ESTROGEN RECEPTOR α MEDIATES 17α-ETHYNYLESTRADIOL-CAUSING HEPATOTOXICITY

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Estrogens are known to cause hepatotoxicity such as intrahepatic cholestasis in susceptible women during pregnancy, after administration of oral contraceptives or during postmenopausal replacement therapy. Enterohepatic nuclear receptors including FXR, PXR and CAR are important in maintaining bile acid homeostasis and protecting the liver from bile acid toxicity. However, no nuclear receptor has been implicated in the mechanism for estrogen-induced hepatotoxicity. Here Era-/-, Erb-/-, Fxr-/-, Pxr-/- and Car-/- mice were employed to show that Era-/- mice were resistant to synthetic estrogen 17α-ethynylestradiol (EE2) induced hepatotoxicity, as indicated by the fact that the EE2-treated Era-/- mice developed none of the hepatotoxic phenotypes such as hepatomegaly, elevation in serum bile acids, increase of alkaline phosphatase activity, liver degeneration and inflammation. Upon EE2 treatment, ERα repressed the expression of bile acid transporters (BSEP, NTCP, OATP1, OATP2, ABCG5 and ABCG8) in the liver. Consistently, biliary secretions of both bile acids and cholesterol were markedly decreased in EE2 treated wild-type mice, but not in the EE2 treated Era-/- mice. In addition, ERα up-regulated the expression of CYP7B1 and down-regulated the CYP7A1 and CYP8B1, shifting bile acid synthesis toward the acid pathway to increase the serum level of β-muricholic acid. ERβ, FXR, PXR and CAR were not involved in regulating the expression of bile acid transporter and biosynthesis enzyme genes following EE2 exposure. Taken together, these results suggest that ERα mediated repression of hepatic transporters and alterations of bile acid biosynthesis may contribute to development of the EE2-induced hepatotoxicity.

Estrogens have long been known to cause intrahepatic cholestasis in susceptible woman during pregnancy, who are using oral contraceptives or who are on postmenopausal hormone replacement therapy (1). Intrahepatic cholestasis of pregnancy (ICP), the most common hepatic disease during pregnancy, starts with modest itching associated with elevated levels of serum bile acids and can lead to spontaneous premature delivery and intrauterine fetal death (2,3). Experimental intrahepatic cholestasis induced by 17α-ethynylestradiol (EE2) treatment in rodents is a widely used in vivo model to examine the mechanisms involved in estrogen-induced cholestasis (4). EE2 treatment decreased the ATP-dependent taurocholate transport in the hepatic canalicular membrane, which was thought to be due to impaired expression of the canalicular bile salt export pump (BSEP) (5). Moreover,
treatment with EE2 also decreased sinusoidal uptake of bile acids by down-regulating the expression of the sodium taurocholate cotransporting polypeptide protein (NTCP) (6). These studies suggest that estrogens induce cholestasis by reducing both the influx and efflux of bile acid in hepatocytes, resulting in a decrease in bile flow. In addition, EE2 treatment was shown to alter bile acid composition, which was associated with cholestatic features (7). However, the molecular mechanism of these EE2-dependent alterations is still not fully understood.

Bile acid homeostasis is tightly regulated by multiple nuclear receptors including FXR, PXR and CAR in physiological and/or pathological conditions (8-10). It is well understood that bile acids repress bile acid biosynthesis by down-regulating transcription of the rate-limiting enzyme CYP7A1 through the FXR-SHP-LRH1 cascade (11-13). NTCP, responsible for bile acid uptake into the hepatocytes, is repressed by FXR activation (11). Simultaneously, FXR up-regulates the expression of BSEP, which increases bile acid efflux from the liver into the bile (11,14). Therefore, FXR regulates transport of bile acids and prevents their over accumulation in hepatocytes. The activation of PXR inhibits production of additional bile acid by inhibiting CYP7A1, and inducing OATP2, which increases the uptake of bile acids from sinusoidal blood to the hepatocytes (15,16). CAR induces SULT2A1 and MRP4 to prevent toxic bile acid accumulation (17). Consistent with preventive roles of PXR and CAR in the cholestatic condition, double null mouse lacking PXR and CAR had a more severe disruption of bile acids and cholesterol homeostasis (18-20). However, no nuclear receptor has been implicated in estrogen-induced hepatotoxicity.

