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Influence of PDZK1 on Lipoprotein Metabolism and Atherosclerosis

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3 figures 1 table

Abstract

PDZK1 is a scaffold protein containing four PDZ protein interaction domains, which bind to the carboxy termini of a number of membrane transporter proteins, including ion channels (e.g., CFTR) and cell surface receptors. One of these, the HDL receptor, scavenger receptor class B type I (SR-BI), exhibits a striking, tissue-specific dependence on PDZK1 for its expression and activity. In PDZK1 knockout (KO) mice there is a marked reduction of SR-BI protein expression (~95%) in the liver, but not in steroidogenic tissues or, as we show in this report, in bone marrow- or spleen-derived macrophages, or lung-derived endothelial cells. Because of hepatic SR-BI deficiency, PDZK1 KO mice exhibit dyslipidemia characterized by elevated plasma cholesterol carried in abnormally large HDL particles. Here, we show that inactivation of the PDZK1 gene promotes the development of aortic root atherosclerosis in apolipoprotein E (apoE) KO mice fed with a high fat/high cholesterol diet. However, unlike complete SR-BI-deficiency in SR-BI/apoE double KO mice, PDZK1 deficiency in PDZK1/apoE double knockout mice did not result in development of occlusive coronary artery disease or myocardial infarction, presumably because of their residual expression of SR-BI. These findings demonstrate that deficiency of an adaptor protein essential for normal expression of a lipoprotein receptor promotes atherosclerosis in a murine model. They also define PDZK1 as a member of the family of proteins that are instrumental in preventing cardiovascular disease by maintaining normal lipoprotein metabolism.

1. Introduction

Hypercholesterolemia causes cardiovascular disease (e.g. myocardial infarction), by promoting atherosclerosis. A number of defects in human genes encoding apoB- the apolipoprotein component of low density lipoprotein (LDL), the LDL receptor, the LDL receptor adaptor ARH (autosomal recessive hypercholesterolemia) or processing enzyme PCSK9 all lead to hypercholesterolemia and atherosclerosis [1-5]. The role of dyslipidemia in promoting atherosclerosis is commonly studied using knockout (KO) mice, in which genes regulating plasma lipoprotein metabolism have been inactivated [6]. Such models include the very widely used apolipoprotein E (apoE) or low-density lipoprotein (LDL) receptor KO mice that exhibit robust aortic atherosclerosis, but little or no occlusive coronary arterial atherosclerosis, and thus usually lack other classic symptoms of coronary heart disease (CHD).

Reverse cholesterol transport, an HDL-mediated process promoting transfer of cholesterol from peripheral tissues to the liver, is thought to play a major role in protecting against the development of atherosclerosis [7]. In mice, this appears to depend, at least in part, on expression of the HDL receptor SR-BI. SR-BI is expressed in hepatocytes, where it mediates selective uptake of cholesterol from HDL and biliary secretion of cholesterol [8-10], and in steroidogenic cells where cholesterol is stored and used for the synthesis of steroid hormones [8, 11]. In the liver, normal expression of SR-BI protein is controlled by its adaptor protein PDZK1 [12-14]. PDZK1 is a protein containing four PDZ protein interaction domains [15]. The most N-terminal of these domains, PDZ1, binds to the carboxy terminus of SR-BI [13, 14]. The carboxy terminus

of SR-BII, a minor splice variant of SR-BI, differs from that of SR-BI and does not interact with PDZK1 [12, 16, 17]. In vivo, murine PDZK1 acts as a tissue-specific, posttranscriptional regulator of SR-BI [12]. In PDZK1 KO mice, there is a dramatic (~95%) reduction in hepatic SR-BI protein expression, which results in a 1.7 fold elevation of plasma cholesterol levels carried in abnormally large HDL particles. The abnormal lipoprotein metabolism in PDZK1 KO mice is similar to, but not as severe as, that observed in homozygous null SR-BI KO mice (e.g., 2.2-fold increase in total plasma cholesterol in even larger, unesterified cholesterol-rich HDL particles) [12, 18-20]. However, there are several marked differences between SR-BI and PDZK1 KO mice: PDZK1 KO mice exhibit essentially normal levels of SR-BI expression in steroidogenic organs, untreated female PDZK1 KO mice are fertile (unlike SR-BI KO mice), and the ratio of plasma unesterified-to-esterified cholesterol in PDZK1 KO mice is not elevated [12].

