Carboxy-terminal deletion of the HDL receptor reduces receptor levels in liver and steroidogenic tissues, induces hypercholesterolemia, and causes fatal heart disease

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<td>As Published</td>
<td><a href="https://doi.org/10.1152/ajpheart.00463.2016">https://doi.org/10.1152/ajpheart.00463.2016</a></td>
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<tr>
<td>Publisher</td>
<td>American Physiological Society</td>
</tr>
<tr>
<td>Version</td>
<td>Author's final manuscript</td>
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<tr>
<td>Accessed</td>
<td>Sun Feb 03 03:06:35 EST 2019</td>
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C-terminal Deletion of the HDL Receptor Reduces Receptor Levels in Liver and Steroidogenic Tissues, Induces Hypercholesterolemia and Causes Fatal Heart Disease.

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Running title: The SR-BIΔCT Mouse: a New Model for Coronary Heart Disease

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Abstract
The HDL receptor SR-BI mediates the transfer of cholesteryl esters from HDL to cells and controls HDL abundance and structure. Depending on the genetic background, loss of SR-BI causes hypercholesterolemia, anemia, reticulocytosis, splenomegaly, thrombocytopenia, female infertility and fatal coronary heart disease (CHD). The C-terminus of SR-BI (505QEAKL509) must bind to the cytoplasmic adaptor PDZK1 for normal hepatic -but not steroidogenic cell- expression of SR-BI protein. To determine if SR-BI’s C-terminus is also required for normal protein levels in steroidogenic cells, we introduced into SR-BI’s gene a 507Ala/STOP mutation that produces a truncated receptor (SR-BIΔCT). As expected, the dramatic reduction of hepatic receptor protein in SR-BIΔCT mice was similar to that in PDZK1 KO mice. Unlike SR-BI KO females, SR-BIΔCT females were fertile. The severity of SR-BIΔCT mice’s hypercholesterolemia was intermediate between those of SR-BI KO and PDZK1 KO mice. Substantially reduced levels of the receptor in adrenal cortical cells, ovarian cells and testicular Leydig cells in SR-BIΔCT mice suggested that steroidogenic cells have an adaptor(s) functionally analogous to hepatic PDZK1. When SR-BIΔCT mice were crossed with apoE KO mice (SR-BIΔCT/apoE KO), pathologies including: hypercholesterolemia, macrocytic anemia, hepatic and splenic extramedullary hematopoeisis, massive splenomegaly, reticulocytosis, thrombocytopenia and rapid onset and fatal, occlusive coronary arterial atherosclerosis and CHD (median age of death: 9 weeks) were observed. These results provide new insights into the control of SR-BI in steroidogenic cells and establish SR-BIΔCT/apoE KO mice as a new animal model for the study of CHD.

New and Noteworthy
Deletion of the mouse HDL receptor’s (SR-BI’s) three carboxy terminal residues induces hypercholesterolemia and decreased receptor expression in liver and steroidogenic tissues, suggesting previously unrecognized C-terminus-binding adaptor protein in steroidogenic cells. SR-BIΔCT/apoE KO fed a regular chow diet show severe coronary heart disease and premature death secondary to myocardial infarction.

Keywords: SR-BI, Steroidogenic Organs, PDZ domains, Atherosclerosis, Myocardial Infarction

Introduction
Scavenger Receptor Class B Type I (SR-BI) is a 509 amino acid cell surface receptor with a large extracellular loop and short intracytoplasmic amino- and carboxy—termini (8 and 45 amino acids long respectively) (49) that is most highly expressed in the liver and in the steroidogenic cells of the adrenal gland, testes and ovary (1). A minor mRNA splicing isoform, SR-BII with a different C-terminus (39 residues replace 45 in SR-BI) has been described (68). As a high density lipoprotein (HDL) receptor, SR-BI plays an important role in the transfer of cholesterol from HDL particles to cells (1, 49), controls HDL abundance and structure in both mice (50) (50% C57BL/6/50% 129-S4 background) and humans (59, 65, 79), serves as a signaling receptor to control eNOS activity and vascular tone (77) and is a co-receptor for hepatitis C virus (8) and malaria (51, 69). Inactivation of murine SR-BI (SR-BI knockout (KO) mice) results in a 2.2-fold increase of plasma cholesterol in abnormally large HDL particles with an abnormally high ratio of unesterified to total cholesterol (UC:TC) (5, 50, 63). Analyses of SR-BI KO mice (50) and mice with hepatic overexpression of total cholesterol (UC:TC) (5, 50, 63). Analyses of SR-BI KO mice (50) and mice with hepatic overexpression of SR-BI (35, 73) have established that SR-BI can influence a variety of physiologic and pathophysiologic systems, including red blood cell maturation and stability (25), platelet stability and function (13), biliary cholesterol secretion (35, 39), reverse cholesterol transport (83), steroidogenesis (37, 58), female fertility (41, 61, 72), deep vein thrombosis (6), and atherosclerosis/coronary heart disease (4, 61).
The expression, localization and function of SR-BI and many other membrane proteins can be regulated by cytoplasmic adaptor proteins (34, 74). A large group of such cytoplasmic proteins consists of the PDZ (PSD-95, Discs-large, ZO-1) domain protein family (64). PDZ domains are globular structures of 80-90 amino acids; usually interacting with the carboxy terminal amino acids of their target protein using a well-defined binding pocket, although some PDZ domains can recognize internal peptide sequences (14, 22, 46) and some bind to anionic lipids in the cytoplasmic leaflets of cellular membranes (9, 62, 85). More than 100 PDZ domain-containing proteins have been described in humans, many of which contain multiple PDZ domains that allow them to function as scaffolds to bring together their target proteins/membranes for signal transduction and complex cellular functions (31, 64, 70). One of these multi-PDZ-domain adaptor proteins, PDZK1, is 519 amino acids long, contains four PDZ domains and interacts with several membrane-associated proteins, mostly ion channels (31, 32).

One of PDZK1’s target proteins is the HDL receptor SR-BI. PDZK1 was first shown to interact with the carboxy terminus of SR-BI by Ikemoto et al. (27) and was the first described tissue-specific adaptor protein of SR-BI (33). The carboxy terminus of SR-BI binds to either the first (PDZ1, high affinity interaction) or the third (PDZ3, low affinity interaction) PDZ domain of PDZK1 (27, 29, 30). The five C-terminal residues of SR-BI (\(505\)QEAKL\(509\)) form hydrogen bonds and hydrophobic contacts with the canonical peptide binding pockets in these two PDZ domains (29, 30). The PDZ4 domain of PDZK1 is also required for full hepatic expression of SR-BI protein. PDZ4 appears to function by mediating PDZK1 binding directly to lipids in the inner leaflet of the plasma membrane rather than by canonical binding to the C-terminus of a target protein (62). Thus bidentate binding of PDZK1 to both SR-BI and membrane lipids is essential for normal hepatic SR-BI expression. This regulation influences the intracellular localization of SR-BI as well as the amount of SR-BI protein, presumably in part as a consequence of reduced protein stability. The results of these and other structure/function studies of the role of PDZK1 in regulating SR-BI are summarized in (62).

In hepatocytes, PDZK1 post-transcriptionally controls the expression, localization and function of SR-BI (33). In PDZK1 KO mice (129SvEv genetic background), hepatic SR-BI protein is reduced by 95% compared to wild-type controls. As a consequence there is increased plasma cholesterol (1.7 fold) in the form of abnormally large HDL particles, a phenotype similar to, but not as severe as, that in SR-BI KO mice (33). Unlike SR-BI KO mice, PDZK1 KO mice do not exhibit an abnormally high ratio of unesterified to total cholesterol (UC:TC) and the females are fertile (33). When PDZK1 KO mice are crossed with apoE KO mice, the atherogenic diet-fed double KO mice (PDZK1/apoE dKO) exhibit increased atherosclerosis relative to apoE KO mice and can develop coronary heart disease that is substantially less severe than that of SR-BI/apoE dKO mice (34, 71).

In PDZK1 KO mice, there is a striking difference in the very low expression of SR-BI protein in hepatocytes (<5%) compared to the essentially wild-type level of SR-BI in steroidogenic cells (100%) (33). It is possible that distinctive features of hepatocytes (polarity, membrane trafficking, etc.) not shared by steroidogenic cells confer a requirement for SR-BI’s C-terminus to bind to an adaptor protein. Alternatively, in steroidogenic cells there may be a distinct adaptor protein(s) that can bind to SR-BI’s C-terminus and play a role analogous to that of PDZK1 in the liver in maintaining normal levels of SR-BI protein expression. To explore these possibilities, we have used Knock-in technology in mice to insert in SR-BI’s gene a Stop codon in place of the codon encoding \(507\)Ala in SR-BI’s C-terminus. The resultant truncated protein, SR-BI ΔCT, is three residues (\(507\)AKL\(509\)) shorter than wild-type SR-BI. Those residues are part of SR-BI’s five residue PDZ-domain binding motif; thus SR-BI ΔCT is not expected to bind to PDZ-domain-containing (e.g. PDZK1) or other adaptors that recognize SR-BI’s most C-terminal residues. Analyses in transfected cell lines have shown that removal, or removal and replacement, of all or part of the PDZ domain-binding motif in SR-BI does not prevent the receptor’s cell surface expression or alter its lipid transport activities (11, 12, 19, 54). As expected from previous studies (29, 30, 33, 54), homozygous Knock-in mice expressing SR-BI ΔCT exhibited a marked reduction of SR-BI ΔCT protein expression in the liver. Strikingly, there was also a marked reduction in receptor expression in steroidogenic cells. Thus, as in the case of the liver, in steroidogenic cells normal SR-BI protein expression requires its three C-
terminal cytoplasmic residues, most likely because those residues are required for binding to a distinct cytoplasmic adaptor required to maintain normal receptor levels.

