenesis, inadequate delivery of nonsteroidal HDL lipids (e.g., lipid-soluble vitamins), or deficiencies in SR-BI functions other than selective cholesterol uptake (lipid efflux, binding of non-HDL ligands). The abnormal structure of plasma HDL in the KO animals [large, apoE-rich (18)] may also contribute to the infertility. Oocyte abnormalities may arise as a result of the inability of cumulus cells to express SR-BI, before or after ovulation, because SR-BI may be needed by these cells to properly nourish the oocyte and/or support its development. SR-BI expression may also be needed in ovarian interstitial and thecal cells surrounding follicles (26–29) during oocyte maturation or in the oviduct environment (at least up to the one-cell stage). SR-BI might also play a role at other stages of reproduction and development. For example, the pattern of expression of SR-BI during later stages of pregnancy (54, 59) and the non-Mendelian (reduced) yield of homozygous mutant offspring from heterozygous mothers (18) suggest it participates in the normal function of the decidua, yolk sac, and/or placenta for nutrient transport and steroid hormone synthesis. Although additional mechanistic studies are necessary, the current data unequivocally establish that SR-BI is important for normal oocyte maturation, embryonic development, and female fertility in mice.

Cardiovascular Pathophysiology. To analyze the effects of SR-BI on atherosclerosis, we crossed SR-BI KO and apoE KO [spontaneously atherosclerotic (40–42)] mice and compared the lipoprotein profiles and development of atherosclerosis in the single and double homozygous KO females at 4–7 weeks of age. Results for males were similar, except as noted. As reported previously, plasma total cholesterol in the single SR-BI KOs was increased relative to controls, because of an increase in large, apoE-enriched HDL particles (18), whereas the even greater relative plasma cholesterol increase in the single apoE KOs (Fig. 2*A Left*) was a consequence of a dramatic increase in cholesterol in VLDL- and intermediate density lipoprotein (IDL)/LDL-sized particles (Fig. 2*B*, diamonds; also see refs. 40 and 42). There was increased plasma cholesterol in the double KOs relative to the single apoE KOs (Fig. 2*A Left*), mainly in VLDL-sized particles (Fig. 2*B*, circles). This might have occurred if SR-BI, which can bind apoB-containing lipoproteins (33–35), directly or indirectly contributes to the clearance of the cholesterol in VLDL-sized particles in single apoE KO mice (reduced clearance in its absence) (20, 21).

The normal-sized HDL cholesterol peak seen in the single apoE KOs virtually disappeared in the double KOs (Fig. 2*B*). However, no statistically significant differences ($P = 0.1$) in plasma levels of HDL's major apolipoprotein, apoA-I, were detected (Fig. 2*A Right*). Based on the analysis of lipoproteins in the single SR-BI KO mice (18), abnormally large, HDL-like particles were expected to appear in the double KOs. Indeed, the loss of normal-sized HDL cholesterol and apoA-I in the double KOs was accompanied by a shift of the apoA-I into the VLDL- and IDL/LDL-sized fractions (Fig. 2*C*). Furthermore, analysis of HDL-like cholesterol in the FPLC fractions by using the EZ HDL assay provides evidence for the presence of abnormally large, HDL-like particles in the double KO mice. In the single apoE KO males (Fig. 2*D Left*), most of this cholesterol was in particles with the size of normal HDL, whereas in their double KO counterparts almost all of this cholesterol was in abnormally large particles. In addition, there was $\approx$3.7-fold more of this HDL-like cholesterol in the double (133 $\pm$ 24 mg/dl) than in the single (36 $\pm$ 16 mg/dl, $P = 0.005$) KO mice. These increases in the amounts and sizes of HDL-like cholesterol by inactivation of the SR-BI gene in an apoE KO background were reminiscent of those seen in a wild-type background [$\approx$2.2-fold increase in cholesterol (18); also see Fig. 2*A*], although the HDL-like particles in the double KO mice were much larger and more heterogeneous than those in the SR-BI single KO mice (18). A similar trend was seen for female mice (Fig. 2*D Right*), except that there were increased levels of abnormally large, HDL-like cholesterol in the single apoE KO females relative to males. Preliminary cholesterol measurements by using magnesium/dextran sulfate precipitation of lipoproteins (40, 45) support the EZ HDL findings of large HDL in the double KO animals (not shown).

