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Bisphenol A induces steatosis in HepaRG cells using a model of perinatal exposure

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ABSTRACT: Human exposure to bisphenol A (BPA) could favor obesity and related metabolic disorders such as hepatic steatosis. Investigations in rodents have shown that these deleterious effects are observed not only when BPA is administered during the adult life but also with different protocols of perinatal exposure. Whether perinatal BPA exposure could pose a risk in human is currently unknown, and thus appropriate in vitro models could be important to tackle this major issue. Accordingly, we determined whether long-term BPA treatment could induce steatosis in human HepaRG cells by using a protocol mimicking perinatal exposure. To this end, the kinetics of expression of seven proteins differentially expressed during liver development was determined during a 4-week period of cell culture required for proliferation and differentiation. By analogy with data reported in rodents and humans, our results indicated that the period of cell culture around day 15 and day 18 after seeding could be considered as the “natal” period. Consequently, HepaRG cells were treated for 3 weeks with BPA (from 0.2 to 2000 nM), with a treatment starting during the proliferating period. BPA was able to induce steatosis with a non-monotonic dose response profile, with significant effects on neutral lipids and triglycerides observed for the 2 nM concentration. However, the expression of many enzymes involved in lipid and carbohydrate homeostasis was unchanged in exposed HepaRG cells. The expression of other potential BPA targets and enzymes involved in BPA biotransformation was also determined, giving answers as well as new questions regarding the mechanisms of action of BPA. Hence, HepaRG cells provide a valuable model that can prove useful for the toxicological assessment of endocrine disruptors on hepatic metabolisms, in particular in the developing liver.

Keywords: bisphenol A; steatosis; HepaRG cells; perinatal exposure; lipids; xenobiotic-metabolizing enzymes; pregnane X receptor; UDP-glucuronosyltransferase; β -glucuronidase; cytochrome P450

INTRODUCTION

Human exposure to the synthetic chemical bisphenol A (BPA) is suspected to favor obesity and related metabolic disorders such as insulin resistance, type 2 diabetes and non-alcoholic fatty liver disease (NAFLD) (Trasande et al., 2012; Khalil et al., 2014; Oppeneer and Robien, 2015; Rancière et al., 2015). NAFLD refers to a large spectrum of hepatic lesions including fatty liver (also referred to as hepatic steatosis), nonalcoholic steatohepatitis and cirrhosis. Actually, similarly to many other environmental contaminants, BPA might exert more harmful effects when exposure occurs during the perinatal period, or in the early postnatal years (Calkins and Devaskar, 2011; Rochester, 2013; Wei et al., 2014).

Although BPA-induced steatosis is likely a secondary consequence of higher body weight and associated metabolic disorders such as insulin resistance, other mechanism(s) could be involved as well. Indeed, several studies have demonstrated that BPA is able to induce lipid accumulation in rat and human hepatoma cells (Huc et al., 2012; Grasselli et al., 2013; Héliès-Toussaint et al., 2014; Peyre et al., 2014). However, investigations in human hepatoma cells were performed so far solely with the HepG2 cell line, which does not have a complete repertoire of xenobiotic metabolizing enzymes (XMEs), in particular regarding some cytochromes P450 (CYPs) and UDP-glucuronosyl transferases (UGTs) (Hewitt and Hewitt, 2004; Westerink and Schoonen, 2007a; Westerink and Schoonen, 2007b; Gerets et al., 2012). Moreover, the duration of BPA treatment in the experiments carried out in HepG2 was relatively short (i.e. 3 or 4 days) (Huc et al., 2012; Héliès-Toussaint et al., 2014; Peyre et al., 2014).

During the last decade, the human hepatoma HepaRG cell line has increasingly been used in the field of toxicology (Jossé et al., 2008; Dumont et al., 2010; Anthérieu et al., 2012; Andersson et al., 2012). Indeed, after differentiation, HepaRG cells express most of the XMEs present in the liver as well as key nuclear receptors regulating their expression including pregnane X receptor (PXR), aryl hydrocarbon receptor (AhR) and constitutive androstane receptor (CAR) (Aninat et al., 2006; Legendre et al., 2007; Anthérieu et al., 2012). The HepaRG cell line, which presents hepatic bipotent progenitor features, is able to enter into a differentiation program towards biliary-like and hepatocyte-like cells (Gripon et al., 2002; Parent et al., 2004). The standard culture protocol allowing to obtain well-differentiated hepatocyte-like cells from the progenitor cells includes 2 weeks of proliferation followed by 2 weeks of differentiation, which is improved by the supplementation of the culture medium with 2% dimethyl sulfoxide (DMSO) (Gripon et al., 2002; Cerec et al., 2007).

Taking all these data into consideration, we determined whether long-term BPA treatment could induce lipid accumulation in HepaRG cells, using a protocol enabling to mimic a perinatal exposure. To this end, we first determined over the 4-week period of cell culture the kinetics of expression of proteins that are known to be differentially expressed during liver development. The kinetics of expression of eight enzymes involved in BPA metabolism was also assessed. In a second step, HepaRG cells were treated for 3 weeks with different concentrations of BPA ranging from 0.2 to 2000 nM, with the BPA treatment starting during the proliferating period.

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MATERIALS AND METHODS

Chemicals

BPA, DMSO and insulin were purchased from Sigma Aldrich (Saint-Quentin-Fallavier, France). William's E medium was obtained from Eurobio laboratories (Les Ulis, France). Fetal Bovine Serum (FBS) was purchased from Lonza (Levallois-Perret, France). Glutamine, penicillin and streptomycin were obtained from Thermo Fisher Scientific (Cergy Pontoise, France). Hydrocortisone hemisuccinate was purchased from Upjohn Pharmacia (Guancourt, France).

