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New insights into the molecular mechanisms underlying effects of estrogen on cholesterol gallstone formation

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Abstract

Epidemiological and clinical studies have found that at all ages women are twice as likely as men to form cholesterol gallstones, and this gender difference begins since puberty and continues through the childbearing years, which highlight the importance of female sex hormones. Estrogen is a crucial hormone in human physiology and regulates a multitude of biological processes. The actions of estrogen have traditionally been ascribed to two closely related classical nuclear hormone receptors, estrogen receptor 1 (ESR1) and ESR2. Recent studies have revealed that the increased risk for cholesterol gallstones in women vs. men is related to differences in how the liver metabolizes cholesterol in response to estrogen. A large number of human and animal studies have proposed that estrogen increases the risk of developing cholesterol gallstones by increasing the hepatic secretion of biliary cholesterol, which, in turn, leads to an increase in cholesterol saturation of bile. Furthermore, it has been identified that hepatic ESR1, but not ESR2, plays a major role in cholesterol gallstone formation in mice in response to high doses of 17 β -estradiol. The mechanisms mediating estrogen's action has become more complicated with the recent identification of a novel estrogen receptor, G protein-coupled receptor 30 (GPR30), a member of the seven-transmembrane G protein-coupled receptor superfamily. In this review, we provide an overview of the evidence for the lithogenic actions of estrogen through ESR1 and discuss the cellular and physiological actions of GPR30 in estrogen-dependent processes and the relationship between GPR30 and classical ESR1 on gallstone formation.

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Keywords

bile; bile salt; crystallization; estrogen; estrogen receptor; female gender; G protein-coupled receptor

Introduction

As found by epidemiological and clinical investigations in every population studied, cholesterol gallstones are more common in women than men, and this gender difference begins since puberty and continues through the childbearing years [1–18]. These observations suggest that estrogen, a major female sex hormone, could be an important risk factor for the formation of cholesterol gallstones. 17β -Estradiol (E_2), a primary female sex steroid, is an important hormone in both health and disease [19,20]. It regulates a broad array of disparate biological processes throughout the body, including reproduction, cardiovascular function, hepato-biliary secretion, metabolic processes, neurological function and inflammation. Estrogens are often used as part of some oral contraceptives for estrogen replacement therapy of postmenopausal women. In addition, the related estrogen-based steroids including estriol, estrone and estrone sulfate, as well as a large variety of natural and synthetic compounds – demonstrate estrogenic activity. Synthetic compounds that selectively mimic or inhibit the activity of estrogen, such as the selective estrogen receptor modulators (SERMs) tamoxifen and raloxifene, or the pure antagonists ICI 162,780 and fulvestrant, have been extensively used to treat cancer and osteoporosis [21]. Accumulated evidence from clinical studies has found that the use of oral contraceptive steroids and conjugated estrogens in premenopausal women significantly enhances the formation of cholesterol gallstones [22–25]. Furthermore, estrogen therapy to postmenopausal women and to men with prostatic carcinoma induces similar lithogenic effects [26–31]. These observations support the concept that higher risks for cholesterol gallstones in women than in men are related to differences in how the liver handles cholesterol in response to estrogen [1].

The actions of estrogen and estrogenic compounds have been traditionally described as occurring through one of the two classical nuclear estrogen receptors (ESRs), ESR1 and ESR2,¹ which function as ligand-dependent transcription factors that bind directly to estrogen response elements (EREs) in the promoter regions of genes and recruit additional transcriptional co-regulators through estrogen receptor activation function domain interactions [32]. In addition to the long-term regulation of gene expression (hours to days), estrogen also mediates rapid signaling events (seconds to minutes) such as the activation of cellular kinases, synthases and ion channels [33–36]. Thus, the ultimate cellular or physiological response to estrogen results from a complex interplay of signaling and transcriptional events. To complicate matters further, a structurally unrelated estrogen receptor, G protein-coupled receptor 30 (GPR30) has recently been identified [37,38]. GPR30, a member of the G protein-coupled receptor (GPCR) superfamily, is characterized by the presence of seven transmembrane helices and expressed predominantly at the endoplasmic reticulum and cell surface although the precise location of GPR30 needs to be further investigated. Some *in vitro* and *in vivo* experiments have shown that GPR30 may be involved in additional estrogen-dependent physiological responses [39–43]. In addition, many studies have found that hepatic hypersecretion of biliary cholesterol is the primary cause of cholesterol gallstones in humans and in several animal models of cholesterol gallstones [44–47]. Despite these observations, the metabolic abnormalities underlying the supersaturation of bile and the formation of cholesterol gallstones induced by estrogen are not yet fully understood. Here, we summarize the biochemistry of estrogen, ESR and GPR30, as well as the molecular pathophysiology of

¹The estrogen receptors alpha and beta are now referred to as estrogen receptors 1 and 2, respectively (official gene symbols are ESR1 and ESR2, respectively).

cholesterol gallstone formation, especially focusing on recent progress on the molecular mechanisms by which estrogen induces lithogenic effects through the ESR1 and the GPR30.

Physiology of estrogen and its receptors

Estrogen is the best-characterized member of the family of steroid hormones, named for its importance in the estrous cycle, and functioning as the primary female sex hormone [48]. Although estrogen is predominantly synthesized in the ovaries, recent studies have revealed that it is also synthesized at multiple discrete sites throughout the body where it may have localized effects. Similar to other steroid hormones highly hydrophobic in nature, estrogen readily passes through cellular membranes by passive diffusion and interacts with its receptors, ESR1 and/or ESR2 within the nucleus. Additionally, estrogen has been shown to activate a G protein-coupled receptor, GPR30.