We compared a variety of characteristics of SR-BIΔCT mice to those of WT, SR-BI KO and PDZK1 KO mice, including composition and size of the HDL, female fertility, red blood cell (RBC) and platelet levels, cholesteryl ester (CE) stores in steroidogenic cells, and atherosclerosis and coronary heart disease susceptibility when combined with apoE deficiency. For some phenotypes, the SR-BIΔCT mice resembled WT mice (female fertility, RBC and platelets, CE stores). Other phenotypes of SR-BIΔCT mice resembled those of either SR-BI KO or PDZK1 KO mice (plasma lipoprotein composition and size, atherosclerosis and coronary heart disease susceptibility). For example, when SR-BIΔCT mice were crossed with apolipoprotein E knockout mice (apoE KO), the SR-BIΔCT/apoE mice fed a standard chow diet exhibited early onset, fatal atherosclerotic coronary heart disease (CHD), and thus provide a novel model for the study of CHD.

Material and Methods

Generation of SR-BIΔCT Knock-in mice: A SR-BIΔCT Knock-in mouse containing a deletion of the last three carboxy terminal amino acids of SR-BI was generated by Ingenious Targeting Laboratory (Fig. 1A). The mouse BAC clone RP23-21D2 (chromosome 5, 125587731-125789903) was used to build the targeting vector, which was constructed using an homologous-based recombination technique. The vector consisted of a 5.0 Kb long arm, including exons 10, 11 and 12 of the SR-BI gene and a 1.9 Kb short arm. The SPEC cassette, containing the ΔCT-SR-BI mutation (replacement of 508Ala by a Stop codon), as well as the Neo cassette were generated by PCR and inserted into the BAC clone by bacterial homologous recombination (middle arm). A DNA fragment containing the long, middle (SPEC and Neo cassettes) and short arms was subcloned from the BAC clone into the targeting vector (total size, 13.05 Kb) (Fig 1A). Ten micrograms of the targeting vector were linearized with NotI prior to electroporation into iTL BA1 (129/SvEv x C57BL/6; 50:50) hybrid embryonic stem cells. After selection with G418, surviving clones were expanded for PCR analysis to identify homologous recombinant ES cell clones. Several clones were identified as positive and selected for further use. Confirmation of the mutation was performed by PCR and DNA sequencing. Further confirmation of the positive ES cell clones was performed by Southern blot. Homologous recombinant ES cells were microinjected into C57BL/6 blastocysts. Embryos were transferred into pseudopregnant mice. Resulting chimeras with high percentage agouti coat color were mated to C57BL/6 FLP mice to remove the Neo cassette. Tail DNA was tested by PCR to determine mouse genotypes and the removal of the Neo cassette using the NDEL1 (CCTCTTCACCCCACCTACTCATAGC) and NDEL2 (GGACACTGAGAAGCAACTGGCCTAAC) oligonucleotide primers. The wild-type allele generated a band at 427 bp, while the SR-BIΔCT-SR-BI ΔCT mutation (replacement of 507Ala by a Stop codon) generated a band at 515 bp (Fig. 1B). Mouse heterozygous for the mutation were mated to 129-Elite Mice (129S2/SvCrI) (Charles River). Heterozygous mice resulting from this mating were used to generate wild-type mice - used as background matched controls - and homozygous Knock-in mutants. Intercrosses of the heterozygous mice resulted in fertile homozygous Knock-in mice (SR-BIΔCT).

Animals: All animal experiments were performed according to IACUC guidelines. Wild-type and SR-BIΔCT mice (both on a mixed C57BL/6 X 129S2/SvCrI (37.5:62.5) genetic background, see above) were maintained on a normal chow diet. Six to ten week old male and female mice were used for experiments. For atherosclerosis studies, apoE deficient mice (C57BL/6 background) (82) were purchased from Jackson Laboratories (Bar Harbor, ME), mated with the SR-BIΔCT mice and maintained on a standard chow diet. Genotypes were determined by PCR using established protocols (see above and Jackson Laboratories web site). After the initial breeding, apoE KO mice heterozygous for the SR-BIΔCT mutation and apoE KO were mated to generate apoE KO and SR-BIΔCT/apoE KO mice with the same proportion of C57/B6 and 129 backgrounds (68.75:31.25, respectively) and were used for experiments. All procedures were performed in accordance with the guidelines of the Beth Israel Deaconess Medical Center and the Massachusetts Institute of Technology Committee on Animal Care. We compare the results described here with results previously reported that employed mice with the following genetic backgrounds: SR-BI KO (mixed C57BL/6 X 129S2/SvCrI (50:50)); PDZK1 KO
Blood and tissue sampling, processing, and analysis: Plasma, liver, spleen, adrenal glands, testis and ovaries were collected and processed as previously described (33, 50). Total and unesterified plasma cholesterol levels and fast protein liquid chromatography (FPLC) cholesterol profiles that separate plasma lipoproteins by size were obtained as previously described (16). The presence of apoA1 and apoE in individual chromatographic fractions was determined by immunoblotting (see below).

Hearts were excised after a short in vivo perfusion with PBS, weighted and frozen in OCT compound. Transverse or sagittal frozen sections (5 µm) were stained with Oil Red O/hematoxylin to assess the presence of atherosclerotic lesions in the aortic root and coronary vessels as previously described (71). Cardiac fibrosis was evaluated on cryosections (5 µm)-stained with Mason’s Trichrome (71).

Erythrocyte and platelet analysis: Blood was collected by cardiac puncture into EDTA tubes (Microvette* 100 from Sarstedt). Hematocrit, erythrocyte measurements, including mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) and platelet counts were determined using an automated Hemavet HV950 analyzer (Drew Scientific) by the Department of Comparative Medicine at MIT. Peripheral smears were performed to assess red blood cell morphology, stained with Wright-Giemsa and examined using standard light and differential interference contrast microscopy (DIC). Reticulocyte counts were performed manually following brief staining in 0.5% methylene blue.

RNA extraction and gene expression evaluation by qPCR: Total RNA was isolated from liver and adrenal glands harvested from 6-8 week old male and female WT and SR-BIΔCT mice (8 WT liver, 6 SR-BIΔCT liver, 14 WT and 14 SR-BIΔCT adrenal glands) using an RNeasy mini kit (Qiagen). Corresponding cDNA was generated by reverse transcription with Superscript III (Invitrogen) using random primers. Quantitative real-time PCR was performed using SYBR Green 1 (Qiagen). MGTP, a form of quantitative real-time PCR, was used to determine mRNA copy numbers. The number of mRNA copies was calculated by normalization to 18S rRNA abundance and expressed as “n” copies/10^6 copies of 18S (53). The DNA primers used were TGGAACGGACTCAGCAGATC and GTCATGAAGGGTGCCCACAT for SR-BI+SR-BII and GCGCAGCCAGGTCCTGAAA and TGGCTGTCTGACCAAGCTA for SR-BII only.

Immunoblotting: Protein samples (~30 µg, as determined using a Biorad DC protein assay) from total tissue lysates of both male and female mice (7 livers, 7 adrenal glands and 3 ovaries per group) were fractionated by 5-20% gradient SDS-PAGE, transferred to nitrocellulose membranes and incubated with either a rabbit polyclonal anti-SR-BI antibody (mSR-BI^495-112) raised against a carboxy terminal peptide of the protein, the anti-SR-BI KKB-1 antibody (19), a rabbit polyclonal antibody that recognizes both SR-BI and SR-BII (16), or a specific rabbit anti-SR-BII (68) antibody (Novus Biologicals) (all used at 1:500 dilution), followed by an anti-rabbit IgG conjugated to horseradish peroxidase (Invitrogen, 1:10,000), and visualized by ECL chemiluminescence (GE Healthcare). Immunoblotting using a polyclonal anti-ε-COP antibody (1:5,000) (20) was used to control for small variations in loading. The relative amounts of proteins were determined quantitatively using a FluorChemQ System Quantitative Western Blot Imaging. Western blot analyses of the FPLC fractions were performed using rabbit anti-apoAI (1:2000) (Biodesign) and rabbit anti-apoE (1:400) (Novus) antibodies and anti-rabbit secondary antibody. For liver samples, longer exposures for chemiluminescence detection of SR-BI/II were used than for adrenal and ovarian samples because of the substantially lower receptor levels in the liver.

