

**Hormonal Regulation of Hepatic CYP Expression: Implications in Altered Drug
Metabolism during Pregnancy**

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Thesis

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LIST OF ABBREVIATIONS

AhR	Aryl Hydrocarbon Receptor
AUC	Area Under the Curve
b5	NADH-Cytochrome b5 Reductase
BNF	β -Naphthoflavone
CAR	Constitutive Androstane Receptor
CITCO	6-(4-Chlorophenyl) imidazo[2,1-b] [1,3]thiazole-5-carbaldehyde O-3,4-dichlorobenzyl) oxime
CYP	Cytochrome P450
DX	Dexamethasone
DME	Drug Metabolizing Enzyme
E1	Estrone
E2	17 β -Estradiol
E3	Estriol
EB	Estradiol Benzoate
ER	Estrogen Receptor
ERE	Estrogen Response Elements
GR	Glucocorticoid Receptor
HH	Human Hepatocyte
PB	Phenobarbital
PBREM	Phenobarbital Responsive Enhancer Module
POR	P450 Oxidoreductase
PRG	Progesterone
PXR	Pregnane X Receptor
XREM	Xenobiotic Responsive Enhancer Module

SUMMARY

Results from clinical studies have suggested that pregnancy alters hepatic drug metabolism in a cytochrome P450 (CYP) isoform-specific manner; elimination of drugs metabolized by CYP2A6, CYP3A4, CYP2D6 and CYP2C9 is increased, while elimination of drugs metabolized by CYP1A2 and CYP2C19 is decreased. While the substantial evidence from clinical and *in vitro* studies have suggested a potential role for increased female hormones [estradiol (E2) and progesterone (PRG)] to result in CYP changes during pregnancy, detailed studies using hepatic tissues of humans or animals for the effects of E2 and PRG on the major drug metabolizing CYPs have been lacking. The goal of this research (Chapter 2 and 3) is to determine the effects of high concentrations (similar to those attainable during pregnancy) of E2 and PRG on hepatic CYPs using *in vitro* (human hepatocytes; chapter 2) and *in vivo* (female rats; chapter 3) model systems.

In chapter 2, freshly isolated human hepatocytes were treated with estrogen, PRG, or known CYP inducers for 72 hours and the mRNA expression and activity levels of CYP were determined. The effects of female hormones on the CYP expression were further characterized regarding the concentration dependent effects and combinatory effects. In addition, we also identified the role of nuclear receptors [i.e., constitutive androstane receptor (CAR), pregnane X receptor (PXR), and estrogen receptor (ER)] for hormone-mediated CYP regulation using a luciferase promoter activity assay.

The results show that E2 enhances CYP2A6, CYP2B6, and CYP3A4 expression whereas PRG enhances CYP2A6, CYP2B6, CYP2C8, CYP3A4, and CYP3A5 expression. The induction was mainly observed when the average hormone concentrations were at the levels reached during pregnancy, suggesting that these effects are likely relevant to those

SUMMARY (continued)

observed during pregnancy. E2 also increased the enzyme activities of CYP2C9 and CYP2E1 without affecting the mRNA expression levels. A combination of estrogens and PRG exhibited non-additive effects on CYP2A6 and CYP2B6 whereas it showed an additive (if not synergistic) effect on CYP3A4 expression. The promoter activity assay showed that E2 is a CAR activator, and PRG is a PXR activator. In addition, the CYP2B6 promoter was activated via ER.

When these results were compared with the clinical observations, increased expression and/or activities of CYP2A6, CYP2C9 and CYP3A4/5 during pregnancy can be, at least in part, explained by increased female hormone levels during pregnancy. E2 and PRG also increased the mRNA expression or activities of CYP2B6, CYP2C8 and CYP2E1, and the clinical significance of these findings remains to be determined. On the other hand, E2 and PRG did not alter the expression and/or activities of CYP2D6, CYP2C19 and CYP1A2, indicating that E2 and PRG are likely not directly responsible for alteration of activities of these CYP isoforms during pregnancy.

In Chapter 3, we characterized effects of E2 on the expression and activities of cytochrome P450 enzymes (CYPs) in an *in vivo* system using rats. To this end, female Sprague-Dawley rats were treated with E2 benzoate (EB) or known CYP inducers. Liver tissues were obtained after 5 days of treatment, and mRNA and protein expression levels, as well as activities, of the major hepatic CYPs were determined by qRT-PCR, immunoblot, and microsomal activity assays. In addition, the effects of E2 on the mRNA levels of nuclear receptors and P450 oxidoreductase (POR) were examined to explore the potential mechanisms underlying the regulation of CYP expression and activity by E2.

SUMMARY (continued)

The results showed that E2 slightly increased CYP1A2 expression and activity. E2 also enhanced CYP2C expression (CYP2C6, CYP2C7, and CYP2C12) to levels comparable to those observed by phenobarbital. E2 upregulated CYP3A9 expression, while expression of CYP3A1 was downregulated. Expression of PXR, CAR, and POR was downregulated suggesting their potential involvement in the regulation of CYP expression and activity by E2. Taken together, in female rats E2 regulates the expression of hepatic CYPs in a CYP isoform-specific manner, but the directional changes are mostly different from those clinically observed during human pregnancy.

In chapter 4, we evaluated Huh7 cells and dimethyl sulfoxide (DMSO)-treated Huh 7 cells as an *in vitro* model system for drug metabolism studies. Although human hepatocytes are considered the gold-standard system for drug metabolism studies, as well as other hepatology research, their variability and availability limit widespread use in research. Recently, it was shown that Huh7 cells, when incubated in the presence of DMSO, obtained a more differentiated hepatocyte state. The aim of the study in this chapter was to characterize the baseline functions of drug metabolizing enzymes in DMSO-treated Huh7 cells and control Huh7 cells. To this end, the basal level of mRNA expression of the major drug metabolizing enzymes (DMEs) and their metabolic activities in Huh7 cells were examined. In addition, the inducibility of the major transcriptional regulator target genes (CYP1A1, CYP2B6, and CYP3A4) was examined by RT-PCR and enzyme catalytic activity studies.

SUMMARY (continued)

The results in Chapter 4 showed that the expression levels of DMEs in control Huh7 cells were very low, but DMSO treatment dramatically induced the mRNA expression levels of most DMEs and liver specific proteins. Enzyme catalytic assays also confirmed the increases in the DME activity. In addition, 3-methylcolanthrane induced CYP1A1 expression in Huh7 cells, suggesting the potential use of Huh7 cells for CYP1A1 regulation studies. However, when compared to human hepatocytes, the overall expression and inducibility of DMEs were low, limiting the utility of DMSO-treated Huh7 cells as a substitution for human hepatocytes. Still, the enhanced expression of DMEs and key transcription factors in the DMSO-treated Huh7 cell culture system potentially makes them an improved *in vitro* hepatocyte model.

1. Introduction

1.1 Drug metabolism and cytochrome P450

1.1.1 Definition of drug metabolism.

Drug metabolism is the process by which drugs are chemically modified by specialized enzyme systems. As a result of metabolism, most drugs become more hydrophilic, lose their pharmacological activities, and are readily excreted from the body. Metabolism occurs in all tissues, but it is especially active in the liver. Hepatic metabolism is mediated by three distinct phases (1, 2) (TABLE I). Phase I metabolism is introduction of polar functional groups such as hydroxylation, dealkylation, hydrolysis, and reduction. The most important enzyme family in phase I metabolism is cytochrome P450 (CYP). Phase II metabolism is conjugation reaction to the parent compound or its phase I metabolites. Glucuronidation, sulfation, acetylation, and glutathione conjugation are examples of Phase II reactions. Phase III is the transport of drugs or their metabolites by drug transport enzymes such as P-glycoprotein.

Metabolism is one of the key factors that determine the fate of an administered drug. Also, it is often associated with toxicity and efficacy of a drug. Unsatisfactory metabolic properties of a drug (e.g, formation of toxic metabolites) have been identified as the major reason for withdrawal of a drug candidate from drug development (2).

TABLE I

LIST OF ENZYMES RESPONSIBLE FOR THREE PHASES OF DRUG METABOLISM

	Phase I	Phase II	Phase III
Function	Oxido-reduction	Conjugation	Transport
List of enzymes	Cytochrome P450 Dehydrogenases (alcohol, aldehyde) Monoamine oxidase (MAO) Flavin-Containing monooxygenase (FMO) Xanthine oxidase Deoxygenase Ketoreductase Esterase Epoxide hydrolase	UDP- Glucuronosyltransferase (UGTs) Sulfotransferase (SULT) Methyltransferase N-acetyltransferase (NAT) Glutathione S-transferase (GST)	Multidrug resistant protein (MDR) Multidrug resistance related protein (MRP) Sodium taurocholate cotransporting polypeptide (NTCP) Organic anion transporting polypeptide (OATP) Organic cation transporter (OCT) Bile salt export pump (BSEP) Breast cancer related protein (BCRP)

Modified from (1).

1.2 Cytochrome P450 as the major drug metabolizing enzymes.

Cytochrome p450 constitutes a superfamily of hemoproteins. The key function of CYP is oxidation of endogenous and xenobiotic molecules. This reaction requires molecular oxygen and two electrons. In humans, electrons are donated by NADPH, and must be channeled by electron transfer proteins such as P450 oxidoreductase (POR) or b5 (3). The CYP superfamily is further classified into families (numbers) and subfamilies (alphabets) based on the homology. Members of each family have at least 40% homology and members of each subfamily have at least 55% homology to one another (4). Currently, 58 human CYP genes have been identified, and categorized into 18 families and 43 subfamilies. Among those, families 1, 2, and 3 (CYP1, CYP2, CYP3) are all located in the endoplasmic reticulum and play important roles for metabolism of xenobiotics, especially drugs used for therapeutic purposes.

Many drugs are subject to CYP-mediated metabolism. About seventy five percent of marketed drugs are subject to CYP-mediated metabolism, and metabolism of a drug is often mediated by specific isoforms (2). It has been estimated that CYP3A4 and CYP3A5 are responsible for at least for 40 % of CYP-mediated hepatic drug metabolism, followed by CYP2D6 and CYP2C9. Fig. 1 shows the relative contribution of CYP isoforms in hepatic drug metabolism and examples of drug substrates of each CYP isoforms.

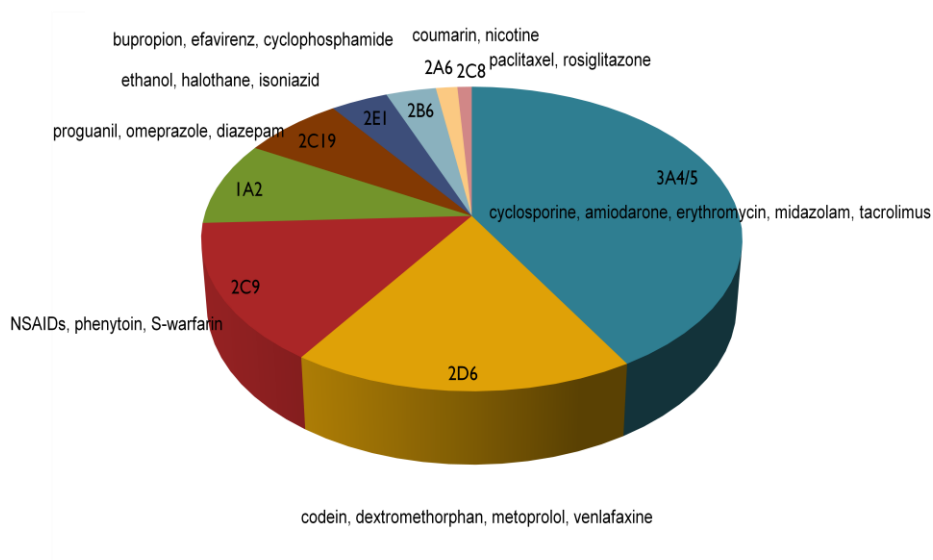


Fig1. Relative contribution of hepatic CYP to drug metabolism and examples of substrates of each CYP isoform.

Modified from (2, 4)

1.1.3 Regulation of CYP expression.

The expression and catalytic activities of CYPs show considerable inter-individual variability and are often altered due to genetic, environmental, and physiological factors. Examples of such factors are exposure to xenobiotics (e.g., drugs, food, or environmental pollutants), pathological conditions (e.g., inflammation, diabetes, or starvation), and physiological changes (e.g., aging or pregnancy) (5-7). Alteration in CYP expression and catalytic activity may have a substantial impact on drug metabolism.

1.1.3.1 Transcriptional regulation.

Various CYP regulators and their mechanism of regulation have been identified over the past decades. Among those, transcriptional regulation of CYP, especially CYP induction, is the most widely studied regulatory mechanism. The two major mechanisms of induction are aromatic hydrocarbon receptor (AhR) pathway-mediated and nuclear receptor-mediated induction (5).

1.1.3.1.1 AhR.

AhR, a member of the basic helix-loop-helix/ Per-Arnt-Sim (bHLH/PAS) family of transcriptional factors, is involved in the transcriptional regulation of many genes including the CYP1 family (5). Upon ligand binding in the cytosol, AhR forms a heterodimer with an AhR nuclear translocator (ARNT), and the dimer binds to response elements (dioxin responsive element; DRE) on the upstream of the CYP1A gene and activates transcription. Of note, a large number of polycyclic aromatic hydrocarbons, such as those found in cigarette smoke or environmental pollutants, are AhR activators and thus induce CYP1 expression (8).

1.1.3.1.2 Nuclear receptors.

The pregnane X receptor (PXR) and constitutive androstane receptor (CAR), members of a group of orphan nuclear receptors, are the key transcriptional regulators of many CYP isoforms. Since they are both nuclear receptors, PXR and CAR share certain structural features (highly conserved DNA-binding domain with two zinc finger motifs, dimerization motif, and trans-activation domains) with other classical steroid hormone receptors such as the estrogen receptor (ER) or the glucocorticoid receptor (GR) (9). However, CAR and PXR have distinctive features compared to classical steroid receptors; a high-affinity endogenous ligand has not been identified for PXR or CAR, thus they are called

as orphan receptors. Both respond to a variety of xenobiotic ligands, including steroids, antibiotics, antifungals, and herbal extracts which have no common structural features (10). For these reasons, PXR and CAR have been identified as “xenobiotic sensors”.

PXR

PXR was identified by using an expressed sequence tag to screen a mouse liver library (11). Discovery of mouse PXR (mPXR) explained the concurrent induction of CYP3A families by high doses of dexamethasone (a glucocorticoid) and pregnenolone-16 α -carbonitrile (PCN, an anti-glucocorticoid) which could not be explained by the glucocorticoid signaling pathway. At approximately the same time, the human steroid X receptor (SXR) was cloned, and later it was identified as the human homologue of mPXR (12).

Soon after, a series of molecular studies revealed the mechanism of PXR-mediated CYP induction. Treatment of an activator (e.g, rifampin) leads to the translocation of PXR from the cytosol to the nucleus. In the nucleus, PXR dimerizes with the retinoid X receptor (RXR), and binds to responsive elements such as the xenobiotic responsive enhancer module (XREM). This recruits other transcriptional factors such as the steroid receptor coactivator-1 (SRC-1) and initiates transcription of the target genes, such as CYP3A4. PXR was later identified as the major regulator of many drug metabolizing enzymes for phase I (CYP 2A, -2B, -2C, and -3A family), phase II (UGT1A and SULT1A), and phase III (MDR1 and OATP2) metabolism (5).

CAR

The role of CAR as a CYP regulator was discovered from studies on the phenobarbital-mediated induction of CYP2B. The effect of phenobarbital on increased

hepatic drug metabolism in rodents was well-known for several decades, but researchers were not able to find out any barbiturate receptors in the liver. In the late 1990's, identification of a phenobarbital-responsive enhancer module (PBREM) in the upstream of the mouse *cyp2b10* gene and its interaction with the newly discovered nuclear receptor CAR led to the elucidation of the mechanism of phenobarbital mediated CYP induction (13, 14). Similarly with PXR, CAR is translocated into the nucleus, dimerizes with RXR. The RXR-CAR dimer binds to the responsive elements such as PBREM, leading to induction of CYP2B in rodents and humans. Later, other CYPs (2A, 2C, 3A families), UGT1A1, and SULT1A1 were identified as CAR-responsive genes (5).

It is of note that CAR activity is modulated by two types of activators, direct and indirect activators. A direct activator involves binding between a ligand and the receptor (ligand binding domain). 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3] thiazole-5-carbaldehyde *O*-[3,4-dichlorobenzyl]oxime (CITCO) for human CAR (hCAR) and 4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) for mouse CAR (mCAR) are examples of direct activators. Indirect activators (e.g, phenobarbital, phenytoin) do not involve ligand binding for CAR activation. The mechanism of indirect CAR activation is thought to involve dephosphorylation of CAR by protein phosphatase 2A (15).

Redundancy between CAR and PXR

CAR and PXR share many ligands, regulatory elements and target genes. Many of the PXR activators are also CAR activators (phenobarbital, phenytoin, and 5 β -pregnane-3,20-dione), but some can play an opposite role. For example, clotrimazole is a PXR activator, but a repressor of CAR (16). Studies on the DNA-binding properties of CAR and PXR showed that they can trans-activate their regulatory elements. CAR can bind to everted

repeat (ER6) or direct repeat (DR3) sequences in XREM on the CYP3A4 upstream. These binding sites were identified as the key sequences for PXR-mediated CYP induction. As a result, activation of CAR can induce CYPs previously identified as PXR target genes such as CYP3A4 (17). Likewise, PXR can bind to CAR binding site in PBREM, and activates CYP genes previously known to be regulated by CAR (18).

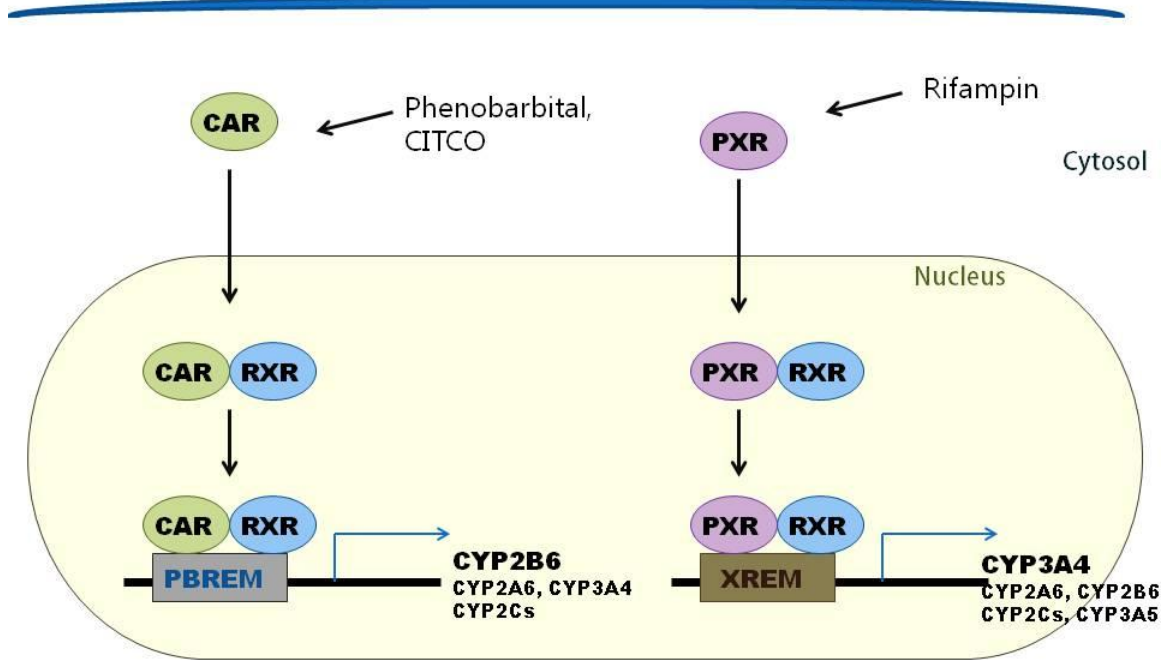


Fig 2. Signaling pathways for CAR and PXR.

Modified from (9)

Other nuclear receptors

Although PXR and CAR are the most studied nuclear receptors for xenobiotic-mediated CYP inductions, some studies have reported that other classical hormone nuclear receptors can activate some of the CYP isoforms. For example, the glucocorticoid receptor (GR) is also known to modulate CYP expression directly and indirectly. Some CYP isoforms (CYP2A6, CYP2C8, CYP2C19, and CYP3A5) are induced via GR and glucocorticoid receptor responsive element (GRE) interaction on the upstream of target genes. The GR also indirectly regulates some inducible CYP genes (e.g CYP2B6 and CYP3A4) by modulating the expression of PXR, CAR and RXR (19). The Vitamin D receptor (VDR) can modulate the expression of CYP3A4 (CYP2B6 and CYP2C9 to a lesser extent) (20). The estrogen receptor alpha (ER α) regulates CYP2A6 expression via interacting with estrogen receptor responsive element (ERE) on CYP2A6 upstream (21).

1.1.3.1.3 Other transcriptional regulators of CYP expression.

While nuclear receptor mediated transcriptional induction has been the most extensively studied regulatory mechanism for CYP, other mechanisms of transcriptional regulation for CYP are also investigated. The expression of CYP can be regulated via membrane signaling pathways; acute inflammation, treatment with epidermal growth factor (EGF) or hepatocyte growth factor (HGF) lead to down-regulation of many CYP isoforms in rat and human hepatocytes, suggesting the global roles of EGF/Ras/MAPK (mitogen-activated protein kinase) signaling pathways in CYP regulation (22). Insulin and glucagon (an endogenous insulin antagonist) regulate the expression of CYP2E1 in an opposing manner, indicating the role of receptor tyrosin kinase and its downstream signaling pathway in CYP regulation (23).

