Hepatic steroid sulfatase critically determines estrogenic activities of conjugated equine estrogens in human cells *in vitro* and in mice

Received for publication, May 6, 2019, and in revised form, June 17, 2019 Published, Papers in Press, June 19, 2019, DOI 10.1074/jbc.RA119.009181

Ye Feng[‡§], Yang Xie[‡], Meishu Xu[‡], Linhao Li[¶], Kyle W. Selcer[‖], Patrick J. Oberly[**], Samuel M. Poloyac[**], Hongbing Wang[¶], Chengjiang Li[§], Fengqin Dong[§], Chaohui Yu[‡‡1], and Wen Xie[‡§§2]

From the [‡]Center for Pharmacogenetics and Department of Pharmaceutical Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, [§]Department of Endocrinology and Metabolic Disease, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, China, [¶]Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, Baltimore, Maryland 21201, [‖]Department of Biological Sciences, Duquesne University, Pittsburgh, Pennsylvania 15282, [**]Department of Pharmaceutical Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, [‡‡]Department of Gastroenterology, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, China, and [§§]Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Edited by Xiao-Fan Wang

Conjugated equine estrogens (CEEs), whose brand name is Premarin, are widely used as a hormone-replacement therapy (HRT) drug to manage postmenopausal symptoms in women. Extracted from pregnant mare urine, CEEs are composed of nearly a dozen estrogens existing in an inactive sulfated form. To determine whether the hepatic steroid sulfatase (STS) is a key contributor to the efficacy of CEEs in HRT, we performed estrogen-responsive element (ERE) reporter gene assay, real-time PCR, and UPLC-MS/MS to assess the STS-dependent and inflammation-responsive estrogenic activity of CEEs in HepG2 cells and human primary hepatocytes. Using liver-specific STS-expressing transgenic mice, we also evaluated the effect of STS on the estrogenic activity of CEEs *in vivo*. We observed that CEEs induce activity of the ERE reporter gene in an STS-dependent manner and that genetic or pharmacological inhibition of STS attenuates CEE estrogenic activity. In hepatocytes, inflammation enhanced CEE estrogenic activity by inducing STS gene expression. The inflammation-responsive estrogenic activity of CEEs, in turn, attenuated inflammation through the anti-inflammatory activity of the active estrogens. *In vivo*, transgenic mice with liver-specific STS expression exhibited markedly increased sensitivity to CEE-induced estrogenic activity in the uterus resulting from increased levels of liver-derived and circulating estrogens. Our results reveal a critical role of hepatic STS in mediating the hormone-replacing activity of CEEs. We propose that caution needs to be applied when Premarin is used in patients with chronic inflammatory liver diseases because such patients may have heightened sensitivity to CEEs due to the inflammatory induction of STS activity.

As the average age of menopause is ~50, and the average life expectancy of women is over 85, women may live more than one-third of their life in the postmenopausal state with dramatic sex hormone deficiencies. Many women during the early phase of menopause experience a number of discomforts, such as vasomotor symptoms (hot flashes), vaginal dryness, insomnia, and mood disturbances. Hormone-replacement therapy (HRT),[3] including estrogens with or without a progestogen, is the most effective therapy for these symptoms (1). Besides relieving menopausal symptoms, HRT has been reported to reduce the incidence of osteoporosis, heart disease, and dementia as well as all-cause mortality. The benefits of HRT have been supported by many clinical and epidemiological studies and meta-analyses (2, 3).

Conjugated equine estrogens (CEEs), whose brand name is Premarin, are one of the most prescribed HRT drugs. CEEs are a complex natural extract from pregnant mare urine, containing nearly a dozen different estrogens with equilin and estrone as the main components (4). The estrogens present in CEEs are the sulfate esters. Unlike estrogens, sulfonated estrogens cannot bind to the estrogen receptor (ER) and thus they are hormonally inactive (5, 6). To the best of our knowledge, how the estrogen sulfates in CEEs exert their pharmacological activity of hormone replacement remains to be defined.

This work was supported in part by National Institutes of Health Grants DK083952, ES023438 (to W. X.), and GM121550 (to H. W.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

[1] To whom correspondence may be addressed. Tel.: 86-571-85093443; Fax: 86-571-87236861; E-mail: zyyyych@zju.edu.cn.

[2] Supported in part by the Joseph Koslow Endowed Professorship from the University of Pittsburgh School of Pharmacy. To whom correspondence may be addressed: Center for Pharmacogenetics and Dept. of Pharmaceutical Sciences, University of Pittsburgh, Pittsburgh, PA 15261. Tel.: 412-648-9941; Fax: 412-648-1664; E-mail: wex6@pitt.edu.

[3] The abbreviations used are: HRT, hormone-replacement therapy; CEE, conjugated equine estrogen; DOX, doxycycline; E_2, estradiol; ER, estrogen receptor; ERE, estrogen-responsive element; EST, estrogen sulfotransferase; FABP, fatty acid–binding protein; HPH, human primary hepatocyte; *Hsp*, heat shock protein; ICI, ICI 182,780; IHC, immunohistochemistry; LPS, lipopolysaccharide; Luc, luciferase; PDTC, pyrrolidine dithiocarbamate; PMA, phorbol 12-myristate 13-acetate; STS, steroid sulfatase; STX, STX64; SULT, sulfotransferase; TFF1, trefoil factor 1; TG, transgenic; TNFα, tumor necrosis factor α; TRE, tetracycline-response element; tTA, tetracycline transactivator; UPLC-MS/MS, ultraperformance liquid chromatography-tandem mass spectrometry; WHI, Women's Health Initiative.

