Hepatocyte estrogen receptor alpha mediates estrogen action to promote reverse cholesterol transport during Western-type diet feeding

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ABSTRACT

Objective: Hepatocyte deletion of estrogen receptor alpha (LKO-ERα) worsens fatty liver, dyslipidemia, and insulin resistance in high-fat diet fed female mice. However, whether or not hepatocyte ERα regulates reverse cholesterol transport (RCT) in mice has not yet been reported.

Methods and results: Using LKO-ERα mice and wild-type (WT) littermates fed a Western-type diet, we found that deletion of hepatocyte ERα impaired in vivo RCT measured by the removal of 3H-cholesterol from macrophages to the liver, and subsequently to feces, in female mice but not in male mice. Deletion of hepatocyte ERα decreased the capacity of isolated HDL to efflux cholesterol from macrophages and reduced the ability of isolated hepatocytes to accept cholesterol from HDL ex vivo in both sexes. However, only in female mice, LKO-ERα increased serum cholesterol levels and increased HDL particle sizes. Deletion of hepatocyte ERα increased adiposity and worsened insulin resistance to a greater degree in female than male mice. All of the changes lead to a 5.6-fold increase in the size of early atherosclerotic lesions in female LKO-ERα mice compared to WT controls.

Conclusions: Estrogen signaling through hepatocyte ERα plays an important role in RCT and is protective against lipid retention in the artery wall during early stages of atherosclerosis in female mice fed a Western-type diet.

Keywords Estrogen; Insulin resistance; Cholesterol metabolism; Sex differences; Reverse cholesterol transport

1. INTRODUCTION

Obese, premenopausal women are more insulin sensitive than body mass index-matched men [1,2], and have lower risk of coronary heart disease (CHD) with a less atherogenic plasma lipid profile [3,4]. Proatherogenic dyslipidemia with obesity is characterized by high levels of LDL-cholesterol and triglycerides and low levels of HDL-cholesterol. Insulin resistance promotes atherosclerosis by worsening dyslipidemia and other metabolic abnormalities [5]. Women have higher VLDL and LDL production rates but lower VLDL and LDL cholesterol levels than men due to enhanced plasma apoB-particle clearance in women that offsets VLDL and LDL production rates [6]. In addition, women have a greater apoA1 synthesis rate associated with higher HDL cholesterol levels [6,7].

One major atheroprotective mechanism of HDL is its ability to promote cholesterol efflux from foam cells and prevent lipid accumulation in the artery wall. To maintain efficient HDL-mediated cellular cholesterol efflux from foam cells, cholesterol and cholesteryl esters in HDL particles are either removed by the liver through the scavenger receptor class B member I (SR-BI) pathway [8] or transferred via cholesteryl ester transfer protein (CETP) to apoB-particles in the blood and subsequently cleared through the LDL receptor (LDLR) or remnant receptor pathways [9]. The process of cholesterol efflux from foam cells to liver for removal from the body is referred to as reverse cholesterol transport (RCT). Impaired regulation of any step in RCT is correlated to CHD risk [9].

After menopause, CHD risk in women approaches that of men of the same age, suggesting that sex differences in CHD risk are in part influenced by estrogen signaling pathways [3,4]. The protective effects of estrogen signaling against insulin resistance are distributed across tissues including the central nervous system, macrophages/immune system, adipose tissues, skeletal muscles, and the liver [2,6,10–12]. However, studies of the tissue-selective actions of estrogen on lipoprotein metabolism and its association to atherosclerosis using mouse models have been limited. Using apoE-null mice, endogenous ovarian hormones suppress atherosclerosis progression with Western

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Abbreviations: ERα, estrogen receptor alpha; LKO-ERα, deletion of hepatocyte estrogen receptor alpha; CHD, coronary heart disease; SR-BI, scavenger receptor class B member I; PDZK1, PDZ domain containing 1; CETP, cholesteryl ester transfer protein; LDLR, LDL receptor; RCT, reverse cholesterol transport; TG, triglyceride; DG, diacylglycerol; WD, Western-type diet; WT, wild type; PLTP, phospholipid transfer protein; SAA1, serum amyloid A1; PON1, paraoxonase 1; FPLC, fast performance liquid chromatography

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type diet in sham-operated mice compared with ovariectomized mice, without changes in plasma cholesterol levels [13]. A similar finding was reported in another study using LDLR-null mice [14]. Although hormone replacement with exogenous estradiol reduced plasma lipids and atherosclerotic burden after ovariectomy, association between lesion area and plasma lipids was weak in studies using apoE- or LDLR-null mice [13,14]. These studies suggest that new mouse models are required to study estrogen signaling pathways in the regulation of lipoprotein metabolism and their potential contribution to the protective effects conferred by being female with regard to atherosclerosis development.

