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Comparative study of bisphenol A and its analogue bisphenol S on human hepatic cells: A focus on their potential involvement in nonalcoholic fatty liver disease

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ABSTRACT

For several decades, people have been in contact with bisphenol A (BPA) primarily through their diet. Nowadays it is gradually replaced by an analogue, bisphenol S (BPS). In this study, we compared the effects of these two bisphenols in parallel with the positive control diethylstilbestrol (DES) on different hepatocyte cell lines. Using a cellular impedance system we have shown that BPS is less cytotoxic than BPA in acute and chronic conditions. We have also demonstrated that, contrary to BPA, BPS is not able to induce an increase in intracellular lipid and does not activate the PXR receptor which is known to be involved in part, in this process. In parallel, it failed to modulate the expression of CYP3A4 and CYP2B6, the drug transporter ABCB1 and other lipid metabolism genes (FASN, PLIN). However, it appears to have a weak effect on GSTA4 protein expression and on the Erk1/2 pathway. In conclusion, in contrast to BPA, BPS does not appear to induce the metabolic syndrome that may lead to non-alcoholic fatty liver disease (NAFLD), *in vitro*. Although we have to pay special attention to BPS, its use could be less dangerous concerning this toxicological endpoint for human health.

1. Introduction

Bisphenol A (BPA) is one of the highest-volume chemicals produced worldwide, and human exposure to BPA is thought to be ubiquitous (Vandenberg et al., 2012). It has been extensively studied since the 1930s and was first used as a synthetic estrogen. Indeed the BPA molecule has structural features that are similar to 17β-estradiol and other natural estrogenic compounds found in food such as daidzein, one of the major soy isoflavones (Csanády et al., 2002). Nowadays, it is mainly found in the composition of a wide variety of polycarbonate plastics, flame retardants, dental sealant resins and liners for food packaging, making humans exposed to BPA from their alimentation (Évaluation des risques du bisphénol A pour la santé humaine, ANSES, 2013). One of the reasons is under certain conditions (sterilization, pH or increased temperature), BPA can be released by the hydrolysis of polymers and thus contaminate the food chain (Howdeshell et al., 2003; Kadoma and Tanaka, 2000).

Over the past ten years, a large number of studies have demonstrated the toxicity of BPA even at very low doses. The potential role of BPA in metabolic abnormalities has been studied in different epidemiological and animal studies (Lang et al., 2008; Lin et al., 2013; Ryan et al., 2010; Somm et al., 2009). It has also been implicated in the onset of breast cancer (Sengupta et al., 2013), abnormal intestinal function and inflammation (Braniste et al., 2010), obesity and diabetes (Shankar et al., 2012) and sterility

Abbreviations: Ab, antibody; BPA, bisphenol A; BPS, bisphenol S; BSA, bovine serum albumin; CAR, constitutive androstane receptor; CsA, cyclosporine A; DES, diethylstilbestrol; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HCS, high content screening; NAFLD, nonalcoholic fatty liver disease; NASH, steatohepatitis; NCI, normalized cell index; PB, phenobarbital; PXR, pregnane X receptor; RIF, rifampicine; TBS-T, Tris-buffered saline with Tween.

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(Cabaton et al., 2011). Moreover, it can be found in human cord blood, placenta, amniotic fluid and breast milk, resulting in the exposure of fetuses and newborns through maternal exposure (Fénichel et al., 2012; Schönfelder et al., 2002). However, epidemiological studies of BPA are still subject to controversy. To date, there are still unresolved problems including the contamination of samples, the methodologies used and statistical analyses making *in vitro* and animal studies the main source of information relating to health outcomes in response to its exposure (Lakind et al., 2014).

Due to increased concerns over the safety of BPA, Health Canada (2009), the European Union (2011) and the US Food and Drug administration (2012) have banned its use in plastic bottles for infants. Its presence in food containers will be totally abolished in France by 2015. Thus more heat-stable bisphenol components have been developed. Among them, the 4,4′-dihydroxyphenyl sulfone, commonly known as bisphenol S (BPS) is already used in polyethersulfone. Its production and use as a chemical additive in pesticides, dyestuffs, color fast agents, dye dispersants and as a monomer in cyclic carbonates is increasing and will exceed that of BPA (Molina-Molina et al., 2013). BPS is mainly characterized by a high thermal stability but a reduced biodegradability. It has been found in the urine of the US and Asian population at concentrations ranging from 0.8 to 84 nM (Liao et al., 2012). Together, these data suggest that BPS could be a serious candidate as an environmental pollutant and for human exposure in the future. As BPS is a novel molecule, very few studies including toxicity and physiological action have been reported to date.

One of the metabolic syndromes associated with exposure to BPA is nonalcoholic fatty liver disease (NAFLD). It is the most prevalent liver disease worldwide in pediatric patients with more than 10% of children and adolescents affected in industrialized countries (Greco et al., 2008; Ibrahim et al., 2011). NAFLD comprises steatohepatitis (NASH) and other liver diseases such as steatosis. NAFLD is associated with increased liver fat content, coagulation factors, glucose overproduction and inflammatory markers. Interestingly, exposure to BPA has also been correlated with the modulation of genes involved in lipid synthesis, increased body weight, lipid accumulation and insulin secretion in mice (Marmugi et al., 2012). This suggests that BPA may influence *de novo* fatty acid synthesis, thereby contributing to hepatic steatosis. In a previous study we showed that BPA promotes lipid accumulation in hepatic cells (HepG2) triggered by the disturbance of mitochondrial function, alteration in lipid metabolism and inflammation that can therefore contribute to steatosis (Huc et al., 2012). This new study aims to compare effects of BPS against such BPA-induced phenomenon.

To provide new insights into the process leading to NAFLD, we focused on the nuclear hormone receptor pregnane X receptor (PXR; also called SXR) which acts as a xenobiotic sensor and coordinates the regulation of xenobiotic metabolism via transcriptional regulation of xenobiotic-detoxifying enzymes and transporters. Over the past few years, research has revealed that PXR can modulate inflammation, vitamin D metabolism, lipid homeostasis, energy homeostasis and cancer (Zhou et al., 2009). The identification of PXR has provided an important tool for studying new mechanisms through which diet, chemical exposure, and environment ultimately impact health and disease. Interestingly, BPA has been shown to activate human PXR (hPXR) leading to the modulation of target genes such as CYP3A4 in humans but not in rodents (Kuzbari et al., 2013; Takeshita et al., 2001). This pathway has been proposed as an alternative to its well described estrogenic activity by which BPA may act (Sui et al., 2012).

