Overexpression of estrogen receptor α increases hepatic cholesterogenesis, leading to biliary hypersecretion in mice

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Abstract We explored whether there is an “estrogen-ERα-SREBP-2” (for estrogen-estrogen receptor subtype α-sterol-regulatory element binding protein-2) pathway for regulating hepatic cholesterol biosynthesis in ovariectomized AKR mice treated with 17β-estradiol (E2) at 6 μg/day or E2 plus the antiestrogenic agent ICI 182,780 at 125 μg/day and on chow or fed a high-cholesterol (1%) diet for 14 days. To monitor changes in cholesterol biosynthesis and newly synthesized cholesterol secreted into bile, incorporation into digitonin-precipitable sterols in mice treated with 25 mCi of [3H]water was measured in extracts of liver and extrahepatic organs 1 h later and in hepatic biles 6 h later. ERα upregulated SREBP-2, with resulting activation of SREBP-2-responsive genes in the cholesterol biosynthetic pathway. The E2-treated mice continued to synthesize cholesterol in spite of its excess availability from high dietary cholesterol, which reflects a loss in controlling the negative feedback regulation of cholesterol synthesis. These alterations augmented biliary cholesterol secretion and enhanced the lithogenicity of bile. However, these lithogenic effects of E2 were fully blocked by ICI 182,780. We conclude that during estrogen treatment, more newly synthesized cholesterol determined by the estrogen-ERα-SREBP-2 pathway is secreted into bile, leading to biliary cholesterol hypersecretion. These studies provide insights into therapeutic approaches to cholesterol gallstones in high-risk subjects, especially those exposed to high levels of estrogen.—Wang, H. H., N. H. Afdhal, and D. Q-H. Wang. Overexpression of estrogen receptor α increases hepatic cholesterogenesis, leading to biliary hypersecretion in mice. J. Lipid Res. 2006. 47: 778–786.

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Epidemiological investigations have found and clinical studies have confirmed that at all ages, women are twice as likely as men to form cholesterol gallstones (1–3), suggesting that estrogen may be an important risk factor for the formation of cholesterol gallstones in humans. Recently, we (4) found that estrogen promotes cholesterol gallstone formation through increasing expression of the hepatic estrogen receptor subtype α (ERα) but not ERβ. Furthermore, the ERα-selective agonist propylpyrazole, but not the ERβ-selective agonist diarylpropionitrile, augments hepatic cholesterol output, resulting in cholesterol-supersaturated bile and gallstones. Similar to estrogen treatment, tamoxifen treatment significantly promotes biliary cholesterol secretion and cholesterol gallstone formation in gonadectomized AKR mice of both genders (4) and increases gallstone prevalence in women (5). In addition, the lithogenic actions of estrogen can be totally abolished by its antagonist ICI 182,780. It is concluded, therefore, that hepatic ERα plays a crucial role in the formation of gallstones in exposure to high levels of estrogen (4). It has already been established that biliary cholesterol hypersecretion is the primary cause of cholesterol gallstones in humans and in several animal models (1, 2). Accumulated evidence from human and animal studies (6–8) showed that high levels of estrogen significantly stimulate the activity of HMG-CoA reductase, the rate-limiting enzyme in hepatic cholesterol biosynthesis, even under high dietary cholesterol loads. These observations suggest that there may be an increased delivery of cholesterol to bile from de novo synthesis in the liver. Furthermore, studies in humans and animals suggested that estrogen could augment the capacity of dietary cholesterol to induce cholesterol supersaturation of bile (4, 8–10). More recently, it was observed that high doses of estrogen significantly enhance intestinal cholesterol absorption, mostly attributable to an upregulated expression of the intestinal sterol influx...
transporter Niemann-Pick C1-like 1 protein via the intestinal ERα pathway (11). Despite these observations, no information is available on the metabolic abnormalities underlying major sources of the excess cholesterol leading to the supersaturation of bile and the formation of cholesterol gallstones induced by estrogen. In this study, to elucidate the molecular mechanism by which estrogen increases the level of cholesterol saturation of bile, we quantitated the contribution of newly synthesized cholesterol to biliary output in a gallstone-resistant strain of the ovariectomized (OVX) AKR mouse treated with high doses of estrogen and fed a high-cholesterol diet. Furthermore, we explored whether the hepatic ERα activated by estrogen interferes with the negative feedback regulation of cholesterol biosynthesis by stimulating sterol-regulatory element binding protein-2 (SREBP-2), which activates the SREBP-2 response genes for the cholesterol biosynthetic pathway. We found that during estrogen treatment, mice continue to synthesize cholesterol in the face of its excess availability from the high-cholesterol diet, suggesting that there is a loss in the negative feedback regulation of cholesterol biosynthesis that results in excess secretion of newly synthesized cholesterol and supersaturation of bile, which predispose to cholesterol precipitation and gallstone formation.