There are three major naturally occurring estrogens in women: estradiol, estriol, and estrone (Figure 1). These hormones are all produced from androgens through actions of a series of enzymes such as aromatase, 17,20 lyase, 3 β -hydroxysteroid dehydrogenase, 17 β -hydrogenase, and 17 β -hydroxysteroid dehydrogenase (Figure 2). From menarche to menopause, the primary estrogen is 17 β -estradiol. In postmenopausal women more estrone is present than estradiol. Estradiol is produced from testosterone and estrone from androstenedione. Estrone displays weaker physiological functions than estradiol. Plasma concentrations of estrogen in women are typically in the 1-nM range, although tissue concentrations in normal breast tissue of postmenopausal women have been reported to be 10 to 20-fold higher than plasma concentrations, suggesting local production of the hormone. In addition to estradiol, the estrogen-based steroids including estriol, estrone and estrone sulfate also modulate biological functions.

(1) Chemistry and biosynthesis of estrogens

Estrogens are synthesized during steroidogenesis, with cholesterol as the starting molecule (Figure 2). Of note is that cholesterol contains 27 carbon atoms. The naturally occurring estrogens are 17 β -estradiol, estrone and estriol, and all of them are C₁₈ steroids; i.e., they do not have an angular methyl group attached to the 10 position or a Δ^4 -3-keto configuration in the A ring. So, the first step in the synthesis of steroid hormones is the removal of a C₆ unit from the side chain of cholesterol to form pregnenolone. The side chain of cholesterol is hydroxylated at C-20 and then at C-22, followed by the cleavage of the bond between C-20 and C-22. Progesterone is synthesized from pregnenolone in two steps. The 3-hydroxyl group of pregnenolone is oxidized to a 3-keto group, and the Δ^5 double bond is isomerized to a Δ^4 double bond. The synthesis of androgens starts with the hydroxylation of progesterone at C-17. The side chain consisting of C-20 and C-21 is then cleaved to yield androstenedione, an androgen. Testosterone, another androgen, is formed by reduction of the 17-keto group of androstenedione. Androgens contain nineteen carbon atoms. Estrogens are synthesized from androgens by the loss of the C-19 angular methyl group and the formation of an aromatic A ring. Androstenedione is a substance of moderate androgenic activity. This compound crosses the basal membrane into the surrounding granulosa cells, where it is converted to estrone or estradiol, either immediately or through testosterone. The conversion of testosterone to estradiol, and of androstenedione to estrone, is catalyzed by the enzyme aromatase.

Although the major estrogen-producing organs in females are the ovary, the corpus luteum and the placenta, some estrogens are also synthesized in smaller amounts by other tissues such as the liver, adrenal glands, and breasts. These secondary sources of estrogens are especially important in postmenopausal women. Of note is that estrogen biosynthesis is regulated by complex interactions between the two gonadotropins and between theca and granulosa cells [49,50]. Although theca and granulosa cells by themselves can synthesize some estrogens,

cooperative actions of both cell types is imperative for optimal hormone production. Cells of the theca interna respond to luteinizing hormone (LH) by forming large amounts of androstenedione and testosterone, which are the precursors of estrogens. However, because these cells have little aromatase activity, they synthesize trace amounts of estrogens. Granulosa cells, which make aromatase in response to follicle-stimulating hormone (FSH), are deficient in enzymes needed to convert C₂₁ precursors to C₁₉ androgens. Thus, granulosa cells can produce progesterone and pregnenolone, but these steroids cannot be used for estrogen synthesis until the side chain at C-17 is removed. When stimulated with FSH, granulosa cells readily transform androgens produced by cells of the theca interna to estrogens [50]. Simultaneously, C₂₁ steroids synthesized in granulosa cells can be converted to androgens by thecal cells. Therefore, the participation of two different cell types, each stimulated by its own gonadotropin, highlights the importance of both pituitary hormones to adequate estrogen production and, hence, to follicular development.

Estrogens circulate in blood loosely bound to albumin in a nonsaturable and nonstoichiometric manner and tightly bound to the testosterone-estrogen-binding globulin, which is also called the sex hormone-binding globulin, a β -globulin [51]. Plasma concentrations of estrogens are considerably lower than those of other gonadal steroids and vary over an almost 20-fold range during the cycle. Estradiol levels vary through the menstrual cycle and almost all of this type of estrogen comes from the ovary. In general, there are two peaks of estrogen secretion: one just before ovulation and the another during the midluteal phase [52–55]. The estradiol secretion rate is 36 μ g/day (i.e., 133 nmol/day) in the early follicular phase, 380 μ g/day just before ovulation, and 250 μ g/day during the midluteal phase. After menopause, estrogen secretion declines to low levels. In addition, the estradiol production rate in men is about 50 μ g/day (184 nmol/day). While estrogens are present in both men and women, they are usually present at significantly higher levels in women of reproductive age. Furthermore, estrogens promote the development of female secondary sex characteristics, such as breasts, and are also involved in the thickening of the endometrium and other aspects of regulating the menstrual cycle. In men, estrogens regulate certain functions of the reproductive system important to the maturation of sperm and may be necessary for a healthy libido. Hyperestrogenemia (elevated levels of estrogens in blood) may be a result of exogenous administration of estrogens or estrogen-like substances, or may be a result of physiologic conditions such as pregnancy.