Numerous studies have established that SR-BI protects against atherosclerosis in the mouse [10, 21, 22]. For example, hepatic overexpression of SR-BI suppresses atherogenesis in LDL receptor KO mice [23, 24], and the combined inactivation of the apoE and SR-BI genes (SR-BI/apoE double KO (dKO)) in mice results in dramatically enhanced aortic root atherogenesis compared to the apoE KO mouse [10, 21]. It appears that SR-BI expression in both hepatocytes and bone marrow derived cells is atheroprotective [22, 23, 25-27]. Strikingly, the SR-BI/apoE dKO mice exhibit CHD-like cardiac phenotypes, not normally seen in apoE KO or other murine models of atherosclerosis [10]. These include occlusive coronary arterial atherosclerosis,

myocardial infarction, heart dysfunction (e.g., cardiomegaly, ECG abnormalities) and premature death (~6 weeks of age) [10, 18, 21].

In the current study, we have shown that complete PDZK1 deficiency did not affect SR-BI expression in two types of extrahepatic cells important for development of atherosclerosis, macrophages and endothelial cells. Furthermore, we examined the effects of PDZK1 deficiency on atherosclerosis in apoE KO mice using PDZK1/apoE dKO mice fed a high fat/high cholesterol (“Western”) atherogenic diet. Our data show that PDZK1 is atheroprotective: inactivation of the PDZK1 gene increased aortic root atherosclerosis. However, unlike complete SR-BI-deficiency in SR-BI/apoE dKO mice, PDZK1 deficiency in apoE dKO mice did not result in development of occlusive coronary artery disease, myocardial infarction or premature death.

2. Materials and methods

2.1 Animals. Animals used for flow cytometric analyses of cell surface SR-BI expression and activity were either on a 50% C57BL/6/50% 129-S4 background (SR-BI KO and matching control wild-type animals [19]) or on a 129 SvEv background (PDZK1 KO and its matching wild-type controls [28]). All animals used for flow cytometric analyses were fed a standard chow diet. ApoE deficient mice (C57BL/6 background) were purchased from Jackson Laboratories (Bar Harbor, ME), mated with PDZK1 KO mice (129 SvEv background). The initial PDZK1/apoE dKO and apoE KO animals from this cross (50:50 C57BL/6:129SvEv background) were used for a pilot atherosclerosis study. Subsequently, we backcrossed these mice for 6 generations into the C57BL/6 background to generate the PDZK1/apoE double knockout (dKO) and apoE single KO mice (~98.5% C57BL/6/1.5% 129 SvEv background) used to collect the data shown in Table 1, Figures 2 and 3 and described in detail in Results and Discussion. Genotypes were determined by PCR using established protocols ([19, 28] and Jackson Laboratories web site). For analyses of lipoproteins and atherosclerosis, approximately 4-week-old animals were fed a “Western diet” from Harlan Teklad (Madison, WI) containing 42% [wt/wt] fat and 0.15% [wt/wt] cholesterol for 3 months.

2.2 Flow Cytometric Analysis. *Measurement of cell surface SR-BI levels:* Marrow from femur bones was collected in Hank’s balanced salt solution (HBSS). Spleens and lungs were ground through a cell strainer in HBSS. Ground lung tissues were further processed by incubation with 2 ml of digestion buffer (HBSS containing 3 mg/ml of collagenase A (Roche, Mannheim, Germany) and 0.5% fatty acid-free BSA) at 37°C for 1 h. Numbers

of total live cells from the lung and spleen were counted by trypan blue exclusion after lysis of red blood cells. Cells (1×10^6) were stained with KKB-1 anti-SR-BI rabbit antiserum³⁵ (a generous gift from Karen Kozarsky from GlaxoSmithKline), or with non-immune normal rabbit serum (Invitrogen, Carlsbad, CA) as a negative control, followed by staining with anti-rabbit IgG-PE (Sigma) in conjunction with cell type-specific, fluorochrome-conjugated antibodies. These cell type-specific antibodies are anti-Gr-1-FITC and anti-CD11b-APC antibodies for macrophages, anti-CD45-Alexa 488 and anti-CD31-APC antibodies for lung endothelial cells, anti-CD19-FITC antibodies for B cells, anti-TCR β -APC antibodies for T cells, and anti-DX5-FITC antibodies for NK cells (purchased from BD Biosciences, San Jose, CA and BioLegend, San Diego, CA). Samples were analyzed on a FACSCaliburTM flow cytometer (BD Biosciences) with FlowJo software (Tree Star Inc., Ashland, OR). Analyses were gated on CD11b positive and Gr-1 medium cells for macrophages and on CD31 high and CD45 negative cells for lung endothelial cells. The relatively high, SR-BI-independent levels of fluorescence detected in macrophages and endothelial cells were due to using flow cytometer setting optimized for detection of the relatively low SR-BI levels and to high intrinsic autofluorescence exhibited by these cell types.