MATERIALS AND METHODS

Chemicals

The radioisotopes [3H]water, dl-[-5-3H]mevalonolactone, and dl-[-3-4C]HMG-CoA were purchased from NEN Life Science Products (Boston, MA). 17β-Estradiol (E2)-releasing pellets were purchased from Innovative Research of America (Sarasota, FL). ICI 182,780 (Fulvestrant; 7α-[9-(4,4,5,5,5-pentafluoropentylsulfinyl)nonyl]estr-1,3,5-(10)-triene-3,17β-diol) was from AstraZeneca Pharmaceuticals (Wilmington, DE).

Animals and diets

Female AKR/J mice, 3 weeks old, were purchased from Jackson Laboratory (Bar Harbor, ME). The gallstone-resistant AKR strain is homozygous for resistant Lith alleles (12). All mice were maintained in a temperature-controlled room (22 ± 1°C) with a 12 h day cycle (6 AM-6 PM) and were provided free access to water and normal mouse chow containing trace (<0.02%) cholesterol (Harlan Teklad Laboratory Animal Diets, Madison, WI). To exclude possible interindividual differences in endogenous estrogen concentration, all experimental mice, after ovariec- tomy, were implanted with E2-releasing pellets (4). In brief, at 4 weeks of age, gen concentration, all experimental mice, after ovariectomy, were

Measurement of newly synthesized biliary cholesterol

The contribution of newly synthesized hepatic cholesterol to biliary output was determined according to the method of Turley and Dietschy (16). In brief, the mice were anesthetized with pentobarbital in a dose of 35 mg/kg. Between 8:30 and 9:30 AM, identical amounts (25 mCi) of [3H]water in 100 μl of 0.9% NaCl were injected via jugular vein as a bolus to chow- or cholesterol-fed OVX AKR mice (n = 4 per group) treated with E2 or with or without ICI 182,780. The animals were left in the cages for 6 h to allow equilibration of the tritiated water in the body compartments of the mice. All of the mice were sexed at 11 days. Female AKR mice with intact ovaries were used as controls. All procedures were in accordance with current National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of Harvard University.

Determination of cholesterol synthesis in vivo

The rates of cholesterol synthesis in the liver and peripheral organs were measured in vivo according to published methods (20, 21). The mice (n = 4 per group) were given an intraperitoneal injection of 25 mCi of [3H]water, and after 1 h, they were anesthetized and exsanguinated. All of the organs were removed and the tissues were assayed for [3H]content. To isolate the cholesterol in each organ, the organs were converted to nanomoles of [3H]water incorporated into digitonin-precipitable sterols measured as digitonin precipitation and pyridine solubilization. The amount of newly synthesized cholesterol secreted into bile was calculated according to the formula (dpm [3H]-labeled sterol) × 1.45/([specific activity of plasma water] × 18). The number 1.45 represents the nanomoles of acetyl-CoA units incorporated into sterols for each nanomole of [3H]water. Because 18 nmol of acetyl-CoA is needed for the synthesis of 1 nmol of cholesterol, the number of acetyl-CoA units incorporated into cholesterol was converted to nanomoles of cholesterol synthesized by dividing the value by 18 (18, 19).