Cholesterol measurements in adrenal glands: Adrenal glands obtained from WT and SR-BIΔCT mice (6 males and 6 females in each group) were homogenized in 0.75 ml of lysis buffer (0.25 M sucrose, 50 mM Tris, pH 7.4) using a dounce homogenizer. The homogenate obtained was mixed with 3 ml of chloroform: methanol (1:2 v/v), vortexed and incubated on a shaker at room temperature for 1 hour. One ml of chloroform and 1 ml of distilled water were added and vortexed. The extract was centrifuged at 1000 rpm for 10 min. at 4°C. The lower organic phase was separated and dried. Dried lipids were resuspended in 0.2 ml of 0.1% Triton X-100 and sonicated. Total cholesterol was measured using a kit from Wako Diagnostics according to manufacturer’s protocol. Protein amounts were determined after drying the aqueous phase, which was reconstituted in 0.3 N NaOH and 0.1% Triton X-100.
**Immunoperoxidase analysis:** Livers, adrenals, ovaries and testis (using tissue samples from 5-6 male or female mice per group) were harvested, fixed in 4% paraformaldehyde in PBS for 4 hours, transferred overnight into 30% sucrose in PBS, frozen, and 5 μm cryosections (2-3 sections per slide) were generated and stained with the anti-SR-BI KKB-1 antibody and biotinylated anti-rabbit IgG, visualized by immunoperoxidase staining, and counterstained with Harris modified hematoxylin, as described previously (33).

**Hemodynamic studies:** Cardiac function was evaluated in 7 week-old mice using left ventricular pressure-volume loop measurements as previously described (3, 10). Maximum and minimum left ventricular volumes, cardiac output, ejection fraction, dP/dt and stroke work were determined.

**Statistical Analysis:** Data are shown as the means ± standard errors. Statistically significant differences were determined by either pairwise comparisons of values using the unpaired t test, or by one-way ANOVA with Tukey post hoc testing. Mean values for experimental groups are considered statistically significantly different for p < 0.05 for both types of tests.

**Results**

**Role of the carboxy terminus of SR-BI on the regulation of SR-BI protein expression and function.**

To determine the impact of the absence of the three carboxy terminal (CT) amino acids of SR-BI on its regulation in the liver and in steroidogenic organs, and the influence of the truncated protein on SR-BI-dependent physiology and pathophysiology, we used Knock-in technology to modify the endogenous mouse SR-BI gene so that the SR-BI protein would contain a deletion ("ΔCT") of the last three C-terminal amino acids (residues 507AKL509) by replacing the codon for 507Ala in exon 12 with a Stop codon (Fig.1A). The ΔCT mutation was designed to prevent the interaction of the C-terminus of SR-BI with cytoplasmic adaptors that recognize the C-terminal residues, such as the PDZ1 and PDZ3 domains in PDZK1 (29, 30). Mice homozygous for this deletion mutation, designated SR-BIΔCT, were generated and have been observed for one year. They develop normally, and show no alteration in gross morphology, weight and size compared to wild-type mice. These mice and their wild-type (WT) controls used for the experiments reported here are on a mixed C57BL/6:129S2/SvCrl (37.5:62.5) genetic background.

**Effects of the SR-BIΔCT mutation on SR-BI mRNA and protein expression in the liver.** The expression levels in the livers of WT and SR-BIΔCT mice of the mRNA for SR-BI and its minor splice isoform SR-BII were evaluated by qPCR. We used oligonucleotide primers that either recognized both SR-BI and its minor splice isoform SR-BII or that were specific for the SR-BII isoform only (see Materials and Methods). Steady state levels of receptor protein were measured by quantitative immunoblot analysis using three specific antibodies: an anti-C-terminus antipeptide antibody (anti-SR-BI495-112) (1, 16) that recognizes SR-BI, but not SR-BII, whose C-terminus differs from that of SR-BI (68). A commercial anti-C-terminus antipeptide antibody (Novus) that recognizes SR-BII, but not SR-BI, and the polyclonal antibody KKB-1 (19) that recognizes the extracellular loops of both SR-BI and SR-BII (33, 68). The KKB-1 antibody is the only one of these three expected to recognize SR-BIΔCT.

The qPCR analysis established that the truncated receptor’s mRNA copy number (expressed as “n” copies/10^6 copies of 18S rRNA, see Materials and Methods) in SR-BIΔCT liver was similar to that of full-length SR-BI in WT liver (WT: 48.0±2.8; ΔCT: 40.5±0.9; ~16% reduction, p=0.04). This small 254 difference might be due to reduced transcription, stability or both. The copy number of the mRNA for the minor splice variant SR-BII was low in WT liver (6.3±0.4) and unexpectedly increased by 2.5 fold in SR-BIΔCT livers (15.9±0.9, p<0.0001).

As demonstrated in Fig. 2A, top panel, the anti-C-terminus anti-SR-BI195-112 antibody (anti-C-term) could readily detect a band corresponding to SR-BI (~82 kDa) in WT liver (and adrenal gland and ovary, see below), but not in tissues from SR-BIΔCT mice carrying the C-terminal, three-residue deletion. An antibody to the cytosolic COPI coat subunit ε-COP was used as a loading control (bottom panel). The absence of signal in the SR-BIΔCT-tissues is likely due to the inability of the anti-C-terminus antibody to
recognize the SR-BIΔCT protein or the complete absence of this protein. Using the KKB-1 antibody, which does not require an intact C-terminus to detect SR-BI or SR-BII, a strong signal could be seen in the immunoblot of WT livers (Fig. 2A, middle panel, left), but there was a more than a 95% reduction in the signal in livers from SR-BIΔCT mice (the relative expression -SR-BIΔCT/WT- was 0.03±0.003 (p<0.0001) (Fig. 2C, left panel). These results were confirmed by immunohistochemical localization of receptors using the KKB-1 antibody. There was robust staining of the sinusoidal membranes of hepatocytes in WT liver (Fig. 2D), but no significant staining in SR-BIΔCT liver (Fig. 2D). Thus, there was a dramatic reduction in the amount of hepatic SR-BIΔCT protein in SR-BIΔCT mice relative to the amount of full-length SR-BI protein in WT livers. This observation was expected because the C-terminus of SR-BIΔCT does not bind to PDZK1 (as predicted by Isothermal Titration Calorimetry which demonstrated that SR-BI 506KGTVLQE506 peptide missing the three carboxy terminal amino acids 507AKL509 did not bind to PDZK1 recombinant protein (data not shown)), and normally high levels of hepatic SR-BI protein expression require SR-BI’s binding to PDZK1 (29, 33).

A small amount of the signal in the WT livers, detected using the KKB-1 antibody (Fig. 2A, middle panel, left), is likely to be contributed by the minor isoform SR-BII. Analysis of PDZK1 KO mice established that the level of hepatic SR-BI protein, whose C-terminus differs from that of SR-BI and is not expected to bind to PDZ domains, is PDZK1 independent (33). Indeed, when a SR-BII-specific, antipeptide antibody was used for immunoblotting (Fig. 2B) the relatively weak signal for SR-BII in WT liver was increased 6.5-fold in SR-BIΔCT livers (relative expression SR-BIΔCT/WT= 6.5±0.7 (p=0.001) (Fig. 2C, right panel). The increased SR-BII protein expression might have been due, at least in part, to the 2.5-fold increase in SR-BII mRNA, increased translation, increased protein stability, or some combination of these. Although there was an increase in the minor splice form of the receptor protein, SR-BII, in SR-BIΔCT compared to WT livers, the overall hepatic levels of all isoforms of this receptor detected by the KKB-1 antibody were dramatically lower in SR-BIΔCT mice compared to WT mice (Fig. 2A, middle panel, left). It is likely that the reduced steady state levels of receptor, primarily the truncated SR-BI isoform, were a consequence of its inability to bind to the adaptor protein PDZK1. Thus, the dramatic reduction in receptor protein in the liver in SR-BIΔCT mice mirrors the loss of hepatic full-length SR-BI in PDZK1 KO mice (33).

**Effects of the SR-BIΔCT mutation on SR-BI mRNA and protein expression in steroidogenic tissues.** We next addressed the question of whether or not there was a requirement for the three C-terminal residues of SR-BI for its normal protein expression in steroidogenic cells. Such a requirement would suggest that an adaptor protein functionally analogous to hepatic PDZK1 would be present in steroidogenic cells. First, we focused on receptor mRNA expression in adrenal glands, where SR-BI is highly expressed in the cortex (1, 37, 48).

The copy number of all isoforms of the receptor’s mRNA was reduced by 27% in SR-BIΔCT adrenal glands compared to WT adrenal glands (WT: 799.4±56.1; SR-BIΔCT: 587.1±55.8, p=0.01). The approximately 15-fold greater number of mRNAs (SR-BI plus SR-BII) in the adrenal gland relative to the liver of WT mice is consistent with previous reports of substantially higher SR-BI mRNA and protein expressed in the adrenal gland (1, 33, 37). The copy number of the SR-BII isoform’s mRNA increased 4.4-fold (WT: 45.2±3.9; SR-BIΔCT: 197.9±40.4, p=0.0009).

