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HepaRG cells as human relevant in vitro model to study the effects of inflammatory stimuli on Cytochrome P450 isoenzymes

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HepaRG cells and effects of IL-6, IL-18 and LPS on P450

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Abbreviations:

BM, Basal medium; C/EBPß, CCAAT enhancer binding protein ß; CAR, Constitutive Androstane Receptor; C/EBPß-LIP, liver-enriched transcriptional inhibitory protein; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; FXR, Farnesoid X receptor; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HepaRG, Human hepatoma cell line; HNF, Hepatic nuclear factor; huPO, Human acidic ribosomal phosphoprotein; IFNγ, interferon-γ; IL, interleukin; LPS, Lipopolysaccharide; LXR, Liver X receptor; MAS,

macrophage activation syndrome; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; PPARa, Peroxisome proliferator activated receptor; PXR, Pregnane X Receptor; RA, Rheumatoid Arthritis; RXRa, retinoid X receptor; IL-6r, IL-6 receptor; IL-18r, IL-18 receptor.

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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 pro-inflammatory 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 and CYP1A2, CYP2B6 and CYP3A4 mRNA levels as well 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 pro-inflammatory 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

Pro-inflammatory cytokines can impact the transcriptional regulation of P450 enzymes. Thus, due to elevated levels of some circulating cytokines in chronic inflammatory disease conditions, expression levels and activity of these drug metabolizing enzymes can be affected (Shedlofsky et al., 1994; Morgan et al., 2001; Kulmatycki and Jamali 2005). This phenomenon is of importance when taking account of pharmacokinetic differences between healthy and diseased subjects, as well as when considering the introduction of novel anti-inflammatory therapies. Depending on the type of therapy, there may be a decrease in circulatory pro-inflammatory 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 the understanding as to the relevance of such events and help in making predictions of 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, 2012). The mechanisms behind this P450 regulation have been investigated and the NFkB dependent inflammatory response and associated re-localization and dimerization of nuclear receptors such as RXRa, LXR, FXR, CAR, PXR and PPARa has been implicated (Jover et al., 2002, Yang et al., 2010; Ghose et al., 2011). However, P450 specific transcriptional regulation has not fully been 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 down regulated (Jover et al., 2002; Aitken and Morgan, 2007, Vee et al., 2009; Dickmann et al., 2011). In HepaRG cells, IL-6 and lipopolysaccharide (LPS), were previously shown to up regulate C-reactive protein (CRP) and down regulate CYP3A4 mRNA (Aninant et al., 2008). Moreover, drug clearance in cancer patients has been shown to inversely correlate with circulating IL-6 levels (Rivory et al., 2002), whilst treatment of rheumatoid arthritis patients with the anti IL-6 antibody drug Tocolizumab 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 playing 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 (COPD), macrophage activation syndrome (MAS) 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 lipopolysaccharide (LPS) on mRNA levels and phenacetin-O-deethylase, bupropion-hydroxylase and midazolam-1'-hydroxylase activities reflecting CYP1A2, CYP2B6 and CYP3A4 function in the human hepatoma cell line, HepaRG. We also investigated these effects of IL-6 and IL-18 on mRNA levels in cryopreserved pooled primary human

hepatocytes. Since up-regulation of acute-phase proteins also forms part of the coordinated pro-inflammatory response mediated through cytokine/cytokine receptor interaction, we investigated the impact of IL-6, IL-18 and LPS treatment on C-reactive protein (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 multi well plate, cell culture media (basal hepatic cell medium, additives for Thaw, Seed and General Purpose HepaRG medium, additives for Serum-free induction HepaRG medium), were purchased from Biopredic International (Rennes, France). Single freeze pooled plateable human hepatocytes Cryotax™, (pool of five individuals, 2 males and 3 females), Hepatocyte Isolation Kit containing Percoll solution and Supplemented Media for isolation and Trypan blue to obtain accurate cell yield and viability (K2000), Resuspension Media, for seeding (K2200) and Hepatocyte Culture Media for dosing cultured hepatocytes (K2300) were purchased from Tebu-bio, (Le Perray-en-Yvelines, Cedex). Type I coated 24-well multi well plate was purchased from Becton Dickinson, (Two Oak Park, Bedford, MA). William's medium E (with $NaHCO_3$, without L-glutamine and phenol red) was purchased from Sigma-Aldrich (St Louis MO, USA). L-glutamine and HEPES, were purchased from Gibco Invitrogen (Paisley, UK). Acetonitrile, methanol and isopropyl alcohol were purchased from Rathburn Chemicals Ltd (Walkerburn, Scotland), formic acid from Sigma Aldrich (St Louis, MO, USA), sodium hydroxide from Akzo Nobel (Bohus, Sweden) and ethanol from Kemetyl (Haninge, Sweden). Chloroform was purchased from Scharlau Chemie S.A (Barcelona, Spain). Recombinant human IL-6 and IL-18 were purchased from R&D (Abingdon, UK), LPS from E.coli was purchased from Sigma Aldrich (St Louis, MO, USA).