Measurement of DiI-HDL uptake: Cells (1×10^6) from bone marrow and spleens were washed twice with RPMI 1640, followed by incubation with 100 μ l of DiI-HDL (5 μ g protein/ml [8]) containing medium (RPMI 1640 with 0.5% fatty acid-free BSA) at 37°C for 5 h. Antibody staining and flow cytometric analysis were performed as described above except the SR-BI antiserum and anti-rabbit IgG antibody were omitted.

2.3 Morphologic and Biochemical Analyses. Mice were anesthetized. Blood was obtained by cardiac puncture using heparinized syringes and centrifuged to obtain plasma. Hearts were excised after a short *in vivo* perfusion with PBS and frozen in OCT compound. Frozen sections (10 μ m) were stained with Oil Red O as previously described [10]. Atherosclerotic lesions were measured by planimetry as the sum of the cross-sectional areas using image measure/SPOT software (Diagnostics Instruments, Sterling Heights, MI) in the aortic root as previously described [10]. Immunoblots were performed as previously described [19]. Briefly, total liver samples (40-50 μ g of protein/sample) were size-fractionated by 10% SDS-PAGE and immunoblotted on nitrocellulose membranes with either polyclonal anti-peptide antibodies for SR-BI [8] or for actin (used as protein loading control, Sigma, St Louis, MO). Antibody binding to protein samples was visualized by enhanced chemiluminescence using Super Signal West Pico Luminal reagents (Pierce, Rockford, IL). To semi-quantitatively determine the relative amounts of SR-BI, tissue lysates were serially diluted, subjected to electrophoresis/immunoblotting and the relative intensities of the signals were compared by visual inspection.

Cholesterol, unesterified cholesterol, phospholipids and triglycerides were measured using kits (Wako Chemical, Richmond, VA). FPLC size fractionation of plasma lipoproteins was performed as previously described [29], except that previously frozen plasma samples were used to determine the UC:TC ratios across FPLC fractions (Figure 2C) (n=4-5). A value of $P < 0.05$ between experimental groups was considered to represent a significant difference using a 2-tailed, unpaired Student's *t* test. Reported values represent mean \pm standard error of the mean.

3. Results

Because macrophages and endothelial cells have been implicated in atherogenesis [30], we compared SR-BI expression on the surfaces of these cells from wild-type and PDZK1 KO mice by flow cytometry using anti-SR-BI polyclonal antibody KKB-1 [31] and antibodies specific for each cell type as described in Methods. To do this, we first compared the intensity of staining with an anti-SR-BI antibody, or a control non-immune serum, of cells from wild-type mice and those isolated from SR-BI KO mice (negative controls) to determine if there were any detectable expression on a variety of types of cells, including NK cells and B- and T-lymphocytes isolated from spleen, monocytes/macrophages from both spleen and bone marrow and endothelial cells isolated from lung. Robust SR-BI expression was detected on macrophages and endothelial cells from wild-type, but not SR-BI KO, mice (Figure 1A and B, left panels), but little or none on B cells, T cells and NK cells (not shown). These results are consistent with previous reports of SR-BI expression in macrophages and endothelial cells [32, 33].

We next compared SR-BI surface expression on macrophage and endothelial cells isolated from wild-type and PDZK1 KO mice using either the specific anti-SR-BI antibody or control non-immune serum. The SR-BI-specific antibody staining intensities of cells from wild-type and PDZK1 KO mice were similar, whereas only a lower, background staining intensity was observed with the control non-immune serum, indicating that the level of SR-BI surface expression was not significantly altered by loss of PDZK1 expression (Figure 1, panels A and B, right panels). [Preliminary studies with dendritic cells gave results similar to those with macrophages.] When bone marrow-

derived (n=3) or splenic (n=2) macrophages were incubated in culture with HDL labeled with a fluorescent lipid, DiI, there was similar uptake of DiI by macrophages isolated from wild-type and PDZK1 KO mice, but significantly lower uptake by macrophages from SR-BI KO mice (not shown and Figure 1C). This result suggests that the SR-BI-dependent uptake of lipid from DiI-HDL was not impaired in macrophages from PDZK1 KO mice, and thus that the SR-BI expressed by PDZK1-negative macrophages exhibited apparently normal lipid transport activity. These data suggest that any effects of PDZK1 deficiency on atherosclerosis (see below) would be unlikely to be a consequence loss of SR-BI activity by macrophages.