TABLE II**TRANSCRIPTION FACTORS INVOLVED IN REGULATION OF CYP EXPRESSION**

	<i>Target gene</i>	<i>Activator</i>	<i>Responsive elements</i>
PXR	CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4, CYP3A5	Rifampin, hyperforin, phenytoin, RU-486, sulfapyrazole, carbamazepine, clotrimazole dexamethason (> 10 µM)	XREM
CAR	CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4, CYP3A5	Phenobarbital, phenytoin, CITCO	PBREM
AhR	CYP1A1, CYP1A2	Polyaromatic hydrocarbons (TCDD, 3-methylcolanthrene, 3,4-benzo(a)pyrene, benzoflavons), halogenated aromatic hydrocarbons (dibenzofurans, dibenzo-p-dioxins)	DRE
VDR	CYP2B6, CYP2C9, CYP3A4	25-Hydroxyvitamine D3	VRE
GR	CYP2A6, CYP2C8, CYP2C19, CYP3A5	Dexamethasone	GRE
ER	CYP2A6	Estradiol	ERE

Adapted from (5, 24, 25)

1.1.3.2 Post-transcriptional regulation of CYP.

Some studies have shown that some CYP proteins are subject to post-translational modification such as phosphorylation and glycosylation. Most studies isolated the modified proteins and identified the site of secondary modification, but its function or significance in drug metabolism is largely unknown (7, 26).

It is of note that CYP enzymatic activities can be altered without any changes in the

level of protein expression or post-translational modification. Catalytic activities can be altered by ligands via reversible (e.g., competitive inhibition) or irreversible (e.g., mechanism-based inhibition) mechanisms. Also, changes in the expression of CYP redox-partners, such as POR and b5, can result in altered activities for some of CYP isoforms.

1.1.4 Model systems to study CYP regulation.

1.1.4.1 Primary human hepatocytes as the gold standard of drug metabolism studies.

Human hepatocytes are the closest model to human liver, and are considered as the gold-standard tool for *in vitro* drug metabolism studies, including *in vitro- in vivo* correlation, drug-drug interaction, and drug toxicity studies (27). After proper isolation and handling, hepatocytes remain differentiated and retain metabolic activities up to one week (28). Hepatocytes retain not only the expression and activities of phase I, II, and III enzymes, but also the expression and activities for transcriptional regulators of drug metabolizing enzymes such as PXR or CAR. This particularly makes human hepatocytes as a favorable model system for studies of CYP regulation.

In hepatocytes, the CYP system can be characterized in terms of expression (mRNA and protein expression), and activity using specific probe substrates (TABLE III). The duration of the drug metabolism studies with a set of human hepatocytes can be anywhere from a couple of hours to as long as a week. When the study involves induction via nuclear receptors, the maximum induction (plateau) observed is usually obtained within 72 hours (29).

TABLE III

PROBE SUBSTRATES AND PROTOTYPICAL INDUCERS USED IN HEPATIC DRUG METABOLISM STUDY IN HUMAN HEPATOCYTES

	Probe substrates	Inducers
CYP1A2	Caffeine 3-N-demethylation Phenacetin O-deethylation 7-ethoxyresorufin O-deethylation	3-methylcolanthrene (0.5-2 μ M) β -naphthoflavone (10-50 μ M)
CYP2A6	Coumarin 7-Hydroxylation Nicotine C-oxidation	Rifampin (10-50 μ M)
CYP2B6	S-mephenytoin N-demethylation Bupropion hydroxylation	CITCO (100 nM–1 μ M) Rifampin (10-50 μ M)
CYP2C8	Taxol 6-hydroxylation	Rifampin (10-25 μ M)
CYP2C9	Diclofenac 4'-hydroxylation Tolbutamide methyl-hydroxylation S-warfarin 4'-hydroxylation	Phenobarbital (0.25–2 mM) Rifampin (10-50 μ M)
CYP2C19	S-mephenytoin 4'-hydroxylation	Rifampin (10-50 μ M)
CYP2D6	Dextromethorphan O-demethylation Bufuralol 1'-hydroxylation	N/A
CYP2E1	<i>p</i> -nitrophenol 3-hydroxylation Hydroxychlorzoxazone 6-hydroxylation	Ethanol (100 mM)
CYP3A4	Midazolam 1-hydroxylation Testosterone 6 β -hydroxylation Nifedipine oxidation	Rifampin (10-50 μ M)

Modified from (27, 30)

There are several limitations of human hepatocytes as a model system for drug metabolism studies. The biggest concern is the scarcity and variability (27); the major sources of primary human hepatocytes are livers discarded for transplantation or waste tissues from partial hepatectomy. Therefore, supply is limited and often unpredictable. Studies using hepatocytes also show significant variability in the metabolic activity as well as the inducibility of CYP isoforms. Variability can be due to the characteristics of donor (e.g., genetic makeup, previous exposure of drugs, or pathological conditions such as hepatic steatosis) or technical aspects (e.g., duration of cold ischemic time, collagen digestion, and use of matrix overlay). Currently, the Food and Drug Administration (FDA) drug-drug interaction industry guideline suggests that at least three sets of hepatocytes from different donors should be used to evaluate drug-drug interactions during new drug development (31).

1.1.4.2 Rats as an *in vivo* model to study drug metabolism.

Animal models are commonly used in the early phase of drug development to test safety, efficacy, and pharmacokinetic profiles. Among model animals, rats have been extensively used as an *in vivo* animal model for drug metabolism studies. Accordingly, its CYP isoform composition, expression, catalytic activities and species differences are well established.

Between rats and humans, the structures and catalytic activities of CYP1A2 and CYP2E1 are well conserved, while remaining CYP isoforms (i.e., CYP2B, CYP2C, CYP2D and CYP3A) show considerable differences in substrate specificity due to the distinct structures of their catalytic site. However, the mechanisms underlying transcriptional regulation of CYP expression appear well conserved between rats and humans regardless of the CYP isoform. For example, in both humans and rats, expression of CYP1A, CYP2B/2C,

and CYP3A is strongly enhanced upon activation of transcription regulators (AhR, CAR, and PXR, respectively), while CYP expression is downregulated by inflammatory mediators. This suggests that rats may serve as a good animal model for studying regulation of CYP expression.

1.2 Altered drug metabolism during pregnancy.

1.2.1 Use of medication during pregnancy.

Medication use during pregnancy is prevalent in the US. Retrospective studies showed that more than sixty percents of pregnant women take at least one prescription medication (excluding vitamin/mineral supplements) (32, 33). The most commonly used classes of drugs are anti-infectives (amoxicillin, nitrofurantoin, cephalexin, and metronidazole), analgesics (acetaminophen, oxycodone, hydrocodone, and ibuprofen), cardiovascular (labetalol, methyldopa, and hydralazine), anti-diabetic (insulin, glyburide, and metformin), anti-depressant (fluoxetine, sertraline, amitriptyline and prochlorperazine), gastrointestinal (omeprazole and metoclopramide), and respiratory drugs (albuterol, hydroxyzine, loratadine, and promethazine). The duration of therapy varies, but some chronic medical conditions such as diabetes or epilepsy require continuous therapy throughout the term.

The choice of a drug and use of appropriate dose is important for the maternal and fetal health as poorly controlled medical conditions such as diabetes, hypertension or epilepsy are associated with exacerbation of maternal health, premature delivery or perinatal death (34). However, the doses used are based on pharmacokinetic studies in the non-pregnant population and they may not be appropriate for the pregnant women. In fact, clinical studies have shown that pregnancy alters clearances of many drugs, but a

comprehensive understanding of altered pharmacokinetics during pregnancy has been lacking.

1.2.2 Pharmacokinetic changes during pregnancy.

Pregnancy is accompanied with extensive physiological changes which can alter pharmacokinetics; gastric motility slows due to an increased concentration of progesterone, and this can delay absorption of some of orally administered drugs. An increased plasma volume during pregnancy is related to decreased plasma albumin concentration, which can alter the clearance of drugs with a high protein binding affinity. An increased cardiac output and subsequent increases in hepatic blood flow can affect the disposition of drugs with a high extraction ratio. An increase in renal blood flow and glomerular filtration rate (GFR) increases the clearance of renally eliminated drugs during pregnancy (6, 35, 36).

Results from clinical studies have suggested that pregnancy influences hepatic drug metabolism in a CYP isoform-specific manner. The clearance of drugs metabolized by CYP2A6, 2C9, 2D6, and CYP3A4 is increased during pregnancy, whereas the clearance of CYP1A2 and CYP2C19 substrates is decreased (6, 36) (TABLE IV). The causative factors responsible for altered hepatic metabolism during pregnancy remain unknown.

CYP1A2

The clearances of CYP1A2 substrates, caffeine and theophylline, are decreased during pregnancy. Several studies reported that the clearance of caffeine or its urinary metabolic ratio (caffeine metabolites to caffeine) is decreased by 50-70% by the third trimester (37-39). Two studies have indicated that activity of CYP1A2 is progressively decreased throughout the pregnancy [35, 50, and 52% during each trimester (37); 30, 50, and 70% during each

trimester (38)]. The hepatic clearance of theophylline, another CYP1A2 substrate, is also decreased by 20-50% in the third trimester and one study showed that some patients required reduced doses due to symptoms of toxicity (insomnia, headache) (40, 41).

CYP2A6

The increased activity of CYP2A6 during pregnancy was reflected in the increased clearance of nicotine and cotinine. The clearance of nicotine and cotinine after nicotine infusion was 60% and 140% higher, respectively, during pregnancy as compared to their post-partum controls (42). Another study indicated that the concentration of cotinine per cigarette was decreased by 55% in pregnant smokers (43). In a study of pregnant smokers using nicotine patch therapy, the effect of pregnancy on nicotine metabolism was inconclusive due to confounding factors and a small sample size (44).

CYP2C9

The increased activities of CYP2C9 during pregnancy were observed with phenytoin and glyburide. The total clearance of phenytoin was decreased over 50- 60% in the third trimester (45, 46). Accordingly, some authors recommended close monitoring of the free fraction of phenytoin during pregnancy, especially in the third trimester. The oral clearance of glyburide was also increased more than 2-fold in pregnant subjects (47).

CYP2C19

Pregnancy is associated with decreases in CYP2C19 activity. Proguanil, an anti-malaria drug used in pregnancy, is a pro-drug and its active metabolite (cycloguanil) is formed by CYP2C19. One study showed a 68% decrease in the plasma concentration of cycloguanil in the third trimester compared to post-partum controls, and the authors

suggested to increase the dose by 50% during pregnancy (48, 49). Another study showed the mean ratio of area under the curve (AUC) of proguanil to cycloguanil was increased by 150% in the third trimester compared to the post-partum control (50). The formation of active metabolite of nelfinavir, hydroxyl-tert-butylamidenelfinavir (M8), is also mainly dependent on CYP2C19 activity. Studies have shown that AUC of M8 is also decreased during pregnancy by 70%, suggesting decreased activity of CYP2C19 (51, 52).

CYP2D6

The induction of CYP2D6 during pregnancy has been extensively studied with various therapeutic agents. The clearance of metoprolol was shown to increase by 4-fold in the third trimester compared to the postpartum controls (53, 54). Urinary metabolic ratio of dextromethorphan to dextrorphan, another marker of CYP2D6 activity, was decreased (indicating the increased activity of CYP2D6) by 50% (55). Another study using dextromethorphan showed that the activity of CYP2D6 increases gradually throughout the term, with an approximate 25%, 40%, and 55% increase in each trimester, respectively (38). The increased activity of CYP2D6 during pregnancy was also indicated by several studies using anti-depressants metabolized by CYP2D6 such as fluoxetine (140% increase in the norfluoxetine to fluoxetine ratio), citalopram (54% increased ratio of didesmethylcitalopram to desmethylcitalopram), and nortriptyline (100% increase in nortriptyline clearance) (56, 57).

CYP3A4

CYP3A4 is responsible for the metabolism of at least 40% of marketed drugs. Determination of the effect of pregnancy on CYP3A4 activity is particularly difficult due to many confounding factors; many of the CYP3A4 substrates are subject to p-glycoprotein mediated elimination. Some causes auto-induction of CYP3A4 (e.g., carbamazepine), and

others are the inducer, inhibitor, and substrate of CYP3A4 at the same time (e.g., efavirenz, and ritonavir). Despite these factors, most studies indicate that pregnancy is associated with increased CYP3A4 activity.

The clearance of nifedipine and midazolam is increased by 30% and 180% respectively in the third trimester compared to their post-partum controls (58, 59). The increased metabolism of methadone was observed in two different studies that showed increased clearance by 90% and the urinary ratio of methadone metabolites to methadone by 140%; the authors concluded that increased metabolism of methadone may explain unexpected maternal methadone withdrawal associated with the late stage of pregnancy (60, 61). Several studies using anti-retrovirals show similar results; clearance of saquinavir, indinavir, and lopinavir was increased by 27%, 175%, and 149%, respectively during pregnancy (62-64).

TABLE IV

ALTERED DRUG METABOLISM DURING PREGNANCY

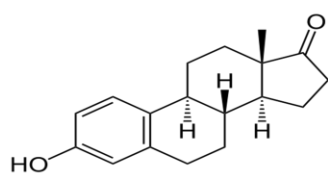
	Changes during pregnancy	Clinical evidence	Substrate drugs can be used during pregnancy
CYP1A2	Decreased	↓ CL: caffeine and theophylline ↓ MF: caffeine	Caffeine, theophylline, clozapine, acetaminophen, olanzapine, ondansetron, cyclobenzaprine
CYP2A6	Increased	↑ CL: nicotine and cotinine	Nicotine, cotinine
CYP2B6	Not studied		Bupropion
CYP2C8	Not studied		Amiodarone, rosiglitazone, verapamil
CYP2C9	Increased	↑ CL: phenytoin and glyburide	Glyburide, phenytoin, diclofenac, ibuprofen, sulfamethoxazole,
CYP2C19	Decreased	↓ MF: Proguanil, nelfinavir	Proguanil, omeprazole, pantoprazole, lansoprazole
CYP2D6	Increased	↑ CL: metoprolol, nortriptyline ↑ MF: dextromethorphan, fluoxetine, citalopram	Metoprolol, dextromethorphan, fluoxetine, paroxetine, duloxetine, citalopram, codeine, ondansetron, venlafaxine
CYP2E1	Not studied		Acetaminophen, chlorzoxazone
CYP3A4	Increased	↑ CL: midazolam, nifedipine, methadone, indinavir, saquinavir, lopinavir ↑ MF: methadone	Midazolam, nifedipine, diltiazem, methadone, anti-retrovirals (indinavir, saquinavir, ritonavir) amlodipine, erythromycin

CL; clearance, MF; metabolite formation, measured by AUC ratio or urinary ratio of parent drug to metabolite)

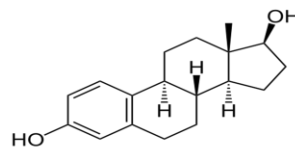
Modified from (6, 36).

1.2.3 Potential roles of female hormones in altered drug metabolism during pregnancy.

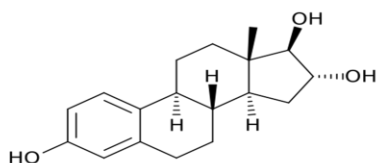
One of the notable changes during pregnancy is a gradual and significant increase in female hormones (65). Estradiol (E2), the major and most potent (for estrogen receptor activation) endogenous estrogen, reaches up to 100 nM during the third trimester of pregnancy. This is up to a 100-fold increase compared to the baseline maintained during a regular menstrual cycle (up to 1 nM during the menstrual cycle) in adult females. Estrone (E1) and estriol (E3), the other two major endogenous estrogens, also increase in a similar way, up to 50 nM at term compared to their non-pregnant controls (Fig. 3). Progesterone (PRG) also gradually increases throughout the gestational period, up to 100-fold during pregnancy (reaching up to 1 μ M in the third trimester). The role of elevated female hormones on hepatic drug metabolism during pregnancy is largely unknown.



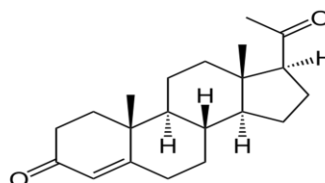
Estrone



Estradiol



Estriol



Progesterone

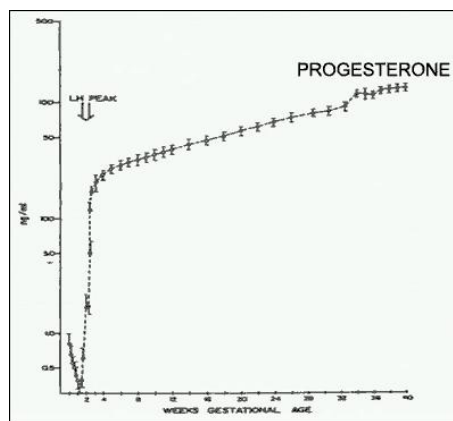
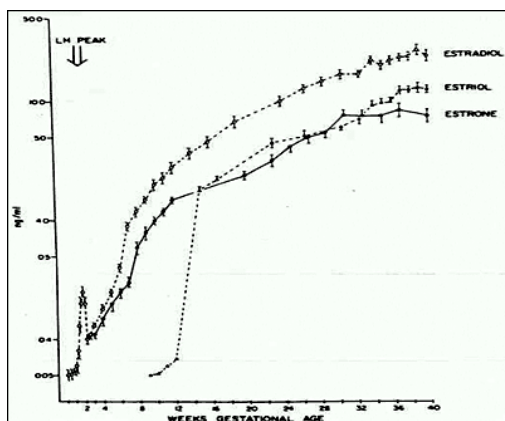


Fig 3. Female hormones: chemical structures and plasma concentration during pregnancy.

Modified from (65).

1.2.4 Clinical evidence for roles of female hormones in altered drug metabolism during pregnancy.

Several lines of clinical evidence suggest that increased female hormone during pregnancy can be responsible, at least in part, for altered hepatic drug metabolism during pregnancy. Pharmacokinetic studies in oral contraceptive users showed that use of oral contraceptives is associated with altered activities of CYP1A2, 2A6, and CYP2C9 in a similar manner with pregnancy; the clearance of CYP1A2 substrates (caffeine, tizanidine, olanzapine) and CYP2C19 substrates (omeprazole, mephenytoin, proguanil) are decreased while that of CYP2A6 substrates (nicotine and cotinine) is increased (66-70). In particular, the increased activity of CYP2A6 and decreased activity of CYP2C19 are associated with the estrogen component (ethinylestradiol) of oral contraceptives, suggesting the roles of estrogen in the regulation of CYP2A6 and CYP2C19 (68, 70). For CYP2D6, use of oral contraceptives does not appear to alter the activity. Interestingly, the hormone replacement therapy in post-menopausal women (0.625 mg conjugated equine estrogen daily) increases CYP2D6 activity, suggesting the possibility of estrogen-mediated CYP2D6 regulation (71).

Clinical studies of sex differences of CYP activities also support the idea of female-hormone mediated CYP-regulation during pregnancy. The plasma concentrations of female hormones are similar between men and women before puberty and after menopause, but show significant differences (up to 10-fold higher in female) depending on the menstrual cycle between adult men and women. Studies in sex differences have shown similar directional changes with those in pregnancy. The activity of CYP1A2 is consistently decreased in women compared to men in several studies using caffeine, clozapine, and olanzapine (72). The activity of CYP2A6 (nicotine and cotinine) is higher in pre-menopausal females than males, but there was no difference between post-menopausal women and men

(70). The activity of CYP2D6 showed no sex differences in some studies (73-75) while other studies showed a small but elevated CYP2D6 activity in females (76, 77). The higher activity of CYP3A4 in females, ranging from a 20 to 50% increase compared to males, was shown with a variety of substrates (72). The higher content of CYP3A4 (about 2-fold) in liver tissue donated from females (compared to males) also supports higher CYP3A4-mediated metabolism in females (78, 79).

In sum, clinical studies in oral contraceptive, hormone replacement therapy, and sex differences show similar changes in CYP-mediated drug metabolism to those shown in pregnant women. This suggests that female hormones may play a role in the CYP regulation in an isoform-specific manner.

1.2.5 *In vitro* evidence for roles of female hormones in altered drug metabolism during pregnancy.

Along with clinical studies, results from several *in vitro* studies also suggest the potential roles of female hormones on CYP regulation. Up to now, most *in vitro* studies examined the role of female hormones on nuclear receptor activation in immortalized cell lines; the effects of female hormones on the expression and activities of the major hepatic CYPs using hepatocytes have been lacking.

Several *in vitro* studies using promoter activity assays suggest potential roles of E2 and PRG via orphan nuclear receptor mediated CYP regulation. E2 is shown to be an activator of PXR at a superphysiological concentration (10 μ M) (80, 81). The effect of E2 on PXR at a physiologically relevant concentration is currently unknown. E2 also activates mouse CAR (mCAR). E2 increases the promoter (PBREM) activity in HepG2 cells and

expression of mouse *cyp2b10* (ortholog of human CYP2B6) in mouse hepatocytes (82). This suggests the possibility of E2-mediated human (hCAR) activation, but the effect of E2 on hCAR has not been evaluated yet.

On the other hand, PRG is shown to be a PXR activator; moderate activation of PXR (20-60% of rifampin-mediated PXR activation) by PRG was observed in several studies at concentration of 1-10 μ M (11, 81, 83, 84). Contrary to E2, PRG is a mCAR suppressor, but the effect of PXR on human CAR is currently unknown.

In addition, emerging evidence suggests the ER may play important roles in CYP regulation. Up to now, estrogen-mediated induction via the ER was observed in a non-hepatic CYP (CYP1B1) or UGT (UGT1A4, UGT2B17) isoforms (85-87). Recently, the activation of the CYP2A6 promoter via ER-ERE binding was reported, suggesting the possibility of an ER involvement in the regulation of other hepatic CYP isoforms (21).

Taken together, various *in vitro* studies also suggested that potential roles of E2 and PRG on CYP regulation, mainly via nuclear receptors. However, the effects of the female hormones on the mRNA expression and activities of major CYPs have not been extensively investigated.

1.2.6 Animal studies in altered drug metabolism by female hormones during pregnancy

1.2.6.1 Animal models to study altered drug metabolism during pregnancy.

Information on pregnancy-associated CYP alteration is mostly available in rat models. Studies using rats have indicated that pregnancy is associated with a global decrease in CYP

expression and activities per gram liver tissue, whereas human pregnancy alters CYP activities in an isoform-specific manner (88-90). It is unclear what pregnancy-specific factor regulates such global down-regulation in rats. However, it is unlikely that female hormone mediated effects, since the increase of female hormones during rat pregnancy is less than 5-fold compared to non-pregnant rats (89, 91). Instead, it is more likely due to the rat-specific physiological changes such as enlarged livers (50-60% increase compared to the non-pregnant controls) and decreased protein concentration per gram tissue (89, 90). In human pregnancy, the liver size is not dramatically altered (92). Overall, pregnant rats may not be an appropriate model system to investigate the effects of increased female hormones on CYPs during pregnancy.