The liver is the major site of metabolic destruction of the estrogens [48]. Estradiol and estrone are completely cleared from blood by a single passage through the liver, and are inactivated by hydroxylation and conjugation with sulfate and glucuronide. Approximately 50% of the protein-bound estrogens in blood are conjugated with sulfate or glucuronide. Although the liver may excrete some conjugated estrogens in bile, they are reabsorbed in the lower part of small intestine and returned to the liver via portal vein by a typical enterohepatic circulatory pattern. Estrogens are also metabolized by hydroxylation and subsequent methylation to form catechol and methoxylated estrogens. Hydroxylation of estrogens yields 2-hydroxyestrogens, 4-hydroxyestrogens, and 16 α -hydroxyestrogens (catechol estrogens). The kidney is the main route of excretion of estrogenic metabolites.

(2) Estrogen receptors and their agonists and antagonists

The receptor for estrogen (termed estrogen receptor (ESR) and later ESR1) was first characterized in the 1960s and was identified in 1973, based on a specific estrogen-binding activity found in rat uterus extracts [56–60]. The DNA sequence of ESR1 was described in 1986, and ten years later, the first crystal structure of an ESR ligand-binding domain was reported. In 1996, on the basis of its DNA-sequence homology to nuclear receptors, a second related estrogen receptor, ESR2, was identified in rat prostate [61]. Between ESR1 and ESR2 encoded by two distinct genes, *ESR1* and *ESR2*, respectively, there is approximately 60%

homology in the ligand-binding domains in humans [62,63]. Furthermore, these encoding genes are located on different chromosomes. The *ESR1* gene is mapped to the long arm q25.1 of chromosome 6, whereas the *ESR2* gene is located on band q23.2 of chromosome 14 in humans. It is clear that the biological effects of estrogen are generally ascribed to transcriptional modulation of target genes through these two subtypes of receptors. Although ESR1 and ESR2 are highly homologous in their DNA- and ligand-binding domains, the relative lack of homology in their transcriptional activation domains, as well as differences in the tissue distribution of the two receptors, suggest that there could be differences in their functions.

ESRs exist predominantly in the nucleus. ESRs could function as ligand-activated nuclear transcription factors that recognize *cis*-acting hormone response elements in the promoters of hormonally regulated genes. Estrogen binds to the ligand-binding domain of the receptor to form the complex of estrogen and ESRs. These estrogen-ESR complexes subsequently bind to specific sequences of DNA called estrogen-response elements (EREs) as homodimers or heterodimers [64]. The estrogen-ESR complexes bind not only to the response elements but also to nuclear-receptor coactivators or repressors. However, estrogen also regulates the transcription of genes that lack functional estrogen-response elements by modulating the activity of other transcription factors. Furthermore, upon ligand binding, conformational changes lead to chaperone dissociation and dimerization of the receptor, followed by DNA binding at proximal promoter sites, where the recruitment of coactivators and/or corepressors results in alterations in the rate of gene expression. However, recent studies find the recruitment of ESR to distal enhancer elements, suggesting that ESR-mediated transcriptional responses to estrogen could be a complicated process. Figure 3 illustrates the major actions of estrogen: these estrogen effects are mainly determined by the structure of the hormone, the subtype or isoform of the estrogen receptor involved, the characteristics of the target gene promoter, and the balance of coactivators and corepressors that modulate the final transcriptional response to the complexes of estrogen and ESRs.

Classically, 17 β -estradiol (E₂) is the most potent agonist at ESRs. It has been reported that propylpyrazole (PPT) is highly selective for ESR1 and binds ESR1 with an affinity that is 400-fold higher than for ESR2 [65–67]. Also, PPT is a potent agonist on ESR1 with no activity on ESR2. In contrast, diarylpropionitrile (DPN) is a full agonist with a 78-fold ESR2 potency selectivity [68]. ICI 182,780 binds with high affinity to both ESR1 and ESR2, and functions as a novel, pure and full estrogen antagonist [21,69,70]. Because tamoxifen, a synthetic, nonsteroidal, *trans*-isomeric derivative of triphenylethylene, has both anti-estrogen and estrogen-like activities [71,72], a possible relationship between the long-term administration of tamoxifen and the formation of gallstones should also be evaluated systematically. These synthetic estrogens that are ESR subtype-selective have the potential to be very useful tools for elucidating the roles of ESR1 and ESR2 in hepato-biliary lipid metabolism and cholesterol gallstone formation.

(3) G protein-coupled receptors

The G protein-coupled receptors (GPCRs) are one of the largest and most diverse protein families in mammalian genomes. On the basis of homology with rhodopsin, they are predicted to contain seven membrane-spanning helices, an extracellular N-terminus and an intracellular C-terminus [73–75]. GPCRs have been defined as receptors that signal through heterotrimeric guanine nucleotide-binding proteins to change the activity of effector proteins [36]. Ligand-activated GPCRs can also bind directly to intracellular proteins, inducing receptor and cellular modification [76,77]. The ligands of GPCRs include hormones, chemokines, biogenic amines, purines, amino acids, peptides, proteins, lipids, nucleotides, and ions, as well as neurotransmitters, light, odorants, and tastants [78,79].