To examine the effects of PDZK1 on lipoprotein metabolism and atherosclerosis in atherosclerosis-prone apoE KO mice, we crossed PDZK1 KO to apoE KO mice and subjected the PDZK1/apoE dKO mice and control apoE single KO mice to an atherogenic Western diet for 3 months. All of the mice remained healthy throughout the feeding period, and at the end of this period their body weights did not differ significantly (36.1 ± 1.1 g (n=9) versus 34.7 ± 1.2 g (n=8) for apoE KO and PDZK1/apoE dKO mice, respectively, $P=0.38$).

We previously reported that hepatic SR-BI protein levels are reduced by ~95% in PDZK1 KO mice relative to wild-type controls [12]. However the influence of PDZK1 deficiency on hepatic SR-BI in apoE KO mice fed an atherogenic Western diet was unknown. The immunoblot in Figure 2A shows that there was a dramatic reduction (~93%) in hepatic SR-BI protein levels by PDZK1 deficiency in apoE KO mice fed either a standard chow diet (left) or an atherogenic Western diet (right). In otherwise wild-type mice fed a standard chow diet, such a large PDZK1-dependent drop in hepatic SR-BI

levels results in an ~1.7-fold increase in total plasma cholesterol in abnormally large lipoprotein particles [12]. This was not the case for apoE KO mice fed the Western diet.

Figure 2B shows representative plasma lipoprotein total cholesterol profiles (FPLC size exclusion chromatography of plasma) of apoE single KO and PDZK1/apoE dKO mice fed with the Western diet. The plasma lipoprotein total cholesterol profiles were virtually identical. They were also similar to previously reported profiles of apoE KO mice fed with atherogenic diets [34, 35], with the largest peak of cholesterol in VLDL-size particles, a broad peak in the IDL/LDL-size range and very little in the HDL-size range. The abundance of the major apolipoprotein of HDL, apoA-I, from the normal-size pooled VLDL, IDL/LDL and HDL fractions (fractions 5-7, 14-16 and 35-37, respectively) was analyzed by immunoblotting and was found to be similar between the two groups (n=3, not shown). This result differs from that for SR-BI/apoE dKOs that do not have apoA-I in the normal HDL size region, because most of the HDL is abnormally large [10]. Figure 2C shows that differences in the UC:TC ratios of the FPLC fractions 6-25 reflected differences in the overall UC:TC ratio difference in plasma (see below). (UC content was below the limit of detection beyond fraction 25, and thus the ratios could not be determined (not shown)). In contrast, SR-BI/apoE dKO animals have very high UC:TC ratios (~0.8) in the VLDL and IDL/LDL size fractions and total plasma (AY, OK, MK, unpublished observations). FPLC analysis of plasma from mice fed a standard chow diet (not shown) showed a total cholesterol profile in apoE single KO mice similar to that previously reported [34, 35] and, as expected [12] a small increase in the apparent size of the HDL peak in the plasma from PDZK1/apoE dKO mice.

Quantitative analysis of plasma lipids from apoE KO and PDZK1/apoE dKO mice fed either a standard chow or Western diets for three months are shown in Table 1. As expected, the plasma cholesterol values were substantially higher in mice fed the Western diet. There were no significant differences in the amounts of plasma total cholesterol between apoE KO and PDZK1/apoE dKO mice with either diet ; however, the level of plasma unesterified cholesterol (UC) was increased by 45% (Western diet, $P=0.026$) or 37% (standard chow diet, $P=0.009$) in the PDZK1/apoE dKO mice relative to apoE single KO mice . For mice fed the Western diet, this difference is reflected in a 36% increase in the UC:TC ratios ($P=0.003$). When fed with a Western diet, the increase in UC:TC was accompanied by a 29% increase in the relative amount of plasma phospholipids ($P=0.01$). There were no statistically significant differences observed for the triglycerides on either diet or for the plasma phospholipids on the chow diet.