We previously reported that ovariectomy in female mice causes fatty liver by increasing lipogenesis and insulin resistance as determined by hyperinsulinemic-euglycemic clamp in high-fat diet fed mice [2]. Estradiol treatment corrected hepatic steatosis and insulin resistance during high-fat diet feeding in a manner that required hepatocyte ERα [2].

In this study, to investigate whether hepatic estrogen signaling regulates RCT and is protective against early stages of atherosclerosis, we fed female and male hepatocyte estrogen receptor α knockout (LKO-ERα) mice on a C57BL/6J background a Western-type (WD) diet. Deletion of hepatocyte ERα increased insulin resistance, impaired HDL capacity for cholesterol transport from foam cells and hepatocyte HDL uptake *ex vivo* for both sexes. However, deletion of hepatocyte ERα decreased RCT *in vivo* accompanied with increases in early atherosclerotic lesion size at the aortic root sinus only in female mice. Thus, we show that hepatocyte ERα plays an important role in RCT to protect against lipid retention in the artery wall at early stages of atherosclerosis during WD feeding in female mice.

### 2. MATERIALS AND METHODS

#### 2.1. In vivo reverse cholesterol transport (RCT) assay

LKO-ERα mice on a C57BL/6J background were made by breeding ERα flox/flox mice with expressing cre recombinase under the control of albumin promoter as described before [2,15]. A cohort of LKO-ERα mice and their wild type (WT) littermates (n = 8) were used for *in vivo* reverse cholesterol transport analysis, which was modified from the method of Tanigawa et al. [16]. To keep the body-weight similar between mice, 8-week old mice were fed a Western-type diet (WD, from Harlan, TD 88137) for a short-term (5 days) before the assay. 5 days of WD did not increase adiposity either make body composition different between LKO and WT mice in both sexes (Table 1). Peritoneal macrophages from WT female mice were collected 72 h after intraperitoneal injection of thioglycollate. Macrophages were first cultured in DMEM with 10% FBS for 2 h to allow for plate surface attachment and then cultured in DMEM with 0.2% BSA overnight. The next day, macrophages were loaded with 3 μCi/ml [3H]-cholesterol and 25 μg/ml of acetylated LDL for 48 h. These labeled foam cells were washed twice, equilibrated in medium with 0.2% BSA for 6 h, centrifuged, and re-suspended in phenol red-free RPMI medium immediately before use. Then mice received intraperitoneal injections of the same amount of [3H]-cholesterol loaded foam cells (5 × 10^6 cells/mouse and 1.1 × 10^6 CPM/mouse) and were caged individually and fed WD ad libitum.

After 48 h, mice were euthanized and blood was collected by cardiac puncture, gallbladder was separated, and the liver was removed and frozen-flash for lipid extraction. Feces were collected continuously over 48 h prior to euthanizing.

Serum and liver lipids were extracted as described before [2]. The lipid layer was collected, evaporated, and re-suspended for scintillation counting. Gallbladders were lysed in 0.1 N NaOH for 4 h, and radioactivity was measured by scintillation counting. Feces of each mouse were soaked in 0.1 N NaOH overnight at 4 °C, then were homogenized the next day. One milliliter of the homogenized samples was subjected to scintillation counting. Protocols for all animal studies were approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center.

#### 2.2. Liver and serum lipid analysis

To further study the mechanism and early atherosclerosis, male and female LKO-ERα mice and their WT littermates on a C57BL/6J background (10 ± 1 weeks, n ≥ 19 for each group) were fed a WD for 7 months. Then mice were sacrificed after a 5-hour fasting. Liver and serum were stored at −80 °C for future analysis.

Liver lipids were analyzed by the Lipid Core Laboratory of the Vanderbilt Mouse Metabolic Phenotyping Center. VLDL, LDL, and HDL were separated from serum using fast performance liquid chromatography (FPLC). Pooled serum from 2 or 3 mice were used for each run of FPLC. Cholesterol in serum and FPLC fractions were determined by enzymatic colorimetric assays (Cholesterol Reagent and Tri-glycerides GPO Reagent kits from Infinity).