The cloning and expression of the G protein-coupled receptor 30 (GPR30) was first reported in the 1990s [37,38]. GPR30 is a member of the rhodopsin-like family of G protein-coupled receptors and is a multi-pass membrane protein that localizes to the endoplasmic reticulum and cell surface although the precise location of GPR30 needs to be further investigated. It has been found that GPR30 transcripts are widely distributed in normal and malignant human tissues. In the normal physiological state, the heart, lung, liver, intestine, ovary, and brain usually display high expression levels of GPR30 [80]. Several primary breast cancers and lymphomas also expressed GPR30 transcripts [81]. In 2000, a potential ligand for GPR30 was identified [38,82], and it was found that GPR30 may be an integral membrane protein with high affinity for estrogen (Figure 3). After binding estrogen, GPR30 induces intracellular calcium mobilization and synthesis of phosphatidylinositol (3,4,5)-trisphosphate in the nucleus. Furthermore, it is found that GPR30 is activated by estrogen and acts independently of the ER to promote activation of the adenylyl cyclase/cAMP-dependent protein kinase A (PKA) pathway [38,83–86]. Thus, GPR30 could play a critical role in the rapid nongenomic signaling events that have been widely observed following stimulation of cells and tissues with estrogen.

The potential interplay between GPR30 and both ESR subtypes has broad implications for the physiology of estrogen-mediated transcription and signaling pathways. The classic ESRs interact with ligands in a remarkably dynamic and plastic manner; they alter the conformation and topology of ligand-bound receptor complexes, thereby mediating interactions with the various co-regulatory proteins that induce a spectrum of estrogen responses. A wide variety of compounds interact with ESRs, including ligands that discriminate between the two subtypes on the basis of affinity or efficacy as transcriptional activators, and considerable effort has focused on the development of subtype selective agents. In addition, GPR30 and the classical ESRs could evolve ligand-binding pockets that display considerable overlap in their ligand-binding profiles, despite the lack of any homology in their primary or secondary structure. Nevertheless, the current view supports the concept that GPR30 may be involved in additional estrogen-dependent physiological responses.

Clinical epidemiology and gender differences

It has been found that in a large study of the Danish population, the five-year incidence rates of gallstones are 0.3%, 2.9%, 2.5%, and 3.3% for Danish men aged 30, 40, 50, and 60 years, respectively; the corresponding rates are 1.4%, 3.6%, 3.1% and 3.7% for Danish women [4]. Some important points can be derived from these data: women have a higher incidence than men do at ages 30 and 40, but this difference reduces with increasing age. The results from these clinical studies could represent real genetic and environmental factors in the specific populations chosen for the epidemiological investigation of gallstones, because these data are appropriate to rates from estimated prevalence rates reported for Denmark and other populations [87].

In the earlier studies of American Pima Indians, the prevalence rates of gallstones were examined by oral cholecystography [12]. With this technique, it is found that the Pima Indians in southern Arizona display very high gallstone prevalence, in which 70% of the women form gallstones after the age of 25. Following the widespread use of real-time ultrasonography for the diagnosis of gallstones, a large study is carried out in nationally representative samples of civilian Mexicans, American Caucasians, non-Hispanic white and non-Hispanic black Americans aged 20–74 of both genders. The cross-sectional prevalence rates of gallstones are found to be highest in certain tribes of American Indians (e.g., Pima Indians), higher in Hispanic Americans than in Caucasians and lowest of all in black Americans. Furthermore, the prevalence rates of gallstones are still higher in women than men in these populations studied [11,12,16].

Ultrasonographic screening or necropsy studies are often used to estimate the prevalence of gallstone disease in different populations. Although ultrasonographic screening cannot distinguish cholesterol from pigment stones, it can be assumed that 70% to 80% of gallbladder gallstones are cholesterol type in the Western countries. Clearly, there is a striking difference in gallstone formation among different populations, highlighting that certain genetic factors could play a key role in the pathogenesis of gallstones, but these are likely to be multifactorial and to vary among populations because many pathophysiologic factors could determine gallstone formation [44]. Furthermore, these differences could represent real genetic and/or environmental factors or specific populations chosen for each study. Especially, most studies have revealed that the prevalence rates for women varied from 5% to 20% between the ages of 20 and 55 and from 25% to 30% after the age of 50. The prevalence rates for men were approximately half of those for women for a given age group.

Clinical studies have found that pregnancy and parity are two important risk factors for the formation of cholesterol gallstones [88–91]. Real-time ultrasonographic investigations have revealed that pregnancy is a greater risk factor for the development of biliary sludge [92–94]. During pregnancy, bile becomes more lithogenic as a result of increased estrogen levels, which results in increased hepatic secretion of biliary cholesterol and cholesterol-supersaturated bile. Additionally, high levels of estrogen could impair gallbladder motility function and consequently induce gallbladder hypomotility [95]. These changes promote the formation of sludge and stones. Increased plasma levels of progesterone also reduce gallbladder motility [96,97]. Since plasma hormone concentrations increase linearly with duration of gestation, the risk of gallstone formation is especially hazardous in the third trimester of pregnancy. Increasing parity could be a significant risk factor for gallstones, especially in younger women.

Most, but not all, studies have found that the use of oral contraceptive steroids and conjugated estrogens in premenopausal women double the incidence of cholesterol gallstones [1,3,22–24]. In addition, the administration of estrogen to postmenopausal women and estrogen therapy to men with prostatic carcinoma display similar lithogenic effects [26–31]. These observations underscore that estrogen is an important risk factor for the formation of cholesterol gallstones.