We have previously reported that SR-BI single KO and SR-BI/apoE dKO mice - but not PDZK1 KO or apoE KO mice - fed with a standard chow diet exhibit abnormally high UC:TC ratios [18], due at least in part to the abnormally large HDL particles failing to serve as effective substrates for the plasma cholesterol esterifying enzyme lecithin: cholesterol acyl transferase [29, 36]. Apparently the additional stress due to the apoE deficiency induces the abnormally high UC:TC ratio (as well as high UC and phospholipid levels) in the PDZK1/apoE dKO mice. The mechanism underlying this effect, however, is not clear.

After feeding the mice a Western diet for 3 months, we measured atherosclerotic plaque areas at the level of the aortic root in Oil Red O stained frozen sections. Both apoE single KO and PDZK1/apoE dKO mice showed significant amounts of

atherosclerosis (Figure 3). Visual inspection of histologic tissue sections revealed that plaques from the aortic sinuses of the apoE single KO and PDZK1/ apoE dKO animals were composed primarily of macrophage-derived foam cells and that smooth muscle cells did not appear to represent a significant cell population in the lesions. There were no apparent differences in the general morphologies of the plaques in the apoE single KO and PDZK1/ apoE dKO mice. However, the areas of plaque were significantly larger (57%) in the PDZK1/apoE dKO mice ($0.493\pm 0.023\text{ mm}^2$) than in the apoE KO mice ($0.314\pm 0.024\text{ mm}^2$, $P<0.0001$). Increased atherosclerosis in PDZK1/apoE dKO compared to apoE KO mice was also observed in a pilot study using mice on a 50:50 C57BL/6:129SvEv background ($P=0.015$). Thus, PDZK1 is atheroprotective in apoE KO mice.

We have previously reported that apoE KO mice bearing an homozygous null mutation in the SR-BI gene (SR-BI/apoE dKO mice) exhibit a number of the hallmarks of CHD not usually observed in apoE single KO mice, including occlusive coronary arterial atherosclerosis, cardiomegaly, myocardial infarctions, cardiac dysfunction and premature death [10, 21]. We therefore examined Masson's trichrome-stained sections of the hearts (mid-portion of the myocardium and level of the aortic root) of Western diet fed PDZK1/apoE dKO ($n=9$) and control apoE KO ($n=8$) mice to determine if the reduced hepatic expression of SR-BI due to the PDZK1 deficiency resulted in similar CHD pathogenesis. Neither PDZK1/apoE dKO or apoE KO mice exhibited fibrosis, myocardial infarction, leukocyte infiltration or coronary arterial occlusions, as do SR-BI/apoE dKO mice (Figure 3 D,E). Furthermore, there was no gross difference in heart size with or without PDZK1 expression. In addition, we did not detect premature death or

gross abnormalities in behavior or general health in either standard lab chow-fed or atherogenic diet-fed PDZK1/apoE dKO mice.

4. Discussion

In the current study, we found that there was a 57% increase in aortic root atherosclerosis in PDZK1/apoE dKO mice relative to apoE KO mice. It seems likely that the reduced hepatic expression of SR-BI in PDZK1/apoE dKO mice relative to apoE KO mice, and the consequent effects on lipoprotein metabolism (potentially reduced reverse cholesterol transport and an increased UC:TC ratio (increased UC and phospholipids) in plasma lipoproteins) contributed to the enhanced atherogenesis in the dKO mice. However, other mechanisms may also contribute to the enhanced atherosclerosis. For example, effects of PDZK1 deficiency on the expression of SR-BI in extrahepatic tissues may have made a contribution. We previously reported [12] that in PDKZ1 KO mice fed a standard chow diet, SR-BI expression is modestly (50%) reduced in the intestines, where SR-BI appears to be expressed primarily on the apical surface of enterocytes [37]. It does not seem likely that a modest reduction in intestinal SR-BI would influence atherogenesis other than via its influence on lipoprotein metabolism.

