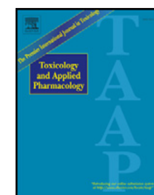
Toxicology and Applied Pharmacology 280 (2014) 224–235

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap



Is bisphenol S a safe substitute for bisphenol A in terms of metabolic function? An *in vitro* study

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ARTICLE INFO

Article history:
Received 5 May 2014
Revised 29 July 2014
Accepted 31 July 2014
Available online 8 August 2014

Keywords:
BPA
BPS
Obesity
Steatosis
Endocrine disruptor
Energy metabolism

ABSTRACT

As bisphenol A (BPA) has been shown to induce adverse effects on human health, especially through the activation of endocrine pathways, it is about to be withdrawn from the European market and replaced by analogues such as bisphenol S (BPS). However, toxicological data on BPS is scarce, and so it is necessary to evaluate the possible effects of this compound on human health. We compared the effect of BPA and BPS on obesity and hepatic steatosis processes using low doses in the same range as those found in the environment. Two *in vitro* models were used, the adipose cell line 3T3-L1 and HepG2 cells, representative of hepatic functions. We analyzed different parameters such as lipid and glucose uptakes, lipolysis, leptin production and the modulation of genes involved in lipid metabolism and energy balance. BPA and BPS induced an increase in the lipid content in the 3T3-L1 cell line and more moderately in the hepatic cells. We also observed a decrease in lipolysis after bisphenol treatment of adipocytes, but only BPS was involved in the increase in glucose uptake and leptin production. These latter effects could be linked to the modulation of SREBP-1c, PPARγ, aP2 and ERRα and γ genes after exposure to BPA, whereas BPS seems to target the PGC1α and the ERRγ genes. The findings suggest that both BPA and BPS could be involved in obesity and steatosis processes, but through two different metabolic pathways.
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Introduction

Bisphenol A (BPA) is a leachable monomer of polymerized polycarbonate plastics that has been extensively used. It is produced in a high volume worldwide and is used to manufacture polycarbonate plastics for food packaging and manufacturing products such as baby bottles, epoxy resins that line most food and beverage cans and dental sealants (Hashimoto and Nakamura, 2000). Thus, BPA is present in the environment as the result of direct release from manufacturing and/or processing facilities. This leads to a global contamination, with human exposure primarily from food and water (Kubwabo et al., 2009).

Human exposure to BPA has been implicated in the development of chronic diseases including obesity (Alonso-Magdalena et al., 2010), diabetes (Grun and Blumberg, 2007), atherosclerosis (Sui et al., 2012b), genital malformations (Gaspari et al., 2011), hepatic disturbances (Marmugi et al., 2012) and cancers (Keri et al., 2007). Moreover, BPA is a lipophilic compound that can accumulate in fat, with detectable levels found in 50% of breast adipose tissue samples from women (Fernandez et al., 2007). BPA has been proven to present biological effects at environmentally relevant concentrations (nanomolar range), exhibiting inverted U-shaped curves and non-monotonic effects, defined as a nonlinear relationship between the dose and the observed effects. These adverse effects have appeared using numerous endpoints including human and animal health, some behaviors and abnormal glucose/insulin homeostasis (Vandenberg et al., 2012, 2013). Consequently it is of considerable interest to examine the effects of BPA at a low concentration range likely to be present in foods and environmental or human samples. All these deleterious effects have led to the development of alternative and more heat-stable bisphenol compounds such as bisphenol S (BPS) (bis-(4-hydroxyphenyl)sulfone), where the two phenolic rings are joined together by a sulfur (Vinas et al., 2010; Liao et al., 2012a; Barrett, 2013; Vinas and Watson, 2013). However, few studies have been carried out on the toxicity of this substitute in terms of food safety, even though it is already largely used in polyethersufones, one of the materials available on the market to replace polycarbonate baby bottles. Indeed, it has already been found in canned soft drinks, canned foods and thermal receipt papers (Vinas et al., 2010; Gallart-Ayala et al., 2011; Liao et al., 2012b). It is worth noting that the production of BPS increases year by year. BPS (free and conjugated) has been detected in 81% of urine samples in American and Asian populations (Liao et al., 2012a; Rosenmai et al., 2014) and the mean daily dietary intakes of BPS (calculated from the mean concentration) were estimated at less than 2 ng/kg bw/day in the

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http://dx.doi.org/10.1016/j.taap.2014.07.025
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United States (Liao and Kannan, 2013). Moreover BPS had been shown to have a longer half-life and a better dermal penetration than BPA; thus this may lead to a longer or higher body burden or bioavailability of BPS *versus* BPA.

Obesity is one of the greatest public health problems to date, representing a major risk factor for serious metabolic diseases and a significant increase in the risk of premature death. It is expected that the resulting health costs will rise dramatically as the number of people suffering from obesity-related illness such as diabetes constantly increases from approximately 371 million individuals worldwide in 2012 to a staggering 552 million people by 2030 (Regnier and Sargis, 2014). It is directly associated with a number of health complications including diabetes, hypertension, heart disease and non-alcoholic fatty liver disease (NAFLD). Obesity arises from an imbalance in energy intake and energy expenditure that eventually leads to the pathological growth of adipocytes. Excess fat accumulated in this tissue results in elevated triglycerides in plasma and other tissues like liver and muscle, which leads to a pathological dysfunction of these tissues. Liver also plays a major role in the regulation of energy metabolism such as neoglucogenesis and lipid mobilization and storage. NAFLD arises from related disorders of energy metabolism of triglyceride (TG) uptake (depending on TG availability in the blood circulation), biosynthesis (de novo lipogenesis, carbohydrate oxidation of fatty acids) and secretion (involving specific transport proteins, VLDL) by the hepatocytes.

Accumulating evidence indicates that the human population is widely exposed to BPA even at low to very low doses and that this continuous exposure can be related to the increase in the obesity pandemic (Carwile and Michels, 2011; Rezg et al., 2014). The obesogenic effects have also been reported in rodents especially after perinatal exposure (Miyawaki et al., 2007; Somm et al., 2009; Vom Saal et al., 2012). In rodents, BPA has been reported to alter several metabolic functions (Sakurai et al., 2004; Masuno et al., 2005; Alonso-Magdalena et al., 2010) which can be related to obesity, type-2 diabetes and NAFLD (Marmugi et al., 2012). It has already been shown that BPA may interfere with cellular energy metabolism resulting in its dysregulation (Masuno et al., 2002, 2005; Sakurai et al., 2004), and to induce lipid accumulation and significant mitochondrial dysfunction such as hyperpolarization and ROS production (Huc et al., 2012). However no information is available on BPS neither on obesogenic effects nor on metabolic functions.

In the present study, we examined the effects of BPS, in comparison with its analogue BPA, on mouse adipocyte and liver cell lines, representative of cell types involved in obesity and NAFLD. The murine preadipose cell line 3T3-L1 that is able to differentiate into adipocytes represents a validated model for studying glucose uptake by fat tissue in response to insulin sensitizing compounds (Sakurai et al., 2004; Zhang et al., 2011; Zhu et al., 2011). We also used HepG2 cells because of their similarities to normal human hepatocytes in terms of physiological function, especially lipid and glucose metabolism (Zhang et al., 2011, 2012; Vidyashankar et al., 2013). In order to be relevant to the environmental exposure and to investigate the potential endocrine effects previously described at low doses with BPA, we used BPS in a range of concentration from femto- to micro-molar.