Molecular mechanisms underlying the effect of estrogen on gallstone formation

Because the prevalence of gallstones is remarkably higher in women than men at all ages as found by epidemiological investigations as well as long-term administration of oral contraceptive steroids and conjugated estrogens markedly increases the risk of cholesterol gallstones, it is hypothesized that estrogen could enhance cholesterol cholelithogenesis by augmenting functions of estrogen receptors in the liver and gallbladder [1]. Although ESR1 and ESR2 have overlapping but not identical tissue expression patterns [48,98], they are both expressed in the hepatocyte [98–100]. Using quantitative real-time PCR techniques, it is found that expression levels of the *Esr1* gene are ~50-fold higher compared with those of *Esr2*, suggesting that ESR1 is a major steroid hormone receptor producing the biological effects of estrogen in the liver [98,100]. By using gonadectomized AKR mice fed the lithogenic diet for 12 weeks, expression levels of *Esr1* in the liver stimulated by various doses (0, 3, or 6 µg/day) of 17β-estradiol (E_2) and its antagonists and agonists are further investigated [100]. It is observed that expression levels of *Esr1* are similar among mice with intact gonads, E_2 -deficient mice, and mice receiving E_2 at 3 µg/day. Moreover, there are significantly increased expression levels of *Esr1* when mice are treated with E_2 at 6 µg/day, the ESR1-selective agonist PPT, and tamoxifen. However, the E_2 effects on expression of the hepatic *Esr1* mRNA can be blocked by the antiestrogenic ICI 162,780. The ESR2-selective agonist DPN does not influence gene expression of *Esr1*, but up-regulates the expression of the *Esr2* in the liver.

To explore the molecular mechanisms of how estrogen influences cholesterol gallstones, a group of mouse models are established with both ovariectomized females and orchidectomized males of gallstone-resistant AKR mice. These gonadectomized mice are subcutaneously implanted with pellets releasing E₂ at 0, 3, or 6 µg/day and fed a lithogenic diet for 12 weeks [100]. To study whether ESRs have a crucial effect on mediating lithogenic actions of estrogen and to dissect the potential pathophysiological roles of each receptor subtype, ESR1 and ESR2, in the formation of gallstones, gonadectomized mice treated with synthetic ESR subtype-selective agonists or antagonists are further investigated [100]. At 12 weeks after feeding the lithogenic diet, the prevalence of gallstones exhibits a dose-dependent increase in gonadectomized mice treated with various doses of exogenous E₂. Obviously, increasing doses (from 0, to 3, to 6 µg/day) of E₂ raise gallstone prevalence from 20%, to 25%, and to 80%, respectively. In contrast, all mice with a combined treatment of E₂ at 6 µg/day and ICI 182,780 at 125 µg/day are gallstone free, highlighting that the lithogenic effects of E₂ are blocked by this antiestrogenic agent. Furthermore, 75% of the mice treated with the ESR1-selective agonist PPT form gallstones, showing that its lithogenic effects at 50 µg/day are as strong as those of E₂ at 6 µg/day. In contrast, the ESR2-selective agonist DPN does not increase gallstone formation markedly, with prevalence rates being 25%. Tamoxifen at 40 µg/day promotes the formation of gallstones with prevalence rates of 50%, indicating that it produces estrogen-like effects on biliary lipid metabolism. These studies strongly support the concept that the receptor-dependent effects of E₂ contribute to biliary cholesterol hypersecretion and cholesterol supersaturation of bile, which significantly enhance the formation of cholesterol gallstones. Furthermore, these findings show that E₂ promotes gallstone formation by up-regulating hepatic expression of ESR1 but not ESR2, and the lithogenic actions of estrogen can be blocked by the antiestrogenic ICI 182,780. The ESR1-selective agonist PPT, but not the ESR2-selective agonist DPN, enhances gallstone formation. Similar to the E₂ treatment, tamoxifen significantly increases gallstone prevalence in both gonadectomized females and males of AKR mice. These observations strongly suggest that the hepatic ESR1, but not ESR2, plays a crucial role in E₂-induced cholesterol gallstones. Furthermore, these results are consistent with the lithogenic effects of estrogen observed in other species, including humans. These findings may offer a new approach to treat gallstones by inhibiting hepatic ESR1 activity with a liver-specific, ESR1-selective antagonist.

Accumulated evidence has revealed that estrogen increases the risk for the formation of cholesterol gallstones by promoting hepatic secretion of biliary cholesterol that induces an increase in cholesterol saturation of bile in humans and in several animal models of cholesterol gallstones [29–31,101–113]. In addition, observations from human and animal studies [101, 105,108,114] have shown that high levels of estrogen significantly enhance the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in hepatic cholesterol biosynthesis, even under high dietary cholesterol loads. These findings suggest that there could be an increased delivery of cholesterol to bile from *de novo* synthesis in the liver. Furthermore, some studies in humans and animals observe that estrogen could augment the capacity of dietary cholesterol to induce cholesterol supersaturation of bile [100–103]. It is also found that high doses of estrogen augment intestinal cholesterol absorption [102], contributable in part to an up-regulated expression of intestinal sterol influx transporter Niemann-Pick C1 like 1 protein (NPC1L1) via the intestinal ESR1 pathway [115]. Because of species differences, NPC1L1 is expressed in the livers of humans and hamsters [116,117, 118], but not in mouse livers [117,119,120]. Together with hepatic lipid transporters such as ABCG5/G8 on cholesterol secretion, ABCB11 on bile salt secretion and ABCB4 on phospholipid secretion [1], NPC1L1 may participate in the regulation of biliary cholesterol secretion. It has been found that ezetimibe treatment can prevent the formation of cholesterol gallstones in mice [117,119] and reduce cholesterol concentrations in bile in humans and hamsters [118,119]. In a preliminary, it is observed that E₂ could promote biliary cholesterol secretion mostly through upregulating expression levels of ABCG5/G8 and ABCB11 in the

liver. However, it needs to further investigate the effect of NPC1L1 on biliary cholesterol secretion under E₂ treatment conditions. Despite these observations, little 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.

