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The deletion of the estrogen receptor α gene reduces susceptibility to estrogen-induced cholesterol cholelithiasis in female mice

Ornella de Bari [a], Helen H. Wang [a], Piero Portincasa [b], Min Liu [c], David Q.-H. Wang [a,*]

[a] Department of Internal Medicine, Division of Gastroenterology and Hepatology, Saint Louis University School of Medicine, St. Louis, MO 63104, USA
[b] Clinica Medica "A. Murri", Department of Biomedical Sciences and Human Oncology, University of Bari Medical School, Bari, Italy
[c] Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, Cincinnati, OH 45237, USA

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ABSTRACT

Compelling evidence has demonstrated that estrogen is a critical risk factor for gallstone formation and enhances cholesterol cholelithogenesis through the hepatic estrogen receptor α (ERα), but not ERβ. To study the lithogenic mechanisms of estrogen through ERα, we investigated whether the deletion of *Erα* protects against gallstone formation in ovariectomized (OVX) female mice fed a lithogenic diet and treated with 17β-estradiol (E_2) at 0 or 6 μg/day for 56 days. Our results showed that the prevalence of gallstones was reduced from 100% in OVX ERα (+/+) mice to 30% in OVX ERα (−/−) mice in response to high doses of E_2 and the lithogenic diet for 56 days. Hepatic cholesterol secretion was significantly diminished in OVX ERα (−/−) mice compared to OVX ERα (+/+) mice even fed the lithogenic diet and treated with E_2 for 56 days. These alterations decreased bile lithogenicity by reducing cholesterol saturation index of gallbladder bile. Immunohistochemical studies revealed that ERα was expressed mainly in the gallbladder smooth muscle cells. High levels of E_2 impaired gallbladder emptying function mostly through the ERα and cholecystokinin-1 receptor pathway, leading to gallbladder stasis in OVX ERα (+/+) mice. By contrast, gallbladder emptying function was greatly improved in OVX ERα (−/−) mice. This markedly retarded cholesterol crystallization and the growth and agglomeration of solid cholesterol crystals into microlithiasis and stones. In conclusion, the deletion of *Erα* reduces susceptibility to the formation of E_2-induced gallstones by diminishing hepatic cholesterol secretion, desaturating gallbladder bile, and improving gallbladder contraction function in female mice.

1. Introduction

Epidemiological and clinical studies have clearly shown that women are twice as likely as men to suffer from cholesterol cholelithiasis at all ages in every population investigated. This gender difference begins since puberty and continues through the childbearing years. Most, but not all, studies have found that the use of oral contraceptive steroids and conjugated estrogens markedly increases cholelithogenesis in premenopausal women, leading to hepatic cholesterol hypersecretion and biliary lithogenicity [1–4]. Because plasma concentrations of female sex hormones, especially estrogen, increase positively and linearly with the duration of gestation, the risk of gallstone formation becomes higher in the third trimester of pregnancy. Increasing parity is also a risk factor for gallstones, especially in younger women. Notably, parity and length of the fertility period increase the incidence of gallstones [5], as well as both the number and frequency of pregnancy are important risk factors for gallstone formation [6–10]. Thus, elevated estrogen levels are a critical risk factor for the formation of cholesterol gallstones during pregnancy. Moreover, biliary sludge (a precursor to gallstones) and microlithiasis are observed to disappear spontaneously after parturition in ~60% of cases mostly because of a significant reduction in estrogen concentrations [11]. Similar lithogenic effects are also found during estrogen therapy in postmenopausal women and in men with prostatic carcinoma [12–16]. All of these findings underscore the importance of estrogen on the pathogenesis of gallstones.