As mentioned above, hPXR is involved in triglyceride homeostasis leading to lipid accumulation and steatosis (Moreau et al., 2009; Moya et al., 2010). In order to determine a possible link between BPA and its substitute BPS, PXR activation and steatosis were designed as possible targets. For this, we used a stable hPXR transfected HepG2 cell line. Indeed, the HepG2 cell line is currently the most used cell line as a human hepatocyte model, but they respond very weakly to the different inducers at the gene expression level, and this translates generally into low CYP450 activities (Gerets et al., 2012). To overcome this, we have previously developed a stable hepatoma cell line which expresses hPXR, CYP3A4 distal and proximal promoters plus the luciferase reporter gene. This cell line was named HepG2-PXR (Lemaire et al., 2004) and provides a useful tool for both studying hPXR activation and identifying environmental xenobiotics capable of inducing CYP3A4 and CYP2B6. Additionally, we used HepaRG cells. Contrary to other human hepatoma cell lines (including HepG2 cells), HepaRG cells express various CYPs (CYP1A2, 2B6, 2C9, 2E1, 3A4) as well as the constitutive androstane receptor (CAR) and PXR, at levels comparable to those found in cultured primary human hepatocytes. This cell line thus represents a good model system for analyzing cyto- and genotoxic compounds. Moreover, the functional activities of HepaRG cells remain relatively stable for 1–2 weeks more after 3–4 weeks at confluency, making the cells a unique model for chronic toxicity studies (Guillouzo et al., 2007).

This study was designed to compare the effects of two environmental pollutants, BPA and its structural analogue BPS, for toxicological and metabolic analyses. DES was chosen as a positive control due to its recognized estrogenic properties. We initially showed that BPA was more toxic than BPS using three hepatocyte models. We then validated that the effects of BPA on PXR activation were consistent with the literature thanks to our HepG2/PXR model whereas BPS does not seem to have a particular affinity for this receptor. In parallel, in hepatic cells we also studied the ability of BPS to induce changes in lipid homeostasis and in the modulation of gene expression linked to lipid metabolism and detoxification. However, BPS did not alter the intracellular lipid level and did not modulate the gene expression of CYP3A4, CYP2B6, ABCB1, FASN and PLIN2. Following 48 h of exposure to BPS, in the HepaRG cell line, we found modulation of the expression of the GSTA4 protein and a weak down regulation of the ERK1/2 pathway. Together these data indicate that BPS would be less hepatotoxic than BPA.

2. Materials and methods

2.1. Cell culture and treatments

The human hepatocellular carcinoma cell line HepG2 was obtained from ATCC (American Type Culture Collection, Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM of stable glutamine (PAA), 0.5% penicillin/streptomycin (Gibco), 1% non-essential amino acids (PAA), sodium pyruvate (Gibco), and 10% fetal bovine serum (FBS from PAA), in a humidified atmosphere at 37 °C containing 5% CO_2. After washing with sterile phosphate buffer saline (PBS), cells were detached by trypsinization (0.05% trypsin/EDTA Gibco) and plated at a concentration of 1×10^4 cells/ml in 96-well plates. The final dimethylsulfoxide (DMSO) concentration was 0.25% (v/v). Experiments were performed between cell passage 10 and 25. The culture media was renewed every 2–3 days.

The HepG2/PXR cell line was derived from a hepatoblastoma and stable expression of the human "pregnane X receptor" (hPXR) was obtained according to the protocol described by Lemaire et al. (2004). As for the HepG2 cells, HepG2/PXR cells were grown in complete DMEM supplemented with 10% FCS but with the addition of 400 μg/ml geneticin (G418, Invitrogen). After trypsinization, cells were seeded in 96-well plates at a density of 1×10^4 cells/well. The culture media was renewed every 2–3 days.

The HepaRG cell line was derived from a liver tumor from a female patient suffering from hepatocarcinoma (Gripon et al., 2002). HepaRG cells were purchased from Biopredic International (Rennes, France) as confluent monolayer (ca. 1×10^4 cells per well) in 96-well plates containing the associated medium. The growing medium was composed of Williams E with Glutamax-I added with 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.1 U/ml human insulin and 50 μM hydrocortisone hemisuccinate. Before shipment, the cells were allowed to proliferate and were then cultured for 2 weeks after reaching confluency in the same medium supple-

mented with 2% DMSO in order to obtain maximum differentiation. After shipment, cells were maintained in media containing DMSO which was renewed every 2–3 days.

BPA, BPS, DES and cyclosporin A (CsA) were all purchased from Sigma–Aldrich. All chemicals were prepared as stock solutions in DMSO. The final concentration of DMSO in the medium was 0.25% in all conditions for the HepG2 and HepG2/PXR cells, and 2% for the HepaRG cells.

For these experiments, we deliberately worked at high doses in order to compare the mechanisms of action leading to both cell death and steatosis. We also replaced traditional cytotoxic tests with a real-time cellular impedance system as described below.

2.2. Real-time cellular impedance

The real-time cell impedance analyzer (xCELLigence® system) was used according to the manufacturers' instructions (Roche Applied Science, Mannheim, Germany and ACEA Biosciences, San Diego, CA, USA). For the HepG2 and HepG2/PXR cell lines, 1×10^4 cells/well were added to 96-well E-plates. Twenty-four hours later, cells were treated with different concentrations of BPA (from 1 pM to 1 mM), BPS (from 1 pM to 1 mM) and DES (from 1 pM to 250 μM) in DMEM. For the HepaRG cells, the same treatments were performed after reaching confluency and differentiation as described above and the medium/treatment was changed every 2–3 days for the chronic experiment. Real-time cellular impedance was measured in each well (cell index values) and the signal was observed through the integrated software (RTCA Analyzer). To observe the influence of chemicals on cells in comparison with the DMSO control, the normalized cell index (NCI) was used, based on the last time point prior to compound addition. Each curve is representative of an experiment performed in triplicate (technical replicates). Data are given as the mean ± standard deviation and are representative of three independent and reproducible experiments.

This technique allowed us to monitor the overall cellular status of adherent cells in culture, i.e. variation in the number of cells and in their cytomorphology. The tests were conducted in a non-invasive environment, without marking or staining, and are compatible for studying both early and late effects. This technology is particularly suitable for the study of the cellular toxicity of chemical entities or mixtures, and is appropriate for assessing rapid morphological changes (activation of membrane receptors) or slower events such as mobility. Here we propose this technology as an alternative to the common tests currently utilized to predict *in vitro* cytotoxicity in human hepatocytes, as it has already been described previously by our team and in other different publications (Atienzar et al., 2011; Crépet et al., 2013; Nawaz et al., 2013).

2.3. Nuclear receptor PXR activation

The transactivation assay of the hPXR was conducted on the HepG2-hPXR cell line, following measurement of the luciferase activity (Steady-Glo® Luciferase Assay) as described by Lemaire et al. (2004). For each experiment, data were normalized compared with the value obtained with the PXR agonist model (Rifampicin 5 μM).

