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Liver-specific Cholesteryl Ester Hydrolase deficiency attenuates sterol elimination in the feces and increases Atherosclerosis in *Ldlr-/-* Mice

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Abstract

Objective—Liver is the major organ responsible for the final elimination of cholesterol from the body either as biliary cholesterol or as bile acids. Intracellular hydrolysis of lipoprotein-derived cholesteryl esters (CE) is essential to generate the free cholesterol (FC) required for this process. We earlier demonstrated that over-expression of human cholesteryl ester hydrolase (Gene symbol CES1) increased bile acid synthesis in human hepatocytes and enhanced reverse cholesterol transport in mice. The objective of the present study was to demonstrate that liver-specific deletion of its murine ortholog, Ces3, would decrease cholesterol elimination from the body and increase atherosclerosis.

Approach and Results—Liver-specific Ces3 knockout mice (Ces3-LKO) were generated and Ces3 deficiency did not affect the expression of genes involved in cholesterol homeostasis and FC or bile acid transport. The effects of Ces3 deficiency on the development of Western diet-induced atherosclerosis were examined in LDLR-/- mice. Despite similar plasma lipoprotein profiles, there was increased lesion development in LDLR-/-Ces3-LKO mice along with a significant decrease in bile acids content of the bile. Ces3 deficiency significantly reduced the flux of cholesterol from [^{3}H]-CE labeled HDL to feces (as FC and bile acids) and decreased total fecal sterol elimination.

Conclusions—Our results demonstrate that hepatic Ces3 modulates the hydrolysis of lipoprotein-delivered CE and thereby regulates FC and bile acid secretion into the feces. Its deficiency, therefore, results in reduced cholesterol elimination from the body leading to significant increase in atherosclerosis. Collectively, these data establish the anti-atherogenic role of hepatic CE hydrolysis.

Keywords

Hepatic cholesteryl ester hydrolysis; atherosclerosis; liver-specific knockout mice; cholesterol elimination from the body; bile acids

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Liver is the major metabolic organ that plays a central role in regulating whole body cholesterol homeostasis (1). Endogenously synthesized cholesterol, along with triglycerides (TG), is packaged into very low density lipoproteins (VLDL) and secreted. Following delivery of TG as a source of energy for the peripheral tissues and cholesterol for membrane and steroid synthesis, the cholesterol-rich and TG-poor low density lipoprotein (LDL) delivers the cholesterol back to the liver. LDL associated cholesterol in the form of cholesteryl esters (CE) is hydrolyzed in the lysosome and the released free cholesterol (FC) is re-esterified by ACAT-2 and stored as CE in lipid droplets. In addition, excess cholesterol from peripheral tissue including artery wall associated macrophage foam cells is also returned to the liver via the high-density lipoproteins (HDL) by the process named reverse cholesterol transport (RCT). To gain access to the intracellular cholesterol pool, HDL-CEs must be hydrolyzed within hepatocytes, and this hydrolysis is thought to occur in the non-lysosomal compartment presumably, by a neutral CE hydrolase (2, 3). Hepatic CE hydrolase is, therefore, required not only to release FC from the intracellular stores of CEs (generated by ACAT2-mediated esterification) but also to hydrolyze CEs delivered via selective uptake of HDL and represents the key enzyme required for releasing the pool of metabolically active FC from intracellular stores of CEs, providing the substrate for bile acid synthesis and for biliary secretion of cholesterol.

Despite its importance in regulating cholesterol elimination from the body, the identity of hepatic CE hydrolase is not completely defined. Several potential CE hydrolases with ability to hydrolyze HDL-CE have been described. Hormone sensitive lipase (HSL) has CE hydrolytic activity but in vitro studies (4) and analyses of HSL-/-mice (5,6) suggest that this enzyme hydrolyses HDL-CE in testes and adrenals but not in liver. A microsomal CE hydrolase has also been purified from hepatocytes but its orientation towards the lumen of the endoplasmic reticulum is not consistent with a role in HDL-CE hydrolysis (7). Camarota et al described the association of bile-salt stimulated lipase or carboxyl ester lipase (CEL) with SR-BI and suggested a role for this enzyme in HDL-CE hydrolysis (8), however, the observed increase in RCT in CEL-/-mice indicate a limited role of this enzyme in hydrolysis of HDL-CE (9). Parathath et al recently characterized carboxylesterase ES4 from rat liver as a hepatic CE hydrolase but its role in the hydrolysis of HDL-CE has not been evaluated as yet (10).