Additional evidence for abnormally large, HDL-like particles in the IDL/LDL size range from both males and females was obtained by using agarose gel electrophoresis and immunoblotting. There was a significant reduction in the amount of immunodetectable apoB present in the IDL/LDL-sized particles from the double KOs relative to the single apoE KOs (Fig. 2*E*, lanes 2 and 4), even though there was as much or more total cholesterol in these fractions in the double KOs (Fig. 1*B*). In addition, there was significantly greater heterogeneity in the electrophoretic mobilities of apoA-I-containing IDL/LDL-sized particles (Fig. 2*E*, compare lanes 1 and 3). This was a result, in part, of the presence of novel apoA-I-containing, apoB-free, HDL-like particles (Fig. 2*E*, lanes 3 and 4, arrow). In contrast, most of the apoA-I in these particles in

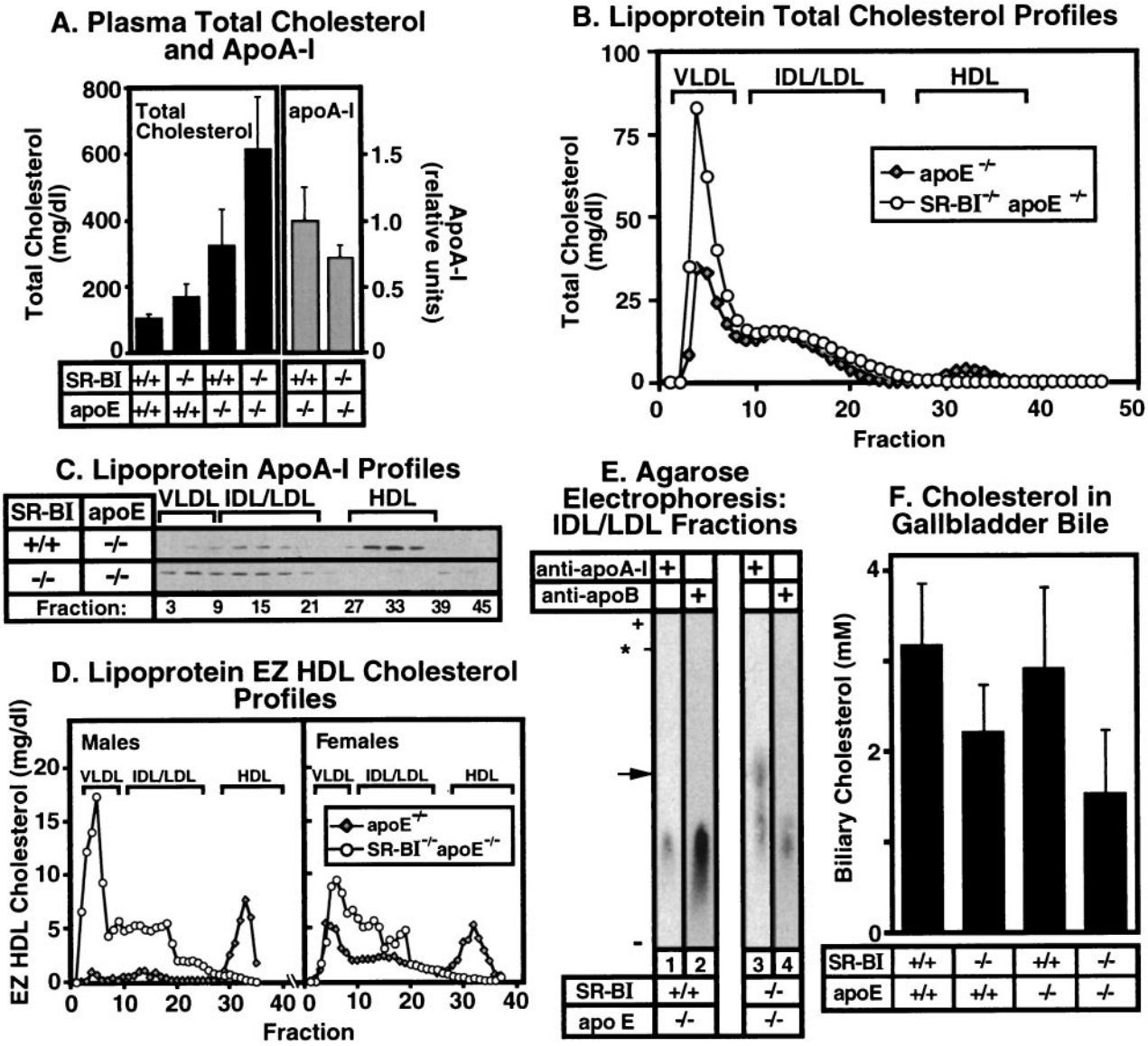

FIG. 2. Effects of SR-BI gene disruption on plasma lipoproteins in apoE KO mice. Mice were 4–7 weeks old. (*A*) Plasma total cholesterol levels (*Left*, mean ± SD) for wild-type ($n = 5$), SR-BI$^{-/-}$ ($n = 12$), apoE$^{-/-}$ ($n = 12$), and SR-BI$^{-/-}$ apoE$^{-/-}$ ($n = 9$) females ($P < 0.0002$). Plasma apoA-I levels (*Right*, mean ± SD, expressed as relative units) were determined by SDS/PAGE (15%) followed by quantitative immunoblotting for apoE$^{-/-}$ ($n = 7$) and SR-BI$^{-/-}$apoE$^{-/-}$ females ($n = 5$) ($P = 0.1$). (*B*) Lipoprotein cholesterol profiles. Plasma lipoproteins from individual apoE$^{-/-}$ (shaded diamonds) or SR-BI$^{-/-}$ apoE$^{-/-}$ (○) females were separated based on size (Superose 6-FPLC), and total cholesterol in each fraction (expressed as mg/dl plasma) was measured. The chromatograms shown are representative of multiple, independent determinations. Approximate elution positions of VLDL, IDL/LDL, and HDL are indicated as described previously (17, 