2.4. Cell imaging microplate assays and cellomics – ArrayScanXTI scanning details

HepG2 and HepG2/PXR cells were seeded at 1×10^4 cells/well in black 96-well cell culture plates and allowed to attach overnight in a humidified incubator at 37 °C, 5% CO_2. The HCS LipidTOX™ Green neutral lipid stain detection kit (Invitrogen, Eugene, Oregon, USA) was used according to the manufacturer's instructions in order to detect intracellular neutral lipids. Briefly, cells were treated for 72 h with different concentrations of xenobiotics (1 pM–0.1 mM). Then the stock concentration of LipidTOX™ contained in DMSO was diluted 2000× in a serum and phenol-red free medium. To each well, 100 μl of the final solution was added and incubated for 30 min with the nuclear marker Hoechst 33342 (2.5 μg/ml final) at 37 °C. The medium was changed and the plates were scanned with the ArrayScanXTI instrument (Cellomics Inc., Pittsburgh, USA). The detection of accumulated lipids was performed with the "compartmental analysis" bio-application (Cellomics Inc., Pittsburgh, USA). The number of cells analyzed was fixed at 500 cells per well. An objective of 20× was used for the imaging analysis. The nuclear dye (Hoechst) was detected in channel 1 (excitation-emission filter set 365/515 nm). The 'objects' targeted for measurement were detected in channel 2 (filter set 549/600 nm) and were defined by expanding the circular region of the nuclei to include the cytoplasm of the cell in order to detect the lipid dye. The Cellomics ArrayScanXTI output feature "mean spot total intensity" was used to analyze the scans. For each plate, the mean of the three control wells (0.25% DMSO) was used as a reference and rescaled to one. Each well value was expressed relative to this reference. A value of two meant a 2-fold induction of the fluorescent signal compared with the control value. CsA (30 μM) was used as a supplementary positive control in this experiment.

2.5. Quantitative RT-PCR analysis

Differentiated HepaRG cells were exposed for 48 h to 1 μM and 0.1 mM of BPA or BPS. The cells were then rinsed with PBS and total RNA was extracted using TriReagent (MRC, Euromedex, Souffelweyersheim, France) according to the manufacturer's protocol. RNA was quantified at 260 nm and the purity was evaluated by measuring the A260/A280 ratio (values close to 2.0) using a NanoDrop (Thermo Scientific, Waltham, MA, USA). Total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Villebon Sur Yvette, France). qPCR mixes were prepared in 384-well plates according to the Power SYBR Green PCR kit protocol (Life Technologies), using automated mixing and loading via a Bravo Robot device (Agilent Technologies, Les Ulis, France). After a 10 min activation at 95 °C of the Taq Polymerase, 40 cycles (15 s at 95 °C + 1 min at 60 °C) were run and analyzed on a Viia7 real-time PCR detection system (Agilent Technologies). Data analysis of the relative gene expression was performed using the $2-\Delta\Delta Ct$ method (Livak and Schmittgen, 2001) after normalization of the raw data with β-actin which was used as a housekeeping gene. Melting curve analysis was carried out to assess the formation of single products. The linearity and efficiency of the PCR reactions were evaluated on the raw data using the LinReg software. The specific primers used are presented in Table 2. Each treatment was performed in triplicate within three separate experiments.

2.6. Western blot analysis

HepaRG cells were lysed with ice-cold specific buffer supplemented with protease inhibitors. Protein lysates were separated on 12% Bis-Tris polyacrylamide gels, then transferred onto nitrocellulose membranes followed by blocking for 60 min in 1% bovine serum albumin (BSA) and 5% nonfat dry milk in 1× Tris-buffered saline with Tween-20 (TBS-T). Membranes were then incubated with the corresponding primary antibodies in 1X TBS-T in the presence of 1% BSA at room temperature for 60 min or overnight at 4 °C under gentle shaking. After three washes in 5% nonfat dry milk in 1× TBS-T, membranes were exposed to corresponding secondary antibodies labeled with near-infrared dyes (IRDyes, Li-COR Biosciences, Cergy Pontoise, France) in blocking buffer for 60 min at room temperature. After three washes in 1× TBS-T followed by one wash in 1× TBS, signals were detected using the Odyssey® Infrared Imaging system (Li-COR). Western blots were probed using primary antibodies against ERK1/2 and alpha-tubulin (Sigma, Saint-Quentin Fallavier, France), phosphorylated-ERK1/2 (Cell Signaling Technologies, Ozyme, Saint Quentin En Yvelines, France) and GSTA4 (Abcam, Paris, France).

2.7. Statistical analyses and modeling of dose–response curves

Each experiment was repeated at least three times. Data shown are the mean ± standard deviation (SD). To determine the statistically significant difference between two groups, a Student's *t* test was used (two-tailed, paired samples for means, and hypothesized difference of 0). Levels of probability are indicated as $^{*}p < 0.05$ or $^{**}p < 0.01$. For the qPCR analysis of gene expression, a *t*-test was performed using the GraphPad Prism 6.02 software. Results are presented as the mean fold change ± SD. Regarding PXR activation, a scatter plot of luciferase activity versus concentration was constructed. All data from the same test compounds were pooled and statistical non-linear regression (dose–response) was conducted after bootstrapping using the Levenberg–Marquardt algorithm. No constraint was applied to the five-parameter equation (equation below). Curve fitting was carryied out using the Statistica software (Statsoft). The mean curve and the associated percentile confidence intervals (2.5–97.5%) were calculated from the whole set of the fitted curve's after bootstrapping.

$$f(x) = D + \frac{A - D}{\left[(1 + (2^{1/G} - 1)) \times \left(\frac{x}{C}\right)^{B}\right]^{G}}$$

3. Results

3.1. BPA is more hepatotoxic than its substitute BPS

To investigate the effects of BPA and BPS on the growth and survival of different cell lines, we used the innovative real-time cellular impedance technology described in Section 2. Of course, this method was complemented with phenotypic visual inspection by microscopy in order to link decreases in cell index with mortality.

Fig. 1 displays the curves for which modifications of the NCI occurred relative to the DMSO control. Concentrations between 1 pM and 50 μM brought about no changes in the NCI regardless of the cell model and the compound tested (data not shown). At 100 μM of BPA and BPS, we observed a decrease in the NCI for both HepG2 and HepG2/PXR cells, reflecting the toxicity of the compounds at this concentration. At the final time point (72 h), this decrease was greater for BPA (see also calculated slopes) suggesting that it is more cytotoxic than BPS. In differentiated HepaRG cells, only cells treated with BPS showed a short increase in NCI,