#### 2.3. Western blots

For liver proteins, frozen livers were lysed in T-PER tissue protein extraction reagent (*Thermo Scientific*) containing protease/phosphatase inhibitors (Sigma), and the protein concentrations were determined using BCA kit (*Thermo Scientific*). For serum proteins, 2 μl of serum or 10 μl of FPLC fractions (fractions of 25–30 for Supplemental Figure 2) were pooled for each sample and denatured in loading buffer (*Invitrogen*) containing reducing buffer (*Invitrogen*) and phospholipase and protease inhibitors (Sigma). Serum proteins were separated with gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated with primary antibody (1:1000) at 4 °C overnight, and with 2nd antibody (1:10,000) at room temperature for 1 h. Rabbit anti-mouse apoA1 antibody was from *Lifespan Biosciences* (LS-C20729); rabbit monoclonal anti-LDL receptor antibody was from abcam (ab52818); rabbit anti-mouse apoA1 antibody was from...
The initial serum level from WT or LKO-ER (K23500R); rabbit anti-SR-BI antibody was from Novus (NB400-104); sheep polyclonal anti-PDZK1 antibody was from R&D (AF4997); rabbit anti-mouse PON1 was from abcam (ab150032); rabbit polyclonal to adiponectin was from abcam (ab3455). IRDye 800CW goat anti-rabbit IgG, goat anti-mouse IgG, and donkey anti-sheep IgG were from LI-COR Biosciences.

2.4. in vitro cellular cholesterol efflux assay
The in vitro cellular cholesterol efflux assay was performed according to Asztalos et al. [17]. For preparation of HDL, 40 µl of 20% PEG600 in 200 mM glycine buffer was added in 100 µl of each serum sample, mixed, and incubated at room temperature for 20 min, then centrifuged at 10000 rpm for 30 min at 4 °C. For cells, peritoneal macrophages from female WT mice were collected 72 h after intraperitoneal injection of thioglycollate. Cholesterol labeled foam cells were prepared as above. These labeled foam cells were washed twice, equilibrated in medium with 0.2% BSA for 2 h, and a set of cells were collected for baseline 3H-radioactivity. The remaining cells were treated with 1% of initial serum level from WT or LKO-ER mice in DMEM with 0.2% BSA, cells treated with DMEM with 0.2% BSA were used as negative control and cells treated with apoA1 (50 µg/ml, Meridian) as positive control. 3H-radioactivities in media were determined using liquid scintillation counting and were used for cholesterol efflux calculation.

2.5. Ex vivo hepatocyte HDL and LDL uptake assays
Primary hepatocytes from LKO-ER and WT mice were isolated and cultured according to Foretz et al. [18]. Cells were plated in a 96-well plate at the density of 4 × 10^4 cells/well. HDL-cholesterol uptake assay was performed using HDL Uptake Assay Kit (Fluorometric) (abcam, Cat#: ab204717), and LDL-cholesterol uptake assay was performed using LDL Uptake Assay Kit (Fluorometric) (abcam, Cat#: ab204716). Assays were performed according to the manufacturer’s protocols. Briefly, primary hepatocytes were incubated in the assay media containing fluorescently-labeled HDL or fluorescently-labeled LDL for 3 h, then were washed, and the fluorescence was measured in a microplate reader at Ex/Em = 540/575 nm. Unlabeled-HDL or -LDL included in the kits is used for assay validation. The cell uptake was normalized by total protein amounts of each well.

2.6. Atherosclerosis analysis
While sacrificing mice that were fed a WD for 7 months (see 2.2.), hearts and aortas were embedded in optimal cutting temperature (OCT) compound and snap-frozen for further analysis. Frozen sections of 10 µm thickness were stained with oil-red-O, and serial 5 µm cryosections of the aortic sinus area were used for immunostaining as previously described [19]. For immunostaining, sections were fixed in cold acetone for 10 min, then washed with PBS for 2 times, blocked in background buster (Innovex) at 37 °C for 1 h, then incubated with rat anti-mouse CD106 (Millipore) for identifying VCAM-1 at 4 °C for overnight. The next morning, sections were washed with PBS for 3 times, then incubated with Alexa Fluor 488 goat-anti rat IgG (Invitrogen) and at 37 °C for 1 h. Slides were washed, and cell nuclei were counterstained with Hoechst (Vector Labs, Burlingame CA). Images were captured using Olympus IX81 microscope. Quantification of images was performed as described before [19]. For quantification of VCAM-1 expression in the lesion, multi-channel images were split first and the density (green component only) of VCAM-1 was measured using Image J software. Blind analysis was performed for all images.