Cell Culture and Treatments

HepaRG cells were cultured according to the standard protocol described by Aninat et al. (2006) but with some adaptations related to the daily change of the culture medium. Briefly, HepaRG cells were seeded at a density of 2.6×10^4 cells/cm² and were first incubated for one week in a William's E medium without phenol red and supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 5 µg/ml insulin and 50 µM hydrocortisone hemisuccinate. After this first week, cells were exposed every day for 3 weeks with different concentrations of BPA (0.2, 2, 20, 200 and 2000 nM). Hence, because of this daily treatment, the William's E medium was supplemented with 5% FBS during this 3-week period. BPA was solubilized in DMSO, which was used at a final concentration of 0.01% for the first week of treatment (i.e. week 2 after seeding). Cells were thereafter cultured during the next two weeks of treatment (i.e. weeks 3 and 4 after seeding) in the presence of 1% DMSO in order to improve hepatocyte differentiation. In addition, experiments were carried out in untreated HepaRG cells cultured during a similar period of 4 weeks in order to determine the kinetics of expression of different genes of interest. For these experiments, the respective concentrations of FBS and DMSO added in the cultures were the same as those used for the BPA protocol treatment.

Measurement of Neutral Lipids and Triglycerides

The HCS LipidTOXTM green neutral lipid stain detection kit (Invitrogen, Eugene, Oregon, USA) was used in order to measure neutral lipids, according to the manufacturer's instructions. Briefly, control and treated cells were rinsed with phosphate buffered saline (PBS) and then fixed for 30 minutes in the dark with PBS containing 4% paraformaldehyde and 2.5 µg/ml of the nuclear dye Hoechst 3342, which was used to detect and count the HepaRG cells. Cells were rinsed three times with PBS and

then incubated for 30 minutes in the dark with the LipidTOX™ stain. The PBS medium was then renewed and the 96-well plates were scanned with an Arrayscan VTI (Cellomics; Thermo Fisher Scientific, Villebon sur Yvette, France). At least 7500 cells were analyzed per well. Cellular triglycerides were measured with a colorimetric kit purchased from Biovision (Milpitas, CA), using the manufacturer's recommendations. The amount of cellular triglycerides was normalized to that of total proteins determined by the bicinchoninic acid (BCA) method.

Isolation of RNA and Real-Time Quantitative PCR Analysis

Total RNA was extracted from ca. 10^6 HepaRG cells with the SV total RNA isolation system purchased from Promega (Charbonnières-les-Bains, France). This RNA isolation kit includes a DNase treatment step. RNA was reverse-transcribed into cDNA using the High-Capacity cDNA Archive kit purchased from Life technologies (Saint-Aubin, France). Real-time quantitative PCR (RT-qPCR) was then performed using the SYBR Green PCR Master Mix on an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystem, Woolston, UK). Expression of the human TATA box binding protein (TBP) was used as reference, and the $2^{-\Delta\Delta Ct}$ method was used to express the relative expression of each selected gene. Sequences of the primers used in this study are available on request.

Statistical Analysis

All results were expressed as mean \pm standard error of mean (SEM). Comparisons between the different conditions were performed with one-way analysis of variance (ANOVA). When ANOVA provided significant differences, individual means were compared with the post-hoc Dunnett's test.

RESULTS

Kinetics of Expression of Enzymes Differentially Expressed during Liver Development

The main goal of this study was to set up a cellular model of perinatal BPA exposure by using the HepaRG cell line. To this end, we first determined over the 4-week period of cell culture after seeding the kinetics of expression of seven different proteins known to be differentially expressed during liver development: pyruvate kinase muscle isoform (PKM), aldolase A (ALDOA), albumin (ALB), aldolase B (ALDOB) as well as the cytochromes P450 3A7 (CYP3A7), CYP2E1 and CYP3A4. Indeed, the differential expression of these proteins has been extensively determined during liver development in rodents but also in humans (Imamura and Tanaka, 1972; Liao et al., 1980; Numazaki et al., 1984; Lacroix et al., 1997; Rich and Boobis, 1997; Lee et al., 2011). Notably, the expression of PKM2, ALDOA and CYP3A7 (Cyp3a16 in mouse) is specific to the fetal liver, whereas the expression of ALB, ALDOB, CYP2E1 and CYP3A4 (Cyp3a1/2 in rat and Cyp3a11 in mouse) increases during the perinatal period, or early after birth. In this study, the highest expression of PKM2, ALDOA and CYP3A7 was observed during the 2 weeks of proliferation, whereas the strongest expression of ALB, ALDOB, CYP2E1 and CYP3A4 was found during the differentiation stage (i.e. weeks 3 and 4 after seeding) (Fig. 1). Hence, by analogy with the *in vivo* situation reported in rodents and humans (Liao et al., 1980; Lacroix et al., 1997; Rich and Boobis, 1997; Hart et al., 2009), the period of cell culture around day 15 and day 18 after seeding could be considered as the “natal” period.

Kinetics of Expression of Enzymes Involved in BPA Metabolism

The expression of the main enzymes involved in BPA metabolism was also determined over the 4-week period of cell culture after seeding. In human liver, BPA is mainly metabolized to BPA-glucuronide by the UDP-glucuronosyltransferase 2B15 (UGT2B15) and to a lesser extent to BPA-sulfate by the sulfotransferases 1A1 (SULT1A1) and 1A3/4 (SULT1A3/4) (Fig. 2) (Hanioka et al., 2008; Quesnot et al., 2014; Nahar et al., 2015; Thayer et al., 2015; Yalcin et al., 2016). Notably, BPA-glucuronide and BPA-sulfate can be converted back to BPA by β -glucuronidase (GUSB) and steroid sulfatase (STS), respectively (Fig. 2) (Nahar et al., 2013; Nahar et al., 2015). A minor fraction of BPA can also be metabolized to catechol-BPA by several CYPs including CYP2C9, CYP2C18 and CYP2C19 (Fig. 2) (Niwa et al., 2001; Ye et al., 2011). In the present study, the expression of UGT2B15, STS, CYP2C9, CYP2C18 and CYP2C19 progressively increased during the

differentiation process and reached a plateau around day 25 after seeding (Fig. 3). In contrast, the expression of SULT1A1 and GUSB was maximal between day 11 and day 13 and plateaued afterward (Fig. 3). Lastly, expression of SULT1A3/4 was maximal around day 13 and decreased later on (Fig. 3). Among these eight enzymes, GUSB, UGT2B15, CYP2C9 and CYP2C19 showed the highest expression in HepaRG cells (Fig. 4).