To investigate whether the high hepatic output of biliary cholesterol observed in E₂-treated mice is the result of a higher rate of hepatic cholesterologenesis, the contribution of newly synthesized hepatic cholesterol to biliary output are measured [105]. Also, biliary secretion rates of total and newly synthesized cholesterol are observed over 4 hours in mice administered with [³H]water for 6 hours before the commencement of total biliary diversion. As a consequence of the surgery that ensures complete interruption of the enterohepatic circulation of bile salts, external biliary drainage in each mouse gave a “washout curve” as evidenced by measurement of bile salt secretion rate. It is observed that during the 4-hour period of interrupted enterohepatic circulation, biliary bile salt output gradually decreases with time. In contrast, cholesterol secretion rate is unaltered during the first 4 hours of biliary washout [121]. Hence, this 4 hour analysis provides biliary cholesterol output during simulation of an intact enterohepatic circulation. In addition, it has been reported 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 the rate of biliary output of both total and labeled cholesterol is constant during the first 4 hours of biliary washout [121]. These experiments further observe hepatic outputs of biliary total and newly synthesized cholesterol in female AKR mice with intact ovaries (i.e., control mice) and in ovariectomized mice treated with E₂ or E₂ plus ICI 182,780, and on chow or fed the high (1%) cholesterol diet for 14 days. In the chow-fed state, hepatic outputs of biliary total and newly synthesized cholesterol are ~5.0 μmol/h/kg and ~0.7 μmol/h/kg, respectively, and the relative contribution of newly synthesized cholesterol to biliary output is ~15% in control mice. E₂ treatment induces a significant increase in hepatic outputs of biliary total (~8.0 μmol/h/kg) and newly synthesized cholesterol (~3.0 μmol/h/kg) and the relative contribution of newly synthesized cholesterol to biliary output is increased to ~45%. In addition, the high (1%) cholesterol diet slightly increases biliary total cholesterol output to ~7.0 μmol/h/kg in control mice, because the AKR mouse is a gallstone-resistant strain. However, the relative contribution of newly synthesized cholesterol to biliary total cholesterol output is reduced to ~6%. In contrast, the secreted newly synthesized cholesterol is 5-fold higher in E₂-treated mice on the high cholesterol diet than those in control mice. Also, E₂ treatment results in a significant increase in biliary total cholesterol outputs (~18.0 μmol/h/kg). Under these circumstances, the origin of biliary cholesterol possibly 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. Furthermore, the biological actions of E₂ are blocked by the antiestrogenic agent ICI 182,780. As a result, hepatic outputs of biliary total and newly synthesized cholesterol are essentially similar between ovariectomized mice treated with E₂ plus ICI 182,780 and control mice, regardless of whether chow or the high cholesterol diet is fed.

To explore whether there is an “estrogen-ESR1-SREBP-2” pathway for the regulation of hepatic cholesterol biosynthesis, expression levels of the sterol regulatory element-binding protein-2 (*Srebp-2*) gene and five major SREBP-2-responsive genes in the liver are investigated by quantitative real-time PCR methods [105]. On the chow diet, high doses of E₂ significantly increase the relative mRNA levels of the *Srebp-2* gene. Furthermore, high dietary cholesterol significantly reduces expression levels of *Srebp-2* by approximately 50% compared with the chow diet in control mice. These observations show that cholesterol biosynthesis may be inhibited by a negative feedback regulation possibly through the SREBP-2 pathway [122, 123]. In contrast, E₂-treated mice still display significantly higher expression levels of *Srebp-2*, even under high dietary cholesterol loads. These results indicate that under conditions

of high levels of E₂, 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 ESR1. Again, these biological actions of E₂ are abolished by the antiestrogenic ICI 182,780, regardless of whether chow or high dietary cholesterol is fed.

When expression levels of five major SREBP-2-responsive genes including HMG-CoA synthase (isoforms 1 and 2), HMG-CoA reductase, farnesyl diphosphate synthase, squalene synthase, and lathosterol synthase in the liver are further investigated under the same experimental conditions as described above, it is observed that in control mice, expression levels of these SREBP-2-responsive genes are significantly reduced by the high cholesterol diet compared with the chow diet. Furthermore, high doses of E₂ significantly up-regulate the relative mRNA levels for the SREBP-2-responsive genes in the liver, regardless of whether chow or high dietary cholesterol is fed. Again, these biological effects of E₂ on expression levels of the SREBP-2-responsive genes are significantly attenuated by ICI 182,780. These findings support the notion that under the 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 attenuated possibly by E₂ via the hepatic ESR1 pathway. Obviously, these findings suggest a possible “estrogen-ESR1-SREBP-2” pathway that regulates hepatic cholesterol biosynthesis [105]. Furthermore, these results show that during estrogen treatment, mice continue to synthesize cholesterol in the face of its excess availability from the high cholesterol diet. It suggests that there is a loss in the negative feedback regulation of cholesterol biosynthesis, which results in excess secretion of newly synthesized cholesterol and supersaturation of bile that predisposes to cholesterol precipitation and gallstone formation [105].