As found from human and animal studies, high levels of estrogen enhance susceptibility to cholesterol cholelithiasis by promoting hepatic hypersecretion of biliary cholesterol and increasing bile lithogenicity [17–22]. Such alterations lead to a dramatic increment in cholesterol saturation of bile, thereby increasing risk of developing gallstones [1, 23–25]. It has been established that estrogen plays a critical role in enhancing cholelithogenesis by activating the hepatic estrogen receptor α (ERα), but not ERβ [26]. While ERα is activated by E_2, mice still keep synthesizing cholesterol in the liver even though excess amounts of cholesterol are available from a high-cholesterol diet. These metabolic abnormalities indicate a disruption in the negative feedback regulation of cholesterol synthesis in the liver, which is determined by an "estrogen-

Abbreviations: ABC, ATP-binding cassette (transporter); CCK, cholecystokinin; CSI, cholesterol saturation index; E_2, 17β-estradiol; ER, estrogen receptor; ERα, ER subtype α; ERβ, ER subtype β; GPR30, the G protein-coupled receptor 30; OVX, ovariectomized.

* Corresponding author at: Department of Internal Medicine, Division of Gastroenterology and Hepatology, Saint Louis University School of Medicine, St. Louis, MO 63104, USA.
E-mail address: dwang15@slu.edu (D.Q.-H. Wang).

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ERα-SREBP-2" pathway [27]. As a result, this induces excess amounts of newly synthesized cholesterol available for biliary hypersecretion, thus leading to cholesterol-supersaturated bile, as well as rapid cholesterol crystallization and gallstone formation in mice treated with high doses of E_2 [26]. Based on these observations, a novel concept has been proposed that estrogen enhances cholesterol cholelithogenesis through the ERα signaling pathway in the liver and that higher risks for gallstone formation in women than in men are related to differences in how cholesterol is handled in the liver under conditions of high estrogen levels [28].

Because ERα has a crucial effect on the formation of E_2-induced cholesterol gallstones, in the current study, we studied whether the targeted disruption of the *Erα* gene protects against the formation of gallstones in mice treated with high doses of E_2 and fed a lithogenic diet for 56 days. Our results show that the susceptibility to E_2-induced cholesterol cholelithiasis is significantly reduced through the ERα-mediated pathways in the liver and gallbladder in ovariectomized (OVX) ERα (−/−) mice even treated with E_2 and fed the lithogenic diet for 56 days.

2. Materials and methods

2.1. Animals and diets

The inbred AKR/J mice of both genders from the Jackson Laboratory (Bar Harbor, ME) were bred to generate female mice for the studies. Although AKR/J mice are a gallstone-resistant strain, they are still susceptible to estrogen-induced cholesterol gallstones [26]. AKR/J mice have intact expression of the *Erα* and *Erβ* genes; however, expression levels of *Erβ* are 50-fold lower than those of *Erα* in the liver [26]. Thus, the AKR/J strain was used as control mice, i.e., ERα (+/+) mice. The ERα (+/−) mice of both genders on a C57BL/6 J genetic background (the Jackson Laboratory) were crossed with AKR/J mice for ten generations to produce a new strain of ERα (+/−) mice on an AKR/J genetic background. We have established breeding colonies of these mice in-house. Of note, ERα (+/−) heterozygotes are healthy and fertile, and they exhibit no obvious phenotypes in association with the disrupted *Erα* genotype. A cross between heterozygous ERα (+/−) mice resulted in the live birth of normal litter sizes of homozygous ERα (−/−) mice.

To exclude possible interindividual differences in endogenous estrogen concentrations, at 4 weeks of age, all female mice were ovariectomized (OVX). At 8 weeks of age, the mice were implanted subcutaneously with pellets releasing E_2 at 0 or 6 μg/day for 56 days (Innovative Research of America, Sarasota, FL) according to our published methods [26]. All animals were maintained in a temperature-controlled room (22 ± 1 °C) with a 12-hour day cycle (0600 h–1800 h) and were provided free access to water and normal mouse chow containing trace cholesterol (<0.02%) (Lab Rodent Diet, St. Louis, MO). For gallstone studies, mice at 8 weeks old were fed a lithogenic diet containing 1% cholesterol, 15% butter fat, and 0.5% cholic acid for 56 days. All procedures were in accordance with current NIH guidelines and were approved by the Institutional Animal Care and Use Committee of Saint Louis University (St. Louis, MO).

