HepaRG Cells as Human-Relevant In Vitro Model to Study the Effects of Inflammatory Stimuli on Cytochrome P450 Isoenzymes

Katarina Rubin, Annika Janefeldt, Linda Andersson, Zsofia Berke, Ken Grime, and Tommy B. Andersson

AstraZeneca R&D, Mölndal, Sweden; and Respiratory, Inflammation and Autoimmunity Innovative Medicines Drug Metabolism and Pharmacokinetics (K.R., K.G.), Cardiovascular and Metabolic Diseases Innovative Medicines (A.J., T.B.A.), Drug Safety and Metabolism Drug Metabolism and Pharmacokinetics (L.A.), Personalized Healthcare and Biomarkers (Z.B.), and Section of Pharmacogenetics, Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden (T.B.A.)

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ABSTRACT

The suppression of hepatic cytochrome P450 (P450) expression during inflammatory and infectious diseases and the relief of this suppression by successful disease treatment have been previously demonstrated to impact drug disposition. To address this clinically relevant phenomenon preclinically, the effect of proinflammatory cytokines on P450 isoenzymes in human hepatocytes has been examined by several researchers. In the present study, we used the human hepatoma cell line (HepaRG) and cryopreserved primary human hepatocytes to investigate the effects of various inflammatory stimuli on P450 levels with the aim of further characterizing HepaRG cells as a useful surrogate for primary hepatocytes. In this study, HepaRG cells were exposed to bacterial lipopolysaccharide (LPS), interleukin-6 (IL-6), and interleukin-18 (IL-18) for 48 or 72 hours. The effects on CYP1A2, CYP2B6, and CYP3A4 mRNA and catalytic activity (phenacetin-O-deethylase, bupropion-hydroxylase, and midazolam-1′-hydroxylase) were measured. Cryopreserved pooled plateable hepatocytes were also exposed to IL-6 or IL-18 for 48 hours, and the effects on CYP1A2, CYP2B6, and CYP3A4 mRNA levels were measured. The exposure of HepaRG cells to IL-6 and LPS resulted in suppression of CYP1A2, CYP2B6, and CYP3A4 mRNA levels as well as their catalytic activities. However, no suppression of P450 activities or mRNA levels was observed after exposure to IL-18. Similar results on CYP1A2, CYP2B6, and CYP3A4 mRNA levels were observed with primary hepatocytes. The present study indicates that different proinflammatory mediators influence the expression of P450 differentially and that HepaRG cells may be used as an alternative to human hepatocytes for studies on cytokine-mediated suppression of drug-metabolizing enzymes.

Introduction

Proinflammatory cytokines can impact the transcriptional regulation of cytochrome P450 (P450) enzymes. Thus, owing to elevated levels of some circulating cytokines in chronic inflammatory disease conditions, expression levels and activity of these drug metabolizing enzymes can be affected (Shedlovsky et al., 1994; Morgan, 2001; Kulmatycki and Jamali, 2005). This phenomenon is of importance when accounting for pharmacokinetic differences between healthy and diseased subjects, and when considering the introduction of novel anti-inflammatory therapies. Depending on the type of therapy, there may be a decrease in circulatory proinflammatory cytokine levels and associated increases in expression and activity of certain P450s. This may have a direct effect on the metabolism of other administered drugs, potentially decreasing their exposure and efficacy (Huang et al., 2010). In vitro studies using human hepatocytes can facilitate understanding of the relevance of such events and help predict clinical outcomes. Cytokine-mediated P450 regulation has been studied previously in primary human hepatocytes in vitro, with different cytokines demonstrating clear effects measured both at the transcriptional and functional activity level (Sunman et al., 2004; Aitken et al., 2006; Christensen and Hermann, 2012). The mechanisms behind this P450 regulation have been investigated, and the nuclear factor κ-light-chain-enhancer of activated B cells–dependent inflammatory response and associated relocation and dimerization of nuclear receptors such as retinoid X receptor, liver X receptor, farnesoid X receptor, constitutive androstane receptor, pregnane X receptor (PXR), and peroxisome proliferator–activated receptor have been implicated (Jover et al., 2002, Yang et al., 2010; Ghose et al., 2011). However, P450-specific transcriptional regulation has not been fully characterized, and the question of common pathways versus specific receptor-dependent intracellular signaling pathways is still to be elucidated.