We used immunoblotting with the KKB-1 antibody, which recognizes both SR-BI and SR-BII and does not require an intact C-terminus to detect the receptors, to assess the steady state receptor protein levels in the adrenal glands and ovaries. Fig. 3A (2nd panel, left and right), shows that, as previously reported (1, 33, 37) in the tissues from the WT mice there was an intense receptor band in the adrenal glands and a weaker one in the ovaries. In contrast, in the tissues from SR-BIΔCT mice compared to those from WT there was an 84% reduction in the intensity of SR-BI/SR-BII protein in adrenal glands (relative expression of SR-BIΔCT/WT= 0.16±0.01 (p<0.0001) (Fig. 3B, left panel) and a ~64% reduction in the ovaries (relative expression SR-BIΔCT/WT= 0.36±0.07 (p=0.0009) (Fig. 3B, left panel). These results were confirmed by immunohistochemical imaging of receptors using the KKB-1 antibody. There was robust staining of the plasma membranes of adrenal cortical cells (Fig. 3C, top left) and ovarian stromal...
cells (Fig. 3C, middle left) in WT mice, but the staining was clearly less intense in SR-BIΔCT mice (Fig. 3C top and middle right), particularly at the cell surfaces. Although we did not perform qPCR or immunoblotting analyses of the testes, immunohistochemical analysis (Fig. 3C bottom) indicated that in the steroidogenic Leydig cells of SR-BIΔCT mice relative to those in WT mice, there was a reduction in SR-BI/SR-BII protein, particularly at the cell surfaces.

A small amount of the receptor protein in SR-BIΔCT adrenal glands and ovaries detected using the KKB-1 antibody (Fig. 3A, 2nd panel, left and right) is likely to have been contributed by the minor isoform SR-BII. When a SR-BII-specific, antipeptide antibody was used for immunoblotting (Fig. 3A, 4th panel) the relatively weak intensities for SR-BII in both WT tissues were increased in SR-BIΔCT tissues, 9.2±2.2-fold (p=0.02) in adrenal glands and 2.7±0.3-fold (p=0.003) in the ovaries (Fig. 3A, fourth panel). The increased SR-BII protein expression might have been due, at least in part, to the increase in SR-BI mRNA, increased translation or increased protein stability, or some combination of these. Despite the increase in SR-BII protein in these steroidogenic tissues in SR-BIΔCT compared to WT mice, the overall levels of both isoforms of this receptor detected by the KKB-1 antibody were substantially lower (84%) in SR-BIΔCT mice compared to WT mice (Fig. 3A, 2nd panel, center and right). These results differed dramatically from those in PDZK1 KO mice in which there is essentially no loss of SR-BI protein expression in steroidogenic tissues compared to WT mice (33). Based on these results, we propose that the steroidogenic cells in the adrenal glands and ovaries, and possibly the testes, express an adaptor protein(s) that is functionally similar to PDZK1 in hepatocytes in that it recognizes the C-terminal three residues of SR-BI and is required for expression of normal SR-BI protein levels in these cells.

Functional consequences of the SR-BIΔCT mutation in SR-BIΔCT KI mice.

Plasma cholesterol and lipoproteins in SR-BIΔCT mice. Figure 4A shows a comparison of the plasma levels of total (unesterified plus esterified, Fig. 4A-a) and unesterified cholesterol (Fig. 4A-b) in male (M) and female (F) wild-type (WT) and SR-BIΔCT (ΔCT) mice. There was a significant 2.1 fold increase in total plasma cholesterol (Fig. 4A-a) in both male and female SR-BIΔCT mice compared to WT mice (male WT: 94.6±3.7 mg/dl, male SR-BIΔCT: 198.6±5.3 mg/dl, female WT: 71.0±4.3 mg/dl, female SR-BI ΔCT: 152.4±6.4 mg/dl, p<0.0001). Total plasma cholesterol levels were significantly different (p=0.008) between male and female WT mice. There was also an ~3-fold increase in unesterified cholesterol (Fig.4A-b) in both male and female SR-BIΔCT mice compared to WT mice (male WT: 26.6±1.6 mg/dl, male SR-BIΔCT: 77.4±2.2 mg/dl, female WT: 16.1±1.0 mg/dl, female SR-BI ΔCT: 54.1±2.5 mg/dl, p<0.0001). A distinctive feature of the plasma of SR-BI KO mice compared to WT and PDZK1 KO mice is a marked, ~65%, increase in the ratio of unesterified-to-total cholesterol (UC:TC: SR-BI KO, 0.515±0.027; WT and PDZK1 KO, ~0.23-0.31; UC:TC can vary depending on sex and genetic background (this study and (5, 33, 63)). The mixed genetic backgrounds of the mice analyzed in this report and of those to which they are compared are provided in the Materials and Methods. The increased UC:TC ratio appears to be responsible for some of the pathophysiology exhibited in SR-BI KO mice (abnormal RBCs and platelets, female infertility) (25, 41, 61, 72). There was an increase in the UC:TC ratio in SR-BIΔCT mice (Fig. 4A-c), although the elevation was not as great as that in SR-BI KO mice (SR-BIΔCT (male/female) 0.39±0.01/0.36±0.01; WT (male/female) 0.28±0.02/0.23±0.02, p<0.0001 for both sexes).

FPLC size fractionation of plasma lipoproteins from WT mice shows that most of the plasma cholesterol (unesterified and esterified) is carried in HDL-size particles that contain the major HDL apolipoprotein apoA-1 as well as some apoE, both detected by immunoblotting (Fig. 4B, squares) (36, 50, 67, 76). In SR-BIΔCT mice the large apoA-1- and apoE-containing HDL peak is partially shifted to the left in the lipoprotein cholesterol profile (Fig. 4B, triangles), indicating a larger and more heterogeneous population of HDL particles. Thus, the dramatic reduction in hepatic SR-BI in SR-BIΔCT mice resulted in plasma cholesterol and lipoprotein phenotypes that were similar to, but not quite as severe as those in SR-BI KO mice (50). It is not clear why these abnormal phenotypes in SR-BIΔCT mice were more severe than those in PDZK1 KO mice (e.g., increased UC:TC ratio in SR-BIΔCT mice); although disruption of
normal SR-BI activity in other organs in the SR-BIΔCT mice (e.g., intestines, steroidogenic tissues, etc.) may play a role.

**Fertility of SR-BIΔCT mice.** It is noteworthy that, despite the modestly elevated UC:TC ratio in female and male SR-BIΔCT mice, these mutant mice were fertile and their litter sizes (5.6 pups/litter, n=12) were comparable to those of WT mice (5.2 pups/litter, n=16). In contrast, SR-BI KO female (but not male) mice, which have a very high UC:TC ratio in their plasma, are infertile due to excess UC deposition in and premature activation of their eggs (41, 61, 72). PDZK1 KO mice are fertile (33).

**Red blood cells (RBC) and platelets in SR-BIΔCT mice.** Table 1 and Fig. 5A show that the hematological characteristics of SR-BIΔCT mice are similar to those of WT mice (RBC morphology - assessed using standard and differential interference contrast microscopy, mean corpuscular hemoglobin (MCH), % reticulocytes and platelet count), with a slightly elevated hematocrit (55.9±3.2% vs 45.8±2.3%, p=0.04) and 21% increase in red blood cell volume (MCV) (p=0.001). As was the case with female fertility, the RBC and platelet phenotypes of SR-BIΔCT mice (UC:TC ratio of 0.36±0.01) were essentially normal, whereas those of SR-BI KO mice exhibit some abnormalities that are likely a consequence of their abnormally high UC:TC ratio (0.515±0.027) (13, 25). Although the SR-BIΔCT mutation alone apparently did not markedly alter the RBCs and platelets, it did have effects on these blood cells when combined with deletion of the apoE gene, as this combination dramatically increased the UC:TC ratio (described below).

**Cholesteryl ester stores in adrenal glands and ovaries of SR-BIΔCT mice.** In WT mice, cholesteryl esters are stored in cytoplasmic lipid droplets in steroidogenic cells to provide cholesterol as feedstock for steroidogenesis, and SR-BI plays an important role in maintaining these cholesteryl ester stores (50, 61), reviewed in (49). The cholesteryl ester stores may be detected by chemical analysis (e.g. extraction and quantitative analysis), staining tissue sections with the neutral lipid staining dye oil red O, or visual inspection in the case of adrenal glands (24, 33, 49, 50, 61). In SR-BIΔCT mice, oil red O staining (Fig. 5B) and visual inspection indicated no substantial reduction in the cellular neutral lipid content in the adrenal cortex the ovarian stroma and testicular Leydig cells compared to WT controls (Fig. 5B). These qualitative histochemical results were confirmed quantitatively in adrenal glands when we measured the total cholesterol content of adrenal glands from male and female (n=6 in each group) WT and SR-BIΔCT mice. In female mice, there was no statistically significant difference (p>0.05) in adrenal gland total cholesterol content between WT and SR-BIΔCT mice (98.67 ± 6.33 and 109.6 ± 6.88 μg cholesterol/mg protein, respectively). The total cholesterol content of the adrenal glands from SR-BIΔCT male mice (99.23 ± 4.44 μg cholesterol/mg protein) was similar to that of their female counterparts (p>0.05). As previously described (57, 66), we observed a significant difference in adrenal gland total cholesterol between male and female WT mice (53.55 ± 3.90 and 98.67 ± 6.33 μg cholesterol/mg protein respectively, p<0.0001). The mechanism(s) responsible for this sexual dimorphism in WT mice remains uncertain.