Quantitative real-time PCR assay

Total RNA was extracted from fresh liver tissues (n = 4 per group) using RNeasy Midi (Qiagen, Valencia, CA). Reverse-transcription reaction was performed using the SuperScript II First-Strand Synthesis System (Invitrogen, Carlsbad, CA) with 5 μg of total RNA and random hexamers to generate cDNA. Primer Express Software (Applied Biosystems, Foster City, CA) was used to design the primers (Table 1) based on sequence data available from GenBank. Real-time PCR assays for all samples were performed in triplicate (22). Relative mRNA levels were calculated using the threshold cycle of an unknown sample against a standard curve with known copy numbers. To obtain a normalized target value, the target amount was divided by the endogenous reference amount of rodent Gap43 as the invariant control (part 4308313; Applied Biosystems).
Determinant of activity of hepatic HMG-CoA reductase

Liver samples were collected from nonfasted mice (n = 4 per group) treated with E2 (6 µg/day) with or without ICI 182,780 (125 µg/day) and on chow or fed the high-cholesterol diet for 14 days. To minimize diurnal variations of hepatic enzyme activities, all procedures were performed between 9:00 and 10:00 AM. Microsomal activities of HMG-CoA reductase were determined by measuring the conversion rate of [14C]HMG-CoA to [14C]mevalonic acid with [3H]mevalonolactone as an internal standard (23).

Lipid analysis

Bile cholesterol, as well as cholesterol content in the chow diet and the high-cholesterol diet, were determined by HPLC (12).

Statistical methods

All data are expressed as means ± SD. Statistically significant differences among groups of mice were assessed by Student’s t-test or the Mann-Whitney U test. If the F value was significant, comparison among groups of mice was further analyzed by a multiple comparison test. Analyses were performed with SuperANOVA software (Abacuss Concepts, Berkeley, CA). Statistical significance was defined as a two-tailed P < 0.05.

RESULTS

Contribution of newly synthesized hepatic cholesterol to biliary output in response to estrogen and its antagonist

To elucidate whether the high hepatic output of biliary cholesterol observed in E2-treated mice is the result of a higher rate of hepatic cholesterologenesis, we quantitated the contribution of newly synthesized hepatic cholesterol to biliary output. We observed biliary secretion rates of total and newly synthesized cholesterol over 4 h in mice administered [3H]water at 6 h before the commencement of total biliary diversion. As a consequence of the surgery that ensured complete interruption of the enterohepatic circulation of bile salts (see Materials and Methods), external biliary drainage in each mouse gave a “washout curve,” as indicated by the measurement of bile salt secretion rate (17). We have found that during the 4 h period of interrupted enterohepatic circulation, bile bile salt output gradually decreases with time (17). In contrast, the cholesterol secretion rate is unaltered during the first 4 h of biliary washout. Hence, our 4 h analysis provided cholesterol secretion out during simulation of an intact enterohepatic circulation. Furthermore, Turley and Dietzchy (16) have observed that the proportion of biliary cholesterol derived from newly synthesized sources is not influenced by the amount of bile salts available for biliary secretion and that the rate of biliary output of both total and labeled cholesterol is constant during the first 4 h of biliary washout.

Figure 1 shows hepatic outputs of biliary total and newly synthesized cholesterol in female AKR mice with intact ovaries (i.e., control mice) and in OVX mice treated with E2 or E2 plus ICI 182,780 on chow or fed the high-cholesterol (1%) diet for 14 days. On the chow diet (Fig. 1A), hepatic outputs of biliary total and newly synthesized cholesterol were 5.1 ± 1.0 and 0.7 ± 0.2 µmol/h/kg, respectively, and the relative contribution of newly synthesized cholesterol to biliary output was 14 ± 3% in the control mice. The E2 treatment induced significant increases in hepatic outputs of biliary total cholesterol (7.7 ± 1.0 µmol/h/kg) and newly synthesized cholesterol (3.4 ± 0.6 µmol/h/kg), and the relative contribution of newly synthesized cholesterol to biliary output was increased to 44 ± 5%. As shown in Fig. 1B, the high-cholesterol diet slightly increased biliary total cholesterol output to 7.2 ± 1.1 µmol/h/kg in control mice, because the AKR mouse is a gallstone-resistant strain (12). However, the relative contribution of newly synthesized cholesterol to biliary total cholesterol output was decreased to 6 ± 2%. In contrast, the secreted newly synthesized cholesterol was 5-fold higher in E2-treated mice on the high-cholesterol diet than in control mice. Also, E2 treatment resulted in a significant increase in biliary total cholesterol outputs (17.7 ± 2.4 µmol/h/kg), which comes mostly from the high-cholesterol diet and partly from lipoproteins such as HDL carrying cholesterol from extrahepatic tissues via a reverse cholesterol transport pathway. Of special note is that the biological actions of E2 were blocked completely by the antiestrogenic agent ICI 182,780. As a result, hepatic
outputs of biliary total and newly synthesized cholesterol were essentially similar between OVX mice treated with E2 plus ICI 182,780 and control mice, regardless of whether chow or the high-cholesterol diet was fed.