IL-6 is a primary mediator of the acute-phase response and is a central inflammatory mediator in several chronic inflammatory diseases, such as rheumatoid arthritis, psoriasis, and atherosclerosis (Arican et al., 2005; Rose-John et al., 2007; Barnes et al., 2011). The effect of IL-6 on primary human hepatocytes has been documented, with the expression of several P450s downregulated (Jover et al., 2002; Aitken and Morgan, 2007; Le Vee et al., 2009; Dickmann et al., 2011). In human hepatoma cell line (HepaRG) cells, IL-6 and lipopolysaccharide (LPS) were previously shown to upregulate C-reactive protein (CRP) and downregulate CYP3A4 mRNA (Aninat et al., 2008). Moreover, drug clearance in cancer patients has been shown to inversely correlate with circulating IL-6 levels (Rivory et al., 2002), whereas treatment of rheumatoid arthritis patients with the anti–IL-6 antibody drug tocilizumab has been shown to directly affect CYP3A4 activity (Schmitt et al., 2011).
Such clinical observations have been further strengthened by preclinical studies assessing the mechanism of IL-6 and IL-6 antagonizing antibody–dependent P450 regulation in primary human hepatocytes (Dickmann et al., 2011).

IL-18 is also a potent inflammatory mediator, belonging to a different family of cytokines, the IL-1 family, and plays a role in chronic inflammatory conditions and autoimmune diseases (Okamura et al., 1995; Smith, 2011). Accordingly, plasma levels of IL-18 are elevated in patients suffering from chronic obstructive pulmonary disease, macrophage-activation syndrome, and atherosclerosis and may be used as a potential independent disease–risk factor indicator (Gracie et al., 2003; Dinarello, 2007). The specific effect of IL-18 on hepatocytes or regulation of P450 enzymes has not been described previously.

In the present study we investigated the effects of IL-6, IL-18, and the bacterial endotoxin LPS on mRNA levels and phenacetin-O-deethylase, bupropion-hydroxylation, and midazolam-1’-hydroxylation activities reflecting CYP1A2, CYP2B6, and CYP3A4 function in the human hepatoma cell line, HepaRG. We also investigated the effects of IL-6 and IL-18 on mRNA levels in cryopreserved pooled primary human hepatocytes. Since upregulation of acute-phase proteins also forms part of the coordinated proinflammatory response mediated through cytokine/cytokine receptor interaction, we investigated the impact of IL-6 and IL-18 treatment on CRP and determined the levels of IL-6 and IL-18 receptors in both the primary and HepaRG hepatocytes. The aim of this study was to characterize the effects of IL-18 and to compare these with the well documented case of IL-6. In doing this we investigated whether cryopreserved HepaRG cells could be an alternative to human hepatocytes for studies of P450 regulation by inflammatory stimuli.

Materials and Methods

Reagents and Chemicals. Cryopreserved differentiated HepaRG cells (batch HPR116062 at passage 16), type I collagen-coated 96-well multiwell plate, basal hepatic cell medium (MIL600), additives for thaw, seed, and general purpose HepaRG medium (ADD670), additives for HepaRG serum-free induction medium (ADD650) were purchased from Biopredic International (Rennes, France). Single-freeze pooled plateable human hepatocytes Cryostax9 (pool of five individuals, two males and three females), Hepatocyte Isolation Kit containing Percoll solution and supplemented media for isolation and Trypan Blue to obtain accurate cell yield and viability, Resuspension Media for seeding, and Hepatocyte Culture Media for dosing cultured hepatocytes were purchased from Tebu-bio, (Le Perray-en-Yvelines Cedex, France). Type I-coated 24-well multiwell plate was purchased from Becton Dickinson (Bedford, MA). Williams’ Medium E (with NaHCO₃, without γ-glutamyl and Phenol Red) was purchased from Sigma-Aldrich (St. Louis MO). L-Glutamine and HEPES were purchased from Gibco-Invitrogen/Life Technologies (Paisley, UK). Acetonitrile, methanol, and isopropyl alcohol were purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland), formic acid from Sigma-Aldrich, sodium hydroxide from AkzoNobel (Bohus, Sweden), and ethanol from Kemetyl (Hanninge, Sweden). Chloroform was purchased from Scharlau Chemie S.A. (Barcelona, Spain). Recombinant human IL-6 and IL-18 were purchased from R&D Systems (Abingdon, UK), LPS from Escherichia coli was purchased from Sigma-Aldrich.