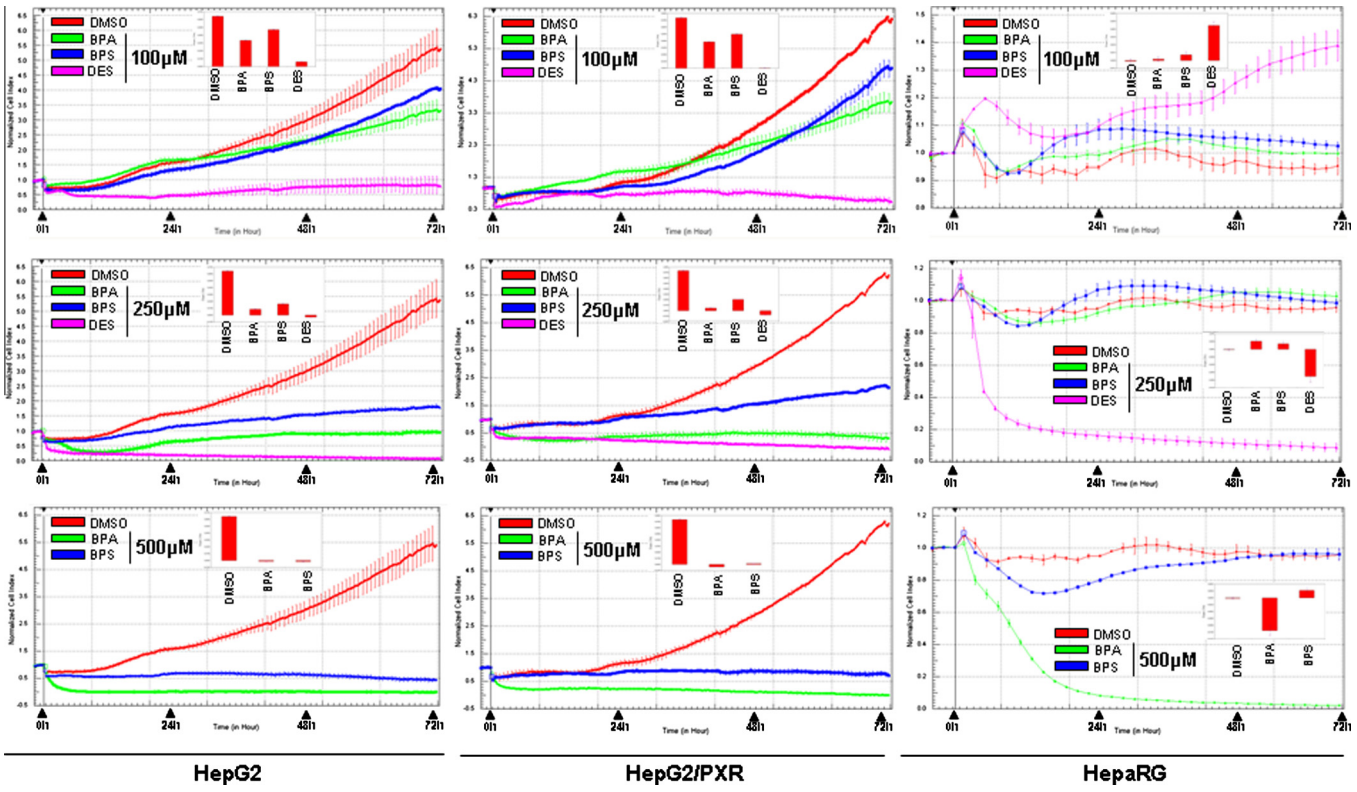

Fig. 1. Acute effects of both bisphenols on real-time cell analyzer curves. HepG2, HepG2/PXR and HepaRG cells were treated for 72 h with different concentrations of bisphenol A (BPA) and bisphenol S (BPS) as indicated in each picture. Diethylstilbestrol (DES) was used as control for this experiment. Cell index was determined using the xCELLigence technology (Roche). All data presented have been normalized and each point was calculated from triplicate values. Data represent the mean ± standard deviation. For each curve, a slope was calculated to compare the effect of xenobiotics on cell index. These results are representative of three independent experiments.

24 h after treatment. DES was highly toxic at this dose except for in HepaRG cells where it induced a strong increase in the cell index (NCI_{DES} = 1.4 whereas NCI_{DMSO} = 0.95 at 72 h).

At 250 µM, the control DES induced an immediate decrease in the NCI on HepG2, HepG2/PXR and HepaRG cells (NCI is close to 0 at 72 h for the three cell lines). At the same concentration, BPA and BPS treatments also led to a decrease in NCI. For example on HepG2 cells, at 72 h the NCI was 5.5 following treatment with the DMSO control compared with 1.0 and 1.8 following treatment with BPA and BPS, respectively. The reduction was therefore more pronounced with BPA and closely resembled the DES response especially in HepG2/PXR cells. However, responses in HepaRG cells to BPA and BPS were similar to the DMSO control at 72 h.

Finally, we observed similar decreases in the NCI (see the histogram representing the average slopes on Fig. 1) when HepG2 and HepG2/PXR cells were treated with 500 µM of BPA and BPS. However, the effects once again occurred earlier and were more severe with BPA than with BPS (at 48 h on HepG2/PXR, NCI_{BPA} = 0, NCI_{BPS} = 0.75 and NCI_{DMSO} = 2.5). However, on the HepaRG cell line, while BPA treatment led to a complete decrease in the NCI after 48 h, BPS induced a short decrease during the first 24 h of exposure before it returned to the same value obtained with the control DMSO.

3.2. Bisphenols exert chronic toxic effects on HepaRG cells

To investigate the chronic toxic impact of BPA and BPS on the liver, HepaRG cells were treated over 3 weeks with increasing concentrations (1 pico- to 1 milli-molar) of each compound. We observed no changes in the NCI following treatment with BPA or BPS at doses ranging from 1 pM to 50 µM (data not shown). The first significant decrease was recorded after exposure to 100 µM of DES (Fig. 2A). This occurred after the second treatment and was complete after 12–15 days.

At 250 µM (Fig. 2B), BPA induced a decrease in the NCI after 72 h of treatment. After 6 days, this corresponded to a loss of 50% in the NCI compared with the control DMSO and 100% at the end of the treatment. At this concentration, BPS treatment led to a significant but stable decrease of about 20% in the NCI between 9 days to 3 weeks.

Treatment with 500 µM of BPA (Fig. 2C) resulted in a 100% decrease in the NCI in less than 72 h (same profile with 250 µM of DES in Fig. 2B), while the BPS led to a slow decrease of nearly 50% after 3 weeks of treatment. As previously demonstrated, BPS therefore appeared to be the least toxic compound. In addition, chronic exposure (made possible by the HepaRG cell model) allowed us to highlight a constant and long-term decrease in the NCI in response to 250 µM of BPA or 500 µM of BPS (Fig. 2B and C) that was not observed in acute conditions in this cell line (Fig. 1).

3.3. Hepatotoxicity evaluation based on cellular impedance monitoring

From the impedance data, we calculated the EC50 values of BPA, BPS and DES on HepG2, HepG2/PXR and HepaRG cells (Table 1), after short-term (from 24 to 72 h) and long-term exposure (1 week and 3 weeks). Firstly, for the same concentrations tested, the results suggest that BPA induces more cytomorphological changes than BPS in the three hepatocyte models tested. Indeed, the EC50 of BPS is 1.5–3 times greater than the EC50 of BPA. Secondly, the EC50 is lower for both BPA and BPS in the HepG2/PXR cell line. For example, the EC50 of BPA after 48 h of treatment was 260 ± 13 µM, 167 ± 24 µM and 380 ± 5 µM on HepG2, HepG2/PXR and HepaRG cells, respectively. At the same time point, the EC50