The objectives of the present study were to compare the *in vitro* effects of low doses of BPS and BPA, on lipid metabolism and storage, glucose uptake and endocrine properties. The effects of these molecules were also studied at the gene level by using RT-qPCR on the cellular mRNAs of treated cells.

Materials and methods

Materials

Bisphenol A (BPA), bisphenol S (BPS), diethylstilbestrol (DES), rosiglitazone (Rosi), cytochalasin B, isoproterenol, wortmannin, insulin, IBMX (3-isobutyl-1-methylxanthine), dexamethasone (DEX), and Dulbecco's modified Eagle's medium (DMEM) were all purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). 3T3-L1 cells were from Tebu-Bio and HepG2 were obtained from ATCC (American Type Culture Collection, Manassas, VA). [^{3}H]-2-deoxyglucose was from PerkinElmer (Boston and Waltham, USA). Dialysed and gold serums were from PAA laboratories (GE Healthcare Science, Vélizy-Villacoublay, France).

Methods. *Cell culture.* The BPA-containing medium was composed of Dulbecco's modified Eagle's medium (DMEM) without phenol red to avoid estrogen contamination. To investigate the effects of both bisphenols on the cellular lipid metabolism, cells were treated for 4 days for the HepG2 and 10 days for the 3T3-L1 cells. BPA and BPS were dissolved in ethanol at 100 mM and used at final concentrations ranging between 0.1 mM and 1 fM. 100 nM DES was used as a reference of estrogenic activity.

Mouse 3T3-L1. Preadipocytes were cultured in DMEM supplemented with 10% dialysed fetal calf serum (PAA) and 0.5% penicillin/streptomycin (Gibco). The cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere. 3T3-L1 preadipocytes were differentiated into adipocytes as previously described (Phrakonkham et al., 2008). The cells were seeded in 6-well plates at a density of 15×10^4 cells/well for RNA extractions, triglyceride (TG) content, lipolysis assays; 24-well plates at a density of 3.5×10^4 cells/well for glucose uptake assays and 3.5×10^3 cells/well in 96-well plates for the cytotoxicity assays. The cells were grown to confluence in a high glucose phenol-red-free DMEM with 10% serum. To induce differentiation, at 2-days post-confluent, the cells were treated with a hormonal cocktail of 0.5 mM IBMX (3-isobutyl-1-methylxanthine), 0.25 μM dexamethasone and 175 nM insulin (day 0 of differentiation) for 48 h. On day 2 (d2), the differentiation medium was replaced by phenol-red-free/10% dialysed serum DMEM containing 175 nM insulin for an additional 2 days. Treatments with different concentrations of bisphenols began at d2, and the culture medium was changed every 48 h with phenol-red free/10% dialysed serum DMEM and the bisphenols. The cells were maintained in the culture medium for an additional 8 days for analysis of lipid accumulation, gene expression, and glucose uptake.

Human HepG2 cell line. The human HepG2 cell line was obtained from ATCC (American Type Culture Collection, Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) 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 95% O_2 and 5% CO_2. After washing with sterile phosphate buffered saline (PBS), the cells were detached by trypsinization (0.05% trypsin/EDTA; Gibco) and plated at 250,000 cells/well in 6-well plates for TG and glucose uptake assays. The media were renewed every 2–3 days.

Determination of cellular toxicity. Following the required incubation period, the wells were gently rinsed with cold PBS, then 20 μL of 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for 4 h. Subsequently, the media from each well was then gently aspirated and 100 μL of dimethylsulfoxide (DMSO) was added to dissolve the formazan crystals. The plates were shaken for 30 min, and absorbance was measured at 570 nm using a Tecan microplate reader (Tecan, USA).

Protein assay. Cellular protein was determined with the Pierce, bicinchoninic acid enzymatic kit (Pierce, France) after cell lysis in 0.1 N NaOH.

Triglyceride assay. The amount of intracellular triglycerides was determined with the TG PAP 150 enzymatic kit (Bio-Merieux, Marcy l'Etoile,

France) after cell lysis (0.1 N NaOH). The TG content was related to protein content to take into account cellular proliferation or toxicity of the molecules. The results are expressed as the percentage of control cells treated only with insulin (50 nM).

Glucose uptake assay. Glucose uptake was measured according to Kim et al. (2010) with some modifications. Briefly, after 4 or 10 days of treatment, depending on the cell type, the cells were washed twice with serum-free DMEM and pre-incubated with this medium for 16 h at 37 °C. After a starvation period, the cells were washed twice with Krebs–Ringer–Bicarbonate (KRB) buffer and incubated further for 30 min at 37 °C with 100 nM insulin (or not, for the negative control). Rosiglitazone (Rosi; 10 nM) was used as positive control. To initiate glucose uptake, 2-deoxy-[1-^{3}H]-glucose (1 µCi/mL) diluted in 0.1 mM D-glucose solution was added to each well and incubated further for 10 min at 37 °C. After incubation, the cells were washed twice with ice-cold KRB buffer and solubilized with 0.1 N NaOH. Half of the content of each well was transferred into scintillation vials, and 10 mL of scintillation cocktail, Ultima Gold LLT, was added. The radioactivity incorporated into the cells was measured using a liquid scintillation counter (Hewlett Packard, USA). The protein content was assayed for each point on the remaining half.

Lipolysis assay. Lipolysis was evaluated after bisphenol treatment in the supernatant of cells used for the TG and protein assays. The enzymatic kit (Abcam AD133115) enables the amount of glycerol released into the medium to be measured, which is proportional to the degree of adipolysis. The cellular supernatant was transferred into a 96-well plate then incubated with the free glycerol assay reagent and incubated for 15 min at room temperature. The absorbance was then read at 540 nm using the Tecan microplate reader (Tecan, USA).

Leptin assay. Leptin was assayed after bisphenol treatment in the supernatant of cells used for TG and protein measurements. The ELISA assay (Mediagnost, Germany) was used according to manufacturer's instructions to detect mouse leptin produced by the cells. The cellular supernatant was transferred into a 96-well plate and then incubated 1 h with the antibodies at room temperature. The absorbance was then read at 450 nm using the Tecan microplate reader (Tecan, USA).

Cell imaging microplate assays and cellomics — ArrayScan^{XTI} scanning details. 3T3-L1 cells were seeded at 5×10^3 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, 3T3-L1 cells were treated for 10 days with different concentrations of bisphenols (1 fM to 1 µM). Then the stock concentration of LipidTOX™ contained in DMSO was diluted 400-fold for the 3T3-L1 cells staining 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 concentration) at 37 °C. The medium was changed and the plates were scanned with the ArrayScan^{XTI} instrument (Cellomics Inc., Pittsburgh, USA). The detection of accumulated lipids was carried out with the "compartmental analysis" bio-application (Cellomics Inc., Pittsburgh, USA). The number of cells analyzed was fixed at 500 cells per well. For each plate, the mean of the three control wells (0.1% EtOH) was used as a reference and rescaled to one. Each well value was expressed relative to this reference. A value of two means a 2-fold induction of the fluorescent signal compared with the control value. Rosiglitazone (2 µM) was used as supplementary positive controls in this experiment.