Trizol Reagent for isolation of RNA, TaqMan Gene Expression Assays, TaqMan Universal Master Mix, SuperScript III First-Strand Synthesis System for reverse transcription–polymerase chain reaction (RT-PCR), and 20× TE buffer (Tris HCL+EDTA) were purchased from Applied Biosystems-Invitrogen/Life Technologies (Eugene Oregon). The primers and probes were purchased from Applied Biosystems (Cheshire, UK). Phenacetin was purchased from Sigma-Aldrich. Bupropion was purchased from Kemprotec Ltd. (Middlesbrough, UK), midazolam from Lipomed (Arlesheim, Switzerland), paracetamol from Sigma-Aldrich, hydroxybupropion from Toronto Research Chemicals (North York, ON, Canada), and 1’-hydroxymidazolam from Ultrafine UFC Limited (Manchester, UK).

The internal standards, paracetamol-d₄ and [13C₅]1’-hydroxymidazolam were purchased from Toronto Research Chemicals and hydroxybupropion-d₄ from Becton Dickinson.

HepaRG Cell Culturing, LPS, and Interleukin Exposure. The cryopreserved HepaRG cells were thawed and washed as follows: Tubes were wiped with 70% ethanol and the cap was briefly twisted and immediately closed under the laminar flow hood to release the internal pressure. The vials were quickly transferred to a 37°C water bath and gently agitated for 1–2 minutes. When only small ice crystals were remaining in the vials, they were removed every 5 min from the water bath. The thawed HepaRG cell suspension was transferred into an aseptic 50-ml Falcon tube containing 9 ml/cryo-vial of prewarmed (37°C) basal hepatocyte cell medium MIL600 supplemented with HepaRG Thaw, Seed, and General Purpose Medium (ADD670). The cryo-vials were rinsed once with approximately 1 ml of the ADD670 and the suspension was returned in to the Falcon tube. The HepaRG cells were centrifuged at 55g for 2 minutes at room temperature. The supernatant was removed and the pellet was resuspended in 5 ml of ADD670 and the number of cells and viability were determined by Trypan Blue cell count analysis. The cell suspension was seeded into flat-bottom multiwell plates, (100 μl/well, 0.72 × 10⁶ cells/ml) coated with rat-tail type I collagen. The cells were maintained at 37°C in an atmosphere of 95% air and 5% CO₂. After 6 hours, the cell morphology was checked and the medium was renewed with prewarmed ADD670. After 72 hours the medium was removed and replaced with basal hepatic cell medium MIL600 supplemented with HepaRG Serum-Free Induction Medium ADD650 including LPS (1.37–333 ng/ml), IL-6 (0.123–30 ng/ml), or IL-18 (2.96–500 ng/ml). Negative controls, exposed to serum-free induction medium ADD650 (basal medium, BM) were run in parallel. The HepaRG cells were exposed for 48 hours (mRNA) or 72 hours (enzyme activity) and the medium was renewed every 24 hours. The medium was always prewarmed to room temperature before addition to the cells (100 μl/well). Activity and mRNA incubations were performed in three plates in each of which two or three wells per concentration were exposed to LPS, IL-6, or IL-18, respectively. For negative control (BM) incubates, six wells per plate were used.

Primary Human Hepatocyte Cell Culturing and Interleukin Exposure. The cryopreserved pooled human hepatocytes were thawed and washed as follows: Tubes were wiped with 70% ethanol under the laminar flow hood. The content of the vials were immediately dispensed into a 50-ml tube containing prewarmed supplemented media, with penicillin (50 IU/ml), streptomycin (50 μg/ml), and isotonic Percoll. The tube was gently inverted until all ice was melted and centrifuged at 100g for 5 minutes at room temperature. The supernatant was discarded and the cell pellet was resuspended in nutrient-rich Hepatocyte Resuspension Media. Number of cells and viability were determined by Trypan Blue cell count analysis. The cell suspension was seeded into flat-bottom multiwell plates, (300 μl/well, 1.3 × 10⁶ cells/ml) coated with rat-tail type I collagen. The cells were allowed to attach for 6 hours in a 37°C humidified static incubator with an atmosphere of 95% air and 5% CO₂. After 6 hours, cell morphology was checked and the cells were washed and replaced with prewarmed Hepatocyte Culture Media. After 24 hours the media was replaced with fresh Hepatocyte Culture Media including IL-6 (0.006–50 ng/ml) or IL-18 (1.95–500 ng/ml). The medium was always prewarmed to 37°C before addition to the cells (350 μl/well) and was renewed every 24 hours. Single (IL-18) or duplicate (IL-6 and negative control) incubations were performed during 48 hours and negative controls were exposed to Hepatocyte Culture Media.