At least two sections from each mouse were analyzed and all the experimental mice (n ≥ 19) were used for analysis.

2.7. Statistical analysis
All measurements passed D'Agostino & Pearson omnibus normality test (alpha = 0.05). For images, data are collected from two sections for each mouse, and mice that were fed a WD for 7 months were all included. Data shown are mean ± SD. Statistical analysis was performed with Student t test or RM (repeated measures by both factors) 2-way ANOVA with Bonferroni’s multiple comparison test.

3. RESULTS

3.1. Reverse cholesterol transport is impaired by the loss of hepatocyte ERz in female mice
To determine whether hepatocyte ERz regulates whole-body cholesterol handling, we performed an in vivo reverse cholesterol transport study. LKO-ERz mice and their WT littermates were fed a WD for a short-term (5 days) and then were injected with 3H-cholesterol loaded foam cells according to the methods of Tanigawa et al. [16]. In female WT mice, serum 3H-radioactivity decreased by 15% from 18 h to 24 h (from 40.3 ± 4.8 to 33.3 ± 5.4 × 10^3 dpm/ml, Figure 1A), further decreased by another 8% till 39 h, then leveled off. Serum 3H-radioactivity decreased by 14% in female LKO-ERz mice (from 41.3 ± 5.8 to 35.9 ± 6.6 × 10^3 dpm/ml, Figure 1A) and did not further decrease at later time points with a disappearance rate slower than their WT controls (Figure 1A). Deletion of hepatocyte ERz did not change the rate of serum 3H-radioactivity disappearance in male mice (Figure 1C). Deletion of hepatocyte ERz reduced 3H-cholesterol uptake by the liver by 22% in female mice (P < 0.01) but did not alter 3H-cholesterol liver uptake in male mice (Figure 1, B and D). Consistently, whole body 3H-cholesterol removal into feces decreased by 18% in female LKO-ERz mice (P < 0.05), but did not alter in male mice (Figure 1, B and D). There were no differences in body weight or adiposity between LKO-ERz mice and their WT littermates at this duration of feeding (Table 1). These results suggest that cholesterol clearance is decreased with the loss of hepatocyte ERz in female mice but not in male mice.

3.2. Deletion of hepatocyte ERz caused the enlargement of HDL particles, an effect more pronounced in female mice
To better understand the mechanism for the impaired RCT seen in LKO-ERz mice, a cohort of mice were fed a WD for 7 months. Body fat composition increased 1.5-fold in female LKO-ERz mice compared to WT controls (19.3 ± 3.6 vs. 29.7 ± 2.1%, P < 0.01, Table 1) but did not change in male mice (23.6 ± 2.6 vs. 25.4 ± 2.5%, Table 1). To understand why loss of hepatocyte ERz caused the increase in adiposity in female mice, we performed indirect calorimetry at the beginning of WD-feeding (Supplemental Figure 1). Energy expenditure was lower during light cycle than dark cycle in both sexes. Energy expenditure was lower in female LKO-ERz mice than their WT controls (Supplemental Figure 1). Fasting insulin level increased 1.4-fold in male LKO-ERz mice in comparison to their respective WT controls (1.5 ± 0.7 vs. 0.9 ng/ml, P < 0.05), and 1.7-fold in female LKO-ERz mice (2.0 ± 0.1 vs. 1.2 ng/ml, P < 0.01). Moreover, fasting cholesterol levels increased 3.6-fold in female LKO-ERz mice (178 ± 11 vs. 214 ± 16 mg/dl, P < 0.05) but did not change by the loss of hepatocyte ERz in male mice (223 ± 7 vs. 222 ± 12 mg/dl, Table 1). The absence of hepatocyte ERz did not significantly alter serum TGs in female or male mice, although there was a trend for higher TGs in LKO-ERz mice (Table 1).
Figure 1: In vivo reverse cholesterol transport was impaired in LKO-ERα female mice. Mice were fed a WD for 5 days to keep body weight similar. 3H-cholesterol loaded foam cells were injected to mice, serum was collected at different time points. Mice were sacrificed 48 h later, liver and gallbladder were collected. Feces were also collected continuously for 48 h. 3H-ractoactivities in serum in female (A) and male (C) mice. 3H-ractoactivities in liver and gallbladder and in feces were determined in female (B) and male (D) mice. Data are expressed as mean ± SD (n = 8). Statistical analysis was performed with RM (repeated measures by both factors) 2-way ANOVA with Bonferroni’s multiple comparisons tests. For Figure 1A, P < 0.05 at 39 h time point and P < 0.01 at 48 h time point.