1.2.6.2 Animal models to study altered drug metabolism by female hormones.

A number of researchers have examined the effects of E2 on hepatic CYP expression in rats. The primary interests in these studies were the effects of E2 on the sexual dimorphism in CYP expression in rats. Rats show prominent a sexual dimorphism in some CYP isoforms. CYP2A2, CYP2C11, and CYP3A2 are expressed only in male rats, while CYP2C7, CYP2C12, and CYP3A9 are female-specific CYPs (3). Studies have shown that the expression of female-specific isoforms are decreased upon ovariectomy and restored by E2 injection (93-95).

While these previous studies provide some information on the effects of E2 on rat hepatic CYPs, the limitations of these studies renders it difficult to conclude the isoform-specific effects of female hormones on CYPs. The effects of female hormones were examined only for the sex specific isoforms and not for the isoforms expressed in both sexes, such as CYP1A2, CYP2B1, CYP2C6, CYP2D2, CYP2E1 and CYP3A1. For most studies,

there was no rationale or pharmacokinetic studies to support the E2 doses used. Therefore, it is unclear whether the effects of female hormones in these studies reflect the pregnancy-related hormonal effects in humans. In addition, these studies often involved surgical manipulation (ovariectomy or hypophysectomy) which can be an additional confounding factor. Some studies were done with less specific molecular probes including RT-PCR primers, antibodies for immunoblot analysis, and less-specific probe substrates for activity measurement. These limitations necessitate a better-designed study on how female hormones influence the expression of the major CYPs, preferably in intact female rats.

1.3 Hypothesis and scope of the study

Pregnancy affects hepatic drug metabolism in a CYP pathway-dependent manner: elimination of drugs metabolized by CYP2A6, CYP3A4, CYP2D6 and CYP2C9 is increased, while elimination of CYP1A2 and CYP2C19 substrates is decreased.

While the substantial evidence from clinical and *in vitro* studies have suggested potential roles of female hormones in pregnancy-associated CYP changes, detailed studies using hepatic tissues of humans or animals to determine the effects of female hormones on the major drug metabolizing CYPs have been lacking. **The ultimate goal of this research is to determine the effects of female hormones at high concentrations on the expression and activities of CYP.** To this end, two model systems, *in vitro* (primary human hepatocytes) and *in vivo* (female rats) were used in this research.

Aim 1. Determine the direct effects of E2 and PRG on the activities and expression of major hepatic CYPs in primary human hepatocytes and determine the roles of nuclear receptors responsible for hormone-mediated CYP regulation.

1. Effects of E2 and PRG on the expression and activities of major CYPs.

Freshly isolated human hepatocytes were incubated with vehicle control (ethanol 0.1%), E2 (1 μ M every 6 hours), PRG (10 μ M every 12 hours) or positive controls (rifampin 10 μ M or CITCO 100 nM) for 72 hours. The concentrations used and frequency of media exchange were designed to reflect the average plasma concentration of E2 and PRG during the third trimester. Cells were harvested for total RNA isolation and qRT-PCR was performed using isoform specific Taqman® primer sets. The effects of hormones on the following CYP isoforms were tested: CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5. The activities of CYPs were determined by measuring the metabolic rates of isoform-specific probe substrates (TABLE IV). Once female hormone responsive CYP isoforms were identified, the concentration-dependent and combination effects of female hormones (E1, E2, E3, and PRG) were examined to determine the clinical relevance and explore the potential mechanisms.

2. Identification of nuclear receptors responsible for hormone-mediated CYP regulation

Our results from Aim 1 part 1 showed that E2 and PRG induced CYP2A6, CYP2B6, CYP3A4, CYP2C8 (PRG only), and CYP3A5 (PRG only). Notably, these genes are induced upon activation of PXR, CAR, or ER signaling pathways. To determine the roles of nuclear receptors, the following experiments were performed: 1) qRT-PCR to determine the effects of female hormones on the expression of PXR and CAR. 2) promoter activity assays using the CYP2B6 promoter (containing the PXR and CAR responsive elements) to examine whether

female hormones are PXR or CAR activators. 3) promoter activity assays using the CYP2B6 promoter to evaluate the role of the ER in E2-mediated CYP2B6 transcriptional activation.

Aim 2. Identify *in vivo* effects of E2 on the expression and activities of CYPs using female rats.

Although hepatocytes are considered as the gold standard for *in vitro* drug metabolism studies, some of the limitations of hepatocytes necessitate *in vivo* studies to determine the effects of female hormones on CYPs. For example, a cell-based system can only examine the direct effects of hormones on CYPs. However, *in vivo*, hormones may exert their effects on a target organ (e.g., liver) indirectly via regulating release of hormones from other organs (e.g., growth hormone or thyroid hormone via the hypothalamus pituitary axis). As the first step of *in vivo* characterization of female hormone-mediated CYP regulation, we examined the effects of E2 on the expression and activities of rat CYPs.

1. Determination of plasma concentration of E2 after estradiol benzoate subcutaneous injection.

To determine the effects of E2 on the expression and activities of CYP *in vivo*, E2 benzoate at 1 mg/kg/day was injected subcutaneously into female Sprague-Dawley rats for 5 days. To ensure the plasma E2 concentrations were comparable to those reached during human pregnancy, we obtained plasma E2 concentration vs. time profiles for day 1 and day 5. An enzyme-linked immunosorbent assay (ELISA) was used for E2 concentration measurement.

2. Effects of E2 on the expression and activities of major CYPs *in vivo* using female rats.

We examined the effects of E2 on the expression and activities of the major hepatic CYPs in female rats (CYP1A2, CYP2B1, CYP2C6, CYP2C7, CYP2C12, CYP3A1, CYP3A9, CYP2D1, and CYP2E1). Estradiol benzoate was subcutaneously administered to female Sprague-Dawley rats for 5 days, and hepatic tissues were prepared for mRNA isolation and hepatic microsomes were isolated for western blot and microsome activity assays. In addition, the effects of E2 on the mRNA levels of major CYP transcriptional regulators (AhR, PXR, CAR) and POR were examined to explore the potential mechanisms underlying the regulation of CYP expression and activity by E2.

2. Effects of Estradiol and Progesterone on the Expression and Activities of Cytochrome P450 in Human Hepatocytes.

2.1 Materials and methods

2.1.1 Chemicals and Reagents.

Acetaminophen, chlorzoxaxone, coumarin, dexamethasone, diclofenac, estrone, estradiol, estriol, ethinylestradiol, indomethacin, ITS, S-mephenytoin, 7-hydroxycoumarin, 4'-hydroxymephenytoin, 4-methylumbelliferone, phenobarbital, s-nirvanol, phenytoin, *p*-nitrophenol, *p*-nitrocatechol, phenacetin, phenytoin, progesterone, rifampin, and William's E medium were obtained from Sigma (St. Louis, MO). Midazolam, 1-hydroxymidazolam, d4-acetaminophen and prazepam were purchased from Cerilliant (Round Rock, TX). 4'-Hydroxydiclofenac was purchased from Axxora (San Diego, CA). CITCO, i.e. 6-(4-Chlorophenyl) imidazo[2,1-b] [1,3]thiazole-5-carbaldehyde O-3,4-dichlorobenzyl) oxime, was purchased from Biomol (Plymouth Meeting, PA). Bufuralol and 1'-hydroxybufuralol were purchased from BD Biosciences (Franklin Lakes, NJ). 6-hydroxychlorzoxazone was purchased from Cayman Chemical Company (Ann Arbor, MI). Formic acid (ACS grade), and methanol (Optima grade) were purchased from Fisher Scientific (Pittsburgh, PA).

2.1.2 Primary human hepatocytes culture.

Freshly isolated human hepatocytes from different donors (age ranging from 29 to 70 years old, no known history of liver diseases, alcohol abuse or hepatitis B or C infection) were obtained from CellzDirect (Pittsboro, NC) or Liver Tissue Cell Distribution System (Pittsburgh, PA). Hepatocytes were shipped overnight in cold preservation media. Upon receipt, media were replaced with serum-free Williams' E media (without phenol red) containing 0.1 μ M dexamethasone, 10 μ g/ml gentamicin, 15 mM HEPES (pH 7.4), 2 mM L-

glutamine, and ITS+ 1X. Cells were maintained at 37 °C in a humidified incubator with 5% CO₂. After overnight incubation, cells were treated with vehicle control (0.1% ethanol), female hormones (E2 1 µM every 6 hours, E1 0.5 µM every 6 hrs, E3 0.5 µM every 6 hours or PRG 10 µM every 12 hours) or known inducers (rifampin 10 µM, CITCO 100 nM, ethanol 100 mM, or phenobarbital 0.5 mM every 24 hours) for 72 hr.

2.1.3 Determination of average concentration of E2 and PRG in media.

Hepatocytes were treated with E2 (1 µM) or PRG (1 and 10 µM), and media were sampled at various time points, ranging from 15 min to 12 hours after treatment. E2 and PRG concentrations in culture media were measured by LC/MS/MS (Agilent 1200 HPLC interfaced with Applied Biosystems Qtrap 3200 using an electrospray ion source). Separation was performed with a Zorbax Eclipse XDB-C8 column (4.6 × 50 mm, 3.5 µm) (Agilent Technologies, Santa Clara, CA) at a flow rate of 0.4 ml/min. For E2, a linear gradient of the mobile phase, consisting of water [0.02% (v/v) ammonium hydroxide] and acetonitrile, was used for separation: 15% acetonitrile at time 0 increased to 90% at 7 min. MRM pairs used for E2 and ethinylestradiol (internal standard) were 271.2/145.1 (negative ion mode) and 295.2/145.1 (negative ion mode), respectively. For PRG, a linear gradient of water [0.1% (v/v) formic acid] and acetonitrile was used for separation: 20% acetonitrile at time 0 increased to 80% at 6 min. MRM pairs used for PRG and prazepam (internal standard) were 315.2/109.2 (positive ion mode) and 325.0/271.1 (positive ion mode), respectively. Average concentrations of E2 or PRG in media were calculated as follows: $C_{ave} = AUC_{0-\tau}/\tau$, where $AUC_{0-\tau}$ is the area under the curve (hormone concentration vs. time from 0 hr to τ hr) (τ : the media change interval: 6 hr for E2, 12 hr for PRG).

2.1.4 RNA isolation and quantitative real time-PCR (qRT-PCR).

Total RNAs were isolated from human hepatocytes using Trizol (Invitrogen, Carlsbad, CA). cDNA was synthesized using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). qRT-PCR was performed using StepOnePlus Real-Time PCR System and CYP isoform-specific TaqMan[®] Gene expression assays (Applied Biosystems) as follows: CYP1A2 (Hs01070369_m1), CYP2A6 (Hs00868409_s1), CYP2B6 (Hs03044634_m1), CYP2C8 (Hs00258314_m1), CYP2C9 (Hs00426397_m1), CYP2C19 (Hs00426380_m1), CYP2D6 (Hs02576167_m1), CYP2E1 (Hs00559367_m1), CYP3A4 (Hs00430021_m1), CYP3A5 (Hs00241417_m1), CAR (Hs00901571_m1), PXR (Hs00243666_m1), POR (Hs00287016_m1), b5 (Hs00157217_m1), and GAPDH (Hs99999905_m1). The relative expression in mRNA levels of CYP upon hormone or known inducer treatment was determined by normalizing the gene expression levels by those of GAPDH ($2^{-\Delta\Delta Ct}$ method). Statistical analysis was performed using the Student's *t*-test.

2.1.5 Western immunoblot analysis.

Total proteins from primary hepatocytes after 72 hr hormone (or vehicle) treatments were extracted in radioimmunoprecipitation assay (RIPA) buffer, and the protein concentration was measured with BCA protein assay kit (Pierce, Rockford, IL). Total proteins were resolved by SDS gel electrophoresis on an 8% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (1.5 hr, 300 mA). The membrane was then blocked at room temperature for 1 hr in 5% (w/v) milk in tris-buffered saline containing 0.05% (v/v) Tween 20 (TBST). Membranes were incubated for overnight at 4 °C in anti-CYP2C9 (1:1000 in 1% milk powder in TBST) (Abcam, Cambridge, MA) or anti-CYP2E1 (1:800 in 1% milk in TBST) (Abcam). After overnight incubation, the membrane was incubated with horseradish

peroxidase-conjugated anti-rabbit IgG secondary antibody at room temperature for 1 hr (1:10,000 in 5% milk in TBST) (Abcam). Protein expression was detected by Supersignal® West Pico Chemiluminescent substrate (Pierce) on Kodak films, and quantitative analysis of the bands was performed using Adobe Photoshop (San Jose, CA)

2.1.6 Determination of CYP Activity.

Hepatocytes were treated with vehicle control (ethanol), female hormones, or known inducers for 72 hours as described in human hepatocytes culture. Then the media were replaced with fresh media containing one of the CYP isoform-specific probe substrates for CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 and sampled at various time points (30 min to 4 hr). The concentration of metabolite of each probe substrate was measured by LC/MS/MS (Agilent 1200 HPLC interfaced with Applied Biosystems Qtrap 3200) using an electrospray ion source. The mobile phase consisted of water (0.1% formic acid) and acetonitrile. Separation was performed with a Zorbax Eclipse XDB-C8 column (4.6 × 50 mm, 3.5 µm) (Agilent Technologies) at a flow rate of 0.4 ml/min except acetaminophen and 7-hydroxycoumarin detection (YMC C8 4.6 × 150 mm, 3.5 µm, Waters, Milford, MA). The CYP isoform-specific substrates, their metabolites, and analytical conditions (i.e., internal standards, MRM pairs, and mobile phase gradients) are summarized in TABLE V. CYP activity was expressed as the rate of metabolite production (pmol/min/million cells), and the relative activity was calculated by comparing the activity of drug treated group to the vehicle treated group.

TABLE V

CYP ISOFORM-SPECIFIC PROBE SUBSTRATES, THEIR METABOLITES, AND ANALYTICAL METHODS

	1A2	2A6	2B6	2C9		2C19	2D6	2E1		3A4
Substrate	phenacetin	coumarin	S-mephenytoin	diclofenac	tolbutamide	S-mephenytoin	bufuralol	<i>p</i> -nitrophenol	chlorzoxazone	midazolam
Conc (μM)	50	100	60	100	100	60	60	100	100	15
metabolite	acetaminophen	7-hydroxy coumarin	nirvanol	4'-hydroxy diclofenac	4-hydroxy tolbutamide	4-hydroxy mephenytoin	1'-hydroxy bufuralol	<i>p</i> -nitro catechol	6-hydroxy chlorzoxazone	1-hydroxy midazolam
Analyte MRM	152.1/110.0	163.1/119.2	205.1/134.2	311.9/230.1	287.1/188.1	235.2/150.1	278.4/186.3	153.8/124.1	183.9/120.0	341.9/324.0
Internal standard	d4-acetaminophen	4-methylumbelliferone	phenytoin	indomethacin	indomethacin	phenytoin	lamotrigine	4'-hydroxy diclofenac	4-hydroxy tolbutamide	prazepam
Internal standard MRM	156.1/114.0	177.1/105.1	253.2/182.2	357.9/139.1	357.9/139.1	253.2/182.2	256.1/211.1	310.1/266.1	285.1/186.0	325.0/271.1
Mobile phase %B (min)	20(0) → 0(7)	20(0) → 0(5.5)	20(0) → 95(5)	30(0) → 100(7)	30(0) → 100(7)	20(0) → 95(5)	20(0) → 95(4)	20(0) → 100(4.5)	20(0) → 0(5)	20(0) → 100(5)

Modified from (96)

2.1.7 HepG2 Cell Culture.

HepG2 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gemini, Woodland, CA), 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% non-essential amino acid at 37 °C under 5% CO₂.

2.1.8 Plasmids.

PXR, ER and CAR expression vectors were prepared as follows: a PXR expression vector in a pCMVsport2 plasmid was kindly provided by Dr. Bingfang Yan (University of Rhode Island, RI). The PXR gene was subcloned into a pcDNA3 vector (Invitrogen) using forward (5'-ACTATAGAAGGTACGCCTG-3') and reverse (5'-CTATGACGTCGCATGCAC-3') primers and a restriction enzyme (EcoRV). Similarly, an ER-expressing vector was prepared by subcloning *ER* from pSG5-HEG0 (a gift from Dr. Pierre Chambon, Illkirch, France) into a pcDNA3 vector using forward (5'-AATACGACTCACTATAGGG-3') and reverse (5'-AGATCTGGATCCGAATTCC) primers and restriction enzymes (*KpnI* and *BamHI*). To construct the CAR expression vector, total RNA was isolated from HepG2 cells and first-strand cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen). With the first-strand cDNA as a template, the CAR gene was PCR-amplified using forward (5'-AAGGATCCGAAAACCAGCAACAGCGTGG-3') and reverse (5'-TTGAATTCTTCCCACTCC CAGTGTATCCAG-3') primers. The PCR product and pcDNA3 were digested by restriction enzymes (*BamHI* and *EcoRI*) and ligated, yielding the CAR expression vector (pcDNA3-CAR). The CAR3 expression vector (pTracerCMV2-CAR3), and the β-galactosidase expression vector were kindly provided by Dr. Curtis J. Omiecinski (Pennsylvania State University, PA), and William T. Beck (University of Illinois at Chicago, IL), respectively. The

luciferase construct containing the phenobarbital-responsive elements (PBREM) of the CYP2B6 promoter (CYP2B6-PBREM) were generous gifts from Dr. Hongbing Wang (University of Maryland, MD).

2.1.9 Promoter activity assay.

HepG2 or HepG2-ER cells were seeded into 12-well plates at 3×10^5 cells/well and incubated overnight. On the next day, cells were transfected with 0.3 μg of a nuclear receptor expression vector (CAR, CAR3, ER, PXR or their respective empty vectors), 0.3 μg of luciferase construct (CYP2B6-PBREM), and 0.1 μg of β -galactosidase expression vector using Fugene 6 transfection reagent (Roche Applied Sciences, Indianapolis, IN) according to the manufacturer's protocol. 24 hr after transfection, cells were treated with vehicle (0.1% ethanol), female hormones (E2; 1 μM or PRG; 10 μM), or known inducers (CITCO; 1 μM or rifampin; 10 μM) for 24 hr. Cells were then harvested for determination of both luciferase and β -galactosidase activities using assay kits from Promega (Madison, WI). The luciferase activity was normalized to the β -galactosidase activity. Each experiment was performed in triplicate and repeated in at least two independent experiments, and statistical analysis was performed using the Student's *t*-test.

2.2. Results

2.2.1 Effect of E2 on the expression and activities of CYPs

2.2.1.1 E2 and PRG are rapidly metabolized in human hepatocytes

E2 and PRG exhibit high clearance when administered to humans due to rapid hepatic metabolism (97, 98). To obtain information that can guide experiments, elimination rates of E2 and PRG in hepatocyte culture medium was determined. To this end, human hepatocytes were treated with E2 (1 μ M) or PRG (1 μ M), and hormone concentrations at various time points were measured by LC/MS/MS. The result (Fig. 4A) showed that E2 was metabolized with a first order elimination rate in human hepatocytes (half-life: 0.57 ± 0.12 hr). The average concentration of E2 over 6 hr was 157 ± 39 nM, corresponding to the average concentration during pregnancy in the third trimester [~ 100 nM, (65)]. Similar results were obtained with different batches of human hepatocytes (data not shown). Based on this result, throughout the rest of the study culture media containing 1 μ M of E2 was replenished every 6 hr during the daytime and every 12 hr for overnight incubations to maintain the average E2 concentration at the level of full term.

PRG was also metabolized rapidly by human hepatocytes with a first order elimination rate (Fig. 4B, half-life: 0.73 ± 0.16 hr). This led to an average concentration of 92 ± 16 nM over 12 hr, corresponding to the PRG concentration at the very early phase of pregnancy, but lower than the concentration at full-term [~ 1 μ M, (65)]. Accordingly, a 10-fold higher concentration of PRG (i.e., 10 μ M) was used throughout the study. After treatment with 10 μ M PRG (Fig. 4C), the average concentration of PRG over 12 hr in culture media was 3.28 ± 0.47 μ M.

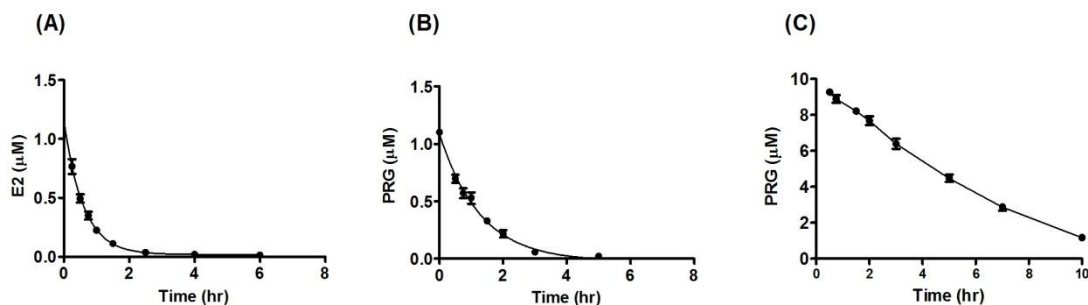


Fig. 4 Elimination of E2 and PRG in a representative batch of human hepatocytes.

Human hepatocytes were treated with E2 (1 μM , A), PRG (1 μM , B; 10 μM , C), and the media were sampled at various time points. The hormone concentrations were determined by LC/MS/MS.

2.2.1.2 E2 induces expression of CYP2A6, CYP2B6, and CYP3A4.

To determine the effects of E2 on CYP expression, human hepatocytes (from five different donors) were treated with E2, and the mRNA expression of the major drug metabolizing CYPs (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5) were determined by qRT-PCR. E2 increased the expression of CYP2A6 (5.9 to 23.4-fold), CYP2B6 (4.8 to 14.4-fold) and CYP3A4 (1.5 to 6.8-fold), but not the expression of the remaining CYP enzymes we examined (Fig. 5). Of note, induction of CYP3A4 by E2 was of a smaller magnitude than that of CYP2A6 and CYP2B6 and was only observed in two of the five hepatocytes.