Although there was a reduction of cell surface HDL receptor protein in the adrenal glands, ovaries and testes of SR-BIΔCT ΔCT mice compared to WT mice (Fig. 3), the residual levels of receptor apparently were sufficient to maintain nearly normal cholesteryl ester stores. While clearly less than that in WT adrenal glands, those residual receptor levels presumably were adequate to maintain the stores under non-stressed conditions. Cai et al. and Hoekstra et al. have shown that adrenal insufficiency develops in stressed SR-BI KO mice (7, 23). Future studies will be required to assess the influence of stress on adrenal cortical cholesteryl ester stores and adrenal function in the SR-BIΔCT mice.

**Influence of the SR-BIΔCT mutation on atherosclerosis and coronary heart disease (CHD).** The apoE KO mouse is a standard model used to study aortic root and aortic atherosclerosis, but typically does not develop robust coronary arterial atherosclerosis or CHD during the first 4 months of life (44).

To assess the effects of the SR-BIΔCT mutation on atherosclerosis, CHD and cardiac physiology, we crossed the SR-BIΔCT mice with apoE KO mice to obtain two mouse populations with matching
The elevated hypercholesterolemia with markedly increased plasma UC:TC ratio in SR-BI\(\Delta\)CT/apoE KO mice raised the possibility that, as is the case with SR-BI/apoE dKO mice (5, 61), these mice might exhibit early onset atherosclerosis. Fig 7A shows that oil red O/hematoxylin staining of heart sections of the SR-BI\(\Delta\)CT/apoE KO mice exhibited substantial lipid-rich (oil red O-positive staining) aortic root atherosclerosis (9 weeks of age, panel b) prior to development of atherosclerosis in apoE KO mice (9 weeks of age, panel a). Furthermore, unlike the case with apoE KO mice, examination of heart sections from 9 week old SR-BI\(\Delta\)CT/apoE KO, stained with either oil red O/hematoxylin (Fig. 7A-d) or Masson’s Trichrome (Fig. 7A-h) exhibited partial or complete, lipid-rich atherosclerotic occlusions in coronary arteries. No coronary arterial atherosclerotic lesions were observed in apoE KO controls (Figs. 7A-c and 7A-g). As one would expect given the occlusive coronary arterial atherosclerosis, trichrome staining of myocardial sections from SR-BI\(\Delta\)CT/apoE KO mice (9 weeks of age) showed evidence of myocardial infarction (MI, fibrosis stained blue, normal myocardium red, Fig. 7A-f), while no fibrosis was observed in the corresponding apoE KO mice (Fig. 7A-e).

There were three pathological phenotypes in SR-BI\(\Delta\)CT/apoE KO mice not observed in apoE KO mice that were likely consequences of the occlusive coronary arterial atherosclerosis and MI. The first is cardiomegaly (Fig. 7B). The hearts in SR-BI\(\Delta\)CT/apoE KO mice (8.24±0.67 mg/g body weight) were abnormally large compared to those of apoE KO (5.21±0.14 mg/g body weight, p<0.0001). There was no statistical difference in the heart:body weight ratios of apoE KO, WT (5.40±0.26 mg/g body weight) and SR-BI\(\Delta\)CT (5.98±0.37 mg/g body weight) mice (p=0.08). Second, the SR-BI\(\Delta\)CT/apoE KO hearts exhibited severe cardiac dysfunction/heart failure. We performed hemodynamic studies using the left ventricular pressure volume loop method on two groups of mice that were analyzed separately because they had different genetic backgrounds: group 1: WT and SR-BI\(\Delta\)CT mice (mixed C57BL/6:129S2/SvCrl (68.75:31.25) genetic background); group 2: apoE KO and SR-BI\(\Delta\)CT/apoE KO mice (mixed C57BL/6:129S2/SvCrl (68.75:31.25) genetic background). Comparison of WT and SR-BI\(\Delta\)CT mice (group 1) established that there were no significant differences in maximum and minimum left ventricular volumes, cardiac output, ejection fraction, dP/dt and stroke work (Table 2). Thus, the SR-BI\(\Delta\)CT mutation alone did not alter these baseline characteristics of heart function. Compared to the control apoE KO hearts, the SR-BI\(\Delta\)CT/apoE KO exhibited significantly lower cardiac output (reduced to 71% of control), ejection fraction (58%) and stroke work (68%) and significantly higher maximum and minimum left ventricular volumes (143% and 183% of control, respectively) (Table 3). The SR-BI\(\Delta\)CT/apoE KO mice also exhibited significantly lower +dP/dt (60% of control) and –dP/dt (66% of control), suggesting both systolic and diastolic dysfunction (Table 3). The ejection fractions determined by PV loop measurement were highly reproducible; however, they were somewhat lower than previously reported for mice (10, 45) . We believe that the lower values are probably a consequence of our using in the present study significantly younger mice (7 weeks old) that those used previously (typically 14-16 weeks old). We analyzed cardiac function in very young mice because the SR-BI\(\Delta\)CT/apoE KO died prematurely.
Based on well-established principles of PDZ domain binding to target peptides (55) and previous studies of mutations in SR-BI, the \textsuperscript{507}AKL text\textsuperscript{509} deletion in SR-BI\textsubscript{ΔCT} will abrogate all receptor activity dependent on C-terminus binding to PDZ domains in PDZK1, but will not alter the surface expression or intrinsic lipid transport activities of SR-BI. The PDZK1-dependent activities include normal SR-BI protein expression in the liver (33), HDL-mediated regulation of endothelial cell physiology (endothelial NO synthase activity, cell migration, reendothelialization following injury (84)) and hepatitis C virus infectivity (8). Intrinsic lipid transport activities include selective lipid (e.g., cholesteryl ester) uptake (1, 42, 56), cellular efflux of unesterified cholesterol (28) and altered accessibility of plasma membrane cholesterol (56). For example, deletion of SR-BI’s C-terminal L\textsuperscript{509} abrogates its binding to PDZK1 and consequent regulation of eNOS activity via a PDZK1-dependent signaling pathway in endothelial cells (2), but does not impair HDL binding and selective lipid uptake in cultured cells (54). Replacement of
either the cytoplasmic C-terminal 45 (or 42) residues of SR-BI with the unrelated cytoplasmic C-terminal 6 (or 14) residues of CD36, another class B scavenger receptor, does not alter HDL binding or lipid transport (11, 12, 19). Indeed, truncation of SR-BI’s C-terminal 42 residues without replacement also does not alter HDL binding or lipid transport (11).

In the current study we found that the C-terminus of SR-BI is required for maintaining normal receptor levels not only in the liver (<5% of normal in SR-BIΔCT mice) but also in the adrenal gland, ovary and testes (e.g., ~84% and ~64% reductions in adrenal gland and ovary, respectively). It is possible that metabolic regulation (52), e.g., cholesterol-stores-mediated suppression of receptor expression (57, 66), may have contributed to reduced receptor protein levels in the steroidogenic cells in SR-BIΔCT mice. However, most of the metabolic (cholesterol stores) and hormonal (ACTH) regulation of SR-BI in steroidogenic cells is thought to occur by controlling mRNA levels (e.g., transcriptional control) (52, 57, 66) and there was only a very small change in receptor mRNA in the SR-BIΔCT mice compared to WT controls. Thus, we conclude that, as is the case in the liver, the reduction in receptor protein levels in SR-BIΔCT mice is primarily a posttranslational process. We propose that, as is the case in the liver with the adaptor protein PDZK1, it is likely that there is an adaptor(s) in steroidogenic cells that is distinct from PDZK1, possibly contains one or more PDZ domains, recognizes SR-BI’s C-terminus and mediates stable SR-BI protein expression. The residual levels of receptor protein in the adrenal glands, ovaries and testes in SR-BIΔCT mice were apparently sufficient to maintain nearly normal cholesteryl ester stores in these chow diet-fed mice under standard housing conditions. In contrast, in SR-BI KO mice that express no SR-BI protein, there is a striking reduction in the cholesteryl ester stores in the adrenal glands (24, 49, 50) and ovarian corpora lutea (61).

