

# Human Placental Lactogen Induces CYP2E1 Expression via PI3-Kinase Pathway in Female Human Hepatocytes<sup>§</sup>

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## ABSTRACT

The state of pregnancy is known to alter hepatic drug metabolism. Hormones that rise during pregnancy are potentially responsible for the changes. Here we report the effects of prolactin (PRL), placental lactogen (PL), and growth hormone variant (GH-v) on expression of major hepatic cytochromes P450 expression and a potential molecular mechanism underlying CYP2E1 induction by PL. In female human hepatocytes, PRL and GH-v showed either no effect or small and variable effects on mRNA expression of CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5. On the other hand, PL increased expression level of CYP2E1 mRNA with corresponding increases in CYP2E1 protein and activity levels. Results from hepatocytes and HepaRG cells indicate that PL does not affect the expression or

activity of HNF1 $\alpha$ , the known transcriptional activator of basal CYP2E1 expression. Furthermore, transient transfection studies and Western blot results showed that STAT signaling, the previously known mediator of PL actions in certain tissues, does not play a role in CYP2E1 induction by PL. A chemical inhibitor of PI3-kinase signaling significantly repressed the CYP2E1 induction by PL in human hepatocytes, suggesting involvement of PI3-kinase pathway in CYP2E1 regulation by PL. CYP2E1-humanized mice did not exhibit enhanced CYP2E1 expression during pregnancy, potentially because of interspecies differences in PL physiology. Taken together, these results indicate that PL induces CYP2E1 expression via PI3-kinase pathway in human hepatocytes.

## Introduction

Cytochrome P450 (P450) enzymes are responsible for eliminating approximately 75% of marketed drugs, and pregnancy is known to alter P450-mediated hepatic drug metabolism. Results from clinical studies indicate that during pregnancy, elimination of drugs metabolized by CYP2A6, CYP3A4, CYP2D6, and CYP2C9 is increased, whereas elimination of the CYP1A2 and CYP2C19 substrate drugs is decreased (Hodge and Tracy, 2007). Pregnancy is also accompanied by increases in plasma concentrations of multiple hormones including estrogen, progesterone, and corticosteroids, and these hormones may be in part responsible for altered drug metabolism during pregnancy. For example, results from previous studies indicate that rising concentrations of estrogen and progesterone may lead to increased elimination of CYP2B6 and CYP3A4 substrate drugs during pregnancy (Koh et al., 2012; Choi et al., 2013; Papageorgiou et al., 2013).

Prolactin (PRL), placental lactogen (PL), and growth hormone variant (GH-v) are peptide hormones belonging to the growth hormone

family, and their plasma concentrations increase during pregnancy. For example, plasma concentration of PRL rises >10-fold during pregnancy compared with the basal level in nonpregnant women. PL and GH-v are pregnancy-specific somatotropins produced from placenta. PL secretion rate is about 1.0 g/day at term, a rate considerably greater than that of any other peptide hormones (Handwerger and Freemark, 2000). GH-v is the predominant growth hormone in the systemic circulation of pregnant women because of decreased pituitary production of native growth hormone during pregnancy. These peptide hormones share similar genetic, structural, binding, and functional properties (Handwerger and Freemark, 2000). PRL, PL, and GH-v show 16, 84, and 93% amino acid homology to the pituitary growth hormone, respectively. The major biologic function of GH-v and PL is to modulate nutrient metabolism (Handwerger and Freemark, 2000), whereas prolactin is primarily involved in mammary gland development and lactation.

In the rat liver, PRL has been shown to modulate drug elimination. In ovariectomized rats, ovine PRL increases the activity of glutathione S-transferase (Luquita et al., 1999) and UDP-glucuronosyltransferase (Luquita et al., 1996). However, significant interspecies differences in amino acid sequences and biologic activities of PRL (Forsyth and Wallis, 2002; Ben-Jonathan et al., 2008) render extrapolation to humans difficult. GH-v was recently shown to have insignificant effects on the expression and activity of the major drug-metabolizing enzyme

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**ABBREVIATIONS:** DCoH, dimerization cofactor for hepatocyte nuclear factor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GH-v, growth hormone variant; HNF1 $\alpha$ , hepatocyte nuclear factor 1 $\alpha$ ; IL, interleukin; P450, cytochrome P450; PBS, phosphate-buffered saline; PL, placental lactogen; PRL, prolactin; PRLR, prolactin receptor; qRT-PCR, quantitative real-time polymerase chain reaction; STAT, signal transducer and activator of transcription; TBST, Tris buffered saline containing 0.1% Tween 20.

CYP3A4 in human hepatocytes (Papageorgiou et al., 2013), but whether GH-v influences expression of other P450 isoforms is unclear. The effects of PL on human P450 expression and activity are completely unknown.

The biologic action of GH-v and PRL is initiated by hormone binding to the growth hormone receptor or the PRL receptor (PRLR), respectively, on cell membranes. Of these two membrane receptors, PL is known to bind preferentially to PRLR (Handwerger and Freemark, 2000). The hormone binding to the receptor triggers activation (i.e., phosphorylation) of a number of signaling proteins, including signal transducers and activators of transcription (STAT). A previous study showed that STAT5a and STAT5b were activated by ovine PRL, mouse PL, and rat growth hormone in primary rat hepatocytes (Cao et al., 2001).

In this study, we examined whether PRL, PL, and GH-v modulate expression and/or activity of major drug-metabolizing P450 enzymes in human hepatocytes. The results demonstrated that PL significantly induces hepatic expression of CYP2E1. Potential molecular mechanisms underlying CYP2E1 induction by PL are presented.

### Materials and Methods

**Reagents.** PRL, PL, and GH-v were obtained from the National Hormone and Peptide Program (Torrance, CA). Antibodies against CYP2E1 and  $\beta$ -actin were purchased from ProteinTech (Chicago, IL) and Sigma-Aldrich (St. Louis, MO), respectively. Antibodies against phosphorylated STAT1, 3, 6, phosphorylated AKT, and native AKT were purchased from Cell Signaling Technology (Danvers, MA). Chlorzoxazone, 6-hydroxychlorzoxazone, and interleukin (IL)-4 were purchased from Sigma-Aldrich. INF $\gamma$  and IL-6 were purchased from Roche Applied Sciences (Indianapolis, IN). Wortmannin, rapamycin, Ro 31-8220, U0126, and SB202190 were purchased from Toronto Research Chemical (Ontario, Canada).

**Animals.** CYP2E1-humanized transgenic mice were previously characterized (Cheung et al., 2005). All mice were housed in temperature-controlled animal facilities with 12-hour light/12-hour dark cycles and were permitted consumption of tap water and standard rodent chow ad libitum. Adult female (8–10 weeks) mice were mated with male mice of similar age. The 2nd day after mating was assigned as gestational day 1 for the mice showing sperm plug. At gestational days 7 and 17 and 30 days after delivery, the female mice were euthanized, and liver tissues were collected. Age-matched virgin female mice were used as controls (prepregnancy). All tissues were gently washed in ice-cold phosphate-buffered saline (PBS) and then stored at  $-80^{\circ}\text{C}$  before use. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago.