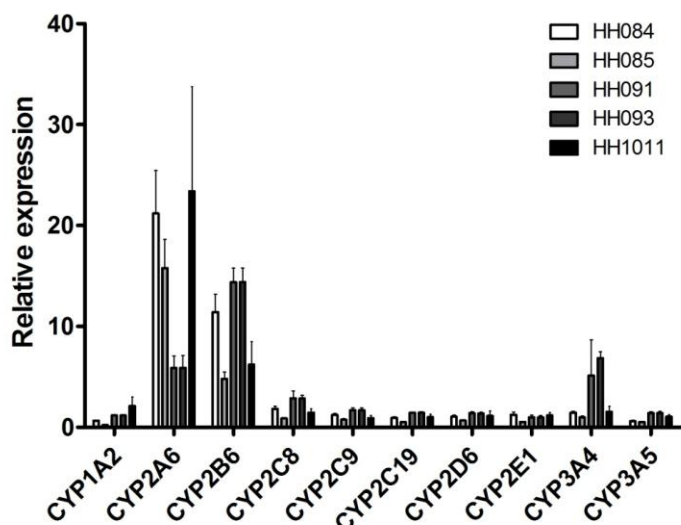


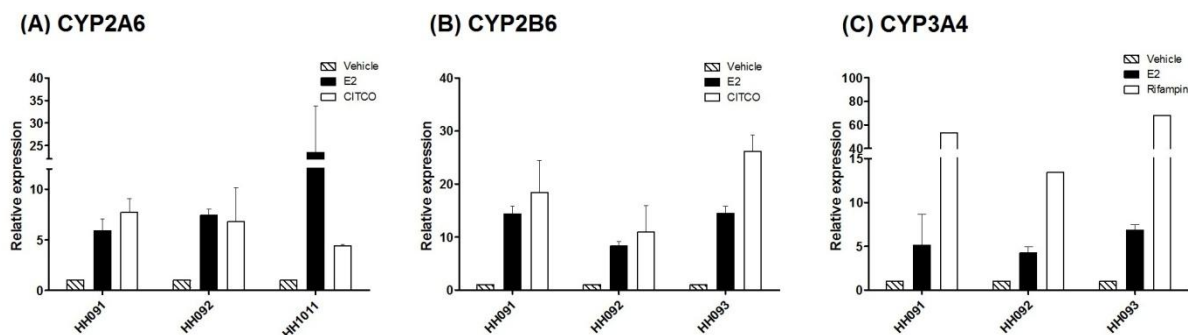
Fig. 5 Effects of E2 on mRNA expression levels of major CYPs.

Human hepatocytes from five different donors (n=3/batch) were treated with E2 (1 μ M) or vehicle (ethanol) for 72 hr (with regular media change; see text for details). mRNA levels of CYP isoforms were measured by qRT-PCR. Results are expressed as relative expression in mRNA levels by E2 treatment relative to vehicle treatment.

When compared to prototypical inducers of CYP expression, the extent of induction of CYP2A6 and CYP2B6 by E2 was comparable with that by CITCO (a CAR activator); induction of CYP2A6 mRNA levels by E2 was 76-530 % of the induction by CITCO while induction of CYP2B6 was 55-78 % of CITCO (Fig. 6A-B). On the other hand, the extent of induction in CYP3A4 expression by E2 was weaker as compared to that by rifampin (a PXR activator) (Fig. 6C), indicating minor effects of E2 on CYP3A4 expression. The effects of E2 on activities of CYP2A6, CYP2B6 and CYP3A4 in comparison with those of known CYP inducers showed similar results (Fig. 6D-F). The effects of E2 on CYP2A6, CYP2B6, and CYP3A4 showed concentration dependency (Fig. 7), and E2 induces these isoforms at high (≥ 1 μ M) concentration, but not at lower concentrations. Taken together, E2 enhances expression of CYP2A6 and CYP2B6 to levels comparable to the prototypical CYP inducer

CITCO. E2 also enhances CYP3A4 expression although the magnitude of induction is small compared to the prototypical inducer, rifampin.

Fold-increases in CYP expression



Fold-increases in CYP activity

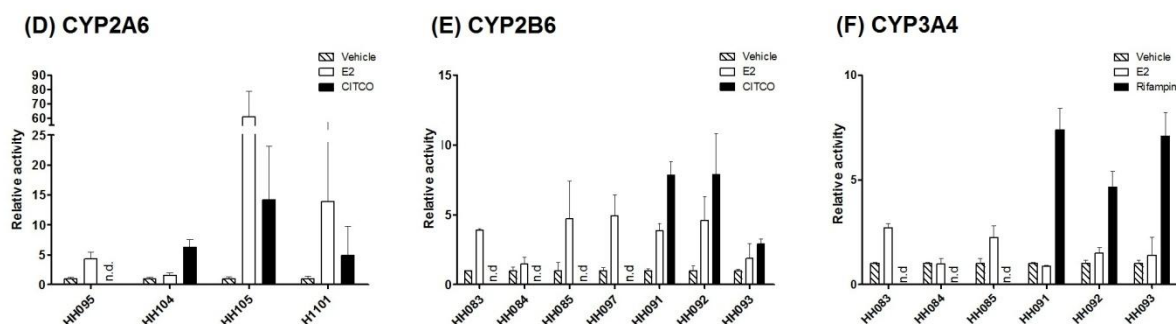


Fig. 6 Effects of E2 on the expression and activities of CYP2A6, CYP2B6, and CYP3A4. Human hepatocytes from three different donors were treated with vehicle (ethanol), E2 (1 μ M), CITCO (100 nM), or rifampin (10 μ M) for 72 hr. (A,B,C) mRNA expression levels of CYP2A6, CYP2B6 and CYP3A4 were determined by qRT-PCR. Results are expressed as relative expression in mRNA levels by drug treatment in comparison to vehicle treatment. (D,E,F) CYP isoform specific probe substrates (100 μ M coumarin for CYP2A6, 100 μ M S-mephenytoin for CYP2B6, 15 μ M midazolam for CYP3A4) were added to media. The rates of metabolite formation were measured in pmol/min/million cells. Results are expressed as relative activity by drug treatment in comparison to vehicle treatment. n.d., not determined.

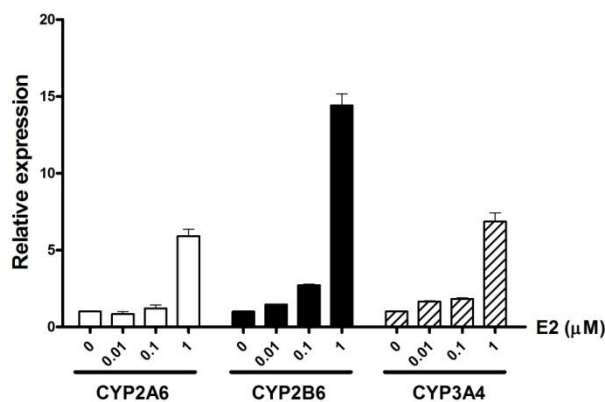


Fig. 7 Concentration-dependent effects of E2 on CYP2A6, CYP2B6, and CYP3A4 expression.

A batch of human hepatocytes (HH093) was treated with E2 (0.01 – 1 μ M) or vehicle (ethanol) for 72 hr. mRNA levels of CYP isoforms were measured by qRT-PCR. Results are expressed as relative expression in mRNA levels by E2 treatment in comparison to vehicle treatment

2.2.1.3 E2 Increased CYP2C9 and CYP2E1 activities

E2 did not significantly affect mRNA expression levels of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 (Fig. 5). To examine the possibility of post-transcriptional regulation for these CYP isoforms, the activities of these isoforms were determined in hormone-treated hepatocytes. Human hepatocytes were treated with E2 (1 μ M) or vehicle for 72 hours, and isoform-specific probe substrates were added to media. Activities were measured as rates of metabolites formation (pmol/min/million cells). E2 did not alter the activities of CYP1A2 (phenacetin *O*-deethylation), CYP2C19 (S-mephenytoin hydroxylation), or CYP2D6 (bufuralol 1'-hydroxylation) (Fig. 11). Interestingly, E2 enhanced the activities of CYP2C9 (diclofenac 4'-hydroxylation) and CYP2E1 (*p*-nitrophenol hydroxylation) by 1.3-2.0 and 1.7-2.1 fold, respectively, as compared to the vehicle treatment (Fig. 8). Induction in the activities of CYP2C9 and CYP2E1 by E2 was comparable to that by known inducers of

CYP2C9 (phenobarbital) (99) and CYP2E1 (ethanol) (100). The enhanced activities of CYP2C9 and CYP2E1 upon E2 treatment were further verified using different probe drugs for the enzymes, tolbutamide and chlorzoxazone. E2 increased tolbutamide 4-hydroxylation (CYP2C9) and chlorzoxazone 6-hydroxylation (CYP2E1) activity by 1.8-fold and 1.4 fold, respectively, similar to the results obtained when using diclofenac and *p*-nitrophenol as the probe drugs.

To determine whether the increased enzyme activity corresponds to increased protein levels, protein expression of CYP2C9 and CYP2E1 were determined by western blotting. The results showed that E2 did not increase the CYP2C9 and CYP2E1 protein expression levels (Fig. 9). Of interest, the activities of CYP2C9 and CYP2E1 are sensitive to the expression level of redox partners such as P450 oxidoreductase (POR) or NADH-cytochrome b5 reductase (b5) (101, 102). To examine whether E2 influences expression of POR and b5 (possibly leading to increased activity of CYP2C9 and CYP2E1), we measured mRNA expression levels of POR and b5 in E2-treated hepatocytes. The results showed that E2 did not alter the expression levels of POR or b5 (Fig. 10). Taken together, E2 increased the activities of CYP2C9 and CYP2E1 without affecting their protein expression or POR/b5 expression levels.

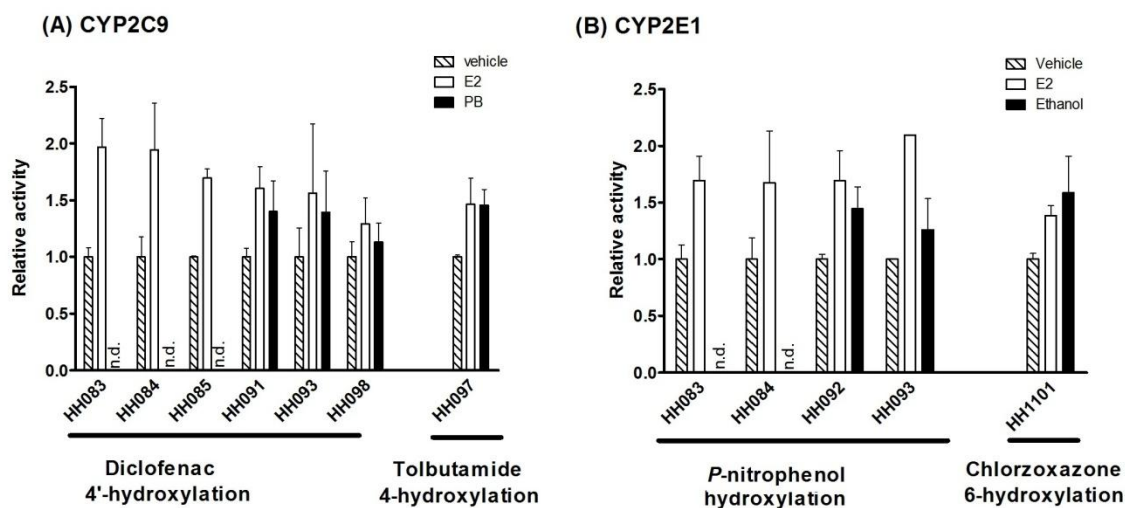


Fig. 8 Effects of E2 on CYP2C9 and CYP2E1 activities.

Human hepatocytes from different donors were treated with vehicle (ethanol; 0.1 %), E2 (1 μ M), phenobarbital (0.5 mM), or ethanol (100 mM) for 72 hr. CYP-isoform specific probe substrates [(A) diclofenac 100 μ M or tolbutamide 100 μ M for CYP2C9; (B) *p*-nitrophenol 100 μ M or chlorzoxazone 100 μ M for CYP2E1] were added to media. The rates of metabolite formation were measured in pmol/min/million cells. Results are expressed as relative activity in metabolite formation rates in E2 treatment in comparison to the vehicle treatment. n.d., not determined.

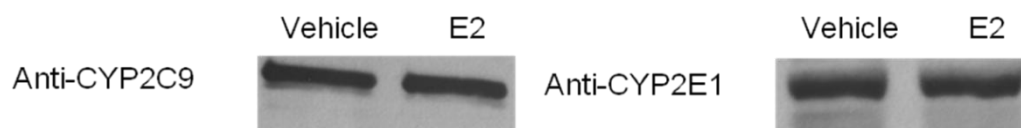


Fig. 9 Effects of E2 on CYP2C9 and CYP2E1 protein expression levels.

A batch of human hepatocytes (HH085) was treated with vehicle (ethanol; 0.1 %) or E2 (1 μ M) for 72 hr. Total protein was isolated and western immunoblot analysis was performed. 20 μ g (CYP2C9) or 50 μ g (CYP2E1) of total protein from each group was resolved on SDS-PAGE gel (8%), transferred to nitrocellulose membrane, and probed with anti human CYP2C9 or CYP2E1 antibodies followed by peroxidase-conjugated secondary antibodies.

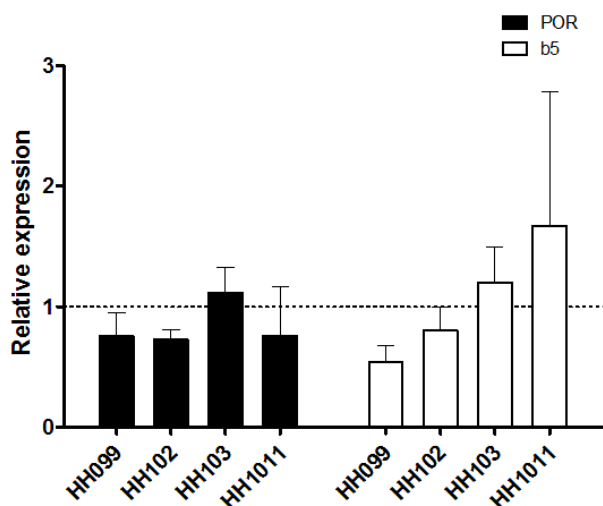


Fig. 10 Effects of E2 on the expression of POR and b5.

Human hepatocytes from four different donors were treated with vehicle (ethanol) or E2 (1 μ M). mRNA expression levels were determined by qRT-PCR. Results are expressed as relative expression in mRNA levels by E2 treatment in comparison to vehicle treatment.

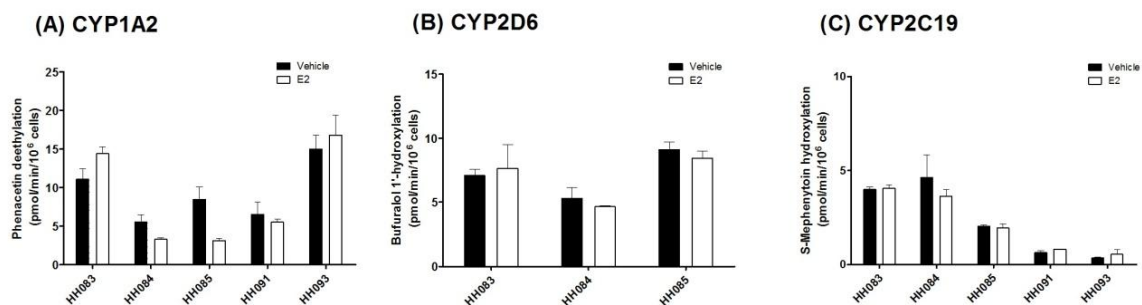


Fig. 11 Effects of E2 on activities of CYP1A2, CYP2D6, and CYP2C19.

Human hepatocytes from different donors were treated with vehicle (ethanol) or E2 (1 μ M) for 72 hr. CYP isoform specific probe substrates (50 μ M phenacetin for CYP1A2, 100 μ M S-mephenytoin for CYP2C19, and 60 μ M bufuralol for CYP2D6) were added to media. Results are expressed as rates of metabolites formation (pmol/min/million cells).

2.2.2 Effects of PRG on the expression and activities of CYPs

2.2.2.1 PRG induces expression of CYP2A6, CYP2B6, CYP2C8, CYP3A4 and CYP3A5

To determine the effects of PRG on the expression of CYP enzymes, we treated human hepatocytes with vehicle or PRG for 72 hr and examined the mRNA expression levels of the major drug metabolizing CYPs (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5) by using qRT-PCR. The results (Fig. 12) showed that PRG increased the expression of CYP2A6 (1.7-3.4 fold), CYP2B6 (1.8-7.6 fold), CYP2C8 (1.4-9.2 fold), CYP3A4 (1.4-12.0 fold), and CYP3A5 (1.4-6.7 fold). PRG exhibited insignificant effects on the mRNA expression of the remaining CYPs (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP2E1).

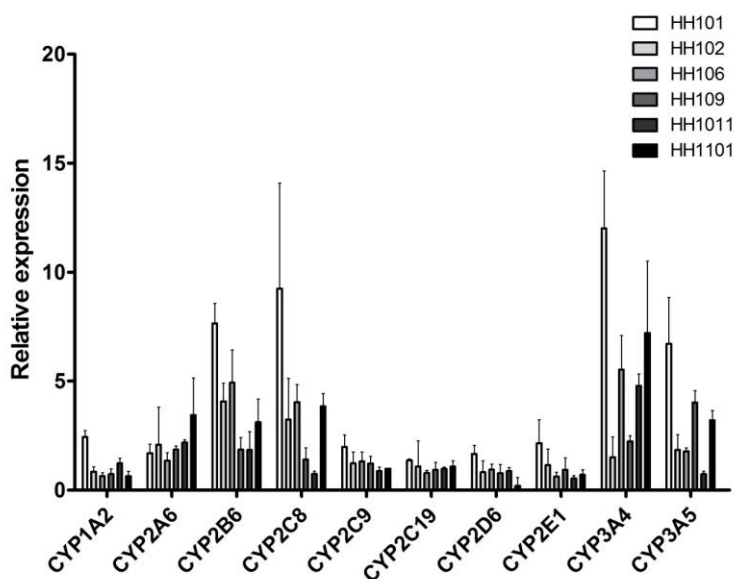
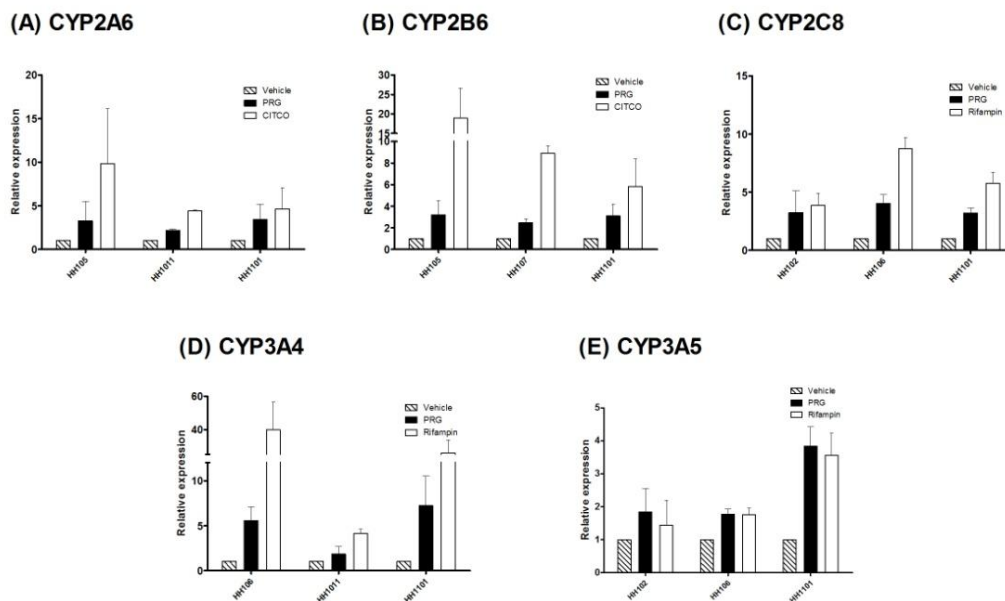


Fig. 12 Effects of PRG on mRNA expression levels of major CYPs.

Human hepatocytes from six different donors were treated with PRG (10 μ M) or vehicle (ethanol) for 72 hr. mRNA expression levels of CYP enzymes were measured by qRT-PCR. Results are expressed as relative expression in mRNA levels by PRG in comparison to vehicle treatment.

When compared to the induction by prototypical inducers (i.e., CITCO and rifampin), the induction in CYP2A6, CYP2B6, and CYP3A4 mRNA levels by PRG was of a smaller magnitude; induction of CYP2A6 and CYP2B6 by PRG was 33-74% and 17-54% of the induction by CITCO respectively while induction of CYP3A4 was 14-45% of the induction by rifampin (Fig. 13). Induction of CYP2C8 and CYP3A5 were comparable to the induction by rifampin (46-108% and 83-107%). PRG-mediated changes in the activities of CYP2A6, CYP2B6, and CYP3A4 appeared to show similar patterns (Fig. 13). Similarly to E2, PRG-mediated induction of CYP2A6, CYP2B6, CYP2C8, CYP3A4 and CYP3A5 exhibited concentration-dependency (Fig. 14), and the induction in CYP expression was prominent at the PRG concentration achieved during pregnancy. Taken together, our data indicate that PRG enhances the expression of CYP2A6, CYP2B6, CYP2C8, CYP3A4 and CYP3A5.

CYP mRNA Expression



CYP Activity

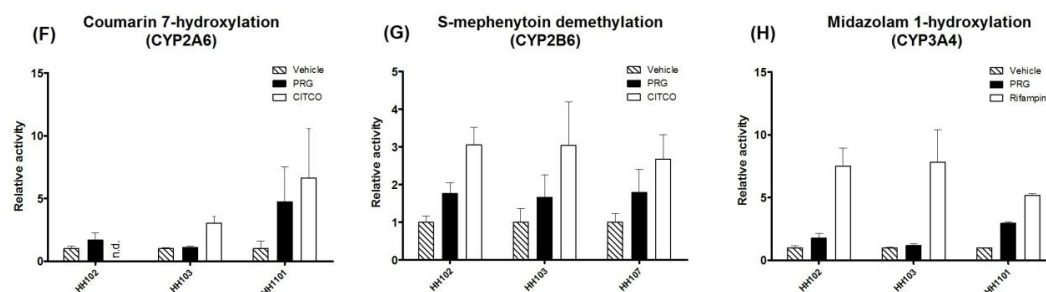


Fig. 13 Effects of PRG and prototypical inducers on the expression (CYP2A6, CYP2B6, CYP2C8, CYP3A4, and CYP3A5) and activities (CYP2A6, CYP2B6, and CYP3A4).

