Human CYP2A6 Is Induced by Estrogen via Estrogen Receptor

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ABSTRACT:

Human CYP2A6, which is predominantly expressed in liver, is a key enzyme responsible for the metabolism of nicotine, coumarin, and some pharmaceutical drugs. CYP2A6 is also expressed in sex steroid-responsive tissues such as breast, ovary, uterus, testis, and adrenal grand. In this study, we examined the regulation of CYP2A6 gene by estrogen. Reverse transcription-polymerase chain reaction (RT-PCR) assays revealed that CYP2A6 mRNA was induced by estradiol in estrogen receptor (ER)-positive MCF-7 (2.9-fold) and HepG2 (1.3-fold) cells, but not in ER-negative MDA-MB-435 cells. Real-time RT-PCR assays revealed the CYP2A6 induction by estradiol in human hepatocytes (1.2- to 1.5-fold). Computer-assisted homology search identified a putative estrogen response element (ERE) at −2436 on the CYP2A6 gene. Electrophoretic mobility shift assays demonstrated specific binding of ERα to this element. Luciferase assays using MCF-7 cells revealed that the transcriptional activity of the CYP2A6 promoter was significantly activated by estradiol in an ERα-dependent manner, in which ERE was responsible for the activation. Chromatin immunoprecipitation assays verified the in vivo association of ERα with the ERE on the CYP2A6 gene. Immunohistochemical analyses using human endometrial tissues indicated that the CYP2A6 protein level in glandular cells was significantly higher in the proliferative phase than in the secretory phase, concomitant with local estrogen secretion during the menstrual cycle. These findings clearly demonstrated that CYP2A6 is directly induced by estrogen in an ERα-dependent manner, implying a biological role of CYP2A6 in estrogen-responsive tissues. Furthermore, this mechanism can also explain clinical aspects of increased nicotine metabolism under estrogen-rich environments.

Cytochrome P450 mediates the metabolism of a variety of endogenous and exogenous compounds. Human CYP2A6 is a major enzyme responsible for the metabolism of nicotine to cotinine (Nakajima et al., 1996b), and further to trans-3′-hydroxycotinine (Nakajima et al., 1996a). In addition, CYP2A6 catalyzes the metabolism of coumarin and pharmaceutical agents such as tegafur, losigamone, letrozole, and valproic acid (Nakajima et al., 2002). It can also metabolically activate tobacco-specific nitrosamines such as 4-methyl-1H-nitrosoamo-1-[(3-pyridyl)-1-butanol and N-nitrosodiethylamine (Yamazaki et al., 1992). Although CYP2A6 is mainly expressed in liver, it is expressed in sex steroid-responsive tissues as well, including breast, ovary, uterus, testis, and adrenal grand (Miksys and Tyn-}

Materials.

Materials. 17β-Estradiol and 4-hydroxytamoxifen (4OHT) were obtained from Sigma-Aldrich (St. Louis, MO). 7α-[9(4,4,5,5,5-Pentafluoropentylsulfonyl)nonyl]ester-1,3,5(10)-trien-3,17β-diol (ICI 182,780) and cycloheximide were purchased from Tocris Cookson (Bullwin, MO) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Blend Taq-Plus- was obtained from Toyobo (Osaka, Japan). Oligonucleotides were commercially synthesized at Toyobo (Osaka, Japan).

ABBREVIATIONS: ERα, estrogen receptor α; 4OHT, 4-hydroxytamoxifen; ChIP, chromatin immunoprecipitation; DMSO, dimethyl sulfoxide; ERE, estrogen response element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICI 182,780, 7α-[9(4,4,5,5,5-pentafluoropentylsulfonyl)nonyl]ester-1,3,5(10)-trien-3,17β-diol; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; ANOVA, analysis of variance.

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Hokkaido System Sciences (Sapporo, Japan). Mouse anti-human ERα monoclonal antibodies and normal mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-human CYP2A6 monoclonal antibodies were obtained from BD Gentest (Woburn, MA). All other reagents were of the highest grade commercially available.