In the present study, we investigated whether nuclear receptor could be involved in the pathogenesis of estrogen induced hepatotoxicity, using Era-/-, Erb-/-, Fxr-/-, Pxr-/- and Car-/- mice. We provide direct in vivo evidence that synthetic estrogen EE2 exposure induces liver damage by activating the ERα signaling pathways leading to an alteration of bile acids biosynthesis and repression of multiple bile acid and cholesterol transporters.

MATERIALS AND METHODS

Materials. 17α-ethynylestradiol and 1,2-propanediol were obtained from Sigma-Aldrich (St. Louis, MO).

Animal treatments. All protocols and procedures were approved by the NIH Animal Care and Use Committee and are in accordance with National Institutes of Health Guidelines. Era-/-, Erb-/-, Pxr-/-, Fxr-/- and Car-/- mice were previously generated and characterized (10,11,21,22). Era-/- and Erb-/- mice on a background of C57BL/6 were obtained from Taconic Farms (Germantown, NY). Pxr-/-, Fxr-/- and Car-/- mice used in these studies were maintained in 129/Sv and C57BL/6 mixed genetic background. All animals were housed in a temperature-controlled environment with 12 hr light/dark cycles with access to standard chow and water ad libitum. Age-matched groups of 8 to 12-week-old mice were used for all experiments. Four to eight mice were used for each treatment group. Adult mice received subcutaneous injections of EE2 (10 mg/kg) or vehicle (80% 1,2-propanediol with 0.15% NaCl) once daily for five successive days. Twenty hours after the last injection, the mice were fasted for 4 hours before harvesting blood from the retro-orbital plexus for subsequent serum analyses and livers for RNA isolation and histology.

Histology and mitosis measurements. Liver samples from each mouse were fixed in 10% neutral buffered formalin. Slides were stained with H&E using standard protocols and examined microscopically for structural changes. Hepatocyte proliferation was evaluated by immunohistochemical staining for Ki67 using rat anti-mouse Ki67 (TEC3) antibody (Dako Corporation). Immunoreactivity was visualized with the Vectastain Elite ABC kit (Vector Laboratories) and slides were counterstained with hematoxylin. To assess proliferative responses to liver lesions, mitotic nuclei were counted in 20 randomly selected fields under 200X magnification and the mitotic index was calculated by dividing the number of mitotic cells by the total number of hepatocytes.
Measurement of serum chemistries. Serum ALP and total bile acids were measured using reagents and controls from Diagnostic Chemicals Limited and the COBAS MIRA Plus CC Analyzer (Roche Diagnosis). Individual bile acid concentrations were measured by LC-MS/MS using a PE SCIEX API2000 ESI triple-quadrupole mass spectrometer (PerkinElmer Life Sciences) controlled by Analyst software, as previously described (23).

Analyses of Biliary Lipids. Biliary lipid secretion was measured following surgery for bile collection. Animals were fasted for 4h before surgery following EE2 (10 mg/kg) or vehicle injection for 5 successive days. Bile collection started between 9:30 to 11:30 AM to minimize influence of circadian variations. Animals were anesthetized with a single dose of pentobarbital (50 mg/kg body weight) and maintained under this condition throughout the experiments. The gallbladder was cannulated with PE-10 tubing after ligation of the common bile duct and secured with a silk suture. Bile collection was measured gravimetrically, assuming a density of the bile of 1.0 g/ml. The initial 15 min of biliary secretion that contained concentrated gallbladder bile were not used for analysis. Bile collection continued for up to 1h and was constant over this period. Body temperature was maintained with a heat pad to prevent hypothermic alterations of bile flow. The biliary concentration of bile acids, cholesterol and phospholipids were measured using the Total Bile Acid Test, Cholesterol E Test and Phospholipids B Test (Wako Chemical). The biliary excretion rates were calculated as the product of the bile flow and the biliary concentration.