We had earlier described purification (11), characterization and cloning of rat liver CE hydrolase (12), a member of the carboxylesterase family and established its role in hepatic cholesterol homeostasis (13). Recently we also cloned and characterized human liver CE hydrolase (CEH) and demonstrated that transient over-expression of CEH increased bile acid synthesis and secretion from primary human hepatocytes (14). Furthermore, adenovirus-mediated over expression of this enzyme enhanced cholesterol elimination by increasing the flux of cholesterol from macrophages to feces (in vivo RCT) (15). More importantly, SR-BI deficiency completely abolished CEH-mediated increase in in vivo RCT demonstrating the requirement of functional SR-BI for CEH to channel HDL derived CE to bile and feces (15).

Because only a small portion (5–20%) of biliary cholesterol is derived from de novo synthesis (16,17) and the bulk is supplied by the hepatic uptake of lipoproteins (18, 19), by enhancing the hydrolysis of lipoprotein derived CE, hepatic CEH regulatesthe removal of cholesterol from the body and should therefore be anti-atherogenic. In the present study we examined the hypothesis that deficiency of hepatic CEH will decrease cholesterol elimination from the body and will, therefore increase diet induced atherosclerosis. The mouse homolog of human CEH is carboxylesterase-3 or Ces3 and we developed mice with liver-specific deficiency of Ces3 and examined the development of Western diet-induced atherosclerosis in these mice in LDLR-/-background. The data presented herein show that

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hepatic Ces3 deficiency increases atherosclerosis and decreased fecal elimination of cholesterol as FC or bile acids is likely the underlying mechanism.

Materials and Methods

Material and methods are provided in the online-only Data Supplement.

Results

Development of Liver-specific Ces3 knockout (Ces3-LKO) mice

Total RNA from multiple tissues was used to determine the expression of Ces3 in WT and Ces3-LKO mice. Consistent with the liver-specific Cre recombinase expression in Alb-Cre transgenic mice, Ces3 expression was reduced by >95% in the liver and no decrease was noted in other tissues tested (Figure 1, Panel A). Ces3 belongs to the carboxylesterase family and several members of this family are expressed in the liver. To evaluate the effects, if any, on the expression of other carboxylesterases by Ces3 deficiency, expression of indicated carboxylesterases was also monitored. Other than a slight decrease in the expression of ES1, no differences were noted in the expression of other genes (Figure 1, Panel B). Based on the observed Ct values (in parentheses), the relative abundance of various carboxylesterases is ES1 (18.61)>ES31 (19.55)>Ces3 = Tgh2 (20.18)>ES22 (20.71)>Ces5 (20.84)>Ces1 (21.59)> KIAA1363 (24.47)>HSL (25.94).

To determine the potential effects of Ces3 deficiency in affecting genes involved in maintaining hepatic cholesterol homeostasis, expression of HMGCR, ACAT-2, Ldlr CYP7A1 and SR-BI was assessed. While a non-significant decrease in the expression of CYP7A1 was observed, there was no change in the expression of other genes (Supplementary Figure I, Panel A).

Since FC released as a result of Ces3-mediated CE hydrolysis can either be secreted directly into bile via the FC transporter AbcG5/G8 or converted into bile acids before secretion via bile acid transporter (BSEP), expression of these transporters was evaluated. Ces3 deficiency did not affect the expression of these transporters (Supplementary Figure I, Panel B). No change was also noted in phospholipid transporter MDR2.

Ces3-LKO mice were crossed into LDLR-/- background for all the subsequent studies.

Hepatic Ces3 deficiency did not affect liver function tests

Fasting plasma from Western-diet fed LDLR-/- and LDLR-/-Ces-LKO mice was analyzed for AST, ALT and Bilirubin to evaluate the effects of Ces3 deficiency on liver function/ toxicity. There was no significant difference in levels of these parameters (Supplementary Figure II) indicating that Ces3 deficiency did not result in hepatic toxicity.

Hepatic Ces3 deficiency did not alter hepatic lipid accumulation

Ces3 catalyzes the hydrolysis of hepatic CE as well as triglycerides (TG) and to determine the effects of Ces3 deficiency on hepatic lipid levels, total lipids extracted from livers from Western diet fed LDLR-/- and LDLR-/-Ces3-LKO mice were analyzed for total cholesterol (TC), FC, CE and TG levels. No significant differences were noted in hepatic lipids (Figure 2, Panel A). However, comparison between genders showed that there is a significant increase in TC and CE in female mice in both LDLR-/- as well as LDLR-/-Ces3-LKO mice (Supplementary Figure III, Panel A).