**Effects of estrogen and its antagonist on rates of cholesterol synthesis in liver and extrahepatic organs**

At 14 days on the chow or the high-cholesterol diet, liver weight (1.3–1.53 g), small intestine weight (1.34–1.43 g), and body weight (23.2–25.6 g) were essentially similar among mice with intact ovarian function (endogenous E2) and OVX mice treated with exogenous E2 (6 μg/day) with or without ICI 182,780 (125 μg/day), indicating that E2 does not alter liver, small intestine, or body weight markedly, consistent with previous results (4). **Figure 2** shows that compared with chow-fed control mice, [3H]water incorporation into hepatic digitonin-precipitable sterols is increased by ~3-fold in the livers and small intestines of E2-treated mice, suggesting that the liver and small intestine are two major organs for cholesterol biosynthesis in mice. On chow, the E2-treated mice displayed significantly (P < 0.001) higher rates of cholesterol synthesis in liver (2,075.4 ± 157.6 nmol/h/g tissue) and small intestine (1,688.3 ± 88.7 nmol/h/g tissue) than control mice (liver, 757.3 ± 96.1 nmol/h/g tissue; small intestine, 768.6 ± 82.4 nmol/h/g tissue). Furthermore, feeding the high-cholesterol diet resulted in essentially a complete repression of cholesterol synthesis in liver (36.7 ± 4.9 nmol/h/g tissue) and small intestine (29.5 ± 4.4 nmol/h/g tissue). In contrast, rates of cholesterol synthesis in liver (1,473.9 ± 188.6 nmol/h/g tissue) and small intes-

**Fig. 1.** The contribution of newly synthesized cholesterol to biliary cholesterol secretion. We measured newly synthesized cholesterol in bile by assaying the specific activity of cholesterol in bile according to classic methods established by Turley and Dietschy (16). Compared with control mice (i.e., female AKR mice with intact ovaries), 17β-estradiol (E2)-treated mice display significantly higher hepatic outputs of biliary total and newly synthesized cholesterol, whether chow (A) or the high-cholesterol (1%) diet (B) is fed. These biological effects of E2 are completely abolished by the antiestrogenic agent ICI 182,780 (ICI). Data are expressed as means ± SD. * P < 0.001, ** P < 0.00001 compared with newly synthesized cholesterol in control mice and in mice treated with E2 plus ICI 182,780 on the same diet. B.W., body weight.

**Fig. 2.** Cholesterol (Ch) synthesis rates in liver and extrahepatic organs in control mice and ovariectomized (OVX) mice treated with E2 or E2 plus ICI 182,780 and on chow or fed the high-cholesterol diet. The mice were administered an intravenous bolus injection of [3H]water and euthanized 1 h later. [3H]sterol content of liver and several extrahepatic organs as well as the whole remaining carcass was then determined as described in Materials and Methods. These contents were taken as a direct measure of the rate of sterol synthesis in each organ, which is expressed as nanomoles of [3H]water incorporated into sterols per hour per organ. As shown in the inset, whole animal sterol synthesis is determined as the sum of synthesis in all organs normalized per kilogram of body weight (B.W.). Data are expressed as means ± SD.
translating, possibly through the SREBP-2 pathway (24, 25). In biosynthesis may be inhibited by a negative feedback regulat-
vation levels of hepatic Srebp-2 (31.1 nmol/h/g body weight in OVX mice receiving E2 at 6 μg/day compared with those in control mice. A: On the chow diet, expression levels of Srebp-2 are significantly increased in OVX mice treated with E2 at 6 μg/day compared with those in control mice. B: Under conditions of high dietary cholesterol, expression levels of Srebp-2 are significantly decreased compared with the chow diet. Moreover, significantly higher expression levels of Srebp-2 are still observed in E2-treated mice. Furthermore, these biological effects of E2 are totally abolished by the antiestrogenic agent ICI 182,780, regardless of whether chow or the high-cholesterol diet is fed.