SR-BI is expressed in many tissues at varying levels and our results with steroidogenic cells raise the possibility that adaptors that recognize its C-terminus may play roles in other nonhepatic and nonsteroidogenic cells as well. For example, in intestines, there is a partial dependence of SR-BI expression on PDZK1 (33) – it is possible that an additional C-terminal adaptor(s) together with PDZK1 in intestines participates in mediating normal levels of SR-BI expression. Future analyses of receptor expression in other tissues of SR-BIΔCT mice will help address this issue. There is precedent for the activities of a lipoprotein receptor depending on different cytoplasmic adaptors in different types of cells. LDL receptors use at least two different adaptors for clathrin-mediated endocytosis, ARH and Dab2. Both adaptors bind to the receptor’s NPXY internalization motif and to phospholipids (18). ARH is required for normal LDL receptor-mediated endocytosis in hepatocytes and lymphocytes, but is not essential for endocytosis in fibroblasts (18, 21, 86). Apparently both ARH and Dab2 can mediate LDL receptor endocytosis in fibroblasts, with endocytosis dramatically reduced in fibroblasts lacking ARH when Dab2 expression is additionally suppressed by siRNA (15, 78). The identity of the putative adaptor(s) for SR-BI in steroidogenic cells is unknown. Azhar and colleagues have reported that two PDZ domain containing proteins, NHERF1 and NHERF2 have the ability to interact with SR-BI, apparently even when its C-terminus is blocked by an epitope tag, and might be involved with the negative regulation of SR-BI in steroidogenic cells (26, 52). As the putative adaptor proposed here is expected to positively regulate SR-BI (i.e., reduced SR-BI when the interaction is blocked by the C-terminal deletion), neither NHERF1 nor NHERF2 is likely to be the putative adaptor proposed here. Future studies will be required to identify the putative adaptor for SR-BI in steroidogenic cells.

In addition to examining receptor protein levels in the livers and steroidogenic tissues in SR-BIΔCT mice, we observed a number of abnormal phenotypes of SR-BIΔCT mice that were intermediate between those of PDZK1 KO mice and SR-BI KO mice. As the SR-BIΔCT, PDZK1 KO and SR-BI KO mice have different genetic backgrounds (see Materials and Methods), caution should be exercised when interpreting some of the phenotypic differences between these mutant mice. The PDZK1 KO mice exhibit limited, tissue specific reduction or loss of SR-BI activity (33), whereas SR-BI KO mice are completely SR-BI/SR-BII negative (50). SR-BI KO, SR-BIΔCT and PDZK1 KO mice are all hypercholesterolemic (2.2-, 2.1- and 1.7-fold plasma cholesterol levels above controls, respectively) with varying amounts of abnormally large HDL particles (32, 33, 50). The absence (SR-BI KO) or dramatic reduction (<5% in SR-
BIΔCT and PDZK1 KO mice) of the hepatic receptor accounts for these striking alterations in plasma
HDL (73). SR-BI KO and SR-BIΔCT mice, but not PDZK1 KO mice, have abnormally high ratios of
unesterified cholesterol:total cholesterol (UC:TC): 0.515, ~0.375 and 0.25, respectively. We do not
understand the mechanism underlying the abnormally high UC:TC ratios in SR-BI KO and SR-BIΔCT
mice (reduced susceptibility of HDL to lecithin:cholesterol acyl transferase-mediated cholesterol
esterification may be involved (38, 60, 75)). However our results raise the possibility that reduced levels
of receptor activity in extrahepatic tissues in SR-BI KO and SR-BIΔCT mice that do not occur in PDZK1
KO mice may contribute to this phenotype.

The abnormally high UC:TC ratio in SR-BI KO mice (0.515) has been linked to a number of
abnormalities, including reticulocytosis (11.9%) (25), thrombocytopenia (13) and female infertility (41,
61, 72), all of which appear to arise, at least in part, because of abnormally high levels of unesterified
cholesterol accumulating in the membranes of red blood cells, platelets and eggs, respectively. These
abnormalities, which are not present in PDZK1 KO mice, were not observed in SR-BIΔCT mice. It seems
likely that the relatively modest increase in UC:TC ratio in standard chow-fed SR-BIΔCT mice (~0.375)
compared to WT and PDZK1 KO mice did not result in accumulation of grossly pathogenic levels of
unesterified cholesterol in susceptible cells. Subjecting the SR-BIΔCT mice to additional stress (e.g.,
additional genetic abnormalities (see below) or possibly an atherogenic diet) might increase the UC:TC
ratio and induce associated pathology.

An additional group of intermediate, pathological phenotypes in SR-BIΔCT mice - relative to SR-
BI KO and PDZK1 KO mice – were observed when the mice were crossed with apoE KO mice, a
standard model for aortic (but not coronary arterial) atherosclerosis (81). ApoE KO mice, which are
hypercholesterolemic, but have an essentially normal UC:TC ratio (~0.29), do not exhibit anemia,
reticulocytosis, thrombocytopenia or female infertility. In SR-BI/apoE double KO (dKO) mice fed a
standard, low fat, chow diet, the total cholesterol is 2.2-fold higher than in apoE KO mice and 4.6-fold
higher than in SR-BI KO mice, and the UC:TC ratio is dramatically elevated to 0.81 (5, 61). The SR-
BI/apoE dKO mice exhibit anemia (hematocrit ~66% of control), severe reticulocytosis (100%), marked
thrombocytopenia (11.9% of control) and splenomegaly (unpublished). Analyses by Fuller et al. (17) of
atherogenic diet-fed SR-BI/LDLR dKO mice (UC:TC ratio as great as 0.81) are generally consistent with
the studies on chow-fed SR-BI/apoE dKO mice; namely, loss of SR-BI can result in anemia, enlarged
RBCs and splenomegaly; although statistically significant thrombocytopenia was not observed in SR-
BI/LDLR dKO mice (also see (40)). SR-BI/apoE dKO mice fed a standard chow diet rapidly develop
severe occlusive coronary arterial atherosclerosis, myocardial infarction (MI), heart dysfunction and
premature death (death between 5-8 weeks of age, median age of death is 6 weeks) (4, 61). PDZK1/apoE
dKO mice exhibit far less severe atherosclerosis-related phenotypes. When PDZK1/apoE dKO mice are
fed a moderately atherogenic (‘Western’) diet for three months, they develop more aortic root
atherosclerosis than apoE KO controls, but no occlusive coronary arterial atherosclerosis, MI or very
early death (34). When the PDZK1/apoE dKO mice are fed a more severe atherogenic (Paigen-or HFC-
15.8% fat, 1.25% cholesterol, 0.5% sodium cholate) diet for three months, they not only exhibit more
aortic root atherosclerosis than the apoE KO, but also develop some occlusive coronary arterial
atherosclerosis and cardiac fibrosis, but do not exhibit premature death (71).

In SR-BIΔCT/apoE KO mice fed a standard chow diet, the total cholesterol was 1.6-fold higher
than in apoE KO controls and ~5.7-fold higher than in SR-BIΔCT mice, and the UC:TC ratio was 0.83.
The SR-BIΔCT/apoE KO mice exhibited severe macrocytic anemia (hematocrit ~66% of control) with
abnormal red blood cell morphology, resulting in hepatic and splenic extramedullary hematopoiesis,
massive splenomegaly, and marked reticulocytosis (31%), as well as thrombocytopenia (57% of control).
In addition, SR-BIΔCT/apoE KO mice rapidly developed extensive aortic root and severe occlusive
coronary arterial atherosclerosis, MI, heart dysfunction and failure (assessed by PV loop method) and
premature death (median age of death was 9 weeks, 65% died between 8.4 and 9.9 weeks of age). As
noted above, our results raise the possibility that reduced levels of receptor activity in extrahepatic tissues
in SR-BI/apoE dKO and SR-BIΔCT/apoE KO mice that do not occur in PDZK1/apoE dKO mice may have contributed to severe, lethal coronary heart disease.

The SR-BIΔCT/apoE KO mice provide a new addition to the limited collection of mouse models of atherosclerotic coronary heart disease (CHD), e.g., see (4, 80). Some of these models involve administration of an atherogenic diet and others do not. In addition to SR-BIΔCT/apoE KO, SR-BI KO/apoE ΔKO, SR-BI KO/LDLR ΔKO (17) and PDZK1 KO/apoE ΔKO mice (71), there is a fifth SR-BI-related atherosclerotic CHD mouse model, HypoE mice (SR-BI KO/ApoeR61Δh/h) (80). In addition to homozygous null mutations in the SR-BI gene, HypoE mice have a severe, but not absolute, deficiency of apoE due to a modification of the apoE gene (ApoeR61Δh/h). ApoeR61Δh/h mice (47) express a mutant murine apoE (Thr61→Arg61) at substantially lower plasma concentrations (2% to 5%) than apoE in control WT mice. When HypoE mice are fed an atherogenic diet (e.g., Pagin/HFC), but not a standard chow diet, they develop atherosclerotic CHD, MI, heart dysfunction and die prematurely (50% mortality ~40 days after initiation of a Paigen diet). The rate of disease progression is environmentally titratable (e.g., severity of the atherogenic diet, substitution of chow diet after short exposure to atherogenic diet, social isolation) (43). CHD progression in SR-BI KO/LDLR ΔKO is also titratable in that it is proportional to the severity of the atherogenic diet (17). It seems likely that a modified HypoE mouse, namely SR-BIΔCT/ApoeR61Δh/h, in which SR-BIΔCT replaces the SR-BI KO, likely would also be an environmentally titratable atherosclerotic CHD model. Because of the fertility of SR-BIΔCT females, SR-BIΔCT/ApoeR61Δh/h mice likely would be an easier and less expensive CHD model than SR-BIΔCT/apoE KO, SR-BI KO/apoE ΔKO, SR-BI/ΔLDLR ΔKO and HypoE mice.