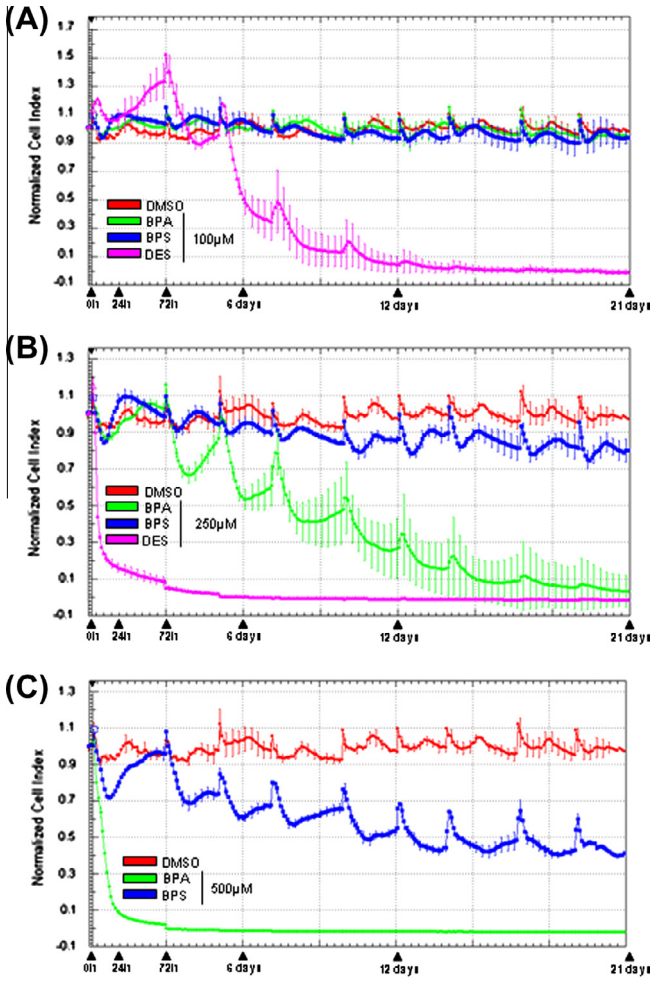

Fig. 2. Chronic effects of both bisphenols on real-time cell analyzer curves. HepaRG cells were treated for 3 weeks with 100 µM (A), 250 µM (B) and 500 µM (C) of bisphenol A (BPA) and bisphenol S (BPS) as indicated in each picture. Diethylstilbestrol (DES) was used as a control for this experiment. Cell index was determined using the xCELLigence technology (Roche). All data presented have been normalized and each point was calculated from triplicate values. Data represent the mean ± standard deviation. These results are representative of three independent experiments.

of BPS was 535 ± 10 µM on HepG2 cells, 437 ± 18 µM on HepG2/PXR cells and 787 ± 13 µM on HepaRG cells. These data suggest that BPA and BPS are more toxic in cell lines stably expressing the PXR receptor (HepG2/PXR). Moreover, it appears that the short-term toxicity of BPA on HepG2/PXR cells is identical regardless of treatment duration (EC50 around 165 µM at 24, 48 and 72 h).

Contrary to BPA and BPS, the positive control DES (which is known to be a PXR activator) appeared to induce more cytomorphological effects on HepG2 cells than on HepG2/PXR cells at the over the shorter exposure time e.g. at 24 h (79 ± 8 µM versus 166 ± 37 µM) yet had a similar impact at 48 and 72 h. With regard to BPA and BPS, and for the same concentrations, DES has more pronounced effects on cellular impedance curves and could be more hepatotoxic for the three cell lines studied.

3.4. Pregnane X receptor activation by the bisphenols

We tested the three compounds over a wide range of concentrations (from 1 pM to 500 µM) and present the results for the high doses in line with previous results. Dose–response curves were calculated from the concentrations devoid of toxicity. Indeed, at high and toxic concentrations, especially for DES and to a lesser extent for BPA, all induce a decrease in activation. The prototypical agonist Rifampicin (5 µM) was used as a positive control of PXR activation in this experiment (Fig. 3A). The results show that BPS is not able to transactivate the PXR receptor, whereas BPA and DES act as agonists of this nuclear receptor at relatively low concentrations. The effects of DES were observed from 2.5 µM and from 5 µM with BPA. Based on modeling data, concentrations inducing 50% of the PXR activation were calculated in comparison with the control conditions. The values obtained (BPA = 11.73 ± 5.14 µM and DES = 25.7 ± 5.73 µM) demonstrate a more powerful transactivation potential of BPA compared with DES.

3.5. BPA but not BPS induces lipid bio-accumulation in HepG2 and HepG2/PXR cells

We used the LipidTOX™ marker to test and compare the effects of BPA and BPS on lipid bioaccumulation in HepG2 and HepG2/PXR cells. We used a new technology (HCS) that combines fluorescence microscopy, flow cytometry and microplate reader measurements in one device. Interestingly, all data collected can be processed via

Table 1
EC50 obtained from real-time cell impedance monitoring on different hepatic cell lines.

	HepG2			HepG2/PXR			HepaRG		
	24 h	48 h	72 h	24 h	48 h	72 h	48 h	1 Week	3 Weeks
BPA	308 ± 8 µM	206 ± 13 µM	252 ± 10 µM	157 ± 15 µM	167 ± 24 µM	175 ± 23 µM	380 ± 5 µM	278 ± 46 µM	180 ± 5 µM
BPS	900 ± 35 µM	535 ± 10 µM	421 ± 19 µM	392 ± 38 µM	437 ± 18 µM	296 ± 48 µM	787 ± 13 µM	630 ± 18 µM	452 ± 28 µM
DES	79 ± 8 µM	58 ± 9 µM	37 ± 4 µM	166 ± 37 µM	74 ± 32 µM	29 ± 5 µM	193 ± 8 µM	93 ± 4 µM	69 ± 18 µM

Table 2
Quantitaive PCR probes list and sequences.

Gene	Forward	Reverse	Source
β-Actin	TTGCTGATCCACATCTGCTG	GACAGGATGCAGAAGGAGAT	Pupo et al. (2012)
FASN	CAGGCACACACGATGGAC	CGGAGTGAATCTGGGTTGAT	Santolla et al. (2012)
GSTA4	ACAGTTGTACAAGTTGCAGGATG	AATTTCAACCATGGGCACTT	UPL[a]
GSTP1	CTCCGCTGCAAATACATCTC	ACAATGAAGGTCTTGCCTCC	UPL[a]
CYP3A4	CTTCATCCAATGGACTGCATAAAT	TCCCAAGTATAACACTCTACACAGACAA	Aninat et al. (2006)
CYP2B6	TTCCTACTGCTTCCGTCTATCAAA	GTGCAGAATCCCACAGCTCA	Aninat et al. (2006)
PLIN2	TCAGCTCCATTCTACTGTTCACC	CCTGAATTTTCTGATTGGCACT	UPL[a]
ABCB1	GCCAAAGCCAAAATATCAGC	TTCCAATGTGTTCGGCATTA	Aninat et al. (2006)

[a] UPL: Universal Public Library (Roche).