**Plasmids.** A luciferase reporter construct containing 1.4-kb upstream regulatory region of human CYP2E1 gene (pGL3-CYP2E1 U1.4kb) was a generous gift from Dr. Magnus Ingelman-Sundberg (Wang et al., 2010). Expression constructs for STAT5a (pCI-STAT5a), PRLR (pcDNA3.1-PRLR), and pGL4-CISH were kindly provided by Drs. Warren Leonard (National Heart, Lung, and Blood Institute), Maria Dufau (National Institute of Child Health and Human Development), and Charles V. Clevenger (Northwestern University), respectively. Expression construct for HNF1 $\alpha$  (pcDNA3.1/HisC WT-HNF1 $\alpha$ ) was a generous gift from Dr. Barbara Triggs-Raine (The Manitoba Institute of Child Health). Four copies of the HNF1 $\alpha$  response element (5'-GGTTA ATATT TACCG GTTAA TGATT ACCGG TTAATC TTAA CCGTT AATTA TTAAC-3') were cloned into pGL3 vector (Promega, Madison, WI) proximal to the thymidine kinase-driven luc gene, and the resulting plasmid was named HNF1 $\alpha$  RE-luc.

**Cell Culture.** HepG2 cells from the American Type Culture Collection (ATCC, Manassas, VA) were cultured in complete Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gemini, Woodland, CA), 2 mM L-glutamine, 100 U penicillin/ml, 100  $\mu\text{g}$  streptomycin/ml, and 1% minimum Eagle's medium nonessential amino acids. HepaRG cells were purchased from Biopredic international (Rennes, France) and maintained in 25-cm<sup>2</sup> culture flask in William's E medium supplemented with 10% fetal bovine serum, 100 U penicillin/ml, 100  $\mu\text{g}$  streptomycin/ml, 5  $\mu\text{g}/\text{ml}$  insulin, 2 mM L-glutamine, and 50  $\mu\text{M}$  hydrocortisone. Then the cells were seeded onto 12-well

plates at a density of  $2 \times 10^5$  cells/ml and cultured for 14 days, after which the cells were used for experiments.

**Primary Hepatocyte Culture.** Freshly isolated human hepatocytes from female donors of age 18–50 years were obtained from Life Technologies (Pittsboro, NC), BD biosciences (San Jose, CA), and Liver Tissue Cell Distribution System (Pittsburgh, PA). Briefly, human hepatocytes were shipped overnight in cold preservation media. Upon receipt, the media were replaced with serum-free Williams' E media (without phenol red) containing 0.1  $\mu\text{M}$  dexamethasone, 100 U penicillin/ml, 100  $\mu\text{g}$  streptomycin/ml, 15 mM HEPES, 2 mM L-glutamine, 5.5  $\mu\text{g}/\text{ml}$  transferrin, and 5 ng/ml sodium selenite. Cells were allowed to recover from shipping for 18 hours at  $37^{\circ}\text{C}$  in an atmosphere containing 5% CO<sub>2</sub> and used for experiments on the next day. Mouse hepatocytes were isolated from the livers of CYP2E1-humanized mice as previously described (Swift and Brouwer, 2010) and cultured as described above for human hepatocytes.

**Quantitative Real-Time Polymerase Chain Reaction.** Total RNA was isolated from human hepatocytes using Trizol (Life Technologies) and used as template for the synthesis of complementary DNA using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). With the cDNA as template, quantitative real-time polymerase chain reaction (qRT-PCR) was performed using StepOnePlus Real-Time PCR System and TaqMan Gene expression assays (Applied Biosystems). The fold change in mRNA levels of P450 isoforms was determined after normalizing the gene expression levels to those of GAPDH ( $2^{-\Delta\Delta\text{Ct}}$  method).

**Precursor RNA Analysis.** RNA was prepared from hormone-treated cells and treated with DNase I. The RNA was then reverse-transcribed using an intron 6-specific CYP2E1 primer (5'-ACACA TCCAT TGACC CATCC-3'), and qRT-PCR was performed using a primer set that can detect intron 5 and exon 6 of CYP2E1 (Life Technologies; Hs01666884\_cn). As a loading control, precursor RNA level of GAPDH was used. To this end, an intron 6-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer (5'-CGGCC ATCAC GCCAC AGTTT-3') and a primer set that can detect exon 5 and intron 5 of GAPDH (Life Technologies; Hs00894322\_cn) were used for reverse-transcription and qRT-PCR, respectively. The fold change in the precursor RNA levels of CYP2E1 was determined after normalizing the gene expression levels to those of GAPDH ( $2^{-\Delta\Delta\text{Ct}}$  method).

**Western Blot Analysis.** Cell lysates were prepared from drug-treated human hepatocytes, and proteins (30  $\mu\text{g}$ ) were separated using 12% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Blots were blocked with 5% skim milk/Tris buffered saline containing 0.1% Tween 20 (TBST) for 1 hour at room temperature and incubated overnight at  $4^{\circ}\text{C}$  with primary antibodies against CYP2E1 or  $\beta$ -actin diluted according to the manufacturer's specifications in 1% skim milk/TBST. The membranes were then washed in TBST before incubation with horseradish peroxidase-coupled anti-rabbit IgG or anti-mouse IgG antibodies for 1 hour at room temperature. The membranes were visualized by an enhanced chemiluminescence detection system (SuperSignal West Pico; Pierce Biotechnology, Rockford, IL) and FluorChem E imager (Cell Biosciences, Santa Clara, CA). The intensities of bands were measured by densitometry using AlphaView Software 3.2.3.0 (Cell Biosciences).

**Measurement of CYP2E1 Activity.** Human hepatocytes were treated with PL for 72 hours, and the media were replaced with fresh media containing chlorzoxazone (100  $\mu\text{M}$ ), a CYP2E1 probe drug (Ogilvie et al., 2008). Media were sampled at various time points (up to 5 hours). The concentrations of metabolite (6-hydroxychlorzoxazone) were determined by liquid chromatography–tandem mass spectrometry. Liquid chromatography–tandem mass spectrometry system consisted of an HP1100 high-performance liquid chromatography system (Agilent Technologies) with an AB Sciex 3200 Q-trap mass spectrometer (Applied Biosystems) equipped with an electrospray ionization source. Hydroxytolbutamide was used as an internal standard. The multiple reaction monitoring detection method was employed for quantification of metabolite and internal standard. Multiple reaction monitoring pairs for 6-hydroxychlorzoxazone and hydroxytolbutamide were  $m/z$  183.9/120.0 and  $m/z$  285.1/186.0, respectively. Standard curves of 6-hydroxychlorzoxazone were linear over the range studied with  $r > 0.998$ . The limit of quantitation for 6-hydroxychlorzoxazone was 27 nM (i.e., 5 ng/ml).

**Luciferase Reporter Assays.** HepG2 cells were seeded in 24-well plates at a density of  $5 \times 10^5$  cells/ml. On the next day, the cells were transfected with 0.2  $\mu\text{g}$  of luciferase construct, 0.15  $\mu\text{g}$  of expression plasmid, and 0.001  $\mu\text{g}$  of CMV-Renilla expression plasmid using Fugene HD transfection reagent (Roche Applied

Sciences) according to the manufacturer's protocol. The transfected cells were treated with PRL (150 ng/ml), PL (6  $\mu$ g/ml), or PBS (vehicle) for 24 hours, and then luciferase assays were performed using the assay kits from Promega. Luciferase activity was normalized to the *Renilla* luciferase activity.