Two types of extrahepatic cells that do directly influence atherosclerosis are macrophages and endothelial cells. We found that surface expression of SR-BI on both macrophages (derived from bone marrow or spleen) and endothelial cells (derived from lung) was not reduced in PDZK1KO mice compared to wild-type controls. Furthermore, the SR-BI expressed by macrophages appeared to function normally, as indicated by its ability to mediate uptake of lipid from HDL. Kimura and colleagues have also reported

that SR-BI surface expression is not impaired in endothelial cells in which PDZK1 expression has been abrogated by siRNA [38]. However, they also reported that, despite the normal level of SR-BI surface expression, at least one activity mediated by SR-BI in endothelial cells, HDL-dependent activation of eNOS, was significantly suppressed in the absence of PDZK1 expression. Thus, it remains possible that loss of SR-BI-mediated activity in endothelial cells may contribute to the increased atherosclerosis observed in apoE KO mice when the PDZK1 gene is inactivated.

Strikingly, PDZK1/apoE dKO mice do not exhibit the severe occlusive atherosclerotic CHD seen in SR-BI/apoE dKO mice. There are several potential –not mutually exclusive– explanations. There is no expression of SR-BI, or its minor alternatively spliced isoform SR-BII [16] (see below), in any tissue of SR-BI KO mice [19]. In contrast, while PDZK1 KO mice express only residual levels of SR-BI in the liver (<5%), they express about 50% of wild-type levels in the intestines and apparently normal levels in steroidogenic organs (e.g. adrenal glands) [12], macrophages and endothelial cells (this study, also see [39]). Loss of SR-BI expression in adrenal glands in SR-BI KO mice results in dramatically reduced adrenal cholesteryl ester stores [19] and thus might influence adrenocortical function [40] and its moderating effects on inflammation [41]. A recent report by Cai et al strongly supports this suggestion [42]. In turn, this potential pathology in SR-BI/apoE dKO, but not PDZK1/apoE dKO, mice may stimulate the development of occlusive coronary arterial atherosclerosis.

Another noteworthy difference is that hepatic expression of the SR-BII splice form, which mediates endocytosis of ligands [16, 17, 43], is normal in PDZK1 KO mice [12], but absent in SR-BI KO mice. Thus, just as differences in SR-BI expression in

hepatic and extrahepatic tissues between PDZK1/apoE dKO and apoE KO mice may account for the increased atherosclerosis in PDZK1/apoE KO mice, so may distinct differences between PDZK1/apoE and SR-BI/apoE dKO mice in SR-BI/SR-BII expression in hepatic and extrahepatic tissues account for their dramatic differences in occlusive CHD. Previous studies have shown that even very low levels of expression of genes involved with atherosclerosis can have a profound effect on pathogenesis. For example, very low expression of apoE has been shown to dramatically alter atherogenesis in several murine systems [44-50].

In conclusion, the current studies show that a functional PDZK1 gene, most likely because of its role as the adaptor protein for SR-BI, is atheroprotective in mice. PDZK1 is the second adaptor protein for an hepatic lipoprotein receptor to be shown to be atheroprotective, the first being the human LDL receptor adaptor ARH [1, 5, 51]. Individuals with inactivating mutations in the ARH gene exhibit phenotypes similar to those of patients with familial hypercholesterolemia (FH), in which the low density lipoprotein receptor (LDLR) is inactivated [1-3, 5]. Further studies will be necessary to determine if genetic variations in PDZK1 in humans influence the incidence of atherosclerosis and CHD.

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Figure legends

Figure 1: Influence of PDZK1 on SR-BI expression and function in macrophages and endothelial cells. SR-BI expression (shown as histograms of relative fluorescence intensity) in splenic macrophages (A) and lung endothelial cells (B) was examined by flow cytometric analysis using as a primary antibody either the anti-SR-BI antibody KKB-1 (' α SR-BI', thick lines) or non-immune control normal rabbit serum ('control', thin lines). SR-BI expression in cells from wild-type (WT) mice is represented by black lines, whereas that from SR-BI KO (left panels) or PDZK1 KO (right panels) mice are represented with gray lines. Results from two pooled mice per group are shown (Panels A, B) and are consistent with independent experiments using two individual mice. In panel A (left) the results from SR-BI KO with control serum (thin gray line) overlaps with SR-BI KO with anti-SR-BI antibody (thick gray line). C. SR-BI function was measured by flow cytometry as the uptake of the lipophilic dye DiI from DiI-labeled HDL by splenic macrophages. SR-BI lipid uptake activity (cellular DiI accumulation) in macrophages from wild-type mice is represented by black lines, whereas that from SR-BI KO (left panels) or PDZK1 KO (right panels) mice are represented with gray lines. Results shown are from representative individual mice (n=2). 100% cell number $\sim 1-2 \times 10^3$.