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Hepatic Ces3 deficiency did not alter cholesterol levels of plasma lipoproteins

Fasting plasma from Western diet fed LDLR-/- and LDLR-/-Ces3-LKO mice was collected and cholesterol distribution between VLDL, LDL and HDL was determined. Hepatic Ces3 deficiency did not alter the cholesterol content of plasma lipoproteins (Figure 2, Panel B). However, comparison of percent distribution of total plasma cholesterol between VLDL, LDL and HDL between genders showed a significantly higher percent of cholesterol associated with VLDL in female mice of both genotypes (Supplementary Figure III, Panel B). In contrast, percent of total plasma cholesterol associated with LDL was higher in males of both genotypes.

Western-diet feeding did not affect the expression of genes involved in hepatic cholesterol homeostasis

Total liver RNA from Western-diet fed LDLR-/- and LDLR-/-Ces3-LKO mice was analyzed for the expression of other members of the carboxylesterase family and other potential hepatic CE hydrolases. As shown in Supplementary Figure IV and similar to the absence of any significant changes in the expression of these genes in chow-fed mice, Western diet feeding did not affect the expression of these genes in LDLR-/-Ces3-LKO mice. Similarly, no significant change was observed in the expression of genes involved in hepatic cholesterol homeostasis and cholesterol/bile acid transport (Supplementary Figure V)

Hepatic Ces3 deficiency increased atherosclerosis

Western diet-induced atherosclerosis was monitored in LDLR-/- and LDLR-/-Ces3-LKO mice after 16 weeks of feeding. No mortality or morbidity was observed in mice with either genotype. The percent area of aorta covered with atherosclerotic lesions was determined by en face analyses and Figure 3 shows representative images of aortas obtained from LDLR-/- and LDLR-/-Ces3-LKO mice (Males – Panel A and Females – Panel B). While very few lesions were noticeable in the thoracic and abdominal aorta as expected with this mouse model, the aortic arch displayed the most profound lesions. While an increase in the atherosclerotic lesion area was observed in the aortic arch in LDLR-/-Ces3-LKO males, this increase was more pronounced in corresponding female mice. The quantification of the surface area occupied by atherosclerotic lesions is shown in Figure 4A. Compared to LDLR-/- mice, liver-specific Ces3 deficiency in LDLR-/-Ces3-LKO mice led to a significant increase in the total area occupied by the lesions in the aortic arch. The magnitude of this increase was higher in females (50.76±8.66%) compared to males (26.44±7.66%, Figure 4). Total area occupied by the lesions in the entire aorta was also determined and liver-specific Ces3 deficiency significantly increased the total area occupied by atherosclerotic lesions and once again the magnitude of this increase was higher in female mice. An overall effect of genotype on total ($P = 0.0012$) and aortic arch ($P = 0.0008$) lesion area was detected by ANOVA. Effects of gender and gender/genotype interactions were not detected. (See Supplementary Table I)

Aortic cholesterol content was also determined and used as another measure of the development of atherosclerosis. As shown in Figure 4B and consistent with en face analyses of the atherosclerotic lesion, Ces3 deficiency resulted in significant increase in TC, FC and CE content of the aorta. Taken together these findings indicate that liver-specific Ces3 deficiency increases diet-induced atherosclerosis in LDLR-/- mice. Similar to the observed differences in lesion area, significant overall effect of the genotype was also noted in total ($P = 0.0039$), free ($P = 0.0011$) and esterified cholesterol ($P = 0.0192$) without any significant gender/genotype interactions. (See Supplementary Table I).

Aortic sinus lesions were compared to determine whether liver-specific Ces3 deficiency altered lesion morphology or affected lesion area at this anatomic site. Supplementary

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Figure VI, Panel A, shows representative images. Total lesion area and percent acellular or the necrotic area was quantified and no significant differences were noted in either of these parameters between the two genotypes in either gender (Supplementary Figure VI, Panel B).