2.2. Microscopic studies of gallbladder bile and gallstones

After anesthetization with pentobarbital, a cholecystectomy was performed on fasted mice. Gallbladder bile was collected before (day 0, on chow) and at frequent intervals after feeding the lithogenic diet for 3, 6, 9, and 12 days for cholesterol crystallization studies (n = 5 per group for each time point), as well as for 56 days for gallstone studies (n = 20 per group). The entire gallbladder bile was studied by phase contrast and polarized light microscopy for observing the presence of mucin gel, liquid crystals, solid cholesterol crystals, sandy stones, and gallstones [29]. The images of cholesterol monohydrate crystals and gallstones were analyzed by a Carl Zeiss Imaging System with an AxioVision Rel 4.6 software (Carl Zeiss Microimaging GmbH, Göttingen, Germany). After microscopic analysis, gallbladder bile was frozen and stored at −20 °C for lipid analyses.

2.3. Measurement of biliary lipid output

During laparotomy, the common bile duct was cannulated with a PE-10 polyethylene catheter. After successful catheterization and flow of fistula bile, cholecystectomy was performed. The first hour sample of hepatic bile was collected by gravity in mice (n = 5 per group) [30]. Subsequently, hepatic bile was examined by polarizing light microscopy and the volume of each bile sample was measured. During surgery and hepatic bile collection, mouse body temperature was kept at 37 ± 0.5 °C with a heating lamp and monitored with a thermometer. All bile samples were frozen and stored at −20 °C for further lipid studies.

2.4. Dynamic measurement of gallbladder emptying function

To explore whether the activation of ERα by E_2 impairs gallbladder contractility, a dynamic measurement of gallbladder motility function was performed in mice in response to a high-fat meal or to exogenously administered sulfated cholecystokinin octapeptide (CCK-8) at 56 days on the lithogenic diet [31]. After mice (n = 5 per group) were fasted overnight but had free access to water, they were anesthetized with pentobarbital. During laparotomy, a PE-10 catheter was inserted into the duodenum. It was externalized through the left abdominal wall and connected to an infusion pump for infusing corn oil. The right jugular vein was cannulated with a PE-10 catheter for intravenous CCK-8 injection. After all surgical procedures were completed, the gallbladder was clearly exposed and its volume was measured with a microcaliper according to previously published methods [31].

After the first group of mice was intraduodenally infused with corn oil (i.e., a high-fat meal) or 0.9% NaCl (as a control) at 40 μL/min for 5 min, postprandial gallbladder volume was determined at 30 min after the infusion. The second group of mice was intravenously injected through the jugular vein with exactly 17 nmol/kg body weight of CCK-8 dissolved in 100 μL of phosphate buffered saline (PBS) solution, or 100 μL of only PBS solution (as a control). Following that, gallbladder volume was measured every 10 min for 30 min. Gallbladder emptying rate was determined by a difference in gallbladder size before and after the duodenal infusion of corn oil or the intravenous injection of CCK-8.

2.5. Biliary lipid analysis

Biliary phospholipids were determined as inorganic phosphorus by the method of Bartlett [32]. Biliary cholesterol was measured using an enzymatic assay [33]. Total bile salt concentration was enzymatically determined by the 3α-hydroxysteroid dehydrogenase method [34]. Individual bile salt species were analyzed by high-performance liquid chromatography (HPLC) [35] and hydrophobicity index of bile samples was calculated according to Heuman's method [36]. Cholesterol saturation index (CSI) of pooled gallbladder bile was calculated from critical tables [37]. Relative lipid composition of pooled gallbladder bile was plotted on a condensed phased diagram. For graphic analysis, the phase limits of the micellar zone and the crystallization pathways were extrapolated from model bile systems developed for taurocholate at 37 °C and at a total lipid concentration of 9 g/dL [38].

2.6. Immunohistochemical staining

The blocks of paraffin-embedded gallbladder tissues were cut at 4-μm thickness, dewaxed, and rehydrated. For ERα staining, antigen retrieval was carried out by boiling in 10 mM citrate buffer (pH 7.0) for 1 min. All the staining processes were performed by using Histostain Plus 3rd Gen IHC Detection Kit according to the