

Short Communication

Effect of estradiol on gene expression profile in cynomolgus macaque liver: implications for drug-metabolizing enzymes

Yasuhiro Uno, Go Kito

Laboratories of Translational Research (YU, GK), Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan; Pharmacokinetics and Bioanalysis Center, Shin Nippon Biomedical Laboratories, Ltd., Kainan, Japan (YU).

Running Title Page

Running title: estradiol-regulated drug-metabolizing enzymes in macaque

Corresponding author: Yasuhiro Uno, D.V.M., Ph.D.

Pharmacokinetics and Bioanalysis Center, Shin Nippon Biomedical Laboratories, Ltd., 16-1 Minami

Akasaka, Kainan 642-0017, Japan

Phone: +81-73-483-8881. Fax: +81-73-483-7377. E-mail address: uno-yasuhiro@snbl.co.jp

Number of Text Pages : 15

Number of Tables : 2

Number of Figures : 1

Number of References : 36

Number of Words in Abstract : 203

Number of Words in Introduction : 517

Number of Words in Result and Discussion : 1590

Abbreviations: CYP, individual forms of cytochrome P450 (EC 1.14.14.1); GH, growth hormone; GO, Gene Ontology; GST, glutathione *S*-transferase; P450, general term for cytochrome P450; PCR, polymerase chain reaction; PXR, pregnane X receptor; qPCR, quantitative PCR; RT-PCR, reverse transcription PCR

Abstract

Estrogen regulation of gene expression is essential for physiological function of estrogen-responsive tissues, such as mammary glands, ovaries, and the uterus. In the liver, estrogen is responsible for sex-dependent gene expression of drug-metabolizing enzymes in rodents. However, the influence of estrogen on hepatic gene expression has not been fully investigated in primates, including human. Macaque, including cynomolgus macaque, is an important species for comparative studies aimed at understanding human physiology due to its evolutionary closeness to human. To identify estrogen-responsive genes in primate liver, therefore, hepatic gene expression was compared, by microarray analysis, in ovariectomized cynomolgus macaques treated with estradiol or solvent (control). The analysis identified 98 estradiol-responsive genes; 47 and 51 were up- and down-regulated by estradiol, respectively (≥ 2.0 -fold, $P < 0.05$). Expression of drug-metabolizing enzyme genes was also influenced by estradiol treatment; estradiol enhanced expression of *GSTM5* (3.8-fold, $P < 0.05$) and *CYP3A8(4)* (2.7-fold, $P < 0.01$), but lowered expression of *CYP4F12* (2.2-fold, $P < 0.01$), as verified by quantitative polymerase chain reaction. Especially, *CYP3A8(4)*, orthologous to human *CYP3A4*, is an essential drug-metabolizing enzyme in cynomolgus monkey liver. These results suggest that expression of hepatic genes, including drug-metabolizing enzyme genes, is at least partly regulated by estradiol in cynomolgus macaque.

Introduction

Estrogen exerts its physiologic effects in estrogen-responsive tissues such as mammary gland, ovary, and uterus. Recent studies indicate that estrogen also exerts its effect in non-classical estrogen-responsive tissues including kidney, bone, and liver (Diel, 2002). In fetal baboon liver, estrogen-regulated genes have been identified by differential display polymerase chain reaction (PCR) (Rosenthal et al., 2004). In mouse liver, microarray analysis revealed that expression of numerous genes is regulated by ethynyl estradiol, including oxidative metabolism and stress, and lipid metabolism and transport genes (Boverhof et al., 2004). Another study also showed that many mouse hepatic genes were regulated by estradiol, including the genes of lipid metabolism and synthesis, such as leptin receptor (LEPR) and stearoyl-CoA desaturase (SCD) (Gao et al., 2006).

The liver is a major drug metabolizing organ, where many drug-metabolizing enzyme genes are expressed including cytochrome P450 (CYP or P450), which is important for the metabolism of various drugs, steroids, fatty acids, and environmental pollutants. In rodent liver, estrogen regulates gene expression; and, is partly responsible for sex-biased gene expression of drug-metabolizing enzymes, including rat CYP3A9 (Anakk et al., 2003) and CYP4Fs (Kalsotra et al., 2002), and mouse CYP3A41 and CYP3A44 (Sakuma et al., 2002). In contrast, estrogen regulation of P450 genes in liver largely remains to be investigated for primate species, including human.

In human, sex has been implicated in drug metabolism differences. For example, activities of erythromycin *N*-demethylation and ifosfamide *N*-dechloroethylation, catalyzed by CYP3A4, are higher in females than males (Hunt et al., 1992; Schmidt et al., 2001). Moreover, females had significantly higher CYP3A4 content (Schmidt et al., 2001), indicating sex dimorphism in CYP3A4-mediated activities. A previous study showed higher gene expression, protein content, and metabolic activity of CYP3A4 in females than in males (Wolbold et al., 2003), suggesting that sex dimorphism in CYP3A4-dependent drug metabolism in human can be, at least partly, explained by sex-based differences in *CYP3A4* gene expression. It is of great importance to investigate the effects of estradiol on CYP3A4 and other drug-metabolizing enzyme genes.