Hepatic Ces3 deficiency decreases removal of HDL associated cholesterol in feces

FC removed from peripheral tissues including artery wall-associated macrophage foam cells is carried back to the liver as CE in the HDL particle. CE delivered to the liver by HDL need to be hydrolyzed to release FC for either direct secretion into the bile or for conversion into bile acids that are also secreted into bile and we have earlier demonstrated that adenovirus-mediated over expression of CEH enhances secretion of bile acids into the feces (15). Fecal excretion of FC or bile acids represents the only route for removal of cholesterol from the body and is, therefore, an anti-atherogenic process. To examine whether hepatic Ces3 deficiency-mediated reduction in removal of cholesterol in feces (either as FC or as bile acids) represents a potential mechanism for the observed increase in atherosclerosis in LDLR-/-Ces3-LKO mice, movement of radiolabeled cholesterol from HDL-CE to FC and bile acids in feces was monitored. There was no significant difference in radioactivity associated with plasma or liver at the end of 48 h (Figure 5A). Ces3 deficiency led to a significant decrease in fecal sterols (FC and bile acids) measured as the percent of injected radioactive HDL-CE (Figure 5B, P=0.0391 and 0.0023, respectively). Total fecal bile acids as well as cholesterol levels were also monitored and as shown in Figure 5C, consistent with decreased flux of HDLCE, there was a significant reduction in total fecal bile acids (P=0.0004) and FC (P=0.0001) in LDLR-/-Ces3-LKO mice. These data demonstrate that Ces3-mediated HDL-CE hydrolysis is required for conversion to bile acids and FC for the subsequent removal from the body in the feces.

To directly test that Ces deficiency reduces the hydrolysis of HDL-CE, accumulation of FC in primary hepatocytes was monitored. Significant reduction (P=0.01) in intracellular FC was observed in primary hepatocytes isolated from LDLR-/- Ces3-LKO mice compared to LDLR-/- controls (Supplementary Figure VII, Panel A) demonstrating reduced hydrolysis of HDL-CE as a result of Ces3 deficiency. To further evaluate whether Ces3 deficiency directly affected bile acid synthesis starting from FC (which will not require Ces3-mediated CE hydrolysis), conversion of [^{3}H]-cholesterol into water-soluble bile acids was monitored using primary hepatocytes obtained from LDLR-/- and LDLR-/-Ces3-LKO mice. No difference was noted between incorporation of [^{3}H]-cholesterol into bile acids, either secreted into the medium or inside the cells (Supplementary Figure VII, Panel B). Taken together with the significantly reduced in vivo flux of HDL-CE to feces, these data suggest that Ces3 deficiency only decreases conversion of [^{3}H]-CE associated with HDL which requires hepatic hydrolysis to generate FC and does not affect the conversion of [^{3}H]-cholesterol to bile acids.

To evaluate whether there is decreased bile acid secretion in vivo, gall bladder bile was collected from Western diet-fed LDLR-/- and LDLR-/-Ces3-LKO mice and analyzed. Bile acid levels normalized to phospholipid content are shown in Figure 6A. A significant decrease in bile acid levels was observed in LDLR-/-Ces3-LKO mice indicating that decreased removal of cholesterol as bile acids represents the potential underlying mechanism for the observed increase in atherosclerosis. Total biliary cholesterol levels remained unchanged in Ces3-deficient mice (Figure 6B).

Discussion

Removal of biliary cholesterol and bile acids in the feces is the only mechanism for elimination of cholesterol from the body and is, therefore, anti-atherogenic. Since bulk of the biliary cholesterol is derived from lipoproteins taken up liver (de novo synthesized

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cholesterol contributes only 5-20% of biliary cholesterol) intracellular hydrolysis of endogenously synthesized CE (from FC released after lysosomal hydrolysis of LDL-CE) or CE delivered by selective uptake from HDL via SR-BI is essential for this process. The data presented herein shows for the first time that deficiency of hepatic CE hydrolase, Ces3, results in decreased movement of cholesterol from HDL-CE to feces resulting in increased diet-induced atherosclerosis in LDLR-/-Ces3-LKO mice. However, liver-specific Ces3 deficiency did not lead to significant changes plasma or hepatic lipids. This apparent lack of correlation between plasma lipids and development of atherosclerosis is consistent with the observed increase in atherosclerosis without altered plasma lipoprotein profiles in several other mouse models (20-22) and underscores the importance of enhancing cholesterol elimination from the body rather than altering plasma lipids alone as an anti-atherogenic strategy. It is noteworthy that although a significant decrease in dyslipidemia was noted, there was no change in the development of atherosclerosis in Xbp1 deficient *ApoE*-/- mice (23).