HepaRG cells were seeded in 12-well plates at a density of  $8 \times 10^5$  cells/ml and cultured for 2 weeks for differentiation. The differentiated cells were transfected with 1  $\mu$ g of luciferase construct, 0.6  $\mu$ g of expression plasmid, and 0.002  $\mu$ g of CMV-*Renilla* expression plasmid using Lipofectamine 2000 transfection reagent (Life Technologies) according to the manufacturer's protocol. The transfected cells were grown for 24 hours and treated with PL (6  $\mu$ g/ml) or PBS (vehicle). On the next day, cells were harvested, and firefly and *Renilla* luciferase activities were measured using assay kits from Promega. Firefly luciferase activity was normalized to the *Renilla* luciferase activity.

**Inhibition of Cell Signaling.** Human hepatocytes were treated with PL or vehicle in the presence or absence of one of the following signaling inhibitors (1  $\mu$ M): wortmannin, rapamycin, Ro 31-8220, U0126, and SB202190 for inhibition of PI3-kinase, p70 S6 kinase, PKC, MEK, and p38 MAPK, respectively. After 72 hours, cell lysates were collected and analyzed.

**Statistical Analysis.** Each experiment with primary human hepatocytes was conducted in triplicate, and data were expressed as mean  $\pm$  S.D. Statistical analysis for human hepatocyte experiments with different hormones was performed by two-way analysis of variance, followed by post hoc Bonferroni test. For luciferase reporter assays, at least two independent experiments were performed in triplicate, and the Student's *t* test was performed for statistically analysis. For cell signaling experiments, one-way analysis of variance was performed, followed by post hoc Dunnett's test.

## Results

**PL Induces CYP2E1 Expression.** To determine the effects of PRL, PL, and GH-v on the expression of major P450 enzymes, human hepatocytes were treated with PRL (150 ng/ml), PL (6  $\mu$ g/ml), GH-v (20 ng/ml), or bicarbonate buffered saline (vehicle), and mRNA expression levels of CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5 were measured by qRT-PCR. The concentrations of hormones chosen for this study reflect the maternal serum levels during the third trimester of pregnancy (MacDonald et al., 1980; Kletzky et al., 1985). The concentrations of PRL and PL in the media did not decrease more than 10% over the treatment period (enzyme-linked immunosorbent assays; data not shown). Furthermore, the treatment with PRL, PL, or GH-v did not cause cytotoxicity to the hepatocytes (lactate dehydrogenase assays; data not shown). PRL and GH-v exhibited either no effect or small and variable effects on P450 expression (Supplemental Fig. 1); in a single batch of human hepatocytes, PRL enhanced CYP2E1 by 1.6-fold, whereas GH-v enhanced CYP2D6 and CYP3A4 by 2.3- and 2.4-fold, respectively. On the other hand, PL significantly induced the expression level of CYP3A5 (1.5- to 4.5-fold induction) in all human hepatocytes. Also, PL significantly enhanced CYP2E1 expression (2.8- to 15.3-fold) in all hepatocytes, whereas its effects on the remaining P450 expression were insignificant or variable (Fig. 1A). CYP2E1 induction by PL was most prominent in hepatocytes #4. Of note, only in the hepatocytes #4, PL inhibited mRNA expression of CYP2A6, CYP2C19, and CYP3A4, demonstrating significant donor-to-donor variability as typically seen in studies using primary hepatocytes (McGinnity et al., 2009). A similar increase in CYP2E1 expression by PL was also shown in HepaRG cells (Fig. 1B); however, the magnitude of CYP2E1 induction by PL in HepaRG was lower than that in human hepatocytes for unknown reasons. Treatment of mouse hepatocytes isolated from *CYP2E1*-humanized mice with human PL did not affect CYP2E1 expression (data not shown), indicating the need to use appropriate experimental systems in studying the physiology of PL.

The CYP2E1 induction by PL was concentration dependent (Fig. 2A). The PL concentration exhibiting 50% of the maximal induction could not be estimated because the effect did not reach a plateau. To

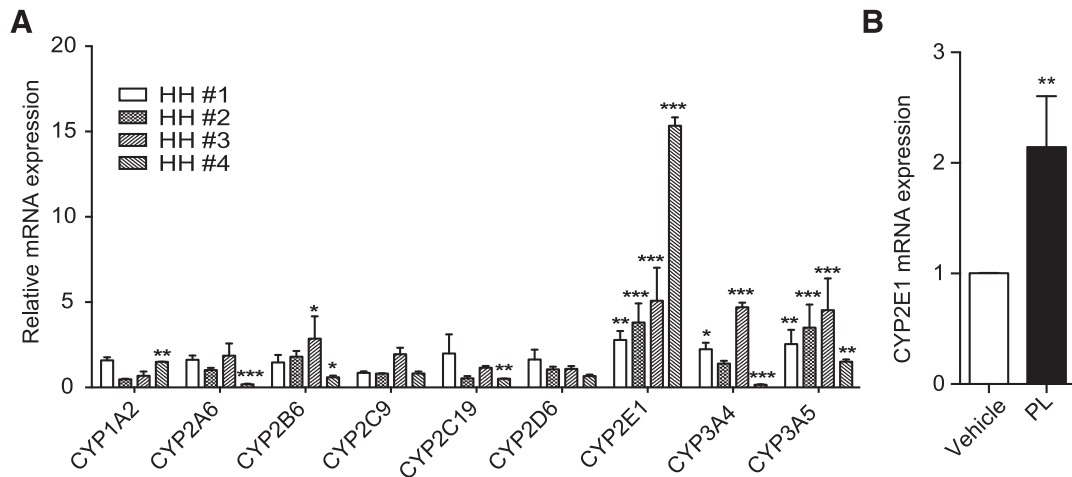
determine whether the increased CYP2E1 mRNA levels led to corresponding increases in protein expression and enzymatic activity, protein and metabolic activity of CYP2E1 were analyzed in the human hepatocytes by using western blot and the CYP2E1 probe drug chlorzoxazone, respectively. The results showed that PL increased CYP2E1 protein (Fig. 2B). The formation of 6-hydroxychlorzoxazone was increased significantly in the hepatocytes pretreated with PL at most of the time points (Fig. 2C). Taken together, these results indicate that at concentrations reached during pregnancy, PL increases CYP2E1 expression.

To determine whether CYP2E1 induction by PL occurs at the transcriptional level, the effects of PL on the level of CYP2E1 precursor RNA (an immature mRNA before processing) were examined in human hepatocytes from a different donor. The hepatocytes were treated with PL or vehicle, RNA isolated, and the level of precursor RNA measured by qRT-PCR using a primer set that can detect the intron regions of CYP2E1. PL significantly increased the level of CYP2E1 precursor RNA compared with vehicle treatment, accompanied by a similar extent of increase in CYP2E1 mRNA level (Fig. 2D). Together, these results indicate that CYP2E1 induction by PL likely occurs at the transcriptional level.