RNA Extraction. Total RNA from HepaRG cells and the primary hepatocytes was prepared after 48 hours exposure to LPS, IL-6, or IL-18 by using TRIzol reagent according to the manufacturer’s protocol. For HepaRG, samples of each concentration of the compounds were pooled, giving a final volume of 200 μl (negative control) and 200 μl or 300 μl (LPS and IL-exposed cells) for mRNA extraction. The final volume for the human hepatocytes was 300 μl. Quantity and purity of the RNA were determined spectrophotometrically using a NanoDrop (Saveen Werner AB, Limhamn, Sweden). On the basis of optimization of linearity, the cDNA was prepared from 800 ng (HepaRG) and 600 ng (hepatocytes) of total RNA using the SuperScript III First-Strand Synthesis System for RT-PCR with random hexamer primers according to the manufacturer’s protocol.

Real-Time RT-PCR. Real-time PCR for human P450 mRNA levels was performed using quantitative real-time PCR employing TaqMan Gene Expression Assay with specific primers and probes for CYP1A2, CYP2B6, CYP3A4, CRP, IL-6, IL-18, and the receptors for IL-6 and IL-18. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The following primers and probes were used for real-time PCR: CYP1A2 (unpublished), CYP2B6 (Life Technologies), CYP3A4 (Life Technologies), CRP (Life Technologies), IL-6 (Life Technologies), IL-18 (Life Technologies), and GAPDH (Life Technologies). The PCR reaction was performed in a 25-μl reaction volume containing 12.5 μl of 2× TaqMan universal master mix, 10 μl of cDNA, 0.5 μl of each primer, and 0.25 μl of each probe.

The PCR reaction was performed in a 25-μl reaction volume containing 12.5 μl of 2× TaqMan universal master mix, 10 μl of cDNA, 0.5 μl of each primer, and 0.25 μl of each probe. The PCR conditions were as follows: 1 cycle of 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The final 10 minutes of the PCR reaction was used for melting curve analysis. The PCR products were analyzed using the SDS software v2.1 (Applied Biosystems). The efficiency of the PCR reaction was determined by regression analysis of the standard curve. The relative expression of each gene was calculated using the 2⁻ΔΔCt method. The results were expressed as the fold change compared to the control group. The experiment was performed in triplicate for each sample.

Conclusion

In conclusion, our results demonstrate that IL-6 and IL-18 have a significant impact on the expression of CYP1A2, CYP2B6, and CYP3A4 in both primary human hepatocytes and HepaRG cells. These findings suggest that IL-6 and IL-18 may be used as potential risk factors for P450-dependent drug metabolism.
dehydrogenase and human acidic ribosomal phosphoprotein (huPO) were used as endogenous gene expression control. For huPO, gene-specific double fluorescent–labeled probes were used as reported previously (Persson et al., 2006). The reaction mixture (25 μl/well) contained 46 ng (HepaRG) or 34 ng (hepatocytes) of cDNA, 2 × Taqman Universal Master Mix, 1.25 μl of Gene Expression Assay Mix, as well as RNease-free water. The thermal cycle conditions were identical for all the genes analyzed and had initial steps of 50°C for 2 minutes and a 10-minute step at 95°C, followed by 40 PCR cycles of 95°C for 15 seconds and 60°C for 1 minute. Each sample was run in duplicate and data were analyzed using the 7500 Sequence Detector Software version 1.3.1 (Applied Biosystems/Life Technologies, Grand Island, NY).

The amount of mRNA was determined relative to that from control samples.

Activity Measurement in HepaRG cells. For cells used for activity measurements, induction medium was renewed every 24 hours and after 72 hours the medium was removed. The cells were washed twice with 100 μl prewarmed (37°C) Williams’ Medium E, supplemented with 2 mM L-glutamine and 25 mM HEPES (pH 7.4). A cocktail of phenacetin (26 μM), bupropion (100 μM), and midazolam (3 μM) (final concentrations) was prepared by dissolving the compounds in methanol. The methanol was taken to dryness under nitrogen gas, so that the activity medium did not include any organic solvent, and the compounds were redissolved in Williams’ Medium E with supplements to reach the intended concentration in the activity assay. The cocktail was prewarmed and added to the wells (50 μl/well). After 60 minutes of incubation, the medium (50 μl) was removed and transferred to a new plate, which was covered with a lid and stored at 4°C. The cells were lysed by adding ice-cold acetonitrile containing 0.8% formic acid (50 μl/well). To completely lyse the cells, the plates were subjected to three freezing-thawing cycles [3 × (20 minutes freezing and 10 minutes thawing)]. The resulting cell lysate was transferred and mixed with the corresponding well containing the supernatant incubation medium. The plates were centrifuged at 3220g for 20 minutes at 4°C. An aliquot (52 μl) of each sample was transferred to a new plate and diluted with 148 μl water supplemented with 25 μl of isotope-labeled internal standards (paracetamol–d4, hydroxybupropion–d3, or [13C3]1’-hydroxymidazolam). The samples were analyzed immediately after preparation.