Macaques, especially cynomolgus macaque and rhesus macaque, are frequently used in biomedical research, including drug metabolism studies, due to their evolutionary closeness to humans. Sequence identity, orthology, and functional characteristics of human P450s are better conserved in macaque P450s (Uno et al., 2011), as compared to P450s of rats or dogs (other animal species used in drug metabolism studies). This fact makes macaques an important species for preclinical studies. In this paper, for cynomolgus CYP2C20, CYP2C43, CYP2C75, CYP3A8, and CYP4F45, the nomenclature recommended by the P450 Nomenclature Committee recommends (Uno et al., 2011) is also indicated.

In this study, the influence of estradiol on hepatic gene expression was assessed in cynomolgus macaque. Microarray analysis was conducted using liver samples collected from ovariectomized animals that were treated with estradiol or solvent. The estradiol-regulated genes identified were subjected to Gene Ontology (GO) analysis, and quantitative PCR (qPCR) was carried out to evaluate microarray data, especially for drug-metabolizing enzyme genes. qPCR was also carried out for 18 P450 genes and *PXR* relevant to drug metabolism, to assess the influence of estradiol on their expression in cynomolgus macaque liver.

Materials and Methods

Materials

17 β -estradiol and estradiol benzoate were purchased from Sigma-Aldrich (St. Louis, MO). Oligonucleotides were synthesized by Invitrogen (Tokyo, Japan), and TaqMan probes were synthesized by Applied Biosystems (Foster City, CA) and Biosearch Technology Japan (Tokyo, Japan). All other reagents were purchased from Wako (Osaka, Japan) unless otherwise specified.

Animals and treatments

Eight female cynomolgus macaques (from Indochina, 14-16 years of age, 3-6 kg) were

ovariectomized according to the standard procedure established at Shin Nippon Biomedical Laboratories, Ltd. (Kagoshima, Japan). Treatment was initiated at least 30 days after the ovariectomy, using daily subcutaneous injections of estradiol benzoate (50 µg/kg body weight, four animals) or sesame oil (two animals as control), and continued for four consecutive days. Similarly, two animals, treated with sesame oil for 1 day (in another study), were also utilized as controls in this study. The initial injection of estradiol benzoate was accompanied by injection of 17β-estradiol (5 µg/kg body weight, i.v.) for a rapid increase in blood estrogen levels, similar to previous study (Robb et al., 2004). Twenty-four hours after the final injection, liver samples were collected and flash-frozen in liquid nitrogen. The study was reviewed and approved by the institutional ethics committee.

RNA preparation and microarray analysis

Total RNA was extracted from liver samples and treated with DNase I as described previously (Uno et al., 2006), and purified using RNeasy Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's protocols, and subjected to microarray analysis. All technical procedures of microarray analysis were performed using the Human Genome U130 GeneChip set (Affymetrix, Santa Clara, CA) by Bio Matrix Research, Inc. (Nagareyama, Japan) in accordance with the procedures established by Affymetrix. These include verification of RNA quality, labeling, hybridization with biotin-labeled cRNAs (15 µg), scanning of the arrays, and data analysis. GeneChip Operating Software (GCOS; Affymetrix) was used for image processing and data acquisition. Differentially expressed genes were selected at a threshold of 2.0-fold difference between estradiol-treated and control animals ($P < 0.05$).

Bioinformatics

Estradiol-responsive genes were subjected to GO classification, which is useful to describe the roles for the genes of interest using structured, precisely defined GO terms (Ashburner et al., 2000). Overrepresented biological processes were determined using DAVID2.0

(<http://david.abcc.ncifcrf.gov/>) according to the protocol. For the analysis, 47 and 51 genes up- and down-regulated, respectively (≥ 2.0 -fold, $P < 0.05$), by treatment with estradiol were used. Significantly overrepresented categories of Biological process, Molecular function, and Cellular process were selected ($P < 0.05$). To identify putative consensus binding sites in the estradiol-responsive genes, sequence upstream of the transcription start site was analyzed using MatInspector program (<http://www.genomatix.de/>).

Quantitative PCR

Expression of cynomolgus P450 genes was measured by real-time reverse transcription (RT)-PCR as described previously (Uno et al., 2006; Uno et al., 2009b), including *CYP1A1*, *CYP2A23*, *CYP2A24*, *CYP2B6*, *CYP2C20(8)*, *CYP2C43(9)*, *CYP2C75(19)*, *CYP2C76*, *CYP2D17*, *CYP2E1*, *CYP3A8(4)*, *CYP3A5*, *CYP3A43*, *CYP4A11*, *CYP4F2*, *CYP4F3*, *CYP4F11*, and *CYP4F12*. Expression of *GSTM5*, *LEPR*, *PXR*, and *SCD*, was also measured. The gene-specific primers and probes, and their final concentrations are listed in Supplemental Material Table 1. Relative expression levels were determined by normalizing the raw data to the 18S ribosomal RNA level for eight animals (four estradiol-treated and four control). Statistical significance of the differences in gene expression level upon treatment with estradiol was determined using a two-tailed unpaired Student's *t* test. Values are presented as mean \pm S.D. of the four animals and were considered significant if P was < 0.05 .