Figure 2: Cholesterol was distributed in enlarged HDL particles in LKO-ERα mice. Serum lipoproteins were separated using FPLC for female mice (A) and male mice (C). HDL-cholesterol, IDL/LDL-cholesterol and VLDL-cholesterol were determined (B and D). Student’s t-test was used for statistical analysis for panels B and D.
Serum lipoproteins were separated by FPLC, and the cholesterol distribution in serum lipoprotein fractions was determined. Cholesterol was distributed in larger particles in female LKO-ERα mice as denoted by the shifted distribution curve toward the left in comparison to WT controls, and HDL and IDL/LDL cholesterol levels were higher in LKO-ERα mice (Figure 2, A and B). This shift in cholesterol distribution in serum lipoprotein fractions caused by the loss of hepatocyte ERα was less pronounced in male mice and only increased IDL/LDL-cholesterol levels in male LKO-ERα mice (Figure 2, C and D). To distinguish HDL from LDL particles, HPLC fractions of 25–30 from each run were pooled for immunoblotting of apoA1 (Supplemental Figure 2). ApoA1 was detected in the pooled fractions for LDL-sized particles from LKO-ERα mice in both sexes, demonstrating an enlargement of HDL particles in those mice (Supplemental Figure 2). The findings of enlarged HDL particles and increased cholesterol levels in LDL fractions suggested that clearance of blood cholesterol is delayed in female LKO-ERα mice, which was less obvious in male LKO-ERα mice.

3.3. HDL-cholesterol clearance is impaired in the absence of hepatocyte ERα in female mice

After 7 months of WD feeding, liver cholesterol content was 20% lower in female LKO-ERα mice than in WT controls (5.87 ± 1.06 vs. 4.39 ± 0.77 μg/mg, P < 0.05, Table 1), but deletion of hepatocyte ERα did not alter liver cholesterol content in male mice (4.75 ± 1.16 vs. 4.54 ± 0.66 μg/mg, Table 1). The absence of hepatocyte ERα did not change liver TGs or diacylglycerol (DG) content in either sex (Table 1). Liver phospholipids were reduced by the deletion of hepatocyte ERα in female and male mice (Table 1), and this reduction did not change liver receptor proteins for serum cholesterol clearance

A. Immunoblots for liver SR-BI, LDLR, and PDZK1. B–D. Quantification of the blots for SR-BI, LDLR, and PDZK1, respectively. Results include all the animals that were fed a WD for 7 months. Data are expressed as mean ± SD. Statistical analysis was performed with RM 2-way ANOVA with Bonferroni’s multiple comparison test.

Figure 3: Changes of liver receptor proteins for serum cholesterol clearance. A. Immunoblots for liver SR-BI, LDLR, and PDZK1. B–D. Quantification of the blots for SR-BI, LDLR, and PDZK1, respectively. Results include all the animals that were fed a WD for 7 months. Data are expressed as mean ± SD. Statistical analysis was performed with RM 2-way ANOVA with Bonferroni’s multiple comparison test.
not change the percent contribution of each phospholipid species (Supplemental Figure 3).

We next compared protein levels of liver receptors for serum cholesterol uptake between WT and LKO-ERα mice. For the HDL receptor, SR-BI protein levels were similar between WT and LKO-ERα mice of both sexes (Figure 3, A and B). In contrast, LDLR protein levels were 22% lower in female LKO-ERα mice and 18% lower in male LKO-ERα mice than their respective WT controls (Figure 3, A and C). PDZK1 is an adaptor protein that plays an important role for HDL-cholesterol uptake mediated by SR-BI [20]. PDZK1 expression is regulated by estrogen [21]. In the present study, deletion of hepatocyte ERα decreased PDZK1 protein levels by 33% in female mice (P < 0.01) and by 22% that was not statistically significant in male mice (Figure 3, A and D).

