Regulation of drug metabolism by the interplay of inflammatory signaling, steatosis, and xeno-sensing receptors in HepaRG cells

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List of non-standard abbreviations: 3-MC; 3-methylcholanthrene; AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; CAR, constitutive androstane receptor; CITCO, 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; CTB, cell titer blue; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; IL, interleukin; LXR, liver-X-receptor; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NRF2, nuclear factor (erythroid-derived 2)-like 2; O/P, equimolar oleate/palmitate mixture; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane-X-receptor; tBHQ, tert.-butylhydroquinone; Wy14,643, 4-Chloro-6-(2,3-xylidino)-2-pyrimidinythioacetic acid
Abstract

Non-alcoholic fatty liver disease (NAFLD), characterized by triglyceride deposition in hepatocytes due to imbalanced lipid homeostasis, is of increasing concern in Western countries, with progression to non-alcoholic steatohepatitis (NASH), liver fibrosis, and cirrhosis. Previous studies suggest a complex, mutual influence of hepatic fat accumulation, NASH-related inflammatory mediators, and drug-sensing receptors regulating xenobiotic metabolism. Here, we investigated the suitability of human HepaRG hepatocarcinoma cells as a model for NAFLD and NASH. Cells were incubated for up to 14 days with an oleate/palmitate mixture (125 µM each), and/or with 10 ng/mL of the inflammatory mediator interleukin-6. Effects of these conditions on the regulation of drug metabolism were studied using xenobiotic agonists of the aryl hydrocarbon receptor (AHR), pregnane-X-receptor (PXR), constitutive androstane receptor (CAR), nuclear factor (erythroid-derived 2)-like 2 (NRF2), and peroxisome proliferator-activated receptor α (PPARα). Results underpin the suitability of HepaRG cells for NAFLD- and NASH-related research and constitute a broad-based analysis of the impact of hepatic fatty acid accumulation and inflammation on drug metabolism and its inducibility by xenobiotics. Interleukin-6 exerted pronounced negative regulatory effects on basal as well as on PXR-, CAR-, PPARα-, but not AHR-dependent induction of drug-metabolizing enzymes. This inhibition was related to diminished transactivation potential of the respective receptors, rather than to reduced transcription of nuclear receptor-encoding mRNAs. Most striking effects of interleukin-6 and/or fatty acid treatment were observed in HepaRG cells following 14 days of treatment, making these cultures appear a suitable model for studying the relationship of fatty acid accumulation, inflammation and xenobiotic-induced drug metabolism.
Introduction

A major part of drug and xenobiotic metabolism is carried out in the liver. The expression and activity of many drug-metabolizing enzymes is regulated via ligand-activated nuclear receptors which function as sensors for xenobiotics. In particular, the induction of cytochrome P450 (CYP) enzymes by exogenous compounds via the aryl hydrocarbon receptor (AHR), constitutive androstane receptor (CAR), and pregnane-X-receptor (PXR) has been studied extensively (Honkakoski and Negishi, 2000). In addition to xenobiotic nuclear receptor ligands, endogenous signaling pathways also affect the regulation of hepatic drug metabolism via nuclear receptors, e.g. see Braeuning et al. (2009), Braeuning and Schwarz (2010), or Schulthess et al. (2015).

Non-alcoholic fatty liver disease (NAFLD) as the hepatic manifestation of the metabolic syndrome is characterized by the (generally reversible) deposition of triglycerides in hepatocytes as a consequence of multiple imbalances in lipid homeostasis (Day and James, 1998). NAFLD is of increasing concern in Western countries, and it is believed that, due to unbalanced dietary intake of fats and carbohydrates, up to one quarter of the population is affected (Weiss et al., 2014). NAFLD may progress to non-alcoholic steatohepatitis (NASH), which is linked to an increase in pro-inflammatory cytokines in the liver, thereby leading to inflammation and oxidative stress, and apoptosis of individual hepatocytes (Day and James, 1998; Hijona et al., 2010). The exact molecular determinants which trigger the transition from NAFLD to NASH are still not fully understood. Perpetuated hepatic cell death and regenerative proliferation in inflamed livers might subsequently give rise to the onset of irreversible hepatic fibrosis and cirrhosis. The latter is considered a major risk factor for the development of hepatocellular carcinoma (Sanyal et al., 2010; Toosi, 2015).

Published data indicate interactions between NAFLD and drug metabolism: different nuclear receptors as well as drug-metabolizing enzymes have been identified as being involved in the formation of liver steatosis based on animal models or patient data; for details see the review by Naik et al. (2013). P450 oxidoreductase knockout-driven loss of CYP activities in a transgenic mouse model has been shown to affect hepatic lipid accumulation as well as CAR
and PXR activities (Finn et al., 2009). Alterations in the activity and abundance of various CYPs and phase II enzymes in rodents and humans have been summarized by Gomez-Lechon et al. (2009) and by Merrell and Cherrington (2011). Their conclusions indicate pleiotropic effects of NAFLD and NASH on drug-metabolizing enzymes; however, not always with consistent results regarding the up- or down-regulation of certain CYP isoforms under different conditions of experimental animal research or clinical studies. Previous studies suggest a negative influence of steatosis on various CYP activities in human primary hepatocytes in vitro (Donato et al., 2006), while in HepaRG cells prolonged PPARα activation counteracts steatosis in these cells (Rogue et al., 2014). Inflammatory mediators, for example interleukin 6 (IL-6) are known to broadly decrease the drug-metabolizing capacities of hepatocytes (Morgan et al., 2008). Mechanistically this phenomenon mainly results from attenuated transcription of drug metabolism-related genes, and seems to involve interference of inflammation signaling with the activity of hepatocyte-enriched transcription factors from the HNF (hepatocyte nuclear factor) family together with downregulation or inactivation of nuclear receptors engaged in the regulation of drug metabolism (Klein et al., 2015; Liu et al., 2012; Merrell and Cherrington, 2011; Pascussi et al., 2000).