Effects of BPA on Neutral Lipids, Triglycerides and Expression of Lipid-Responsive Genes

HepaRG cells were treated daily with different concentrations of BPA (from 0.2 to 2000 nM) between day 7 and day 28 after seeding in order to determine whether chronic BPA exposure could induce steatosis in our cellular model. Our results showed that BPA significantly enhanced cellular neutral lipids and triglycerides at the 2 nM concentration (Fig. 5). Interestingly, a non-monotonic dose response (NMDR) was observed for triglycerides and to a lesser extent for neutral lipids (Fig. 5). We also determined the mRNA levels of apolipoprotein A4 (APOA4), since we recently found that APOA4 expression was a sensitive marker of lipid accumulation in HepaRG cells incubated with fatty acids (Michaut et al., 2016). In the present study, APOA4 expression was also modulated by BPA in a non-monotonic manner with a significant increase observed at the 2 nM concentration (Fig. 5). In contrast, BPA was not found to modify the expression of several proteins that coat lipid droplets, namely perilipin 1 (PLIN 1), perilipin 2 (PLIN2 also known as ADFP and ADRP) and perilipin 3 (also referred to as TIP47) (Fig. 5 and data not shown). These results were in keeping with our previous data showing that, compared with APOA4, expression of PLIN1 and PLIN 2 was significantly lower in fatty acid-loaded HepaRG cells (Michaut et al., 2016).

Effects of BPA on the Expression of Genes Involved in Carbohydrate and Lipid Homeostasis

In order to determine the mechanism whereby BPA could induce lipid accumulation in HepaRG cells, we further assessed the expression of different genes involved in carbohydrate and lipid homeostasis: ATP citrate lyase (ACLY), acetyl-CoA carboxylase alpha (ACACA), fatty acid synthase (FASN), thyroid hormone responsive protein (THRSP, also known as SPOT14), patatin-like phospholipase domain containing 3 (PNPLA3), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), peroxisome proliferator-activated receptor gamma (PPARG), solute carrier family 2 member 2 (SLC2A2, also known as GLUT2), pyruvate dehydrogenase kinase 4 (PDK4), carnitine palmitoyltransferase 1A (CPT1A), apolipoprotein B (APOB) and microsomal triglyceride transfer protein (MTTP). However,

the expression of these different genes was not significantly modified by BPA treatment (data not shown).

Effects of BPA on the Expression of Genes Involved in Oxidative Stress

Because steatosis can be associated with oxidative stress (Begrache et al., 2013; Tariq et al., 2014), we also assessed the mRNA expression of the following genes: heme oxygenase 1 (HMOX1), NAD(P)H dehydrogenase quinone 1 (NQO1), nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NFKB1), heat shock 70kDa protein 1A (HSPA1A, also known as HSP70-1A), glutathione S-transferase alpha 1/2 (GSTA1/2), GSTA3 and tribbles pseudokinase 3 (TRIB3). TRIB3 was investigated because its mRNA expression can be markedly enhanced in different conditions of stress, including when the cellular stores of the antioxidant glutathione are reduced (Ord and Ord, 2005; Aubert et al., 2012; Michaut et al., 2016). The expression of these genes was however unchanged (data not shown), thus indicating that BPA did not induce significant oxidative stress in our experimental conditions.

Expression of ERR γ , PXR and their Targets Genes

BPA is able to activate different nuclear receptors such as estrogen receptor (ER), estrogen related receptor γ (ERR γ), glucocorticoid receptor (GR), AhR and PXR but BPA binding affinity varies greatly from one receptor to another (Takayanagi et al., 2006; Krüger et al., 2008; Vandenberg et al., 2009; Riu et al., 2011; Rubin, 2011; Sui et al., 2012; Peyre et al., 2014; Rezg et al., 2014). Notably, BPA strongly binds to human ERR γ with a binding affinity constant (K_d) of ca. 5 nM, while its affinity for ER α and ER β was reported to be 80- and 100-fold lower, respectively (Takayanagi et al., 2006; Okada et al., 2008). Hence, it has been postulated that ERR γ activation could significantly mediate some toxic effects induced by BPA in different experimental models (Héliès-Toussaint et al., 2014; Tohmé et al., 2014; Song et al., 2015). Importantly, ERR γ is able to regulate different key metabolic processes such as gluconeogenesis, fatty acid oxidation and oxidative phosphorylation (Alaynick et al., 2007; Eichner et al., 2010; Kim et al., 2012). Taking these data into consideration, we assessed the effects of BPA on the expression of three ERR γ target genes, namely phosphoenolpyruvate carboxykinase 1 (PCK1), succinate dehydrogenase complex subunit D (SDHD) and estrogen-related receptor alpha (ESRRA) (Alaynick et al., 2007; Eichner et al., 2010; Kim et al., 2012). However, the expression of these genes was unchanged in treated HepaRG cells, whatever the BPA concentration (Fig. 6A). In addition, BPA did not alter the expression of PDK4 (data not shown), another target

gene of ERR γ (Lee et al., 2012; Poidatz et al., 2012). Altogether, these data suggested that BPA did not activate ERR γ in HepaRG cells in our experimental conditions, despite additional investigations showing that the mRNA levels of ERR γ (ESRRG) increased sharply during HepaRG proliferation/differentiation, to rapidly reach a plateau around day 13 after seeding (Fig. 6A).