Q-PCR. The 3T3-L1 cells (n = 3 per sample, two independent experiments) were treated as indicated in the Material and methods section. Only the two most effective concentrations were tested: 1 nM and 1 pM. Total RNA was extracted with TRIzol reagent (Invitrogen, Cergy Pontoise, France). Total RNA samples (2 µg) were reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Courtaboeuf, France). Primers for SYBR Green assays are shown in Supporting Table 2. Amplifications were performed on a FX96 Real Time PCR System (BioRad). qPCR data were normalized to TATA-box binding protein (TBP) messenger RNA (mRNA) levels.

Statistical analysis. All data were expressed as the mean ± the standard error of the mean (SEM) of three (or more) independent experiments. Statistical significance was determined by one-way analysis of variance (ANOVA), followed by a Newman–Keuls post hoc testing, using GraphPad Prism software.* = $p < 0.05$ was considered to be statistically significant, ** = $p < 0.01$. In the case of the cell imaging assays, 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 **$p < 0.01$.

Results

The concentrations used were non-cytotoxic

In our experimental conditions, the MTT test showed that neither BPA nor BPS was cytotoxic whatever the cell line (Table 1). Only the highest concentration (0.1 mM) showed a decrease in viability (60% viability for BPA in adipocytes, 77% for BPS in hepatocytes). Throughout the study the concentration range chosen was between 1 µM and 1 fM which fits with environmental concentrations. HepG2 cells were treated for 4 days whereas 3T3-L1 cells were treated for 10 days after the differentiation process, to be relevant to all following experiments.

BPA and BPS increase the triglyceride and neutral lipid content in adipocytes

Firstly, the triglyceride (TG) content was evaluated in 3T3-L1 cells after treatments with increasing concentrations of either BPA or BPS (Fig. 1A). Data showed that BPA and BPS induced a significant increase in intracellular triglycerides (around 10%, Fig. 1A). The DES treatment (10 nM) also increased intracellular triglycerides in the same order of magnitude. These results confirm that low doses of bisphenol A promote accumulation of intracellular triglycerides in developing fat cells. Bisphenol S had the same properties at the same concentrations as BPA.

LipidTOX staining (Figs. 1B and C), was carried out to complete the first observations and to examine the effects of bisphenols on neutral lipid accumulation in adipocytes. BPA increased the intracellular lipids whatever the concentrations (around 1.35 fold increase). BPS was also active but only at 1 nM and 1 µM. These increases (around 1.4-fold) were significant with respect to the negative EtOH control and the undifferentiated condition, with 50% less lipids detected in adipocytes for the latter. As shown in Fig. 1C, the neutral lipids were grouped into droplets in the cytoplasm (green staining), and the number of cells with a high staining was greater after DES (1 µM), rosiglitazone (1 and

Table 1
Cytotoxicity of BPA and BPS.
Cytotoxic effects of BPA or BPS were investigated in 3T3-L1 and HepG2 cells. Results are expressed as percentage of non-treated control cells.

	3T3-L1		HepG2	
	BPA	BPS	BPA	BPS
Control	100	100	100	100
10^{-12} M	112.81 ± 6.25	118.75 ± 1.40	88.84 ± 4.61	91.94 ± 3.25
10^{-9} M	99.75 ± 4.16	107.01 ± 3.69	95.16 ± 6.72	83.98 ± 4.74
10^{-6} M	102.30 ± 5.47	103.50 ± 4.80	87.83 ± 3.98	90.38 ± 7.25
10^{-4} M	60.38 ± 2.04	81.33 ± 3.91	78.89 ± 5.78	77.41 ± 1.07

Table 2
Primers for SYBR Green assays.

Genes	Primers
SREBP1c-F	GGAGCCATGGATTGCACATT
SREBP1c-R	GCTTCCAGAGAGGAGGCCAG
Pparγ2-F	ATGGGTGAAACTCTGGGAGATTCT
Pparγ2-R	CTTGGAGCTTCAGGTCATATTTGTA
aP2-F	TTCGATGAAATCACCGCAGA
aP2-R	GGTCGACTTTCCATCCCACTT
PGC1α-F	GGCACGCAGCCCTATTCAT
PGC1α-R	CACGGAGAGTTAAAGGAAGAGCAA
ERRα-F	GGAGTACGTCCTGCTGAAAGCT
ERRα-R	CACAGCCTCAGCATCTTCAATG
ERRγ-F	GACCCTACTGTCCCCGACAGT
ERRγ-R	AACTCTCGGTCAGCCAAGTCA
LPL-F	TTATCCCAATGGAGGCACTTTC
LPL-R	CACGTCTCCGAGTCCTCTCTCT
LHS-F	GGCTTACTGGGCACAGATACCT
LHS-R	CTGAAGGCTCTGAGTTGCTCAA
FASN-F	ATCCTGGAACGAGAACACGATCT
FASN-R	AGAGACGTGTCACTCCTGGACTT
GPR30-F	CGGCACAGATCAGGACACCC
GPR30-R	TGGGTGCATGGCAGAAATGA
PGC1β-F	CATCTGGGAAAAGCAAGTACGA
PGC1β-R	CCTCGAAGGTTAAGGCTGATATCA

2 μM) and bisphenol treatments than in the ETOH condition. Our data showed that both BPA and BPS induced an accumulation of lipids in differentiated 3T3-L1 cells.

BPA and BPS induced a decrease in lipolysis

The amount of TG stored in adipocytes depends both on the cellular capacities of storage and release of TG. The release is due to lipolysis and can be evaluated by glycerol release into the cellular medium. As shown in Fig. 2, compared to insulin alone (0), both bisphenols significantly reduced lipolysis in 3T3-L1 cells treated with low concentrations of BPA (non-significant for 1 μM) or BPS in the presence of 50 nM of insulin. The effect was much greater when cells were treated with BPS (around of 65% of control instead of 80% with BPA) and this effect was always more pronounced at concentrations under 1 μM. DES treatment did not show any significant effect.

BPS but not BPA increased glucose uptake. Glucose uptake was evaluated using deoxyglucose, a non-metabolizable glucose analogue, combined with a tritium radiolabeled deoxyglucose tracer. Cytochalasin B was used to block cell glucose uptake, and DES was used as the reference for estrogenic activity. Rosiglitazone, a thiazolidinedione, which is a PPARγ ligand was used to increase insulin sensitivity, and to promote the storage of glucose in adipocytes. As expected, glucose uptake in the presence of the inhibitor cytochalasin B was equivalent to cellular uptake of glucose in undifferentiated cells (18% of control *vs* 28% for non-differentiated cells). Moreover, rosiglitazone induced a major increase in glucose uptake (182% of the control) (Fig. 3C). Data showed that neither BPA nor DES had a significant effect on glucose uptake (Figs. 3A, C). On the contrary, BPS significantly increased glucose uptake in the 3T3-L1 cells (up to +130%) (Fig. 3B). This effect was dose dependent with a major effect at lower doses.