While primary hepatocytes are still considered as a gold standard for in vitro research in liver metabolism (Godoy et al., 2013), their use is often limited by scarce availability and large inter-donor variation. Human HepaRG hepatocarcinoma cells constitute an alternative to primary human hepatocytes, due to the fact that this permanent cell line is able to differentiate into hepatocyte-like cells which possess many physiological functions of hepatocytes, especially with regard to drug metabolism (Aninat et al., 2006). Therefore, HepaRG cells have been used successfully for in vitro experimentation in basic research and drug development for a few years. Some previous studies have used HepaRG cells as an in vitro model for hepatic steatosis (Brown et al., 2013; Rogue et al., 2014) or inflammation (Dubois-Pot-Schneider et al., 2014; Klein et al., 2015; Rubin et al., 2014), thus demonstrating the general suitability of this cell line for NAFLD and NASH research.
Previous studies have addressed selected endpoints, while comparative systematic analyses of basal and xenobiotic-induced drug metabolism in HepaRG cells under different experimental conditions representing NAFLD and NASH are still not available. The present study was aimed to systematically characterize the impact of steatotic and inflammatory conditions on the basal and inducible expression of drug-metabolizing enzymes in HepaRG cells.
Materials and Methods

Cell culture and treatment

HepaRG human hepatocarcinoma cells (Biopredic, Rennes, France) were differentiated and cultivated as recently described (Luckert et al., 2017), including 2 weeks incubation with 1.7% dimethyl sulfoxide (DMSO) for the induction of differentiation into hepatocyte-like cells. Fetal calf serum concentrations were reduced to 1% 24h prior to treatment with the compounds listed below. Standard 2-dimensional cultivation of HepG2 human hepatocarcinoma cells has been described previously (Luckert et al., 2017). Cells were treated according to the scheme depicted in Figure 1 with the following chemicals: xenobiotic-sensing receptors were activated by treatment with either 5 µM of the aryl hydrocarbon receptor (AHR) agonist 3-methylcholanthrene (3-MC; Enzo Life Sciences, Lörrach, Germany), 5 µM of the constitutive androstane receptor (CAR) agonist 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime (CITCO; Enzo Life Sciences), 10 µM of the pregnane-X-receptor (PXR) agonist rifampicin (Rif; Sigma, Taufkirchen, Germany); 30 µM of the NRF2 (nuclear factor (erythroid-derived 2)-like 2) activator tert.-butylhydroquinone (tBHQ; Sigma), or 50 µM of the peroxisome proliferator-activated receptor α (PPARα) agonist Wy14,643 (Wy; 4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid; gift from Dr. M. Schwarz, Tübingen, Germany). All compounds were dissolved in dimethyl sulfoxide (DMSO). Additional cell treatment was performed with 10 ng/mL of the inflammatory mediator interleukin 6 (IL-6; PromoCell, Heidelberg, Germany), with equimolar mixtures of the fatty acids oleate and palmitate (O/P; dispersed in cell culture medium by sonication; both compounds purchased from Sigma), or with 10 µM of the LXR agonist GW 3965 (Sigma). Oleate and palmitate were chosen as representatives of unsaturated and saturated fatty acids, respectively, and are the two fatty acids that show the highest abundance in NAFLD and NASH patients (Aray et al., 2004; please also refer to the discussion section). For details on the treatment regimen, please also refer to the legend to Figure 1.
The concentrations of receptor agonists were chosen based on initial cytotoxicity testing and respective literature values (Braeuning et al., 2015), in order to ensure robust and substantial activation of the respective receptor and transcription of its downstream targets in the absence of toxic injury. The selected concentration of interleukin-6 (IL-6) as a model inflammatory mediator (10 ng/mL) is substantially higher than IL-6 serum levels in NASH patients (~10 pg/mL in NASH patients vs. ~6 pg/mL in healthy controls; e.g. see Kugelmas et al. (2003)). A supra-physiological concentration, however, is needed to provoke the full response of HepaRG cells to IL-6 with regard to inhibitory effects on the transcription of selected CYP genes or induction of the acute-phase marker CRP (Klein et al., 2014; Rubin et al., 2015). The selected concentration of 10 ng/mL was tested for cytotoxicity to ensure the absence of non-specific effects due to cell stress. The concentration of a 1:1 mixture of oleate and palmitate (O/P) was based on initial cytotoxicity testing and pre-tests for cellular lipid accumulation.

**Transfection and luciferase reporter assay**

Transfections were carried out in HepG2 human hepatoma cells due to low transfection efficiency in differentiated HepaRG cells. HepG2 cells were transfected using the methodology described in Luckert et al. (2015). For monitoring the activities of the nuclear receptors PPARα and LXR, luciferase reporter systems based on fusion constructs of the GAL4 ligand binding domain with the ligand binding domain of the respective receptor were used as described in more detail in Luckert et al. (2015). The fusion constructs were encoded by pSG5-derived plasmids expressing the respective proteins under the control of the SV40 promoter. The activities of CAR and PXR were analyzed by the use of a luciferase reporter driven by a fragment of the CAR- and PXR-responsive human CYP2B6 gene (Zukunft et al., 2005), in combination with co-transfected expression vectors for human CAR (pSG5-based, SV40 promoter-driven) or PXR (pCR3-based, CMV promoter-driven). Wildtype HepG2 cells are deficient in both aforementioned receptors. For monitoring AHR activity, a pT81luc-based luciferase reporter system driven by an array of 3 AHR-binding DREs (dioxin
response elements) from the human *CYP1A1* gene promoter was transfected (Schulthess et al., 2015). A plasmid encoding Renilla luciferase under the control of a constitutively active promoter was co-transfected for normalization purposes. Cells were treated 24h after transfection for 24h and then assayed for firefly and Renilla luciferase activities as recently described (Luckert et al., 2015).

**Cytotoxicity testing**

Cytotoxicity of IL-6 and equimolar mixtures of oleate and palmitate (O/P) was investigated in 96-well plates using the Cell Titer Blue (CTB; Promega, Mannheim, Germany) and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) tests according to standard protocols. Triton X-100 (0.1%) was used as a positive control. Only concentrations yielding cell viabilities of >80% were considered as non-cytotoxic and used for subsequent cell treatment.