18). (*C*) Lipoprotein apoA-I profiles. Pooled Superose 6-FPLC fractions (see above, ≈21 μl per pool) from females in an independent experiment were analyzed by SDS-polyacrylamide gradient (3–15%) gel electrophoresis and immunoblotting with an anti-apoA-I antibody (18). Each pool contained three fractions, and lanes are labeled with the number of the middle fraction in each pool. (*D*) Average EZ HDL cholesterol FPLC profiles for apoE$^{-/-}$ (diamonds) or SR-BI$^{-/-}$ apoE$^{-/-}$ (○) males (*Left*, $n = 3$) or females (*Right*, $n = 3$). (*E*) Agarose gel electrophoresis and immunoblotting. Pooled fractions (fractions 11–21; 3.5 μl) from the IDL/LDL region of the lipoprotein profile from individual apoE$^{-/-}$ or SR-BI$^{-/-}$ apoE$^{-/-}$ females were analyzed by using either anti-apoA-I or anti-apoB antibodies. The position of migration of normal-sized HDL is indicated by the asterisk. The arrow on the left indicates the mobility of the apoA-I-containing, apoB-free particles from double KOs (lane 3). Migration was upward from negative to positive (origin not shown). (*F*) Gallbladder biliary cholesterol (mean ± SD). Total gallbladder biliary cholesterol from both male and female mice of the indicated genotypes ($n = 10$ or 11 per genotype) was measured. Except for the wild-type and apoE$^{-/-}$ values, all pairwise differences were statistically significant ($P < 0.025$–0.0005).

the single apoE KOs appeared to comigrate with apoB (Fig. 2*E*, lanes 1 and 2). Thus, it appears that normal-sized HDL in the single apoE KO animals was replaced by very large (VLDL/IDL/LDL-sized), HDL-like particles in the double KO animals. It is possible that normal-sized HDL is converted into these large, HDL-like particles in the absence of both apoE and SR-BI because of substantially reduced, selective (SR-BI-mediated) and apoE-mediated uptake or transfer of cholesterol from HDL particles.