Trizol® Reagent for isolation of RNA, TaqMan® Gene Expression Assays, TaqMan® Universal Master Mix, SuperScript III First-Strand Synthesis System for RT-PCR, 20xTe buffer (Tris HCL+EDTA) were purchased from Applied Biosystems, Invitrogen (Eugene Oregon, USA). The primers and probes were purchased from Applied Biosystems (Cheshire, UK). Phenacetin was purchased from Sigma-Aldrich (St Louis, MO, USA). Bupropion was

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purchased from Kemprotec Ltd (Middlesbrough, UK), midazolam from Lipomed (Switzerland), paracetamol from Sigma-Aldrich (St Louis, MO, USA), hydroxybupropion from Toronto Research Chemicals (North York, Canada) and 1'-hydroxymidazolam from Ultrafine UFC Limited (Manchester, UK).

The internal standards, paracetamol-d_4 and 1'-hydroxymidazolam-$^{13}C_3$ were purchased from Toronto Research Chemicals (North York, Canada) and hydroxybupropion-d_6 from Becton Dickinson, (Two Oak Park, Bedford, MA).

HepaRG Cell culturing, LPS and Interleukin exposure. The cryopreserved HepaRG cells were thawed and washed as follows:

The 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 from the water bath. The thawed HepaRG cell suspension was transferred into an aseptic 50 mL Falcon tube containing 9 mL/cryo vial of pre-warmed (37ºC) HepaRG Thaw, Seed and General Purpose Medium (MIL670). The cryo vials were rinsed once with approximately 1 mL of the MIL670 and the suspension was returned in to the Falcon tube. The HepaRG cells were centrifuged at 357g for 2 minutes at room temperature. The supernatant was removed and the pellet was re-suspended in 5 mL of MIL670 and amount of cells and viability were determined by Trypan Blue cell count analysis. The cell suspension was seeded into flat–bottom multi-well plates, (100 µL/well, $0.72x10^6$ cells/mL) coated with rat tail type I collagen. The cells were maintained at 37°C in an atmosphere of 95% air and 5% CO_2. After 6 hours, the cell morphology was checked and the medium was renewed with pre-warmed Thaw, Seed and General Purpose Medium (MIL670). After 72 hours the HepaRG Thaw, Seed, and General

Purpose Medium 670 was removed and replaced with the HepaRG Serum-free Induction Medium 650 including LPS (1.37-333 ng/mL), IL-6 (0.123-30 ng/mL) or IL-18 (2.06-500 ng/mL). Negative controls, exposed to Serum-free Induction Medium 650 (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 pre-warmed to room temperature before addition to the cells (100 µL/well). Activity and mRNA incubations were performed in three plates respectively, where two or three wells per plate where exposed to LPS, IL-6, or IL-18. 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:
The 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 U/mL), Streptomycin (50 µg/mL) and isotonic Percoll^{TM} (K2000). The tube was gently inverted until all ice was melted and centrifuged at 100 g for 5 minutes at room temperature. The supernatant was discarded and the cell pellet was re-suspended in nutrient rich Hepatocyte Re-Suspension media (K2200). Amount of cells and viability were determined by Trypan Blue cell count analysis. The cell suspension was seeded into flat–bottom multi-well plates, (300 µL/well, 1.3x10^6 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% CO_2. After 6 hours, cell morphology was checked and the cells were washed and replaced with pre-warmed Hepatocyte Culture Media (2300). 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 pre-warmed 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 manufactures 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). Based on 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 reverse transcription-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 receptor for IL-6 and IL-18. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and human acidic ribosomal phosphoprotein (huPO) were used as endogenous gene expression control. For human acidic ribosomal phosphoprotein (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, 2xTaqman Universal Master Mix, 1.25 µL Gene Expression Assay Mix as well as RNase free water. The thermal cycle conditions were identical for all the genes analyzed and had initial steps of 50°C for 2 min and a 10 minutes 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, Foster City, CA). 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 pre-warmed (37°C) William's 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 re-dissolved in William's medium E with supplements to reach the intended concentration in the activity assay. The cocktail was pre-warmed 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 4ºC. 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 x (20 min freezing and 10 min thawing)). The resulting cell lysate was transferred and mixed with the corresponding well containing the supernatant incubation medium. The plates were centrifuged at 3220 g 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 isotope labeled internal standards (IS) (paracetamol d_4, hydroxybupropion-d_6 or 1' hydroxymidazolam-$^{13}C_3$). The samples were analyzed immediately after preparation.