Results and Discussion

To investigate the effect of estradiol on gene expression in cynomolgus macaque liver, microarray analysis was conducted to measure gene expression in estradiol-treated animals and control animals (corn oil-treated). All the animals were ovariectomized at least 1 month prior to the experiments. For microarray analysis, human microarrays were used, since cynomolgus macaque

microarrays were not commercially available at the time of the experiment. Human microarrays have been successfully utilized to analyze gene expression in monkeys (Cáceres et al., 2003; Marvanová et al., 2003; Walker et al., 2006). The microarray analysis successfully identified 47 and 51 genes up- and down-regulated by estradiol treatment (≥ 2.0 -fold, $P < 0.05$), and those showing ≥ 2.5 -fold change in gene expression are listed in Table 1. Some of these genes were previously reported as estradiol-responsive genes in mouse liver, such as *LEPR* and *SCD* (Gao et al., 2006), with changes in expression of 7.1-fold ($P < 0.05$) and 9.0-fold ($P < 0.05$), respectively, as confirmed by quantitative PCR analysis, partly suggesting that our microarray analysis was successful.

To better understand functional categories of estradiol-responsive genes, GO analysis was carried out with level 5 GO annotation using DAVID2.0. Sixteen, 2, and 10 functional categories were enriched for Biological process, Molecular function, and Cellular component ($P < 0.05$), respectively (Table 2). The categories of Biological process were related to lipid metabolism, protein complex assembly, cell death, and cell cycle signaling, while the categories of Molecular function were related to leukotriene-B4 20-monooxygenase activity and iron ion binding. Based on the Cellular component categories, many of the estradiol-responsive genes encode proteins localized in endoplasmic reticulum and cytoplasm.

Microarray analysis indicated that expression of drug-metabolizing enzyme genes (*CYP2D*, *CYP3A*, *CYP4F*, and *GSTM5*) was altered (≥ 1.5 -fold, $P < 0.05$) by estradiol treatment (Table 1), and thus expression of these genes were analyzed by qPCR analysis. The analysis confirmed that expression of *GSTM5* increased 3.8-fold ($P < 0.05$) (Fig. 1). qPCR using gene-specific primers and probes for cynomolgus *CYP3A* genes, including *CYP3A8(4)*, *CYP3A5*, and *CYP3A43*, showed that *CYP3A8(4)* was up-regulated by estradiol treatment (2.7-fold, $P < 0.01$) (Fig. 1). *CYP3A7* has not been identified in cynomolgus monkey, and thus was excluded from the analysis. *CYP3A43* was also up-regulated (2.9-fold), but the difference was not statistically significant. Moreover, expression of *CYP3A43* was substantially lower than that of *CYP3A8(4)*, as described previously (Uno et al., 2009a). Estradiol treatment did not change *CYP3A5* expression substantially. qPCR using gene-specific primers and probes for cynomolgus *CYP4F* genes (*CYP4F2*, *CYP4F3*, *CYP4F11*,

and *CYP4F12*) showed that among these *CYP4F* genes, *CYP4F12* was up-regulated by estradiol treatment (2.2-fold, $P < 0.01$) (Fig. 1), whereas differential expression (≥ 1.5 -fold) was not observed for *CYP4F3* and *CYP4F11*. *CYP4F2* was down-reduced (1.8-fold), but the difference did not reach statistical significance. Similarly, qPCR analysis showed that cynomolgus *CYP2D17* (highly identical to human *CYP2D6*) was down-regulated (1.4-fold), but the difference did not reach statistical significance. These results prompted further qPCR analysis for other P450 genes relevant to drug metabolism: *CYP1A1*, *CYP2A23*, *CYP2A24*, *CYP2B6*, *CYP2C20(8)*, *CYP2C43(9)*, *CYP2C75(19)*, *CYP2C76*, *CYP2E1*, and *CYP4A11*. *PXR*, an important regulator for expression of some P450 genes, was also included in this analysis. Among these genes, differential expression (≥ 1.5 -fold) in liver between estradiol-treated and control animals, was observed in five P450 genes; *CYP2C20(8)* (2.3-fold), *CYP2C43(9)* (1.8-fold), *CYP2C76* (1.7-fold), *CYP2E1* (1.9-fold) were up-regulated, whereas *CYP4A11* (1.6-fold) was down-regulated; however, these differences were not statistically significant. These results suggest that expression of drug-metabolizing enzyme genes, *GSTM5*, *CYP3A8(4)*, and *CYP4F12*, are regulated by estradiol in cynomolgus macaque liver.

GSTM5 belongs to the glutathione-S-transferase (GST) family of drug metabolizing enzymes, which plays an important role in detoxification of xenobiotics including carcinogens, drugs, and environmental toxins, by conjugating such electrophilic compounds with glutathione (Hayes et al., 2005). Microarray analyses showed that expression of several GST genes was regulated by estradiol in mouse liver (Boverhof et al., 2004; Singhal et al., 2009). Human *CYP3A4* is one of the most important drug-metabolizing enzymes, involved in the metabolism of more than half of all prescription drugs (Thummel and Wilkinson, 1998). Sex-biased expression of human *CYP3A4* has been shown in liver; 1.91-fold higher in females than in males (Wolbold et al., 2003). A previous study showed that human *CYP3A4* expression was regulated by estradiol in endometrium (Williams et al., 2004). These results suggest that hepatic expression of some GST and P450 genes are regulated by estradiol in primates as well as rodents, which might affect metabolism and detoxification of drugs and toxins, depending on hormonal status, such as pregnancy and menopause. Indeed, the rate and extent of hepatic drug metabolism (by drug-metabolizing enzymes) changes during pregnancy (Hodge

and Tracy, 2007).