In addition to examining plasma cholesterol, we measured biliary cholesterol in the mice. Cholesterol levels in gallbladder bile were reduced significantly in SR-BI single KO (30%, $P < 0.005$) and SR-BI/apoE double KO (47%, $P < 0.0005$) mice relative to their SR-BI$^{+/+}$ controls (Fig. 2*F*). This is consistent with the previous finding that hepatic overexpression of SR-BI increases biliary cholesterol levels (17, 32) and indicates that SR-BI normally may play an important role in the last stage of reverse cholesterol transport—transfer of plasma HDL cho-

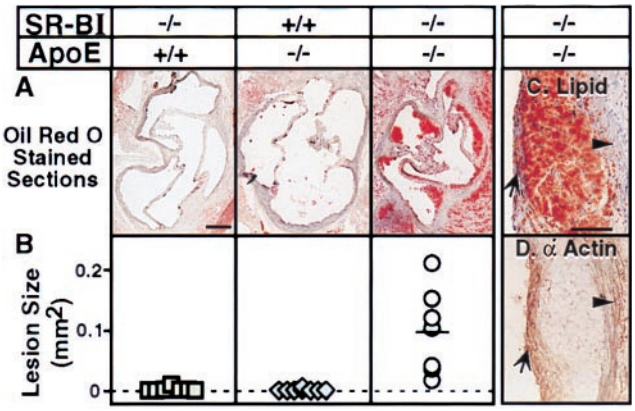

FIG. 3. Effects of SR-BI gene disruption on atherosclerosis in apoE KO mice. Atherosclerosis in SR-BI$^{-/-}$ (n = 8, 4–6 weeks old), apoE$^{-/-}$ (n = 8, 5–7 weeks old), or SR-BI$^{-/-}$ apoE$^{-/-}$ (n = 7, 5–6 weeks old) female mice was analyzed in cryosections of aortic sinuses stained with oil red O and Mayer's hematoxylin as described in *Materials and Methods*. (*A*) Representative sections through the aortic root region. (Bar = 200 μm.) (*B*) Sizes (cross-sectional areas) of oil red O-stained lesions in the aortic root region (see *Materials and Methods*). Average lesion areas (mm^2 ± SD) for SR-BI$^{-/-}$apoE$^{-/-}$, apoE$^{-/-}$, or SR-BI$^{-/-}$ mice, respectively, were as follows: 0.10 ± 0.07 (horizontal line), 0.002 ± 0.002, and 0.001 ± 0.002 (P = 0.0005). Also see Table 1. (*C* and *D*) High-magnification views of serial sections of plaque from the aortic sinus of a 7-week-old SR-BI/apoE double KO male, stained either with oil red O and Mayer's hematoxylin (*C*) or with an anti-α actin antibody that recognizes smooth-muscle cells (*D*). The lumen is to the left of the plaque. The smooth-muscle wall (arrowheads) and cellular cap (arrows) are indicated. (Bar = 100 μm.)

lesterol into bile. The data in Fig. 2*F* also suggest that apoE expression can regulate biliary cholesterol content in a SR-BI KO, but not SR-BI$^{+/+}$, background.

Atherosclerosis in the animals was assessed by analyzing plaque areas in aortic sinuses [Fig. 3 and Table 1, quantitative analysis of females; qualitative analysis of a smaller sample of males gave similar results (not shown)]. There were virtually no detectable lesions in the single KO animals at this relatively young age (4–7 weeks; refs. 40–42). However, there was substantial, statistically significant lesion development in the double KOs in the aortic root region, elsewhere in the aortic sinus (Fig. 3 and Table 1), and in coronary arteries (not shown). The lipid-rich lesions were cellular (hematoxylin-stained nuclei were seen at high magnification; Fig. 3*C*) and, in some cases, had a cellular cap that stained with antibodies to smooth-muscle actin (Fig. 3*D*). Thus, the atherosclerotic plaques were relatively advanced.