Reporter assays. HepG2 cells, grown in phenol red-free MEM medium supplemented with 5% charcoal-dextran stripped FBS, were transfected with a Gal4-responsive luciferase reporter and Gal4-DBD fused ERα-LDB, ERβ-LDB, FXR-LDB, PXR-LDB, and CAR-LDB (24) using the calcium phosphate method (Amersham Pharmacia Biotech). At 16 hours after transfection, cells were treated with 10 nM 17β-estradiol, 10 µM 17α-ethynylestradiol or DMSO for 24 hours and assayed for luciferase activity with the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was normalized for transfection efficiency using the phRL-TK vector as an internal control.

Analyses of gene expressions. Total RNAs were isolated from mouse hepatic tissue using the TRizol method (Invitrogen) and cDNAs were synthesized from total RNAs with the Superscript First-Strand Synthesis System (Invitrogen) and random hexamer primers. Real Time PCR measurements of individual cDNAs were performed with the ABI prism 7700 sequence detection system. Gene-specific primers and probes were designed using Prime Express Software (PE Applied Biosystems) or purchased as pre-designed TaqMan Gene Expression Assays gene-specific probe and primers mixture (PE Applied Biosystems). Assay ID number of pre-designed TaqMan Gene expression Assay (gene, assay ID number) and sequences of the probes and primers (gene, probe/forward primer/reverse primer, 5’ to 3’) used in this study are as follows: Bsep, Mm00445168_m1; Mdr2, Mm00435630_m1; Ntcp, Mm00444241_m1; Oatp1, Mm00649796_m1; Oatp2, Mm00453126_m1; Abcg5, Mm00446249_m1; Abcg8, Mm00445970_m1; Abca1, Mm00442646_m1; Cyp7a1, Mm00484152_m1; Cyp8b1, Mm00501637_s1; Cyp7b1, Mm00484157_m1; Shp, Mm00442278_m1; Srebp-1, Mm00550338_m1; Fabp, Mm00662319_m1; Spot14, Mm01273967_m1; Sr-bi, Mm00450236_m1; Vlacsrm, Mm00447768_m1; Cyp27, Mm00486606_m1; 6FAM-CCTCACCTATGGGATCTTCATCGACA-TAMRA / CCACCTTTGGAGCAAGTGATGA / CAAAAGCTGAGCAGATGTA; Fxr, 6FAM-CTGCAGAGCCCTCCCC-MGB / GCACAGAGGCCTTGGAGAA / GCACAGCTTGTGATGACACATCAA. The TaqMan rodent GAPDH control reagent (PE Applied Biosystems) was used as an internal control. All Real Time PCR data were obtained using RNA isolated from tissues of individual animals.

Statistical analyses. Values were reported as mean ± SE. Statistical differences were determined by a Student’s t test. P values less than 0.05 were considered to be statistically significant. Statistical significances relative to the corresponding vehicle treated control are displayed as #.
RESULTS

Lack of EE2-induced hepatotoxicity in ERα-null mice. To study the mechanism of estrogen-induced hepatotoxicity in vivo, Era−/−, Erb−/−, Fxr−/−, Pxr−/−, Car−/− and corresponding wild-type (Wt+/+) mice were treated with EE2. While body weights were similar between EE2 treated mice and vehicle control mice (data not shown), liver weights were significantly increased in EE2 treated Wt+/+, Erb−/−, Fxr−/−, Pxr−/− and Car−/− mice, but no hepatomegaly was evident in EE2 treated male and female Era−/− mice (Figure 1A). Serum bile acid levels were also elevated in EE2-treated Wt+/+, Erb−/−, Fxr−/−, Pxr−/− and Car−/− mice but not in Era−/− mice (Figure 1B). Serum ALP activities, a marker for hepatocyte damage, were significantly increased in EE2 treated mice, but not in Era−/− mice (Figure 1C). Era+/+ mice are shown as representative wild-type (Wt+/+) mice. The other corresponding wild-type mice also showed similar responses following EE2 treatment to those of Era+/+ mice (Supplemental Figure 1). As shown in previous studies (11,25), Fxr−/− mice exhibited increased basal bile acid levels. Importantly, levels of bile acids and ALP activity were further increased by EE2 in Fxr−/− mice, suggesting that EE2 treatment induces hepatotoxicity via an FXR-independent mechanism. These results clearly demonstrate that ERα is required for the development of EE2 induced hepatotoxicity.