In addition to estradiol, growth hormone (GH) possibly regulates *CYP3A4* expression also. In rodents, expression of some P450 genes is sex-biased, which is partly regulated by GH (Waxman and O'Connor, 2006). The different secretion patterns of GH cause sex-dependent gene expression; a pulsatile and continuous secretion pattern activates male and female-specific gene expression, respectively, and this sex-dependent effect of GH on hepatic gene expression is at least partly mediated through STAT5b, belonging to the signal transducers and activators of transcription (STAT) family of transcription factors, in a JAK-dependent manner (Waxman and O'Connor, 2006). Similarly, in human, hepatic *CYP3A4* activity decreased with pulsatile GH infusion, but increased with continuous GH infusion (Jaffe et al., 2002). Moreover, in human primary hepatocytes, continuous GH treatments induce expression of *CYP3A4* and its protein (Liddle et al., 1998), which is suppressed by pulsatile GH treatment (Dhir et al., 2006). In *CYP3A4* transgenic mice, *CYP3A4* expression was detected in adult liver only for females, but can be induced in adult males by continuous GH treatment (Cheung et al., 2006). Therefore, *CYP3A4* expression is possibly regulated by GH as well as estradiol.

To gain mechanistic insight into the estradiol-responsive genes identified, the upstream region of each gene was searched for estrogen response element and STAT5-binding element using MatInspector. Since cynomolgus macaque genome data have not been available, sequence information on the human orthologs of the estradiol-responsive genes identified were used for the analysis. As expected, estrogen response element was found in the upstream regions of most estradiol-responsive genes identified (≥ 2.5 -fold change) (Table 1), including 15 of 19 up-regulated genes and 12 of 14 down-regulated genes. The genes that did not contain estrogen response element in the upstream region might be regulated by different mechanism. For example, estrogen exerts its effect not only via estrogen receptors, but also through signal transduction by activating cytosolic signaling effectors (e.g. p38 mitogen-activated protein kinase, phosphoinositol-3-kinase, and cAMP) (Leung et al., 2004). Moreover, there is an evidence that estrogen can activate STAT signaling in a JAK-independent manner (Leung et al., 2004). In the upstream region of at least one

down-regulated gene, STAT5-binding element, but not estrogen response element, was found. The STAT5-binding element was found in 9 of 19 up-regulated genes and 3 of 14 down-regulated genes. It is of great interest to investigate the role of STAT5 in the regulation of these estradiol-responsive genes.

In this study, macaque *CYP2A* expression was not significantly changed by estradiol. In contrast, human *CYP2A6* expression is induced by estradiol in hepatocytes, but only 1.2- or 1.5-fold, depending on hepatocyte lot (Higashi et al., 2007); therefore, a small induction of macaque *CYP2A*, if any, might not be apparent *in vivo*. Alternatively, macaque *CYP2As* might not be estradiol responsive due to divergence in the regulatory region of *CYP2As*. *CYP2As* are located in the *CYP2ABFGST* cluster of the genome, and the arrangement of the *CYP2As* in the *CYP2ABFGST* cluster is somewhat different even between macaque and human (Hoffman and Hu, 2007). This divergence of *CYP2As* in cynomolgus macaque and human genomes might have led to the differences in the regulatory region of the *CYP2As*, thus accounting for the disparity in *CYP2A* response to estradiol between the two species.

Unlike the *CYP2ABFGST* cluster, the *CYP3A* cluster is conserved well between macaque and human; the location and direction of each orthologous gene in this cluster correspond well in the two species (Qiu et al., 2008). Macaque *CYP3A8(4)* metabolizes typical human *CYP3A4* substrates such as midazolam and nifedipine (Iwasaki et al., 2010; Uno et al., 2010) and is induced by the typical human *CYP3A4* inducer, rifampicin (Kim et al., 2010), indicating that *CYP3A8(4)* has drug-metabolizing enzyme properties similar to human *CYP3A4*. Induction of cynomolgus macaque *CYP3A8(4)* and human *CYP3A4* by rifampicin is mediated via PXR (Kim et al., 2010), which binds to the regulatory element in the upstream region of *CYP3A4*, suggesting that the regulatory region of cynomolgus macaque *CYP3A8(4)* and human *CYP3A4* is conserved well between macaque and human. Induction of *CYP3A8(4)* expression in macaque liver by estradiol, therefore, raises the possibility that *CYP3A4* is also regulated by estradiol in human liver. Investigation of sex differences in human drug-metabolizing enzyme genes is a challenge, due to many confounding factors such as drug intake, smoking, diet supplements, exposure to toxins, and

menopause, which could influence gene expression of many drug-metabolizing enzymes. Such factors can be controlled well in macaques. Together with the similarities in drug-metabolizing enzyme properties, macaques could be useful for investigating human CYP3A4-related drug metabolism.