To further understand the role of hepatic ERα in cholesterol uptake by the liver, primary hepatocytes were isolated from each group of mice and cultured to define hepatic HDL- and LDL-cholesterol uptake. As shown in Figure 4, deletion of hepatocyte ERα decreased HDL-cholesterol uptake by 15% in females (40.1 ± 2.5 vs. 34.2 ± 3.7 ng/µg/hr, P < 0.05), and by 16% in males (27.1 ± 3.8 vs. 21.9 ± 1.7 ng/µg/hr, P < 0.05). However, deletion of hepatocyte ERα did not alter LDL uptake in either female or male mice (Figure 4).

3.4. HDL capacity for cellular cholesterol efflux is decreased by the loss of hepatocyte ERα

Since HDL-cholesterol levels increased and HDL particle size was enlarged in LKO-ERα mice, we evaluated the HDL function with regard to cholesterol efflux from foam cells. HDL was isolated from long-term WD fed mice and was incubated with foam cells loaded with 3H-cholesterol for 6 h. HDL capacity for cholesterol efflux was reduced by 20% by deletion of hepatocyte ERα in female mice (9.74 ± 0.46% vs. 7.83 ± 1.1%, P < 0.05) and by 33% in male mice (9.65 ± 1.2% vs. 6.47 ± 0.98%, P < 0.01, Figure 5A).

We examined changes in serum apolipoproteins that play key roles in HDL cholesterol efflux capacity between groups. Deletion of hepatocyte ERα decreased serum apoE in both female and male mice (Figure 5, B and D). Levels for an acute inflammatory marker, serum amyloid A1 (SAA1), were significantly higher in male than in female mice. Deletion of hepatocyte ERα did not change SAA1 in female mice but decreased SAA1 in male mice (Figure 5, B and F). In addition, serum paraoxonase 1 (PON1) and adiponectin levels were higher in female mice but were not affected by the deletion of hepatocyte ERα (Figure 5, B, C and E). Both adiponectin and PON1 are protective against the development of insulin resistance [22,23]. The lower levels of PON1 and adiponectin in serum were consistent with the higher fasting insulin levels in male mice (Table 1). However, changes of serum PON1 and apoE were only partially related to protein amounts of PON1 and apoE in the liver (Supplemental Figure 4). Deletion of hepatocyte ERα significantly increased serum phospholipid transfer protein (PLTP) levels in both female and male mice (Figure 5, B and G). This observed change may be due to changes in liver phospholipids (Table 1).

Then we compared early stages of atherosclerosis to verify whether the impaired RCT seen in LKO-ERα mice would contribute to the lipid

Figure 4: Hepatocyte uptake of HDL and LDL. Hepatocytes were isolated from female (A) and male (B) LKO-ERα and WT mice. HDL and LDL uptake assay were performed after an overnight culturing. Data are from two independent experiments performed in duplicates (n = 4 for each group). Data are expressed as mean ± SD. Statistical analysis was performed with Student’s t-test.
retention in the artery wall. Atherosclerotic lesion size at the aortic root sinus was determined after the oil-red-O staining. Atherosclerotic lesions developed in all LKO-ERα female mice, while the early lesion was not consistently detectable in WT controls (Figure 6A). The lesion size was increased 5.6-fold in female mice without hepatocyte ERα compared to WT controls (2981 ± 1684 vs. 529 ± 718 × 10^3 μm^2, P < 0.001, Figure 6, A and B). There was no increase in atherosclerotic lesions in male LKO-ERα mice compared to WT controls (data not shown). Expression of VCAM-1 over early foam cell lesions plays critical role for atherosclerosis progression by regulating the monocyte recruitment to artery walls [24]. As shown in Figure 6, VCAM-1 expression on the surface of the lesion area in female LKO-ERα mice was 1.44-fold of its expression in artery walls in female WT mice (Figure 6, C and D).