Only BPS increased adipocyte leptin production

In order to understand the cellular effects of bisphenols, leptin secretion was evaluated in 3T3-L1 cells treated with a range of increasing concentrations of either molecule, using isoproterenol as a positive control. The results showed (Figs. 4A and B) that BPS induced a high release of leptin by the cells (about 200% of control cells) with a level

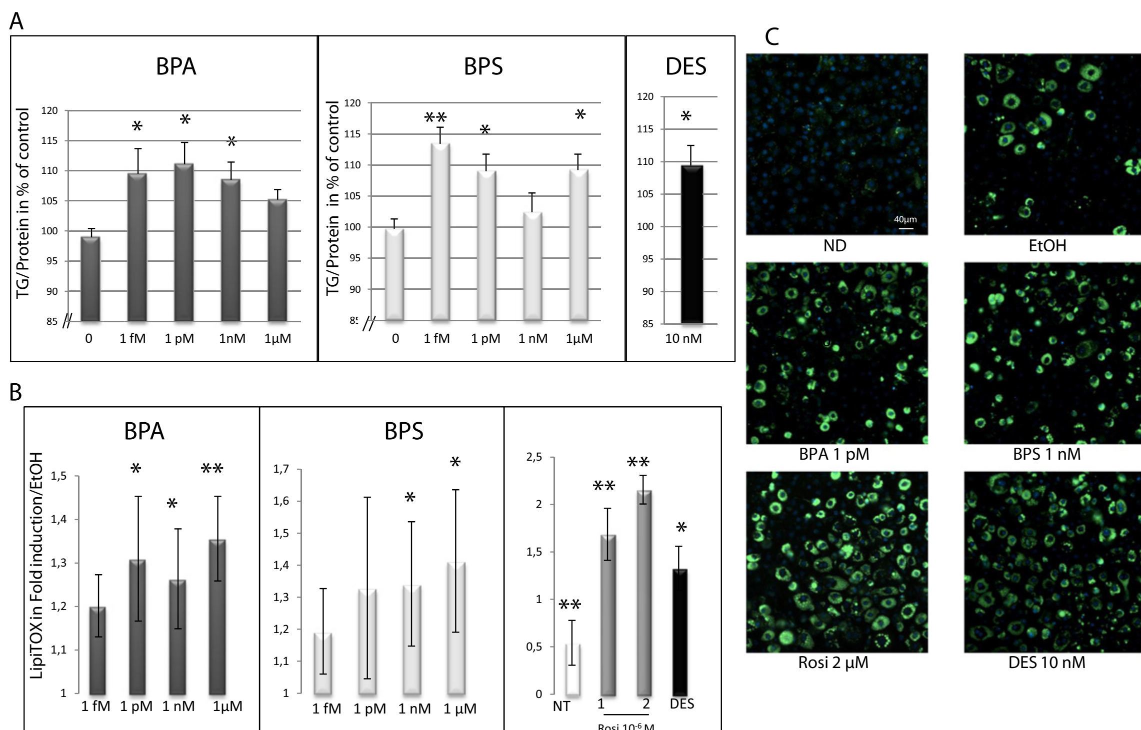

Fig. 1. Effects of BPA and BPS on triglyceride and lipid content in 3T3-L1 adipocytes. A, Cells were seeded in 6 well-plates and treated by indicated concentrations of BPA or BPS or for 10 days after the induction of differentiation. Triglyceride content, normalized to the protein content is expressed as the percentage of control cells treated only with insulin 50 nM (0). Data represent 3 different wells and 3 independent experiments. Results shown are mean ± SEM. *p < 0.05; **p < 0.01. B and C, Cells were seeded onto 96-well plates, treated with indicated concentrations of BPA or BPS for 10 days after induction of differentiation. They were stained with Hoechst 33342 (blue) and with the LipidTOX Neutral Lipid kit (green) for 30 min. Spot intensity was detected using the ArrayScan^{XTI} ThermoScientific and expressed as a ratio relative to the negative control EtOH. Rosiglitazone (Rosi) at 1 and 2 μM and 10 nM of diethylstilbestrol (DES) were used as positive controls for the lipid accumulation. The undifferentiated condition (ND) was used as supplementary control. Error bars represent the standard deviation of three independent experiments. *p < 0.05, **p < 0.01.

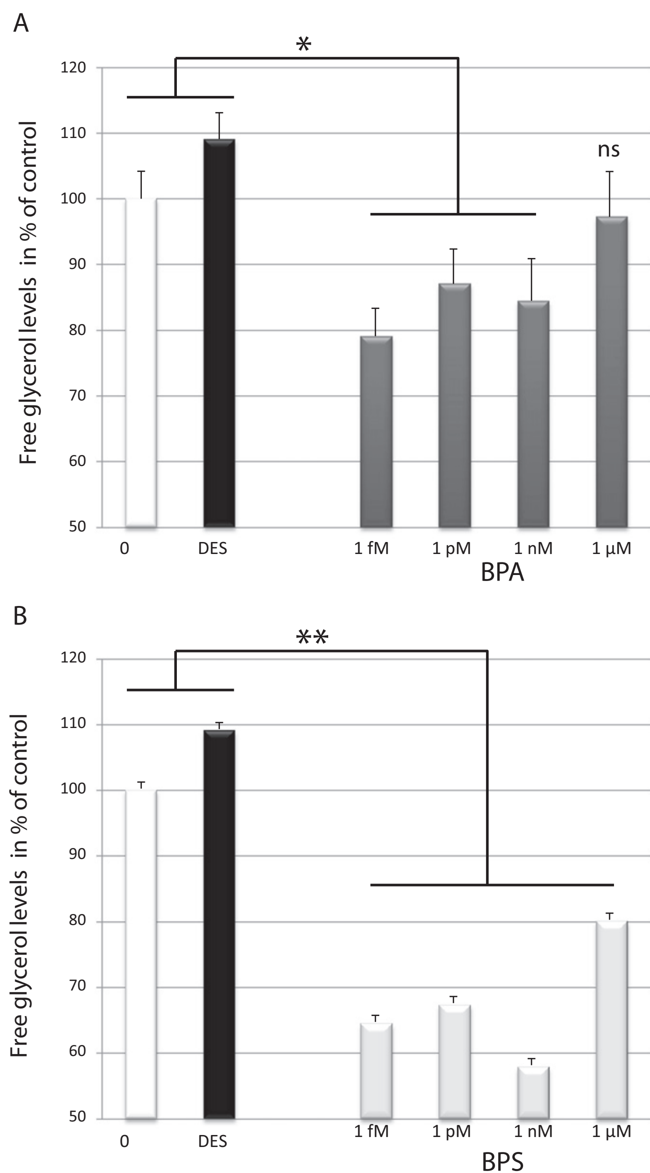

Fig. 2. Effects of BPA and BPS on 3T3-L1 adipocyte lipolysis. Cells were seeded in 6 well-plates and treated by indicated concentration of BPA (A) or BPS (B) or for 10 days after the induction of differentiation. Lipolysis was evaluated as free glycerol released into the culture medium. Results shown are expressed as the percentage of control cells treated only with 50 nM insulin (0) and are mean ± SEM. $^*p < 0.05$; $^{**}p < 0.01$, ns = not significantly different from control.

equivalent to that of isoproterenol (206%). The increase in leptin secretion produced by BPA was less and thus not significantly different from the control (Fig. 4A). DES significantly decreased leptin production in our experimental conditions.