In conclusion, microarray analysis has successfully identified 47 and 51 genes, up- and down-regulated, respectively, by estradiol (≥ 2.0 -fold, $P < 0.05$), including genes encoding the drug-metabolizing enzymes, P450 and GST. Notably, *CYP3A8(4)*, orthologous to human *CYP3A4*, was up-regulated by estradiol in cynomolgus macaque liver. The results presented in this paper should help in the understanding of liver physiology, especially drug metabolism in cynomolgus macaque, which could be extrapolated, partly, to human.

Acknowledgements

We greatly thank Mr. Masahiro Utoh, Dr. Koichiro Fukuzaki, and Dr. Ryoichi Nagata for their support of this work, and Mr. Patrick Gray for reviewing the paper.

Authorship Contribution

Participated in research design: Uno, Kito.

Conducted experiments: Uno.

Performed data analysis: Uno.

Wrote or contributed to the writing of the manuscript: Uno, Kito.

References

- Anakk S, Ku CY, Vore M and Strobel HW (2003) Insights into gender bias: rat cytochrome P450 3A9. *J Pharmacol Exp Ther* **305**:703-709.
- Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM and Sherlock G (2000) Gene ontology: tool for the unification of biology. *Nat Genet* **25**:25-29.
- Boverhof DR, Fertuck KC, Burgoon LD, Eckel JE, Gennings C and Zacharewski TR (2004) Temporal- and dose-dependent hepatic gene expression changes in immature ovariectomized mice following exposure to ethynyl estradiol. *Carcinogenesis* **25**:1277-1291.
- Cáceres M, Lachuer J, Zapala MA, Redmond JC, Kudo L, Geschwind DH, Lockhart DJ, Preuss TM and Barlow C (2003) Elevated gene expression levels distinguish human from non-human primate brains. *Proc Natl Acad Sci U S A* **100**:13030-13035.
- Cheung C, Yu AM, Chen CS, Krausz KW, Byrd LG, Feigenbaum L, Edwards RJ, Waxman DJ and Gonzalez FJ (2006) Growth hormone determines sexual dimorphism of hepatic cytochrome P450 3A4 expression in transgenic mice. *J Pharmacol Exp Ther* **316**:1328-1334.
- Dhir RN, Dworakowski W, Thangavel C and Shapiro BH (2006) Sexually dimorphic regulation of hepatic isoforms of human cytochrome P450 by growth hormone. *J Pharmacol Exp Ther* **316**:87-94.
- Diel P (2002) Tissue-specific estrogenic response and molecular mechanisms. *Toxicol Lett* **127**:217-224.
- Gao H, Bryzgalova G, Hedman E, Khan A, Efendic S, Gustafsson JÅ and Dahlman-Wright K (2006) Long-term administration of estradiol decreases expression of hepatic lipogenic genes and improves insulin sensitivity in ob/ob mice: a possible mechanism is through direct regulation of signal transducer and activator of transcription 3. *Mol Endocrinol* **20**:1287-1299.

- Hayes JD, Flanagan JU and Jowsey IR (2005) Glutathione transferases. *Annu Rev Pharmacol Toxicol* **45**:51-88.
- Higashi E, Fukami T, Itoh M, Kyo S, Inoue M, Yokoi T and Nakajima M (2007) Human CYP2A6 is induced by estrogen via estrogen receptor. *Drug Metab Dispos* **35**:1935-1941.
- Hodge LS and Tracy TS (2007) Alterations in drug disposition during pregnancy: implications for drug therapy. *Expert Opin Drug Metab Toxicol* **3**:557-571.
- Hoffman SM and Hu S (2007) Dynamic evolution of the *CYP2ABFGST* gene cluster in primates. *Mutat Res* **616**:133-138.
- Hunt CM, Westerkam WR and Stave GM (1992) Effect of age and gender on the activity of human hepatic CYP3A. *Biochem Pharmacol* **44**:275-283.
- Iwasaki K, Murayama N, Koizumi R, Uno Y and Yamazaki H (2010) Comparison of cytochrome P450 3A enzymes in cynomolgus monkeys and humans. *Drug Metab Pharmacokinet* **25**:388-391.
- Jaffe CA, Turgeon DK, Lown K, Demott-Friberg R and Watkins PB (2002) Growth hormone secretion pattern is an independent regulator of growth hormone actions in humans. *Am J Physiol Endocrinol Metab* **283**:E1008-1015.
- Kalsotra A, Anakk S, Boehme CL and Strobel HW (2002) Sexual dimorphism and tissue specificity in the expression of CYP4F forms in Sprague Dawley rats. *Drug Metab Dispos* **30**:1022-1028.
- Kim S, Dinchuk JE, Anthony MN, Orcutt T, Zoekler ME, Sauer MB, Mosure KW, Vuppugalla R, Grace JE, Jr., Simmermacher J, Dulac HA, Pizzano J and Sinz M (2010) Evaluation of cynomolgus monkey pregnane X receptor, primary hepatocyte, and in vivo pharmacokinetic changes in predicting human CYP3A4 induction. *Drug Metab Dispos* **38**:16-24.
- Leung KC, Johannsson G, Leong GM and Ho KK (2004) Estrogen regulation of growth hormone action. *Endocr Rev* **25**:693-721.
- Liddle C, Goodwin BJ, George J, Tapner M and Farrell GC (1998) Separate and interactive regulation of cytochrome P450 3A4 by triiodothyronine, dexamethasone, and growth hormone in cultured hepatocytes. *J Clin Endocrinol Metab* **83**:2411-2416.