**Oil Red O staining**

Accumulation of fatty acids was monitored by staining with the dye Oil Red O (Sigma). Cells were fixed by 3.7% formaldehyde for 1 hour followed by 1 hour of staining with a sterile-filtered (0.45 µm pore size), freshly prepared 0.18% Oil Red O solution in 84% isopropanol. Cells were washed with distilled water and with 60% isopropanol and then either analyzed by light microscopy, or cells were lysed in 0.7% sodium dodecylsulfate in isopropanol for quantitative analysis of Oil Red O absorption at λ=500 nm in a multi-well plate reader (Infinite M200 Pro, Tecan Group Ltd., Männedorf, Switzerland).

**RNA isolation and gene expression analysis**

Gene expression analysis was performed according to a previously published approach (Braeuning et al., 2015). Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions and including an on-column genomic DNA digestion step with RNase-Free DNase. Reverse transcription of RNA was carried out.
using TaqMan Reverse Transcription Reagents (Applera GmbH, Darmstadt, Germany). A selection of 90 transcripts (Supplementary Table 1) related to functions in xenobiotic metabolism, fatty acid metabolism and inflammation was quantified using a Fluidigm BioMark HD high-throughput quantitative chip platform (Fluidigm, San Francisco, CA, USA) as recently described (Braeuning et al., 2015). Levels of mRNA expression were normalized to the expression of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Calculation of relative gene expression changes was done using the \( \Delta \Delta C_t \) method.

Verification of selected gene expression changes was performed for the cell culture conditions outlined in Figure 1 A-B by conventional SYBR Green I-based real-time RT-PCR with the Maxima SYBR Green/ROX qPCR Mastermix (Thermo Fisher Scientific, Berlin, Germany) on a LightCycler 96 machine (Roche, Mannheim, Germany) following reverse transcription by the help of the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany), using the \( \Delta \Delta C_t \) quantification method with GAPDH as a reference gene, for which no systematic treatment effects were recorded. The following primers were used for RT-PCR analysis:

- **CRP**: fwd 5'-GGCGAAGTGTTCACCAAACC-3', rev 5'-AAAGCGGGAGGTACCAGAGA-3'
- **CYP2B6**: fwd 5'-TTCTACTGCTTCCGTCTATCAA-3', rev 5'-GTGCAGAATCCCACAGCTCA-3'
- **CYP3A4**: fwd 5'-TCAGCCTGGTGCTCCTCTATCTAT-3', rev 5'-AAGCCCTTATGTTAGGACAAATATTT-3'
- **FABP1**: fwd 5'-CAAGTTCACCATCACCGCTGGGTC-3', rev 5'-TCATTGTCTCCAGCTCATTCTCTC-3'
- **GAPDH**: fwd 5'-ATTTGGCTACAGCAACAGGG-3', rev 5'-CAACTGTGAGGAGGGGAGA-3'.

**CYP activity assays**

Determination of CYP activities was performed using a multiplex LC-MS/MS detection method of metabolic conversion of model CYP substrates. Analyses were carried out according to Luckert et al. (2017) with minor modifications as follows: cells were incubated for 2h with a substrate mix containing model CYP substrates (50 µM phenacetin (CYP1A2),...
25 µM bupropion (CYP2B6), 5 µM amodiaquine (CYP2C8), 100 µM tolbutamide (CYP2C9), 100 µM S-mephenytoin (CYP2C19), 5 µM propafenone (CYP2D6), 10 µM midazolam (CYP3A4)). Cell culture supernatant samples were mixed with 10% of 250 mM formic acid, immediately frozen and then used for mass spectrometry-based analysis of metabolites according to Luckert et al. (2017) and references therein. Data were normalized to cell viability values.

**Statistical analysis**

Gene expression, reporter assay, and CYP activity data were analyzed using SigmaPlot software (version 13). For comparing the means between different groups we used the parametric one-way ANOVA (analysis of variance) test assuming normal distribution of data. ANOVA is insensitive to deviations from normal distribution and thus can also be used if the criteria of normality are not strictly met for all data (McDonald, 2014). Furthermore, parametric tests are to be preferred for parametric data as they are more powerful than non-parametric tests where the original parametric data have to be first converted to ranks leading to a loss of information resulting in a loss of power. If statistically significant differences between treatment groups were detected by ANOVA analyses, the Bonferroni post hoc test was subsequently used to identify which treatment groups differ from the control group. For CYP activity and gene expression data statistical analysis was independently performed for treatment scenario 1 (only NR agonists), 2 (NR agonists and IL-6), 3 (NR agonists and O/P) and 4 (NR agonists, IL-6 and O/P) within the treatment schemes A, B and C (see Figure 1) and the five treatments with the different NR agonists were compared to the solvent control group. Additionally, the solvent control groups of treatment scenario 2, 3 and 4 were compared to solvent control group of treatment scenario 1 to analyze the effect of IL-6, O/P or a combination of both IL-6 and O/P in the absence of NR agonists. For reporter gene assay data statistical analysis was independently performed for the non-induced and the induced nuclear receptors and the three treatment groups (II-6, O/P
and a combination of both IL-6 and O/P) were compared to solvent control group. A p-value < 0.05 was considered significant for ANOVA and post hoc analysis.
Results

In initial experiments, cell viability testing revealed no indication of cytotoxic effects of the nuclear receptor agonists, the 1:1 mixture of oleate and palmitate (O/P), and of interleukin-6 (IL-6) as inflammatory mediator (Supplementary Figure 1). This was important to exclude unspecific effects in subsequent experiments caused by cell stress. Dose-dependent induction of steatosis by O/P treatment was verified by triglyceride staining of HepaRG cells using Oil Red O (Supplementary Figure 2).