4. DISCUSSION

In the present study, we show that hepatocyte ERα contributes to several key steps governing the development of atherosclerosis in female mice: cholesterol efflux from foam cells to HDL, HDL-delivery of cholesterol to hepatocytes, and removal of cholesterol from the body into feces, collectively termed reverse cholesterol transport. When hepatocyte ERα signaling was absent in female LKO-ERα mice, clearance of blood cholesterol through liver was decreased, leading to cholesterol accumulation in the circulation. Deletion of hepatocyte ERα increased early-stage atherosclerosis in female mice but not in male mice, indicating an important role of hepatocyte ERα for the protective effects of estrogen signaling with regard to atherosclerosis.

We show that the loss of hepatocyte ERα impairs HDL capacity for cholesterol efflux from foam cells. Although higher levels of HDL-cholesterol in plasma are associated with a lower risk of CHD, pharmacologic approaches to raise HDL-cholesterol have not reduced CHD risk [25–27]. Therefore, efforts to enhance HDL cholesterol efflux capacity or increase the whole-body reverse cholesterol transport, regardless of HDL level, may be of therapeutic interest. Indeed, cholesterol efflux capacity is inversely correlated with CHD independent of HDL-cholesterol level [28,29]. We previously reported that loss of ovarian hormones influences HDL protein composition with high-fat diet feeding [30]. Both PLTP and apoE play important roles in HDL capacity for cellular cholesterol efflux, and their activities are associated with CHD risk [31–33]. In the present study, HDL capacity for cholesterol efflux was impaired in both sexes mice by the loss of hepatocyte ERα, which was associated with exacerbated insulin resistance with WD, and changes of serum PLTP and apoE.

We report that deletion of hepatocyte ERα impairs blood cholesterol clearance and RCT, which leads to early stages of lipid retention in the artery wall. Humans carry the majority of blood cholesterol in HDL particles, thus delayed clearance of LDL cholesterol results in increased CHD risk [3]. Additionally, impairment of HDL-cholesterol clearance from the circulation increases CHD risk in humans with loss of function variant SCARB1 (P376L). These individuals have a profound HDL-related phenotype with increases in large HDL particles [34]. Mice carry the majority of blood cholesterol in HDL particles and
are resistant to atherosclerosis. ApoE- or LDLR-null mouse models have increased LDL-cholesterol and been frequently used for atherosclerosis studies. However, apoE- and LDLR-null mice have impaired cholesterol clearance and are not ideal models for studying liver cholesterol uptake and RCT. Thus, clearance of blood cholesterol through HDL receptor, SR-BI, in the liver is important in mice, but this pathway is also applicable to humans. Impaired HDL-cholesterol uptake in SR-BI knockout mice with LDLR-null background increases plasma cholesterol and enlarges HDL particle size, leading to increased atherosclerotic burden [35–37]. In the present study, HDL-cholesterol uptake by the liver was impaired and led to early stages of atherosclerosis in female LKO-ERα mice in which hepatocyte estrogen signaling was reduced. Deletion of hepatocyte ERα decreased LDLR in both sexes mice, in line with our previous report that hepatocyte ERα signaling is involved in VLDL metabolism [2,15]. It has been reported that estrogen up-regulates mRNA expression of SR-Bi, the HDL receptor, and promotes HDL-cholesterol uptake [38,39]. We did not see that liver SR-Bi protein abundance was altered by the deletion of hepatocyte ERα. We did see that the loss of hepatocyte ERα down-regulated the expression of PDZK1, the adaptor protein for SR-Bi mediated HDL-cholesterol uptake. Mice without PDZK1 have increased plasma cholesterol, enlarged HDL particles, and increased atherosclerosis with WD [20,40,41], a similar phenotype to the female LKO-ERα mice in our study. PDZK1 is required for endocytosis of SR-Bi from the basolateral membrane and traffics to the apical membrane to mediate rapid transport of cholesterol and cholesteryl ester from HDL into bile [42,43]. We cannot rule out that PDZK1 regulates the subcellular location of SR-Bi in the present study, which might functionally reduce SR-Bi action in LKO-ERα mice. Recently, SR-Bi has been reported to be mainly expressed in sinusoidal endothelial cells in the liver and a transcytosis mechanism might be required for SR-Bi function [44]. This study is consistent with our observation that SR-Bi from liver tissues did not significantly decrease in LKO-ERα mice, in which ERα is deleted only in hepatocytes [2]. Future studies are required to clarify the underlying mechanism. Our studies suggest that estrogen signaling through hepatocyte ERα protects against atherosclerosis by at least two means. First, hepatocyte ERα protects female mice from obesity and insulin resistance.