The effect of bisphenols on adipocyte transcription factors

In order to gain insight into the transcriptional mechanisms that could contribute to the effects of bisphenols on adipocyte gene expression, we measured the expression of different transcription factors involved in the regulation of adipocyte differentiation. We chose to test early differentiation genes (SREBP-1c, PPARγ2 and aP2 (adipocyte lipid binding protein) and genes involved later in the differentiation (LPL (lipoprotein lipase), HSL (hormone-sensitive lipase) and FAS (fatty acid synthase)). BPA was able to induce a slight but significant increase in the transcription level of early genes (Fig. 5, left panel), but had no effect on LPL, LHS, and FAS (Complementary figure, left panel). Interestingly, this significant increase in the expression of these genes was observed only at 1 nM, without a dose-effect. In contrast, BPS did not show any modification of transcript levels whatever the genes (Fig. 5 and Complementary figure, right panel). BPA was shown to affect the GPR30 estrogenic pathway in endocrine responsive tissues and cells, so we also tested if bisphenols could affect the GPR30 transcripts. Under our conditions neither BPA nor BPS changed this gene expression (Complementary figure), suggesting that the GPR30 pathway was not implicated in the effects of bisphenols on 3T3-L1 differentiation.

Bisphenols' implication in energy balance

We also examined the implication of bisphenols in energy balance through the modification of PGC-1α, PGC-1β, ERRα and γ gene expression (Fig. 6). The PPARγ coactivator α (PGC-1α) gene expression was up-regulated by BPS (significantly at 1 pM) but BPA had no effect. Contrariwise PGC-1β expression was not modified (Complementary figure). The two estrogen-related receptors ERRα and γ, known to be involved in energy balance and to bind BPA were slightly but significantly enhanced by the two concentrations of BPA tested (Fig. 6). BPS had no effect on ERRα and while BPA enhanced the expression of ERRγ, BPS had the opposite effect.

Effects observed in a human hepatoma cell line

To obtain complementary data in terms of lipid homeostasis deregulation in response to bisphenol exposure, we used HepG2 cells. We evaluated triglyceride storage in hepatic cells exposed to a range of concentrations of bisphenol A and S. Both molecules increased the storage of triglycerides in the hepatocytes: to a small extent with BPS and only at 1 pM for BPA (Fig. 7A). Glucose uptake in HepG2 cells treated with different concentrations of both bisphenols produced the same effects as with adipocytes, *i.e.* no effect of BPA and a considerable increase in glucose uptake induced by low BPS concentrations (+145%) (Fig. 7B).

Discussion

A growing number of studies report adverse effects of bisphenol A involved in many chronic diseases such as metabolic syndrome and hormone-dependent cancers. Therefore the increasing concern of government agencies and environmental security groups has led to the development of some potential substitutes for BPA, such as BPS. BPS leaching from plastic containers had been shown to occur to a lesser extent; nevertheless it was present in small amounts in the diet (Vinas et al., 2010) and resulted in a very low concentration (less than 2 ng/kg bw/day) in the American population (Liao and Kannan, 2013). However, even though there is very little data on the effects of low doses of BPS, it is already widely used to replace BPA. This study was designed to analyze the effects of low doses of BPS on cellular metabolism in two models of adipocytes and hepatocytes, in comparison with the effects of BPA in the same range of concentrations. In this study we have shown that BPS has many properties in common with BPA, although the activation pathways of energy metabolism are different. BPS appears to have less deleterious properties compared to BPA as regards insulin resistance.

Bisphenol A has been implicated in the development of metabolic syndrome at low and very low doses compatible with environmental concentrations (Hugo et al., 2008; Ben-Jonathan et al., 2009; Marmugi et al., 2012; Cabaton et al., 2013; Boucher et al., 2014). As a first step, we investigated the intracellular levels of triglycerides in adipocytes treated with low doses of BPA during their differentiation (ten days after induction of the differentiation process) and clearly showed that at very low doses (femtomolar), BPA induces an increase in cellular triglycerides. The same results were observed with BPS. It had already been reported that BPA induced TG storage in adipose cells, but at