- Marvanová M, Ménager J, Bezard E, Bontrop RE, Pradier L and Wong G (2003) Microarray analysis of nonhuman primates: validation of experimental models in neurological disorders. *FASEB J* **17**:929-931.
- Qiu H, Taudien S, Herlyn H, Schmitz J, Zhou Y, Chen G, Roberto R, Rocchi M, Platzer M and Wojnowski L (2008) CYP3 phylogenomics: evidence for positive selection of CYP3A4 and CYP3A7. *Pharmacogenet Genomics* **18**:53-66.
- Robb VA, Pepe GJ and Albrecht ED (2004) Acute temporal regulation of placental vascular endothelial growth/permeability factor expression in baboons by estrogen. *Biol Reprod* **71**:1694-1698.
- Rosenthal MD, Albrecht ED and Pepe GJ (2004) Estrogen modulates developmentally regulated gene expression in the fetal baboon liver. *Endocrine* **23**:219-228.
- Sakuma T, Endo Y, Mashino M, Kuroiwa M, Ohara A, Jarukamjorn K and Nemoto N (2002) Regulation of the expression of two female-predominant CYP3A mRNAs (CYP3A41 and CYP3A44) in mouse liver by sex and growth hormones. *Arch Biochem Biophys* **404**:234-242.
- Schmidt R, Baumann F, Hanschmann H, Geissler F and Preiss R (2001) Gender difference in ifosfamide metabolism by human liver microsomes. *Eur J Drug Metab Pharmacokinet* **26**:193-200.
- Singhal R, Shankar K, Badger TM and Ronis MJ (2009) Hepatic gene expression following consumption of soy protein isolate in female Sprague-Dawley rats differs from that produced by 17 β -estradiol treatment. *J Endocrinol* **202**:141-152.
- Thummel KE and Wilkinson GR (1998) *In vitro* and *in vivo* drug interactions involving human CYP3A. *Annu Rev Pharmacol Toxicol* **38**:389-430.
- Uno Y, Fujino H, Kito G, Kamataki T and Nagata R (2006) CYP2C76, a novel cytochrome P450 in cynomolgus monkey, is a major CYP2C in liver, metabolizing tolbutamide and testosterone. *Mol Pharmacol* **70**:477-486.
- Uno Y, Iwasaki K, Yamazaki H and Nelson DR (2011) Macaque cytochromes P450: nomenclature, transcript, gene, genomic structure, and function. *Drug Metab Rev* **In press**.

- Uno Y, Matsuno K, Nakamura C, Utoh M and Yamazaki H (2009a) Cloning, expression, and characterization of CYP3A43 cDNA in cynomolgus macaque (*Macaca fascicularis*). *Drug Metab Lett* **3**:228-233.
- Uno Y, Matsushita A, Osada N, Uehara S, Kohara S, Nagata R, Fukuzaki K, Utoh M, Murayama N and Yamazaki H (2010) Genetic variants of *CYP3A4* and *CYP3A5* in cynomolgus and rhesus macaques. *Drug Metab Dispos* **38**:209-214.
- Uno Y, Ohuchi T, Uehara S, Kito G, Kamataki T and Nagata R (2009b) Sex-related differences in the expression of *mfGSTA2*, a novel *GST* identified in cynomolgus monkey (*Macaca fascicularis*). *Drug Metab Dispos* **37**:453-456.
- Walker SJ, Wang Y, Grant KA, Chan F and Hellmann GM (2006) Long versus short oligonucleotide microarrays for the study of gene expression in nonhuman primates. *J Neurosci Methods* **152**:179-189.
- Waxman DJ and O'Connor C (2006) Growth hormone regulation of sex-dependent liver gene expression. *Mol Endocrinol* **20**:2613-2629.
- Williams ET, Leyk M, Wrighton SA, Davies PJ, Loose DS, Shipley GL and Strobel HW (2004) Estrogen regulation of the cytochrome P450 3A subfamily in humans. *J Pharmacol Exp Ther* **311**:728-735.
- Wolbold R, Klein K, Burk O, Nüssler AK, Neuhaus P, Eichelbaum M, Schwab M and Zanger UM (2003) Sex is a major determinant of CYP3A4 expression in human liver. *Hepatology* **38**:978-988.

Figure legends

Fig. 1. qPCR analysis of drug-metabolizing enzyme genes. Differential expression of *CYP3A8(4)*, *CYP4F12*, and *GSTM5* was confirmed by real-time RT-PCR using liver total RNAs from estradiol-treated (E2) and control animals as described in *Materials and Methods*. Values are presented as mean \pm S.D. of the four animals. The expression level of each gene was arbitrarily adjusted to 1, and all other expression levels were adjusted accordingly. *, $P < 0.05$. **, $P < 0.01$.

Table 1

Genes up- and down-regulated in estradiol-treated macaque liver (≥ 2.5 -fold, $P < 0.05$).

Drug-metabolizing enzyme genes up- and down-regulated by estradiol treatment (≥ 1.5 -fold, $P < 0.05$) are also listed.