The livers of EE2 treated Wt+/+ and Era−/− mice were histologically examined. Liver sections of vehicle-treated mice showed normal morphology (Figure 2A), whereas EE2 treated Wt+/+ mice exhibited hepatocyte degeneration and infiltration of inflammatory cells in hepatic sinusoids. However, EE2-treated Era−/− mice did not show any liver damage. Accordingly, livers from the EE2-treated Wt+/+ mice but not the EE2-treated Era−/− mice contained many Ki67 positive proliferating hepatocytes. To further quantify the response to liver damage, we counted the number of mitotic nuclei in these samples. The percentage of mitotic nuclei was markedly increased with EE2 treatment in Wt+/+ mice. However, no such increase was observed in EE2-treated Era−/− mice (Figure 2B).

To confirm the specificity of EE2 in receptor activation, we performed gene reporter assays by co-transfecting a Gal-luciferase reporter gene construct with Gal-fused ERα, ERβ, FXR, PXR and CAR-LBD in HepG2 cells (Figure 3). As shown in previous reports, both ERα and ERβ were exclusively activated by EE2 as well as by the endogenous ligand 17β-estradiol (E2) (26). Unexpectedly, EE2 treatment activated FXR and PXR approximately two-fold, while there was no activation by E2. CAR was slightly activated by E2 treatment (24), but not by EE2 treatment. Both ERα and ERβ are predominantly expressed in reproductive organs. However, ERα is significantly expressed in liver while the level of ERβ mRNA in liver is very low (27). Although E2 and EE2 activate both ERα and ERβ, ERβ probably does not play a significant role in hepatocytes because of its low expression level, which is in agreement with the hepatotoxic phenotypes in Erb−/− mice following EE2 treatment.

Alteration of bile acid biosynthesis. We examined the expression of genes involved in bile acid biosynthesis; Cyp7a1, Cyp8b1, Cyp7b1, Cyp27, Fxr and Shp (Figure 4A). The biosynthesis of the primary bile acids, cholic acid (CA) and β-muricholic acid (β-MCA), is mediated by either the neutral or acidic pathway (28,29). The rate-limiting reaction in the neutral pathway is catalyzed by CYP7A1. The expression of CYP7A1 was decreased in EE2-treated Wt+/+, Erb−/−, Fxr−/−, Pxr−/−, and Car−/− mice, but was not altered in the EE2-treated Era−/− mice. Since basal expression of CYP7A1 remained unchanged in Era−/− mice compared with that of Wt+/+ mice (Supplemental Figure 2), the repression of CYP7A1 reflected the response activity of ERα to EE2. Similarly, Erb−/− and Pxr−/− mice did not change basal expression of CYP7A1. Fxr−/− and Car−/− mice showed increase in basal expression of CYP7A1 mRNA of 4- and 1.6-folds, respectively. However, FXR and CAR did not contribute to the repression of CYP7A1 by EE2 treatment. The expression of CYP8B1, another enzyme involved in the neutral pathway, was also decreased in all EE2 treated mouse lines, except
that the Era−/− mice retained the original level of CYP8B1. In contrast, the expression of CYP7B1, an important enzyme in the acidic pathway, was markedly induced in all EE2 treated mouse lines but not in the Era−/− mice. In addition, the expression of the gene encoding sterol 27-hydroxylase CYP27 was not changed in any of the mouse lines treated with EE2. Furthermore, no differences were found in the expression of a key regulator of bile acid homeostasis FXR and short heterodimer partner (SHP), suggesting that the FXR-SHP negative feedback is not involved in the regulation of these biosynthesis enzymes by EE2 treatment.

To investigate the effect of altered CYPs expressions by EE2, we measured the composition of bile acids in male and female Wt+/+ and Era−/− mice using LC-MS/MS. Indeed, consistent with the expression of CYP7A1, CYP7B1 and CYP8B1, the ratios of β-MCA to CA were significantly increased in Wt+/+ mice following EE2 treatment, with no alteration in Era−/− mice (Figure 4B). These results suggest that alterations in bile acid biosynthesis may contribute to the development of hepatotoxicity by EE2 treatment.