The samples were analyzed by LC/MS for the metabolites of the marker substrates paracetamol (CYP1A), hydroxybupropion (CYP2B6) and 1'-hydroxymidazolam (CYP3A) and for the internal standards. HPLC separation was achieved using a 10 µL injection volume on to a reversed-phase Halo C18 column (3.0x50 mm, 2.7 µm), Advanced Materials

Technology, DE, USA) with a flow rate of 750 µL/min. The mobile phase consisted of (A) 50 mL acetonitrile, 1.0 mL formic acid and 950 mL water and (B) 950 mL acetonitrile, 1.0 mL formic acid and 50 mL 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, Canada). The MS 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 basal medium (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 ΔC_T and the $\Delta\Delta C_T$ method respectively:

The expression in each sample was normalised by subtracting the endogenous control Ct values (GAPDH and huPO) from the target Ct value (CYP, IL, CRP) (Equation 1).

$$\Delta Ct = Ct_{CYP,\ IL,\ CRP} - Ct_{GADPH,huPO} \quad (1)$$

Relative gene expression level was calculated as $2^{-\Delta Ct}$ (2)

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

$$\Delta\Delta CT = \Delta Ct_{compound\ treated} - \Delta Ct_{basal\ medium\ treated} \quad (3)$$

Fold changed = $2^{-\Delta\Delta Ct}$ (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 CYP activity and IL-6 and LPS concentration was determined using a four-parameter logistic non-linear regression analysis in Xlfit:

$$v = A + \frac{B - A}{1 + ([I] / IC_{50})^{D}}$$

where v is the fold change to control in activity or mRNA, [I] is the log of IL-6 or LPS concentration, A and B are the lowest and highest activity or mRNA response (% of control) and D is the Hill slope of the curve.

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_{min}) and the concentration 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 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-6r mRNA in both cell systems. Whilst the same was true also for IL-18r and IL-6 mRNA, the data suggest a 4 fold higher level of IL-18 mRNA in the hepatocytes as compared with HepaRG cells (Figure 1). The expression of IL-6 was 16- and 7-fold lower than the expression of IL-18 in primary hepatocytes and HepRG cells respectively, whilst the expression of IL-6r was 13- and 29-fold higher in hepatocytes and HepaRG cells, respectively compared to IL-18r (Figure 1).

The degree of inflammatory response by IL-6 and IL-18 was assessed by measuring the mRNA of C-reactive protein, (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 concentrations tested of IL-6 in HepaRG treated cells and primary cells (Fig. 3). The EC_{50} and the E_{min} 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 (Table 1, Table 2 and Table 3). IL-6 caused the greatest effect on both activity in the HepaRG cells and mRNA levels in the two cell systems with an EC_{50} below 10 ng/mL for all P450 isoforms (Table 1, Table 2 and Table 3). Similar potency was noted for LPS treated HepaRG cells on CYP3A4, with an EC_{50} 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 pro-inflammatory mediators on CYP gene regulation and transcription, primary human hepatocytes are regarded as the system of choice, especially when supporting the clinical development of therapeutic agents. Cell lines such as HepaRG are considered as useful for providing supportive data (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf;http://www.ema.europa.eu/docs/enGB/document_library/Scientific_guideline/2012/07/WC500129606.pdf). However, limited availability of high-quality, plateable human hepatocytes coupled with the inter-individual variability in P450 levels and response has encouraged the investigation of hepatic cell line utility. Although recent progress in the availability of large batches of pre-characterized 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 (Schrem et al. 2002; Schrem et al. 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 down-regulation of CYP3A4 by IL-6 is the translational induction of a truncated form of CCAAT enhancer binding protein ß (C/EBPß), the 20kDa liver-enriched