In conclusion, the new SR-BIΔCT mouse is likely to be a powerful mouse model which will provide a better understanding of steroid hormone production and the mode of regulation of SR-BI in steroidogenic organs. It also provides the research community with a new convenient mouse model, the SR-BIΔCT/apoE KO mouse, which closely recapitulates the findings observed in human cardiovascular disease.

Footnotes:

Abbreviations: CHD, coronary heart disease; HDL, high density lipoprotein; LDLR, low-density lipoprotein receptor; SR-BI, scavenger receptor class B type I; ΔCT, deletion of the last three carboxy terminal amino acids; Ala, alanine; AKL, alanine-lysine-leucine; WT, wild-type; KO, knockout; PDZ, PSD-95/Discs-large/ZO-1; FPLC, fast protein liquid chromatography; RBC, red blood cell; Ht, hematocrit, MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration

Acknowledgments:

This work was supported by grants from the National Institutes of Health: HL077780 (OK), and HL127174 (MK) and a Pre-Doctoral Training Grant T32GM007287 (LW). We thank Robert Farese, Jr. and Tobias Walther for generously making their facilities available and supporting Chandramohan Chitraju, and Laura Liscum for help in performing preliminary experiments.

Conflict of Interest: The authors declare no competing financial interest.

Author contributions:

OK and MK conceived the study. OK, MK and PMK designed experiments. OK, RP, QK, GAP, MLP, AY, CC and LW performed experiments. OK and MK wrote the paper. All authors analyzed the results and approved the final version of the manuscript.
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**Figure legends**

Figure 1. Generation of Knock-in mutation into exon 12 of the SR-BI (Scarb1) gene encoding the truncated receptor SR-BIΔCT (A) and genotyping of SR-BIΔCT mice by PCR analysis (B). A. Top - The organization of a portion of the wild-type (WT) SR-BI (Scarb1) allele showing the corresponding positions in the targeting vector of the long arm (LA, red line), including exons 10-12, the middle arm (MA, green) and the short arm (SA, blue), as well as the sites for the NDE1 and NDE2 primers used for genotyping. **Middle** – The initially targeted allele including the 507Ala/STOP mutation (*) and the Neo cassette with FRT sites (F). **Bottom** - organization of a portion of the final, Neo cassette deleted, SR-BIΔCT mutant allele. B. PCR analysis using the NDE1 and NDE2 primers of genomic DNA from wild-type (WT), heterozygous Knock-in (HT) and homozygous Knock-in (ΔCT) mutant mice. The WT allele generates a 427 base pair (bp) band while the SR-BIΔCT mutant allele generates a 515 bp band.

Figure 2. Effects of the SR-BIΔCT mutation on the hepatic expression of SR-BI and SR-BII proteins. A-B. Immunoblotting analyses of liver lysates (~30 µg of protein) from male WT and SR-BIΔCT (ΔCT) six to ten week old mice. Results using female mice were virtually identical. Bands were visualized by chemiluminescence. A. Proteins were detected using either a SR-BI-specific anti-C-terminal peptide antibody (SR-BI (anti-C-term)), a rabbit polyclonal antibody that recognizes the extracellular domains of both SR-BI and its minor splice isoform SR-BII (SR-BI/SR-BII (KKB-1)), ~82 kDa, or polyclonal rabbit anti-ε-COP (~34 kDa) that was used as a loading control. B. Immunoblotting analysis of the expression of SR-BII using a rabbit polyclonal, SR-BI-specific anti-C-terminal peptide antibody (SR-BII (anti-C-term)) and anti-ε-COP. C. Quantitative analyses of immunoblots was used to determine the relative expression of SR-BI/SR-BII(KKB-1) and SR-BII in the livers of WT and SR-BIΔCT (ΔCT) mice. *, value significantly different (p<0.0001 for SR-BI/SR-BII(KKB-1) and p=0.001 for SR-BII). D. Livers from male WT and SR-BIΔCT mice were fixed, frozen, sectioned and the sections stained with the polyclonal anti-SR-BI/SR-BII KKB-1 antibody and a biotinylated anti-rabbit IgG secondary antibody, and visualized by immunoperoxidase staining. (Magnification, X600; bar: 50 µm).

Figure 3. Effects of the SR-BIΔCT mutation on the expression of SR-BI and SR-BII proteins in steroidogenic tissues. A. Immunoblotting analyses of adrenal gland and ovary lysates (~30 µg of protein) from WT and SR-BIΔCT six to ten week old mice. Bands were visualized by chemiluminescence, but using exposure times significantly shorter than those used in Fig 2A because of the higher protein expression in these steroidogenic tissues than in the liver. Proteins were detected using an SR-BI-specific (SR-BI (anti-C-term)) antibody, an anti-SR-BI/SR-BII (KKB-1) antibody or an SR-BI-specific (SR-BII (anti-C-term)) antibody, as described in Fig 2. An anti-ε-COP antibody was used as a loading control. B. Relative expression of SR-BI/SR-BII(KKB-1) and SR-BII in WT and SR-BIΔCT (ΔCT) mouse adrenal gland and ovary. *, value significantly different (p<0.0001 for SR-BI/SR-BII(KKB-1) and p=0.001 for SR-BII). C. Adrenal glands, ovaries and testes from WT and SR-BIΔCT mice were fixed, frozen, sectioned and the sections stained with the polyclonal anti-SR-BI/SR-BII KKB-1 antibody and a biotinylated anti-rabbit IgG secondary antibody, and visualized by immunoperoxidase staining. Results for male and female mouse adrenal glands were identical (Magnification, X300; Bar: 100 µm).

Figure 4. Effects of the SR-BIΔCT (ΔCT) mutation on plasma total (TC), unesterified cholesterol (UC) levels and the UC:TC ratio (A) and on plasma lipoprotein size distribution profiles (cholesterol and apolipoproteins) (B). Plasma samples were harvested from male (M) and female (F) WT and SR-BIΔCT (ΔCT) six to ten week old mice. A. Plasma total (a) and unesterified (b) cholesterol levels and the UT:TC ratio (c) were determined in individual samples by enzymatic assay, and mean values (+/- standard error) from the indicated numbers of animals (n) are shown. *, value significantly different (p<0.0001) between ΔCT mice and the corresponding WT controls of the same sex. B. Pooled
plasma samples from three males of each genotype were size-fractionated by FPLC, and the total cholesterol content of each fraction was determined by an enzymatic assay (upper panel). The chromatograms (WT: squares; SR-BIΔCT: triangles) are representative of multiple individually determined profiles. Approximate elution positions of native VLDL, IDL/LDL and HDL particles are indicated by brackets and were determined as previously described (50). The FPLC fractions were analyzed by immunoblotting (lower panels) to determine the distribution of the apolipoproteins apoA-I and apoE.

Figure 5. Red blood cell morphology (A) and Oil Red O (neutral lipid) staining of steroidogenic tissues (B) in WT and SR-BIΔCT mice. A. Blood samples from six to ten week old WT (a,c) and SR-BIΔCT (b,d) mice were stained with Wright-Giemsa and visualized using standard light microscopy (a,b) or Differential Interference Contrast (DIC) optics (c,d). (Magnification, X1000; bar: 20 µm). B. Adrenal, ovarian and testicular tissues from WT and SR-BIΔCT mice were frozen and frozen sections (5 µm) were stained with Oil Red O/hematoxylin. Neutral lipids (e.g., cholesteryl esters) stain red. Representative sections are shown and results for male and female mouse adrenal glands were essentially identical. (Magnification, x25 adrenal gland, x50 ovary, and x250 testis; bars: 0.5 mm, 0.25 mm and 125 µm)

Figure 6. Effects of the SR-BIΔCT (ΔCT) mutation in apoE KO mice on plasma total (TC) and unesterified cholesterol (UC) levels and the UC:TC ratio (A) and on the plasma lipoprotein cholesterol size distribution profile (B). A. Plasma samples were harvested from apoE KO (black) and SR-BIΔCT/apoE KO (gray) six to eight week old mice. No significant differences were observed between males and females. Total plasma and unesterified cholesterol levels were determined in individual samples by enzymatic assay, and mean values (+/- standard error) from the indicated numbers of animals (n) are shown for each genotype. * p<0.0001, for SR-BIΔCT/apoE KO compared to apoE KO mice. B. Pooled plasma samples from three male mice were size-fractionated by FPLC, and the total cholesterol content of each fraction was determined by an enzymatic assay. The shapes of the chromatograms (apoE: diamonds; SR-BIΔCT/apoE KO: squares) are representative of multiple individually determined profiles. Approximate elution positions of native VLDL, IDL/LDL and HDL particles are indicated by brackets and were determined as previously described (50).