**HNF1 $\alpha$  Is Not Involved in CYP2E1 Induction by PL.** HNF1 $\alpha$  is a known transcriptional activator of the *CYP2E1* gene (Liu and Gonzalez, 1995). To determine whether PL affects expression of HNF1 $\alpha$  and its dimerization cofactor DCoH (Mendel et al., 1991), their mRNA levels were determined by qRT-PCR in human hepatocytes treated with vehicle or PL for 72 hours. The result showed that PL did not affect the mRNA expression of HNF1 $\alpha$  or DCoH in human hepatocytes (Fig. 3, A and B, respectively). To determine whether the transcriptional activity of HNF1 $\alpha$  is modulated by PL, HepaRG cells were transiently transfected with a luciferase construct HNF1 $\alpha$  RE-luc where *luc* gene expression is driven by four copies of HNF1 $\alpha$  response element, and its response to PL was examined. HepaRG cells were chosen because the expression levels of hepatic genes (likely including DCoH) in these cells are comparable to those in primary human hepatocytes (Lubberstedt et al., 2011). In the vehicle-treated cells, overexpression of HNF1 $\alpha$  significantly increased the luciferase activity, indicating that components for proper HNF1 $\alpha$  actions are present and functional in HepaRG cells (Fig. 3C). However, PL did not induce HNF1 $\alpha$  RE-luc activity in the cells (Fig. 3C), indicating a lack of PL effects on HNF1 $\alpha$  activity. Together, these results indicate that PL does not affect the expression or activity of HNF1 $\alpha$  and that HNF1 $\alpha$  likely plays an insignificant role in CYP2E1 induction by PL.

**STATs Are Not Involved in CYP2E1 Induction by PL.** To examine potential involvement of STAT5 pathway in PL-mediated CYP2E1 induction, luciferase reporter assays were performed. HepG2 cells were cotransfected with expression vectors for PRLR, STAT5a, and a luciferase construct (pGL3-CYP2E1 U1.4kb or pGL4-CISH), treated with vehicle or PL (6  $\mu$ g/ml), and luciferase assays were performed. pGL3-CYP2E1 U1.4kb harbors 1.4-kb upstream region of CYP2E1 where multiple putative STAT5 binding sites were found (data not shown). pGL4-CISH contains four copies of a verified STAT5 binding site of cytokine-inducible SH-2-containing protein (encoded by *CISH* gene) that drives *luc* expression and was used as a positive control. The results showed that PL increased the promoter activity of CISH but not that of CYP2E1 (Fig. 3D). These results suggest that STAT5 does not transactivate the CYP2E1 promoter.

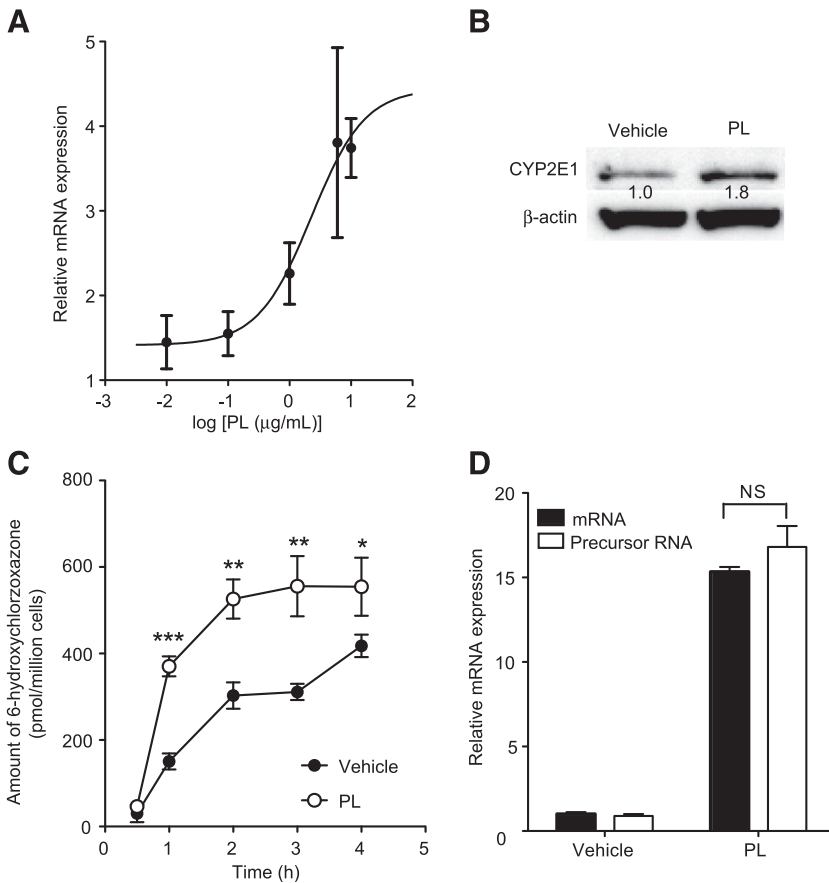
To investigate whether other STATs are involved in PL action on CYP2E1 expression, the effect of PL on activation of multiple STATs was examined in human hepatocytes. Human hepatocytes starved of serum for 18 hours were treated with PL or vehicle for 10 or 30 minutes, and phosphorylated STAT proteins were detected by using Western blot. Previously known activators of individual STAT