Gene	Fold Change	Description
Up-regulated:		
<i>ANO4</i>	12.6	Anoctamin 4
<i>LEPR</i>	9.7	Leptin receptor
<i>GLYAT</i>	4.6	Glycine-N-acyltransferase
<i>IGFBP1</i>	4.2	Insulin-like growth factor binding protein 1
<i>KIAA0101</i>	3.4	KIAA0101
<i>FDX1</i>	3.1	Ferredoxin 1
<i>PRNP</i>	3.1	Prion protein
<i>PDSS2</i>	3.0	Prenyl (decaprenyl) diphosphate synthase, subunit 2
<i>HPGD</i>	2.9	Hydroxyprostaglandin dehydrogenase 15-(NAD)
<i>ATP6V1H</i>	2.9	ATPase, H ⁺ transporting, lysosomal 50/57kDa, V1
<i>HEMK</i>	2.8	HemK methyltransferase family member 1
<i>TSPAN12</i>	2.8	Tetraspanin 12
<i>TFPI</i>	2.8	Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)
<i>HSPA13</i>	2.6	Heat shock protein 70kDa family, member 13
<i>NUP50</i>	2.6	Nucleoporin 50kDa
<i>NUDT21</i>	2.5	Nudix (nucleoside diphosphate linked moiety X)-type motif 21 (NUDT21)
<i>FNDC3B</i>	2.5	Fibronectin type III domain containing 3B
<i>F2R</i>	2.5	Coagulation factor II (thrombin) receptor
<i>CHORDC1</i>	2.5	Cysteine and histidine-rich domain (CHORD)-containing 1 (CHORDC1)
Down-regulated:		
<i>SCD</i>	-6.9	Stearoyl-CoA desaturase (delta-9-desaturase)
<i>ALDH1B1</i>	-5.1	Aldehyde dehydrogenase 1 family, member B1
<i>IGF2</i>	-4.8	Insulin-like growth factor 2 (somatomedin A)
<i>ORM1</i>	-4.5	Orosomucoid 1
<i>FASN</i>	-3.9	Fatty acid synthase
<i>ELOVL6</i>	-3.6	ELOVL family member 6, elongation of long chain fatty acids
<i>SELENBP1</i>	-3.4	Selenium binding protein 1
<i>PIGR</i>	-3.2	Polymeric immunoglobulin receptor
<i>TRIM14</i>	-3.0	Tripartite motif-containing 14
<i>MOV10L1</i>	-2.8	Mov10l1, Moloney leukemia virus 10-like 1, homolog (mouse)
<i>KIAA0802</i>	-2.8	KIAA0802
<i>ADSSL1</i>	-2.6	Adenylosuccinate synthase like 1
<i>TAPBP</i>	-2.6	TAP binding protein (tapasin)
<i>PSAT1</i>	-2.6	Phosphoserine aminotransferase 1
Drug-metabolizing enzyme genes:		
<i>CYP3A7</i>	2.4	Cytochrome P450, family 3, subfamily A, polypeptide 7
<i>GSTM5</i>	2.3	Glutathione S-transferase mu 5
<i>CYP3A4</i>	1.8	Cytochrome P450, family 3, subfamily A, polypeptide 4
<i>CYP4F2</i>	-2.2	Cytochrome P450, family 4, subfamily F, polypeptide 2
<i>CYP2D6</i>	-1.5	Cytochrome P450, family 2, subfamily D, polypeptide 6

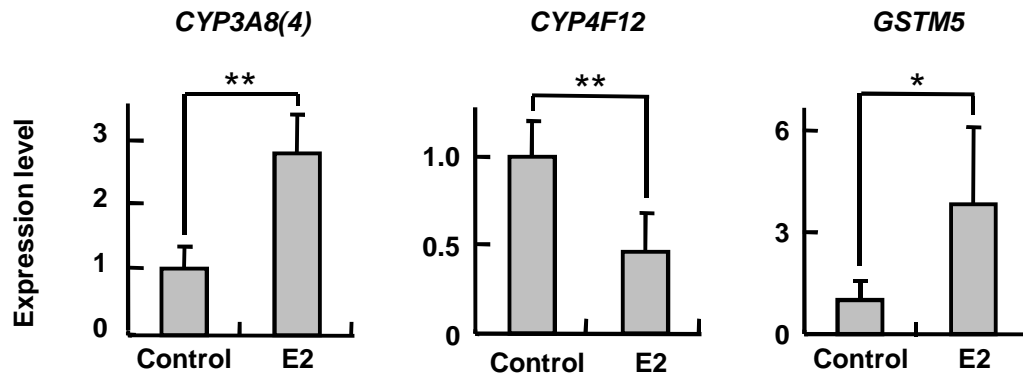
Table 2

Biological GO categories of up- and down-regulated genes by estradiol treatment in macaque livers.

Biological process	Molecular function	Cellular component
fatty acid metabolic process	leukotriene-B4 20-monooxygenase activity	cytoplasm
monocarboxylic acid metabolic process	iron ion binding	cytoplasmic part
lipid biosynthetic process		endoplasmic reticulum
fatty acid biosynthetic process		endoplasmic reticulum membrane
regulation of apoptosis		nuclear envelope-endoplasmic reticulum network
carboxylic acid biosynthetic process		endoplasmic reticulum part
regulation of programmed cell death		mitochondrion
apoptosis		microsome
cell death		vesicular fraction
icosanoid metabolic process		intracellular membrane-bound organelle
regulation of progression through cell cycle		
negative regulation of apoptosis		
negative regulation of programmed cell death		
protein oligomerization		
negative regulation of amine metabolic process		

Functional categories of up- and down-regulated genes were identified with level 5 GO terms using DAVID2.0 ($P < 0.05$) as described in *Materials and Methods*.