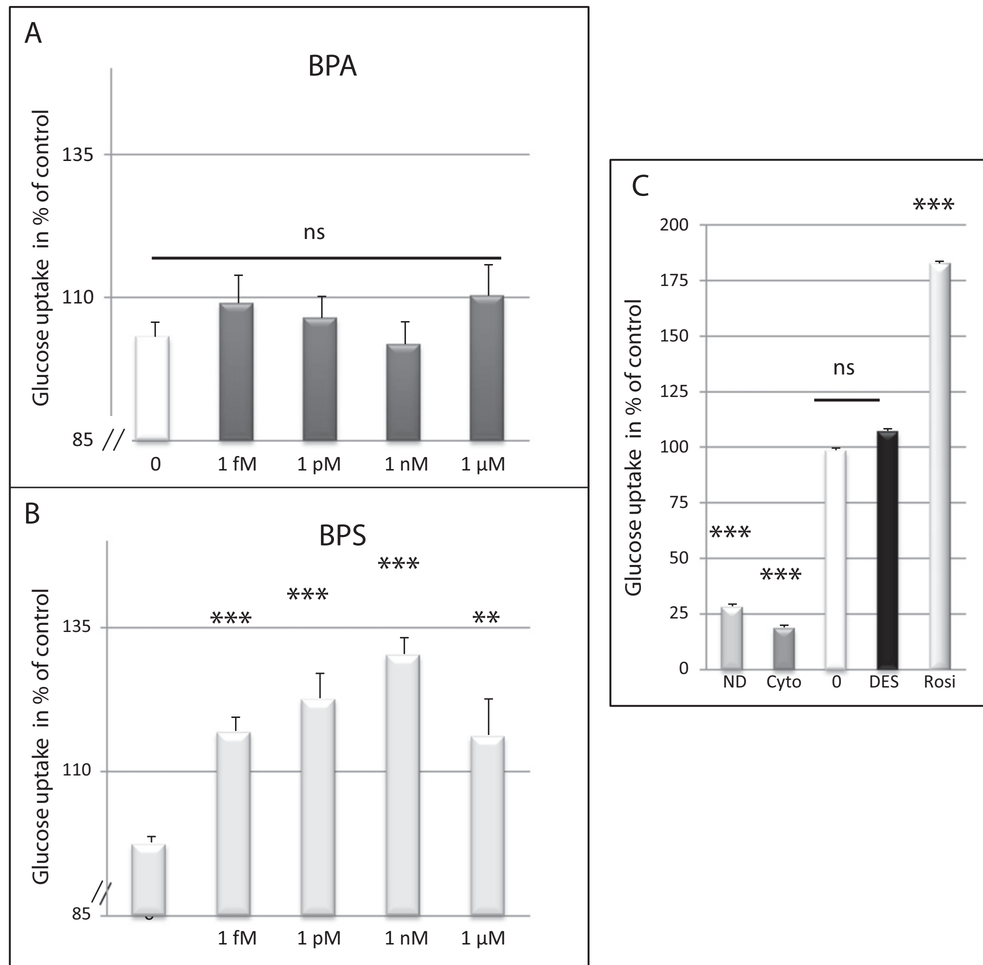

Fig. 3. Effects of BPA and BPS on glucose uptake in 3T3-L1 adipocytes. Cells were seeded in 6 well-plates and treated with the indicated concentrations of BPA (A) or BPS (B) or positive (DES, Rosi) and negative (NT, Cyto) controls (C) or for 10 days after the induction of differentiation. 2-deoxy-[1-^{3}H]-glucose was added to each well and incubated for 10 min. Glucose uptake was evaluated as the radioactivity incorporated into the cells, normalized to the protein content. Results are expressed as the percentage of control cells treated only with insulin 50 nM. Cytochalasin B (Cyto, 10 μM) and nondifferentiated cells (ND) were used as negative controls, DES (10 nM) and rosiglitazone (10 μM) as positive controls. Results shown are expressed as the percentage of control cells treated only with 50 nM insulin (0) and are mean ± SEM. *p < 0.05; **p < 0.01, ***p < 0.001, ns, not significantly different from control.

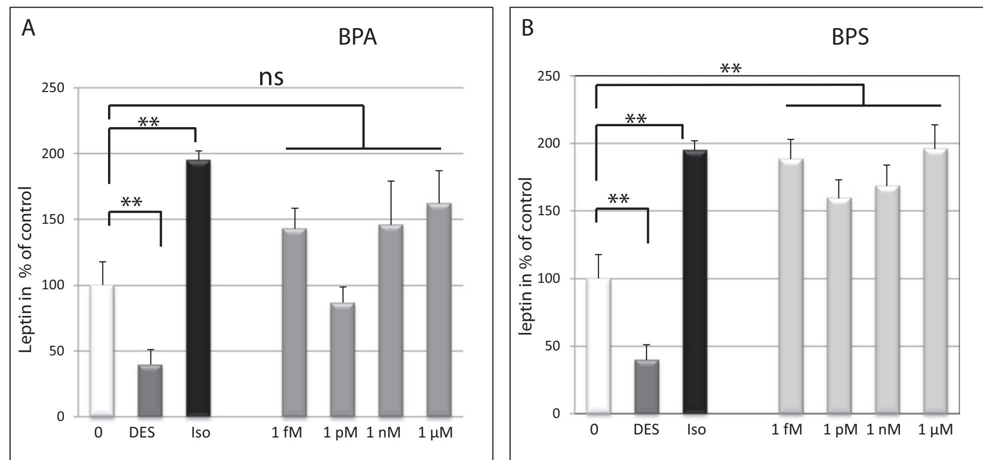

Fig. 4. Effects of BPA and BPS on leptin production from 3T3-L1 adipocytes. Cells were seeded in 6 well-plates and treated with the indicated concentrations of BPA (A) or BPS (B) or for 10 days after the induction of differentiation. The supernatants were transferred into a 96-well plate then incubated with the antibodies at room temperature. DES (10 nM) and isoproterenol (Iso, 10 μM) were used as positive controls. Results shown are expressed as the percentage of control cells treated only by 50 nM insulin (0) and are mean ± SEM. *p < 0.05; **p < 0.01, ns, not significantly different from control.

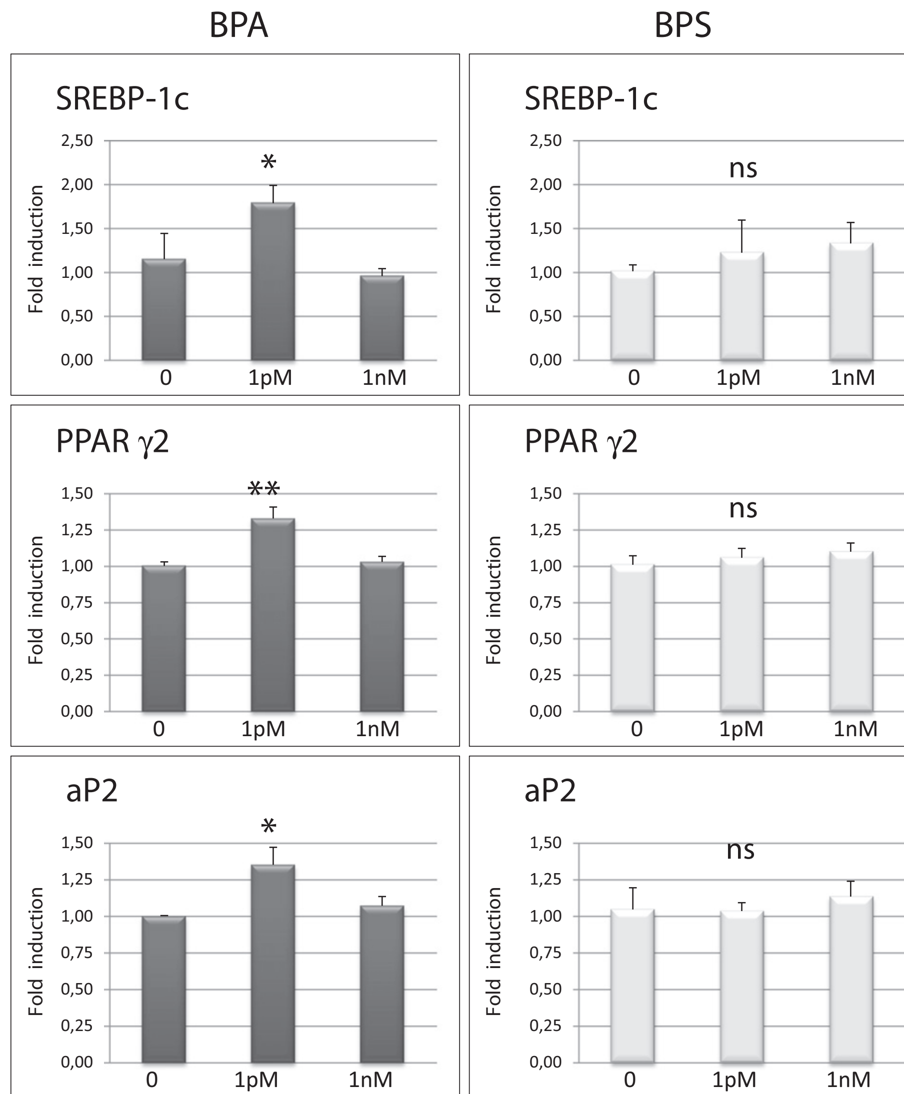

Fig. 5. Effects of bisphenols on gene expression of transcription factors in 3T3-L1 adipocytes. Relative mRNA levels (SREBP-1c; PPARγ2; aP2) from cells treated with indicated BPA (left panel) or BPS (right panel) concentrations. Results are means ± SEM and expressed as fold induction of control cells (0) treated only by 50 nM insulin. *$p < 0.05$; **$p < 0.01$; ns, not significantly different from control.

higher concentrations (Masuno et al., 2002, 2005). To our knowledge, this is the first time it is reported that BPS at low doses, in concentrations compatible with those found in food contamination, produces the same increase in triglycerides in adipocytes.