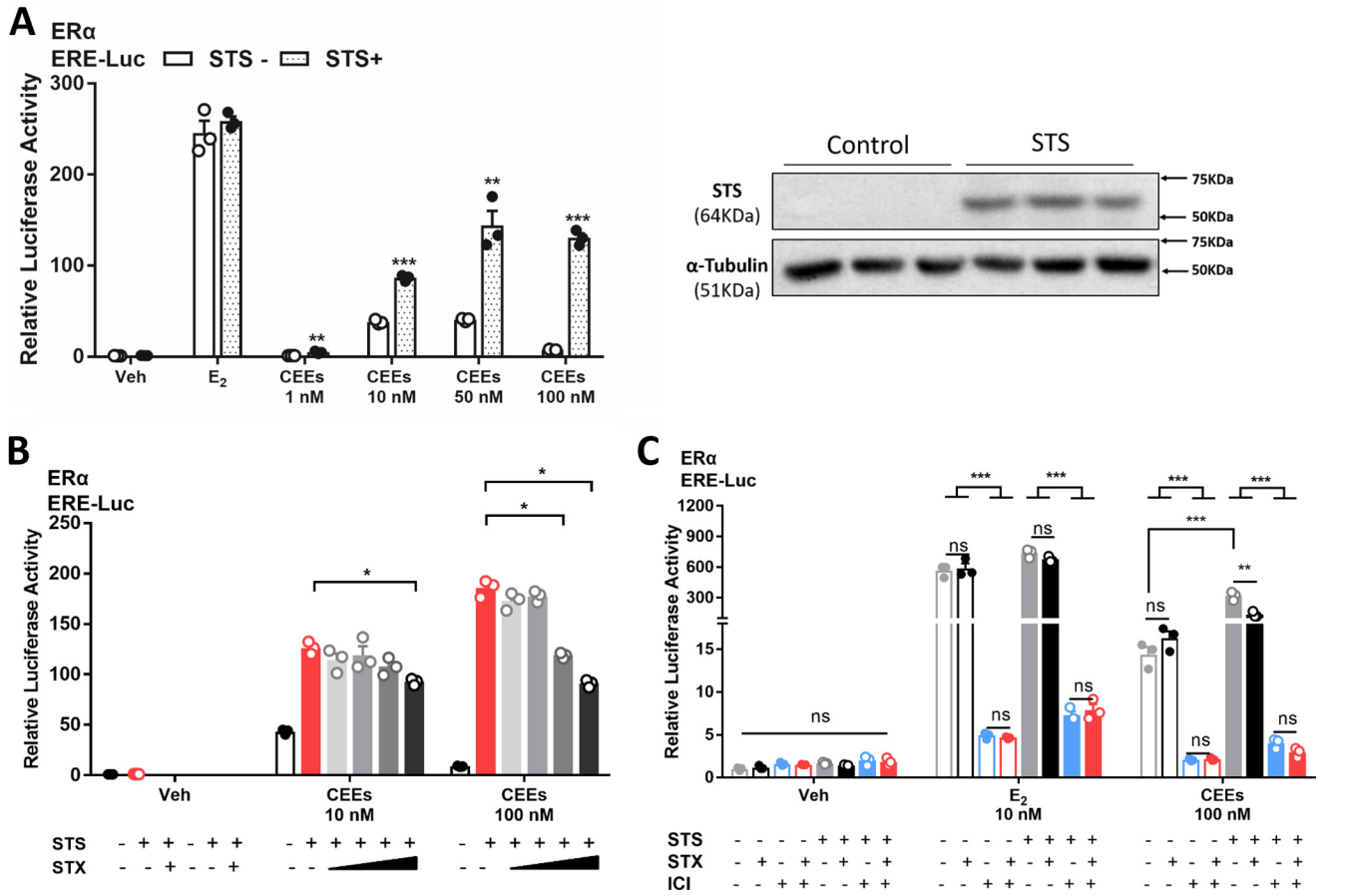

Figure 1. The expression and activity of STS are required for the estrogenic activity of CEEs in reporter gene assay. 293T cells were transfected with ERα expression vector and the ERE-luciferase reporter in the absence or presence of cotransfection of STS expression vector. Transfected cells were treated with the indicated drugs or their combinations at the indicated drug concentrations for 24 h before cell lysis and luciferase assay. *A*, treatment with CEEs induced the ERE-luciferase reporter activity in a concentration-dependent manner. E_2 was included as the positive control (*left*). Shown on the *right* is the verification of STS protein expression in STS-transfected cells by Western blotting. *B*, treatment with the STS inhibitor STX inhibited CEE-responsive ERE-luciferase reporter activity in a dose-dependent manner. *C*, treatment with the ER antagonist ICI (100 nM) abolished the CEE-responsive and STS-dependent ERE-luciferase reporter activity. Results are -fold induction over the vehicle controls from triplicate assays. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with vehicle (*Veh*) (*A*). Other comparisons are labeled. *ns*, not significant. *Error bars* represent S.E.

Steroid sulfatase (STS) is the enzyme responsible for the hydrolysis of steroid sulfates to their unconjugated forms (7). Although the tissue distribution of STS varies considerably among mammalian species, the liver is one of the organs rich in this enzyme. STS mediated desulfation and sulfotransferase (SULT) mediated sulfation are critical metabolic mechanisms to regulate the chemical and functional homeostasis of endogenous chemicals, including the estrogens. Estrogen sulfotransferase (EST; or SULT1E1) is the primary SULT that sulfonates and deactivates estrogens at their physiological concentrations (8). Compared with the parent estrogens, estrogen sulfates have higher concentrations and prolonged half-life in the circulation. The desulfation of steroid sulfate is catalyzed by STS, which is believed to be the only enzyme responsible for the activation of conjugated estrogens. Although it is known that CEEs are a natural mixture of sulfated estrogens, the specific role of STS in the hormone-replacement effect of CEEs has yet to be established. Here, we provide genetic and pharmacological evidence that hepatic STS and its inflammatory regulation in hepatocytes are essential for the hormone-replacement therapy efficacy of CEEs.

Results

The expression and activity of STS are required for the estrogenic activity of CEEs in a reporter gene assay

To evaluate the estrogenic activity of CEEs and its dependence on STS *in vitro*, 293T cells were transfected with ERα expression vector and the estrogen-responsive luciferase reporter gene ERE-Luc in the absence or presence of the cotransfection of STS expression vector. The transfected cells were then treated with vehicle or increasing concentrations of CEEs. Without the STS cotransfection, treatment with the positive control, estradiol (E_2), efficiently induced the reporter activity as expected, whereas CEEs elicited little reporter activity (Fig. 1*A*), consistent with the notion that conjugated estrogens are hormonally inactive. In contrast, cotransfection of STS markedly elevated CEE-induced reporter activity at all of the tested concentrations, and CEEs induced the reporter activity in a concentration-dependent manner (Fig. 1*A*). Cotransfection of STS had little effect on the activity of E_2 (Fig. 1*A*). In STS-cotransfected cells, treatment with STX64, a pharmacological inhibitor of STS (9), concentration-dependently inhibited CEE-elicited reporter activity (Fig. 1*B*), suggesting that the

STS activity was required for STS-dependent estrogenic activity of CEEs. The CEE-elicited reporter activity in STS-transfected cells was estrogen-dependent because the stimulated reporter activity was abolished in cells cotreated with the ERα antagonist fulvestrant (also called ICI 182,780 (ICI)) (Fig. 1*C*).

Inflammation enhances the estrogenic activity of CEEs by inducing the expression of STS in hepatoma HepG2 cells

We have previously reported that inflammation can induce the expression of STS in human liver cells, and the human *STS* gene was established as an NF-κB target gene (10). Indeed, treatment of human hepatoma HepG2 cells with phorbol 12-myristate 13-acetate (PMA), but not tumor necrosis factor α (TNFα) or LPS, induced mRNA expression of *STS* (Fig. 2*A*), and the induction of STS protein in PMA-treated HepG2 cells was confirmed by Western blot analysis (Fig. 2*A*, *inset*) and enzymatic activity assay (Fig. 2*B*). In HepG2 cells, CEEs had little effect on the basal or PMA-inducible expression of STS (Fig. 2*C*). The effect of PMA was enzyme-specific because PMA had little effect on the expression of the estrogen-deactivating enzyme EST (Fig. 2*C*).

Having shown that STS can promote the estrogenic activity of CEEs, we hypothesized that inflammation may enhance the estrogenic activity of CEEs by inducing STS. To test this hypothesis, HepG2 cells were treated with CEEs for 24 h, in the absence or presence of PMA and/or STX, before being measured for the intracellular or medium concentrations of estrone and estradiol by ultraperformance LC-tandem mass spectrometry (UPLC-MS/MS). As shown in Fig. 2*D*, the intracellular (*left panel*) and medium (*right panel*) concentrations of estrone and estradiol were increased in CEE-treated cells even in the absence of PMA, likely due to basal endogenous expression of STS in these cells. Cotreatment with PMA elevated the CEE-responsive estrogen levels, but the PMA effect was completely abolished by cotreatment with STX. At the functional level, treatment with CEEs alone had little effect on the expression of the estrogen-responsive gene trefoil factor 1 (*TFF1*) (11), but cotreatment with PMA resulted in a marked activation of *TFF1* (Fig. 2*E*). The PMA effect on CEE-responsive induction of *TFF1* was abolished when STS was knocked down by siRNA (Fig. 2*F*). The efficiency of STS knockdown was verified by real-time PCR and Western blotting (Fig. 2*G*). The effect of PMA on CEE-elicited estrogenic activity in HepG2 cells was also confirmed by a reporter gene assay. The ERE reporter activity induced by cotreatment with CEEs and PMA was abolished by treatment with STX (Fig. 2*H*), ICI (Fig. 2*I*) or the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) (12) (Fig. 2*J*). The effect of PDTC was consistent with the notion that NF-κB mediates the inflammatory induction of STS (10).