**Cell Culture.** Human breast adenocarcinoma cell lines, MCF-7 and MDA-MB-435, and human hepatocarcinoma cell line HepG2 were obtained from American Type Culture Collection (Manassas, VA). MCF-7 and HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Melbourne, VIC, Australia) and 0.1 mM nonessential amino acids (Invitrogen) at 37°C under 5% CO2. Before estradiol treatment, these cells were grown in phenol red-free DMEM (Invitrogen) containing 10% dextran-coated, charcoal-treated FBS for 48 h. Three lots of human cryopreserved hepatocytes, lot 79 (Caucasian, male, 9 months), lot 82 (Hispanic, female, 23 years), and lot 100 (Caucasian, female, 74 years), were purchased from In Vitro Technologies (Baltimore, MD) and maintained in HCM hepatocyte culture medium (Cambrex, East Rutherford, NJ) on collagen-coated plates at 37°C under 5% CO2. Human hepatocytes were seeded into collagen-coated six-well plates at 106 cells/well. After 24 h, the culture medium was changed to HCM medium (epidermal growth factor- and antibiotic-free) containing a 0.4% sodium dodecyl sulfate (SDS) (Invitrogen) buffer for 20 h. The DNA-protein complexes were detected by Western blot analysis using a mouse monoclonal antibody against ERα and normal mouse IgG were obtained from Santa Cruz (CA). Detection of the day of the menstrual cycle. Immunohistochemical analysis of the day of the menstrual cycle was performed using formalin-fixed, paraffin-embedded tissues. The day of the menstrual cycle was determined by histological examination of the tissues and also confirmed by determination of the day of the menstrual cycle. Immunohistochemical analyses of CYP2A6 were performed using formalin-fixed, paraffin-embedded tissues.

**Electrophoretic Mobility Shift Assays.** Human ERα protein was synthesized in vitro using the ERα expression plasmid (pSG5-HD) (Tsukuiya et al., 2004) and TNT T7 Quick Coupled Transcription/Translation Systems (Promega, Madison, WI) following the manufacturer’s protocol. The oligonucleotide sequences used for electrophoretic mobility shift assays are shown in Table 1. Double-stranded oligonucleotides were labeled with [γ-32P]ATP using T4 polynucleotide kinase (Toyobo) and purified by Microspin G-50 columns (GE Healthcare Bio-Sciences). The labeled probe (20 fmol, ~10,000 cpm) was applied to each binding reaction in 25 mM HEPES-KOH (pH 7.9), 0.5 mM EDTA, 50 mM KCl, 10% glycerol, 0.5 mM dithiothreitol, 0.5 mM (p-aminomethyl)phenylmethanesulfonyl fluoride, 2 μg of poly(dI-dC), and 5 μl of in vitro transcribed/translated proteins to a final reaction volume of 15 μl. To determine the specificity of the binding to the oligonucleotides, competition experiments were conducted by coincubation with the unlabeled competitors at 5-, 25-, and 100-fold molar excesses. For supershift experiments, 2 μg of anti-ERα antibodies or normal mouse IgG (control) were preincubated with in vitro transcribed/translated proteins on ice for 30 min. The mixtures were incubated on ice for 15 min and then loaded on 4% acrylamide gels in 0.5X Tris-borate EDTA buffer. The gels were dried and exposed to X-ray film for 20 h. The DNA-protein complexes were detected with a Fuji Bio-Image Analyzer BAS 1000 (Fuji Film, Tokyo, Japan).

**Luciferase Reporter Plasmids.** Plasmids termed pGL3-2A6/ERE and pGL3-3(2A6/ERE) were constructed by single and triple insertion of the double-stranded oligonucleotides of 2A6/ERE into the pGL3-promoter vector (Promega). A pGL3 plasmid containing the 5′-flanking region from −4102 to −1 of the CYP2A6 gene was previously constructed in our laboratory (Itoh et al., 2006). Using this plasmid, various plasmids were constructed by digestion with restriction enzymes and ligation. A plasmid mutated in the ERE of pGL3-2907/-898 was constructed by site-directed mutagenesis with a QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The forward primer was 5′-gtgatcagaGTTTCGcTaCTGgccgaggagggT3′, in which hexamer half-sites are capitalized and mutated nucleotides are underlined.