While formation of CE-laden macrophage foam cells is the key step in the development of arterial plaques, extracellular acceptor (e.g., ApoA1 or HDL) mediated removal of FC is critical for reduction in cellular CE content and thus reduction/reversal of plaque formation. Intracellular hydrolysis of CE is the obligatory first step in this mobilization of cellular CE and we have earlier demonstrated that macrophage-specific transgenic expression of human CEH enhances macrophage CE mobilization and attenuates diet-induced atherosclerosis in LDLR-/- mice (17). Cholesterol removed from arterial plaque-associated macrophages returns to the liver and while HDL-FC may be directly secreted into bile (24), HDL-CE need to be hydrolyzed within the hepatocyte and uptake of CE from HDL is efficiently coupled to bile acid synthesis (25). The observed decreased in bile acid secretion and flux of labeled cholesterol from HDL to feces in liver specific Ces3 deficiency clearly demonstrates the critical role of hepatic CE hydrolysis in regulating this last step in the process of RCT. It should be noted that the ability of primary hepatocytes to synthesize and secrete bile acids starting from [^{3}H]-cholesterol was not affected by Ces3 deficiency indicating that decreased availability of FC due to Ces3 deficiency (Supplementary Figure S7) is responsible for the observed reduction in the bile acid content of the bile as well as flux of HDL-CE to bile acids in the feces. These data are consistent with the reported increase in fecal bile acids by adenovirus-mediated CEH over-expression (14). The "flux" of cholesterol from macrophage foam cells via plasma lipoproteins (e.g., HDL) to liver and finally to feces is increasingly being considered more important than atherogenic lipoprotein profiles in determining the progression of atherosclerosis. Consistently, strategies aimed at enhancing the removal of cholesterol from macrophage foam cells (first step in RCT) lead to decrease in plaque burden or atherosclerosis in both mice (26, 27) and humans (28). Similarly, enhancing the uptake of HDL-CE by increasing hepatic SR-BI expression increased cholesterol elimination into feces (last step in RCT) (29). The data presented herein identifies hepatic CE hydrolysis as another important step in the regulation of the last step in RCT namely, the fecal elimination of total sterols (FC+bile acids).

Development of atherosclerosis is higher in male mice compared to females and this gender difference is also observed in humans where the risk for the development of cardiovascular disease is lower in pre-menopausal women. While treatment with estrogen (30) or estrogen-agonists (31) reduces atherosclerosis without affecting plasma lipids, the underlying mechanism(s) is not completely understood. Gender specific differences in hepatic cholesterol content have recently been reported (32). Several studies have demonstrated an increase in CE hydrolase activity by estradiol (33-35). Chiba et al suggested that estradiol-dependent activation of CE hydrolase is the underlying mechanism for the gender differences of atherogenesis in ApoE-/- mice (36). The magnitude of effects on reduction in bile acid content, flux of cholesterol from HDL-CE to feces and ultimately development of

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atherosclerosis was higher in females than males in the present study. Furthermore, significant gender effects were also noted in liver TC and CE, plasma VLDL-C/LDL-C and fecal bile acids. It is also noteworthy that total bile acid synthesis as well as fecal bile acid excretion is higher in females and using CYP7A1 deficient mice, Schwarz et al demonstrated that this difference is attributable to classic bile acid synthetic pathway despite similar enzyme activity of CYP7A1 in the two genders suggesting a regulatory role of FC substrate availability (37). Taken together with these earlier studies, it is likely that in the absence of Ces3, the protection rendered by increased CE hydrolytic activity and resulting increased FC availability for bile acid synthesis is lost resulting in higher magnitude of differences in females. Additional studies in the future may completely establish whether hepatic CE hydrolysis represents the mechanism for the gender difference in susceptibility to atherosclerosis.

Mouse Ces3 is a member of carboxylesterase family which includes enzymes with esterase activity towards a wide range of substrates and therefore, members of this family have been named based on the first substrate used for characterization. Based on its initial characterization using triglyceride as a substrate, Ces3 is also known as triglyceride hydrolase (Tgh). Focusing on its role as a Tgh, Ces3 is thought to provide triglycerides for VLDL assembly and Wei et al reported a decrease in plasma lipids in Tgh deficient 129sv mice (38) and this decrease in plasma lipids (30-50%) was considered as the underlying mechanism for the observed decrease in atherosclerotic lesions in the aortic root of Tgh-/-LDLR-/- mice; development of lesions in the aorta/aortic arch were not examined in this study (39). The data presented herein demonstrates an increase in atherosclerotic lesion in the entire aorta in LDLR-/-Ces3-/-LKO mice and Ces3 deficiency-mediated inability to hydrolyze endogenous or lipoprotein derived CE and thereby reducing cholesterol elimination from the body was established as the underlying mechanism. It should be noted that Ces3 deficiency in the present study is liver-specific. Intestinal deficiency of Ces3 in Tgh-/-Ldlr-/- mice led to a significant decrease in chylomicron secretion (39) likely contributing to decrease in intestinal cholesterol absorption which is shown to result in attenuated atherosclerosis in LDLR-/- mice (40).