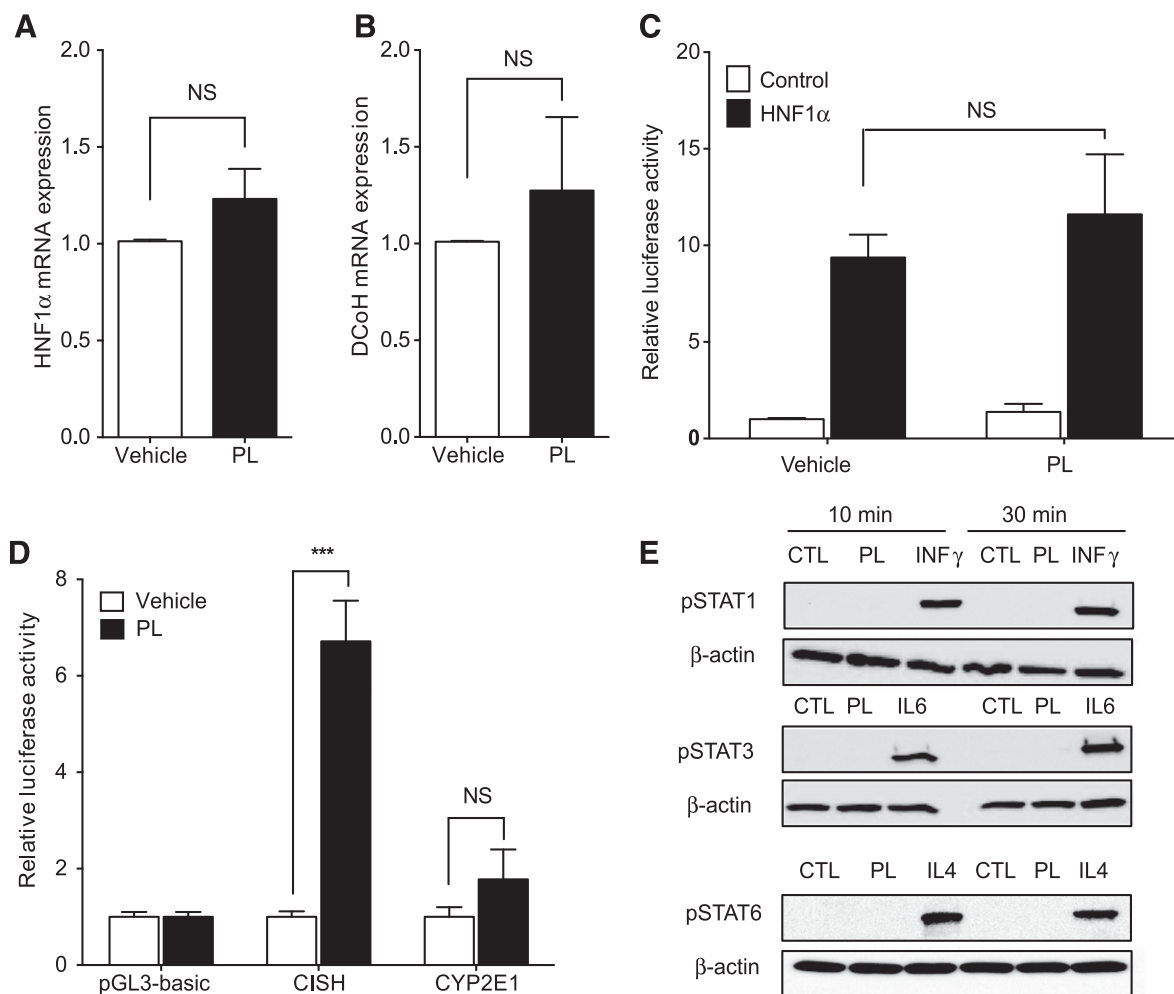
**Fig. 1.** Effects of PL on mRNA expression of major hepatic P450 enzymes. (A) Human hepatocytes (HH) from different donors were treated with PL (6  $\mu$ g/ml) or vehicle (PBS) for 72 hours. mRNA levels of CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5 were determined by qRT-PCR. Results represent mRNA levels of each P450 relative to those in vehicle control (mean  $\pm$  S.D.;  $n = 3$  or 4). The dotted line represents the relative expression level of 1.0. (B) HepaRG cells were treated with PL (6  $\mu$ g/ml) or vehicle (PBS) for 72 hours. mRNA levels of CYP2E1 were determined by qRT-PCR \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  versus vehicle-treated group.

pathways (i.e.,  $\text{INF}\gamma$  for STAT1, IL-6 for STAT3, and IL-4 for STAT6 activation) were used as positive controls. The results showed that  $\text{INF}\gamma$ , IL-6, and IL-4 phosphorylated the respective STAT proteins as expected; however, PL did not activate any of the STATs (Fig. 3E). Taken together, these results indicate that STAT signaling is likely not responsible for CYP2E1 induction by PL.

**PI3-Kinase Plays a Key Role in CYP2E1 Induction by PL.** In primary rat hepatocytes, insulin was shown to modulate CYP2E1 mRNA expression via activation of PI3-kinase and p70 S6 kinase signaling pathways (Woodcroft et al., 2002). To examine whether one or more intracellular signaling pathways mediating insulin reaction are involved in CYP2E1 induction by PL, human hepatocytes were



**Fig. 2.** Transcriptional regulation of CYP2E1 expression by PL. (A) Human hepatocytes from HH #2 were treated with various concentration of PL (0–10  $\mu$ g/ml) for 72 hours, and mRNA expression level of CYP2E1 was determined by qRT-PCR. Results represent fold changes in mRNA levels of P450 isozyme relative to vehicle control. (B) CYP2E1 protein expression was determined by using Western blot in human hepatocytes from HH #2 treated with PL (6  $\mu$ g/ml) or vehicle for 72 hours. The numbers represent the signal of CYP2E1 normalized by that of  $\beta$ -actin. (C) the amount of 6-hydroxychlorzoxazone was measured in human hepatocytes from HH #1 treated with PL (6  $\mu$ g/ml) or vehicle for 72 hours. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  versus vehicle-treated group. (D) The level of CYP2E1 precursor RNA was measured in human hepatocytes from HH #4 treated with PL (6  $\mu$ g/ml) for 72 hours. NS, not significant.



**Fig. 3.** Insignificant role of HNF1 $\alpha$  and PRLR/STAT5 in CYP2E1 induction by PL. (A and B) Human hepatocytes were treated with PL (6  $\mu$ g/ml) or vehicle (PBS) for 72 hours, and mRNA expression levels of HNF1 $\alpha$  (A) and DCoH (B) were determined by qRT-PCR (mean  $\pm$  S.D.;  $n = 3$ ). NS, not significant. (C) HepaRG cells were transiently cotransfected with expression vectors for HNF1 $\alpha$  (or empty vector), HNF1 $\alpha$  RE-luc harboring four copies of HNF1 $\alpha$  response element, and CMV-*Renilla* expression plasmid. The transfected cells were treated with vehicle or PL (6  $\mu$ g/ml) for 24 hours, and luciferase assays were performed. (D) HepG2 cells were transiently cotransfected with expression vectors for hPRLR and STAT5a together with a luciferase vector (promotorless pGL3-basic, pGL4-CISH, or pGL3-CYP2E1 U1.4kb) and CMV-*Renilla* expression plasmid. The transfected HepG2 cells were treated with PL (6  $\mu$ g/ml) or vehicle for 24 hours, and luciferase assay was performed. Results represent fold changes in luciferase activity by drug treatment relative to vehicle treatment. \*\*\* $P < 0.001$ . (E) Human hepatocytes were cultured with serum- and dexamethasone-free medium for 18 hours and treated with control medium (CTL) or medium containing PL (6  $\mu$ g/ml) or positive controls for each STAT [INF $\gamma$  (10 ng/ml), IL-6 (20 ng/ml), and IL-4 (33 ng/ml) for STAT1, STAT3, and STAT6, respectively] for 10 or 30 minutes. Phosphorylated STAT proteins were determined by Western blot analysis.

treated with vehicle or PL for 72 hours in the presence of signaling inhibitors (wortmannin for PI3-kinase, rapamycin for p70 S6 kinase, Ro 31-8220 for PKC, U0126 for MEK, and SB202190 for p38 MAPK inhibition), and CYP2E1 mRNA levels were determined by qRT-PCR. In human hepatocytes, the enhanced CYP2E1 expression by PL was significantly attenuated by wortmannin but not by any of the remaining inhibitors (Fig. 4A), suggesting significant roles of PI3-kinase in mediating CYP2E1 induction by PL.