Repression of bile acid transporters and cholesterol transporters. To further understand the molecular basis of EE2-induced hepatotoxicity, we examined the expression of key hepatic transporters involved in bile acids, cholesterol and phospholipids transport. We first examined the expression of canalicular transporters BSEP and MDR2 that have been identified as the genes responsible for familial intrahepatic cholestasis PFIC-2 and PFIC-3, respectively (30). Expression of BSEP was decreased in EE2-treated Wt+/+, Erb−/−, Fxr−/−, Pxr−/−, and Car−/− mice, but was not altered in the EE2-treated Era−/− mice (Figure 5). In contrast, MDR2 levels were not significantly changed in any of the mouse lines treated with EE2. NTCP is responsible for bile acid uptake into hepatocytes and OATP transporters are also involved in sodium-independent hepatic uptake of bile acids. A reduction in NTCP and OATP-C (the human homologue of rodent OATP2) was observed in patients with cholestasis (31). Indeed, NTCP expression was decreased in all EE2 treated mouse lines except the Era−/− mice. OATP1 and OATP2 levels were also decreased by EE2 treatment in all mouse lines except Era−/− mice. We also analyzed the expression of cholesterol efflux transporters; ABCG5, ABCG8 and ABCA1. Expression of ABCG5 and ABCG8 mRNAs was significantly decreased by EE2 treatment in all mouse models, except the Era−/− mice. In contrast, EE2 treatment had no effect on ABCA1 expression. ABCG5 and ABCG8 play a crucial role in cellular cholesterol efflux from hepatocytes to bile (32). EE2 decreased both ABCG5 and ABCG8 levels, suggesting abrogation of cholesterol homeostasis. To evaluate whether the repression of hepatic transporters reflected biliary lipids secretion, biliary bile acids, cholesterol and phospholipids secretion were determined directly by cannulating the gallbladder after ligation of the common bile duct (Figure 6). In agreement with the decreased expression of hepatic transporters, the secretion of bile acids and cholesterol were significantly decreased by EE2 in male and female Wt+/+ mice, but not in Era−/− mice. The secretion of biliary phospholipids was also decreased by EE2. While the degree of repression of the major phospholipids transporter MDR2 was small, a significant decrease of phospholipids secretion was observed. These data are consistent with the hypothesis that biliary phospholipids secretion is coupled to biliary bile acids secretion. Thus, these data indicate that the activation of ERα is associated with a marked impairment in biliary secretion.

DISCUSSION

We have provided direct evidence that ERα can become a risk factor for the development of hepatotoxicity following estrogen exposures. Using various nuclear receptor KO mice, we have now shown that ERα is the receptor responsible for repressing multiple hepatic bile acid and cholesterol transporters; repressing hepatic transporters reduces biliary lipids secretions resulting in liver damage (Figure 7). In addition, the ERα-mediated alteration of bile acids biosynthesis may have also contributed to the EE2-induced development of hepatotoxicity. None of the other nuclear receptors (ERβ, FXR, PXR and CAR) was shown to be involved in developing hepatotoxicity. Our present study with
Era-/- mice is the first to directly demonstrate the involvement of ERα in estrogen-induced hepatotoxicities, although it has been reported that treatment with ERα agonists causes adverse effects such as gallstone formation in humans and experimental animal models (33,34).

The molecular mechanism of how ERα regulates CYPs and transporters remains an open question at the present time. Some of the genes subjected for our present study can also be regulated by other nuclear receptors such as LXR, LRH-1 and HNF4 (9,35). Since expression of the known HNF4-regulated gene (very long chain acyl-CoA synthase-related gene; Vlacsr) was found to be repressed in the EE2-treated Wt+/+ mice but not in EE2-treated Era-/- mice (Supplemental Figure 3), the possibility remains that ERα indirectly repressed NTCP and OATP1 through antagonizing HNF4 activity. However, BSEP that was repressed by EE2 in our study was up-regulated in HNF4-null mice (35). Hence even an indirect mechanism such as HNF4-mediated repression may explain some but not all of ERα-mediated regulation. Neither LXR targets (SREBP-1, FAS and SPOT14) nor the LRH-1 target, SR-BI, were affected by EE2 treatment, suggesting that no involvement of these two nuclear receptors in EE2 actions. We have performed promoter analyses for both Cyp7b1 and Cyp8b1 genes to delineate ERα response elements but failed to identify these elements. The molecular mechanism of EE2-induced hepatotoxicity may be complex and could require both direct and indirect regulations.