In order to allow for a comprehensive analysis of the interplay of nuclear receptor-mediated induction of drug-metabolizing enzymes, steatosis and inflammatory signaling, HepaRG human hepatocarcinoma cells were treated according to the scheme depicted in Figure 1. The experimental setup comprised both, short-term (24h) as well as long-term (14 days) treatments with the fatty acids and/or the inflammatory mediator. It was thus aimed to mimic three different conditions: (i) acute, short-term fatty acid overflow and/or inflammation, (ii) conditions of prolonged fatty acid overflow resulting in steatosis as observed in early fatty liver disease, and (iii) conditions of steatotic liver cells in an inflammatory environment as observed in steatohepatitis patients.

Effects on inflammation- and fat metabolism-related genes

In total, expression of a panel of 91 genes was measured, resulting in >20,000 individual data points. The gene set was selected based on literature data and consisted of genes related to hepatic drug metabolism, fatty acid metabolism, and inflammation. These data are summarized in Supplementary Table 1. Treatment with O/P barely affected the transcription of inflammation-related genes (Supplementary Figure 3). Short-term as well as long-term treatment with IL-6 strongly induced the transcription of the model inflammation marker CRP (c-reactive protein), an acute-phase protein and frequently-used marker for NASH in vivo (Fierbinteau-Braticevic et al., 2011). Similarly, the mRNA for serum amyloid A1/2 (SAA1/2), another acute-phase protein, was consistently upregulated by IL-6, thus confirming that IL-6 had in fact exerted the desired biological effects in HepaRG cells (Supplementary Figure 3).
While no effects on the mRNAs encoding the cytokines CCL20, IL-6, and IL-8 were observed with 24h of IL-6 treatment, these transcripts were upregulated following long-term incubation with IL-6 for 14 days, together with an upregulation of the mRNA encoding PTGS, an enzyme engaged in prostaglandin biosynthesis (Supplementary Figure 3). Of the nuclear receptor agonists, only the AHR activator 3-MC influenced a number of inflammation-related genes to a remarkable degree (Supplementary Figure 3).

When inspecting alterations in the expression of fat metabolism-related genes, it was observed that IL-6 treatment led to a downregulation of most of these genes (Supplementary Figure 4). The presence of O/P for 24h or 14 days, as well as incubation of HepaRG cells with the different nuclear receptor agonists did not substantially affect fat metabolism-related gene expression, with the exception of 3-MC, which downregulated a number of relevant mRNAs (Supplementary Figure 4). However, these alterations by IL-6 and 3-MC were not accompanied by remarkable changes in steatosis induction by O/P, since Oil Red O staining revealed unchanged steatosis induction by O/P in the presence of IL-6 and nuclear receptor agonists, both after 24h and 14 days (data not shown).

**Effect of inflammatory conditions and fat overload on CYP expression and activities**

Data for the 12 different CYP isoforms contained in the expression analysis are presented in Figure 2 (for additional non-CYP genes involved in phase I of drug metabolism see Supplementary Figure 5). Treatment with IL-6 for 24h or 14 days led to pronounced downregulation of basal expression (i.e., in solvent (DMSO)-treated cells when xenobiotic activators of nuclear receptors were absent) of most CYP isoforms (Figure 2A-C, left two panels), an effect which was especially pronounced after repeated cell treatment over a period of 2 weeks (Figure 2C; see also Supplementary Table 1 for underlying data). Treatment with O/P for 24h or 14 days exerted only minor effects, with a tendency for downregulation of some CYP isoforms (Figure 2A-B, third panel from left). Alterations in CYP expression after combined treatment with O/P and IL-6 for 24h (Figure 2A, right panel) or 2 weeks (Figure 2C, right panel), or following 24h of IL-6 treatment subsequent to 13 days of
pre-treatment with O/P (Figure 2B, right panel) were similar to the corresponding alterations monitored with IL-6 treatment alone. Downregulation of CYPs was confirmed by analyses of the enzymatic activities of 7 different CYPs, which revealed a pronounced effect on drug metabolism-related CYP activities by IL-6, and also slight downregulation of basal CYP enzyme activities in steatotic cells following 14 days of O/P treatment (Figure 3). For detailed data underlying the latter figure please refer to Supplementary Table 2.

**Inflammatory conditions diminish xenobiotic induction of CAR- and PXR-, but not of AHR-dependent CYP isoforms**

The presence of xenobiotic activators of ligand-activated receptors in HepaRG cells not treated with O/P or IL-6 led to the expected induction of target genes of the respective receptors, with comparable patterns of CYP induction following 24h or 14 days cultures (Fig 2A-C, left panels): activation of the mRNAs encoding the AHR-dependent CYP isoforms CYP1A1 and CYP1A2 by 3-methylcholanthrene (3-MC) was striking; CITCO induced the expression of the CAR target genes CYP1A2, CYP2B6, and CYP3A4, while the PXR activator rifampicin (Rif) induced CYP2B6 and CYP3A4 mRNAs (Figure 2). The latter CYP isoform was also induced when cells were treated with the PPARα agonist WY14,643, whereas the NRF2 activator tert.-butylhydroquinone (tBHQ) did not exert strong effects on CYP expression (Figure 2). Treatment with IL-6, again visible more pronouncedly after 2 weeks, efficiently counteracted the inductive effects of nuclear receptor agonists with the exception of 3-MC-mediated induction of CYP1A1/CYP1A2 which remained rather stable under these conditions (Figure 2). Short-term treatment with O/P exerted only minor effects on nuclear receptor-mediated CYP induction, as evidenced by a very similar CYP induction pattern in O/P-treated cells and solvent controls (Figure 2A). As for basal CYP expression, data on xenobiotic-induced transcription of selected CYP isoforms also indicated a broad-spectrum inhibitory effect of IL-6 treatment especially on CAR and PXR target CYPs, which was most obvious after 2 weeks of repeated IL-6 treatment (Figure 2). However, an interesting finding was that 3-MC- and thus AHR-mediated induction of CYP1A1 and
CYP1A2 mRNAs was not remarkably diminished following incubations of cells in the presence of IL-6 (Figure 2).