time (878.7 ± 67.0 nmol/h/g tissue) were still significantly (P < 0.001) higher in the E2-treated mice. As a result, in the chow-fed state, the rate of cholesterol synthesis in the whole animal (Fig. 2, inset) was significantly increased to 548.9 ± 31.1 nmol/h/g body weight in OVX mice receiving E2 at 6 μg/day compared with control mice (229.0 ± 34.4 μmol/h/kg body weight). Furthermore, even under high dietary cholesterol loads, E2-treated mice displayed significantly higher levels of sterol synthesis (336.4 ± 16.6 μmol/h/kg body weight). It should be emphasized that the biological effects of E2 on cholesterol biosynthesis were blocked fully by the antagonist ICI 182,780. Consequently, there were no significant differences in cholesterol synthetic rates between mice treated with E2 plus ICI 182,780 and control mice.

Regulation of expression of Srebp-2 and the SREBP-2-responsive genes by estrogen via ERα

To explore whether there is an "estrogen-ERα-SREBP-2" pathway for regulating hepatic cholesterol biosynthesis, we investigated expression levels of the hepatic Srebp-2 gene (Fig. 3) and five major SREBP-2-responsive genes (Fig. 4) by quantitative real-time PCR. In the chow-fed state (Fig. 3A), high doses of E2 significantly increased relative mRNA levels for the hepatic Srebp-2 gene. Furthermore, we found that high dietary cholesterol significantly decreased expression levels of hepatic Srebp-2 by ~50% compared with the chow diet in control mice (Fig. 3B). This indicates that cholesterol biosynthesis may be inhibited by a negative feedback regulation, possibly through the SREBP-2 pathway (24, 25). In contrast, E2-treated mice still displayed significantly higher expression levels of Srebp-2 even under high dietary cholesterol loads. These results suggest that under conditions of high levels of E2, the mice continue to synthesize cholesterol in the liver because the negative feedback regulation of cholesterol synthesis by the SREBP-2 pathway may be inhibited by E2 through the hepatic ERα. Again, these biological actions of E2 were completely abolished by the antiestrogenic agent ICI 182,780, whether chow or high dietary cholesterol was fed.

We further investigated the expression levels of five major SREBP-2-responsive genes [HMG-CoA synthase (isoforms 1 and 2), HMG-CoA reductase, farnesyl diphosphate synthase, squalene synthase, and lathosterol synthase] in the liver under the same experimental conditions described for Fig. 3. Of special note is that in each panel of Fig. 4, the left side shows data for mice on chow and the right side shows data for mice on the high-cholesterol diet. We found that in control mice, expression levels of these SREBP-2-responsive genes were significantly decreased by the high-cholesterol diet compared with the chow diet. Furthermore, high doses of E2 significantly increased relative mRNA levels for the SREBP-2-responsive genes in the liver, regardless of whether chow or high dietary cholesterol was fed. However, these biological effects of E2 on the expression levels of the SREBP-2-responsive genes were fully blocked by ICI 182,780. Again, these results support the concept that under normal physiological conditions, there is a negative feedback regulation of cholesterol biosynthesis by cholesterol; however, under conditions of high levels of E2, these important regulatory effects are retarded, possibly by E2 via the hepatic ERα pathway. Obviously, these studies suggest that there is a possible estrogen-ERα-SREBP-2 pathway that regulates hepatic cholesterol biosynthesis. More importantly, high levels of E2 may increase cholesterol synthesis in the liver via this pathway by inhibiting the negative feedback regulation of cholesterol biosynthesis. Consequently, these alterations may augment biliary cholesterol secretion and enhance the litho-
genicity of bile.