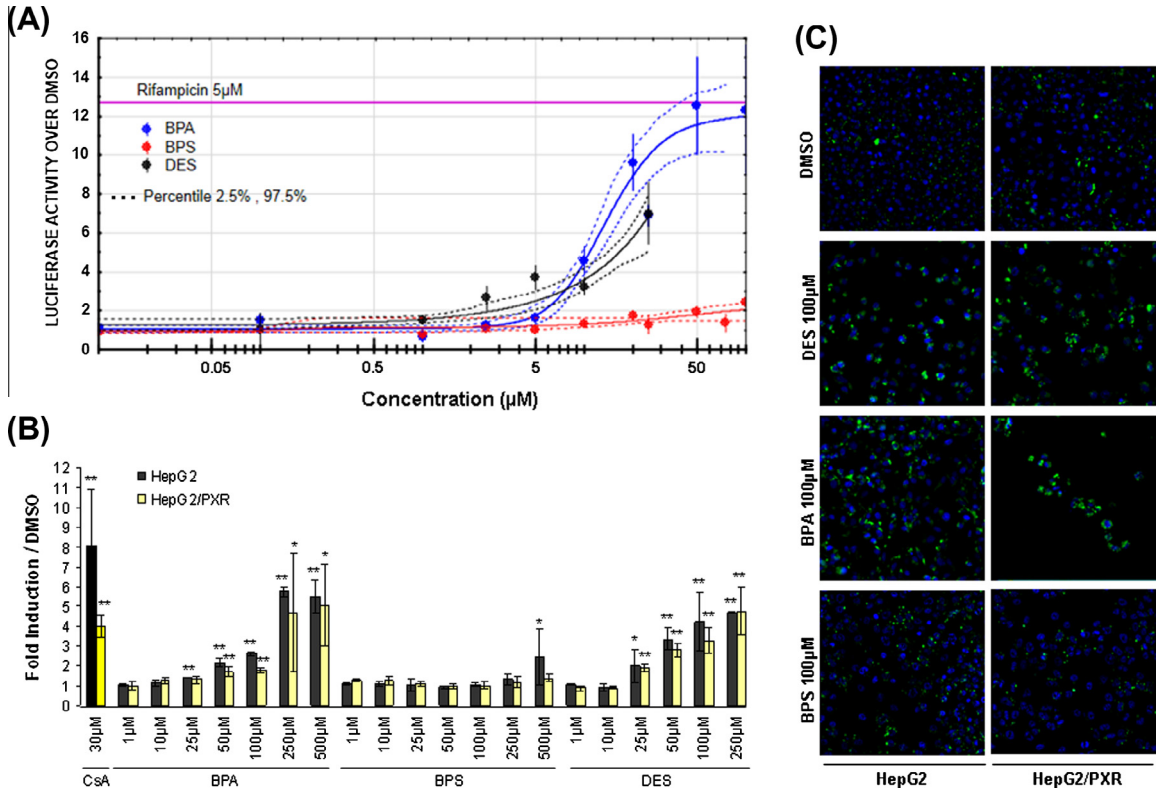

Fig. 3. PXR activation and lipid bioaccumulation in response to both bisphenols. (A) PXR agonist activity of bisphenol A (BPA), bisphenol S (BPS) and diethylstilbestrol (DES) in stable hPXR/HepG2 cells was performed. Cells were incubated for 24 h in the presence of each compound at the stated concentrations. The values represent the mean ± standard deviation of three separate experiments in duplicate. Curves were fitted after bootstrapping of the whole data set (four experiments in triplicate). The mean curves with corresponding 2.5 and 97.5 percentile confidence intervals are shown. (B and C) HepG2 and HepG2/PXR cells were seeded onto 96-well plates and treated for 72 h with increasing concentrations of BPA, BPS and DES. Cells were then stained with Hoechst 33342 and LipidTOX Neutral Lipid kit over 30–45 min. Intensity of the spots were detected with the ArrayScan^{XTI} and expressed as a ratio relative to the negative control DMSO. 30 µM of cyclosporine A (CSa) was used as a positive control for the lipid accumulation process. Error bars represent the standard deviation of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$.

a simultaneous high-throughput analysis. This innovative and multi-functional "cytomic" technology enables the assessment of live cells in response to chemical stress (proliferation, apoptosis, metabolism, activation of receptors and signaling pathways, etc.).

Our data highlighted the pro-steatotic effect of BPA (Fig. 3B and C) with a significant 1.4 to 2-fold increase in intracellular lipids at the non-toxic concentrations of 25 and 50 µM ($p < 0.01$). Regarding the potential cytotoxic doses (100–500 µM), we observed a 2 to 6-fold gain in the neutral lipid stain in the cell lines tested. These dose-dependent results were similar to those observed with the control DES which showed a similar induction, which however started at lower concentrations. BPS did not induce a dose-dependent increase in intracellular lipids, except for at the highest and most toxic concentration used (500 µM) in HepG2 cells.

Activation of the PXR affects various processes as described in the introduction, particularly lipid metabolism. Moreover, this receptor is also involved in the process of steatosis in the liver (Zhou et al., 2008). To investigate the role of PXR in the pro-steatotic effect of bisphenols, we hypothesized that the action of CsA, BPA and DES could be dependent on PXR induction. Indeed, as shown in Fig. 3B, we noticed that the average effects on HepG2 cells were more important than on HepG2/PXR cells. For example, CsA induced a 8-fold induction on HepG2 cells whereas a 4-fold induction was found on HepG2/PXR cells. We also found a significant difference between HepG2 and HepG2/PXR cells ($p < 0.05$) at 100 µM of BPA. However, because of the high variability observed, no other significant differences were found between the HepG2 and HepG2/PXR cell lines regarding the impact of bisphenols on lipid metabolism.

3.6. Modulation of metabolic and detoxification genes by BPA and BPS

We investigated the expression of different genes and/or proteins of interest in response to exposure with both bisphenols (Figs. 4 and 5). For this, we used two prototypical activators of the PXR and CAR, rifampicine (RIF) and phenobarbital (PB). Both significantly increased the expression of CYP3A4 and CYP2B6 (Fig. 4A and B), whereas RIF only modulated the expression of ABCB1 (Fig. 4D) and PB downregulated FASN (Fig. 5A). We then examined the impact of BPA and BPS and we found that 0.1 mM of BPA increased the expression of CYP3A4 by about 1.4-fold and in contrast decreased the expression of the CYP2B6 gene by 20%. No modulations were observed regarding GSTA4 gene however BPS seemed to modulate its protein expression at low doses (Fig. 4E). No changes were observed at those concentrations in ABCB1 gene expression coding for the MDR1 transporter (Fig. 4D). In an additional qPCR experiment, we also tested the gene expression of perilipin 2 (PLIN2), a protein associated with intracellular lipid storage droplet membranes, and FASN which catalyzes the synthesis of palmitate from acetyl-CoA and malonyl-CoA into long-chain saturated fatty acids (Fig. 5A and B). We found PLIN2 expression was unchanged neither by BPA nor by BPS treatments in our conditions whereas FASN was downregulated following treatment with 100 µM of BPA. Finally, activation of the ERK1/2 pathway was assessed through its phosphorylation (Fig. 4F). We found that 1 µM of BPA increased ERK1/2 phosphorylation whereas BPS slightly decreased its phosphorylation at 100 µM.