The inhibition of CAR- and PXR-dependent xenobiotic-induced CYP enzyme activities by IL-6 was confirmed by mass-spectrometric analyses (Figure 3; Supplementary Table 2) and, in line with the results from the mRNA expression analyses, only the induction of the AHR-dependent enzyme CYP1A2 by 3-MC was exempt from the repressive action of IL-6 on the CYPs (Figure 3). Of note, elevated CYP2D6 enzyme activity levels were measured in the supernatants of 3-MC-treated cells. This finding, however, should be interpreted with care due to the fact that overall CYP2D6 activities in the HepaRG cell lines are extremely low.

The non-IL-6- and non O/P-treated cells in Figures 2B and 2C stem from independent experiments performed under identical conditions, i.e. cultivation of the HepaRG cells in a differentiated stage for 14 days followed by treatment with nuclear receptor agonists for the last 24h of cultivation. Presented data show very similar patterns of CYP induction at both, the mRNA and enzyme activity levels (Figure 2B-C, Figure 3B-C). This demonstrates the high reproducibility and validity of the data set. In addition, selected data on xenobiotic- and IL-6-mediated alterations in gene expression (CYP2B6, CYP3A4, CRP, FABP1) were independently verified using a standard real-time RT-PCR system. Data were well in agreement with the Fluidigm gene expression analysis results (data not shown).

**Impact of O/P and IL-6 on mRNA expression related to phase II and III**

Expression of a broad spectrum of mRNAs encoding proteins involved in phase II and phase III of xenobiotic metabolism was also analyzed (Supplementary Figure 6). Similar to what had been observed with CYP expression, the inflammatory mediator IL-6 reduced the expression of most of the genes analyzed (Supplementary Figure 6). O/P treatment for 24h did not exert remarkable effects, while a slight tendency for down-regulation of some genes was observed after 14 days of incubation in the presence of O/P (Supplementary Figure 6). Induction of phase II/phase III-related genes by xenobiotics was, as expected, much less pronounced than for most CYP genes, and as it was observed with phase I enzymes,
xenobiotic effects on phase II/phase III-related gene transcription were also dampened by IL-6 (Supplementary Figure 6). As an exception, SULT1B1 mRNA levels were increased by IL-6 after 14 days of treatment, but not following 24h of IL-6 treatment (Supplementary Figure 6).

**Impact of O/P and IL-6 on xenobiotic-activated transcription factor expression and activity**

A selection of nuclear receptors and transcription factors engaged in the regulation of drug-metabolizing enzymes was analyzed at the mRNA level in response to the different treatments (Figure 4). Generally, effects of exogenous agonists and O/P treatment were not very prominent with the exception of AHR and vitamin D receptor (VDR) upregulation by the AHR agonist 3-MC, coupled to a downregulation of other nuclear receptors such as CAR and PXR (Figure 4). IL-6 resulted in downregulation of most genes of this category, including the important xeno-sensors CAR, PXR. The latter effect was consistently observed with short- as well as long-term IL-6 treatment (Figure 4). Downregulation of AHR mRNA was statistically significant following 24h of IL-6 treatment but failed our criteria of statistical significance in the long-term experiments (Figure 4).

In principle, downregulation of nuclear receptor target genes, e.g. the CYPs, by IL-6 might be a consequence of diminished availability of the nuclear receptors themselves, or due to an interference of IL-6-dependent signaling with the transactivation potential of the receptors. In order to follow up the above findings of the impact of IL-6 treatment on nuclear receptor-encoding mRNAs as well as on nuclear receptor target genes, the human hepatoma cell line HepG2 was chosen for subsequent reporter gene analyses of the transcriptional activity of nuclear receptors under IL-6 and O/P treatment, due to the fact that HepaRG cells showed poor transfection efficiency with different plasmids. Basal as well as ligand-induced activities of the receptors AHR, CAR, PXR, PPARα, and LXR were assessed using luciferase-based assays. In these assays, physiological effects of IL-6 on nuclear receptor mRNA expression were eliminated by the use of appropriate vectors expressing the receptors (native or in the form of GAL4-based fusion proteins) under the control of the constitutively active CMV or
SV40 promoters. As presented in Figure 5A, inspection of luciferase reporter data on basal (i.e. in the absence of xenobiotic inducers) nuclear receptor activities revealed that the transactivation potential of the nuclear receptors CAR, PXR, and LXR was significantly inhibited by IL-6. O/P treatment also weakly inhibited basal CAR activity, while a remarkable induction of LXR and PPARα activities was recorded following treatment with O/P (Figure 5A). None of the treatment protocols exerted pronounced effects on the AHR (Figure 5A).

For the xenobiotic-regulated receptors AHR, CAR, PXR, and PPARα, the assays were repeated in the presence of strong model activators of the respective receptors (Figure 5B). Treatment of HepG2 cells with O/P barely affected xenobiotic-induced reporter activities driven by these receptors (Figure 5B). By contrast, IL-6 substantially inhibited the activities of the CAR-, PXR-, and PPARα-dependent reporter systems, but did not affect 3-MC-induced AHR activity at the CYP1A1 promoter-derived 3xDRE reporter (Figure 5B).
Discussion

The present study provides novel insights into the effects of fatty acid accumulation and/or inflammatory signals on drug metabolism in HepaRG cells. This work thereby confirms, extends and complements earlier investigations on the effects of fatty acid accumulation on the regulation of drug-metabolizing enzymes in human primary hepatocytes, HepaRG cells, or rodent cells by inflammatory mediators (Abdel-Razzak et al., 1995; Abdel-Razzak et al., 1993; Klein et al., 2015; Pascussi et al., 2000; Rubin et al., 2014). In extension of the aforementioned studies, which focused on the influence of inflammation on drug-metabolism in its basal (this means in the absence of xenobiotic inducers of drug metabolism) state and under short-term treatment conditions, the present study aims to approach the role of interactions between steatosis, inflammation, and nuclear receptors on drug metabolism and its inducibility.