AKT, a serine/threonine specific protein kinase, is known to be a key mediator for PI3-kinase signaling pathway (Fayard et al., 2010). To further verify the involvement of PI3-kinase in PL action, whether PL activates AKT was examined. Human hepatocytes or HepaRG cells were treated with vehicle or PL for 30 minutes, and activated (i.e., phosphorylated) AKT was detected by Western blot. The result showed that the level of phosphorylated AKT was increased upon treatment with PL (Fig. 4B). Wortmannin abolished AKT phosphorylation, verifying that AKT is a downstream effector of PI3-kinase. Together, these data indicate that PL activates PI3-kinase/AKT signaling pathway.

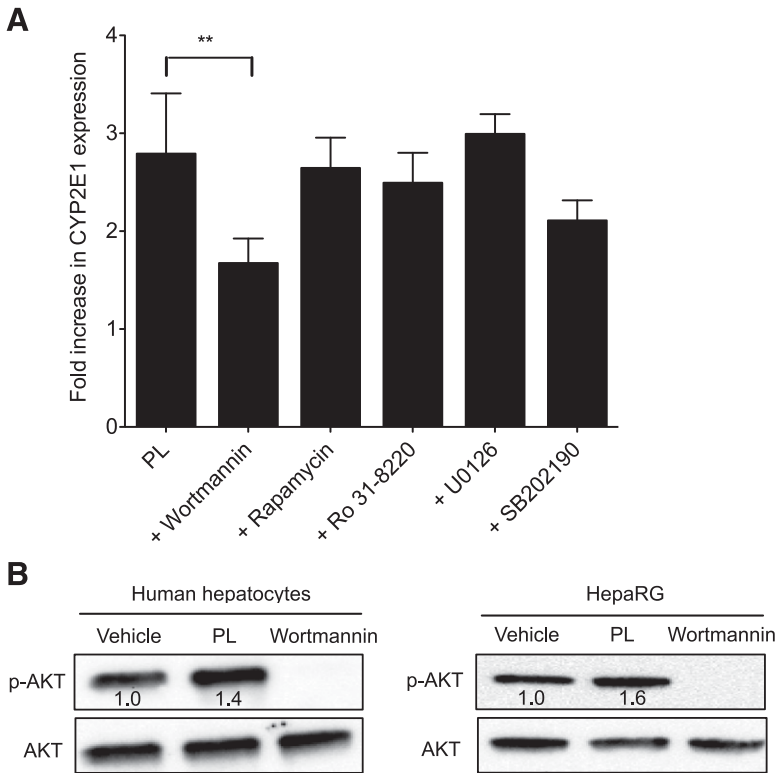
**CYP2E1 Expression Is Not Increased in CYP2E1-Humanized Mice during Pregnancy.** To determine whether PL effects on

CYP2E1 lead to enhanced CYP2E1 expression during pregnancy, a CYP2E1-humanized mouse line was used. The genome of CYP2E1-humanized mice harbors the complete human CYP2E1 gene along with 2.7-kb of upstream region of the CYP2E1 gene (Cheung et al., 2005). Liver samples were collected from the mice at pre-pregnancy, 7 or 17 days of pregnancy, and 30 days postpartum, and the level of CYP2E1 mRNA was determined by qRT-PCR. Results showed that CYP2E1 mRNA level was significantly decreased at 7 days of pregnancy in CYP2E1-humanized mice (Fig. 5), apparently inconsistent with the PL-mediated induction of CYP2E1 in human hepatocytes.

## Discussion

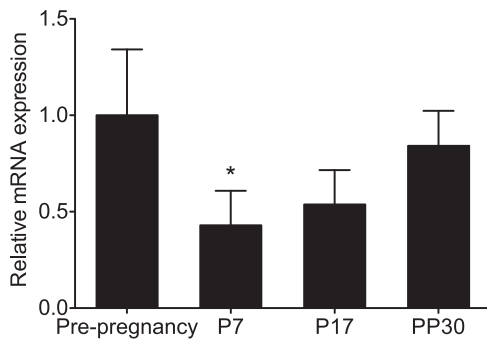
In this study, we examined the effects of PRL, PL, and GH-v on expression of hepatic drug-metabolizing enzymes in human hepatocytes and investigated the underlying regulatory mechanisms. The results demonstrated that PL enhances CYP2E1 expression in primary human hepatocytes, in part by activation of PI3-kinase signaling pathways.

CYP2E1 contributes to a small portion (i.e., 5–10%) of overall P450-mediated metabolism, but the significance of this enzyme cannot



**Fig. 4.** Role of PI3-kinase signaling pathway in CYP2E1 induction by PL. (A) Human hepatocytes were treated with vehicle or PL (6  $\mu\text{g/ml}$ ) [alone or in combination with an inhibitor (1  $\mu\text{M}$ ) of signaling pathways] for 72 hours. CYP2E1 mRNA was determined by using qRT-PCR.  $^{***}P < 0.01$ . (B) Human hepatocytes or HepaRG cells were treated with vehicle, PL (6  $\mu\text{g/ml}$ ), or wortmannin (1  $\mu\text{M}$ ) for 30 minutes. Protein expression of native and phosphorylated AKT was determined by using Western blot, and the numbers represent the signal of phosphorylated AKT normalized by that of native AKT.

be overlooked because of its toxicological role in the metabolic activation of many carcinogenic and toxic chemicals as well as the production of reactive oxygen species (Lu and Cederbaum, 2008). Potentially because of its physiologic significance, CYP2E1 expression is highly regulated. Several regulatory mechanisms for CYP2E1 expression have been reported, including transcriptional regulation (Umeno et al., 1988; Vieira et al., 1996), post-transcriptional mechanisms (Song et al., 1987; Yang and Cederbaum, 1997; Khalighi et al., 1999), and post-translational protein stabilization (Song et al., 1989). Our results showed that in human hepatocytes, PL increases the precursor RNA levels of CYP2E1, suggesting that PL induces CYP2E1 via transcriptional activation of the gene.



**Fig. 5.** The effect of pregnancy on CYP2E1 expression in CYP2E1-humanized mice. Livers were collected from CYP2E1-humanized mice at pre-pregnancy, 7 or 17 days of pregnancy (P7 or P17), and 30 days postpartum (PP30) ( $n = 4/\text{group}$ ). The level of CYP2E1 mRNA was measured by using qRT-PCR.  $^{*}P < 0.05$  versus pre-pregnancy group.

It was previously demonstrated that HNF1 $\alpha$  is a transcription activator of CYP2E1 through direct binding to the promoter (Liu and Gonzalez, 1995; Cheung et al., 2003). Of note, the transcriptional activity of HNF1 $\alpha$  can be modulated without any changes in HNF1 $\alpha$  expression, by post-translational modification of HNF1 $\alpha$  (Lim et al., 2002) or by altered expression of its cofactor DCoH (Mendel et al., 1991). Our results from qRT-PCR and luciferase reporter assays indicate that PL does not influence expression of HNF1 $\alpha$  or DCoH or the transcriptional activity of HNF1 $\alpha$ . This suggests that although HNF1 $\alpha$  may be a key transcriptional regulator of basal CYP2E1 expression, HNF1 $\alpha$  does not play a role in CYP2E1 induction by PL in human hepatocytes. This finding is in part consistent with previously published results indicating the presence of other regulatory mechanisms for CYP2E1 expression (Hakkola et al., 2003; Sekine et al., 2006). For example, transgenic mice with liver-specific disruption of the  $\beta$ -catenin gene showed an almost complete loss of CYP2E1 mRNA expression (>90%), although HNF1 $\alpha$  expression did not change (Sekine et al., 2006).