**Transfection and Luciferase Assay.** MCF-7 cells were transfected with 250 ng of the ERα expression plasmid and were incubated with 1 μM ICI 182,780 or 40HT in the absence or presence of 10 nM estradiol for 24 h.

**Chromatin Immunoprecipitation Assay.** MCF-7 cells were cultured in a 100-mm dish to 80% confluence. HepG2 cells cultured in 100-mm dish to 60% confluence were transfected with 5 μg of the ERα expression plasmid and incubated for 24 h. These cells were treated with 10 nM estradiol or DMSO vehicle (0.1%) for 12 h. The chromatin immunoprecipitation (ChIP) assay was performed using a ChIP assay kit (Upstate, Charlottesville, VA) according to the manufacturer’s protocol. Anti-ERα antibodies and normal mouse IgG were used for immunoprecipitation of protein-DNA complex. PCR was performed using primer sets: region 1, forward, 5′-ctcactctgaaagcctgttcc-3′ and reverse, 5′-gtccaaagtcAGGTCAcagTGACCTgatcaaagtt-3′; region 2, forward, 5′-gtgatcagaGTTTCGcTaCTGgccgaggagggT3′, in which hexamer half-sites are capitalized and mutated nucleotides are underlined. The consensus ERE in the Xenopus vitellogenin A2 promoter (Tsuchiya et al., 2004). The relative luciferase activities were normalized with the Renilla luciferase activities. For the studies using ERα antagonists, MCF-7 cells were transfected with reporter construct without the ERα expression plasmid and were incubated with 1 μM ICI 182,780 or 40HT in the absence or presence of 10 nM estradiol for 24 h.

**Immunohistochemistry of CYP2A6 in Human Endometrial Tissues.** Human endometrial tissues were obtained from 19 patients undergoing hysterectomy as a treatment for benign neoplasms not associated with endometrial disease at Kanazawa University Hospital. Written informed consent was obtained from all patients. The collected endometrial tissues were sampled for histopathological diagnosis, and the remaining normal portions of the samples were used for immunohistochemistry. The phase of the menstrual cycle was determined by histological examination of the tissues and also confirmed by determination of the day of the menstrual cycle. Immunohistochemical analyses of CYP2A6 were performed using formalin-fixed, paraffin-embedded tissues.
specimens of normal endometrial tissues. The sections were incubated with L.A.B. reagent (Polysciences, Warrington, PA) at room temperature for 8 min and then with anti-CYP2A6 antibodies at 4°C for 16 h. Staining reactions were performed using the ABC-elite kit (Vector Laboratories, Burlingame, CA). Staining of glands in functional layers was evaluated by three independent pathologists as positive when >10% of the constituting glandular epithelial cells were stained. The results were judged as − (<10%) of the glands at the region of interest), + (>10%), or ++ (>10% with high intensity).

Statistical Analysis. Data are expressed as mean ± S.D. Comparison of two groups was made with two-tailed Student’s t test. Comparison of multiple groups was made with ANOVA followed by Tukey’s or Dunnett’s test. In immunohistochemistry, the statistical significance of differences in the extent of staining in the different phases of the menstrual cycle was tested by Fisher’s exact test. $P < 0.05$ was considered statistically significant.