Figure 6: Deletion of hepatocyte ERα increased atherosclerosis in female mice after a long-term of Western-type diet. Female LKO-ERα mice and their wild-type (WT) littermates were fed a Western-type diet for 7 months. A. Cross-sectional area of proximal aortas was stained with oil-red-O, which was used for the quantification of atherosclerosis in B. C–D. VCAM-1(green) positive staining is stronger in LKO-ERα mice than in WT controls and the quantification is shown in C. Nuclei were counterstained with Hoechst (blue). Data are collected from two sections for each mouse, and all experimental mice that were fed a WD for 7 months were included. Data are expressed as mean ± SD. n ≥ 19, statistical analysis was performed with Student’s t-test.
after long-term WD. Obesity and insulin resistance may contribute to the development of atherosclerosis through multiple pathways [5]. HDL composition and binding to SR-BI for liver cholesterol uptake are impaired in obese mice [45]. In line with this, we observed that HDL capacity for cholesterol efflux was decreased in female LKO-ERα mice, which might be related to increased obesity and insulin resistance in these mice. Second, the absence of hepatocyte ERα decreases liver cholesterol uptake by downregulating its target gene PDZK1. In BMI-matched female LKO-ERα and WT mice after short-term WD, in vivo cholesterol efflux assays showed that liver cholesterol uptake was diminished in female LKO-ERα mice. Results from ex vivo studies, i.e. cholesterol efflux capacity and hepatocyte HDL- and LDL-cholesterol uptake assays, showed the effects of the loss of hepatocyte ERα in both sexes. The diminished effects in vivo in male mice may be due to the difference of estrogen levels between male and female mice. We previously showed that blood estradiol levels are increased in male LKO-ERα mice but not in female LKO-ERα mice [2,15]. Estrogen’s action in peripheral tissues such as macrophages/immune-system is also protective against atherosclerosis [12]. Furthermore, SAA1 is a contributor for atherosclerosis progression [46]. In current study, the loss of hepatic ERα decreased serum SAA1 only in male mice, which may have protected male LKO-ERα mice against atherosclerosis.

There may be discordance between the effects of naturally cycling estrogens and estrogen treatment approaches after menopause with regard to lipoprotein metabolism and atherosclerosis risk. Treatment of menopausal women with oral estrogens, which have a larger hepatic effect, increases HDL and lowers LDL. This effect is less potent with transdermal estradiol, which is delivered to the peripheral circulation and has less hepatic effects [47,48]. Our studies are limited to defining the effect of endogenous estrogen signaling through hepatocyte ERα, deficiency of which resulted in the accumulation of cholesterol in circulation, presumably because of impairment in HDL’s ability to deliver cholesterol. In vivo, the effects of estrogens to limit liver fat content are likely distributed among tissues, including estrogen signaling in adipose tissues, muscles, and the liver. In our previously published work, we showed that mice with ERα deletion from hepatocytes develop steatosis on high-fat diet [2]. Those mice were obese because they had undergone ovariectomy which leads to weight gain. By contrast, Hart-Unger et al. recently showed that female LKO-ERα mice with intact ovaries and without E2 treatment did not worsen fatty liver during high fat diet feeding compared to controls, but these mice were only minimally heavier than controls [49]. Comparison of these studies suggests the role of hepatocyte ERα to limit steatosis is more important in the setting of more severe obesity when fatty acid load to the liver would be higher [2,49]. Additionally, mice lacking ERα in skeletal muscle have severe metabolic dysfunction and impaired fatty acid utilization [50], which would be expected to worsen fatty liver by increasing fatty acid delivery to liver.

5. CONCLUSION

Hepatocyte ERα promotes HDL’s ability for cholesterol efflux from foam cells by modulating molecular signaling within the liver in both female and male mice. Hepatocyte ERα promotes cholesterol uptake by the liver and whole-body reverse cholesterol transport, which process is especially important in females. Our results suggest that hepatocyte ERα is required for the actions of endogenous estrogens to protect against atherosclerosis. Given that hepatocyte ERα contributed to steps of RCT in males as well, this pathway may be beneficially targeted to improve steps of reverse cholesterol transport in both males and females.

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DISCLOSURES

All authors have nothing to disclose.

CONFLICT OF INTEREST

None.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.molmet.2017.12.012

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