BPA is also an effective agonist of human PXR (Sui et al., 2012; Kuzbari et al., 2013), as many other xenobiotics (Zhang et al., 2008; Tolson and Wang, 2010). Notably, PXR regulates not only the expression of numerous XMEs such as CYPs, UGTs and SULTs (Zhang et al., 2008; Tolson and Wang, 2010), but also that of enzymes and other proteins involved in carbohydrate and lipid homeostasis (Moreau et al., 2008; Wada et al., 2009; Gao and Xie, 2012; Koutsounas et al., 2013; Li et al., 2015). Accordingly, we assessed the effects of BPA on the expression of different PXR target genes, namely CYP3A4, CYP2C9 and the CD36 molecule (CD36, also known as FAT) (Zhang et al., 2008; Tolson and Wang, 2010). However, the mRNA levels of these genes were not modified in HepaRG cells treated with the different concentrations of BPA (Fig. 6B). Contrasting with ESRRG expression, the mRNA levels of PXR (NR112) increased progressively during the proliferation and differentiation stages of HepaRG, and plateaued around day 25 after seeding (Fig. 6B).

Effects of BPA on the Expression of Genes Involved in its Own Biotransformation

Numerous studies carried out in different *in vitro* and *in vivo* models have reported that BPA is able to modulate the expression and/or the activity of numerous XMEs including CYPs, UGTs and SULTs (Hanioka et al., 2000; Nahar et al., 2014; Quesnot et al., 2014). Hence, in a last series of investigations we wished to determine whether BPA altered in HepaRG cells the expression of the main enzymes involved in its own biotransformation, namely UGT2B15, GUSB, SULT1A1, SULT1A3/4, STS, CYP2C9, CYP2C18 and CYP2C19 (Fig. 2). However, the expression of these enzymes was unchanged in treated HepaRG cells, whatever the BPA concentration (data not shown). It is noteworthy that in addition to CYP2C9 (see above), other BPA metabolizing enzymes such as CYP2C19 and SULT1A1 are also PXR targets (Tolson and Wang, 2010). Thus, the lack of BPA effects on CYP2C19 and SULT1A1 expression was fully consistent with our finding that PXR was not activated in treated HepaRG cells.

DISCUSSION

There is an increasing concern regarding the potential harmful effects of human BPA exposure on carbohydrate and lipid homeostasis. Indeed, BPA is greatly suspected to favor obesity, insulin resistance, type 2 diabetes and liver steatosis (Trasande et al., 2012; Khalil et al., 2014; Oppeneer and Robien, 2015; Ranci re et al., 2015). Regarding BPA-induced steatosis, different hypotheses can be put forward including the indirect metabolic effects of obesity and insulin resistance (Marmugi et al., 2012; Polyzos et al., 2012), or direct effects of BPA on lipid homeostasis in hepatocytes (Huc et al., 2012; Grasselli et al., 2013; Peyre et al., 2014; H li s-Toussaint et al., 2014). However, despite a wealth of experimental studies, the mechanisms whereby BPA is able to alter carbohydrate and lipid homeostasis are still poorly understood. Notably, BPA-induced adverse effects could be more important when BPA exposure occurs during the perinatal period, or in the early postnatal years (Calkins and Devaskar, 2011; Rochester, 2013).

Taking all these elements into consideration, a first aim of the present study was to determine whether BPA could induce steatosis in HepaRG cells using a model of perinatal exposure. To this end, we first assessed the expression of several genes known to be differentially expressed during liver development such as PKM, ALDOA, ALDOB, ALB, and three different CYPs (Liao et al., 1980; Lacroix et al., 1997; Rich and Boobis, 1997; Hart et al., 2009). According to the different profiles of gene expression, our results indicated that the period of HepaRG cell culture around day 15 and day 18 after seeding could be considered as the “natal” period (Fig. 1). Consequently, HepaRG cells were treated daily with different concentrations of BPA (ranging from 0.2 to 2000 nM) between day 7 and day 28 after seeding, in order to determine whether chronic BPA exposure could induce steatosis in our cell model. We found that BPA increased neutral lipids and triglycerides in treated cells in a NMDR manner, although these effects were statistically significant only for the 2 nM concentration (Fig. 5). Notably, this concentration is within the range of concentrations found in human serum in the context of environmental exposure (Alonso-Magdalena et al., 2010a; Vandenberg et al., 2010). The NMDR profile observed for neutral lipids and triglycerides was also observed for APOA4 expression (Fig. 5). Interestingly, a recent review of the literature reported that NMDR curves are rather common with BPA (Vandenberg, 2014). It has been proposed that a NMDR profile can occur whenever two or more opposite monotonic dose response curves overlap (Vandenberg et al., 2009; Vandenberg, 2014). Thus, depending of the concentrations, BPA might have contrary effects on triglyceride synthesis on the one hand and on triglyceride disposal on the other hand.

Despite our attempt to decipher how BPA induced steatosis in HepaRG cells, none of our results gave us a starting hypothesis. Importantly, abnormal accumulation of triglycerides within hepatocytes can result from an unbalance between different metabolic fluxes including the entry of fatty acids via specific transporters (e.g. CD36), their esterification into triglycerides, their degradation by the peroxisomal and mitochondrial β -oxidation pathways and their output from the cells after VLDL assembly (Cohen et al., 2011; Begriche et al., 2013). In this study, the expression of several key factors involved in these metabolic processes was however found unchanged. In addition, we found no modification in the expression of different known targets of ERR γ and PXR, thus suggesting that these nuclear receptors are not involved in BPA-induced steatosis, either directly or indirectly. A microarray analysis could be helpful in order to determine whether the dysregulation of other genes might play a role in fat accumulation. It would be also interesting to study the metabolic fluxes linked to lipogenesis, β -oxidation and VLDL output and the activity of key enzymes involved in these metabolic pathways.