The samples were analyzed by liquid chromatography–mass spectrometry for the metabolites of the marker substrates paracetamol (CYP1A), hydroxybupropion (CYP2B6), and 1’-hydroxymidazolam (CYP3A) and for the internal standards. High-performance liquid chromatography separation was achieved using a 10-μl injection volume onto a reversed-phase Halo C18 column (3.0 × 50 mm, 2.7 μm) (Advanced Materials Technology, Wilmington, DE) with a flow rate of 750 μl/min. The mobile phase consisted of (A) 50 ml of acetonitrile, 1.0 ml of formic acid, and 950 ml of water and (B) 950 ml of acetonitrile, 1.0 ml of formic acid, and 50 ml of water and was run for 3.10 minutes (5% B to 95% B over 2.3 minutes; 0.2 minutes at 95% B; 95% B to 5% B over 0.2 minutes, and 0.4 minutes equilibration at 5% B before the next injection). Detection was performed with a triple quadrupole mass spectrometer, API4000, equipped with electrospray interface (Applied Biosystems/MDS Sciex, Concord, ON, Canada). The mass spectrometry parameters were optimized using each analyte. Instrument control, data acquisition and data evaluation were performed using Applied Biosystems/MDS Sciex Analyst 1.4 software.

Data Evaluation of Activity Results. Fold reduction was calculated for IL-6, IL-18, and LPS-treated cells using BM-treated cells as a control. Mean values from treated cells were used for calculation. Mean values were calculated from three different plates and three replicates per plate.

Data Evaluation of Gene Expression Results. Quantification of relative gene expression and fold induction was performed using the ΔΔCt and the ΔCt method, respectively.

The expression in each sample was normalized by subtracting the endogenous control Ct values (glyceraldehyde-3-phosphate dehydrogenase and huPO) from the target Ct value (P450, IL, CRP) (eq. 1).

\[ ΔCt = Ct_{P450, IL, CRP} - Ct_{GAPDH, huPO} \] (1)

Relative gene expression level was calculated as 2^ΔCt (2).

Fold change was calculated for IL-6, IL-18, and LPS-treated cells using basal medium–treated cells as a calibrator (eqs. 3 and 4).

\[ ΔΔCt = ΔCt_{compound \ treated} - ΔCt_{basal \ medium \ treated} \] (3)

\[ \text{Fold change} = 2^{ΔΔCt} \] (4)

Mean values from treated cells involving three different plates, with two or three pooled replicates per plate, were used in the calculations for HepaRG. Individual and mean results were used for the cryopreserved primary human hepatocytes, with mRNA levels measured from one plate with single (IL-18) or duplicate (IL-6) incubations. The response relationship between mRNA or P450 activity and IL-6 and LPS concentrations was determined using a four-parameter logistic nonlinear regression analysis in Xlfit (IDBS, Guildford, Surrey, UK):
Results

The human hepatoma cell line HepaRG was exposed to a range of concentrations of LPS (1.37–333 ng/ml) and the cytokines IL-6 (0.123 ng/ml to 30 ng/ml) and IL-18 (2.06–500 ng/ml) for 48 hours (mRNA analyses) and 72 hours (enzyme activity measurements). The primary hepatocytes were exposed to IL-6 (0.006–50 ng/ml) and IL-18 (1.95–500 ng/ml) for 48 hours prior to mRNA analysis. The results refer to enzyme activity and mRNA levels relative to the negative control (BM-treated cells) at the highest nominal LPS or cytokine concentration tested (E_{max}) and the concentrations of IL-6, IL-18, or LPS required to cause 50% decrease in P450 activity or mRNA (EC_{50}). The levels of IL-6 and IL-18 mRNAs and the expression of their