Inflammation enhances the estrogenic activity of CEEs by inducing the expression of STS in human primary hepatocytes (HPHs)

We then determined whether the inflammation-responsive enhancement of CEE estrogenic activity can be verified in HPHs. Interestingly, the induction of STS in HPHs was more responsive to LPS than to PMA as confirmed by real-time PCR (Fig. 3*A*) and Western blotting (Fig. 3*B*). It is unclear why PMA and LPS showed different effects on STS expression in HepG2 cells and primary human hepatocytes, but a similar pattern of difference was observed when we compared Huh7 cells and primary human hepatocytes in their inflammatory induction of STS (10). CEEs had little effect on the basal or LPS-inducible expression of STS (Fig. 3*C*). The expression of EST was suppressed by LPS but restored by the cotreatment with CEEs (Fig. 3*C*). At the biochemical level, treatment of HPHs with CEEs alone led to detectable intracellular (Fig. 3*D*, *left panel*) and medium (Fig. 3*D*, *right panel*) levels of estrone and estradiol because of the basal expression of STS. At the same dose of CEEs, cotreatment with LPS substantially increased the intracellular and medium concentrations of estrogens, and the sensitizing effect of LPS was abolished by STX (Fig. 3*D*). At the functional level, CEEs were effective to induce the expression of estrogen-responsive genes *TFF1*, X-box binding protein-1 (*XBP1*), and growth-regulating estrogen receptor–binding 1 (*GREB1*) (13, 14) only when HPHs were cotreated with LPS (Fig. 3*E*).

The inflammation-responsive and STS-mediated estrogenic activity of CEEs attenuates inflammatory response in liver cells

Given the known anti-inflammatory effect of estrogens (15), we hypothesized that the inflammation-responsive and STS-mediated estrogenic activity of CEEs may in return attenuate inflammation. Indeed, the PMA-responsive induction of inflammatory marker genes, including monocyte chemotactic protein-1 (*MCP1*), *TNFα*, and interleukin-8 (*IL-8*), was attenuated in cells cotreated with CEEs (Fig. 4*A*). The inflammation-attenuating effect of CEEs was abolished by treatment with STX or ICI (Fig. 4*A*), suggesting that the effect of CEE and PMA cotreatment was mediated by STS and ER, respectively. In an independent assay using an NF-κB–responsive luciferase reporter gene, the PMA-induced activation of the NF-κB reporter was attenuated by CEEs with an inhibitory effect similar to that of estradiol (Fig. 4*B*). Again, the attenuating effect of CEEs on the NF-κB reporter activity was abolished by STX or ICI (Fig. 4*C*).

The estrogenic activity of CEEs is increased in primary hepatocytes isolated from the liver of STS transgenic mice

To understand the function of STS in CEE activation *in vivo*, we used the FABP-STS transgenic (TG) mice generated by crossing the FABP-tTA mice that express the tetracycline transactivator (tTA) in liver under the fatty acid–binding protein (FABP) gene promoter, and the TRE-STS mice expressing STS under the control of the tetracycline-response element (TRE) as outlined in Fig. 5*A* (16). The protein expression of the transgene in the liver was confirmed by Western blotting (Fig. 5*B*) and enzymatic assay (Fig. 5*C*). We found that the intracellular (Fig. 5*D*, *left*) and medium (Fig. 5*D*, *right*) concentrations of estrone and estradiol were substantially higher in CEE-treated primary hepatocytes isolated from the TG mice compared with their wildtype (WT) counterparts. The basal and CEE-responsive expression of *Hsp*, a hepatic estrogen-responsive gene, was increased in hepatocytes isolated from the TG mice (Fig. 5*E*).