Effects of estrogen and its antagonist on the hepatic activity of HMG-CoA reductase

We also examined the hepatic activity of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, under the same experimental conditions described for Fig. 3, by an independent biochemical method (23). On the chow diet (Fig. 5A), HMG-CoA reductase activities (84 ± 9 pmol/min/mg) were significantly increased in E2-treated mice compared with control mice (54 ± 7 pmol/min/mg). Under conditions of high dietary cholesterol (Fig. 5B), HMG-CoA reductase activities (36 ± 3 pmol/min/mg) in control mice were significantly decreased compared with the chow diet. However, E2-treated mice still displayed significantly higher HMG-CoA reductase activities (66 ± 8 pmol/min/mg) in response to the high-cholesterol diet, suggesting that these mice continue to synthesize cholesterol in the liver. Again, these biological effects of E2 on the hepatic activity of HMG-CoA reductase were completely abolished by the antiestrogenic agent ICI 182,780.

Fig. 3. Relative mRNA levels of the hepatic sterol-regulatory element binding protein-2 gene (Srebp-2) as analyzed by real-time PCR. For each mRNA expression level, the fold change for the OVX mice treated with E2 or E2 plus ICI 182,780 (ICI) is expressed relative to chow-fed control mice, the value for which was arbitrarily set at 1.0. The values represent means ± SD of the fold changes found in these mice. A: On the chow diet, expression levels of Srebp-2 are significantly increased in OVX mice treated with E2 at 6 μg/day compared with those in control mice. B: Under conditions of high dietary cholesterol, expression levels of Srebp-2 are significantly decreased compared with the chow diet. However, significantly higher expression levels of Srebp-2 are still observed in E2-treated mice. Furthermore, these biological effects of E2 are totally abolished by the antiestrogenic agent ICI 182,780, regardless of whether chow or the high-cholesterol diet is fed.
DISCUSSION

A large number of human and animal studies have proposed that estrogen increases the risk of developing cholesterol gallstones by augmenting the hepatic secretion of biliary cholesterol that leads to an increase in cholesterol saturation of bile (4, 8–10, 26–32). The major findings of this study are that i) high physiological doses of E₂ delivered via subcutaneous hormone-release pellets significantly enhance the hepatic output of newly synthesized cholesterol, thereby increasing biliary total cholesterol output in a gallstone-resistant strain of OVX AKR mice; ii) during E₂ treatment, even under high dietary cholesterol loads, mice continue to synthesize cholesterol in the liver because the negative feedback regulation of cholesterol biosynthesis determined by the SREBP-2 pathway is inhibited by E₂ through the hepatic ERα; and iii) these biological effects of E₂ can be completely blocked by the antiestrogenic agent ICI 182,780 through a mechanism involved in the inhibition of hepatic ERα activity. Together, these results strongly suggest that there is a possible estrogen-ERα-SREBP-2 pathway regulating hepatic cholesterol biosynthesis and that this pathway plays a crucial role in promoting the excess secretion of newly synthesized cholesterol, particularly in response to high levels of E₂, which leads to the cholesterol supersaturation of bile and the formation of cholesterol gallstones (4).

It has been found (24, 25, 33–36) that lipid homeostasis in vertebrate cells and mice is regulated by a family of membrane-bound transcription factors ascribed to SREBPs. The mammalian genome encodes three SREBP isoforms, designated SREBP-1a, SREBP-1c, and SREBP-2. At normal levels of expression, SREBP-2 preferentially activates hepatic cholesterol biosynthesis, which regulates the production of cholesterol for export into bile containing vesicles and micelles. The major SREBP-2-responsive genes in the cholesterol biosynthetic pathway include those for the enzymes HMG-CoA synthase, HMG-CoA reductase, farnesyl diphosphate synthase, squalene synthase, and lathosterol synthase. On the basis of studies by Goldstein, Brown, and colleagues (37–39), the mechanism for the regulation of SREBP-2

Fig. 5. Hepatic HMG-CoA reductase activities measured under the same experimental conditions described for Fig. 3. A: On the chow diet, activities of HMG-CoA reductase are significantly increased in OVX mice treated with E₂ at a dose of 6 µg/day compared with those in control mice. B: Under high dietary cholesterol loads, activities of HMG-CoA reductase are significantly decreased compared with the chow diet. However, activities of HMG-CoA reductase are still significantly higher in E₂-treated mice. These biological effects of E₂ on the hepatic activity of HMG-CoA reductase are fully abolished by ICI 182,780 (ICI). Data are expressed as means ± SD.