White adipose tissue (WAT) stores excess energy as triacylglycerols in the lipid droplet of adipocytes. When energy is needed between meals or during physical exercise, WAT delivers fatty acids (FAs) to be oxidized in peripheral tissues. Lipolysis is the process by which stored TGs are released as non-esterified fatty acids whereas lipogenesis results in triglyceride storage by WAT. In this study we wondered whether treatment with bisphenols could lead to an imbalance of these two processes leading to an excess storage of TG in fat cells. We showed that lipolysis was diminished by both bisphenols tested, with a greater decrease with BPS (−35% *vs* −20% for BPA). The higher concentrations of BPA and BPS gave a less significant decrease in lipolysis which could be attributed to non-monotonic effects of the molecules. Lower lipolysis could be related to a higher TG storage in adipose cells, which was observed in this study; a diminution in WAT lipolysis could favor the development of obesity, through the storage of TG in adipocytes. However, it could also be postulated (Girousse et al., 2013), that limiting fatty acid release and inducing *de novo* lipogenesis, may contribute to improve insulin sensitivity and improve adipocyte insulin-stimulated glucose uptake. Thus, this effect on lipolysis has to be related to other metabolic cellular processes in adipocytes and other insulin-sensitive tissues. Insulin-stimulated glucose uptake, which was defined as insulin sensitivity, is considered to be the gold standard for evaluating insulin resistance at the cellular level. Cells with a significant reduction in insulin-stimulated glucose uptake have commonly been accepted to be in an insulin-resistant state (Goldstein, 2002). In order to understand the effects of bisphenols on insulin resistance linked to obesity, we measured glucose uptake in cells treated with low concentrations of BPA or BPS. Very interestingly, while we did not observe any effect with BPA, BPS induced a significant increase in the uptake of glucose, specifically at low doses. Sakurai et al. (2004) previously showed that BPA was able to induce a very low increase in glucose transport. They used a different mouse adipose cell line (3T3-F442A) and the concentrations tested for BPA corresponded to high concentrations that were cytotoxic in our conditions. BPS induced a lower increase in glucose uptake but in the same order of magnitude as rosiglitazone used as a positive control, suggesting that BPS could potentiate insulin sensitivity in adipose cells. Increasing glucose uptake in adipocytes could contribute to lower plasma glucose levels. One of

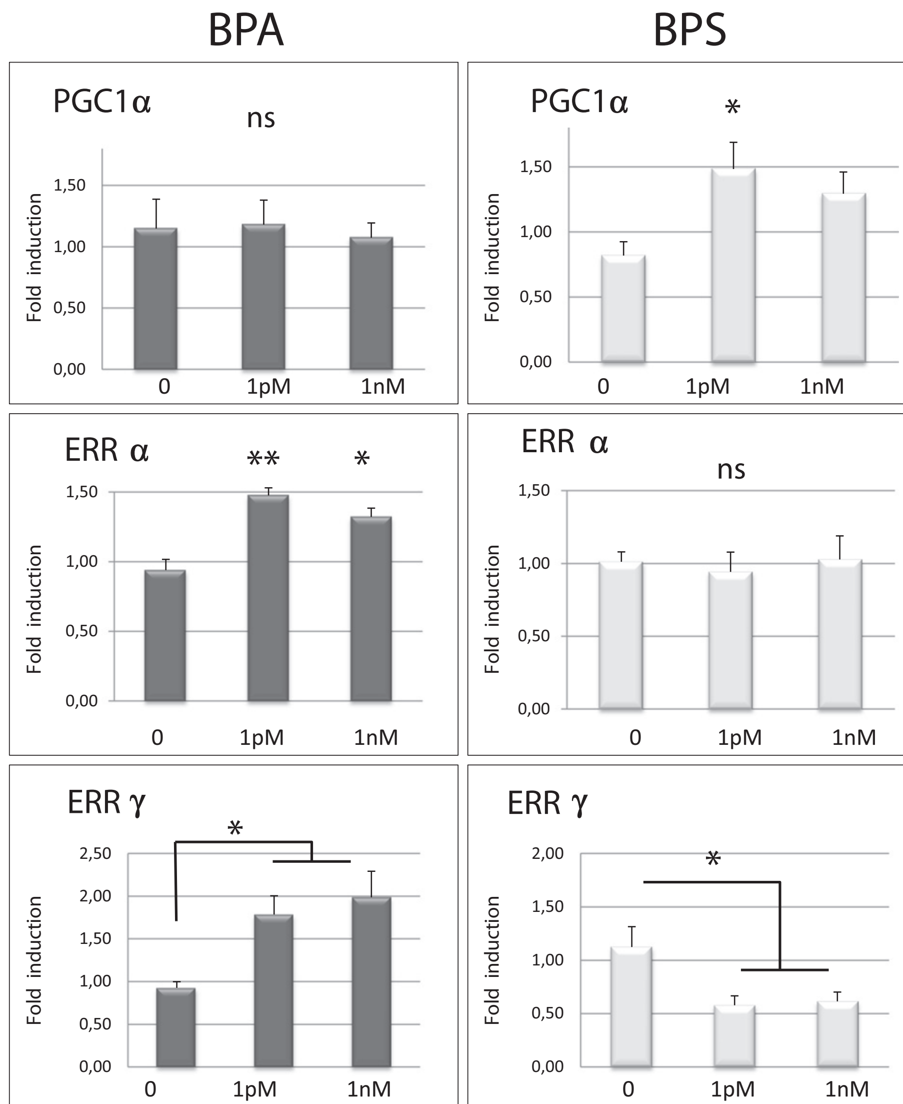

Fig. 6. Effects of bisphenols on energy metabolism gene expression in 3T3-L1 adipocyte. Relative mRNA levels (PGC1α; ERRα; ERRγ) from cells treated with indicated concentrations of BPA (left panel) or BPS (right panel). Results are means ± SEM and expressed as fold induction compared with control cells (0) treated only by 50 nM insulin. *p < 0.05; **p < 0.01; ns, not significantly different from control.

the strategies considered to manage diabetes is to stimulate glucose uptake by the liver, skeletal muscles or adipocytes, inducing improved insulin sensitivity and reduced insulin resistance (Girousse et al., 2013). Many studies reporting different effects on glucose uptake and on the cellular effects of BPA are linked to the insulin receptor signaling (IRS) pathways (Sakurai et al., 2004; Zhang et al., 2011, 2012; Song et al., 2013). It could be of interest to analyze these pathways in the adipocytes and to analyze changes in GLUT4 transporters in response to bisphenols.