Fig. 3. The effect of IL-6 on CYP1A2 (A and B), CYP2B6 (C and D), and CYP3A4 (E and F) mRNA expression in human hepatoma cell line HepaRG (A, C, and E) and primary hepatocytes (B, D, and F). The results represent HepaRG cells treated for 48 hours with IL-6 (0.123–30 ng/ml) and primary hepatocytes treated with IL-6 (0.006–50 ng/ml). Results are expressed as mean values from three plates with three pooled replicates per plate (HepaRG) or one plate with duplicate incubations (hepatocytes).
receptors were measured in the negative control samples. The slopes of amplification indicate equal amplification efficiencies and the delta Ct values were thus used to compare the expression levels in the two cell types. These delta Ct values suggest very similar levels of IL-6 receptor (IL-6r) mRNA in both cell systems. Although the same was true also for IL-18 receptor (IL-18r) and IL-6 mRNA, the data suggest a 4-fold higher level of IL-18 mRNA in the hepatocytes compared with HepaRG cells (Fig. 1). The expression of IL-6 was 16- and 7-fold lower than the expression of IL-18 in primary hepatocytes and HepaRG cells, respectively, whereas the expression of IL-6r was 13- and 29-fold higher in hepatocytes and HepaRG cells, respectively, compared with IL-18r (Fig. 1).

The degree of inflammatory response by IL-6 and IL-18 was assessed by measuring the mRNA of CRP. IL-6 caused an increase of CRP mRNA level in HepaRG cells and primary human hepatocytes, but no induction of CRP mRNA could be seen in cells exposed to IL-18 in the two cell systems (Fig. 2).

A decrease in mRNA levels, with a clear dose response was observed for all P450s studied within the tested concentrations of IL-6 in HepaRG-treated cells and primary cells (Fig. 3). The EC50 and the Emax estimates showed that the potency and the extent of activity and gene suppression varied among the P450 isoforms for both HepaRG and primary cells treated with LPS and IL-6 (Tables 1, 2 and 3). IL-6 caused the greatest effect on both activity in the HepaRG cells and mRNA levels in the two cell systems with an EC50 below 10 ng/ml for all P450 isoforms (Tables 1, 2 and 3). Similar potency was noted for LPS-treated HepaRG cells on CYP3A4, with an EC50 below 10 ng/ml. CYP1A2 and CYP2B6 were less sensitive to suppression by LPS, and no significant decrease in enzyme activity or gene expression was seen for IL-18-treated cells at the concentrations tested in HepaRG cells or in the cryopreserved human hepatocytes.

**Discussion**

For the in vitro study of the impact of test compounds or external stimuli such as proinflammatory mediators on P450 gene regulation and transcription, primary human hepatocytes are regarded as the system of choice, especially when studying the clinical development of therapeutic agents. Cell lines such as HepaRG are considered useful for providing supportive data (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf; http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf). However, limited availability of high-quality, plateable human hepatocytes coupled with the interindividual variability in P450 levels and response has encouraged the investigation of hepatoma–cell-line utility. Although recent progress in the availability of large batches of precharacterized inducible cryopreserved primary human hepatocytes has somewhat mitigated the issues, cost is a serious consideration, at least in a drug discovery setting. Thus, the pursuit of close surrogates to primary cells that can mimic relevant cellular responses, drug-metabolizing capacity, and regulation of the enzymes involved continues to be important. Basal expression of hepatic P450 isoenzymes and response to P450 gene regulators is complex and involves many ligand-activated transcription factors such as PXR, as well as direct binding transcription factors (Schreim et al., 2002, 2004). A separate level of control lies in chromatin conformation, which dictates transcription factor binding (Phillips et al., 2005). Both processes play key roles in regulating P450 expression and alterations in both are important in understanding not only the decline in P450 expression after isolation of hepatocytes from the intact liver but also the disparity in P450 expression between isolated primary hepatocytes and some hepatic cell lines (Phillips et al., 2005; Castell et al., 2006). An important factor in the downregulation of CYP3A4 by IL-6 is the translational induction of a truncated form of CCAAT-enhancer binding protein β (C/EBPβ), the 20-kDa liver-enriched transcriptional inhibitory protein (C/EBPβ-LIP). C/EBPβ-LIP heterodimerizes with the full-length C/EBPβ to form a dominant negative regulator of P450, since it contains a DNA-binding domain but not a transcriptional domain (Jover et al., 2002; Castell et al., 2006). LPS reduces the DNA binding activities of several hepatic nuclear factors and represses constitutive androstane receptor, PXR, and retinoid X receptor through activation of the nuclear factor k-light-chain-enhancer of activated B cells pathway (Martinez-Jimenez et al., 2007).