transcriptional inhibitory protein (C/EBPß-LIP). C/EBPß-LIP hetero-dimerises with the full length C/EBPß to form a dominant negative regulator of P450, since it contains a DNA-binding domain but not a trans-activation domain (Jover et al., 2002; Castell et al., 2006). LPS reduces the DNA binding activities of several Hepatic Nuclear Factors (HNF) and represses CAR, PXR and RXR through activation of the NFkB 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 (Philips et al, 2005; Castell et al., 2006; Hariparsad et al., 2008; Martin et al., 2008). On the other hand, HepaRG cells are emerging as having wide ranging utility with expression of some, but not all, drug metabolizing enzymes and transporters at levels comparable to primary cells (Kanebratt and Andersson 2008) and CYP 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 (Dickmann et al., 2011; Aitken and Morgan, 2007). In the present investigation we used HepaRG cells and cryopreserved primary human hepatocytes to investigate the effects of the previously studied inflammatory stimuli in order 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 (IFNγ) to amplify the immune response. The same IL-18 preparation was used in all studies. The concentration range used was chosen based on the observed effects of LPS and IL-18 to induce IFNγ production in primary human leukocytes in

vitro (Puren et al, 1998, Berke, unpublished observations). Physiological concentrations of these cytokines measured in plasma can be quite variable, depending on disease status and possibly 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 (RA 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 tocolizumab treated RA patients (Schmitt et al, 2011).

When comparing our HepaRG IL-6 EC50 values with those previously reported for primary hepatocytes (Dickmann et al., 2011; Aitken and Morgan, 2007) there is a good degree of agreement between the results. For CYP1A2 phenacetin O-deethylation activity, Dickmann and co-workers reported EC50 values ranging from 0.14 to 4 ng/mL whereas the EC50 determined for CYP1A2 mRNA down regulation 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 - 10-fold more potent than Aitken and Morgan (0.5 ng/mL EC50 compared to an estimated value of 2 - 5ng/mL). Our primary hepatocyte EC50 indicated a slightly more potent suppression by IL-6, with an EC50 value of 0.18 ng/mL. Dickmann et al determined an IL-6 EC50 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 EC50 value in the range < 100 pg/mL for HepaRG mRNA and activity. Aitken and Morgan were also unable to report an IL-6 EC50 value in their study, but

observed a very high level of suppression of CYP3A4 mRNA at 10 ng/mL. Whilst we found reasonable agreement in the Emin and EC50 values obtained based on 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 EC50 (our value of 5 ng/mL compared to 0.07 ng/mL). Similarly we estimated 0.5 ng/mL to be the EC50 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 indicating a lack of effect of IL-18 on P450 expression, which we first determined in HepaRG cells. Taking this into account and the inter-subject variability in response to IL-6 evident in the P450 activity data presented by Dickmann et al, the inter-lab 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 down-regulation of drug metabolising enzymes by pro-inflammatory cytokines, acute-phase proteins including CRP are similarly synthesized in response to infection and tissue injury. In arterial 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 stimulation with LPS and IL-6 stimulation. Interestingly there was no elevation in CRP mRNA in response to IL-18 treatment, further indicating that different pro-inflammatory cytokines exert different effects on hepatocytes. Whilst 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 difference 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 down-regulating effects of IL-1 and IL-6 in human hepatocytes dependent on distinct mechanisms (Assenat et al., 2004, Jover et al., 2002, Martinez-Jimenez et al., 2005).

In summary, this work provides further evidence that many of the complex and multi-faceted control mechanisms for maintaining liver-specific function are operational in HepaRG cells and that this hepatic 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 pro-inflammatory LPS and IL-6 in not causing down-regulation of CYP1A2, CYP2B6 and CYP3A4.

Authorship Contributions

Participated in research design: TB Andersson, K Rubin, K Grime, Z Berke, and A Janefeldt.