Figure 2: Immunoblot analysis of hepatic SR-BI expression and lipoprotein cholesterol profiles from apoE KO and PDZK1/apoE dKO mice. Mice were fed a standard chow (panel A) or a high fat/high cholesterol "Western" diet (panels A, B and

C) for three months. A. Livers were harvested and subjected to immunoblotting using anti-SR-BI and anti-actin antibodies as described in Materials and methods. B. Plasma harvested from individual Western diet-fed animals was size fractionated using FPLC and the total cholesterol (note change of scale in inset) contents of the fractions (mg/dL plasma) were determined by enzymatic assay. Profiles averaged from six apoE KO (filled circles) and six PDZK1/apoE dKO (open circles) mice are shown. Approximate elution positions of human VLDL, IDL/LDL and HDL are indicated. C. Unesterified cholesterol (UC) and total cholesterol (TC), and the corresponding unesterified-to-total (UC:TC) cholesterol ratios, were determined for FPLC fractions 6-25 (averages from five apoE KO (filled circles) and four PDZK1/apoE dKO (open circles) mice). UC values beyond fraction 25 were below the limits of detection. Error bars represent SEM. See also Materials and methods.

Figure 3: Aortic root atherosclerosis in Western diet-fed apoE KO and PDZK1/apoE KO mice. A-B. Representative cross-sections of Oil red O-stained aortic root lesions. Magnification, x 20. C. Average lesion areas (mm^2 , horizontal lines) were: apoE KO, 0.314 ± 0.024 (n=9); PDZK1/apoE dKO, 0.493 ± 0.023 (n=8), *P<0.0001. D-E. Representative sections of hearts stained with Masson's trichrome, showing no evidence of either fibrosis or myocardial infarction. Magnification, x 4.5.

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Table 1. Plasma Lipids in Chow and Western-diet fed apoE KO and PDZK1/apoE dKO Mice

Diet	Genotype (sample size)	TC (mg/dl)	UC (mg/dl)	UC/TC	TG (mg/dl)	PL (mg/dl)
Chow	APOE KO (n=3)	451 ± 16	149 ± 5 ^a	0.33 ± 0.02 ^b	41 ± 5	280 ± 15
	PDZK1/APOE dKO (n=6)	529 ± 35	204 ± 10 ^a	0.39 ± 0.02 ^b	54 ± 10	323 ± 16
Western	APOE KO (n=9)	1424 ± 106	473 ± 56 ^c	0.33 ± 0.02 ^d	50 ± 9	511 ± 32 ^e
	PDZK1/APOE dKO (n=8)	1521 ± 126	688 ± 67 ^c	0.45 ± 0.03 ^d	59 ± 10	661 ± 42 ^e