Results

Estradiol Induces CYP2A6 mRNA Expression in an ER-Dependent Manner. We first examined the effects of estradiol on the expression level of CYP2A6 mRNA using various cancer cell lines and human hepatocytes (Fig. 1). RT-PCR assays revealed that CYP2A6 mRNA was significantly increased by the treatment with estradiol for 12 h in ERα-positive MCF-7 (2.9-fold, $P < 0.01$) but not in ERα-negative MDA-MB-435 cells. To further investigate whether this activation is direct or indirect, MCF-7 cells were treated with estradiol in the presence of cycloheximide, and RT-PCR assays were performed. The induction of CYP2A6 mRNA was not abolished by cycloheximide, indicating that estradiol directly regulates CYP2A6. A significant induction was also observed in HepG2 cells (1.3-fold, $P < 0.001$), which are known to be estrogen-responsive, although the ERα level is low (Marino et al., 2001). Furthermore, real-time RT-PCR assays revealed that CYP2A6 mRNA was significantly increased by the treatment with estradiol in three lots of human hepatocytes (1.5-fold in lot 79 ($P < 0.001$) and lot 82 ($P < 0.01$) and 1.2-fold in lot 100 ($P < 0.05$)). These results suggested that CYP2A6 mRNA was induced by estradiol in an ERα-dependent manner.

EαR Directly Binds to the ERE on the CYP2A6 Gene. A computer-assisted homology search identified a putative ERE on the CYP2A6 gene at −2436. The sequence of the putative ERE (AGGT-CAtgCACA) has 2-base pair mismatches compared with the consensus ERE (AGGTCAnnnTGACCT). We therefore sought to determine whether ERα directly binds to the putative ERE of the CYP2A6 gene. Electrophoretic mobility shift assays were performed using in vitro transcribed ERα and consensus ERE or the putative ERE on the CYP2A6 gene (2A6/ERE) as a probe. We first confirmed that the ERα we transcribed with the in vitro system certainly binds to the consensus ERE sequence (Tsuchiya et al., 2004), indicating that the ERα we produced worked well (Fig. 2A). This binding was supershifted by ERα antibodies and competed out by 2A6/ERE, but not by the mutant sequence (2A6/ERE-mut). When ERα was incubated with 2A6/ERE as a probe, we found a specific retarded band, which was supershifted by ERα antibodies and competed out by the consensus ERE as well as by the homologous competitor (Fig. 2B). No significant band was observed with 2A6/ERE-mut as a probe (Fig. 2C). These results indicated that ERα specifically binds to the ERE on the CYP2A6 gene.

Fig. 1. Effects of estradiol treatment on the CYP2A6 mRNA level in various cell lines and human hepatocytes. The cells were treated with 10 nM estradiol or DMSO vehicle (0.1%) for 12 h. The MCF-7 cells were treated with cycloheximide (CHX) at 10 μg/ml for 1 h before treatment with estradiol. The expression levels of CYP2A6 mRNA in cell lines and human hepatocytes were determined by semiquantitative RT-PCR and real-time RT-PCR, respectively. The values are the CYP2A6 mRNA level normalized with the GAPDH mRNA level relative to that in DMSO-treated MDA-MB-435 cells determined by semiquantitative RT-PCR. Each column represents the mean ± S.D. of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; and ###, $P < 0.001$ compared with DMSO treatment.

Fig. 2. Electrophoretic mobility shift assays of the binding of ERα to the putative ERE on the CYP2A6 gene. The sequences of oligonucleotides of consensus ERE (A), 2A6/ERE (B), and 2A6/ERE-mut (C) used as probes are shown in Table 1. Unlabeled oligonucleotides were used as a nonradioactive competitor at 5-, 25-, and 100-fold molar excess. For supershift analyses, 2 μg of the anti-ERα antibodies or normal mouse IgG were preincubated with the in vitro transcribed/translated ERα proteins on ice for 30 min. The lower arrow indicates the position of the ERα-dependent shifted band and the upper arrow indicates the supershifted ERα complex.
Estradiol Activates CYP2A6 Promoter in ER-Positive Cells. To examine the transcriptional activation of the CYP2A6 gene by estradiol, luciferase assays were performed with a series of reporter plasmids containing the 5'-flanking region of CYP2A6 in MCF-7 cells. We confirmed that the luciferase activity of pGL3-3EREc38 plasmid used as a positive control increased up to 37-fold by estradiol treatment, which was further enhanced by the overexpression of ERα (67-fold) (Fig. 3A). Unexpectedly, estradiol treatment did not activate the transcriptional activities of any CYP2A6 constructs. However, in the presence of overexpressed ERα, the pGL3-3046 plasmid containing the ERE responded to estradiol treatment (1.4-fold), whereas the pGL3-2157 plasmid lacking it did not.