Human hepatocytes from three different donors were treated with vehicle (ethanol), PRG (10 μ M), CITCO (100 nM), or rifampin (10 μ M) for 72 hr. (A-E). mRNA expression levels of CYP2A6, CYP2B6 and CYP3A4 were determined by qRT-PCR. Results are expressed as relative expression in mRNA levels by drug treatment in comparison to vehicle treatment. (F-H). CYP isoform specific probe substrates (100 μ M coumarin for CYP2A6, 100 μ M S-mephenytoin for CYP2B6, 15 μ M midazolam for CYP3A4) were added to media. The rates of metabolite formation were measured in pmol/min/million cells. Results are expressed as relative activity in metabolite formation rates by drug treatment in comparison to vehicle treatment.

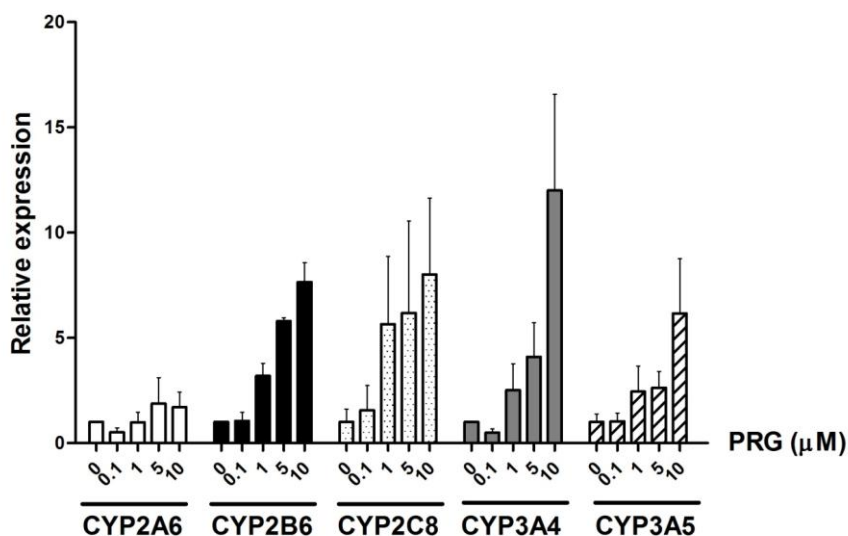


Fig. 14 Concentration-dependent effects of PRG on CYP2A6, CYP2B6, CYP2C8, CYP3A4 and CYP3A5 expression.

A batch of human hepatocytes (HH101) was treated with PRG (0.1 – 10 μM) or vehicle (ethanol) for 72hr. mRNA levels of CYP isoforms were measured by qRT-PCR. Results are expressed as relative expression in mRNA levels by PRG in comparison to vehicle treatment.

2.2.2.2 PRG does not alter the activities of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP2E1.

PRG did not significantly affect the mRNA expression levels of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 (Fig. 12). To examine the possibility of post-transcriptional regulation for these CYP isoforms, the activities of these isoforms were determined in hormone-treated hepatocytes. Several batches of human hepatocytes were treated with PRG (10 μM) or vehicle for 72 hours, and isoform-specific probe substrates were added to the media; metabolites in the media were measured using LC/MS/MS. The results showed that PRG did not alter the activities of CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP2E1 (Fig. 15).

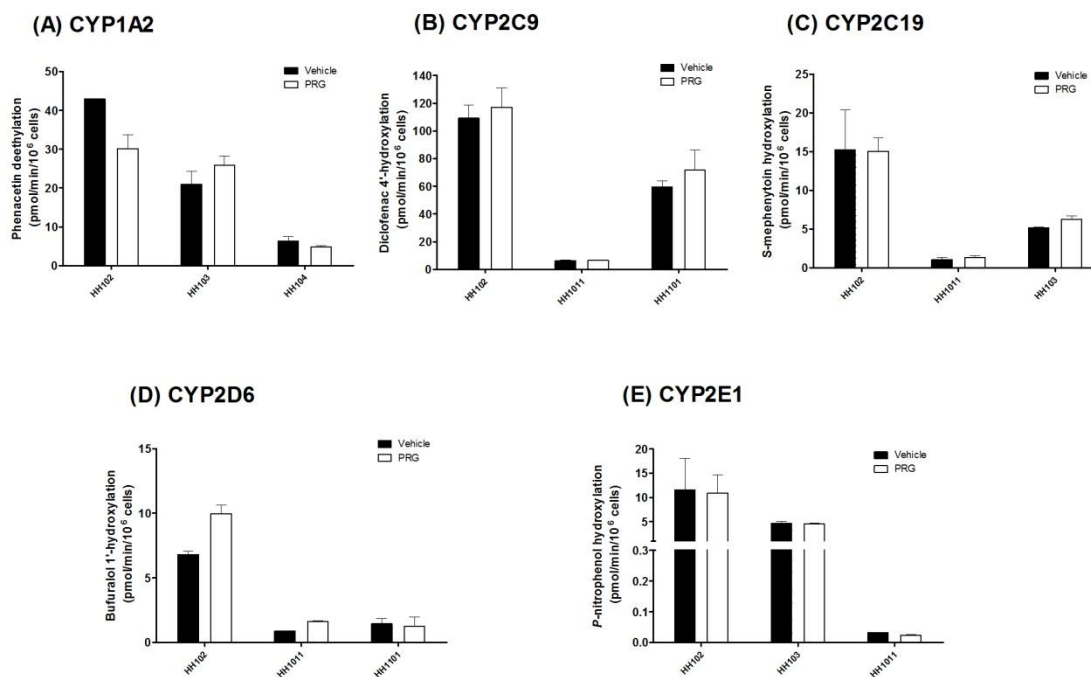


Fig. 15 Effects of PRG on activities of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP2E1.

Human hepatocytes from different donors were treated with vehicle (ethanol) or PRG (10 μ M) for 72 hr. CYP isoform specific probe substrates (50 μ M phenacetin for CYP1A2, 100 μ M diclofenac for CYP2C9, 100 μ M S-mephenytoin for CYP2C19, 60 μ M bufuralol for CYP2D6, and 100 μ M *p*-nitrophenol for CYP2E1) were added to the media. Concentrations of corresponding metabolites in the media were determined by LC/MS/MS. Results are expressed as rates of metabolites formation (pmol/min/million cells).

2.2.3. Estrogens and PRG exhibit additive effects on CYP3A4, but not on CYP2A6 and CYP2B6 expression

Pregnancy is accompanied by concurrent increases in the concentration of estrogens (E1, E2, E3) and PRG (65). In this study, we determined whether E1 and E3, the other two major endogenous female hormones, also induce CYP expression at the concentrations reached during pregnancy, as was shown with E2. We also examined combined effects of the estrogens and PRG on CYP expression. To this end, human hepatocytes were treated

with the female hormones, individually or in combination, and examined the mRNA expression of CYP2A6, CYP2B6, and CYP3A4 (i.e., the CYP enzymes whose expression is upregulated by both E2 and PRG) by using qRT-PCR.

The results (Fig. 16) showed that the induction of CYP2A6 and CYP2B6 by E1 was similar or weaker as compared to the induction by E2, while E3 was constantly a much weaker inducer of CYP2A6 and CYP2B6. The mRNA expression of CYP2A6 and CYP2B6 in the hepatocytes treated with a combination of female hormones did not show any additive effects of the hormones. In contrast, the hormones showed additive effects on expression of CYP3A4 in the HH1011 hepatocytes; individual treatment with E2, E3 and PRG led to 1.5-, 2.3-, and 4.8-fold increases in CYP3A4 expression, respectively, while co-treatment with the combination induced CYP3A4 by about 10-fold. These results suggest that regulatory mechanisms underlying upregulation of CYP3A4 are potentially distinct from those of CYP2A6 and CYP2B6.

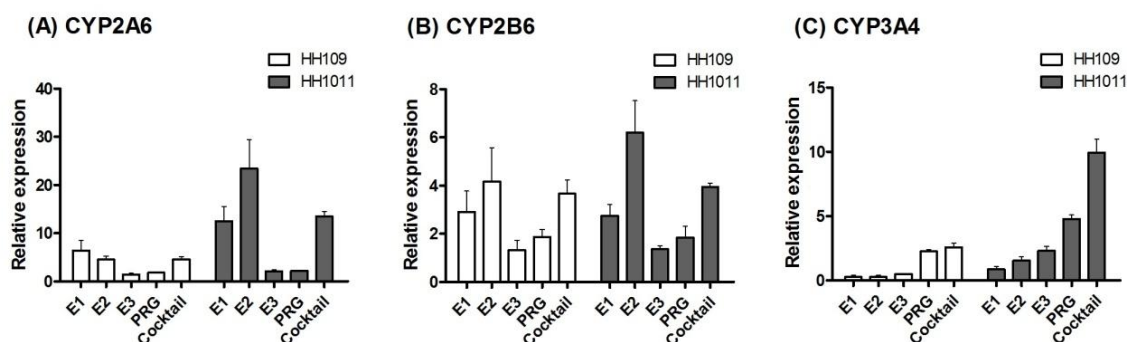


Fig. 16 Combined effects of estrogens and PRG on expression of CYP2A6, CYP2B6, and CYP3A4.

Two different batches of human hepatocytes (HH109 and HH1011) were treated with estrogens (E1, E2, and E3), PRG (10 μ M), or their combination for 72 hr. mRNA expression levels were determined by qRT-PCR. Results are expressed as relative expression in mRNA levels by drug treatment in comparison to vehicle treatment.

2.2.4 Role of CAR, PXR and ER in the female hormone-mediated CYP regulation

2.2.4.1 E2 and PRG do not induce the expression of CAR and PXR.

We observed that E2 and PRG increased the expression of CYP2A6, CYP2B6, and CYP3A4 and in addition, PRG enhanced the expression of CYP2C8 and CYP3A5. Notably, these genes are regulated via orphan nuclear receptors (PXR and/or CAR). Previous studies have shown that the expression of PXR/CAR target genes can be regulated not only by activation of PXR/CAR but also by changes in the expression levels of PXR or CAR (102). Accordingly, to determine whether hormone-mediated CYP induction is attributable to changes in PXR and CAR expression, the expression level of PXR and CAR were examined in hormone-treated hepatocytes. Contrary to its ability to induce many CYPs, E2 suppressed the expression of PXR and CAR (0.59 to 0.88-fold, 0.47 to 0.86-fold) (Fig. 17). In contrast, PRG did not alter the mRNA expression levels of PXR and CAR. Therefore, it appears that

E2 and PRG mediated CYP induction is not mediated by altered expression levels of PXR and CAR.

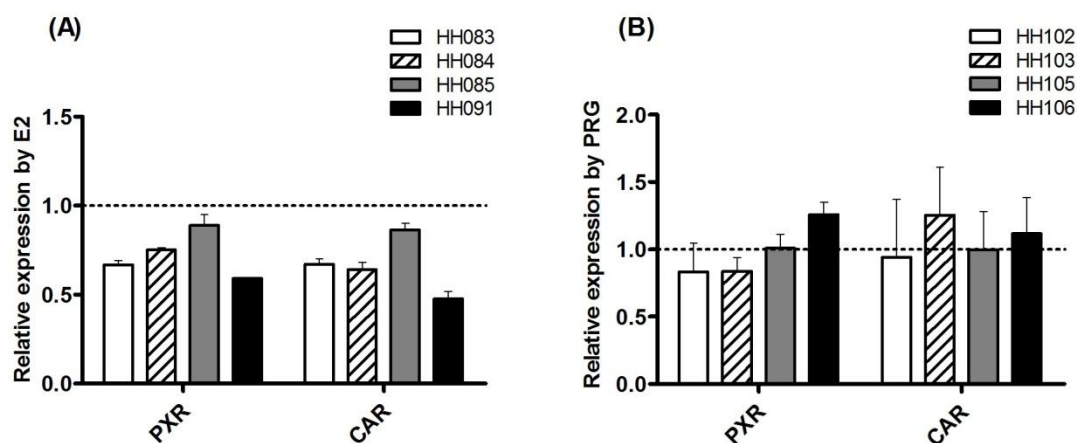


Fig. 17 Effects of E2 (A) and PRG (B) on the mRNA expression of PXR and CAR.

Four different batches of human hepatocytes were treated with vehicle, E2 (1 μ M), or PRG (10 μ M) for 72 hr. mRNA expression levels of PXR and CAR were determined by qRT-PCR. Results shown are relative mRNA expression in hormone-treated cells in comparison with vehicle treated cells.

2.2.4.2 Role of E2 and PRG in CAR and PXR activation

To investigate the mechanism of female hormone-mediated CYP induction, we examined whether E2 and PRG activate CAR and PXR by using a luciferase vector harboring the -1839 to -12 region of CYP2B6 proximal to *luc* (i.e., pGL3-CYP2B6). The regulatory region contains a phenobarbital responsive enhancer module (PBREM; -1733/-1683) where both CAR and PXR can bind for upregulation of downstream genes (103). HepG2 cells were transfected with pGL3-CYP2B6, β -galactosidase, and an expression vector for CAR, CAR3 or PXR (or an empty vector). The transfected cells were treated with

vehicle, E2, PRG, CITCO, or rifampin for 24 hr, and luciferase activity was measured.

The results showed that E2 is a CAR activator, but not a PXR activator (Fig. 18). E2 increased PBREM activity via CAR by 2.2-fold, comparable to the activation by CITCO. The result was further verified using the CAR3 expressing vector, a CAR splicing variant to rule out the effect of basal CAR activation on the E2 action. CAR3 contains an extra 5 amino acid residues inserted within the ligand-binding domain, which abolishes the constitutive activation of CAR in HepG2 (104). E2 increased CYP2B6 promoter activity by 4.2-fold, comparable with activation by CITCO (4.7-fold) via CAR3. E2 had no effects on PXR mediated promoter activation. On the other hand, PRG activated the CYP2B6 promoter via PXR (4.5-fold), although to a lesser extent compared to rifampin (8.1-fold). PRG did not increase the promoter activity of CYP2B6 via CAR and CAR3. In sum, E2 is a CAR activator and PRG is a PXR activator.

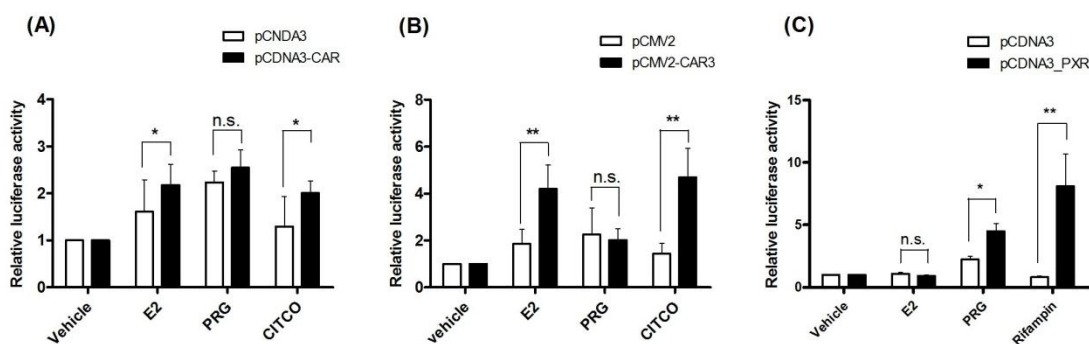


Fig. 18 Female hormone mediated PXR and CAR activation.

HepG2 cells were transfected with pGL3-CYP2B6, β -galactosidase expression vector, and an expression vector for CAR, CAR3, PXR, or a respective empty vector (pCDNA3 or pCMV2). At 24 hr post-transfection, cells were treated with vehicle (ethanol), E2 (1 μ M), PRG (10 μ M), CITCO (1 μ M) or rifampin (10 μ M) for 24 hours. Results shown are relative luciferase activity in drug treated group in comparison with vehicle treated group. n.s., not statistically significant.

* $p < 0.05$, ** $p < 0.01$

2.2.4.3 Role of ER on CYP2B6 promoter activation.

Among the E2-responsive CYPs, CYP2A6 and CYP2B6 showed consistent and strong induction by E2. CYP2A6 was shown to be regulated by ER via direct ER-ERE interaction at the CYP2A6 promoter (21), but ER involvement in CYP2B6 expression was unknown. We investigated the role of ER on E2-mediated CYP2B6 activation by luciferase assay in HepG2 cells. HepG2 cells were transfected with pGL3-CYP2B6, ER expression vector (or empty vector) and β -galactosidase expression vector. Transfected cells were treated with vehicle control, E2, or CITCO.

E2 increased CYP2B6 promoter activity by 6.4-fold upon ER transfection (Fig. 19). CITCO did not increase CYP2B6 activity in this experiment, ruling out the possibility of CAR

involvement in this experiment. This result suggests that ER plays a role for E2 mediated CYP2B6 induction.

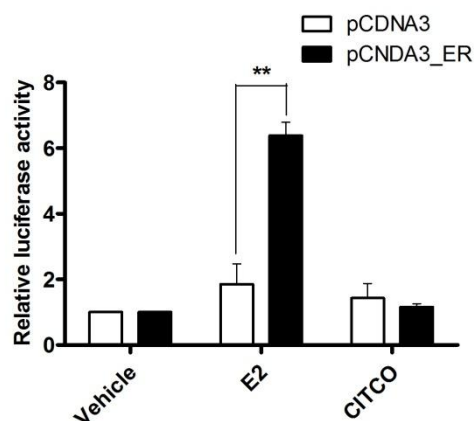


Fig. 19 ER mediated CYP2B6 promoter activation.

HepG2 cells were transfected with pGL3-CYP2B6, β -galactosidase expression vector, and an ER expression vector (or an empty vector; pCDNA3). At 24 hr post-transfection, cells were treated with vehicle (ethanol), E2 (1 μ M), or CITCO (1 μ M) for 24 hours. Results shown are relative luciferase activity of drug treated group in comparison with vehicle treated group

** $p < 0.01$

2.3 Discussion

Results from clinical studies have suggested that increased plasma levels of female hormones are potentially responsible for altered drug metabolism during pregnancy. However, there have been no comprehensive studies to examine the direct effects of female hormones on major drug metabolizing CYPs using human liver tissues. In this study, we characterized the effects of major female hormones, estrogens and PRG, on the expression of 10 different CYP isozymes using human hepatocytes, and we defined the potential mechanisms underlying hormonal regulation of CYP.

Our results indicate that E2 induces the expression CYP2A6, CYP2B6, and CYP3A4 in human hepatocytes. The induction was mainly observed when the average E2 concentration was at the levels reached during pregnancy (i.e., 100 nM), suggesting that the hormonal effects on CYP expression are likely pregnancy-specific. Induction of CYP2A6 by E2 is in agreement with a previous study where E2 increased the transcriptional activity of CYP2A6 via ER (21). The induction in CYP2A6 mRNA levels by E2 shown in our study was to a similar extent to that by CITCO, a known inducer of CYP2A6 (105). This suggests that induction of CYP2A6 by E2 potentially leads to clinically significant outcomes, and may be in part responsible for the increased metabolism of some CYP2A6 substrates (e.g., nicotine) during pregnancy (42). Similarly, CYP2B6 induction by E2 was comparable to that by CITCO, suggesting potential increases in elimination of CYP2B6 substrates (such as bupropion, an antidepressant) during pregnancy. Whether pregnancy indeed influences CYP2B6-mediated drug metabolism remains to be determined clinically. Induction of CYP3A4 expression by E2 was relatively weak.

The activities of CYP2C9 and CYP2E1 were unexpectedly increased by E2 without

any change in the expression levels. This was only observed in E2-treated hepatocytes, not in PRG-treated ones. For CYP2C9, this is unlikely due to changes in other CYPs or phase II enzymes since 4'-hydroxydiclofenac is the major urine and plasma metabolites *in vivo* and it is mostly formed by CYP2C9 in human microsomes (106). *P*-nitrophenol, a CYP2E1 substrate, is in part metabolized by CYP3A4 and UGT, but activities were increased regardless of CYP3A4 activities. Co-treatment with a phase II enzyme inhibitor (salicylamide 2 mM) did not abolish the increased activity indicating it is likely not due to the changes in phase II enzymes (data not shown). Notably, these two genes are known to be sensitive to the expression levels of redox partners such as POR or b5 (101, 102). However, the level of POR and b5 were not altered by E2. These results suggest that E2 may increase CYP2C9 and CYP2E1 activities by post-transcriptional mechanisms such as phosphorylation or protein-protein interaction. Future study is warranted to identify the mechanism of E2-mediated post-translational regulation.

PRG enhanced the mRNA expression of CYP2A6, CYP2B6, CYP2C8, CYP3A4, and CYP3A5. Similarly to E2, the induction was mainly observed when the average PRG concentration was at the levels reached during pregnancy. Although this list of CYP isozymes partly overlaps with those induced by E2, the pattern of CYP induction by PRG is different; PRG exhibits greater effects on CYP3A4, CYP3A5, and CYP2C8, while it leads to less induction of CYP2A6 and CYP2B6 compared to E2. It is of note that PRG also induces CYP3A5 and CYP2C8 while E2 does not. As CYP3A5 shares many ligands with CYP3A4, CYP3A5 induction by PRG may have contributed in part to the observed increases in CYP3A4 activities during human pregnancy (107). CYP2C8 is responsible for oxidative metabolism of at least 5% of the drugs cleared by phase I processes, and its substrates include repaglinide and arachidonic acids (108). Whether pregnancy influences

pharmacokinetics of these substrates is unknown, and the clinical significance of PRG-mediated CYP2C8 induction is yet to be determined.

All the CYPs induced by PRG and/or E2 in this study are known to be regulated by PXR and/or CAR signaling pathways. Accordingly, we examined the effects of E2 and PRG on PXR and CAR activation and expression. Our study revealed that E2 activates CAR, but not PXR. This is in part consistent with results from a previous study where E2 activated a mouse CAR (82). On the other hand, PRG activated PXR but not CAR. PXR activation by PRG on CYP2B6 promoter is consistent with the previous findings where PRG activates UGT1A1 or CYP3A4-XREM promoter activity via PXR (11, 84). Interestingly, PXR is reported to repress CAR activity in mice, but we did not observe CAR suppression by PRG (82), suggesting inter-species differences in the role of PRG on CAR suppression.