The utility of some hepatic cell lines in the study of drug-metabolizing enzymes and hepatic function has been shown to be limited by the deficit in one or more of the extremely complex pathways that make up the fully functioning primary hepatic parenchymal cells (Phillips et al., 2005; Castell et al., 2006; Hariparsad et al., 2008; Martin et al., 2008). On the other hand, HepaRG cells appear to have wide-ranging utility and can express some, but not all, drug-metabolizing enzymes and transporters at levels comparable to primary cells (Kanebratt and Andersson 2008) and have P450-inducer response closely mimicking primary cells (McGinnity et al., 2009).

Previous reports have described the direct effect of LPS and IL-6 on the expression and activity of P450 in primary human hepatocytes (Aitken and Morgan, 2007; Dickmann et al., 2011). In the present investigation we used HepaRG cells and cryopreserved primary human hepatocytes to investigate the effects of the previously studied inflammatory stimuli to further characterize HepaRG cells as a potentially useful surrogate for primary hepatocytes. Additionally, we added IL-18 to the list of investigated mediators, since IL-18 is produced by macrophages and other cells of the immune system to elicit pro-inflammatory effects following infection with microbial products such as LPS. After stimulation with IL-18, T-cells release interferon-γ to amplify the immune response. The same IL-18 preparation was used in

**TABLE 1**

<table>
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<th>Enzyme</th>
<th>LPS</th>
<th>IL6</th>
<th>IL18 EC50</th>
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<td>CYP1A2</td>
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<td>CYP2B6</td>
<td>31.3 ± 6.12</td>
<td>19.8</td>
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<td>CYP3A4</td>
<td>4.39 ± 2.73</td>
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<td>0.998 ± 0.418</td>
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</tbody>
</table>

Mean and standard deviation values are from three different plates. Data were normalized to untreated cells and fitted to a four-parameter dose-response model. Mean and standard deviation values are from three different plates. For IL-18, no E<sub>max</sub> is quoted owing to the lack of any dose-response.

**TABLE 2**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IL6</th>
<th>IL18 EC50</th>
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<td>IL18 E&lt;sub&gt;max&lt;/sub&gt; ng/ml</td>
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<tr>
<td>CYP1A2</td>
<td>19.3 ± 9.46</td>
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<td>CYP2B6</td>
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<tr>
<td>CYP3A4</td>
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</table>

Data were normalized to untreated cells and fitted to a four-parameter dose-response model. Mean and standard deviation values are from two different wells. For IL-18, no E<sub>max</sub> is quoted owing to the lack of any dose-response.

Data were normalized to untreated cells and fitted to a four-parameter dose-response model. Mean and standard deviation values are from two different wells. For IL-18, no E<sub>max</sub> is quoted owing to the lack of any dose-response.
all studies. The concentration range used was chosen on the basis of the observed effects of LPS and IL-18 to induce interferon-$\gamma$ production in primary human leukocytes in vitro (Puren et al., 1998; Berke, unpublished observations). Physiologic concentrations of these cytokines measured in plasma can be quite variable, depending on disease status and possibly on medication (Arican et al., 2005). Indeed, plasma concentrations of IL-18 range from around 0.1–0.2 ng/ml in healthy controls but up to 1–2 ng/ml in disease state (Blankenberg et al., 2003; Berke, unpublished observations). IL-6 levels in plasma from non-diseased subjects are typically around 5 pg/ml but can be as high as 50–60 pg/ml in rheumatoid arthritis patients (Robak et al., 1998). Thus, the concentration range used in previous publications of in vitro models extends beyond the values measured in clinical plasma samples, but it is difficult to correlate such measurements to local concentrations in the liver (both IL-6 and IL-18 are locally produced and can act in the tissue), and there is a clear correlation between the in vitro measured effects of IL-6 on P450 and the observations in tocilizumab-treated rheumatoid arthritis patients (Schmitt et al., 2011).