Intrahepatic cholestasis of pregnancy (ICP) is the most common liver disease during pregnancy. Although numerous studies have investigated ICP, the molecular basis for its development remains unknown. Since hepatic transporters BSEP and MDR3 (the human homologue of rodent MDR2) were identified as the genes responsible for progressive familial intrahepatic cholestasis (PFIC2 and PFIC3, respectively) (30), several studies have investigated the association of these cholestatic genes with ICP. Mutations in the BSEP gene were not implicated in sporadic case of ICP in Swiss patients (36). In addition, common mutations in MDR3 were not found in familial and sporadic cases of ICP in Finnish patients (37). Although mutations in these genes may raise the susceptibility to ICP, the results of these studies did not confirm a direct correlation between mutational events in these candidate genes and ICP. However, it has been reported that BSEP expression is decreased in livers of ICP patients (4,31). In addition, the deletion of Bsep gene caused intrahepatic cholestasis in mice (38). Our current finding that ERα plays a key role to repress multiple transporters in the development of cholestasis suggests that mutations or polymorphisms in the ERα gene may be implicated in some form of ICP in humans.

Primary biliary cirrhosis (PBC) is a chronic liver disease that results in cholestasis, which predominantly affects women and is characterized biochemically by elevated serum alkaline phosphatase (ALP). Interestingly, tamoxifen, an ERα antagonist used to treat breast cancer, was found to dramatically decrease ALP levels in PBC patients, suggesting that tamoxifen can also be used to treat cholestasis (39,40). These clinical reports support the notion that the hepatic gene regulation through ERα plays a key role in estrogen-induced cholestasis. Our present findings that ERα regulates bile acid transporters including BSEP have provided the molecular basis of ERα-mediated estrogen-induced hepatotoxicity. FXR is also known to regulate the same genes involved in bile acid homeostasis. Although treatment with the synthetic FXR agonist GW4064 was shown to be effective in improving cholestasis and reducing gallstone formation in experimental animal model (41,42), no FXR agonists such as GW4064 has been proved as therapeutic drug. Treatment of cholestasis with ursodeoxycholic acid is widely used and reduces pruritus in ICP. However, ursodeoxycholic acid therapy is not completely effective in preventing fetal death or premature delivery associated with fetal distress (43). The development of liver specific ERα antagonists could yield alternative therapeutics for treatment of chronic cholestasis patients as well as ICP patients.
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FOOTNOTES

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The abbreviations used are: alkaline phosphatase (ALP); ATP-binding cassette transporter (ABC); bile salt export pump (BSEP); cholic acid (CA); β-muricholic acid (β-MCA); constitutive active/androstane receptor (CAR); cytochrome P450 (CYP); 17β-estradiol (E2); 17α-ethynylestradiol (EE2); estrogen receptor (ER); fatty acid synthase (FAS); farnesoid X receptor (FXR); intrahepatic cholestasis of pregnancy (ICP); hepatocyte nuclear factor 4 (HNF4); liver receptor homolog 1 (LRH-1); liver X receptor (LXR); multidrug resistance associated protein (MDR); organic anion transporting polypeptide (OATP); primary biliary cirrhosis (PBC); progressive familial intrahepatic cholestasis (PFIC); pregnane X receptor (PXR); short heterodimer partner (SHP); scavenger Receptor BI (SR-BI); Na+/taurocholate cotransporting polypeptide (NTCP); Sterol regulatory element binding protein-1 (SREBP-1); sulfotransferase (SULT); very long chain acyl-CoA synthase-related gene (VLACSR).
**FIGURE LEGENDS**

**Fig. 1.** *Era-/-* mice are resistant to EE2 induced hepatotoxicity. Wild type and ERα, ERβ, FXR, PXR and CAR null mice were daily injected with vehicle or 10 mg/kg EE2 for 5 days. A) Percentage of liver weight over body weight ratio. B) Measurements of serum bile acid concentration. C) Measurements of serum ALP activity. White bars indicate vehicle-treated mice and black bars indicate EE2-treated mice. # indicates statistical significance between vehicle and EE2-treated mice of same genotype (p<0.05).