As previously mentioned, the human hepatoma HepaRG cell line has increasingly been used in order to study drug metabolism and toxicity. Indeed, this cell line expresses most of the XMEs present in the liver as well as different key nuclear receptors regulating their expression (Aninat et al., 2006; Legendre et al., 2007; Jossé et al., 2008; Dumont et al., 2010; Andersson et al., 2012; Anthérieu et al., 2012). In this study, we found that HepaRG cells expressed relatively high levels of different enzymes involved in BPA biotransformation (Fig. 2), in particular GUSB, UGT2B15, CYP2C9 and CYP2C19 (Fig. 4). Interestingly, the kinetics of expression of UGT2B15, CYP2C9 and CYP2C19 over a 32-day period of cell culture after seeding (Fig. 3) was almost similar to that of ALDOB (Fig. 1), with a progressive expression between day 6 and day 25 subsequently followed by a plateau. These data suggest that the developmental expression of these XME genes could be regulated by factors similar to those involved in ALDOB expression such as the liver-enriched transcription factor hepatocyte nuclear factor 1 α (HNF-1 α) (Tsutsumi et al., 1989; Ito et al., 1995). Our data regarding UGT2B15 and GUSB (Fig. 3) also suggest that their developmental expression could be very different, which might have important consequences on BPA biotransformation. For instance, the combination of relatively high expression of GUSB and low expression of UGT2B15 during early development could favor BPA accumulation and the occurrence of deleterious effects.

Taking all these data into consideration, the HepaRG cell line could be a valuable tool in order to gain further information regarding BPA biotransformation in human hepatocytes, in particular during the developmental period. Our results also underline the relevance of the HepaRG model in order to

investigate the hepatic effects of endocrine disruptors that are expected to occur during the perinatal period. However, it should be kept in mind that the HepaRG cell line is derived from a female patient (Gripon et al., 2002). This feature might be important because previous investigations carried out in rodents reported that perinatal exposure of BPA could induce differential effects on lipid and carbohydrate homeostasis between male and female offspring (Miyawaki et al., 2007; Somm et al., 2009; Alonso-Magdalena et al., 2010b). Moreover, it is well-known that the expression and activity of different XMEs can significantly vary depending on the sex (Mugford and Kedderis, 1998; Waxman and Holloway, 2009). Thus, one cannot exclude the possibility that our results might not have been similar with cells from a male donor.