Comparison our HepaRG IL-6 EC$_{50}$ values with those previously reported for primary hepatocytes (Aitken and Morgan, 2007; Dickmann et al., 2011) shows a good degree of agreement between the results. For CYP1A2 phenacetin O-deethylation activity, Dickmann and coworkers reported EC$_{50}$ values ranging from 0.14 to 4 ng/ml, whereas the EC$_{50}$ determined for CYP1A2 mRNA downregulation was 0.3 ng/ml. The values we report here for HepaRG are 9 ng/ml for activity and 0.4 ng/ml for the mRNA analysis. For suppression of CYP2B6 transcription in HepaRG cells, we found IL-6 to be approximately 4- to 10-fold more potent than did Aitken and Morgan (0.5 ng/ml EC$_{50}$ compared with an estimated value of 2–5 ng/ml). Our primary hepatocyte EC$_{50}$ indicated a slightly more potent suppression by IL-6, with an EC$_{50}$ value of 0.18 ng/ml. Dickmann et al. determined an IL-6 EC$_{50}$ value of 3 pg/ml for CYP3A4 mRNA and values of 4–176 pg/ml for activity. From our data we are only able to place the EC$_{50}$ value in the range <100 pg/ml for HepaRG mRNA and activity. Aitken and Morgan were also unable to report an IL-6 EC$_{50}$ value in their study but observed a very high level of suppression of CYP3A4 mRNA at 10 ng/ml. Although we found reasonable agreement in the $E_{\text{min}}$ and EC$_{50}$ values obtained on the bases of CYP1A2, 2B6, and 3A4 mRNA analysis following HepaRG and primary hepatocyte experiments in our laboratory, the correspondence does not hold so well when comparing our primary hepatocyte CYP1A2 mRNA data to that of Dickmann et al., there being a 70-fold difference in IL-6 EC$_{50}$ (our value of 5 ng/ml compared with 0.07 ng/ml). Likewise, we estimated 0.5 ng/ml to be the EC$_{50}$ value in our primary hepatocyte CYP3A4 mRNA assay, considerably less potent than the value estimated by Dickmann et al. (3 pg/ml). Our primary hepatocyte study was limited in replicates; however, our principal aim was to corroborate our novel findings that indicated a lack of effect of IL-18 on P450 expression, as we first determined in HepaRG cells. Taking this into account and the intersubject variability in response to IL-6 evident in the P450 activity data presented by Dickmann et al., the interlaboratory IL-6 potency differences described above cannot undermine the significance of our findings for HepaRG cells as a substitute for primary hepatocytes and, indeed, the novel finding that IL-18 does not impact on P450 expression.

In addition to downregulation of drug-metabolizing enzymes by proinflammatory cytokines, acute-phase proteins including CRP are likewise synthesized in response to infection and tissue injury. In arteriolar smooth muscle cells both IL-6 and LPS have been shown to induce CRP production (Calabró et al., 2003). IL-6 in particular has been shown to be a potent regulator of acute-phase protein synthesis in primary hepatocytes (Castell et al., 1988). Here we also demonstrated that primary hepatocytes and HepaRG cells produced increased levels of CRP mRNA after IL-6 stimulation. Interestingly, there was no elevation in CRP mRNA in response to IL-18 treatment, further indicating that different proinflammatory cytokines exert different effects on hepatocytes. Although there is considerable literature assessing plasma CRP and IL-18 levels as independent risk factors in several chronic inflammatory diseases and CRP has been implicated as a potential cause of increased circulating IL-18 under inflammatory conditions (Yamaoka-Toyo et al., 2003), the direct effect of elevated IL-18 levels on CRP expression has not been studied previously.

The observation that different cytokines may have diverse effects on P450 isoenzyme transcription is in line with previous data by (Aitken and Morgan 2007). Whether this is a consequence of differences in intracellular signaling pathways from the different cytokine receptors is still to be investigated. However, it has been demonstrated previously that nuclear receptors implicated in P450 transcriptional modulation are differentially regulated by cytokines, with the downregulating effects of IL-1 and IL-6 in human hepatocytes dependent on distinct mechanisms (Jover et al., 2002; Assenat et al., 2004; Martinez-Jimenez et al., 2005).

In summary, this work provides further evidence that many of the complex and multifaceted control mechanisms for maintaining liver-specific function are operational in HepaRG cells and that this hepatoma cell line can, in certain circumstances, be used with full confidence as a surrogate system for primary human hepatocytes. Additionally, IL-18 is distinct from proinflammatory LPS and IL-6 in not causing downregulation of CYP1A2, CYP2B6, and CYP3A4.

### Authorship Contributions

**Participated in research design:** T. Andersson, Rubin, Grime, Berke, Janefeldt.

**Conducted experiments:** Rubin, Janefeldt.

**Contributed new reagents or analytic tools:** Berke.

**Performed data analysis:** Rubin, L. Andersson.

**Wrote or contributed to the writing of the manuscript:** Rubin, Grime, T. Andersson, Berke.