Conducted experiments: K Rubin and A Janefeldt

Contributed new reagents or analytic tools: Z Berke

Performed data analysis: K Rubin, L Andersson

Wrote or contributed to the writing of the manuscript: K Rubin, K Grime, TB Andersson and Z Berke.

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Legend for Figures

Fig.1. The relative gene expression level of IL-6, IL-18 and their receptors IL-6r and IL18r, measured in untreated HepaRG cells (gray bar) and primary hepatocytes (black bar). The results are presented as the mRNA expression in each sample normalised by subtracting the endogenous control according to equation 1 in Material and Methods. HepaRG results are expressed as mean values from 3 plates with three pooled replicates per plate and the hepatocyte from one plate with two pooled replicates.

Fig. 2. The effect of proinflammatory cytokines on CRP gene expression in HepaRG cells (dark gray bar) and primary hepatocytes (black bar) after treatment with the highest concentration of IL-6 (30 and 50 ng/mL respectively) and IL-18 (500 ng/mL) for 48 hours. Data was normalized to untreated cells (light gray bar) and calculated as described under Materials and Methods, equation 1, 3 and 4. The HepaRG results are expressed as mean values from three plates with two or three pooled replicates per plate and the hepatocyte from one plate with duplicate (IL-6) or single (IL-18) incubations.

Fig. 3.The effect of IL-6 on CYP1A2 (A, B), CYP2B6 (C, D) and CYP3A4 (E, F) mRNA expression in human hepatoma cell line HepaRG (A, C, E) and primary hepatocytes (B, D, F). The results represent HepaRG cells treated for 48 hours with IL-6 (0.123 ng/mL-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).

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Tables

Table 1.Suppression of gene expression for CYP1A2, CYP2B6 and CYP3A4 after treating HepaRG cells with increasing concentrations of LPS (1.37 -333 ng/mL), IL-6 (0.123-30 ng/mL) and IL-18 (2.06-500 ng/mL). 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_{min} is quoted due to the lack of any dose-response.

Enzyme	LPS		IL6		IL18
	E_{min} (% of control)	**EC_{50} ng/mL**	**E_{min} (% of control)**	**EC_{50} ng/mL**	**EC_{50} ng/mL**
CYP1A2	28.8 ± 20.0	37.9	7.83 ± 5.30	0.452	>500
CYP2B6	31.3± 6.12	19.8	4.64 ± 0.98	0.388	>500
CYP3A4	4.39± 2.73	<1.37	0.998 ±0.418	<0.123	>500

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Table 2. Suppression of gene expression for CYP1A2, CYP2B6 and CYP3A4 after treating primary hepatocytes with increasing concentrations of IL-6 (0.006-50 ng/mL) and IL-18 (1.95-500 ng/mL). 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_{min} is quoted due to the lack of any dose-response.

Enzyme	IL6		IL18
	E_{min} (% of control)	**EC_{50} ng/mL**	**EC_{50} ng/mL**
CYP1A2	19.3 ± 9.46	5.49	>500
CYP2B6	22.4 ± 2.27	0.177	>500
CYP3A4	7.65 ± 3.43	0.454	>500

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Table 3. Suppression of paracetamol (CYP1A2), hydroxybupropion (CYP2B6) and 1' hydroxymidazolam (CYP3A4) formation after treating HepaRG cells with increasing concentrations of LPS (1.37-333 ng/mL), IL-6 (0.123 -30 ng/mL) and IL-18 (2.06-500 ng/mL). Data were normalized to untreated cells and fit to a four parameter dose-response model. Mean and standard deviation values are from three different plates. For IL-18, no E_{min} is quoted due to the lack of any dose-response.