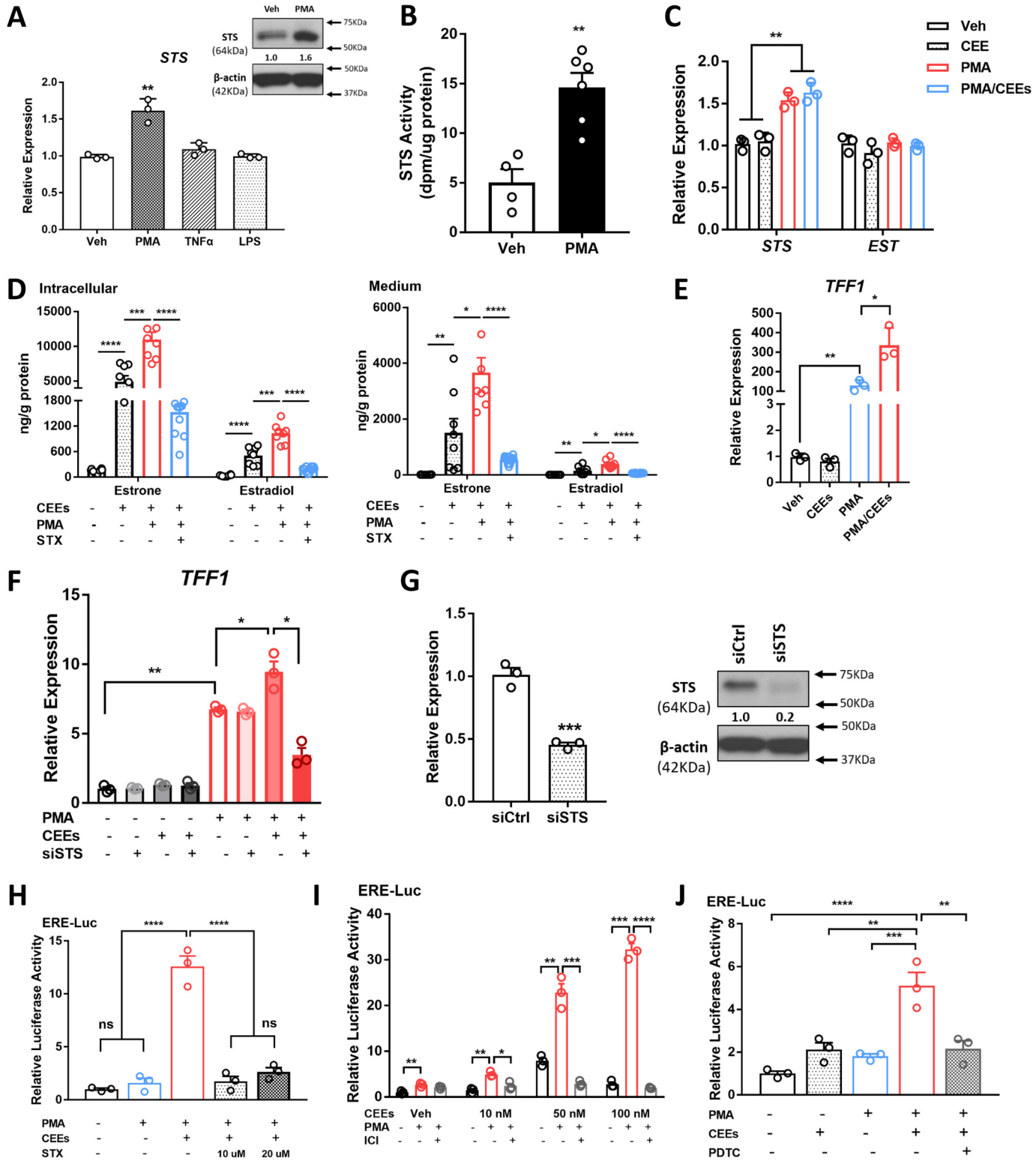

Figure 2. Inflammation enhances the estrogenic activity of CEEs by inducing the expression of STS in HepG2 cells. *A*, HepG2 cells were treated with vehicle (*Veh*), PMA (50 ng/ml), TNFα (40 ng/ml), or LPS (1 μg/ml) for 24 h before measuring the mRNA expression of *STS* by real-time PCR. Shown in the *inset* is the protein expression of STS measured by Western blotting in HelpG2 cells treated with PMA for 48 h. *B*, STS enzymatic activity in HepG2 cells treated with vehicle or PMA (50 ng/ml) for 24 h. *C*, mRNA expression of *STS* and estrogen sulfotransferase (*EST*) in HepG2 cells treated with PMA (50 ng/ml) and CEEs (100 nM) individually or in combination for 24 h. *D*, intracellular (*left*) and medium (*right*) estrone and estradiol concentrations in HepG2 cells treated with CEEs (500 nM) alone or in combination with PMA (50 ng/ml) or STX (10 μM). *E*, mRNA expression of the estrogen-responsive gene *TFF1* in HepG2 cells treated with PMA (50 ng/ml) and/or CEEs (100 nM). *F*, induction of *TFF1* by cotreatment with CEE and PMA was abolished by siRNA knockdown of STS. *G*, efficiency of STS siRNA knockdown was verified by real-time PCR (*left*) and Western blotting (*right*). *H–J*, HepG2 cells were transfected with ERE-luciferase reporter. Shown are STX (*H*), ICI (*I*), or PDTC (25 μM) (*J*) inhibition of reporter activity induced by combined treatment with CEEs (100 nM) and PMA (50 ng/ml). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; *ns*, not significant. *Ctrl*, control. *Error bars* represent S.E.

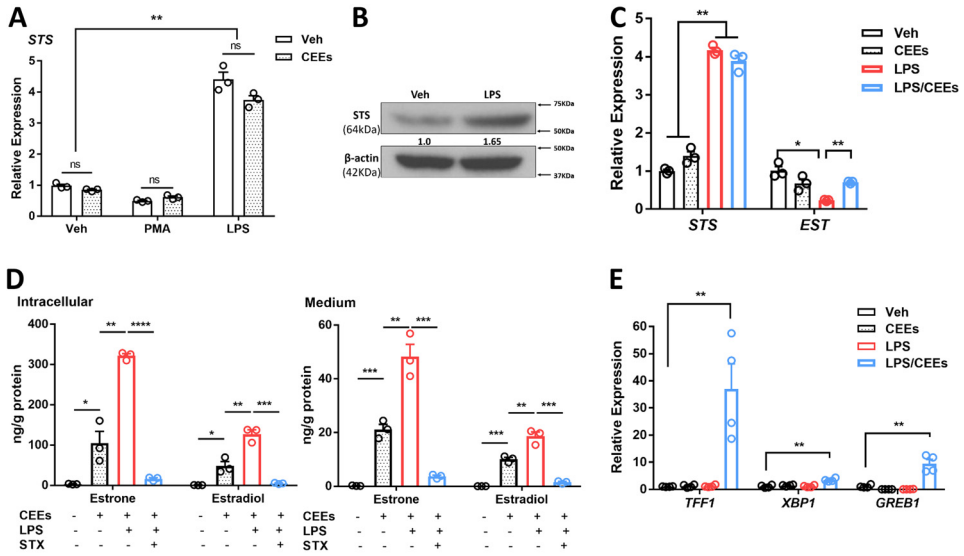

Figure 3. Inflammation enhances the estrogenic activity of CEEs by inducing the expression of STS in human primary hepatocytes. *A*, mRNA expression of *STS* in HPHs treated with vehicle (*Veh*), PMA (50 ng/ml), or LPS (1 μg/ml) in the absence or presence of CEEs (100 nM) for 24 h. *B*, protein expression of STS in HPHs with vehicle or LPS treatment for 48 h was measured by Western blotting. *C*, mRNA expression of *STS* and *EST* in HPHs treated with LPS (1 μg/ml) or CEEs (100 nM) alone or in combination for 24 h. *D*, intracellular (*left*) and medium (*right*) estrone and estradiol concentrations in HPHs treated with CEEs (500 nM) alone or in combination with LPS (1 μg/ml) or STX (10 μM). *E*, mRNA expression of estrogen-responsive genes in HPHs treated with LPS (1 μg/ml) or CEEs (100 nM) alone or in combination for 24 h. *XBP1*, X-box–binding protein-1; *GREB1*, growth-regulating estrogen receptor–binding 1. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; *ns*, not significant. Comparisons are labeled. *Error bars* represent S.E.

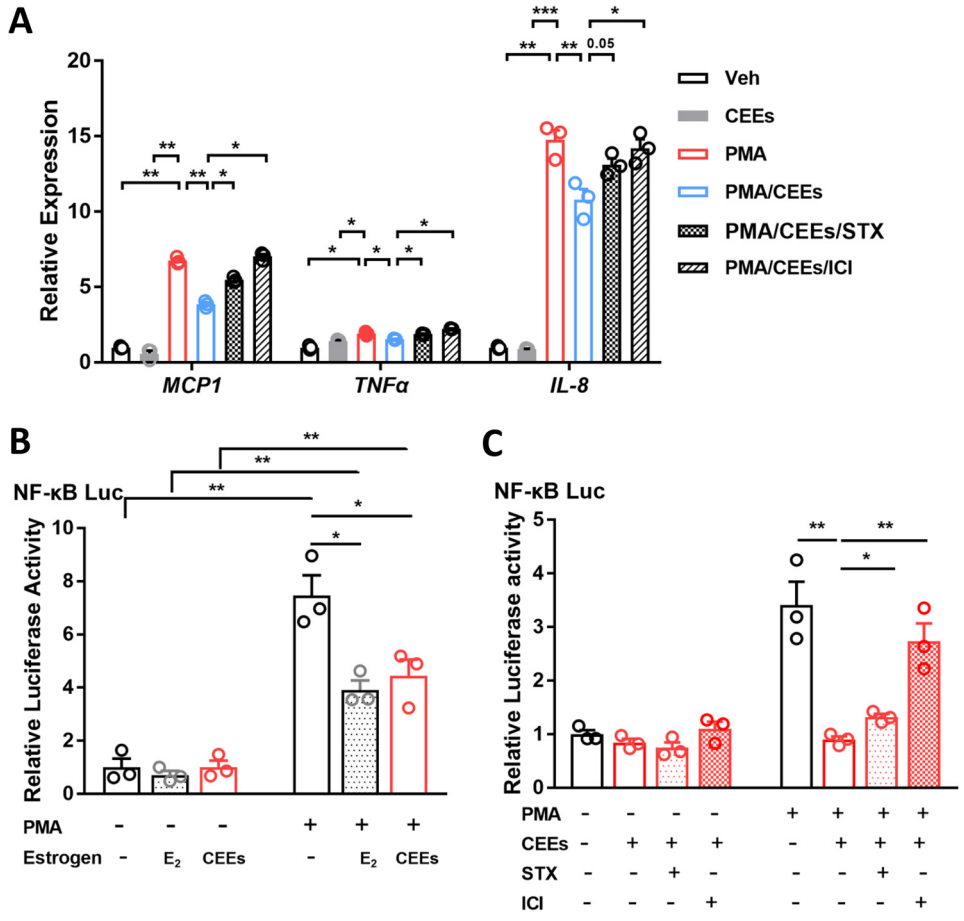

Figure 4. The inflammation-responsive and STS-mediated estrogenic activity of CEEs attenuates inflammatory response in liver cells. *A*, mRNA expression of proinflammatory marker genes in HepG2 cells treated with PMA (50 ng/ml) and CEEs (100 nM) individually or in combination in the absence or presence of cotreatment with STX (10 μM) or ICI (100 nM). *MCP1*, monocyte chemotactic protein-1; *IL-8*, interleukin-8. *B* and *C*, HepG2 cells were transfected with the NF-κB luciferase reporter gene. Shown are the inhibition of PMA-induced NF-κB reporter activity by cotreatment with CEEs (100 nM) with E_2 (10 nM) treatment as the positive control (*B*) and the abolishment of the inhibitory effect of CEEs by cotreatment with STX (10 μM) or ICI (100 nM) (*C*). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The comparisons are labeled. *Veh*, vehicle. *Error bars* represent S.E.