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LEGENDS TO THE FIGURES

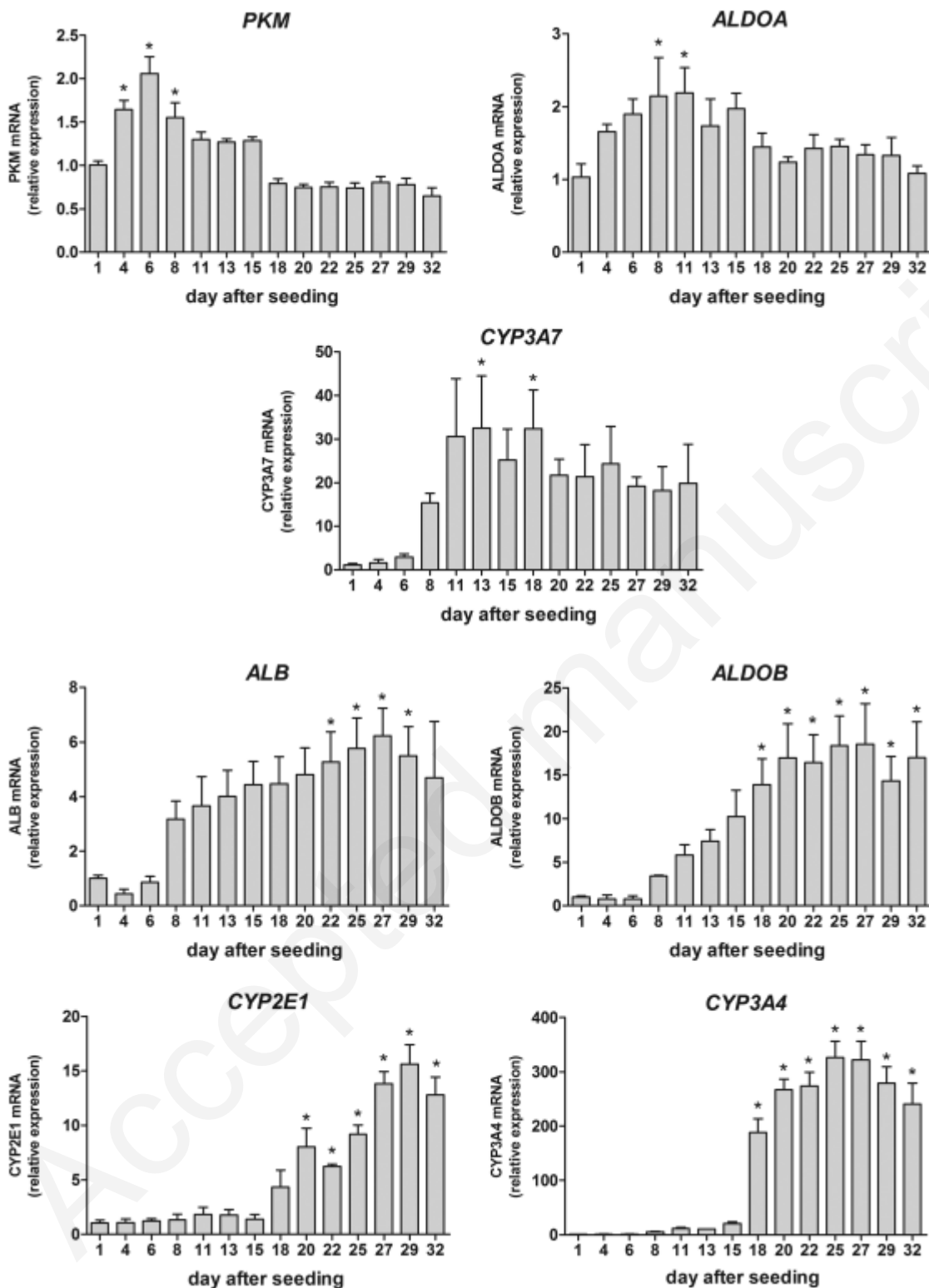


Figure 1. Kinetics of expression in HepaRG cells of seven different proteins known to be differentially expressed during liver development. The mRNA levels of each protein were determined

over a 32-day period of cell culture after seeding. Expression of TBP was used as reference. Results are means \pm SEM for 3 independent cultures. *Significantly different from HepaRG cells at day 1 after seeding ($P < 0.05$).

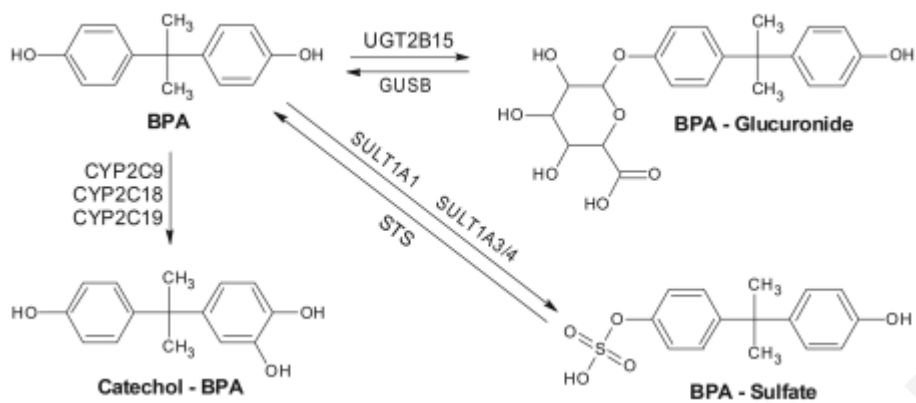


Figure 2. Schematic representation of BPA metabolism in human according to the current literature. The correspondence of the different abbreviations and further information is given in the text.