**Fig. 2.** Histology of livers from *Wt*+/+ and *Era-/-* mice treated vehicle or EE2. A) Representative histological liver section stained with H&E and anti-Ki67 antibody. Arrows indicate hepatocyte degeneration with cytoplasmic vacuolation; arrowheads indicate infiltration of inflammatory cells. Scale bar, 100 µm. B) Percentage of the number of hepatocytes showing mitotic nuclei over total number of hepatocytes. White bars indicate vehicle-treated mice and black bars indicate EE2-treated mice. # indicates statistical significance between vehicle and EE2-treated mice of same genotype (p<0.05).

**Fig. 3.** Activation of nuclear receptors by E2 or EE2. HepG2 cells were transiently cotransfected with a Gal responsible luciferase reporter gene construct and Gal-fused ERα, ERβ, FXR, PXR or CAR-LBD. Cells were treated with DMSO, 10 nM E2 or 10 µM EE2 and assayed for luciferase activity. Luciferase values were normalized to that obtained for co-transfected Renilla luciferase and expressed as fold activation compared with control DMSO treated cells transfected with the same reporter and receptor.

**Fig. 4.** Effects of ERα agonist on bile acids biosynthesis. A) Expression patterns of genes involved in bile acids biosynthesis. Real time PCR was performed on RNA from individual livers of vehicle or EE2 treated mice. mRNA values were normalized to the vehicle treated mice for each genotype. B) Relative serum concentration of β-MCA over CA. Individual bile acid concentrations were measured by LC-MS/MS. White bars indicate vehicle-treated mice and black bars indicate EE2-treated mice. # indicates statistical significance between vehicle and EE2-treated mice of same genotype (p<0.05).

**Fig. 5.** Hepatic gene expression of bile acid, cholesterol and phospholipids transporters in EE2 treated mice. Expression patterns of genes involved in bile acids transport (BSEP, NTCP, OATP1 and OATP2), cholesterol transport (ABCG5, ABCG8 and ABCA1) and phospholipids transport (MDR2). Real-time PCR analysis was performed on RNA from individual livers of vehicle or EE2 treated mice. mRNA values were normalized to the vehicle treated mice for each genotype. White bars indicate vehicle-treated mice and black bars indicate EE2-treated mice. # indicates statistical significance between vehicle and EE2-treated mice of same genotype (p<0.05).

**Fig. 6.** Effect of ERα agonist on biliary lipids secretions. Wild type and ERα null mice were daily injected with vehicle or EE2 for 5 days. The common bile duct was ligated, the gallbladder was cannulated and hepatic bile was measured gravimetrically for 60 min. The concentrations of biliary bile acids, cholesterol and phospholipids were determined, and the secretion rate of each lipid was determined from measurements of bile flow. White bars indicate vehicle-treated mice and black bars indicate EE2-treated mice. # indicates statistical significance between vehicle and EE2-treated mice of same genotype (p<0.05).

**Fig. 7.** Role of ERα in the estrogen-induced hepatotoxicity. Activation of ERα by estrogen receptor agonist (EE2) leads to repression of bile acid (NTCP, OATP1/2 and BSEP) and cholesterol (ABCG5/8) transporters and alteration of bile acid biosynthesis enzymes (CYP7A1, CYP7B1 and CYP8B1). This results in repression of biliary lipids secretion and alteration of bile acid composition, causing pathogenesis of hepatocyte degeneration and inflammation such as intrahepatic cholestasis.
Yamamoto et al. Figure 3

Fold LUC activation vs. receptor type with different treatments (DMSO, E2, EE2).
Figure 6

Bar charts showing biliary secretion rates (nmol/min/g) for different groups: Wt +/+, Era +/−, Wt +/+ (Male), and Era +/− (Female). The bars represent Phospholipids (20-10), Cholesterol (1-0.5), and Bile Acids (0-10). The error bars indicate variability.
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