Fig. 4. Expression levels of five major SREBP-2-responsive genes [HMG-CoA synthase (isoforms 1 and 2), HMG-CoA reductase, farnesyl diphosphate synthase, squalene synthase, and lathosterol synthase] in the liver under the same experimental conditions described for Fig. 3. In each panel, the left side shows data for mice on chow and the right side shows data for mice on the high-cholesterol diet. For each mRNA expression level, the fold change for OVX mice treated with E₂ or E₂ plus ICI 182,780 (ICI) is expressed relative to Chow-fed control mice, the value for which in each case was arbitrarily set at 1.0. The values represent means ± SD of the fold changes detected in these mice. * P < 0.05, ** P < 0.01, *** P < 0.001 compared with control mice and mice treated with E₂ plus ICI 182,780 on the same diet.
activity was elucidated through studies of cultured fibroblasts and some “manufactured” mouse strains. When the cultured fibroblasts and hepatocytes in these mice are deprived of cholesterol, a two-step proteolytic process releases the NH2-terminal domains of SREBP-2 so that it can enter the nucleus. There, it binds to sterol-regulatory elements in the promoter regions of genes encoding the above-mentioned enzymes for cholesterol biosynthesis. Binding to the promoters of all of these genes leads to transcriptional activation, which results in the increased synthesis of cholesterol. In contrast, when cholesterol overaccumulates in these cells, the proteolytic release of SREBP-2 is blocked, the cholesterol. In contrast, when cholesterol overaccumulates in these cells, the proteolytic release of SREBP-2 is blocked, the

NH2-terminal fragments remain bound to membranes, and the transcription of all target genes declines. Overall, these studies indicate that the proteolytic release of the SREBP-2 NH2-terminal domains is subject to negative feedback regulation by cholesterol, which has a critical effect on the regulation of cholesterol biosynthesis.

More recently, results from a mouse model of cholesterol gallstones showed that hepatic ERα, but not ERβ, plays a crucial role in E2-induced cholesterol gallstones (4). Furthermore, ERα is an important member of the steroid hormone receptor superfamily of ligand-activated transcription factors (40). Assayed semiquantitatively by Northern blot techniques, it was found that ERα is expressed highly in uterus, ovary, testis, epididymis, pituitary, kidney, and adrenal gland and moderately in liver, small intestine, colon, and heart in rats (41). Using more accurate quantitative real-time PCR methods, we found that liver and small intestine, two major organs for cholesterol biosynthesis, show moderate ERα mRNA expression levels in mice (4, 11). We found that except of liver, small intestine, colon, and kidney (Fig. 2), the rates of cholesterol biosynthesis in many organs in response to high doses of E2 are not changed markedly. One possible explanation is that organs such as uterus, ovary, testis, and adrenal gland are very small, so that total rates of cholesterol biosynthesis are very low, although their ERα mRNA expression levels are high. Another possible reason is that organs such as lung, stomach, and spleen show weak or no expression of ERα mRNA. Nevertheless, it has been observed that ERα could function as a major ligand-activated transcription factor for regulating hepatic and intestinal cholesterol metabolism as well as hepatic bile salt metabolism (4, 11). Most notably, results from human and animal studies (6–8) demonstrated that high levels of E2 increase hepatic HMG-CoA reductase activity, even under high dietary cholesterol loads, so that more total body cholesterol may be produced. It appears that there is an increased delivery of cholesterol to bile from de novo synthesis in the liver in response to increased estrogen. Thus, these alterations should provide a source of excess cholesterol, resulting in a significant increase in biliary secretion and supersaturation of bile.