In conclusion, the present study establishes the anti-atherogenic role of hepatic CE hydrolysis which is required for mobilizing endogenously synthesized or HDL derived CE to generate FC for biliary secretion and bile acid synthesis. Hepatic Ces3 deficiency decreased fecal elimination of cholesterol (as FC and bile acids) and thereby increased diet-induced atherosclerosis in LDLR-/-Ces3-LKO mice without affecting plasma lipid levels. While modifying plasma lipoprotein profile and, specifically increase in HDL cholesterol, has been the focus of therapies targeted to reduce cardiovascular risk, recent developments such as use of Niacin and CETP inhibitors have clearly established the need for the change of paradigm from HDL cholesterol to HDL flux (41). Demonstrating the role of hepatic Ces3 in modulating cholesterol flux from HDL to feces is, therefore, significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

An imbalance between cholesterol intake/synthesis and cholesterol elimination from the body results in accumulation of cholesterol in artery wall associated macrophage foam cells leading to the development of atherosclerosis. Liver plays a central role in the final elimination of cholesterol from the body in feces either as cholesterol or as bile acids. Cholesterol is removed from peripheral tissues including artery wall-associated macrophage foam cells and carried to the liver as lipoprotein (HDL) associated-cholesteryl esters (CE). Hepatic CE hydrolysis is, therefore, an obligatory step for the generation of free cholesterol, either for direct secretion into bile or for conversion to bile acids. This study demonstrates that liver-specific deficiency of CE hydrolase (Ces3) results in decreased hydrolysis of HDL-CE, bile acid secretion and elimination of cholesterol and bile acids in feces leading to increased development of diet-induced atherosclerosis in LDLR-/- mice. Therefore, similar to macrophages, intracellular hepatic CE hydrolysis is also anti-atherogenic.

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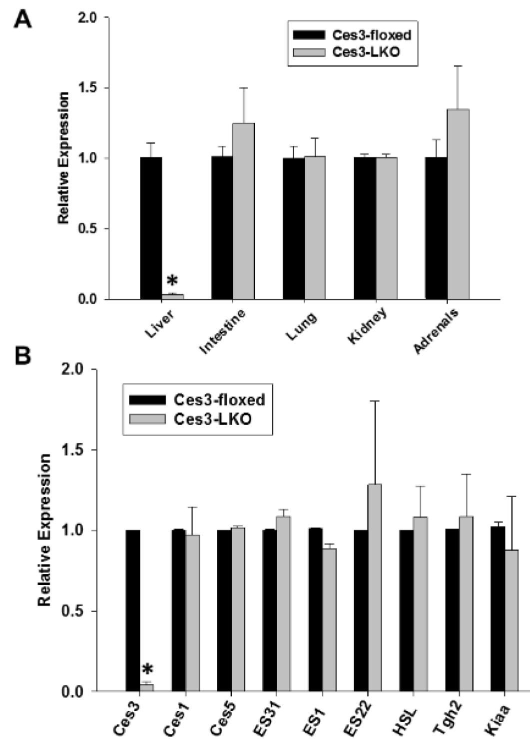

Figure 1. Liver-specific deletion of Ces3 and hepatic gene expression

Panel A: Total RNA was extracted from liver, intestine, lung, kidney and adrenals and expression of Ces3 was determined by Real Time PCR as described under "Methods". Panel B: Total liver RNA was also used to determine the expression of indicated genes and expression in Ces3-LKO mice relative to Ces3-floxed is shown (Mean±SD, n=3). *P<0.05

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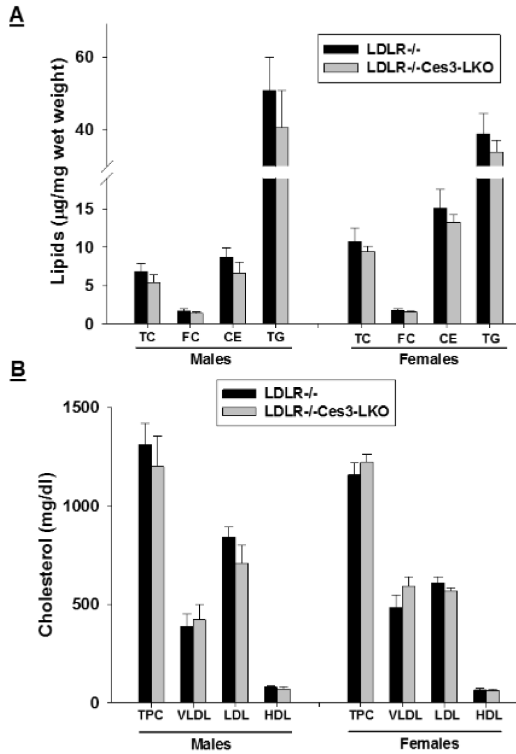