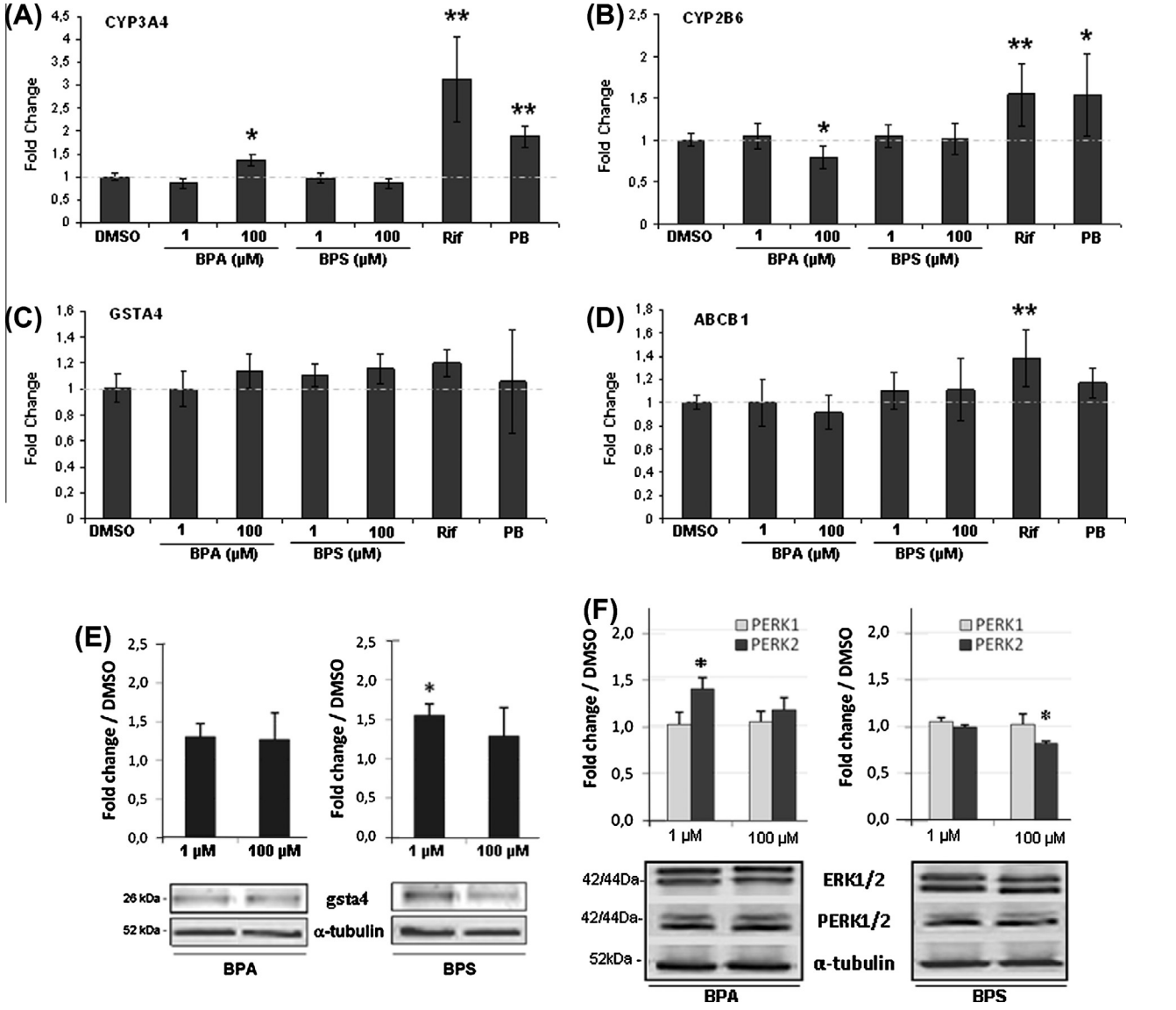

Fig. 4. Analysis of toxicity-, stress- and transport-related genes and protein expression. (A–D) Differentiated HepaRG cells were exposed for 48 h to 1 and 100 µM of BPA or BPS. Real-time quantitative PCR was used to quantify the relative mRNA levels of CYP3A4, CYP2B6, GSTA4 and ABCB1, respectively. Each experiment was performed in triplicate within three separate experiments. Linearity and efficiency were checked and β-actin was used as housekeeping gene to normalize. Results are presented as the mean fold change ± SD. Levels of probability are indicated as $^{*}p < 0.05$ or $^{**}p < 0.01$. (E and F) Differentiated HepaRG cells were treated for 48 h with 1 and 100 µM of BPA or BPS. Cells were lysed and levels of phosphorylated GSTA4, Erk1/2 and Erk1/2 proteins were analyzed by Western blotting. Band densitometry was performed and the results are defined as the ratio between treated cells versus DMSO-treated cells normalized by α-tubulin. Levels of probability are indicated as $^{*}p < 0.05$ or $^{**}p < 0.01$.

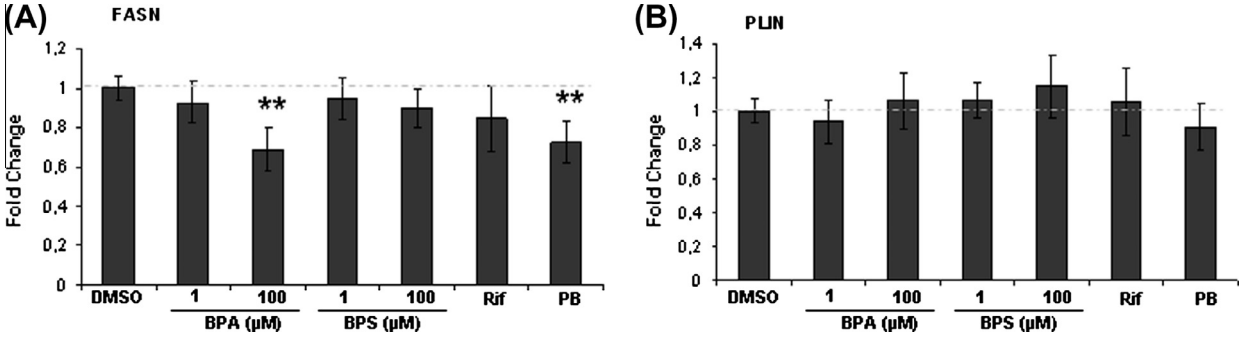

Fig. 5. Analysis of the expression of genes related to lipid metabolism. (A and B) Differentiated HepaRG cells were exposed for 48 h to 1 and 100 µM of BPA or BPS. Real-time quantitative PCR was used to quantify the relative mRNA levels of FASN and PLIN2. Each experiment was performed in triplicate within three separate experiments. Linearity and efficiency were checked and β-actin was used as housekeeping gene to normalize. Results are presented as the mean fold change ± SD. Levels of probability are indicated as $^{*}p < 0.05$ or $^{**}p < 0.01$.

4. Discussion

To date, numerous data associate exposure to BPA with various cellular and molecular dysfunctions that could subsequently lead to the development of pathologies as highlighted in the introduction. Its use was initially limited and it will be permanently banned in French food packaging in 2015. Faced with being unable to use BPA in industry, new candidates such as BPS are already used. In the presence of scarce toxicological data on this compound, there is a real need to investigate the impact of BPS on cell metabolism, detoxification capacities and homeostasis. The aim of this study was to compare these two bisphenols in different hepatic models and to identify a possible action associated with NAFLD which has already been partially described in response to BPA (Khalil et al., 2014).