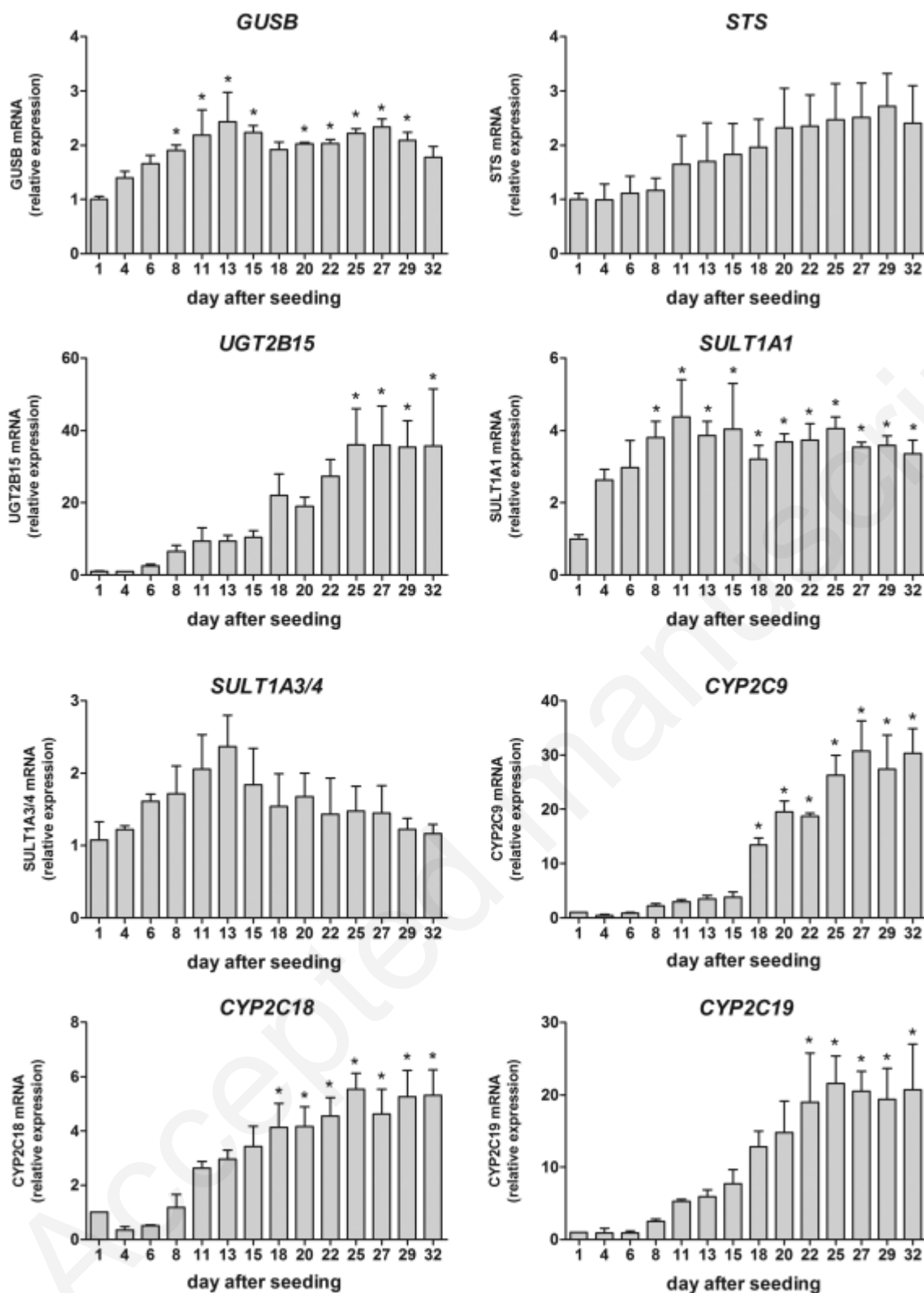


Figure 3. Kinetics of expression in HepaRG cells of eight different enzymes involved in BPA metabolism in human according to the current literature. The mRNA levels of each protein were determined over a 32-day period of cell culture after seeding. Expression of TBP was used as

reference. Results are means \pm SEM for 3 independent cultures. *Significantly different from HepaRG cells at day 1 after seeding ($P < 0.05$).

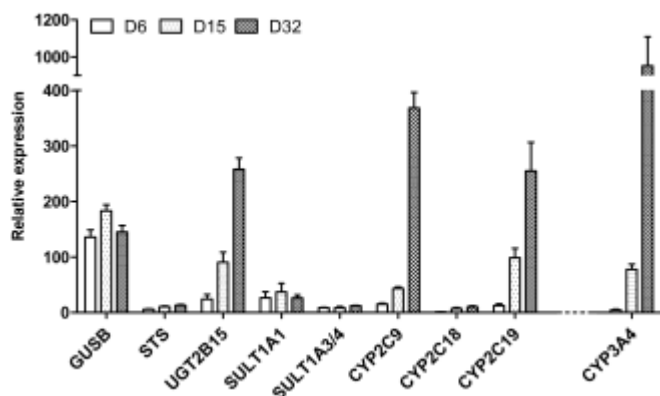


Figure 4. Comparative mRNA expression in HepaRG cells of eight different enzymes involved in BPA metabolism. The relative expression are shown for day 6 (D6), day 15 (D15) and day 32 (D32) after seeding. The relative expression of CYP2C18 at D6 was arbitrary set to 1. For proper comparison, the figure also shows the expression of CYP3A4, a prototypical XME that is highly expressed in human liver.

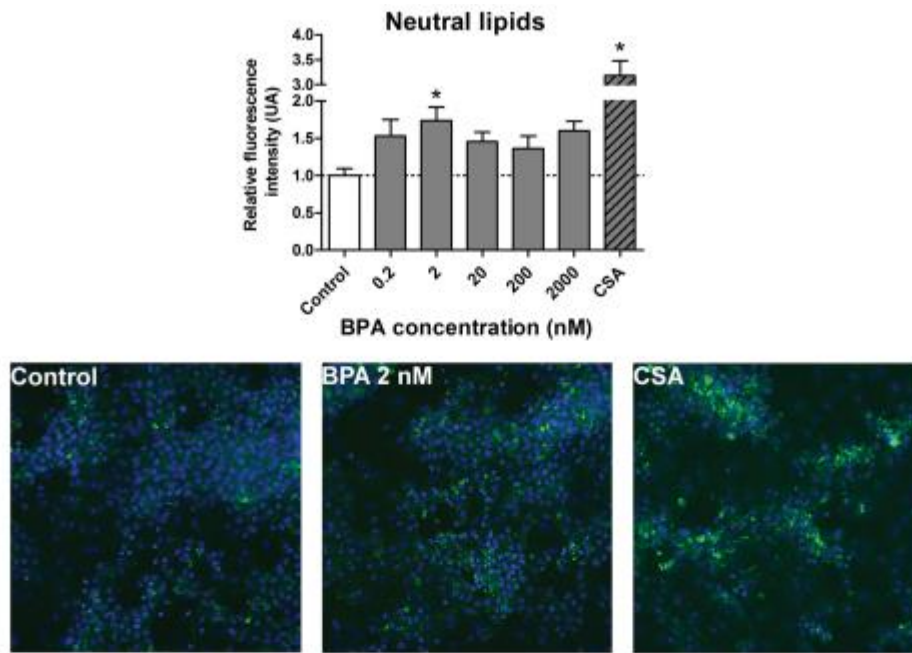
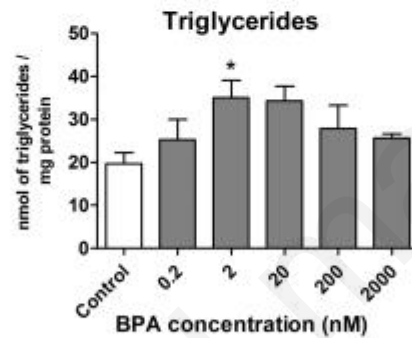
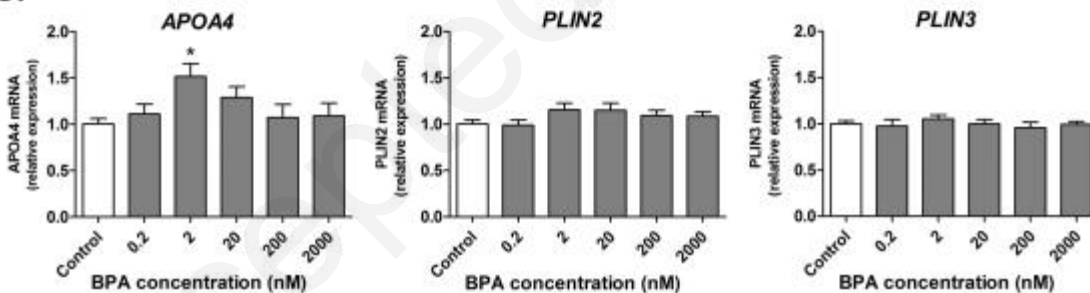
A.**B.****C.**