Figure 2. Comparison of the plasma lipoprotein profiles and hepatic lipids

LDLR-/- and LDLR-/-Ces3-LKO mice were fed a Western Diet for 16 weeks. At the time of necropsy, fasting plasma was collected and analyzed as described under "Methods". Panel A: Total lipids were extracted from liver and analyzed to determine total cholesterol (TC), unesterified or free cholesterol (FC) cholesteryl esters (CE) and triglyceride levels, and normalized to wet weight. Data are presented as Mean±SD (n=8 for males and n=6 for females). Panel B: Distribution of total plasma cholesterol (TPC) between VLDL, LDL and HDL is shown. Data are presented as (Mean±SD, n=8 for males and n=6 for Females).

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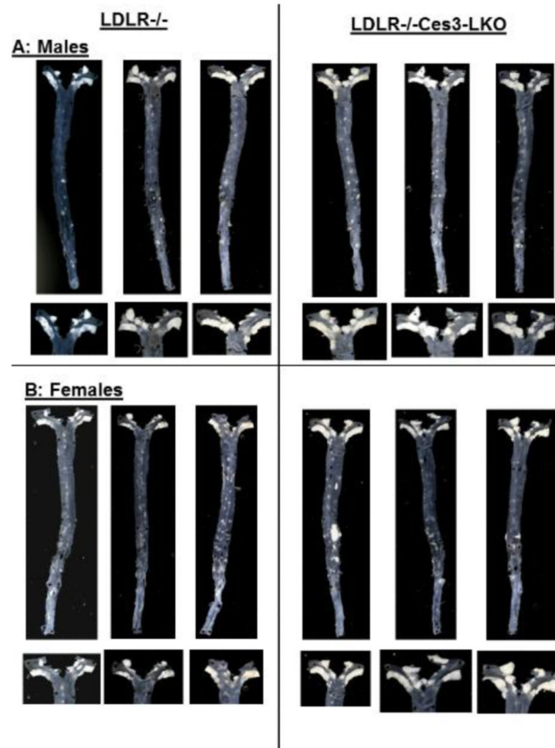

Figure 3. LDLR-/-Ces3-LKO mice have increased diet-induced atherosclerosis compared to LDLR-/- controls

LDLR-/- and LDLR-/-Ces3-LKO mice were fed a Western Diet for 16 weeks. Aorta (from aortic sinus to the iliac bifurcation) was isolated, opened and pinned to expose the lesions, and imaged on a black background. Representative images of the entire aorta or aortic arch from male (Panel A) and female (Panel B) mice are shown.

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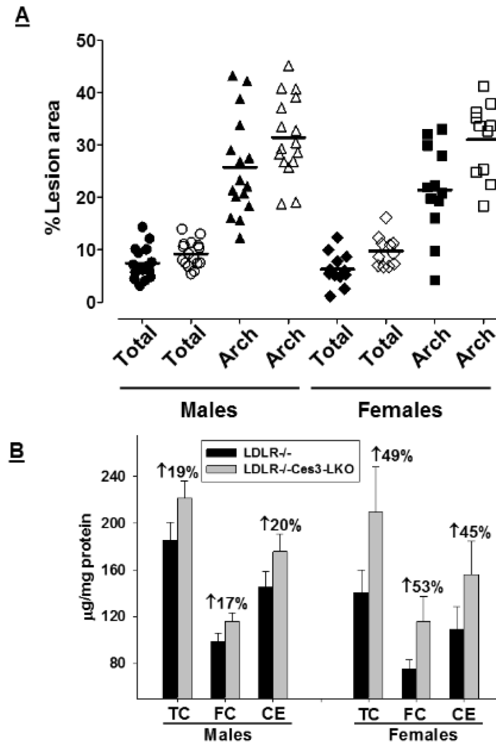

Figure 4. LDLR-/-Ces3-LKO mice have increased diet-induced atherosclerosis compared to LDLR-/- controls

Panel A: The quantification of the surface area occupied by the lesions in the total aorta and aortic arch is shown. Data (% area occupied by the lesions) for individual mice in the group are shown as a scatter plot and the mean is shown by the horizontal line. LDLR-/- (Solid symbols) and LDLR-/-Ces3-LKO (open symbols). Panel B: Total (TC), unesterified or free cholesterol (FC) and Cholesteryl ester (CE) content of the aortas was determined and normalized to total protein. Data are shown as Mean±SD. The effects of genotype (or Ces3 deficiency) on lesion area and aortic cholesterol content were determined to be significant by Two-way ANOVA analysis and individual P values are included in the Supplementary Table 1. Numbers above the bars indicate the mean percent increase between the two genotypes.