Potential causes of the dramatically accelerated atherosclerosis in the double KOs include (*i*) changes in relative amounts of cholesterol in proatherogenic (e.g., increased VLDL-sized or abnormally large HDL-like particles) and antiatherogenic (e.g., loss of normal HDL) lipoproteins, (*ii*) altered flux of cholesterol into or out of the aortic wall, perhaps directly because of SR-BI-mediated efflux (17, 38, 39), (*iii*) decreases in RCT, suggested by the generation of abnormally large, HDL-like particles and decreased biliary cholesterol levels because of the absence of hepatic SR-BI activity, and (*iv*) changes in other metabolic/organ systems that might influence the cardiovascular system. For example, there was significant accumulation of oil red O-staining lipids in other tissues, including the myocardium, in the double, but not single, KO animals (Fig. 3; data not shown). In addition, at 5–6 weeks of age when the double KOs were studied, they were somewhat smaller (≈20% lower weight) than single apoE KO controls. Although most did not exhibit overt signs of illness at that time, they all died suddenly around 8–9 weeks of age (see *Note*).

The antiatherosclerotic effect of SR-BI expression in apoE KO mice is consistent with the recent reports that adenovirus-mediated (K. Kozarsky, M. Donahee, J. Glick, M.K., and D. Rader, unpublished data) or transgene-mediated (60) hepatic overexpression of SR-BI in the cholesterol and fat-fed LDL receptor KO mouse reduces atherosclerosis. Thus, pharmacologic stimulation of endogenous SR-BI activity may be antiatherogenic, possibly because of its importance for RCT. The accelerated atherogenesis and loss of normal-sized HDL cholesterol in the double KOs (Figs. 2 and 3) resembles that reported for high-fat diet-fed single apoE KO mice (refs. 41 and 42; data not shown), although those mice have far higher total plasma cholesterol levels (1,800–4,000 vs. ≈600 mg/dl). Perhaps the similarities arise in part because the very high levels of large lipoproteins in the fat-fed single apoE KO might block the ability of SR-BI to interact with HDL and other ligands (functional SR-BI deficiency because of competition) or because of dietary suppression of hepatic SR-BI expression (61).

Taken together with earlier work (23), the current study provides compelling evidence for the proposal that, at least in rodents, SR-BI is an HDL receptor that mediates physiologically relevant, selective cholesterol transport and plays a key role in controlling plasma lipoprotein and biliary cholesterol concentrations and RCT. It also influences HDL's structure, cholesterol's delivery to and utilization by cells (including those in steroidogenic tissues), reproductive and cardiovascular physiology, and possibly other aspects of lipid metabolism (62). Because the *in vitro* activity, tissue distribution, and regulation of human SR-BI (34, 63–65) resemble those of the mouse, SR-BI may become an attractive target for prevention of or therapeutic intervention in a variety of human diseases (13, 17, 18, 22, 23).

Note. Electrocardiographic studies indicated that premature death of the double KOs was due to progressive heart block (cardiac conduction defects), and histology revealed extensive cardiac fibrosis and narrowing or occlusion of the coronary arteries, suggesting myocardial infarction (MI) because of advanced atherosclerotic disease (B.T., A.B., J. Edelberg, M.P., M.S., R. Rosenberg, and M.K., unpublished data). Independently, Caligiuri *et al.* (66) have reported MIs for stressed apoE/LDL receptor double KO mice after a 7-month, high-fat diet.

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Table 1. Average lesion sizes in the aortic sinuses of mice deficient in SR-BI, apoE, or both

Genotype	Mean lesion size, mm^2				
	Aortic root	Partial-valve cusps	Valve-attachment sites	Proximal aorta	Overall mean*
SR-BI$^{-/-}$	0.001 ± 0.002 (8)	0.0003 ± 0.0008 (8)	0 ± 0 (8)	0 ± 0 (6)	0.0004 ± 0.001 (6)
apoE$^{-/-}$	0.002 ± 0.002 (9)	0.0006 ± 0.0009 (9)	0.001 ± 0.002 (9)	0.0002 ± 0.0003 (9)	0.001 ± 0.002 (9)
SR-BI$^{-/-}$ apoE$^{-/-}$	0.10 ± 0.07 (7)	0.07 ± 0.07 (7)	0.02 ± 0.01 (6)	0.02 ± 0.02 (6)	0.04 ± 0.04 (6)
P value†	0.0005	0.006	0.002	0.003	0.001

Values are the means ± SD (number of animals indicated in parentheses).
*Means of combined values from the regions of the aortic root, partial-valve cusps, valve-attachment sites, and proximal aorta.
†Lesion sizes in each region were compared by using the Kruskall–Wallis test.

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