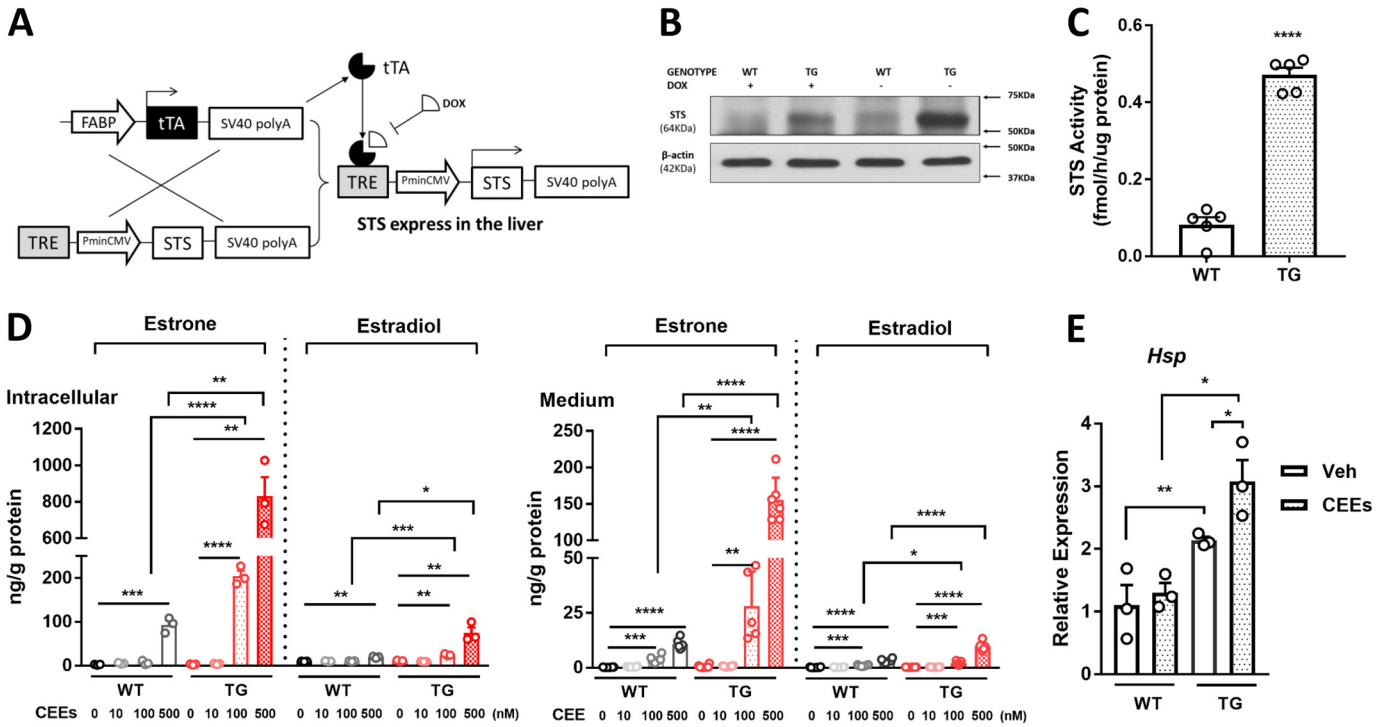

Figure 5. The estrogenic activity of CEEs is increased in primary hepatocytes isolated from the liver of STS transgenic mice. *A*, schematic representation of the Tet-Off FABP-STS transgenic system. *SV40 polyA*, simian virus 40 polyadenylation signal; *PminCMV*, minimal cytomegalovirus promoter. *B*, hepatic expression of STS protein was measured by Western blotting. *C*, hepatic STS enzymatic activity was determined by an estrone sulfate conversion assay normalized against protein concentrations. *D*, intracellular (*left*) and medium (*right*) estrone and estradiol concentrations in mouse primary hepatocytes isolated from WT or TG mice treated with the indicated doses of CEEs. *E*, hepatic expression of the estrogen-responsive gene *Hsp* was determined by real-time PCR. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. The comparisons are labeled. *Veh*, vehicle. *Error bars* represent S.E.

Hepatic transgenic expression of STS enhances the estrogenic activity of CEEs in vivo

The uterus is one of the organs most sensitive to estrogen stimulation. Therefore, we used the uterus to determine the estrogenic activity of CEEs *in vivo*. As outlined in Fig. 6*A*, ovariectomized virgin female WT mice or TG mice were treated with a single gavage of vehicle or CEEs (20 or 50 μg/kg of body weight) and sacrificed 20 h later. The treatment with CEEs, especially at the dose of 50 μg/kg, which was the boundary and most sensitive dose of CEEs showing the estrogenic difference between WT and TG mice, caused obvious uterine enlargement with hyperemia in the TG mice (Fig. 6*B*). Consistent with the gross appearance, CEE-treated TG mice showed a dramatically increased ratio of uterus to body weight, whereas only a mild increase of the ratio was observed in WT mice (Fig. 6*C*). The estrogen-responsive uterine epithelial proliferation was evaluated by bromodeoxyuridine (BrdU) labeling and immunostaining. A single dose of CEEs (50 μg/kg) results in a BrdU labeling index of ~15% in TG mice compared with 5% in the WT mice (Fig. 6*D*). The transgenic effect on the uterine response to CEEs was abolished when the TG mice were treated with doxycycline (DOX) to silence the transgene expression (Fig. 6, *B–D*). The uterotropic effect of CEEs was also confirmed by estrogen-responsive gene expression in that CEEs had little effect on the expression of uterine estrogen-responsive genes, including progesterone receptor (*Pgr*), lactoferrin (*Ltf*), and insulin-like growth factor 1 (*Igf-1*), in the WT mice, likely because the boundary dose of CEEs (50 μg/kg) was used, but it caused a marked induction of these genes in the TG mice (Fig. 6*E*).

The uteri of the TG mice did not express the STS transgene, and the expression of endogenous STS in the uteri of the TG mice was not affected either (data not shown), suggesting that the increased CEE-elicited estrogenic activity in the uterus was derived from the liver and through the circulation. Indeed, the liver concentrations of estrone and estradiol in CEE-treated TG mice were significantly higher than those in their WT counterparts (Fig. 6*F*). CEEs induced the expression of hepatic estrogen-responsive genes insulin-like growth factor–binding protein 4 (*Igfbp4*), sulfatase 2 (*Sulf2*), and *Hsp* in TG mice but not in WT mice (Fig. 6*G*). The circulating concentration of estrone was elevated in CEE-treated TG mice but not in WT mice (Fig. 6*H*).

Discussion

HRT is the most effective treatment for menopausal symptoms, but the safety profile of these hormone products, especially the risk of breast cancer, has been a concern (17). It is now widely accepted that the window of opportunity is crucial for balancing the benefit and risk of HRT, meaning the women's age and the time to initiate HRT relative to menopause onset are crucial (2). It has been reported that benefits exceed risk for women starting HRT before 60 years of age or during 10 years since the onset of menopause (18).