Figure 5. BPA-induced steatosis in HepaRG cells. Cells were treated with different concentrations of BPA (from 0.2 to 2000 nM) during 3 consecutive weeks and different parameters linked to steatosis were subsequently assessed. **A.** Determination of neutral lipids with the LipidTOX™ stain. Results are means \pm SEM for 3 independent cultures. The pictures (magnification \times 200) are representative of the respective conditions. Cyclosporin A (CSA, 30 μ M) was used as a positive control to induce lipid accumulation, as recommended by LipidTOX™ manufacturer. *Significantly different from control HepaRG cells ($P < 0.05$). **B.** Determination of cellular triglycerides with a colorimetric assay.

Results are means \pm SEM for 4 independent cultures. *Significantly different from control HepaRG cells ($P < 0.05$). **C.** mRNA expression of APOA4, PLIN2 and PLIN3. Expression of TBP was used as reference. Results are means \pm SEM for 3 independent cultures. *Significantly different from control HepaRG cells ($P < 0.05$).

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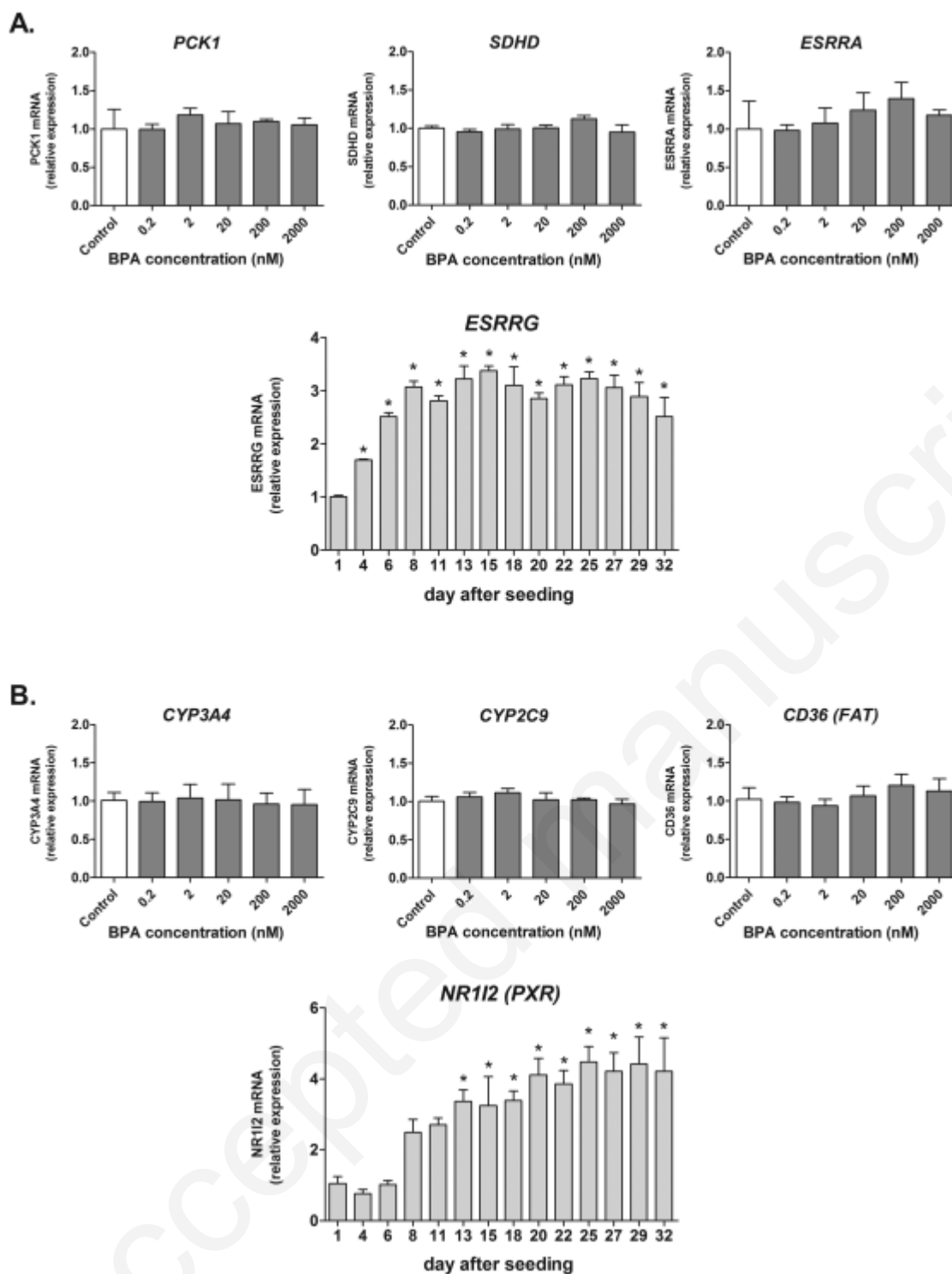


Figure 6. Effects of BPA in HepaRG cells on the expression of known targets of $ERR\gamma$ (ESRRG) and PXR (NR112) and kinetics of expression of these nuclear receptors over a 32-day period of cell culture after seeding. **A.** Expression of 3 different ESRRG target genes and kinetics of ESRRG expression. Results are means \pm SEM for 3 independent cultures. *Significantly different from HepaRG cells at day 1 after seeding ($P < 0.05$). **B.** Expression of 3 different PXR target genes and kinetics of PXR

expression. Results are means \pm SEM for 3 independent cultures. *Significantly different from HepaRG cells at day 1 after seeding ($P < 0.05$).

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