Our previous study found that there is a potential suppressor region from −1013 to −185 on the CYP2A6 gene (Itoh et al., 2006). Deletion from −897 to −1 did not result in significant induction of transcriptional activity in response to estradiol treatment (Fig. 3B). However, in the presence of overexpressed ERα, their transcriptional activities were enhanced up to 3.7-fold in pGL3-3046/-898 plasmid and 4.3-fold in pGL3-2697/-898 plasmid. Substitution mutations introduced into the ERE of the pGL3-2697/-898 plasmid abrogated the estradiol-induced activation. In the presence of overexpressed ERα, the transcriptional activity of the pGL3-2697/-2157 plasmid containing the ERE was moderately activated by estradiol (2.7-fold), whereas that of the pGL3-2157/-898 lacking the ERE was weakened (1.6-fold). To further verify the role of the ERE, we constructed a reporter plasmid containing three copies of consensus ERE and transiently transfected it into MCF-7 cells with ERα expression plasmid (pSG5 empty vector). The pGL3-3EREc38 plasmid, which contains three copies of consensus ERE, was used as a positive control. After 24 h, the cells were treated with 10 nM estradiol or DMSO vehicle (0.1%) for 24 h. The relative luciferase activities were normalized with the Renilla luciferase activities. The right part of the figure shows the fold-induction of the transcriptional activity by estradiol treatment. Each column represents the mean ± S.D. of three independent experiments. *, P < 0.05, and ***, P < 0.001 compared with DMSO treatment by ANOVA and Tukey’s test.

![Graphs showing relative promoter activities of CYP2A6 constructs and effects of estradiol treatment and overexpression of ERα in MCF-7 cells.](image1)

![Graphs showing effects of ICI 182,780 (ICI) and 4OHT on estradiol-dependent transcriptional activation in MCF-7 cells.](image2)
REGULATION OF CYP2A6 BY ESTROGEN

pGL3-3(2A6/ERE), in which tandem-repeated ERE was linked to the SV40 promoter. This plasmid dramatically responded to estradiol treatment exhibiting a 38-fold induction of transcriptional activity even in the absence of overexpressed ER/\textsubscript{H9251}, which was largely inhibited by the treatment with ER antagonists ICI 182,780 or 4OHT (Fig. 4). These results clearly indicate that the ERE on the CYP2A6 gene works as a functional element in an ER/\textsubscript{H9251}-dependent manner.

ER/\textsubscript{H9251} Is Associated with the ERE on CYP2A6 Gene in Intact Cells. To further investigate the association of ER/\textsubscript{H9251} with the CYP2A6 gene in intact cells, ChIP assays were performed using MCF-7 and ER/\textsubscript{H9251}-overexpressed HepG2 cells. PCR was performed with a primer set for region 1 including the ERE, or for region 2 as a negative control. Normal mouse IgG was used as a negative control of immunoprecipitation for the ChIP assays. As shown in Fig. 5, the immunoprecipitants of estradiol-treated MCF-7 cells obtained with anti-ER/\textsubscript{H9251} antibodies generated a distinct PCR product for region 1, but those obtained with mouse IgG did not. The immunoprecipitants of HepG2 cells obtained with anti-ER/\textsubscript{H9251} antibodies generated a distinct PCR product for region 1, irrespective of estradiol or DMSO treatment. Using the primer set for region 2, no PCR products were generated. These results suggested that ER/\textsubscript{H9251} binds to the CYP2A6 gene in intact cells.