The results from this study demonstrate that PL enhances CYP2E1 expression by activating PI3-kinase in human hepatocytes. Of interest, a previous study showed that activation of PI3-kinase by insulin leads to repressed CYP2E1 expression (Woodcroft et al., 2002), whereas in this study, activation of PI3-kinase by PL led to enhanced CYP2E1 expression. Although detailed underlying mechanisms for the differential actions of PL and insulin warrant comparative studies, this seemingly conflicting data may be explained by the complexity of intracellular signaling pathways. For example, insulin is known to activate PI3-kinase and the downstream AKT signaling, but transgenic mice with decreased PI3-kinase function (via liver-specific deletion of a major regulatory subunit of PI3-kinase) exhibit increased AKT activation (Taniguchi et al., 2006), suggesting that insulin may

modulate other signaling pathways, the net result of which is AKT activation. Similarly, it appears plausible that PL activates a distinct set of multiple intracellular signaling pathways (including PI3-kinase), a net result of which is the CYP2E1 induction. The additional signaling pathways activated by PL remain to be identified.

Humans and mice have shown significant interspecies differences in the directional changes in P450-mediated drug metabolism during pregnancy. For example, CYP2D6-mediated drug metabolism increases during pregnancy in humans (Hodge and Tracy, 2007), whereas expression of CYP2D homologs decreases in mice (Koh et al., 2011). This is potentially caused by DNA sequence differences in the upstream regulatory region of P450 genes between humans and mice. In this regard, CYP-humanized mice whose genomes harbor the human structural gene along with the upstream regulatory region have been proven to be useful as an *in vivo* model by recapitulating altered P450 expression during human pregnancy (Koh et al., 2014). Based on data indicating CYP2E1 induction by PL in human hepatocytes, we hypothesized that pregnancy enhances CYP2E1 expression. In CYP2E1-humanized mice, however, the CYP2E1 expression was decreased during pregnancy. Interspecies differences in hormone physiology may have contributed to the finding. For example, although the human placenta produces one form of PL, the mouse placenta produces two different forms of PL (mPL1 and mPL2) whose amino acid sequences share less than 50% homology with those of human PL. Whether pregnancy indeed affects CYP2E1 expression and activity remains to be determined in humans.

Many CYP2E1 substrates are known to produce toxic metabolites. For example, CYP2E1 metabolizes isoniazid to acetylhydrazine that triggers hepatitis (Yue et al., 2004). Of interest, pregnant women are four times more likely to develop isoniazid-mediated hepatitis than are nonpregnant women (Kawamoto et al., 1999), suggesting that expression and activity of CYP2E1 may increase during pregnancy. CYP2E1 plays a major role in metabolizing acetaminophen (a commonly used analgesic during pregnancy) to *N*-acetyl-*p*-benzoquinone, the toxic metabolite causing fatal liver injury (Lee et al., 1996). Of note, a recent study demonstrated that acetaminophen elimination to oxidative metabolites was significantly increased in women at delivery compared with that in postpartum women, suggesting clinically relevant increase in CYP2E1 expression and/or activity during pregnancy (Kulo et al., 2013). Taken together, caution should be advised when acetaminophen is prescribed to pregnant women.

In summary, the present study showed that PL increases CYP2E1 expression and activity in human hepatocytes in part by PI3-kinase signaling pathway. The results underscore significant interspecies differences in PL physiology between humans and mice. This represents the first report of *human* PL affecting hepatic functions and should shed light on physiologic and molecular basis of human PL action.

#### Authorship Contributions

Participated in research design: Lee, Chung, Jeong.

Conducted experiments: Lee, Chung, L. Fischer.

Contributed new reagents or analytic tools: Gonzalez.

Performed data analysis: Lee, Chung, Jeong.

Wrote or contributed to the writing of the manuscript: Lee, Chung, J. Fischer, Gonzalez, Jeong.