In the study presented here, the cellular impedance data in response to acute exposure to BPA and BPS revealed two important features. Firstly, bisphenols have more toxic effects on growing cells (HepG2, HepG2/PXR cells) than on differentiated HepaRG cells. This first observation revealed that BPA and BPS could be processed by the detoxification pathway present in the HepaRG cells but is almost missing in the other two models (Andersson et al., 2012; Gerets et al., 2012). Their potential biotransformation (phases I, II and III) into inactive metabolites should make them less toxic. This idea is supported by a second observation which showed that BPA is more toxic than BPS and its toxicity is enhanced in HepG2 cells which over-express PXR (Table 1). This supports the role of the PXR in BPA bio-toxication (Kuzbari et al., 2013; Sui et al., 2012) via the formation of various CYP-dependent secondary derivatives such as hydroquinone (Nakamura et al., 2011) that could lead to further hepatotoxicity. However, this aspect related to BPA metabolites needs to be further explored.

We also showed that BPA and to a lesser extent BPS exerts chronic toxic effects on the HepaRG cell line. Their toxicity increases with the time of exposure, and appears to be maximal after 21 days for concentrations that were previously not considered to be hepatotoxic in acute conditions (i.e. 250 μM BPA or 100 μM DES). This underlines the importance of working with the HepaRG cell line for toxicological studies. Another consideration concerns the effects of DES on hepatic cells. Contrary to BPA and BPS, the positive control DES, which is known to be a PXR activator appeared to be more toxic to HepG2 cells than to HepG2/PXR cells at the earlier time of exposure e.g. at 24 h (79 ± 8 μM versus 166 ± 37 μM) yet had similar toxicity at 48 h and 72 h. This singular result can be explained by a short and transient effect observed which consists of an increase in the NCI of the HepG2/PXR cells in comparison with the NCI of cells treated with the control DMSO. This was observed at low doses ranging from 1 nM to 10 μM (data not shown) and could be associated with metabolic and developmental deregulation (Besteman et al., 2007; Swedenborg et al., 2012) leading to changes in the NCI. In this context, such a positive variation in the NCI could lead to the overestimation of IC50 at 24 h via the cellular impedance monitoring. That kind of effect was not observed after exposure to BPA and BPS.

We then focused on the PXR receptor and its possible involvement in the NAFLD process. BPA has already been described as a potential agonist for this receptor (Barrett, 2012; Kuzbari et al., 2013; Takeshita et al., 2001). We tried to characterize the transactivation potency of BPS towards the PXR in comparison with BPA and DES. The results demonstrate that BPA can be regarded as the most powerful PXR agonist among the three molecules tested whereas BPS failed to significantly activate this receptor, as already observed by Sui et al. (2012) thus indicating an important difference between BPA and BPS regarding the mechanism of pharmaco-toxicological action. In parallel, we investigated the role of both BPA and BPS on steatosis. The cell imaging data demonstrated a dose-dependent lipid accumulation in HepG2 and HepG2/PXR cells in response to BPA which seems consistent with previous findings both *in vitro* (Huc et al., 2012) and *in vivo* (Marmugi et al., 2012). This detection method did not allow us to highlight the pro-steatotic effects of BPA for values below 25 μM. Moreover, BPS did not induce the accumulation of neutral lipids in liver cells as it was observed after DES and BPA treatments. We also noted that the bioaccumulation observed is more important in HepG2 cells than in HepG2/PXR cells. Indeed, the mean value found was always higher in HepG2 cells, but the standard deviations do not allow us to conclude to a significant effect except for those observed with 100 μM of BPA ($p < 0.05$). We cannot definitively conclude that the PXR acts here on the steatotic process by limiting the bioaccumulation of lipids.

At this stage, we found differences in terms of the mechanism of action and toxicity between BPA and BPS. Whereas BPS had no effect on lipid accumulation and PXR activation, BPA was shown to be the most toxic and induced increases in intracellular neutral lipid storage, a process which may involve the PXR receptor and lead to the observed disruption of lipid homeostasis. Several lines of evidence allowed us to support this hypothesis. Indeed, only BPA was able to downregulate FASN gene expression, a PXR target which encodes for the fatty acid synthase involved in lipogenesis (Moreau et al., 2009). This modulation has already been observed in HepaRG cells following oleic-acid treatment (Anthérieu et al., 2011) and has been proposed to be a negative feedback regulator of lipid accumulation. Secondly, BPA positively modulated the expression of the CYP3A4 gene but negatively modulated the CYP2B6 gene. Whereas increases in CYP3A4 have previously been associated with BPA dependant-PXR activation (DeKeyser et al., 2011; Kuzbari et al., 2013), no data exist concerning the decrease in CYP2B6 expression. Different explanations have been proposed to correlate such a decrease with biological processes. Indeed, CYP2B6 has been shown to be downregulated during inflammation (Aitken et al., 2008; Chen et al., 2011; Pascussi et al., 2000), steatosis (Donato et al., 2006) and alterations of the signal transduction cascade GR-[PXR/CAR/RXR]-P450 (Dvorak et al., 2003). BPA could lead at least to this negative regulation in parallel with PXR activation.

The gene expression of PLIN2 (a gene encoding protein involved in the formation of droplets), ABCB1 which codes for MDR1 transporter and GSTA4 which encodes the glutathione s-transferase A4, remained unchanged after 48 h of exposure to both BPA and BPS in HepaRG cells. This supports recent data showing that BPA is not a substrate for the human MDR1 (Mazur et al., 2012). Moreover, accumulation of lipids is not necessarily associated with the formation of droplets in hepatocytes and hepatoblastoma cells (Donato et al., 2009; Moya et al., 2010). For GSTA4, modulation of its expression can be controlled by Nrf2 and is associated with the production of reactive oxygen species (ROS) leading to lipid peroxidation (Desmots et al., 2001; Lu et al., 2010) and also with liver regeneration (Desmots et al., 2002). In cultured hepatocytes, the tumor necrosis factor α (TNFα), interleukin-6 (IL-6) and epidermal growth factor (EGF) have been shown to be involved in the regulation of GSTA4. The authors propose that among the three factors, TNFα could act at a post-transcriptional level. As we only observed an increase in GSTA4 protein expression after BPS treatment, especially at low doses (nano- and pico-molar, data not shown), we hypothesize that BPS could act at the post-transcriptional level through an unidentified pathway.

Finally, differing modulation in ERK1/2 activation was observed in response to BPA and BPS. BPA appears to induce increases in MAPK phosphorylation, this gain being more important at lower doses (data not shown). These results on HepaRG cells are

consistent with those found on other human cell lines (Izumi et al., 2011; Li et al., 2012; Park et al., 2009; Xu et al., 2014). The weak decrease in ERK1/2 phosphorylation observed in response to high concentrations of BPS allows us to suspect its ability to disrupt the MAPK pathway which has already been described in a rat pituitary cell line (Viñas and Watson, 2013).

In conclusion, our study demonstrates that BPS is not able to disrupt the main hepatic functions *in vitro* and could not be associated with a potential lipogenic deregulation leading to NAFLD. However, even if the hepato-toxicity of BPS seems to be less important than its analogue the BPA, complementary studies are required to investigate its impacts at lower doses i.e. closer to concentrations that mimic human exposure.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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