Short Communication

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

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Running Title Page

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

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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

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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.

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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

17β-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 ($P < 0.05$).

Bioinfomatics

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 ± 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 ($\geq$ 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

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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.

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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.

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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 ± 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. *, $P < 0.05$. **, $P < 0.01$.

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

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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*.

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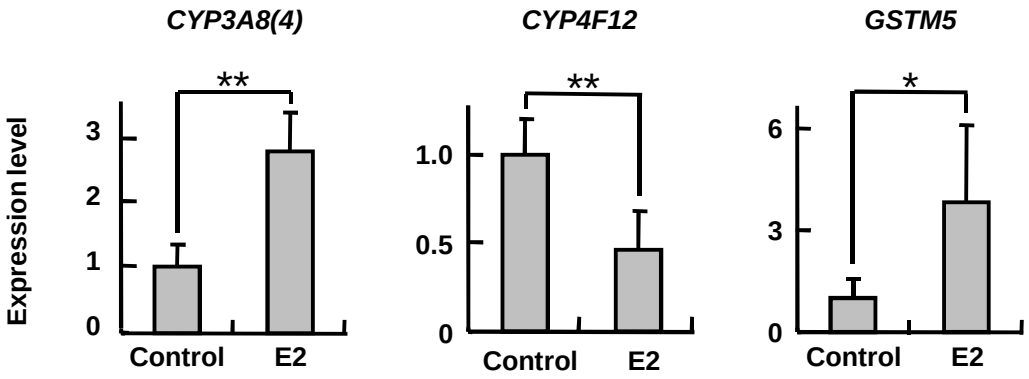
Fig. 1



Supplemental Material Table 1

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

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

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

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