CEEs are one of the most prescribed HRT agents. Indeed, CEEs were used in the Women's Health Initiative (WHI), the

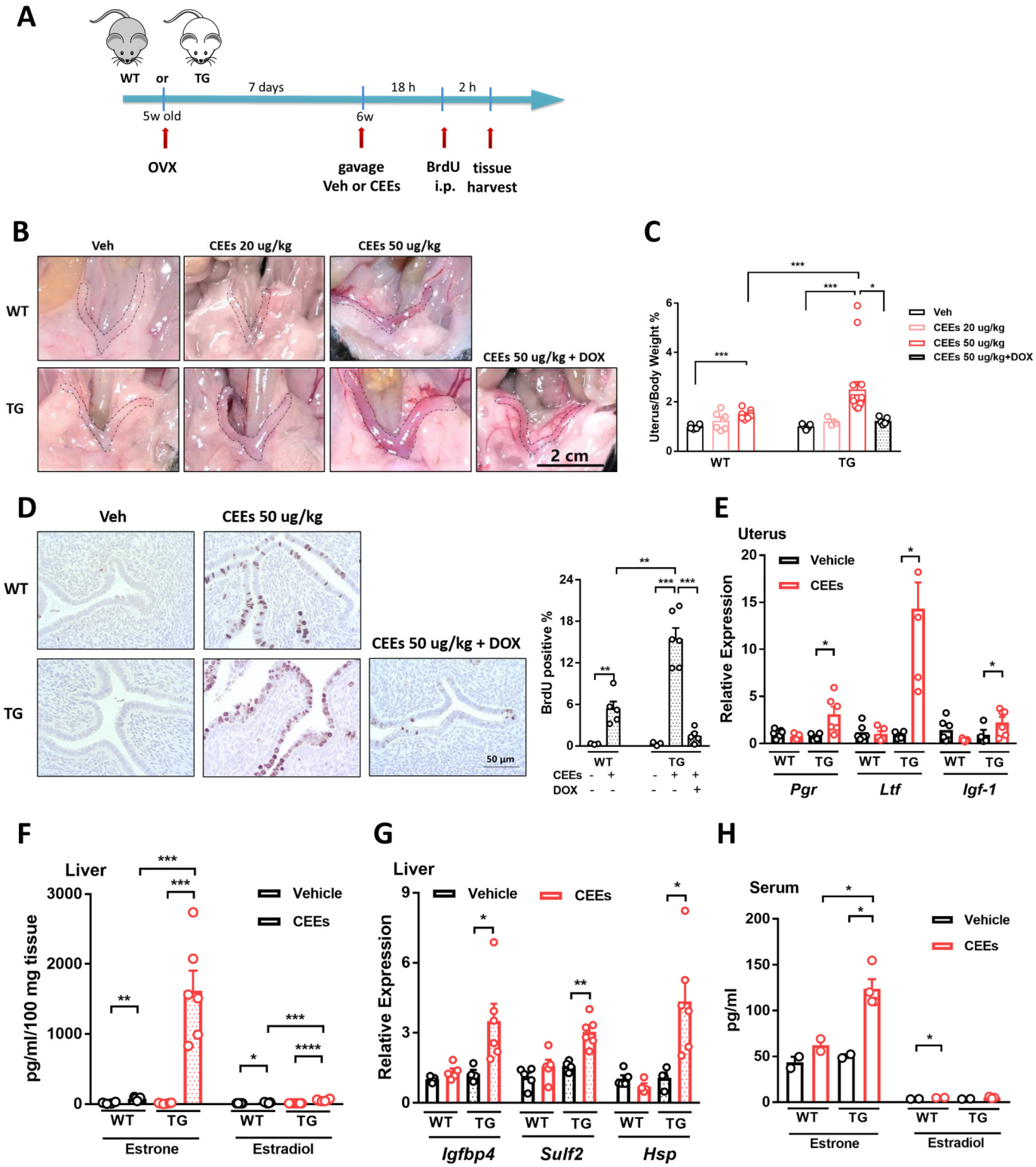

Figure 6. Hepatic transgenic expression of STS enhances the estrogenic activity of CEEs *in vivo*. *A*, experimental scheme for vehicle (*Veh*) or CEE treatment in ovariectomized (*OVX*) WT or TG mice. *B*, gross appearance of the uterus after drug treatment. When applicable, DOX (2 mg/ml) was given in drinking water 1 day before the OVX surgery and until the completion of the experiment. *C*, quantification of uterus to body weight ratio. *D*, BrdU labeling and immunostaining on uterine paraffin sections of mice that received 50 μg/kg body weight CEEs. Shown on the *right* is the quantification of BrdU-positive nuclei. The original *magnification* is 200× for all panels. *E*, expression of uterine estrogen-responsive genes in mice that received 50 μg/kg body weight CEEs. *Pgr*, progesterone receptor; *Ltf*, lactoferrin; *Igf-1*, insulin-like growth factor 1. *F*, liver estrone and estradiol concentrations in mice that received 50 μg/kg body weight CEEs. *G*, expression of hepatic estrogen-responsive genes in mice that received 50 μg/kg body weight CEEs. *Igfbp4*, insulin-like growth factor–binding protein 4; *Sulf2*, sulfatase 2. *H*, serum concentrations of estrone and estradiol in mice that received 50 μg/kg body weight CEEs. $n = 3$–5 for each group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. The comparisons are labeled. *Error bars* represent S.E.

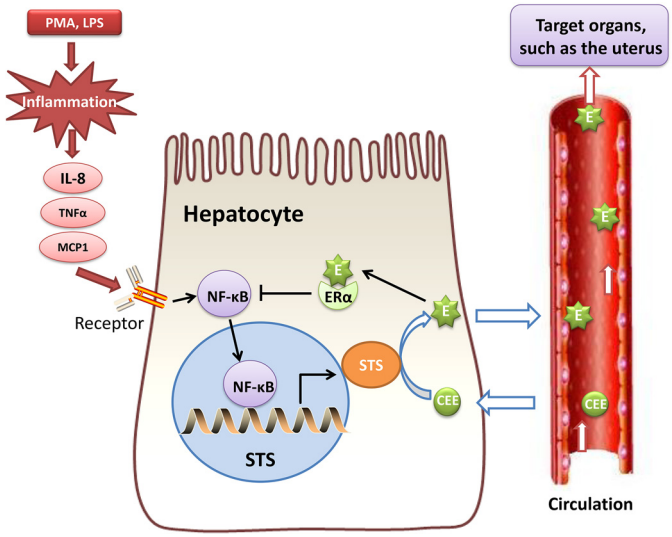

Figure 7. Proposed model by which the hepatic STS and its inflammatory regulation dictate the hormone replacement therapy efficacy of CEEs. STS is required for the bioactivation and estrogenic activity of CEEs. Hepatic inflammation promotes the hormone-replacement therapy efficacy of CEEs by transcriptional activation of STS, an NF-κB target gene. Hepatic STS and its inflammatory regulation affect CEE-elicited estrogenic activity in the liver and distal organs such as the uterus through the circulation. *E*, estrogen; *IL-8*, interleukin-8; *MCP1*, monocyte chemotactic protein-1.

largest clinical trial to date, that was initiated in 1991. Based on the results from the WHI, women aged 50–59 years with CEE-alone treatment had a lower incidence of the combined end points of coronary heart disease and coronary revascularization compared with women older than 60 years (19). Additionally, the WHI study showed that unlike CEEs plus medroxyprogesterone acetate, the regimen of CEEs alone did not increase the risk of breast cancer (20). It was probably due to a decreased level of total insulin-like growth factor-1 in the CEE-alone subjects (21), which is a risk factor for breast cancer (22). These clinical observations verify that CEEs are a safe estrogen product for women in the early phase of postmenopause.