In addition, estrogen could decrease plasma low-density lipoprotein (LDL) cholesterol and increase plasma high-density lipoprotein (HDL) cholesterol because high doses of E₂ amplify expression levels of HDL receptor SR-BI and LDL receptor [124–127]. The decrease in plasma LDL is a result of increased hepatic LDL receptor expression, which increases the clearance of plasma LDL. Therefore, the increased uptake of LDL by the liver may result in increased secretion of cholesterol into the bile. These alterations could induce an apparent increase in hepatic output of biliary cholesterol derived from circulating lipoproteins such as HDL and LDL, although LDL cholesterol could have a less effect on biliary secretion.

Although estrogen has been proposed to be one important risk factor for cholesterol gallstones, the predominant mechanisms of lithogenic action of E₂ depend on the ESR subtypes and the dose of hormone administered. Obviously, these observations suggest a critical role for ESR1 in E₂-induced cholesterol gallstones. To explore whether deletion of the *Esr1* gene decreases susceptibility to estrogen-induced cholesterol gallstones, ESR1 deficient mice are challenged to the lithogenic diet and treated with high doses of estrogen [128]. The ESR1 knockout mice in an AKR genetic background of gallstone-resistant strain are generated by targeting deletion of the *Esr1* gene. At 4 weeks of age, ESR1 knockout and wild-type mice are gonadectomized. At 8 weeks, these mice are implanted subcutaneously with pellets designed to release E₂ at 6 µg/day for 8 weeks. Under conditions of the lithogenic diet feeding, cholesterol crystallization and gallstone formation are greatly accelerated in wild-type mice when challenged to high levels of E₂, mainly through up-regulating expression levels of the *Esr1* gene in the liver. Compared with ESR1 knockout mice, E₂-treated wild-type mice display significantly increased expression levels of mRNAs of SREBP-2 and other four major genes for cholesterol biosynthesis pathway, no matter if the chow or the lithogenic diet is fed. The increase in hepatic cholesterol synthesis could be associated with a significant increase in hepatic secretion of biliary cholesterol. However, the E₂ effects on increasing cholesterol biosynthesis and promoting cholesterol gallstone formation are partially blocked by deletion of the *Esr1* gene. The marked reduction in cholesterol synthesis correlates with the significant decrease in the

amount of mRNAs of SREBP-2 and multiple genes for cholesterol biosynthesis and biliary cholesterol secretion in ESR1 knockout mice. These observations suggest that the hepatic ESR1 is an important, selective target for the treatment of cholesterol gallstones. Figure 4 illustrates the potential lithogenic mechanisms of estrogen through the ESR1 pathway in the liver.

New directions for future studies

Clinical studies have found that in the U.S. cholesterol cholelithiasis is one of the most prevalent and most costly digestive diseases with at least 20 million Americans (12% of adults) affected [129,130]. Although many gallstones are “silent”, approximately a third eventually cause symptoms and complications. Each year, approximately one million new cases are diagnosed and 700,000 cholecystectomies are performed, as well as the unavoidable complications result in 3,000 deaths (0.12% of all deaths). Furthermore, medical expenses for the treatment of gallstones exceeded \$6 billion in the year 2000. The prevalence of cholesterol gallstones seems to be rising because of the world-wide obesity epidemic with insulin resistance being part of the metabolic syndrome. Therefore, it is crucial to systematically investigate pathophysiology of cholesterol gallstones and understand the molecular pathogenesis that underlies the formation of cholesterol gallstones. Especially, there are gender differences in cholesterol gallstones and high levels of estrogen are a significant risk factor for this disease.

With the recent identification of GPR30 for the activation of rapid cellular signaling pathways induced by estrogen, our understanding of the molecular mechanisms of estrogen action has become more complicated. A series of *in vitro* and *in vivo* studies have clearly demonstrated that GPR30 is a novel estrogen-responsive receptor that functions alongside the classical ESR1 and ESR2 to influence the physiological responses to estrogen. Not only does GPR30 activate rapid kinase signaling pathways, it also mediates transcriptional regulation of genes and regulates gene expression in response to estrogen, which is previously thought to be the domain of the classical ESR1 and ESR2 only (although not necessarily through the estrogen response elements (EREs)). It is unclear why the ligand-binding specificity of GPR30 overlaps substantially with that of ESR1 and ESR2, and whether there are ligands that display high selectivity for each type of ESR, or GPR30. Furthermore, it is imperative to explore what signals may result from the interaction of these two receptors, either directly or indirectly via their downstream signaling events.

For future studies, although hepatic ESR1 has been identified to play a crucial role in the formation of cholesterol gallstones [100], the individual contributions of ESR1 and GPR30 to the lithogenic effects of estrogen need to be defined and the individual roles of ESR1 and GPR30 in the rapid signaling effects of estrogen in the liver and gallbladder should be characterized. In addition, the molecular mechanisms by which ESR1 and GPR30 interact to regulate the hepatic metabolism of cholesterol and bile salts as well as hepatic secretion of biliary lipids, are likely to be complex. It is reasonable to hypothesize that either receptor may augment or alternatively inhibit signaling through the other receptor, with such interactions depending on the cellular context. In addition, estrogen, through ESR1 and/or GPR30, may mediate rapid cellular signaling via the suppression of gallbladder CCK-1 receptor gene expression, possibly resulting in impaired binding activity of CCK with its receptor and dysfunctional gallbladder motility. Obviously, all of these will require extensive investigation. These studies may provide an efficacious novel strategy for the prevention of cholesterol gallstones by inhibiting the activities of both hepatic ESR1 and GPR30 with a liver-specific antagonist, particularly for postmenopausal women and patients who are exposed to high doses of estrogen.