Considering concurrent rises in plasma concentration of estrogens (E1, E2, and E3) and PRG, it is of interest to examine whether any interaction between estrogens and PRG exists in modulating CYP expression. We focused on CYP2A6, CYP2B6, and CYP3A4 as they are the CYP enzymes whose expression is enhanced by both E2 and PRG. When all major female hormones (E1, E2, E3 and PRG) were given together, estrogens and PRG showed antagonistic (if not nonadditive) effects on CYP2A6 and CYP2B6 expression whereas the same combination led to additive (if not synergistic) induction in CYP3A4 expression. The lack of additive effects on CYP2A6 and CYP2B6 expression can be in part explained by competition for shared coactivators between PXR and CAR (24). Furthermore, PXR and CAR are known to bind common *trans*-elements in upstream regulatory regions of the target genes (24), suggesting that PXR and CAR may also compete for binding to the same response elements when PXR and CAR are concurrently activated by PRG and

estrogens, respectively. These negative cross-talks between PXR and CAR may lead to nonadditive effects of estrogens and PRG on CYP2A6 and CYP2B6. On the other hand, PRG and estrogens show additive (if not synergistic) effects on CYP3A4 induction, suggesting a distinctive underlying mechanism for CYP3A4 induction by female hormones. The underlying mechanism and its clinical significance on the additive effects of estrogens and PRG remain to be identified.

When these results are compared with the clinical observations, increased expression and/or activities of CYP2A6, CYP2C9 and CYP3A4/5 during pregnancy can be, at least in part, explained by increased female hormone levels during pregnancy. E2 and PRG also increased the mRNA expression and activities of CYP2B6, CYP2C8 (PRG) and CYP2E1 (E2) and the clinical significance of this induction remains to be determined. On the other hand, E2 and PRG did not alter the expression and/or activities of CYP2D6, CYP2C19 and CYP1A2, indicating that E2 and PRG are likely not directly responsible for alteration in the activities of these CYP isoforms. Examination of the effects of other pregnancy-specific hormones (such as prolactin or placental lactogen) may shed further light on the factors responsible for altered drug metabolism during pregnancy.

In summary, the effects of E2 and PRG on the expression and activities of CYP were comprehensively characterized using human hepatocytes. Both E2 and PRG regulate CYP expression and activities in an isoform-specific manner via PXR (PRG), CAR (E2), or ER (E2). While further molecular mechanism and clinical studies are needed, our findings provide a basis in understanding altered drug metabolism during pregnancy.

3. Effects of E2 on Cytochrome P450 Expression and Activities in Female Rats

3.1 Materials and methods

3.1.1 Chemicals and reagents.

Resorufin, 7-ethoxyresorufin, dexamethasone (DX), β -naphthoflavone (BNF), estradiol benzoate (EB), potassium fluoride, sodium arsenate, NADP⁺, isocitric acid, magnesium chloride and isocitric acid dehydrogenase were obtained from Sigma (St. Louis, MO). The remaining chemicals and reagents used in this chapter are listed in chapter 2 (2.1.1).

3.1.2 Drug treatment.

Adult female Sprague-Dawley rats weighing 180-200 g (8-week old) were purchased from Harlan (Indianapolis, IN). After one week of acclimation, rats were administered EB (1 mg/kg/day subcutaneous injection), BNF (40 mg/kg/day intraperitoneal injection), PB (60 mg/kg/day intraperitoneal injection), DX (60 mg/kg/day intraperitoneal injection), or vehicle (corn oil subcutaneous injection) [n = 4 for each group except for control and DX groups (n = 3)]. On day 5, the livers were removed and weighed. Microsomes and total RNA were prepared from the liver tissues. In parallel, two separate groups of rats (n = 3 per group) were treated with EB (1 mg/kg subcutaneous injection) for either 1 day or 5 days. After EB administration, 200 μ l of blood was collected at various time points (pretreatment, 0.5, 1, 2, 4, 7, 11, and 24 hr post-injection). Plasma samples were obtained by immediate centrifugation of the blood and 50 μ l of 2 M sodium arsenate and 10 μ l of 50% potassium fluoride/ml plasma (plasma esterase inhibitors) was added.

3.1.3 Pharmacokinetic analysis.

The concentration of E2 in plasma was determined by an enzyme-linked immunosorbent assay (ELISA) kit following manufacturer's protocol (Cayman Chemical, Ann Arbor, MI). The maximum plasma concentration ($C_{\max}$) and the time to reach $C_{\max}$ after injection ($T_{\max}$) were determined by visual examination of the concentration vs. time profile. The area under the curve over the dosing interval ($AUC_{0-24\text{ h}}$) was estimated by using the linear trapezoidal rule. The average plasma concentration (C_{ave}) over the dosing interval (τ , 24 hr) was estimated by using the equation: $C_{\text{ave}} = AUC_{0-24\text{ h}} / \tau$.

3.1.4 Hepatic microsomal assays.

Hepatic microsomes were prepared by differential ultracentrifugation of hepatic tissues as previously described (109). The protein concentration of the prepared microsomes was determined by using BCA Protein Assay kits (Pierce, Rockford, IL), and the total CYP amount was measured by the method of Omura and Sato (110). For microsomal reactions, an NADPH-generating system (1 mM NADP⁺, 5 mM isocitric acid, 0.2 U/ml isocitric acid dehydrogenase, and 5.0 mM MgCl₂) was used. The reactions were initiated by adding NADP⁺ to drug-containing reaction media and terminated by adding three volumes of ice-cold acetonitrile. A control reaction was performed in the absence of NADP⁺. Preliminary experiments were conducted for each substrate to determine the microsomal protein concentration and the incubation time that lead to proportional increases in metabolite production. For each substrate compound, $V_{\max}$ and K_m were determined using Prism 5 software (GraphPad, La Jolla, CA). Statistical analysis was performed using Student's t-test.

3.1.5 Determination of metabolite concentrations.

The microsomal samples were analyzed by LC/MS/MS (Agilent 1200 HPLC

interfaced with Applied Biosystems Qtrap 3200) using an electrospray ion source. The mobile phase consisted of water (0.1% formic acid) and methanol. Separation was performed with a Zorbax Eclipse XDB-C8 column (4.6 × 50 mm, 3.5 μm) (Agilent Technologies, Santa Clara, CA) at a flow rate of 0.4 ml/min. MS detection of metabolites and internal standards was performed in positive ion mode by examining multiple MRM pairs as described in chapter 2. The concentration of resorufin, the metabolite of 7-ethoxyresorufin, was measured by a fluorescence plate reader (Synergy 4) (BioTek, Winooski, VT) at excitation and emission wavelengths of 530 and 582 nm, respectively.

3.1.6 RNA isolation and quantitative real time-PCR (qRT-PCR).

Total RNAs were isolated from liver tissues using Trizol® (Invitrogen, Carlsbad, CA). cDNA was prepared using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. qRT-PCR was performed using StepOnePlus Real-Time PCR System (Applied Biosystems). TaqMan® Gene expression Primers (Applied Biosystems) were used for CYP1A2 (Rn00561082_m1), CYP2B1 (Rn01457880_m1), CYP2D2 (Rn00562419_m1), CYP2E1 (Rn00580624_m1), CYP3A1 (Rn01412959_g1), CYP3A9 (Rn00595977_m1), and β-actin (Rn0066789_m1). Expression levels of CYP2C6, CYP2C7, CYP2C12, pregnane X receptor (PXR), constitutive androstane receptor (CAR), aryl hydrocarbon receptor (AhR), and P450 oxidoreductase (POR) were determined using a SYBR® green expression master mix (Applied Biosystems). The following primers were designed by using Primer 3 software: CYP2C6 (F: 5'-ATGGCAGCCT GCCTCCTCT-3', R: 5'-GGCATGCGGCTCCTGTCC TG-3'), CYP2C7 (F: 5'- TGCCTTTCTC AGCAGGAAAACGAGC-3', R: 5'-ACAAC TGCATGCGGGCCAGG-3'), CYP2C12 (F: 5'- TGATTGGGAGACACCGCAGCC-3', R: 5'-AGGGCATGTGGATCCTGTCCAAC-3'), PXR (F: 5'-GGAGGGCAGGGGCTGACAGA-3', R: 5'-GAAACACCGAGGTAGCCGGA-3'), CAR (F: 5'-

GGCGCCACACTCGTCATGT-3', R: 5'-GCCGGAGGCCTGAACTGCAC-3'), AhR (F: 5'-ATGAGCAGCGGCGCCAACAT-3', R: 5'-ACTGTTTTCTGCACCGGC TTGC-3'), POR (F: 5'-AACCCGCCACGCACCAATGT -3', R: 5'-ACAGCTCCTTGCCCTCGC CT -3'), and β -actin (F: 5'-AAGTCCCTCACCCTCCCAAAAG-3', R: 5'-AAGCAATGCTGTCACCTTCCC-3'). The fold change in mRNA levels of CYP upon drug treatment was determined by normalizing the gene expression levels by those of β -actin ($2^{-\Delta\Delta Ct}$ method).

3.1.7 Western immunoblot analysis.

Liver microsomes were resolved by SDS gel electrophoresis on an 8% polyacrylamide gel (8 μ g microsomal protein/lane). Proteins were transferred to a nitrocellulose membrane (1.5 hr, 300 mA); loading of equal sample amounts was ensured by comparing signals after Ponceau S staining. The membrane was then blocked at room temperature for 1 hr in 5% (w/v) milk in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBST). The membranes were incubated overnight at 4 °C in anti-CYP1A2 (1:1000 in 2% milk powder in TBST) (Chemicon International, Billerica, MA), anti-CYP3A1 (1:1500 in 2% milk in TBST) (Chemicon International), or anti-CYP2C (1:1000 in 2% milk in TBST) (Abcam, Cambridge, MA). Then, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody at room temperature for 1 hr (1:10,000 in 5% milk in TBST) (Abcam). Protein expression was detected by Supersignal[®] West Pico Chemiluminescent substrate (Pierce, Rockford, IL) on Kodak films, and the signals were quantitated by using Adobe Photoshop.

3.2 Results

3.2.1 Effects of E2 on liver size, microsomal protein and CYP contents.

To investigate the effects of E2 on CYP expression and activity, female rats were administered with vehicle, EB, or known inducers of CYP enzymes for 5 days. BNF, PB, and DX were used as prototypical inducers for CYP1A, CYP2C, and CYP3A, respectively. EB treatment did not affect liver size or microsomal CYP contents as compared to the vehicle treatment. However, EB treatment increased the concentration of total protein in the microsome by unknown mechanisms. BNF, DX, and PB increased both the total microsomal proteins and CYP contents, as expected.

TABLE VI

LIVER WEIGHT, MICROSOMAL PROTEIN AND TOTAL CYTOCHROME P450 CONTENTS AFTER DRUG TREATMENT

	<i>Control</i>	<i>EB</i>	<i>BNF</i>	<i>PB</i>	<i>DX</i>
n ^a	3	4	4	4	3
Liver (g)	10.4 ± 1.8	9.2 ± 0.7	11.3 ± 0.5	10.3 ± 0.6	11.9 ± 1.9
Microsomal protein (mg/m)	6.7 ± 0.8	9.6 ± 1.4*	8.3 ± 2.4*	9.3 ± 1.6*	8.1 ± 1.5*
CYP (pmol/mg protein)	0.32 ± 0.10	0.44 ± 0.04	0.78 ± 0.15*	0.45 ± 0.13	0.63 ± 0.24*

^a n is the number of animals used in each group.

* $p < 0.05$ vs. control

3.2.2 Plasma concentration of E2 after EB injection.

Plasma E2 concentration vs. time profiles obtained after a single or multiple doses (over 5 consecutive days) of EB are shown in Fig. 20. A single subcutaneous injection of EB led to a $C_{\max}$ of 1.68 ± 0.45 ng/ml and a $T_{\max}$ of 2 hr. The E2 concentration at 24 hrs (0.34 ± 0.09 ng/ml) was higher than the pretreatment basal level (0.023 ± 0.001 ng/ml; Fig. 20A). After multiple dosing, the $C_{\max}$ was 1.84 ± 0.46 ng/ml and the $T_{\max}$ was 2 hr. The E2 concentration before the 5th dose was 0.83 ± 0.12 ng/ml, showing an accumulation of E2 in the bodies. The average concentration of E2, estimated from the $AUC_{0-24 \text{ h}}$, was also elevated from 0.56 ± 0.15 ng/ml on day 1 to 0.99 ± 0.04 ng/ml on day 5 (Fig. 20B). These concentrations are comparable to the plasma E2 concentrations attainable during human pregnancy.

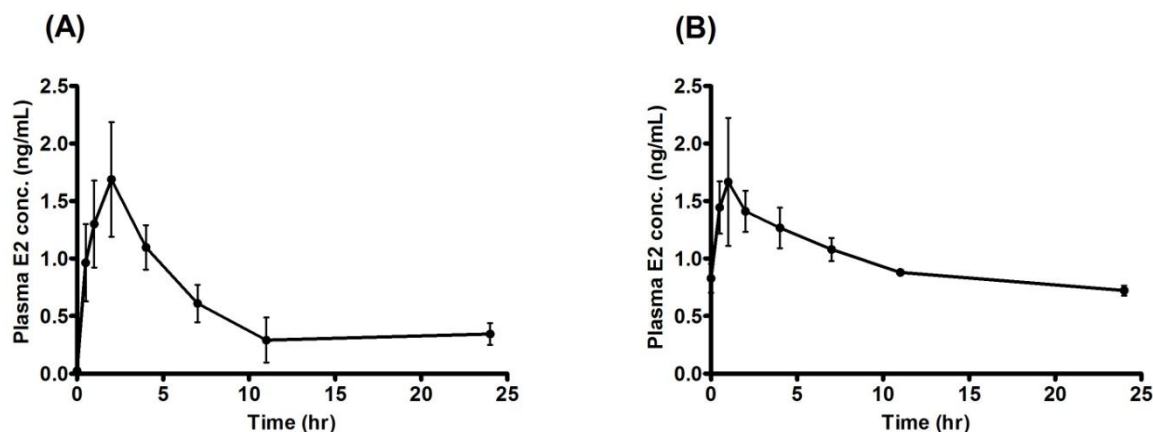


Fig. 20 Plasma concentration of E2 vs. time profile after a single injection (A) and 5-day injections (B) of EB.

Rats were administered with EB (1mg/kg subcutaneous injection), and plasma samples were collected at various time points after EB administration. The concentration of E2 in plasma was determined by ELISA. The values are mean $\pm$ SEM (ng/ml).

3.2.3 Effects of E2 on mRNA expression of major CYPs.

qRT-PCR was performed to investigate the effects of E2 on the mRNA expression of the major hepatic CYPs in rat livers: CYP1A2, CYP2B1, CYP2C6, CYP2C7, CYP2C12, CYP2D2, CYP3A1, CYP3A9 and CYP2E1. The results showed that E2 differentially regulated the expression of individual CYP isoforms (Fig. 21). EB treatment increased the expression of CYP1A2 by 2.0-fold ($p = 0.049$) as compared to vehicle treatment, whereas BNF increased CYP1A2 expression by over 20-fold. EB treatment led to upregulation of CYP2C isoforms by 2.2-, 2.7- and 4.0-fold for CYP2C6, CYP2C7, and CYP2C12, respectively. The induction in CYP2C expression by EB treatment was to a similar extent as the induction by PB (2.7-, 3.0- and 4.6-fold for CYP2C6, CYP2C7, and CYP2C12, respectively). EB treatment also increased CYP3A9 expression by 2.3-fold ($p = 0.007$) while downregulating CYP3A1 expression by 8.0-fold ($p < 0.001$). DX increased CYP3A1 expression (by 2.8-fold) but had no effect on CYP3A9 expression. EB treatment had insignificant effects on the mRNA expression of CYP2B1, CYP2E1, and CYP2D2.

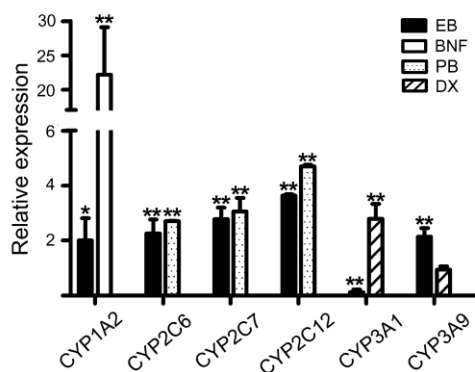


Fig. 21 Effects of E2 on mRNA levels of rat CYPs.

Rats were administered with EB (1 mg/kg/day), BNF (40 mg/kg/day), PB (60 mg/kg/day), DX (60 mg/kg/day), or vehicle (corn oil) for 5 days ($n = 3-4/\text{group}$). mRNA levels were determined by qRT-PCR. Data shown are relative CYP expression as compared to the control group (corn oil).

*, $p < 0.05$; **, $p < 0.01$ vs. control.

3.2.4 Immunoblot analysis.

To determine whether the E2-mediated mRNA changes in CYPs led to corresponding changes in the protein levels, immunoblot analysis was performed for CYP1A2, CYP3A1, and CYP2C. The results showed that EB treatment increased the protein levels of CYP1A2 by 1.4-fold ($p = 0.022$), but to a much smaller extent than the increase observed with BNF (4.0-fold, $p < 0.001$) (Fig. 22A). EB treatment also increased CYP2C protein expression by 3.1-fold ($p < 0.001$), an increase comparable to that by PB (3.5-fold, $p < 0.001$) (Fig. 22B). On the other hand, EB treatment markedly decreased CYP3A1 expression (by 4.8-fold, $p < 0.001$) (Fig. 22C), consistent with the qRT-PCR results. The increased mRNA level of CYP3A9 by EB treatment was not confirmed by immunoblot analysis due to a lack of commercially available antibodies that specifically detect rat CYP3A9.

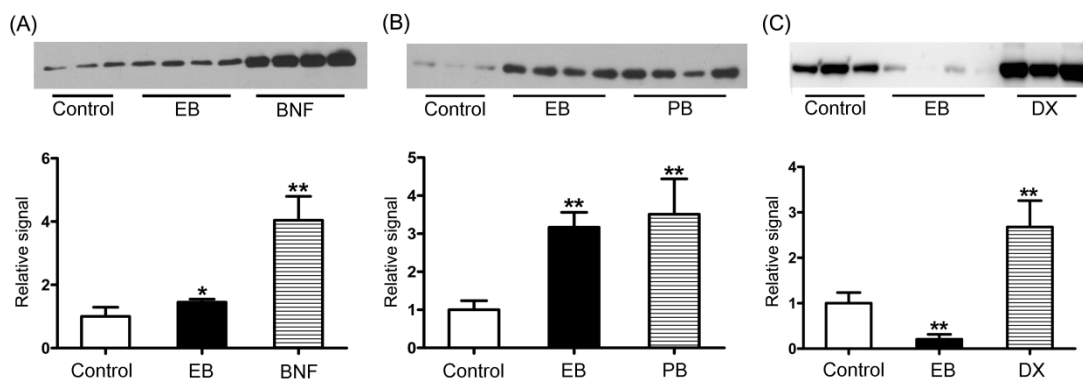


Fig. 22 Effects of E2 on protein levels of CYP1A2 (A), CYP2C (B), and CYP3A1 (C).

Western blot analysis was performed using hepatic microsomes prepared from rats administered with EB (1 mg/kg/day), BNF (40 mg/kg/day), PB (60 mg/kg/day), DX (60 mg/kg/day), or vehicle (corn oil) for 5 days. Eight micrograms of microsome from each treatment group were resolved on SDS-PAGE gel (8%). Results from quantitative analysis of the blots were shown at the bottom panel, expressed as relative signals in comparison with the control group (corn oil).

*, $p < 0.05$; **, $p < 0.01$ vs. control.

3.2.5 Effects of E2 on CYP activities.

To examine the effects of E2 on CYP activities, microsomal assays were performed using the CYP-isoform specific probe substrates: 7-ethoxyresorufin (CYP1A2), diclofenac (CYP2C6/7), bufuralol (CYP2D2), *p*-nitrophenol (CYP2E1) and midazolam (CYP3A). EB treatment increased the $V_{\max}$ and intrinsic clearance (CL_{int}) of 7-ethoxyresorufin *O*-dealkylation activity as compared to the vehicle treatment (by 1.5-fold and 1.7-fold, respectively) (TABLE VII); however, it did not affect the K_m , suggesting that the intrinsic function of CYP1A2 was likely not influenced by EB treatment. The CL_{int} and $V_{\max}$ of *p*-nitrophenol hydroxylation were decreased by EB treatment by 70% and 50%, respectively. EB treatment had insignificant effects on diclofenac 4'-hydroxylation, bufuralol 1'-hydroxylation, and midazolam 1'-hydroxylation activities.

TABLE VII

KINETIC PARAMETERS FOR METABOLITE FORMATION FROM EACH SUBSTRATE

	K_m (μM)	$V_{\max}$ (pmol/min/mg protein)	CL_{int} (ml/min/mg protein)
Ethoxyresorufin <i>O</i> -dealkylation (CYP1A2)			
Control	0.48 ± 0.06	53 ± 2	109 ± 13
EB	0.45 ± 0.05	$80 \pm 2^*$	$183 \pm 37^*$
BNF	$1.35 \pm 0.33^*$	$631 \pm 4^{**}$	$463 \pm 54^{**}$
Diclofenac 4'-hydroxylation (CYP2C6/7)			
Control	18.6 ± 5.5	432 ± 40	23.9 ± 5.2
EB	20.0 ± 4.0	434 ± 27	24.1 ± 8.6
PB	28.0 ± 0.6	$902 \pm 197^*$	$32.3 \pm 7.7^*$
Midazolam 1'-hydroxylation (CYP3A)			
Control	8.0 ± 6.8	178 ± 42	16.1 ± 9.2
EB	10.7 ± 3.0	71 ± 10	7.1 ± 2.4
DX	8.6 ± 4.3	$643 \pm 72^{**}$	$85.2 \pm 20.9^*$
<i>P</i> -Nitrophenol hydroxylation (CYP2E1)			
Control	4.8 ± 1.5	1308 ± 94	296 ± 139
EB	7.8 ± 1.9	$649 \pm 38^*$	$89 \pm 24^*$
Bufuralol 1'-hydroxylation (CYP2D2)			
Control	5.6 ± 1.3	383 ± 30	97 ± 7
EB	4.0 ± 0.8	492 ± 23	120 ± 16

*, $p < 0.05$; **, $p < 0.01$ vs. controlResults are expressed as mean $\pm$ S.D. (n = 3-4/group)

3.2.6 Effects of E2 on modulators of drug metabolism.

PXR, CAR, and AhR are transcriptional regulators that play key roles in modulating CYP expression. On the other hand, POR is the obligate redox partner of CYP enzymes. To explore potential mechanisms underlying E2-mediated regulation of CYP expression and activity, we examined whether EB treatment affects expression of PXR, CAR, AhR and POR. The results from qRT-PCR (Fig. 23) showed that EB treatment downregulated the mRNA levels of PXR (3.4-fold, $p < 0.001$), CAR (2.9-fold, $p < 0.001$), and POR (8.6-fold, $p < 0.001$) as compared to the vehicle treatment, whereas EB treatment had an insignificant effect on AhR expression. These results suggest that in rats, E2 may influence CYP expression and activities by downregulating transcription factors and/or the redox partner.