As a natural hormone product extracted from pregnant mare urine, containing unique ring B unsaturated estrogens such as equilin and equilenin (4), the metabolism and therapeutic effect of CEEs are more complex than single-component natural or synthetic estrogen. STS is believed to be the only enzyme responsible for the deconjugation and activation of conjugated estrogens. In the current study, we have provided compelling *in vitro* and *in vivo* evidence that the expression and activity of STS are sufficient and necessary for the effective hormonal activity of CEEs. The induction of STS in hepatocytes had little effect on the expression of the estrogen-deactivating enzyme EST, suggesting that STS is the only enzyme responsible for the estrogenic activity of CEEs. Our transgenic studies showed that the effect of the hepatic expression of STS on the estrogenic activity of CEEs can be systemic because transgenic expression of STS in the liver can distally enhance CEE-induced estrogenic activity in the uterus.

The effect of inflammation on estrogenic activity of CEEs is interesting and could have a major clinical significance. Estrogen is an anti-inflammatory hormone. As summarized in Fig. 7, inflammation can enhance the estrogenic activity of CEEs through the induction of STS in an NF-κB–dependent manner, and the inflammation-responsive estrogenic activity of CEEs in return can inhibit inflammation, completing a negative feedback loop to attenuate inflammation. It will be interesting to determine whether chronic inflammation, including that of the liver, such as viral and nonviral hepatitis, steatosis, steatohepatitis, and cirrhosis, can sensitize postmenopausal women to the HRT effect of CEEs. In addition, a systemic, low-grade, and subacute inflammatory state is commonly and closely associated with metabolic dysfunctions, including obesity, type 2 diabetes, and nonalcohol fatty liver disease (23). It remains to be determined whether metabolic syndrome should be a factor considered for the precise use of CEEs.

Besides the oral tablets, Premarin is also used in the form of vaginal cream to treat the vaginal symptoms of menopause such as dryness, burning, irritation, and painful sexual intercourse (24). Future studies are necessary to examine the expression and regulation of STS in the vaginal wall and whether vaginal inflammation can sensitize patients to Premarin vaginal cream.

In summary, the current study has uncovered an essential function of hepatic STS in activating CEEs. Caution needs to be applied in the clinical use of CEEs/Premarin in patients with chronic inflammation in the liver because these patients may be more sensitive to CEE therapy due to STS induction.

Experimental procedures

Chemicals

CEEs from Pfizer was dissolved in DMSO. The recombinant human TNFα was purchased from R&D Systems (Minneapolis, MN). All other chemicals were purchased from Sigma.

Cell culture, plasmid constructs, and reporter gene assay

The HepG2 and HEK293T cell lines were obtained from the American Type Culture Collection (Manassas, VA). HepG2 and HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. In experiments in which exogenous estrogen or anti-estrogen were added, the cells were maintained in phenol red–free and serum-free Dulbecco's modified Eagle's medium with high glucose (4.5 mg/liter) before the drug treatment. The TATA-ERE-Luc or TATA-NF-κB-Luc reporter genes were described previously (10). Cells were transiently transfected with the reporter constructs followed by specific chemical treatment for 24 h and then measured for luciferase and β-gal activities. Transfection efficiency was normalized against β-gal activity from the cotransfected pCMX-β-gal plasmid.

For knockdown experiments, the human STS siRNA and control scrambled siRNA were purchased from Dharmacon Research (Lafayette, CO). After siRNA transfection, the cells were collected at 24 h for mRNA expression analysis or at 48 h for protein expression analysis.

Animals

The FABP-STS transgenic mice in C57BL/6J background were generated by cross-breeding the TRE-STS mice with the FABP-tTA mice as described before (10). Five-week-old virgin females C57BL/6J mice (WT) or TG mice were subjected to ovariectomy. The ovariectomized mice were rested for 7 days

before being given a single dose of vehicle or CEEs (20 or 50 μg/kg of body weight) by gavage (25). 18 h after treatment, mice were given a single i.p. injection of BrdU (60 mg/kg of body weight) and killed 2 h later. When necessary, DOX (2 mg/ml) was given in drinking water 1 day before surgery and until the completion of the experiment. The use of mice in this study complied with all relevant federal guidelines and institutional policies and was approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Immunohistochemistry, real-time PCR, and Western blot analysis

One uterine horn was harvested for paraffin section and BrdU immunostaining using an anti-BrdU antibody (1:200; ab6326, Abcam, Cambridge, MA) as described before (26), and the result was quantified using ImageJ software. Total RNA was prepared using TRIzol reagent. For Western blotting, the anti-STS antibody (1:200; ab34781) was purchased from Abcam.

Mouse and human primary hepatocyte isolation

Mouse primary hepatocytes were isolated by collagenase perfusion from age- and body weight–matched female WT or TG mice as reported previously (27). Human primary hepatocytes were provided by BioIVT (Baltimore, MD). The cells were cultured as described previously (28) and treated with LPS (1 μg/ml) or PMA (50 ng/ml) with or without CEEs (100 nM) for 24 h before cell harvest.

STS enzymatic activity assay and UPLC-MS/MS detection of estrogens

The hepatic STS enzymatic activity was determined from cytosol by an estrone sulfate conversion assay as described before (29). UPLC-MS/MS was used to quantify estrone (E_1) and E_2 in the serum or liver of mice and in cell lysate or tissue culture medium (30).

Statistical analysis

Data represent mean ± S.E. GraphPad Prism software (San Diego, CA) was used for statistical analysis. Student's *t* test was used to calculate statistical significance between two group means. One-way analysis of variance with Dunnett's test or Tukey's test was used to compare multiple group means. Two-way analysis of variance with Bonferroni post hoc test was used to compare two groups with multiple data sets. The statistical significance threshold was set at $p < 0.05$.

Author contributions—Y. F. and W. X. conceptualization; Y. F., Y. X., M. X., L. L., K. W. S., and P. J. O. data curation; Y. F. formal analysis; Y. F., Y. X., M. X., L. L., K. W. S., and P. J. O. investigation; Y. F., Y. X., L. L., K. W. S., and P. J. O. methodology; Y. F., Y. X., S. M. P., H. W., C. L., F. D., C. Y., and W. X. writing-original draft; Y. F., Y. X., S. M. P., H. W., C. L., F. D., C. Y., and W. X. writing-review and editing; S. M. P., C. Y., and W. X. supervision; W. X. resources; W. X. funding acquisition; W. X. project administration.