Fig. 1



Supplemental Material Table 1

Primer and probe sequences for real-time RT-PCR.

Gene		Sequence (5' → 3')	Conc. (nM)
<i>CYP1A1</i>	F	AAACCTTTGAGAAGGGCCACA	300
	R	TCATCCAGCTGCTTCTCCTGA	300
	P	FAM-CCGGGACATCACAGAC-MGB	200
<i>CYP2A23</i>	F	CAAGAAGGTGGAGCACAACAG	300
	R	GGTTCAGCGAGGTCAGCAC	300
	P	FAM-ACGCTGGATCCCAACT-MGB	200
<i>CYP2A24</i>	F	ACGCTGAACCTCTTCATTGC	600
	R	CTGCGGGCCAAACTCAT	600
	P	FAM-CTTGACCCGGTCCT-MGB	200
<i>CYP2B6</i>	F	CAACATCATCTGCTCCATCG	200
	R	TGTGTGCCCCAGGAAAGTA	200
<i>CYP2C8</i>	F	TTTCTGGAAGAGGCATTTTGC	300
	R	TCCATCTCTTTCCATTGCTGG	300
	P	FAM-AACGGACTTGGAATCA-MGB	250
<i>CYP2C9</i>	F	GCCATTTCCCACTGTTTGAAA	300
	R	GCAGCGTCATGAGGGAGAA	300
	P	FAM-ACAATTCCAAATCTTCT-MGB	250
<i>CYP2C19</i>	F	TTCCATTGGCTGACAGAGCTAA	900
	R	CCGCAGTGTCATGAGGGAA	900
	P	FAM-CGATTCCAAATCCT-MGB	250
<i>CYP2C76</i>	F	TGGCCGAGGGAGTTTTCC	100
	R	AGAGAGAAACGCCGAATTTGC	100
	P	FAM-CCAAGGATTCGGAGTTA-MGB	250
<i>CYP2D17</i>	F	CACCGACCAAGCCGGAC	300
	R	AGGCGATCACGTTGCTCACT	300
	P	FAM-ACCCTTTCGCCCAAAC-MGB	200
<i>CYP2E1</i>	F	TCCTGATTCTCATGAAATACCCTGA	600
	R	TGAATCTCATGCACCACAGCA	600
	P	FAM-AGCTCCATGAAGAAATTGACAGGGTGATTG-BHQ	200
<i>CYP3A4</i>	F	CACCCTGGTGCTCCTCTATT	600
	R	CAGGCTGTCGACCATCATAA	600
	P	FAM-CTTTTGGACGTTTGATATG-MGB	200
<i>CYP3A5</i>	F	CAGCCTGGTGCTCCTCTATC	300
	R	TGTCGGGATCTGTGATGGT	300
	P	FAM-TGTGGAGAACGCAAG-MGB	200
<i>CYP3A43</i>	F	ACATGGGTTCCTCGTGGCTA	300
	R	CCAGCATGGGCTGTTGT	300
	P	FAM-CTCATAACAGCCCCAC-MGB	200

<i>CYP4A11</i>	F	CGAAATCCCATGGTTCCTACAG	300
	R	GAACCATGTCTGCCCATTCAA	300
	P	FAM-TCCATGGATTGGGTACGGCTTGCT-BHQ	200
<i>CYP4F2</i>	F	AAGACGGGAAGGAGTTGTCC	300
	R	GCACAATGTCCTGGGTGAC	300
	P	FAM-ATCAGGAACGTTGCCGA-MGB	200
<i>CYP4F3</i>	F	AGAGCCTGCGGTTGCAC	300
	R	GAACACTGATGTGGCAGATAACA	300
	P	FAM-AGTTCCTGCCATCTC-MGB	200
<i>CYP4F11</i>	F	CGCGAGAGCTTCTGAAGGACT	300
	R	GGTGCAGCATCGGGAGAC	300
	P	FAM-TTGAGATTGAATGGGATGACT-MGB	200
<i>CYP4F12</i>	F	CGAAATCCCATGGTTCCTACAG	300
	R	GAACCATGTCTGCCCATTCAA	300
	P	FAM-TCCATGGATTGGGTACGGCTTGCT-BHQ	200
<i>GSTM5</i>	F	TTGGAGGAACTCCCTGAAAAG	200
	R	CTGGACTTCATGTAGGCAGAGA	200
<i>LEPR</i>	F	GTGAATGTCTTGTGCCTGTGC	600
	R	GGTGGATCAGGCTTCACCAT	600
<i>PXR</i>	F	TGAATGCAATCGGCCCC	300
	R	CGGAGCTCGGTGAGCATAGC	300
	P	FAM-CACAAGCGTGGAGGTCTATGAGCACATC-BHQ	200
<i>SCD</i>	F	GTGGGTTGGCTGCTTGTG	100
	R	AGCAAGCCAGGTTTGTAGTACCT	100

* F, forward primer, R, reverse primer, P, probe.