#### References

- Ben-Jonathan N, LaPensee CR, and LaPensee EW (2008) What can we learn from rodents about prolactin in humans? *Endocr Rev* **29**:1–41.
- Cao J, Gowri PM, Ganguly TC, Wood M, Hyde JF, Talamantes F, and Vore M (2001) PRL, placental lactogen, and GH induce NA(+)/taurocholate-cotransporting polypeptide gene expression by activating signal transducer and activator of transcription-5 in liver cells. *Endocrinology* **142**:4212–4222.
- Cheung C, Akiyama TE, Kudo G, and Gonzalez FJ (2003) Hepatic expression of cytochrome P450s in hepatocyte nuclear factor 1-alpha (HNF1alpha)-deficient mice. *Biochem Pharmacol* **66**:2011–2020.
- Cheung C, Yu AM, Ward JM, Krausz KW, Akiyama TE, Feigenbaum L, and Gonzalez FJ (2005) The cyp2e1-humanized transgenic mouse: role of cyp2e1 in acetaminophen hepatotoxicity. *Drug Metab Dispos* **33**:449–457.
- Choi SY, Koh KH, and Jeong H (2013) Isoform-specific regulation of cytochromes P450 expression by estradiol and progesterone. *Drug Metab Dispos* **41**:263–269.
- Fayard E, Xue G, Parcellier A, Bozulic L, and Hemmings BA (2010) Protein kinase B (PKB/Akt), a key mediator of the PI3K signaling pathway. *Curr Top Microbiol Immunol* **346**:31–56.
- Forsyth IA and Wallis M (2002) Growth hormone and prolactin—molecular and functional evolution. *J Mammary Gland Biol Neoplasia* **7**:291–312.
- Hakkola J, Hu Y, and Ingelman-Sundberg M (2003) Mechanisms of down-regulation of CYP2E1 expression by inflammatory cytokines in rat hepatoma cells. *J Pharmacol Exp Ther* **304**:1048–1054.
- Handwerker S and Freemark M (2000) The roles of placental growth hormone and placental lactogen in the regulation of human fetal growth and development. *J Pediatr Endocrinol Metab* **13**:343–356.
- Hodge LS and Tracy TS (2007) Alterations in drug disposition during pregnancy: implications for drug therapy. *Expert Opin Drug Metab Toxicol* **3**:557–571.
- Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K, and Negishi M (1999) Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. *Mol Cell Biol* **19**:6318–6322.
- Khalighi M, Brzezinski MR, Chen H, and Juchau MR (1999) Inhibition of human prenatal biosynthesis of all-trans-retinoic acid by ethanol, ethanol metabolites, and products of lipid peroxidation reactions: a possible role for CYP2E1. *Biochem Pharmacol* **57**:811–821.
- Kletzky OA, Rossman F, Bertolli SL, Platt LD, and Mishell DR, Jr (1985) Dynamics of human chorionic gonadotropin, prolactin, and growth hormone in serum and amniotic fluid throughout normal human pregnancy. *Am J Obstet Gynecol* **151**:878–884.
- Koh KH, Jurkovic S, Yang K, Choi SY, Jung JW, Kim KP, Zhang W, and Jeong H (2012) Estradiol induces cytochrome P450 2B6 expression at high concentrations: implication in estrogen-mediated gene regulation in pregnancy. *Biochem Pharmacol* **84**:93–103.
- Koh KH, Pan X, Shen HW, Arnold SL, Yu AM, Gonzalez FJ, Isoherranen N, and Jeong H (2014) Altered expression of small heterodimer partner governs cytochrome P450 (CYP) 2D6 induction during pregnancy in CYP2D6-humanized mice. *J Biol Chem* **289**(6):3105–13.
- Koh KH, Xie H, Yu AM, and Jeong H (2011) Altered cytochrome P450 expression in mice during pregnancy. *Drug Metab Dispos* **39**:165–169.
- Kulo A, Peeters MY, Allegaert K, Smits A, de Hoon J, Verbesselt R, Lewi L, van de Velde M, and Knibbe CA (2013) Pharmacokinetics of paracetamol and its metabolites in women at delivery and post-partum. *Br J Clin Pharmacol* **75**:850–860.
- Lee SS, Buters JT, Pineau T, Fernandez-Salguero P, and Gonzalez FJ (1996) Role of CYP2E1 in the hepatotoxicity of acetaminophen. *J Biol Chem* **271**:12063–12067.
- Lim S, Jin K, and Friedman E (2002) Mirk protein kinase is activated by MKK3 and functions as a transcriptional activator of HNF1alpha. *J Biol Chem* **277**:25040–25046.
- Liu SY and Gonzalez FJ (1995) Role of the liver-enriched transcription factor HNF-1 alpha in expression of the CYP2E1 gene. *DNA Cell Biol* **14**:285–293.
- Lu Y and Cederbaum AI (2008) CYP2E1 and oxidative liver injury by alcohol. *Free Radic Biol Med* **44**:723–738.
- Lübberstedt M, Müller-Vieira U, Mayer M, Biemel KM, Knöpsel F, Knobloch D, Nüssler AK, Gerlach JC, and Zeilinger K (2011) HepaRG human hepatic cell line utility as a surrogate for primary human hepatocytes in drug metabolism assessment *in vitro*. *J Pharmacol Toxicol Methods* **63**:59–68.
- Luquita MG, Catania VA, Sánchez-Pozzi EJ, and Mottino AD (1996) Ovine prolactin increases hepatic UDP-glucuronosyltransferase activity in ovariectomized rats. *J Pharmacol Exp Ther* **278**:921–925.
- Luquita MG, Catania VA, Sánchez-Pozzi EJ, Vore M, and Mottino AD (1999) Prolactin increases the hepatic content of mu-class subunits of glutathione S-transferase in the rat. *Drug Metab Dispos* **27**:122–124.
- MacDonald DJ, Nicol KM, Belfield A, Shah MM, and Mack DS (1980) Enzyme-linked immunoassay for placental lactogen in human serum. *Clin Chem* **26**:745–749.
- McGinnity DF, Zhang G, Kenny JR, Hamilton GA, Otmani S, Stams KR, Haney S, Brassil P, Stresser DM, and Riley RJ (2009) Evaluation of multiple *in vitro* systems for assessment of CYP3A4 induction in drug discovery: human hepatocytes, pregnane X receptor reporter gene, and Fa2N-4 and HepaRG cells. *Drug Metab Dispos* **37**:1259–1268.
- Mendel DB, Khavari PA, Conley PB, Graves MK, Hansen LP, Admon A, and Crabtree GR (1991) Characterization of a cofactor that regulates dimerization of a mammalian homeodomain protein. *Science* **254**:1762–1767.
- Ogilvie BW, Usuki E, Yerino P, and Parkinson A (2008) *In vitro* approaches for studying the inhibition of drug-metabolizing enzymes and identifying the drug-metabolizing enzymes responsible for the metabolism of drugs (reaction phenotyping) with emphasis on cytochrome P450, in *Drug-Drug Interactions* (Rodrigues AD ed), pp 231–358, Informa Healthcare, New York.
- Pan X, Koh KH, Yu AM, and Jeong H (2013) Induction of CYP2D6 expression during pregnancy is associated with an increased activity of hepatocyte nuclear factor 4, in *Experimental Biology*, pp 1102–1103, The FASEB Journal, Boston, MA.
- Papageorgiou I, Grepper S, and Unadkat JD (2013) Induction of hepatic CYP3A enzymes by pregnancy-related hormones: studies in human hepatocytes and hepatic cell lines. *Drug Metab Dispos* **41**:281–290.
- Sekine S, Lan BY, Bedolli M, Feng S, and Hebrok M (2006) Liver-specific loss of beta-catenin blocks glutamine synthesis pathway activity and cytochrome p450 expression in mice. *Hepatology* **43**:817–825.
- Song BJ, Matsunaga T, Hardwick JP, Park SS, Veech RL, Yang CS, Gelboin HV, and Gonzalez FJ (1987) Stabilization of cytochrome P450j messenger ribonucleic acid in the diabetic rat. *Mol Endocrinol* **1**:542–547.
- Song BJ, Veech RL, Park SS, Gelboin HV, and Gonzalez FJ (1989) Induction of rat hepatic N-nitrosodimethylamine demethylase by acetone is due to protein stabilization. *J Biol Chem* **264**:3568–3572.
- Swift B and Brouwer KL (2010) Influence of seeding density and extracellular matrix on bile Acid transport and mrp4 expression in sandwich-cultured mouse hepatocytes. *Mol Pharm* **7**:491–500.

- Taniguchi CM, Tran TT, Kondo T, Luo J, Ueki K, Cantley LC, and Kahn CR (2006) Phosphoinositide 3-kinase regulatory subunit p85alpha suppresses insulin action via positive regulation of PTEN. *Proc Natl Acad Sci USA* **103**:12093–12097.
- Umeno M, McBride OW, Yang CS, Gelboin HV, and Gonzalez FJ (1988) Human ethanol-inducible P450IIE1: complete gene sequence, promoter characterization, chromosome mapping, and cDNA-directed expression. *Biochemistry* **27**:9006–9013.
- Vieira I, Sonnier M, and Cresteil T (1996) Developmental expression of CYP2E1 in the human liver. Hypermethylation control of gene expression during the neonatal period. *Eur J Biochem* **238**:476–483.
- Wang J, Hu Y, Nekvindova J, Ingelman-Sundberg M, and Neve EP (2010) IL-4-mediated transcriptional regulation of human CYP2E1 by two independent signaling pathways. *Biochem Pharmacol* **80**:1592–1600.
- Woodcroft KJ, Hafner MS, and Novak RF (2002) Insulin signaling in the transcriptional and posttranscriptional regulation of CYP2E1 expression. *Hepatology* **35**:263–273.
- Yang MX and Cederbaum AI (1997) Characterization of cytochrome P450E1 turnover in transfected HepG2 cells expressing human CYP2E1. *Arch Biochem Biophys* **341**:25–33.
- Yue J, Peng RX, Yang J, Kong R, and Liu J (2004) CYP2E1 mediated isoniazid-induced hepatotoxicity in rats. *Acta Pharmacol Sin* **25**:699–704.

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