Figure 7. Effects of the SR-BIΔCT mutation in chow-diet-fed apoE KO mice on aortic root (A, a-b) and coronary atherosclerosis (A, c-d and g-h), cardiac fibrosis (A, e-f), heart-to-body weight ratio (B) and survival (C). Hearts were harvested from six to eight week old standard chow diet-fed apoE KO (A a,c,e,g) and SR-BIΔCT/apoE KO (A, b,d,f,h) mice as described in Methods and representative images are shown. a-d: Oil red O-stained aortic root (a,b) and coronary artery (c,d) lesions (magnifications, X20 and X100). e-f: Masson’s trichrome stained cross section of myocardium at low magnification (e,f, magnification, X10) and higher magnification (g,h, magnification, X100). Fibrotic tissue is stained blue. A patent coronary arteriole is seen in g (apoE KO), whereas a totally occluded arteriole is seen in h (SR-BIΔCT/apoE KO). B. Heart-to-body weight ratios of are expressed as mg of heart weight/g of body weight. * p<0.0001, for SR-BIΔCT/apoE KO compared to apoE KO hearts (p<0.0001). p=0.24, for WT compared to SR-BIΔCT hearts. C. Kaplin-Meier survival curves for chow-fed apoE KO (n= 16, red line) and SR-BIΔCT/apoE KO (n= 22, black line, median age of death of 63 days) mice.

Figure 8. Morphological evaluation of red blood cells (A), liver (B) and spleen (B and C) from WT, SR-BIΔCT, apoE KO and SR-BIΔCT/apoE KO mice. A. Blood samples from apoE KO (a,c) and SR-BIΔCT/apoE KO (b,d) six to eight week old mice were stained with Wright-Giemsa and visualized using standard light microscopy (a,b) or Differential Interference Contrast (DIC) optics (c,d). (bar = 20 µm). B. Livers (a,c,e,g) and spleens (b,d,f,h) from 6-8 week old mice of the indicated genotypes were embedded in paraffin, sectioned and stained with hematoxylin and eosin (bar = 100 µm). Extramedullary hematopoiesis is observed in panels g and h (arrows). C. Representative photographs of spleens from mice of the indicated genotypes. Spleen-to-body weight ratios from mice of the indicated genotypes (n=4-
12) are expressed as mg of spleen weight/g of body weight. *p<0.0001, for SR-BI ΔCT/apoE KO compared to apoE KO spleens; p=0.27, for WT compared to SR-BI ΔCT spleens.
Table 1. Hematological Data for Wild-type and SR-BIΔCT mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Hematocrit, %</th>
<th>MCV, fL</th>
<th>Reticulocytes %</th>
<th>MCH concentration, g/dL</th>
<th>MCH, pg</th>
<th>Platelets, x 10⁶/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (n=5)</td>
<td>45.8 ± 2.3</td>
<td>55.9 ± 2.4</td>
<td>2.7 ± 0.3</td>
<td>27.3 ± 1.4</td>
<td>15.8 ± 0.1</td>
<td>687.8 ± 44.1</td>
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<tr>
<td>SR-BIΔCT (n=6)</td>
<td>55.9 ± 3.2</td>
<td>67.8 ± 1.0</td>
<td>2.8 ± 0.5</td>
<td>24.8 ± 0.2</td>
<td>16.8 ± 0.2</td>
<td>608.4 ± 51.0</td>
</tr>
</tbody>
</table>

p value p=0.04 p=0.001 p>0.05 p>0.05 p=0.005 p>0.05

Table 2. Left Ventricular pressure volume loop measurements for Wild-type and SR-BIΔCT mice

<table>
<thead>
<tr>
<th></th>
<th>Maximum Left Ventricular Volume (μL)</th>
<th>Minimum Left Ventricular Volume (μL)</th>
<th>Cardiac output (µL/min)</th>
<th>Ejection Fraction (%)</th>
<th>Stroke Work (mm Hg*µL)</th>
<th>dP/dt MAX (mmHg/Sec)</th>
<th>dP/dt MIN (mmHg/Sec)</th>
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<tr>
<td>Wild-type (n=7)</td>
<td>45.9 ± 2.4</td>
<td>27.2 ± 1.5</td>
<td>5.9 ± 0.3</td>
<td>31.7 ± 1.3</td>
<td>1703 ± 96</td>
<td>8438 ± 653</td>
<td>-7661 ± 630</td>
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<tr>
<td>SR-BIΔCT (n=6)</td>
<td>45.3 ± 1.4</td>
<td>27.3 ± 0.8</td>
<td>5.4 ± 0.3</td>
<td>28.8 ± 1.5</td>
<td>1718 ± 116</td>
<td>8638 ± 645</td>
<td>-8199 ± 593</td>
</tr>
</tbody>
</table>

p value p>0.05 p>0.05 p>0.05 p>0.05 p>0.05 p>0.05
### Table 3. Left Ventricular pressure volume loop measurements for apoE KO and SR-BIΔCT/apoE KO mice

<table>
<thead>
<tr>
<th></th>
<th>Maximum Left Ventricular Volume (μL)</th>
<th>Minimum Left Ventricular Volume (μL)</th>
<th>Cardiac output (μL/min)</th>
<th>Ejection Fraction (%)</th>
<th>Stroke Work (mm Hg*μL)</th>
<th>+dP/dt (mmHg/Sec)</th>
<th>-dP/dt (mmHg/Sec)</th>
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<tbody>
<tr>
<td>apoE KO (n=6)</td>
<td>41.5 ± 1.9</td>
<td>22.3 ± 1.1</td>
<td>6.6 ± 0.5</td>
<td>36.5 ± 1.5</td>
<td>1947 ± 137</td>
<td>10737 ± 454</td>
<td>-8084 ± 400</td>
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<tr>
<td>SR-BIΔCT/apoE KO (n=8)</td>
<td>59.5 ± 6.8</td>
<td>40.9 ± 4.6</td>
<td>4.7 ± 0.3</td>
<td>21.0 ± 1.6</td>
<td>1331 ± 120</td>
<td>6404 ± 654</td>
<td>-5318 ± 574</td>
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<tr>
<td>p value</td>
<td>0.04</td>
<td>0.005</td>
<td>0.005</td>
<td>&lt;0.0001</td>
<td>0.006</td>
<td>0.0003</td>
<td>0.0032</td>
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### Table 4. Hematological Data for apoE KO and SR-BIΔCT/apoE KO mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Hematocrit, %</th>
<th>MCV, fL</th>
<th>Reticulocytes %</th>
<th>MCH concentration, g/dL</th>
<th>MCH, pg</th>
<th>Platelets, x 10⁶/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoE KO (n=6)</td>
<td>43.7 ± 2.2</td>
<td>49.6 ± 1.7</td>
<td>2.3 ± 0.8</td>
<td>31.9 ± 0.4</td>
<td>15.8 ± 0.4</td>
<td>777.5 ± 55.8</td>
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<tr>
<td>SR-BIΔCT/apoE KO (n=6)</td>
<td>29.0 ± 2.4</td>
<td>86.4 ± 3.2</td>
<td>31.0 ± 6.3</td>
<td>27.2 ± 0.7</td>
<td>23.5 ± 0.6</td>
<td>444.7 ± 31.0</td>
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<tr>
<td>p value</td>
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<td>p&lt;0.0001</td>
<td>p=0.001</td>
<td>p=0.0003</td>
<td>p&lt;0.0001</td>
<td>p=0.0004</td>
</tr>
</tbody>
</table>
A. Mutagenesis Strategy

B. PCR Genotype Analysis

Figure 1
Figure 2
Figure 3
A. Plasma Total and Unesterified Cholesterol

a. WT & ΔCT

b. WT & ΔCT

c. WT & ΔCT

B. Plasma Lipoprotein Cholesterol Profiles

VLDL, IDL/LDL, HDL

Total Cholesterol (mg/dL)

Fraction number

17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47

apoA-I

apoE

Wild-type

SR-BIΔCT

Figure 4
<table>
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<tr>
<th></th>
<th>WT</th>
<th>SR-BiACT</th>
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<td>Adrenal</td>
<td><img src="image5" alt="Image of Adrenal WT" /></td>
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<td>Ovary</td>
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<td><img src="image8" alt="Image of Ovary SR-BiACT" /></td>
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<tr>
<td>Testis</td>
<td><img src="image9" alt="Image of Testis WT" /></td>
<td><img src="image10" alt="Image of Testis SR-BiACT" /></td>
</tr>
</tbody>
</table>

Figure 5
A. Plasma Total and Unesterified Cholesterol

\[ \text{Plasma Total Cholesterol (mg/dL)} \]

- **a.**
  - \[n = 6\] [apoE KO]
  - \[n = 8\] [SR-BI ΔCT/apoE KO]

\[ \text{Plasma Unesterified Cholesterol Ratio} \]

- **b.**
  - \[n = 6\] [apoE KO]
  - \[n = 8\] [SR-BI ΔCT/apoE KO]

B. Plasma Lipoprotein Cholesterol Profiles

- **VLDL**
- **IDL/IDL**
- **HDL**

\[ \text{Total Cholesterol (mg/dL)} \]

- **apoE KO**
- **SR-BI ΔCT/apoE KO**

Figure 6
Figure 7
Figure 8