Expression of CYP2A6 Protein in Human Endometrial Tissues Is Dependent on the Menstrual Cycle. The endometrium would be a suitable tissue to investigate regulation by estrogen. Immunohistochemistry of CYP2A6 was performed in normal endometrial tissues obtained from 19 patients at different phases of the menstrual cycle. Immunostaining of CYP2A6 was detected in both the glandular epithelial and stromal cells. We evaluated the staining in the glandular epithelial cells in functional layers, because they are mostly susceptible to estrogen. Strong staining was observed in the glandular epithelial cells during the proliferative phase (Fig. 6A) and in the early secretory phase, but the staining was diminished in the late secretory phase (Fig. 6B). Of 11 samples in the proliferative phase, 7 and 4 samples were judged as (+) and (+), respectively (Table 2). Of 5 samples in the early secretory phase, 3 samples were judged as (+) and (+), respectively. In contrast, 3 samples in the late secretory phase were judged as (−). A statistically significant difference in the staining was observed among the different phases of

<table>
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<tr>
<th>Menstrual Cycle</th>
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<td>Proliferative (n = 11)</td>
<td>7</td>
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<tr>
<td>Early secretory (n = 5)</td>
<td>3</td>
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<tr>
<td>Late secretory (n = 3)</td>
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TABLE 2

CYP2A6 staining of normal human endometrial tissues in the proliferative and secretory phases

When >10% of the constituting glandular epithelial cells were stained, the specimen was evaluated as CYP2A6-positive (+) and that with high intensity was judged as (+). The statistical significance of differences in the extent of staining in the different phases of the menstrual cycle was tested by Fisher’s exact test (\(P < 0.001\)).

A total of 10% of the constituting glandular epithelial cells were stained, the specimen was evaluated as CYP2A6-positive (+) and that with high intensity was judged as (+). The statistical significance of differences in the extent of staining in the different phases of the menstrual cycle was tested by Fisher’s exact test (\(P < 0.001\)).
the menstrual cycle (P < 0.001). Although there are genetic polymorphisms in the CYP2A6 gene, we confirmed that none of the 19 patients were homozygotes or combined heterozygotes of the CYP2A6*4 (entire gene deleted) allele and CYP2A6*9 (promoter activity-decreased) allele (data not shown). Thus, the expression levels of CYP2A6 in the glandular cells appeared to be tightly regulated in a menstrual phase-dependent manner, concomitant with the secretion of estradiol.

**Discussion**

In the present study, we found that CYP2A6 is a direct target of estradiol, supporting the clinical findings that CYP2A6 is up-regulated under estrogen-rich circumstances (Iscan et al., 1994; Dempsey et al., 2002; Benowitz et al., 2006) in which ERα plays a central role, because the induction of CYP2A6 mRNA was observed only in ERα-positive cells and estradiol-dependent transactivation of the promoter was substantially increased when ERα was overexpressed in MCF-7 cells. Furthermore, ERα antagonists were able to inhibit the estradiol-induced transactivation of CYP2A6.

The ERE we identified at −2436 on the CYP2A6 gene had 2-base pair mismatches compared with the consensus ERE. Cumulative data from various ER-target genes indicated that some nucleotide differences in the ERE sequence allow the ERα to bind. However, the nucleotide changes in both hexamer half-sites decrease the binding of ERα compared with those in only one half-site (Klinge, 2001). The fact that the ERE on the CYP2A6 gene has the mismatches in only one half-site supports the view that this element functions as the ERα binding site. The luciferase analyses showed that the estradiol-dependent transactivation was not fully abolished by the mutation or deletion of the ERE on the CYP2A6 gene, indicating that some other element(s) may also be responsible for the regulation. It is known that ERα modulates the activities of other transcription factors such as activator protein-1 (Kusher et al., 2000), specificity protein-1 (Saville et al., 2000), or nuclear factor-κB (Stein and Yang, 1995; Ray et al., 1997) by stabilizing their DNA binding and/or recruiting coactivators to the complex. Moreover, ERβ could bind an ERE half-site cooperating with specificity protein-1 (Petz and Nardulli, 2000; Vyhildal et al., 2000). Interestingly, we found two ERE half-sites on −2079 and −2500 of the CYP2A6 gene. Further studies are needed to clarify whether the coactivator-like function of ERα is additionally involved in the estradiol-dependent induction of CYP2A6.