The present results underline the suitability of HepaRG cells as an in vitro model of hepatic steatosis. This cell line has previously been used for steatosis induction with an O/P mixture (Brown et al., 2013) or with oleic acid alone (Antherieu et al., 2011; Rogue et al., 2014). Oleate and palmitate constitute the most abundant fatty acids in NAFLD and NASH patients and the relative composition of liver fatty acids significantly shifts towards oleate and palmitate, when NAFLD or NASH livers are compared to healthy controls (Araya et al., 2004). Thus, the chosen way of inducing steatosis in vitro by an overload with these two fatty acids is considered to be in line with the physiological development of steatosis in vivo. In general, and as compared to the effects of IL-6 or nuclear receptor agonists, transcriptional alterations induced by O/P were rather moderate. Despite pronounced accumulation of fatty acids, as measured by Oil Red O staining, only few transcriptional responses were recorded following 24h of O/P treatment. Thus long-term cultivation and treatment might be more suited for in vitro simulation of NAFLD than a short-term fatty acid overload approach. O/P did not substantially affect basal and xenobiotic-induced activities of AHR, CAR, and PXR, and also of their target mRNAs, in contrast to PPARα and LXR. Receptor activation by xenobiotics did also not significantly influence fatty acid accumulation in our own
experiments, where we used HepaRG cells under cell culture conditions identical with the experimental setup from this study (see Figure 1, all conditions from panels A and B) in combination with Oil Red O staining as a readout for fatty acid accumulation (own unpublished data). This lack of impact of xenobiotic receptor agonists on steatosis of HepaRG cells might appear to be in contrast to previous results from Rogue et al. (2014), who have shown that PPARα activation in HepaRG cells is capable of reducing liver steatosis. However, also in that study no effects of PPARα agonists on steatosis were observed when the cells were treated with nuclear receptor agonists for only 24h. Thus, our observations are well in agreement with the data from Rogue et al. (2014) and support the hypothesis that only long-term presence of a PPARα activator is able to substantially affect fatty accumulation in HepaRG cells. In another study with HepG2 cells, linoleic acid weakly induced a CAR-driven luciferase reporter system (Finn et al., 2009), whereas a tendency for down-regulation was observed with O/P as fatty acids in our analyses. Differences between the fatty acids used for cell treatment (linoleic acid vs. equimolar mixture of O/P), as well as differences between the concentrations used (up to 12.5 µM in Finn et al. (2009) vs. 125 µM each of O and P in this work) might explain the discrepancies in the results between the two studies.

Previous data suggest a generally down-regulating effect of IL-6 treatment on different players in drug metabolism, including metabolic enzymes at the mRNA and activity levels, as well as mRNAs encoding important nuclear receptors (Klein et al., 2015; Pascussi et al., 2000; Rubin et al., 2014) or hepatic transcription factors (Klein et al., 2015; Liu et al., 2012; Merrell and Cherrington, 2011) in primary human hepatocytes and/or HepaRG cells. Our own unpublished data similarly show that IL-6 downregulates many drug-metabolizing enzymes in HC-AFW1 human hepatocarcinoma cells, another well-suited in vitro model for studying the regulation of human drug metabolism (Braeuning et al., 2015; Petzuch et al., 2015). The present data corroborate the findings on IL-6-mediated inhibition of drug metabolism and add the important aspect of long-term IL-6 treatment, which exerted much more pronounced effects on drug metabolism in HepaRG cells than short-term IL-6 treatment. Thus, repeated
treatment with IL-6 might be considered as the superior HepaRG in vitro model for representing NASH, as compared to shorter treatment. However, it should be noted that despite a strong and consistent response of HepaRG cells to IL-6 treatment which was observed with regard to the induction of cytokine production and acute-phase markers of inflammation, no substantial evidence for an IL-6-dependent induction of an oxidative stress response (such as e.g. HMOX-1 or NQO1 gene transcription) has been recorded in the course of our analyses. Potentially, the long-term HepaRG inflammation model might thus further be optimized by developing it into a co-culture model with immune cells which might enhance reactive oxygen species formation and resulting stress to hepatocytes.

Previous literature data on the effect of IL-6 treatment on nuclear receptors are not entirely consistent: while observations on reduced cytochrome P450 activities indirectly suggest an involvement of nuclear receptors (Rubin et al., 2014), their downregulation at the mRNA level has been convincingly shown (Klein et al., 2015; Pascussi et al., 2000). Here we confirm nuclear receptor downregulation by IL-6 and furthermore provide new insight by analyzing the effects of IL-6 on the transcriptional activities of the nuclear receptors AHR, CAR, PXR, PPARα, and LXR. Interestingly, it appears that xenobiotic-induced gene expression via the AHR is not substantially affected by IL-6, whereas the other xeno-sensing receptors studied displayed remarkable inhibition. In contrast to our analyses, no effects on CAR-dependent reporter gene transactivation have been observed in a previous study (Pascussi et al., 2000). One might speculate that the response of HUH-7 cells used in the study by (Pascussi et al., 2000) to IL-6 might not correspond to effects consistently observed in primary human hepatocytes, HepaRG cells, and HepG2 cells at the mRNA, enzyme activity, and reporter assay levels. The inhibition of nuclear receptor mRNA transcription described here and by others (Klein et al., 2015; Pascussi et al., 2000) might be connected to lower levels of the mRNAs or activities of HNF family members (Klein et al., 2015; Liu et al., 2012; Merrell and Cherrington, 2011). This hypothesis is consistent with the findings of the present study. However, it appears unlikely that the observed moderate loss of nuclear receptor mRNAs is solely responsible for decreased CYP expression and especially diminished xenobioto-
stimulated CYP induction in the presence of IL-6: nuclear receptors (or their GAL4 fusion
construct variants) used for the reporter assays in this study were expressed from
constitutively active promoters and have nonetheless revealed pronounced inhibition of
reporter gene transactivation by IL-6, especially for CAR and PXR. Instead, the findings from
the reporter gene analyses suggest that inhibition of the transactivation potential of certain
nuclear receptors, rather than reduced nuclear receptor-encoding mRNA levels, is causative
for the observed effects. This applies to CAR, PXR, PPARα, and LXR, but not for the AHR,
the only receptor not substantially affected by IL-6 treatment in our analyses. With the
exception of the bHLH/PAS family protein AHR, which uses ARNT (aryl hydrocarbon
receptor nuclear translocator) as its dimerization partner, the other investigated receptors
dimerize with their common partner RXRα. Using an in silico modeling approach based on
transcriptomic and phosphoproteomic data, it has recently been proposed that RXRα plays a
decisive role in mediating the downregulation of hepatic detoxification under inflammatory
conditions (Keller et al., 2016). The present experimental data fit well into these model
predictions by demonstrating that the transcriptional activity of different nuclear receptors
using RXRα is diminished when hepatic cells are treated with the inflammatory mediator IL-6,
whereas the activity of the AHR, which acts independent of RXRα, is not affected. The exact
mechanism of action by which IL-6 affects RXRα remains to be elucidated.
Taken together, the present data provide a comprehensive overview of the influence of
NAFLD- and NASH-like conditions on hepatic drug metabolism and its regulation by
xenobiotics in human HepaRG cells in vitro. The cell system is well-suited for this type of
analysis, in particular when long-term effects are in the focus of the study. According to the
present data, it might be more appropriate to study liver disease conditions in vitro in long-
term culture than by using single short-term incubation of the cells with the various effectors.
While demonstrating that fatty acid accumulation alone does not substantially influence the
drug-metabolizing capacity of these liver cells, our results demonstrate massive inhibition of
basal drug metabolism by the inflammatory mediator IL-6, and show that xenobiotic-induced
levels of drug-metabolizing enzymes regulated by CAR and PXR, but not AHR, are similarly influenced by the cytokine.