These observations (6–8) especially are in agreement with our findings here that the activity of HMG-CoA reductase and the expression levels of Srebp-2 and major SREBP-2-responsive genes for the cholesterol biosynthetic pathway are increased significantly in the livers of mice treated with high doses of E2, regardless of whether chow or high dietary cholesterol is fed. Furthermore, they are supported by the fact that the rate of cholesterol synthesis in the liver and extrahepatic organs is increased significantly in E2-treated mice, as determined by measuring rates of incorporation of 3H-labeled water into sterols. These results also suggest that a negative feedback regulation of cholesterol biosynthesis in the liver may be lost during E2 administration, because E2-treated mice persist in synthesizing more cholesterol, even under conditions of high dietary cholesterol feeding. However, these biological effects of E2 can be totally abolished by the antiestrogenic agent ICI 182,780. Thus, our observations strongly support the notion that ERα could interfere with the negative feedback regulation of hepatic cholesterol biosynthesis by stimulating SREBP-2, which activates the above-mentioned cholesterol biosynthetic pathway. These data imply that the estrogen-ERα-SREBP-2 pathway may play a unique role in regulating cholesterol metabolism and in the formation of cholesterol gallstones, particularly in response to high levels of E2 (Fig. 6).

It is widely appreciated that hypersecretion of biliary cholesterol from the liver into the bile is a prerequisite for cholesterol gallstone formation in humans and mice (1, 2). Biliary cholesterol secretion derives from either dietary or newly synthesized cholesterol or both (42–45). Obviously, in an animal receiving no dietary cholesterol, all biliary cholesterol must ultimately be derived from de novo synthesis. As indicated by in situ perfused rat liver (46) and human studies (47), plasma HDL preferentially provides cholesterol for secretion into bile. Therefore, HDL is crucial to reverse cholesterol transport (48), whereby newly synthesized cholesterol is transferred from peripheral tissues to the liver for biliary secretion. Furthermore, we found that the contribution of de novo cholesterol synthesis to biliary cholesterol secretion is ~15%, consistent with previous studies (16, 42–45). Under high dietary cholesterol

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**Fig. 6.** Model for a possible estrogen-ERα-SREBP-2 pathway enhancing hepatic cholesterol biosynthesis and the hepatic output of biliary cholesterol. We found that hepatic estrogen receptor subtype α (ERα) activated by E2 interferes with the negative feedback regulation (as shown by the dashed line) of cholesterol biosynthesis by stimulating SREBP-2, with the resulting activation of the SREBP-2-responsive genes for the cholesterol biosynthetic pathway. Consequently, these alterations result in the excess secretion of newly synthesized cholesterol and supersaturation of bile that predispose to cholesterol precipitation and gallstone formation. It should be emphasized that these lithogenic effects of E2 are completely blocked by the antiestrogenic agent ICI 182,780. See text for further description.
loads, the metabolism of exogenous chylomicrons (i.e., lipoproteins of intestinal origin) also plays a major regulatory role in the response of biliary cholesterol secretion to high dietary cholesterol and greatly contributes to the formation of cholesterol gallstones (49, 50). It has been found that ERα activated by E2 enhances cholesterol absorption by mediating intestinal sterol transporters favoring the influx of intraluminal cholesterol molecules across the apical membrane of the enterocyte in mice (11). In this study, we found that during E2 treatment, an increase in the hepatic output of biliary total and newly synthesized cholesterol (Fig. 1) is associated with a significant increase in hepatic cholesterol biosynthesis (Fig. 2), whether mice are fed trace or high levels of dietary cholesterol. However, a marked reduction in cholesterol synthesis correlates with a significant decrease in the amount of mRNAs for Srebp-2 and SREBP-2-responsive genes for cholesterol biosynthesis in mice treated with E2 plus ICI 182,780. These results indicate that during E2 treatment, the contribution of newly synthesized cholesterol to biliary cholesterol secretion and gallstone formation is appreciable (4). It should be emphasized that high doses of E2 also upregulate expression levels of the HDL receptor scavenger receptor class B type I (51, 52) and LDL receptor (53, 54). These alterations could induce an apparent increase in the hepatic output of biliary cholesterol derived from circulating lipoproteins such as HDL and LDL, although LDL cholesterol has less effect on biliary secretion.

We conclude that during estrogen treatment, the contribution of de novo cholesterol biosynthesis in the liver to biliary secretion, as determined possibly by the estrogen-ERα-SREBP-2 pathway, is of major quantitative importance in mice. Further investigation into how estrogen regulates hepatic cholesterol metabolism may give insights into therapeutic approaches to modulating the cholesterol biosynthesis pathway as well as preventing the formation of cholesterol gallstones in high-risk subjects, especially those exposed to high levels of estrogen.

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