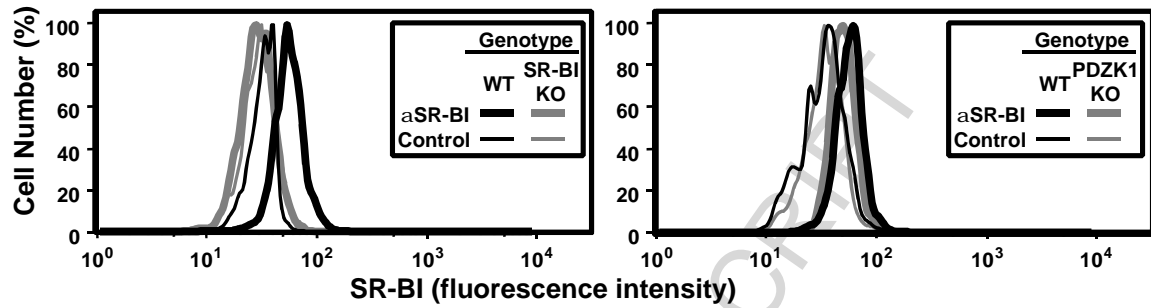
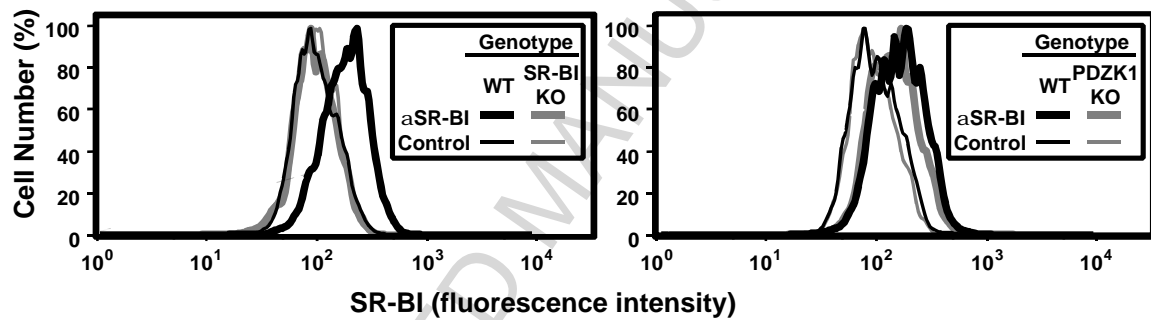
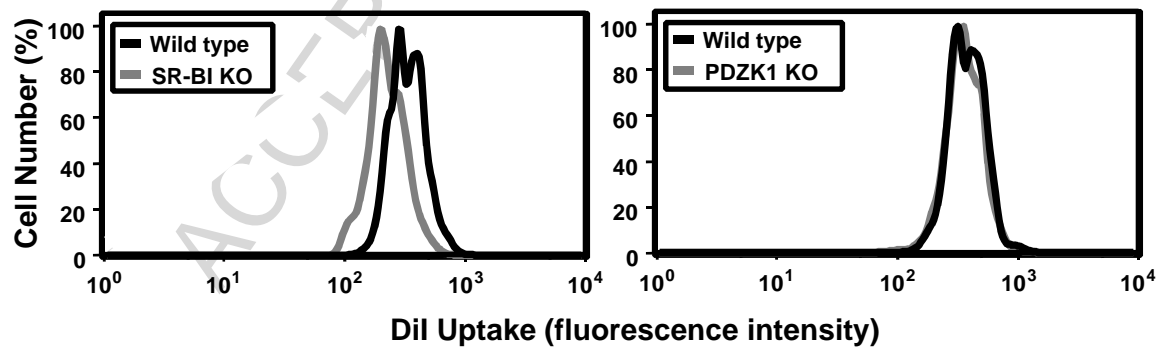
Four-to-five month old animals were fasted for 4 hours prior to sample collection. TC:

total cholesterol, UC: unesterified cholesterol, TG: triglycerides and PL: phospholipids.

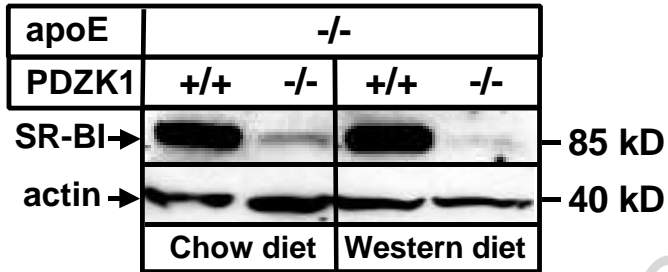
Values are represented as mean ± standard error. Statistical significance was determined by pairwise comparisons of each value from dKO mice with apoE controls within the same diet by using unpaired Student's t test.

a, b, c, d, e p<0.05

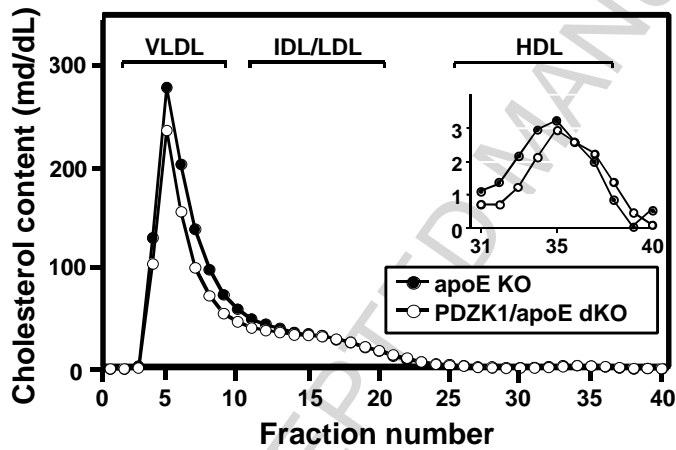
A

A. SR-BI Expression in Macrophages**B. SR-BI Expression in Endothelial Cells****C. Dil Uptake in Macrophages**

A. Immunoblot Analysis of SR-BI



B. Lipoprotein Cholesterol Profiles



C. UC:TC Ratios