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Authorship contributions

Participated in research design: L.B., M.T., A.B.

Conducted experiments: N.T., L.K., M.T., U.H.

Performed data analysis: N.T., L.K., M.T., C.L.

Wrote or contributed to writing of the manuscript: L.B., U.M.Z., A.L., A.B.
References


**Footnotes**

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Please send reprint requests to: Albert Braeuning, German Federal Institute for Risk Assessment, Dept. Food Safety, Max-Dohrn-Str. 8-10, 10589 Berlin, Germany; phone: +49-(0)30-18412-3758; fax: +49-(0)30-18412-63758; email: Albert.Braeuning@bfr.bund.de
Figure legends

Figure 1. Schematic setup of cell treatment. HepaRG cells were treated with different nuclear receptor agonists (or DMSO as a control) as detailed in the Materials and Methods section (5 µM 3MC, 5 µM CITCO, 10 µM Rif, 30 µM tBHQ, 50 µM Wy), alone or in combination with 10 ng/mL IL-6 to mimic inflammatory conditions, and/or with oleate/palmitate (O/P; 125 µM each) to induce steatosis of hepatocyte-like HepaRG cells. Different approaches were followed to analyze short- as well as long-term effects of treatment. (A) For analysis of short-term effects, differentiated HepaRG cells were incubated for 24h prior to harvest. (B) Long-term steatosis effects were analyzed using a treatment regimen based on 2 weeks of pre-treatment with O/P, prior to 24h of treatment with nuclear receptor agonists and/or IL-6. (C) Long-term inflammatory effects were analyzed using a treatment regimen based on 2 weeks of pre-treatment with IL-6, and long-term combined inflammatory and steatotic effects (mimicking steatohepatitis) were analyzed using a treatment regimen based on 2 weeks of pre-treatment with IL-6 and O/P.

Figure 2. Heat-map visualization of the expression of cytochrome P450 (CYP) genes in HepaRG cells and their regulation by nuclear receptor agonists (5 µM 3MC, 5 µM CITCO, 10 µM Rif, 30 µM tBHQ, 50 µM Wy), O/P (125 µM each), and IL-6 (10 ng/mL). Cells were treated according to the scheme presented in Figure 1 and mRNA expression levels were analyzed using a Fluidigm PCR system. Differentiated HepaRG cells were either incubated with the test compounds for 24h prior to harvest (A), or using a treatment regimen based on 2 weeks of pre-treatment with O/P prior to 24h of treatment with nuclear receptor agonists and/or IL-6 (B), or using a treatment regimen based on 2 weeks of pre-treatment with IL-6 and/or IL-6 and O/P (C). Mean relative values (n=3 independent biological replicates) are given; asterisks indicate statistical significance in comparison to solvent control (p<0.05); hashmarks indicate statistical significance compared to solvent control of cells not treated with IL-6 and/or O/P.
**Figure 3.** Heat-map visualization of the activities of CYP enzymes in HepaRG cells and their regulation by nuclear receptor agonists (5 µM 3MC, 5 µM CITCO, 10 µM Rif, 30 µM tBHQ, 50 µM Wy), O/P (125 µM each), and IL-6 (10 ng/mL). Cells were treated according to the scheme presented in Figure 1 and CYP activities were assayed using model substrate compounds and LC-MS/MS-based analytics. Differentiated HepaRG cells were either incubated with the test compounds for 24h prior to harvest (A), or using a treatment regimen based on 2 weeks of pre-treatment with O/P prior to 24h of treatment with nuclear receptor agonists and/or IL-6 (B), or using a treatment regimen based on 2 weeks of pre-treatment with IL-6 and/or IL-6 and O/P (C). Mean relative values (n=3 independent biological replicates) are given; asterisks indicate statistical significance in comparison to solvent control (p<0.05); hashmarks indicate statistical significance compared to solvent control of cells not treated with IL-6 and/or O/P. For raw data on CYP activities given in pmol/mL cell culture supernatant, please refer to Supplementary Table 2.