References

1. North American Menopause Society (2012) The 2012 hormone therapy position statement of: The North American Menopause Society. *Menopause* **19,** 257–271 CrossRef Medline
2. Lobo, R. A., Pickar, J. H., Stevenson, J. C., Mack, W. J., and Hodis, H. N. (2016) Back to the future: hormone replacement therapy as part of a prevention strategy for women at the onset of menopause. *Atherosclerosis* **254,** 282–290 CrossRef Medline
3. Nelson, H. D., Humphrey, L. L., Nygren, P., Teutsch, S. M., and Allan, J. D. (2002) Postmenopausal hormone replacement therapy: scientific review. *JAMA* **288,** 872–881 CrossRef Medline
4. Bhavnani, B. R., and Stanczyk, F. Z. (2014) Pharmacology of conjugated equine estrogens: efficacy, safety and mechanism of action. *J. Steroid Biochem. Mol. Biol.* **142,** 16–29 CrossRef Medline
5. Pan, C. C., Woolever, C. A., and Bhavnani, B. R. (1985) Transport of equine estrogens: binding of conjugated and unconjugated equine estrogens with human serum proteins. *J. Clin. Endocrinol. Metab.* **61,** 499–507 CrossRef Medline
6. Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Häggblad, J., Nilsson, S., and Gustafsson, J. A. (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology* **138,** 863–870 CrossRef Medline
7. Ferrante, P., Messali, S., Meroni, G., and Ballabio, A. (2002) Molecular and biochemical characterisation of a novel sulphatase gene: Arylsulfatase G (ARSG). *Eur. J. Hum. Genet.* **10,** 813–818 CrossRef Medline
8. Falany, C. N. (1997) Enzymology of human cytosolic sulfotransferases. *FASEB J.* **11,** 206–216 CrossRef Medline
9. Stanway, S. J., Purohit, A., Woo, L. W., Sufi, S., Vigushin, D., Ward, R., Wilson, R. H., Stanczyk, F. Z., Dobbs, N., Kulinskaya, E., Elliott, M., Potter, B. V., Reed, M. J., and Coombes, R. C. (2006) Phase I study of STX 64 (667 Coumate) in breast cancer patients: the first study of a steroid sulfatase inhibitor. *Clin. Cancer Res.* **12,** 1585–1592 CrossRef Medline
10. Jiang, M., Klein, M., Zanger, U. M., Mohammad, M. K., Cave, M. C., Gaikwad, N. W., Dias, N. J., Selcer, K. W., Guo, Y., He, J., Zhang, X., Shen, Q., Qin, W., Li, J., Li, S., and Xie, W. (2016) Inflammatory regulation of steroid sulfatase: a novel mechanism to control estrogen homeostasis and inflammation in chronic liver disease. *J. Hepatol.* **64,** 44–52 CrossRef Medline
11. Barkhem, T., Haldosén, L. A., Gustafsson, J. A., and Nilsson, S. (2002) pS2 gene expression in HepG2 cells: complex regulation through crosstalk between the estrogen receptor α, an estrogen-responsive element, and the activator protein 1 response element. *Mol. Pharmacol.* **61,** 1273–1283 CrossRef Medline
12. Schreck, R., Meier, B., Männel, D. N., Dröge, W., and Baeuerle, P. A. (1992) Dithiocarbamates as potent inhibitors of nuclear factor κB activation in intact cells. *J. Exp. Med.* **175,** 1181–1194 CrossRef Medline
13. Lee, Y. J., Jin, Y. R., Lim, W. C., Park, W. K., Cho, J. Y., Jang, S., and Lee, S. K. (2003) Ginsenoside-Rb1 acts as a weak phytoestrogen in MCF-7 human breast cancer cells. *Arch. Pharm. Res.* **26,** 58–63 CrossRef Medline
14. Sengupta, S., Sharma, C. G., and Jordan, V. C. (2010) Estrogen regulation of X-box binding protein-1 and its role in estrogen induced growth of breast and endometrial cancer cells. *Horm. Mol. Biol. Clin. Investig.* **2,** 235–243 CrossRef Medline
15. Straub, R. H. (2007) The complex role of estrogens in inflammation. *Endocr. Rev.* **28,** 521–574 CrossRef Medline
16. Zhou, J., Zhai, Y., Mu, Y., Gong, H., Uppal, H., Toma, D., Ren, S., Evans, R. M., and Xie, W. (2006) A novel pregnane X receptor-mediated and sterol regulatory element-binding protein-independent lipogenic pathway. *J. Biol. Chem.* **281,** 15013–15020 CrossRef Medline
17. Miller, V. M., and Harman, S. M. (2017) An update on hormone therapy in postmenopausal women: mini-review for the basic scientist. *Am. J. Physiol. Heart Circ. Physiol.* **313,** H1013–H1021 CrossRef Medline
18. Stuenkel, C. A., Gass, M. L., Manson, J. E., Lobo, R. A., Pal, L., Rebar, R. W., and Hall, J. E. (2012) A decade after the Women's Health Initiative—the experts do agree. *Menopause* **19,** 846–847 CrossRef Medline
19. Hsia, J., Langer, R. D., Manson, J. E., Kuller, L., Johnson, K. C., Hendrix, S. L., Pettinger, M., Heckbert, S. R., Greep, N., Crawford, S., Eaton, C. B., Kostis, J. B., Caralis, P., Prentice, R., and Women's Health Initiative Investigators (2006) Conjugated equine estrogens and coronary heart disease: the Women's Health Initiative. *Arch. Int. Med.* **166,** 357–365 CrossRef Medline

20. Anderson, G. L., Limacher, M., Assaf, A. R., Bassford, T., Beresford, S. A., Black, H., Bonds, D., Brunner, R., Brzyski, R., Caan, B., Chlebowski, R., Curb, D., Gass, M., Hays, J., Heiss, G., *et al.* (2004) Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. *JAMA* **291,** 1701–1712 CrossRef Medline
21. Cardim, H. J., Lopes, C. M., Giannella-Neto, D., da Fonseca, A. M., and Pinotti, J. A. (2001) The insulin-like growth factor-I system and hormone replacement therapy. *Fertil. Steril.* **75,** 282–287 CrossRef Medline
22. Christopoulos, P. F., Msaouel, P., and Koutsilieris, M. (2015) The role of the insulin-like growth factor-1 system in breast cancer. *Mol. Cancer* **14,** 43 CrossRef Medline
23. Hotamisligil, G. S. (2006) Inflammation and metabolic disorders. *Nature* **444,** 860–867 CrossRef Medline
24. Suwanvesh, N., Manonai, J., Sophonsritsuk, A., and Cherdshewasart, W. (2017) Comparison of *Pueraria mirifica* gel and conjugated equine estrogen cream effects on vaginal health in postmenopausal women. *Menopause* **24,** 210–215 CrossRef Medline
25. Della Torre, S., Biserni, A., Rando, G., Monteleone, G., Ciana, P., Komm, B., and Maggi, A. (2011) The conundrum of estrogen receptor oscillatory activity in the search for an appropriate hormone replacement therapy. *Endocrinology* **152,** 2256–2265 CrossRef Medline
26. Gong, H., Guo, P., Zhai, Y., Zhou, J., Uppal, H., Jarzynka, M. J., Song, W. C., Cheng, S. Y., and Xie, W. (2007) Estrogen deprivation and inhibition of breast cancer growth *in vivo* through activation of the orphan nuclear receptor liver X receptor. *Mol. Endocrinol.* **21,** 1781–1790 CrossRef Medline
27. Mu, Y., Zhang, J., Zhang, S., Zhou, H. H., Toma, D., Ren, S., Huang, L., Yaramus, M., Baum, A., Venkataramanan, R., and Xie, W. (2006) Traditional Chinese medicines Wu Wei Zi (*Schisandra chinensis* Baill) and Gan Cao (*Glycyrrhiza uralensis* Fisch) activate pregnane X receptor and increase warfarin clearance in rats. *J. Pharmacol. Exp. Ther.* **316,** 1369–1377 CrossRef Medline
28. Faucette, S. R., Zhang, T. C., Moore, R., Sueyoshi, T., Omiecinski, C. J., LeCluyse, E. L., Negishi, M., and Wang, H. (2007) Relative activation of human pregnane X receptor *versus* constitutive androstane receptor defines distinct classes of CYP2B6 and CYP3A4 inducers. *J. Pharmacol. Exp. Ther.* **320,** 72–80 CrossRef Medline
29. Selcer, K. W., and Difrancesca, H. M. (2012) Characterization of steroid sulfatase in the MC3T3-E1 mouse pre-osteoblastic cell line. *Steroids* **77,** 696–702 CrossRef Medline
30. Li, J., Oberly, P. J., Poloyac, S. M., and Gibbs, R. B. (2016) A microsomal based method to detect aromatase activity in different brain regions of the rat using ultra performance liquid chromatography-mass spectrometry. *J. Steroid Biochem. Mol. Biol.* **163,** 113–120 CrossRef Medline