In the present data, there is some discrepancy between in vitro and in vivo findings regarding ERα dependence for the regulation. The ChIP assay showed that endogenous ERα in MCF-7 cells binds to the ERE on CYP2A6 gene in intact cells (Fig. 5), whereas the CYP2A6 promoter was not transactiviated by estradiol in MCF-7 cells and overexpressed ERα was required for activation in the luciferase reporter assay (Fig. 3). In contrast, the CYP2A6 promoter was not transactivated by estradiol in HepG2 cells, even though ERα was overexpressed (data not shown), in contrast to the fact that CYP2A6 mRNA was significantly induced by estradiol in both MCF-7 and HepG2 cells. Therefore, cell type-related conformational and/or epigenetic events may play important roles in this regulation.

Another notable finding we identified is that the expression levels of CYP2A6 protein in endometrial glandular cells was exquisitely regulated in a menstrual phase-dependent manner. The expression pattern of CYP2A6 in endometrial glands is consistent with changes in the local concentration of estradiol (Alsbach et al., 1983) as well as the ERα expression (Lecce et al., 2001), again supporting our regulatory model of CYP2A6. This regulatory mechanism may occur in liver, where CYP2A6 is abundantly expressed and estradiol and ERα are also distributed, supported by the induction of CYP2A6 by estradiol in human hepatocytes (Fig. 1). Hukkanen et al. (2005), however, reported that the menstrual cycle phase did not affect the nicotine and cotinine clearances. Because these clearances are determined by CYP2A6-dependent metabolism in liver, a small change of the estrogen level during the menstrual cycle might not affect the CYP2A6 expression in liver. The order of changes in the estrogen level and that in the nicotine clearance are the same: sex difference < oral contraceptive use < pregnancy, indicating their association. Thus, the observed sex differences in CYP2A6 activity might be due to the induction by estradiol, although other unknown mechanisms may also be involved. In contrast, oral contraceptive use and pregnancy, which strikingly increase the estrogen level, would result in the significant increases of nicotine clearance by induction of CYP2A6.

What is the biological role of CYP2A6 in estrogen-responsive tissues? At present, we have no conclusive explanation. Many cytochrome P450 isofoms are likely to be induced by the substrates themselves. It has been reported that CYP2A6 catalyzes 16β-hydroxylation of estradiol and has substantial activity for the conversion of estradiol to estrone and a few dehydro-estradiol metabolites (Lee et al., 2003). Therefore, the regulation of CYP2A6 by estradiol is consistent with this theory. In sex steroid-responsive tissues, sex hormones play pivotal roles in their functions. In particular, estrogen is indispensable for endometrial proliferation to maintain the menstrual cycle. However, such hormonal actions sometimes trigger unexpected molecular events including carcinogenesis when they are overproduced. In the endometrium, excess exposure to estradiol is well recognized as a major etiologic risk factor for endometrial carcinogenesis. The presence and induction of estradiol-metabolizing enzyme(s), including CYP2A6, may play some role in protecting against such adverse effects of estrogen in estrogen-responsive tissues. On the other hand, CYP2A6 catalyzes the metabolic activation of tobacco-specific nitrosamines. It has been reported that female smokers have a higher risk of lung cancer than male smokers (Zang and Wynder, 1996; Henschke and Miettinen, 2004). Smoking is also a risk factor in breast and uterine cervix cancers. Thus, the induction of CYP2A6 by estrogen may be involved in the tobacco-related carcinogenesis in estrogen-responsive tissues.

In summary, we found that human CYP2A6 is induced by estradiol via ERα. This study could provide insights into the molecular mechanism of increased nicotine metabolism accompanied by an increased estrogen level. The regulation by estrogen may imply some biological role of CYP2A6 in estrogen-responsive tissues.

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