**Figure 4.** Heat-map visualization of the expression of nuclear receptors genes and genes encoding other transcriptional regulators with relevance for the regulation of xenobiotic metabolism in HepaRG cells. Their regulation by nuclear receptor agonists (5 µM 3MC, 5 µM CITCO, 10 µM Rif, 30 µM tBHQ, 50 µM Wy), O/P (125 µM each), and IL-6 (10 ng/mL) following treatment of cells according to the scheme presented in Figure 1 is depicted. mRNA expression levels were analyzed using a Fluidigm PCR system. Differentiated HepaRG cells were either incubated with the test compounds for 24h prior to harvest (A), or using a treatment regimen based on 2 weeks of pre-treatment with O/P prior to 24h of treatment with nuclear receptor agonists and/or IL-6 (B), or using a treatment regimen based on 2 weeks of pre-treatment with IL-6 and/or IL-6 and O/P (C). Mean relative values (n=3 independent biological replicates) are given; asterisks indicate statistical significance in comparison to solvent control (p<0.05); hashmarks indicate statistical significance compared to solvent control of cells not treated with IL-6 and/or O/P.
Figure 5. Heat-map visualization of the transcriptional activities of nuclear receptors engaged in the regulation of drug and lipid metabolism, as determined by luciferase reporter analyses in HepG2 cells. Regulation of the basal activities of AHR, CAR, PXR, LXR and PPARα by O/P (125 µM each) and IL-6 (10 ng/mL) in the absence of xenobiotic receptor activation (A), as well as regulation of xenobiotic-induced activities of AHR (5 µM 3MC), CAR (5 µM CITCO), PXR (10 µM Rif), and PPARα (50 µM Wy) by O/P (125 µM each) and IL-6 (10 ng/mL) (B) is shown. Mean values (n=6-9 independent biological replicates; each in 2-3 technical replicates) are given relative to solvent controls (A) or receptor agonists (B); asterisks indicate statistical significance in comparison to solvent control (p<0.05). Treatment with receptor agonists led to a mean induction of the respective reporter signals by the following factors, thus demonstrating functionality of the assays: AHR, 5 µM 3MC, 50-fold; CAR, 5 µM CITCO, 2.8-fold; PXR, 10 µM Rif, 3.1-fold; PPARα, 50 µM Wy, 103-fold; LXR, 10 µM GW3965, 472-fold.
differentiated HepaRG every 2nd day treatment with IL-6 (groups 2-4) and with O/P (group 4)

1) + NR agonists
2) + NR agonists and IL-6
3) + NR agonists and O/P
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FIGURE 2

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O/P (24h)

IL-6 (14d)

O/P (14d)

Fold expression:

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This article has not been copyedited and formatted. The final version may differ from this version.
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fold expression

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## FIGURE 5

### AHR (induced)

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### CAR (induced)

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### PPARα (induced)

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### LXR

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### IL-6 (24h) vs. O/P (24h)

### Fold Activity

- 0
- 0.25
- 0.5
- 0.75
- 1
- 1.5
- 3
- 7
Regulation of drug metabolism by the interplay of inflammatory signaling, steatosis, and xeno-sensing receptors in HepaRG cells

Norman Tanner, Lisa Kubik, Claudia Luckert, Maria Thomas, Ute Hofmann, Ulrich M. Zanger, Linda Böhmert, Alfonso Lampen, Albert Braeuning

Supplementary figures and legends
Supplementary Figure 1. Cytotoxicity testing of HepaRG cells. Cells were incubated in the presence of increasing concentrations of an equimolar O/P (1:1) mixture (A, C) or in the presence of increasing concentrations of IL-6 (B, D). Incubation was performed for either 24h (A, B) or for 14 days with medium change and repeated treatment every 2nd day (C,D). Data from the MTT and CTB tests are depicted as means + SD (two independent biological replicates, each assayed in quintuple technical replicates) and given relative to solvent-treated control cells (NC). PC (positive control): cells treated with 0.1% Triton X-100. The non-toxic concentrations of 10 ng/mL IL-6 and 125 μM O/P were selected for further analyses.
Supplementary Figure 2. Verification of cellular steatosis by Oil Red O staining. HepaRG cells were treated for 24h with control medium (A) or with 125 µM of O/P (125 µM of each of the fatty acids) (B) and images were taken under a light microscope. The scale bars in the images denote a distance of 200 µm. (C) For quantitative analysis of the dose-dependency of fat incorporation Oil Red O staining was measured on a multi-well plate reader following incubation of differentiated HepaRG cells with the indicated concentrations of O/P for 24h. Means + SD (assayed in quintuple determination) are given relative to untreated cells (set to 1).” A non-toxic and steatosis-inducing concentration of 125 µM O/P was selected for further analyses.
Supplementary Figure 3. Heat-map visualization of the expression of inflammation-related genes in HepaRG cells and their regulation by nuclear receptor agonists, O/P, and IL-6. Cells were treated according to the scheme presented in Figure 1 and mRNA expression levels were analyzed using a Fluidigm PCR system. For more details, please refer to the legend to Figure 2 and the raw data in Supplemental Table 1. Mean values (n=3 independent biological replicates) are given relative; asterisks indicate statistical significance in comparison to solvent control (p<0.05); hashmarks indicate statistical significance compared to solvent control of cells not treated with IL-6 and/or O/P.
Supplementary Figure 4. Heat-map visualization of the expression of fat metabolism-related genes in HepaRG cells and their regulation by nuclear receptor agonists, O/P, and IL-6. For more details, please refer to the legend to Figure 2 and the raw data in Supplemental Table 1. Mean values (n=3 independent biological replicates) are given; asterisks indicate statistical significance in comparison to solvent control (p<0.05); hashmarks indicate statistical significance compared to solvent control of cells not treated with IL-6 and/or O/P.
**Supplementary Figure 5.** Heat-map visualization of the expression of non-CYP genes related to phase I of drug metabolism in HepaRG cells and their regulation by nuclear receptor agonists, O/P, and IL-6. For more details, please refer to the legend to Figure 2 and the raw data in Supplemental Table 1. Mean values (n=3 independent biological replicates) are given; asterisks indicate statistical significance in comparison to solvent control (p<0.05); hashmarks indicate statistical significance compared to solvent control of cells not treated with IL-6 and/or O/P.
**Supplementary Figure 6.** Heat-map visualization of the expression of genes related to phase II or phase III of drug metabolism in HepaRG cells and their regulation by nuclear receptor agonists, O/P, and IL-6. For more details, please refer to the legend to Figure 2 and the raw data in Supplemental Table 1. Mean values (n=3 independent biological replicates) are given; asterisks indicate statistical significance in comparison to solvent control (p<0.05); hashmarks indicate statistical significance compared to solvent control of cells not treated with IL-6 and/or O/P.