Abbreviations

DPN, diarylpropionitrile
 E₂, 17 β -estradiol
 ESR, estrogen receptor
 ERE, estrogen response element
 FSH, follicle-stimulating hormone
 GPCR, G protein-coupled receptor
 GPR30, G protein-coupled receptor 30
 HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A
 LH, luteinizing hormone
 NPC1L1, Niemann-Pick C1 like 1
 PKA, protein kinase A
 PPT, propylpyrazole
 SERM, selective estrogen receptor modulator

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Estradiol
 (8R,9S,13S,14S,17S)-13-methyl-
 6,7,8,9,11,12,14,15,16,17-
 decahydrocyclopenta[a]phenanthrene-
 3,17-diol
 (C₁₈H₂₄O₂)
 m.w. 272.38

Estriol
 (8R,9S,13S,14S,16R,17R)-13-methyl-
 7,8,9,11,12,13,14,15,16,17-
 decahydro-6H-cyclopenta[a]phenanthrene-
 3,16,17-triol
 (C₁₈H₂₆O₃)
 m.w. 288.38

Estrone
 (8R,9S,13S,14S)-3-hydroxy-13-methyl-
 7,8,9,11,12,13,15,16-octahydro-6H-
 cyclopenta[a]phenanthren-17(14H)-one
 (C₁₈H₂₂O₂)
 m.w. 270.37

Figure 1.

Three major naturally occurring estrogens in women: formal chemical names (IUPAC), molecular formulae and molecular weights (left panel); molecular structures (middle panel); 3-dimensional models (right panel) of estradiol, estriol and estrone.

Figure 2.

The estrogen biosynthesis pathway. The naturally occurring estrogens include 17 β -estradiol, estrone and estriol, and all of them are C₁₈ steroids. 17 β -Estradiol is the most potent estrogen of the three, and estriol is the least. These hormones are steroids and are derived from cholesterol by the series of reactions. The principal hormones secreted by the ovary are estrogens (17 β -estradiol and estrone) and progesterone. See text for details.

Figure 3.

The potential mechanisms of estrogen-mediated signaling through estrogen receptor (ESR) and G protein-coupled receptor 30 (GPR30). Estrogen plays a role in the regulation of cellular functions through two signaling pathways previously broadly classified as “genomic” and “nongenomic”. More recently, the FASEB steroid signaling work group suggested that “nuclear-initiated steroid signaling” and “membrane-initiated steroid signaling” are more appropriate terminologies. The nuclear-initiated signaling of estrogen via ESR1 and ESR2, i.e., the “classical” signaling pathway of estrogen, exert diverse effects on a variety of tissues that involves gene stimulation as well as gene repression. Estrogen is freely cell permeable, passively diffusing into the cell to act as a transcription factor by binding to its receptor, ESR1 and ESR2, which causes a change in receptor tertiary and quaternary structure to form the active complex. The active complex then bound a unique DNA sequence known as the estrogen response elements (ERE) on the DNA upstream from estrogen responsive genes, i.e., in estrogen responsive gene promoters. Subsequently, transcription and translation of these genes results in proteins that ultimately are responsible for mediating estrogen’s effects.

Alternatively, the complex may induce the production of a specific protein more indirectly through the activation of its transcription factor. In addition, estrogen regulates a plethora of biological processes through the membrane-initiated signaling, i.e., GPR30 pathway. Upon estrogen binding, GPR30 activates heterotrimeric G proteins that stimulate multiple effectors, including production of cyclic adenosine monophosphate (cAMP) as well as steroid receptor coactivator (SRC), a family of nuclear receptor coactivators. The latter pathway in turn activates matrix metalloproteinase (MMP), which cleaves pro-heparin-binding epidermal

growth factor-like growth factor (pro-HB-EGF) and promotes a release of free HB-EGF. Subsequently, HB-EGF transactivates epidermal growth factor receptor (EGFR). EGFR activation induces multiple downstream events, including the activation of phospholipase C (PLC), mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K). PLC activation results in intracellular calcium mobilization by inositol triphosphate receptor (IP3R) via the actions of inositol triphosphate (IP3). The activation of MAPK and PI3K stimulates numerous cytosolic pathways and some transcription factors. These estrogen stimulations could induce the transcription of target genes whose promoters do not contain estrogen response elements.

Figure 4.

The proposed model underlying the potential lithogenic mechanisms of estrogen through the estrogen receptor 1 (ESR1) pathway in the liver. In the liver there is a possible “estrogen-ESR1-SREBP-2” pathway promoting cholesterol biosynthesis and hepatic secretion of biliary cholesterol. The negative feedback regulation of cholesterol biosynthesis (as shown in a dashed line) is inhibited by ESR1 that is activated by estrogen, mostly through stimulating the activity of sterol regulatory element-binding protein-2 (SREBP-2) with the resulting activation of the SREBP-2 responsive genes for the cholesterol biosynthetic pathway. Consequently, these alterations induce excess secretion of newly synthesized cholesterol and supersaturation of bile that predisposes to cholesterol precipitation and gallstone formation. Moreover, the hepatic

ESR1 activated by estrogen could stimulate the activity of ATP-binding cassette (ABC) transporter ABCG5 and ABCG8 on the canalicular membrane of the hepatocyte and promote biliary cholesterol secretion. Of special note is that these lithogenic effects of estrogen are inhibited by the antiestrogenic ICI 182,780. In addition, the estrogen effects on increasing cholesterol biosynthesis and promoting cholesterol gallstone formation are, in part, blocked by deletion of the *Esr1* gene. See text for details. Modified from Reference [128], with permission.