Enzyme	Metabolite	LPS		IL6		IL18
		E_{min} (% of control)	EC_{50} ng/mL	E_{min} (% of control)	EC_{50} ng/mL	EC_{50} ng/mL
CYP1A2	paracetamol	47.6 ± 7.2	>333	35.2 ± 7.7	8.96	>500
CYP2B6	hydroxybupropion	47.4 ± 2.18	116	20.4 ± 1.28	0.970	>500
CYP3A4	1'-hydroxymidazolam	39.1 ± 4.63	7.85	41.1 ± 3.44	2.89	>500

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Figures

Figure 1

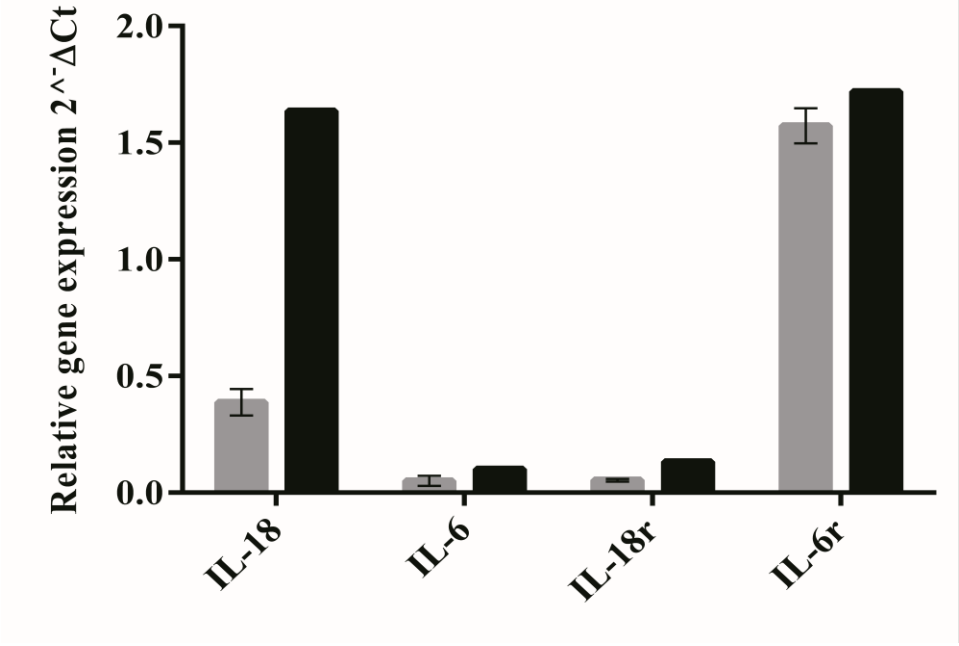

Figure 2

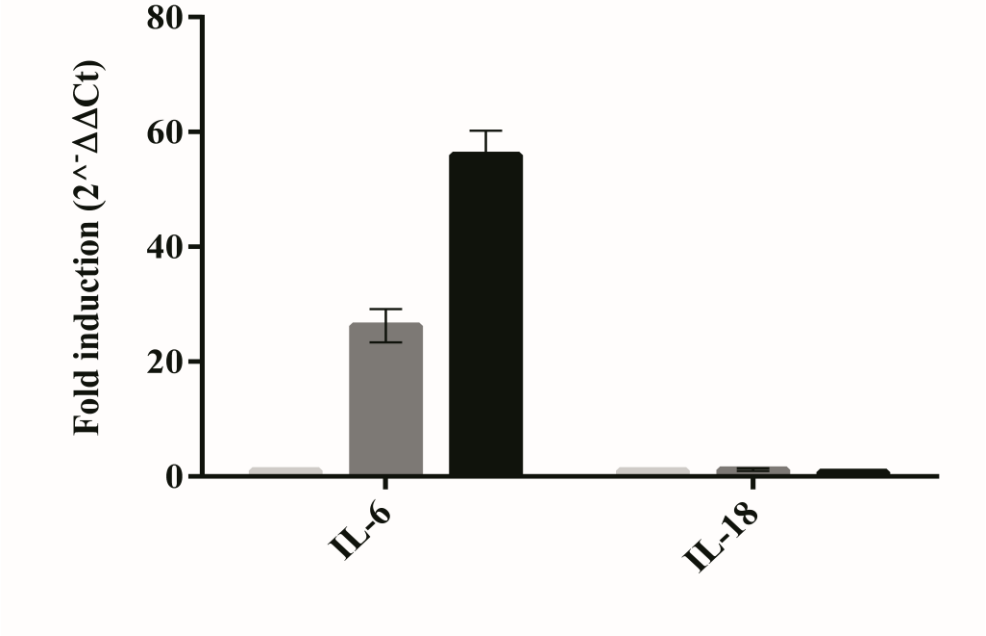

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Figure 3