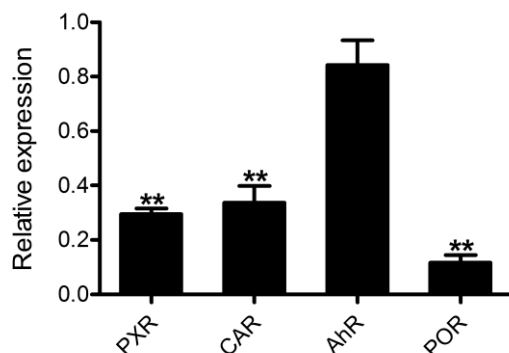


Fig. 23 Effects of E2 on mRNA levels of transcription factors and POR.

Female rats were administered with EB (1 mg/kg/day) or vehicle (corn oil) for 5 days ($n = 3-4$ /group). mRNA expression levels of PXR, CAR, AhR, and POR in the livers were determined by qRT-PCR. Data shown are relative CYP expression as compared to the control group (corn oil).

*, $p < 0.05$; **, $p < 0.01$ vs. control.

3.3 Discussion

Pregnancy influences hepatic drug metabolism in a CYP isoform-specific manner in humans. However, the responsible factors or the underlying mechanisms remain largely unknown. The objective of this study was to determine whether increasing plasma concentration of E2 during pregnancy is potentially responsible for the altered drug metabolism using rats as an *in vivo* model.

In the present study, to achieve the plasma E2 concentrations attainable during human pregnancy, we administered 1 mg/kg of EB by subcutaneous injection into female rats. This led to a $C_{\max}$ and a C_{ave} of 1.84 ± 0.46 ng/ml and 0.99 ± 0.04 ng/ml, respectively (Fig. 20). The C_{ave} corresponds to the plasma E2 level in pregnant women during the first trimester (65), which is 40-fold higher than the baseline E2 concentration in non-pregnant rats and women. Of note, high doses of estrogen are known to negatively influence hepatic functions in rats, e.g., increasing liver sizes and cholestasis (111), which may indirectly affect hepatic drug metabolism. At the dosage used in this study, EB treatment did not change liver weight or CYP concentration in the hepatic microsomes of rats (TABLE VI), suggesting an apparent lack of E2-mediated effects on normal liver physiology.

Our results indicate that E2 modulates CYP expression in an isoform-specific manner: upregulation of CYP1A2, CYP2Cs, and CYP3A9 expression and downregulation of CYP3A1. The increased expression and activity of rat hepatic CYP1A2 by EB treatment are, in part, in agreement with a previous study where activity of rat intestinal CYP1A2 is enhanced by EB treatment (112). The induction of CYP1A2 expression may be attributed to activation of an AhR-mediated regulatory mechanism which is involved in upregulation of hepatic and intestinal CYP1A expression (113). mRNA of NADPH dehydrogenase quinone 1 (NQO1), another representative target gene of AhR signaling pathway (113), was increased by E2 (by

2-fold; data not shown). These results suggest E2 can activate AhR signaling pathway in rats. Based on previous studies and our results from chapter 2, it is unlikely E2 activates AhR pathways in a direct manner (being an AhR ligand in hepatic tissue). Rather, it may potentiate the induction of CYP1A by regulating endogenous (yet to be identified) AhR activators. On the other hand, the induction of CYP1A2 expression in rats by EB treatment did not correspond to the clinically reported reduction in metabolism of CYP1A2 substrates during human pregnancy.

EB treatment significantly upregulated expression of CYP2C isoforms (CYP2C6, CYP2C7, and CYP2C12) (Fig. 21 and Fig. 22B), comparable to the induction observed from phenobarbital treatment. This result appears consistent with previous studies where administration of E2 enhanced expression of CYP2C7 and CYP2C12 in male rats (94) and increased mRNA expression and activity of CYP2C6 in rat hepatocytes (114, 115). The global upregulation of CYP2C shown in our study as well as others suggests that E2 may be responsible for the increased metabolism of CYP2C9 substrates in pregnant women (36, 38). Interestingly, despite significant induction in CYP2C expression by E2, diclofenac 4'-hydroxylation, a marker for CYP2C6 and CYP2C7 activities (116), did not increase in the EB-treated rats as compared to the vehicle-treated rats (TABLE VII). Downregulation of POR expression by E2 (Fig. 23) and subsequent decrease in CYP activity may provide a potential explanation. This is the opposite of the results from primary human hepatocytes studies where activity of CYP2C9 was increased by E2 without changing the level of mRNA, protein expression, or POR. Although their underlying mechanism appear different, both studies (human hepatocytes in chapter 2 and the *in vivo* rat study in this chapter) support that E2 has a significant role on CYP2C9 regulation across the species and is potentially responsible for increased CYP2C9 activity during pregnancy.

EB treatment had differential effects on the expression of CYP3A1 and CYP3A9 (two major female hepatic CYP3A isoforms): marked downregulation of CYP3A1 and upregulation of CYP3A9 (Fig. 21). The opposing effects of E2 on CYP3A1 and CYP3A9 expression are potentially responsible for the minimal changes in CL_{int} of midazolam 1'-hydroxylation (TABLE VII) in the EB-treated group as compared to the control because the reaction is mediated by both CYP3A1 and CYP3A9 enzymes (117). The downregulation of CYP3A1 expression in the EB-treated rats may be attributed to the decreased expression of PXR (Fig. 23), a key transcription factor in modulating CYP3A1 expression (118). On the other hand, CYP3A9 expression is not governed by PXR (119). Interestingly, consistent with our data indicating increased CYP3A9 expression upon EB treatment, CYP3A9 expression has been shown to be female-specific and enhanced by ethinylestradiol in rats (117, 120). These directional changes in CYP3A9 expression are similar to those reported for CYP3A4 expression in pregnant women. The mechanism underlying estrogen-responsive expression of CYP3A9, although currently unclear, may help us determine how E2 is potentially involved in the increased CYP3A4 expression during pregnancy.

Our results show that EB treatment has insignificant effects on the expression and activity of CYP2D6, suggesting a lack of involvement of E2 in the increased metabolism of CYP2D6 substrates in pregnant women. EB treatment also shows minimal effects on CYP2E1 expression while decreasing the V_{max} and CL_{int} of *p*-nitrophenol hydroxylation as compared to the vehicle-treated group. Although *p*-nitrophenol hydroxylation is mainly mediated by CYP2E1 in humans, in rats CYP3A1 also mediates the reaction with an efficiency ~40% that of CYP2E1 (121). Potentially, the decreased *p*-nitrophenol hydroxylation may reflect the reduced CYP3A1 expression in the EB-treated rats. Our immunoblot results also show that the protein levels of CYP2E1 were not affected by EB

treatment (data not shown).

Taken together, we have characterized the *in vivo* effects of E2 on hepatic CYP expression and activities in rats. Our results show that E2 modulates CYP expression in an isoform-specific manner, leading to downregulation of CYP3A1 expression and upregulation of CYP1A2, CYP2C6, CYP2C7, CYP2C12, and CYP3A9. It is of note that E2-mediated CYP regulation in rats is significantly different from that in primary human hepatocytes. Some discrepancies may have resulted from *in vivo/in vitro* differences, while other may be due to species differences. Similar experiments using rat primary hepatocytes may be useful to distinguish the species differences. On the other hand, results from the current study cannot explain the changes in CYP1A2 and CYP2D6 observed during pregnancy which were also not explained by the *in vitro* E2 study in chapter 2. This suggests changes in CYP1A2 and CYP2D6 are likely due to pregnancy-specific factors other than E2. Further studies are required to identify these pregnancy-specific factors.

4. Characterization of Increased Drug Metabolism Activity in DMSO-Treated Huh7 Cells

4.1 Introduction

4.1.1 Liver models used in drug metabolism research: hepatocytes and hepatoma cell lines

Primary human hepatocytes are the “gold standard” system for examining hepatic metabolism of drugs, drug toxicity, and the potential for drug-drug interactions, as well as to investigate progression of liver diseases such as hepatitis B, C (122). However, variability and availability limit its widespread use in research.

On the other hand, hepatoma cell lines have indefinite proliferative capacity and thus could potentially serve as the basis of more practical, alternative experimental systems. However, while different hepatoma cell lines do retain certain liver-specific functions, these cells typically fail to exhibit sufficient hepatocyte function. For example, in HepG2 cells, a hepatocellular carcinoma cell line that has been widely used in the study of liver physiology, the expression levels of various liver-specific drug metabolizing enzymes and transcription factors are low, making them a less than ideal substitute for primary hepatocytes (123-126).

Recently, HepaRG cells have been characterized as a potential *in vitro* hepatocytes model. The HepaRG cell line was derived from a hepatocellular carcinoma. HepaRG cells have been reported to express Phase I and II enzymes at levels comparable to those in human hepatocytes when cultured in the presence of 2% DMSO (127-129). In addition, the expression of drug-metabolizing enzymes in these cells were also shown to be readily inducible by typical enzyme inducers (127), rendering HepaRG as a possible alternative hepatocyte model for drug metabolism studies. However, use of HepaRG has so far been

limited due to its availability and the technical difficulties faced to maintain differentiation (e.g., requiring costly culture media available from the supplier of HepaRG cells).

4.1.2 Potential applicability of Huh7 hepatoma cell line in drug metabolism research

Huh7 is another continuous hepatoma cell line which was established in 1982 from a 57-year-old male with a well-differentiated hepatocellular carcinoma (130). Unlike HepaRG cells, Huh7 cells are widely distributed, commercially available (Japan Health Science Research Resources Bank, cat# JCRB0403), and cheaper to culture. Huh7 cells have been used to investigate the liver toxicity of drugs (131), the molecular mechanisms of hepatic gene regulation and drug efficacy (132-134) as well as the pathogenesis of hepatitis virus infection (135, 136). These cells retain the functional activities of various carbohydrate-metabolizing enzymes and are capable of secreting plasma proteins such as albumin (130); however, despite their widespread use in hepatology research, the expression and function of drug-metabolizing enzymes in Huh7 cells has not yet been extensively characterized.

Interestingly, it was recently shown that Huh7 cells, when incubated in the presence of dimethyl sulfoxide (DMSO), stopped dividing, entered a G0 state, and remained viable in culture without splitting for over 60 days. Under these conditions the Huh7 cells obtained a more “differentiated” hepatocyte state characterized by up-regulation of liver-specific genes such as albumin, transthyretin, HNF4 α , and α 1-antitrypsin (137). This suggests that expression of drug metabolizing enzymes, only highly expressed in differentiated primary hepatocytes but not in hepatoma cell lines, can be upregulated by DMSO-mediated differentiation in Huh7 cells.

4.1.3 Aim of the Study

The ultimate goal of this study is to evaluate Huh7 cells and DMSO-treated Huh 7 cells as an *in vitro* model system for drug metabolism studies, particularly as a substitution of primary human hepatocytes. To this end, the basal level of mRNA expression of major drug metabolizing enzymes (CYPs and UGTs) and their metabolic activities in Huh 7 cells as well as DMSO-treated Huh7 cells were examined. In addition, the inducibility of the major transcriptional regulator target genes (CYP1A1, CYP2B6, and CYP3A4) was examined by qRT-PCR and enzyme catalytic activity study.

4.2 Materials and methods

4.2.1 Chemicals and reagents.

Dextrorphan, levallorphan, caffeine, and paraxanthine were obtained from Sigma (St. Louis, MO). Morphine, morphine 6-glucuronide, and [$^2\text{H}_3$] morphine-3-Glucuronide were purchased from Cerilliant (Round Rock, TX). Dextromethorphan was purchased from Axxora (San Diego, CA). P450-Glo™ CYP1A2 assay system was purchased from Promega (Madison, WI). Lamotrigine and lamotrigine-N2-glucuronide were generous gifts from GlaxoSmithKline (Research Triangle Park, NC). The remaining chemicals and reagents used in this chapter are listed in chapter 2 (2.1.1).

4.2.2 Cells and treatments.

Huh7 cells were cultured in complete DMEM which consists of 10% fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin, 10 mM hepes, 2 mM L-glutamine, and nonessential amino acid. For all DMSO experiments, Huh7 cells were seeded in 12-well collagen-coated BioCoat dishes (BD Biosciences) at a cell density of 8×10^4 cells/well. At 95% confluence, culture medium was replaced with 3 ml complete DMEM containing 1% DMSO (Sigma-Aldrich). Cultures were incubated for 20 days, as previously described (137) during which time complete DMEM containing 1% DMSO was replenished every three days. Non-DMSO treated Huh7 cells were seeded 24 hr prior to use at a cell density of 2×10^4 cells/well in 12-well BioCoat dishes in 2 ml complete DMEM. For comparison of gene expression in Huh7 cells with primary human hepatocytes, pooled hepatocytes were purchased from Celsis (Baltimore, MD; catalog number X008052; 5 donors of mixed gender).

4.2.3 Isolation of RNA and Quantitative Reverse Transcription Real-Time (RT) PCR.

Total cellular RNA was isolated from Huh7 cells as well as from pooled human hepatocytes using Trizol (Invitrogen). One µg of RNA was used for cDNA synthesis using TaqMan reverse transcription reagents (Applied Biosystems) followed by real-time PCR quantification using an Applied Biosystems 7300 real-time thermocycler (Applied Biosystems). Thermal cycling consisted of an initial denaturation step for 10 min at 95 °C followed by 40 cycles of denaturation (15 s at 95 °C) and annealing/extension (1 min at 60 °C). Expression levels were estimated using the $\Delta\Delta C_t$ method and normalized to β -actin. $\Delta\Delta C_t$ was transformed into fold-induction (compared to non-DMSO treated, growing Huh7 cells) with the following formula: fold change = $2^{-\Delta\Delta C_t}$. The PCR primers used are listed in TABLE VIII.

TABLE VIII
PRIMER SEQUENCES FOR SEMI-QUANTITATIVE RT-PCR.

Gene	Forward (5' to 3')	Reverse (5' to 3')
CYP1A1	TCC-AGA-GAC-AAC-AGG-TAA-AAC-A	AGG-AAG-GGC-AGA-GGA-ATG-TGA-T
CYP1A2	GCT-TCT-ACA-TCC-CCA-AGA-AAT	TCC-CAC-TTG-GCC-AGG-ACT
CYP2B6	ATG-GGG-CAC-TGA-AAA-AGA-CTG-A	AGA-GGC-GGG-GAC-ACT-GAA-TGA-C
CYP2C8	AGA-TCA-GAA-TTT-TCT-CAC-CC	AAC-TTC-GTG-TAA-GAG-CAA-CA
CYP2C9	CAG-ATC-TGC-AAT-AAT-TTT-TCT-C	CTT-TCA-ATA-GTA-AAT-TCA-GAT-G
CYP2C19	TTG-AAT-GAA-AAC-ATC-AGG-ATT-G	GAG-GGT-TGT-TGA-TGT-CCA-TC
CYP2D6	GGT-GTG-ACC-CAT-ATG-ACA-TC	CTC-CCC-GAG-GCA-TGC-ACG
CYP3A4	CCA-AGC-TAT-GCT-CTT-CAC-CG	TCA-GGC-TCC-ACT-TAC-GGT-GC
CYP3A5	AGT-GTT-CTT-TCC-TTC-ACT-TTA	TTC-AAC-ATC-TTT-CTT-GCA-AGT
UGT1A1	GGC-CTC-TCT-CCT-CTC-ATT-CA	GGA-ATT-CTG-AGG-CAA-GGG-TT
UGT1A4	TAC-CCT-CTG-GCC-CTG-TCC-TA	GAA-CAG-CCA-CAC-GGA-TGC
UGT1A6	GCA-GAA-GCC-CAG-ACC-CT	GGG-CTC-CAA-CAA-ATT-AAC-AA
UGT1A9	GGT-TGT-AGT-CAT-GCC-AGA-GG	ACT-CCC-GGT-CCA-GAT-CC
UGT2B7	CTG-GGG-TCA-ATG-GTC-AGT-AA	GAA-CCT-TTT-GTG-GGA-TCT-GG
CAR	AGT-GCT-TAG-ATG-CTG-GCA-TGA-GGA	TGC-TCC-TTA-CTC-AGT-TGC-ACA-GGT
PXR	CAA-GCG-GAA-GAA-AAG-TGA-ACG	CTG-GTC-CTC-GAT-GGG-CAA-GTC
AhR	GGC-CGT-GTC-GAT-GTA-TCA-GTG	GTA-CTG-GAT-TGT-ACT-GCA-TCT-GAC
RXR α	AAG-GAC-CGG-AAC-GAG-AAT-GA	ATC-CTC-TCC-ACC-GGC-ATG-T
β -actin	ATC-CTG-GCC-TCG-CTG-TCC	CTC-CTG-CTT-GCT-GAT-CCA-CAT
Albumin	CGC-CTG-AGC-CAG-AGA-TTT-C	GCC-CTG-TCA-TCA-GCA-CAT-TC
TTR	CCG-GTG-AAT-CCA-AGT-GTC-CT	GCA-CGG-CCA-CAT-TGA-TG
HNF4 α	ACA-TTC-GGG-CGA-AGA-AGA-TT	ACT-TGG-CCC-ACT-CAA-CGA-G
A1AT	TGC-TGC-CCA-GAA-GAC-AGA-TA	GGC-GGT-ATA-GGC-TGA-AGG

CAR; constitutive androstane receptor, PXR; pregnane X receptor, AhR; aromatic hydrocarbon receptor, RXR α ; retinoid X receptor, TTR; transthyretin, HNF4 α ; hepatocytes nuclear factor 4 α , A1AT; α 1-antitrypsin

4.2.4 Determination of drug-metabolizing enzyme activities.

Various probe drugs were added to growing or DMSO-treated Huh7 cells and culture media were sampled at various time points for up to 48 hr post treatment. For all metabolic pathways, the concentration of metabolites was determined by LC/MS/MS (Agilent 1200 HPLC interfaced with Applied Biosystems Qtrap 3200) using an electrospray ion source. The mobile phase consisted of water [0.1% (v/v) formic acid] and methanol. For most analytes, separation was performed with a Zorbax Eclipse XDB-C8 column (4.6 × 50 mm, 3.5 µm; Agilent Technologies) at a flow rate of 0.4 ml/min. For morphine, Eclipse XDB-18 (4.6 × 50 mm, 3.5 µm; Agilent Technologies) was used at a flow rate of 0.6 ml/min. MS detection of the parent drugs and metabolites was followed by examining multiple MRM pairs in the positive ion mode. At the end of the 48-hr experiment, the cells were trypsinized and cell numbers were counted to normalize the rate of metabolite formation. The limit of quantification for most analytes was 1 ng/ml.

For enzyme induction studies, growing or DMSO-treated Huh7 cells were incubated with enzyme inducers [rifampin (10 µM), 3-MC (1 µM), or CITCO (100 nM)] or vehicle control (DMSO, in a final concentration of 0.1%) for 48 hr. Then, the media were changed to contain caffeine, S-mephenytoin, or midazolam. Rate of metabolite formation was determined by measuring concentrations of relevant metabolites in the media by using LC/MS/MS as described above.

4.3 Results

4.3.1 Baseline expression of drug-metabolizing enzyme genes in Huh7 cells.

Huh7 cells have been widely used in hepatology research, including drug metabolism studies such as measuring drug toxicity and determining the molecular mechanism of drug metabolizing enzyme regulation. However, the basal expression of drug metabolizing enzymes in Huh7 cells has not been extensively characterized yet. In this study, the basal expression of the major hepatic drug metabolizing enzymes in Huh7 cells were determined and compared to expression levels in human hepatocytes. To this end, total RNAs were prepared from Huh7 cells and human hepatocytes, and qRT-PCR was performed for major CYPs (CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and 3A5) and UGTs (UGT1A1, UGT1A4, UGT1A6, UGT1A9 and UGT2B7).

The results showed that the mRNA expression levels in Huh7 cells are low as compared to the levels in human hepatocytes. The mRNA expression levels of CYPs were less than 1% of the level of mRNA in primary human hepatocytes, except for CYP1A1 (25%). Likewise, the mRNA levels of Phase II enzymes were low, ranging from 0.74 to 1.7% of the mRNA content in hepatocytes (Fig. 24).

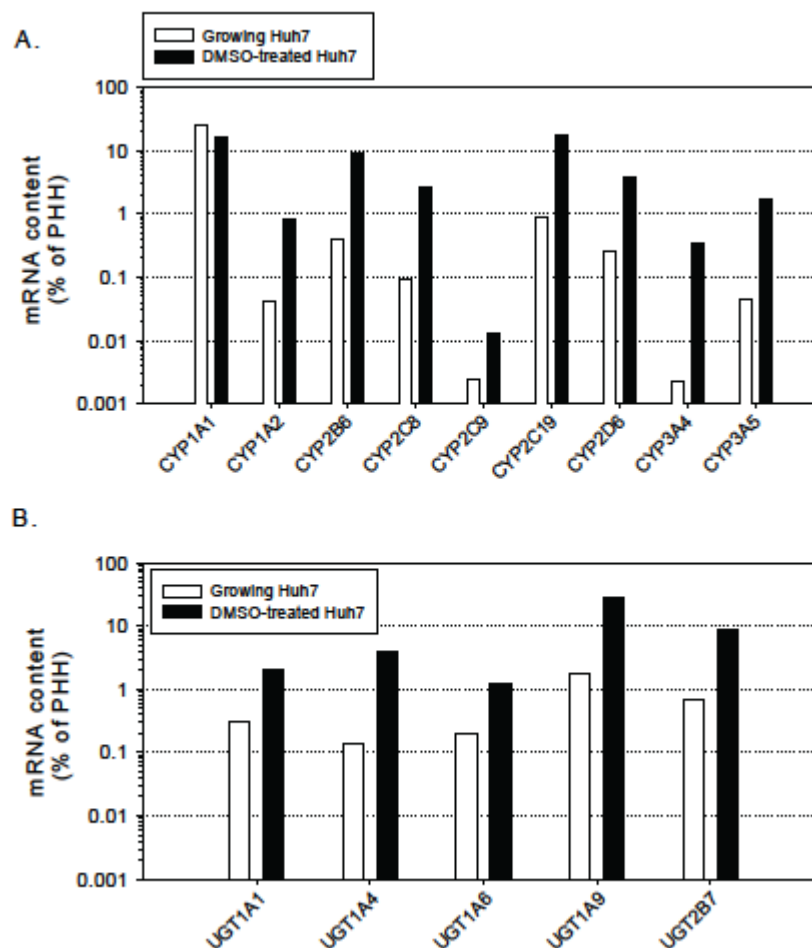


Fig. 24 Expression of CYP (A) and UGT (B) in growing and DMSO-treated Huh7 cells.