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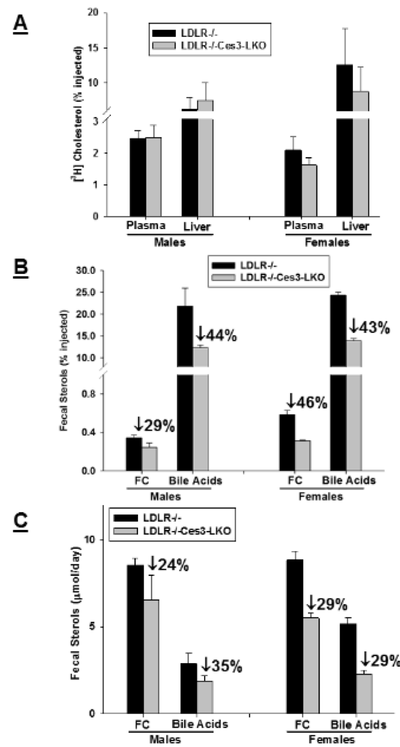

Figure 5. Decreased removal of HDL associated cholesterol in LDLR-/-Ces3-LKO mice

In vivo movement of [^{3}H]-cholesterol HDL-CE to feces was monitored as described under Methods. Total radioactivity (dpm) of [^{3}H]-cholesterol was determined and data (mean ± SD; n = 3) are presented as percentage of the total dpm injected. Panel A: Radioactivity associated with Plasma and liver homogenates was determined and normalized to 1 ml or 1 g wet weight, respectively. Panel B: Radioactivity associated with fecal bile acids and FC was determined following extraction of dried feces and normalized to weight of total feces. Panel C: Total bile acids and cholesterol was extracted from feces and analyzed as described under "Methods". Data werenormalized to dry weight of feces/day and presented as (Mean ±SD, n=6). The effects of genotype (or Ces3 deficiency) on flux of [^{3}H] cholesterol from HDL to fecal FC and bile acids as well as total fecal sterol excretion were determined to be statistically significant and individual P values are included in the Supplementary Table 1. Numbers above the bars indicate the mean percent decrease between the two genotypes.

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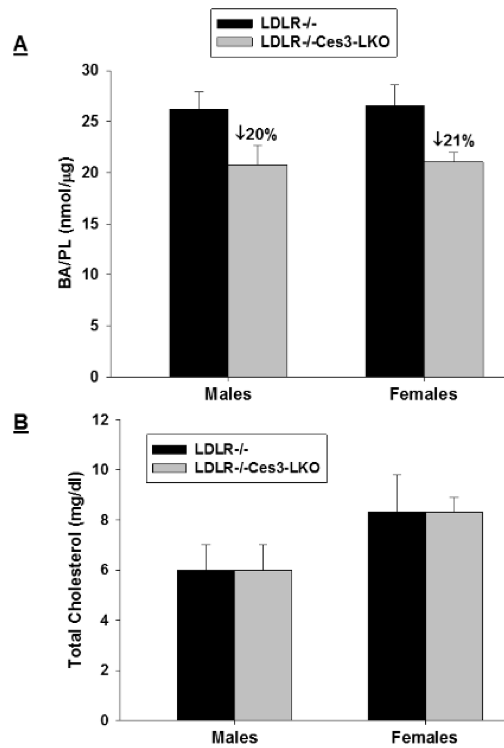

Figure 6. Reduced bile acid secretion in Ces3-deficient mice

Gall bladder bile was collected and analyzed for total bile acid (BA), phospholipid (PL) and cholesterol content. Panel A: BA/PL ratios (BA and PL concentrations varied from 69.55 to 157.25 nmole/µl and 3.39 to 7.11 µg/µl, respectively); Panel B: Total biliary cholesterol. Data are presented as (Mean±SD, n=6). The effects of genotype (or Ces3 deficiency) were significant for BA/PL ratios and individual P values are included in the Supplementary Table 1. Numbers above the bars indicate the mean percent decrease between the two genotypes.