Original Article

MOLECULAR METABOLISM

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 ^{3}H-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 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.

Table 1 — Sex dimorphism and genotype differences in physiology related to lipid metabolism.

	Female		Male	
	WT	LKO-ERα	WT	LKO-ERα
1st cohort_before WD				
Body weight (g)	18.2 ± 1.7	18.3 ± 1.6	24.2 ± 1.1^	23.6 ± 2.3
Adiposity (%)	17.0 ± 1.7	16.3 ± 1.6	13.8 ± 1.8^	13.2 ± 1.2
1st cohort_5-day WD				
Body weight (g)	18.1 ± 1.3	18.5 ± 1.2	24.3 ± 1.1^	23.2 ± 1.4
Adiposity (%)	17.1 ± 2.8	16.9 ± 1.3	13.4 ± 2.0^	12.4 ± 1.6
2nd cohort_7-month WD				
Body weight (g)	24.1 ± 2.2	26.3 ± 2.8	34.7 ± 3.9^	34.6 ± 2.4
Adiposity (%)	19.3 ± 3.6	29.7 ± 2.1**	23.6 ± 2.6^	25.4 ± 2.5
Fasting insulin (ng/ml)	0.3 ± 0.1	0.7 ± 0.2*	1.5 ± 0.7^	2.1 ± 0.9*
Fasting glucose (mg/dl)	142 ± 14	158 ± 20	169 ± 28	161 ± 25
Fasting cholesterol (mg/dl)	178 ± 11	214 ± 16*	223 ± 7	222 ± 12
Fasting TGs (mg/dl)	70 ± 2	81 ± 7	78 ± 22	95 ± 31
Liver TGs (μg/mg)	79.5 ± 18.5	81.5 ± 11.7	103.9 ± 14.4	93.8 ± 22.9
Liver DG (μg/mg)	0.31 ± 0.06	0.36 ± 0.15	0.49 ± 0.23	0.47 ± 0.34
Liver cholesterol (μg/mg)	5.87 ± 1.06	4.39 ± 0.77*	4.75 ± 1.16	4.54 ± 0.66
Liver phospholipids (μg/mg)	16.7 ± 5.7	12.6 ± 2.5*	16.1 ± 4.3	13.8 ± 3.76*

Data shown are mean ± SD. Statistical analysis was performed by RM 2-Way ANOVA with multiple comparison test.
*$P < 0.05$; **$P < 0.01$ in comparison to respective WT controls.
^$P < 0.05$, WT male mice in comparison to WT 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 ^{3}H-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 ^{3}H-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 Triglycerides 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 apoB antibody was from *Lifespan Biosciences* (LS-C20729); rabbit monoclonal anti-LDL receptor antibody was from *abcam* (ab52818); rabbit anti-mouse apoA1 antibody was from