Total RNA was isolated from growing Huh7 cells, DMSO-treated Huh7 cells and pooled human hepatocytes. Specific mRNA levels were determined by qRT-PCR, and normalized by β -actin. Results are expressed as the percentage of mRNA content of each enzyme in growing or DMSO-treated Huh7 as compared to the mRNA content in pooled human hepatocytes.

4.3.2 DMSO-mediated induction of drug-metabolizing enzyme expression.

DMSO-treatment in Huh7 cells (1% over 20 days) leads to differentiation and up-regulation of liver-specific genes such as albumin. To examine whether the mRNA expression levels of CYPs and UGTs in DMSO-treated Huh7 cells are up-regulated, qRT-PCR was performed.

DMSO-treatment increased the mRNA levels of most CYP and UGT enzymes as compared to the levels in growing Huh7 (no DMSO-treatment; control), except no enhancement was found for the already relatively highly expressed CYP1A1. The extent of up-regulation was isoform-specific; the expression of CYP3A4 and CYP3A5 was up-regulated by 155-fold and 38-fold respectively while the expression of CYP2C9, UGT1A1 and UGT1A6 was up-regulated by less than 10-fold (5.5-, 6.5-, and 6.5-fold, respectively). The extent of up-regulation of the remaining isoforms ranged from 13.7-fold (UGT2B7) to 27.9-fold (CYP2C8). When the mRNA expression levels in DMSO-treated Huh7 cells are compared with the levels in primary human hepatocytes, the expression level is still relatively low, but some isoforms reached near or more than 10% of the mRNA levels in primary human hepatocytes (CYP1A2, CYP2B6, CYP2C9, UGT1A9, and UGT2B7).

4.3.3 Increased activities of CYPs and UGTs in DMSO-treated Huh7 cells

To examine whether DMSO-treatment in Huh7 cells lead to increase in enzymatic activities of CYPs and UGTs, the metabolic activities of major CYPs (CYP1A1/2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and UGT1A4) were examined in growing Huh7 cells and DMSO-treated Huh7 cells. To this end, isoform-specific probe substrates were added to Huh7 cells, and metabolite production rates (pmol/min/million cells) were determined.

DMSO-treatment led to an increase in the activities of CYP2C9, CYP2D6, CYP3A4 and UGT1A4. For these isoforms, the metabolites were undetectable in growing Huh7 cells for 24 hr while DMSO-treatment showed significant accumulation of metabolites all above the quantitation limits starting from 1 hr after substrate treatment. Despite the relatively high mRNA expression in DMSO-treated Huh7 cells, the metabolic activities of CYP2B6 and CYP2C19 were not detectable in both growing Huh 7 and DMSO-treated Huh 7 cells, potentially due the inhibitory effects of DMSO on the reaction (138). Unexpectedly, metabolite production rates of CYP1A1/2 (Caffeine N-demethylation) appeared to be decreased in DMSO-treated Huh7 cells as compared to the growing Huh7 cells. However, further characterization of CYP1A2 activities in these cells using luciferin-ME indicated an enhanced CYP1A2-mediated metabolite production in the DMSO-treated Huh7 cells (Data not shown).

The metabolic activities in DMSO-treated cells were also compared to the activities in primary hepatocytes. Similarly to the mRNA results, the activities of CYP1A1/2, CYP2C9, CYP2D6, CYP3A4, and UGT1A4 were significantly low as compared to activities in primary hepatocytes (0.5-6.7%, 0.3-6.6%, 0.2-1.7%, 8.9-65%, and 21% respectively, as compared to activities in primary human hepatocytes).

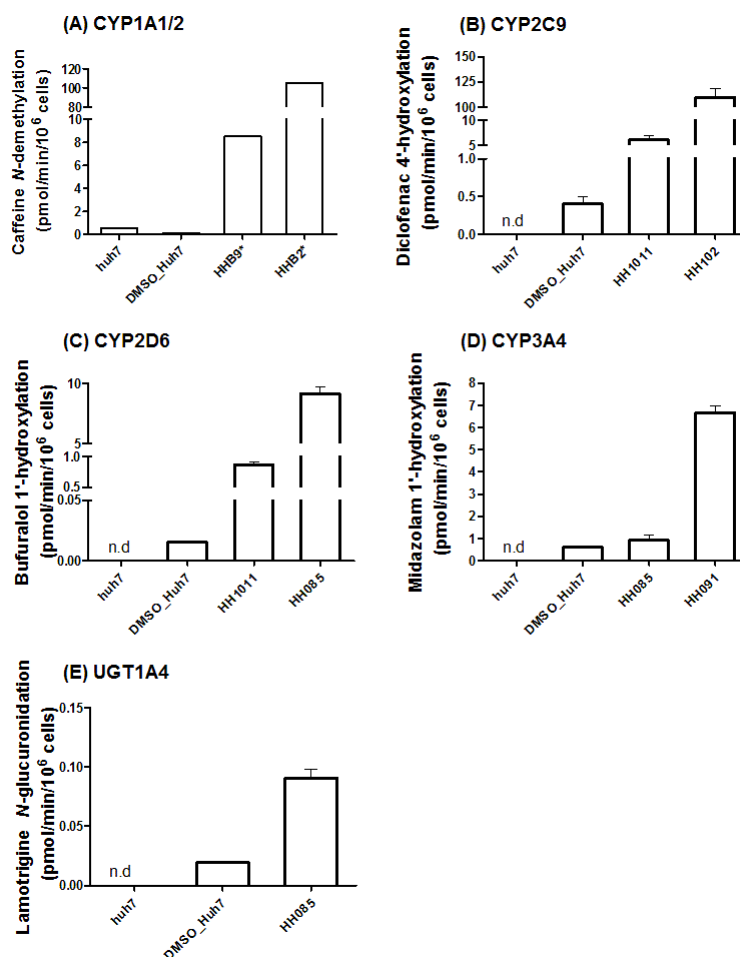


Fig. 25 Catalytic activities of major drug-metabolizing enzymes in growing and DMSO-treated Huh7 cells.

Growing or DMSO-treated Huh7 cells were incubated with various probe drugs. The media were sampled at the indicated time points and metabolic activities of CYP1A1/2, CYP2C9, CYP2D6, CYP3A4, and UGT1A4 were determined by measuring metabolite formation rates of probe substrates (75 μ M caffeine, 100 μ M diclofenac, 60 μ M bufuralol, 15 μ M midazolam, and 40 μ M lamotrigine, respectively). The metabolic activities of those isoforms were obtained in several batches of human hepatocytes as described in chapter 2. The results are expressed as the rates of metabolite formation, expressed in pmol/10⁶ cells/min.

4.3.4 Baseline expression of liver specific receptors and other differentiation-associated genes.

Expression levels of transcription regulators involved in regulation of drug metabolizing enzymes (AhR, CAR, PXR, and RXR α), and four common markers of liver differentiation (albumin, transthyretin, HNF4 α , and α 1-antitrypsin) were determined in growing and DMSO-treated Huh7 cells and compared to levels in primary human hepatocytes (Fig. 26).

DMSO treatments led to an increase in the mRNA levels of all the nuclear receptors. The increase was most dramatic for CAR which showed a 92-fold enhancement of its expression in DMSO-treated cells; however, the transcript level was still significantly lower compared to that detected in primary human hepatocytes. The DMSO-mediated induction of PXR was 2-fold, also significantly low compared to the expression level in primary human hepatocytes. On the other hand, the expression levels of AhR in DMSO-treated Huh7 cells were comparable to those in primary human hepatocytes. The expression levels of RXR α , which were only marginally higher in primary human hepatocytes relative to the growing Huh7 cells, was not induced by DMSO-treatment (Fig. 26A). The expression levels of the hepatocyte differentiation markers were comparable to those detected in the primary human hepatocytes confirming the effectiveness of our DMSO treatment (Fig. 26B).

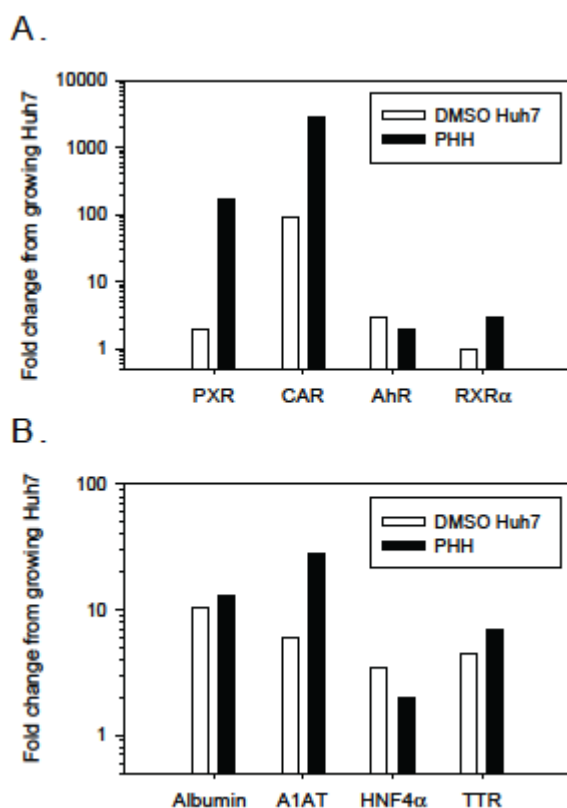


Fig. 26 Expression levels of hepatic transcription factors in Huh7 cells.

Expression levels of transcription factors (A) and liver-specific genes (B) were compared among Huh7 cells (growing and DMSO-treated) and pooled human hepatocytes (PHH), by qRT-PCR. Results are expressed as fold changes of the gene expression relative to growing Huh7 cells.

PXR: pregnane X receptor, CAR: constitutive androstane receptor, AhR: aromatic hydrocarbon receptor, RXR α : retinoid X receptor α , A1AT: α 1-antitrypsin, HNF4 α : hepatocyte nuclear factor 4 α , TTR: transthyretin

4.3.5 Effects of enzyme inducers on expression of drug-metabolizing enzymes.

We observed the up-regulation of CYP transcription regulators by DMSO treatment. To examine whether upregulation of CYP transcriptional regulators results in regulation of their target genes (CYP1A1, CYP2B6, and CYP3A4, respectively) upon inducer treatment,

prototypical inducers (3-MC for AhR, CITCO for CAR, and rifampin for PXR) were added to Huh7 cells, and the mRNA expression level and metabolic activities were determined.

3-MC exhibited the greatest effect among the three inducers (Fig. 27A), as expected from the relatively high AhR expression levels in the Huh7 cells (both growing and DMSO-treated) compared to primary human hepatocytes (Fig. 26A). The mRNA levels were increased by 9.4- and 22.7-fold in growing and DMSO-treated Huh7 cells, respectively; Rifampin led to a marginal induction in the expression of CYP3A4 in both growing and DMSO-treated Huh7 cells (by 2-fold). Despite the significant induction of CAR (92-fold) in DMSO-treated Huh7 cells, the mRNA expression of CYP2B6 was not induced by CITCO.

The metabolic activities of CYP1A1/2, CYP2B6, and CYP3A4 showed similar results; the activity of CYP1A1/2 (caffeine N-demethylation) was enhanced by 3-MC treatment in DMSO-treated Huh7 cells (Fig. 27B). On the other hand, the activity of CYP3A4 (midazolam 1-hydroxylation) was not altered by rifampin treatment (Fig. 27C). S-nirvanol, a CYP2B6-mediated metabolite of S-mephenytoin, was undetectable in the media before and after CITCO treatment in DMSO-treated Huh7 cells (data not shown).

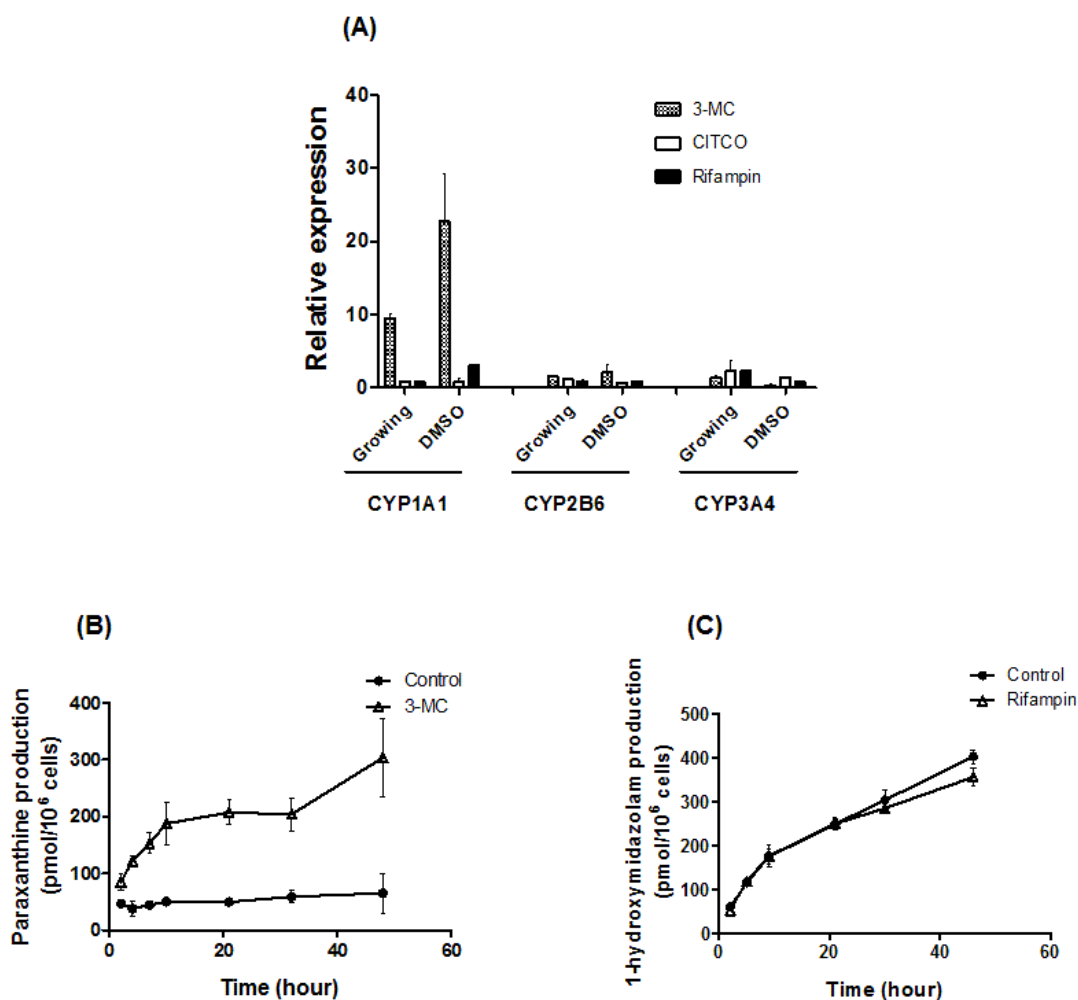


Fig. 27 Effects of prototypical enzyme inducers on mRNA (A) and catalytic activity levels of CYPs (B,C) in growing and DMSO-treated Huh7 cells.

(A) Growing and DMSO-treated Huh7 cells were treated with 3-MC (AhR ligand, 1 μ M), CITCO (CAR ligand, 100 nM), rifampin (PXR ligand, 10 μ M), or vehicle (DMSO in a final concentration of 0.1%) for 48 hr. mRNA levels of CYP1A1, CYP2B6, and CYP3A4 (target genes of AhR, CAR, and PXR, respectively) were determined by real-time PCR. Relative expression was calculated by comparing β -actin-normalized mRNA levels of the relevant genes between the vehicle-treated and inducer-treated groups. (B, C): DMSO-treated Huh7 cells were incubated with 3-MC or rifampin, and the differences in metabolite production were determined. Production of paraxanthine from caffeine (CYP1A1/2; B) and 1-hydroxymidazolam from midazolam (CYP3A4; C) are shown.

4.4 Discussion

The use of primary human hepatocyte, although it is the closest system to the liver physiologically, is limited by their scarce availability, inherent donor-based variability, and inconsistent differentiation state. On the other hand, low expression levels of drug metabolizing enzymes in the more accessible, stable hepatoma cell lines limit their use in drug metabolism studies. Recently, it was reported that DMSO treatment of the widely used Huh7 hepatoma cells induces expression of four differentiation markers of the liver (137). Here, we characterized the baseline functions of drug metabolizing enzymes in the widely used Huh7 hepatoma cell line, as well as parallel DMSO-treated Huh7 cells.

The baseline mRNA expression levels of most CYP and UGT genes investigated were very low in growing Huh7 as compared to the levels of primary human hepatocytes, consistent with previous studies (139, 140). DMSO treatment of the cells significantly enhances xenobiotic metabolism and the inducibility of some major drug metabolizing enzymes by known enzyme inducers. This validates that DMSO significantly increases the state of Huh7 cell differentiation both at the level of gene expression and function. This also suggests that DMSO-treated Huh7 cells might serve as an improved *in vitro* hepatocyte model for a wide range of studies in which hepatocyte physiology is of relevance (e.g. hepatitis C virus-host cell interactions, hepatocyte differentiation-specific gene expression and drug-drug interaction studies). Notably, however, it was clear that CYP and UGT expression was still higher (~10-fold or more) in the primary human hepatocytes leaving primary human hepatocytes as the “gold standard” for drug metabolism studies.

Interestingly, the basal expression level of CYP1A1 in growing Huh7 cells was relatively high, about 25% of the mRNA level of CYP1A1 in human hepatocytes. Also,

CYP1A1 expression was not influenced by DMSO treatment in Huh7 cells, as were the other CYP enzymes which showed significant increases in their expression in DMSO-treated Huh7 cell cultures (Fig. 24). Relatively high expression level of CYP1A1 is also observed in HepG2 cells, another most commonly used hepatocarcinoma cell lines (141). These findings suggest that expression of CYP1A1 is not governed by differentiation state of the hepatocytes. Consistent with this idea, a previous report did note that after isolation, hepatocytes maintained CYP1A1 expression in contrast to expression of other major CYP enzyme, such as CYP3A4, which were rapidly lost (142).

The basal expression level of transcriptional regulators (AhR, CAR, and PXR) and DMSO-mediated effects on their expression show differential effects on the regulation of transcriptional regulators. In growing Huh7, CAR showed the lowest expression level, less than 0.1 % of the expression level in human hepatocytes. DMSO-treatment lead to dramatic increase expression in CAR, nearly a 100-fold induction compared to the basal expression in growing Huh7. CAR has been shown to be a very sensitive differentiation marker in hepatocytes (139). Therefore, dramatic increases in CAR additionally confirm the effects of DMSO on differentiation of hepatoma cell lines. However, treatment of CAR specific agonist (CITCO) failed to induce mRNA of CYP2B6 (a representative CAR target gene) in DMSO-treated Huh7. The metabolic activity of CYP2B6, formation of S-nirvanol, was below the detection level in both groups as well. It is unclear at this point why CITCO did not induce CYP2B6 despite a dramatic increase of CAR. Potentially, it is due to the fact that the CAR expression level in DMSO-treated Huh7 was still too low (< 10%) compared to the basal expression level in human hepatocytes. Another possibility is that other transcriptional factors that play a role in the CAR-signaling pathway are not properly expressed in DMSO-growing Huh7 cells. In contrast, the expression level of PXR was marginally increase (~2-

fold) by DMSO-treatment in Huh7 cells. Accordingly, the expression and activities of CYP3A4 (the representative target gene of the PXR signaling pathway) were not induced by rifampin (a prototypical PXR activator). Interestingly, AhR, the major transcriptional regulator of the CYP1A family, has higher basal expression level in growing Huh7 cells (~50% of hepatocytes). Accordingly, 3-MC (AhR activator) treatment lead to a 9-fold induction in the mRNA expression level in growing Huh7 cells. DMSO-treatment enhanced the expression of AhR comparably to the expression level in human hepatocytes. In DMSO-treated Huh7 cells, treatment of 3-MC induced the mRNA expression level and activity of CYP1A1 more than 20-fold. This result suggests the potential use of Huh7 cells as a model for AhR-mediated drug metabolism induction studies. The extent of induction is comparable to the level of induction typically observed in primary human hepatocytes (6 to 20-fold) (27, 96).

In summary, we characterized the baseline expression and function of drug metabolizing enzymes in Huh7 cells and demonstrated that DMSO treatment of Huh7 cells induces their capability to metabolize drugs. Our results indicate that a highly differentiated state can be achieved in Huh7 cells by DMSO treatment with the expression of liver specific genes, such as drug-metabolizing enzymes and liver-enriched transcription factors. When compared to primary hepatocytes, the activities and expression levels of most drug metabolizing enzymes in DMSO-treated Huh7 cells were still significantly lower, limiting the utility of Huh7 cells for drug metabolism studies, except for the AhR-signaling pathway. Still, the enhanced expression of drug metabolism enzymes as well as the differential regulation of major drug-metabolizing enzymes and key transcription factors in the DMSO-treated Huh7 cell culture system makes them a potential improved *in vitro* hepatocyte model for a variety of other studies such as: (1) metabolite profiling of new drug candidates, (2) characterization of potential drug-drug interactions, especially ones involving AhR, (3) cytotoxicity testing of

bioactivated compounds for both short term and long term effects (the plated DMSO-treated Huh7 cells are viable for over a few months), (4) investigation of hepatocyte differentiation process and molecular details of certain hepatocyte-specific functions.

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