Leptin is a secreted peptide produced primarily by white adipose tissue. It plays a vital role in controlling body weight. Leptin serves as a major 'adipostat' by repressing food intake and promoting energy expenditure (Barr et al., 1997). Circulating concentrations of leptin increase in proportion to the size of white fat depots. In rodents, Miyawaki et al. showed a possible positive correlation between serum leptin levels and adipose tissue mass (Miyawaki et al., 2007). Increases in leptin expression and secretion from adipocytes have been linked to insulin stimulation (Barr et al., 1997), cell glucose uptake (Mueller et al., 1998) and the availability of energy substrates (Cammisotto et al., 2005), all of which are indicative of an anabolic state. In addition to its well-described role in energy balance, leptin has notable effects on glucose homeostasis, as it reverses hyperglycemia in ob/ob mice before body weight is corrected (Rosen and Spiegelman, 2006). In our study we checked the effects of bisphenols on leptin secretion by the adipocytes. Our results showed that BPS but not BPA induced significantly leptin secretion from adipocytes, and whatever the concentration tested, the increase induced by BPA is present but is weaker and thus not significant. In the same cell line but at the preadipocytes stage, Phrakonkham et al. had shown that BPA treatment augmented leptin gene expression (Phrakonkham et al., 2008). However the concentration used (80 μM) was much higher than that used in our study and no information was provided regarding leptin at the protein level. In their study, Mueller et al. (1998) reported that the secretion of leptin was directly proportional to the amount of glucose taken up by the adipocytes. They concluded that glucose transport and metabolism are important factors in the regulation of leptin expression and secretion and that the effect of insulin to increase adipocyte glucose utilization is likely to contribute to insulin-stimulated leptin secretion. For Perez-Matute et al., leptin production by adipocytes is linked to glucose transport and glucose metabolism (Perez-Matute et al., 2005). In our experiments, only BPS induced a significant increase in adipocyte glucose uptake as well as an increase in leptin secretion. BPS could then be implicated in glucose metabolism regulation. Leptin secretion is currently known to induce lipolysis (Perez et al., 2004; Kim et al.,

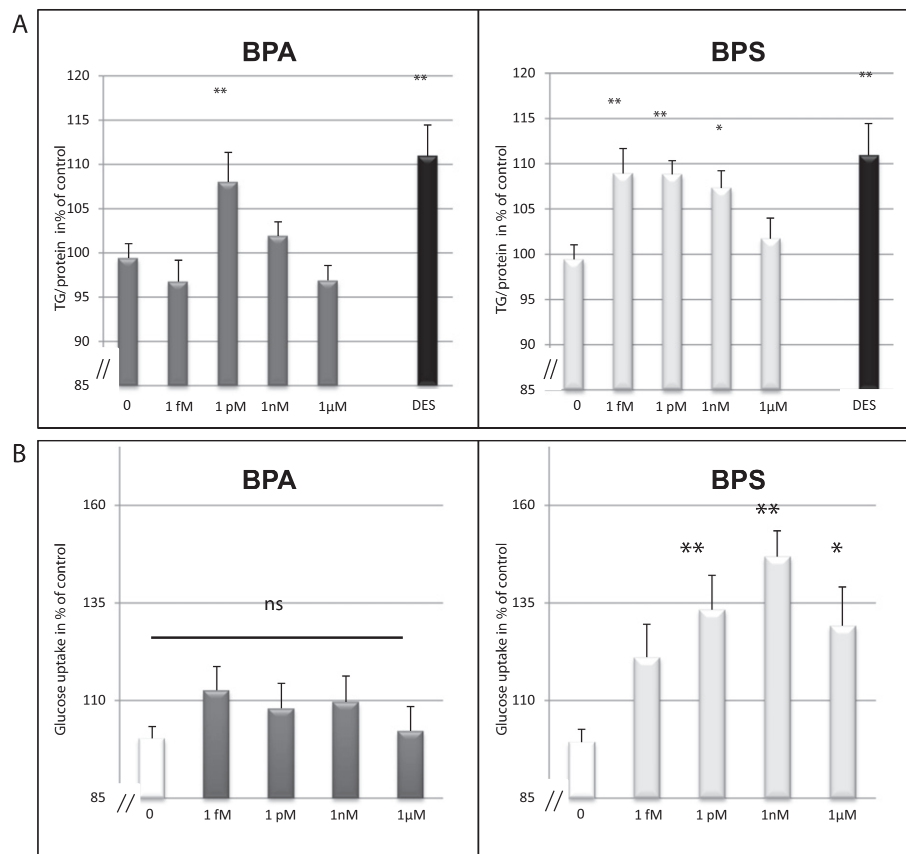

Fig. 7. Effects of bisphenols on HepG2 cells. A: Effects of BPA and BPS on triglyceride content. Cells were seeded in 6 well-plates and treated with the indicated concentrations of BPA or BPS or for 4 days. Triglyceride content, normalized to the protein content is expressed as the percentage of control cells (0). DES (10 nM) was used as a positive control. Data represent 3 different wells and 3 independent experiments. Results shown are mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$. B: Effects of BPA and BPS on glucose uptake. Cells were seeded in 6 well-plates and treated with the indicated concentrations of BPA or BPS. 2-deoxy-[1-^{3}H]-glucose was added to each well and incubated for 10 min. Glucose uptake was evaluated as the radioactivity incorporated into the cells, normalized to the protein content. Results shown are expressed as the percentage of control cells treated only by 50 nM insulin (0) and are mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$.

2008; Harris, 2014) however, the correlation between leptin production and lipolysis was not found in our experiments. In their work, Kim et al. reported that leptin added to the culture medium enhanced glycerol release (50–500 ng/mL) but had no effect at lower concentration (5 ng/mL) (Kim et al., 2008). Thus the concentration of leptin released from cells treated by BPS in our conditions could be below the effective concentration. Furthermore, our results resulted from *in vitro* data and may not take into account the *in vivo* regulations.

Although BPA and BPS have equivalent properties and related molecular formulae, our results show that they do not have the same effects in a model of adipose cells. In order to obtain information on their modes of action, we looked beyond their molecular effects, by analyzing the expression of genes involved in adipocyte differentiation and in the regulation of energy homeostasis. PPARγ is expressed at high levels in adipose tissue (Picard and Auwerx, 2002) where it plays an important role in the regulation of genes involved in adipocyte differentiation, lipid storage, glucose metabolism, and insulin sensitivity. PPARγ expression in adipocytes is associated with adipogenesis and activation of insulin-sensitive glucose transport. Sterol regulatory element binding transcription factor 1 (SREBP-1c) was identified as a pro-adipogenic transcription factor that induces PPARγ expression. SREBP-1c also mediates the induction of lipid biosynthesis by insulin in adipocytes, increasing the gene expression of the main lipogenic genes, such as fatty acid synthase (FAS). Treatment with BPA (1 pM) slightly increased the expression of the SREBP-1c, PPARγ-2 and aP2 mRNAs which are early genes involved in adipocyte differentiation. These results can be related to the observed improvement in triglyceride levels in adipocytes under low doses of BPA. These slight effects were in the same order of magnitude as already shown (Hugo et al., 2008) and could be related to the environmentally relevant doses used. In addition, BPS showed no effects on the expressions of these genes. As BPS also showed an increased level of intracellular TG, we had to find another pathway to explain our results. BPA is one of several environmental estrogens that have the ability to bind to GPER (Thomas and Dong, 2006) and to activate transduction pathways (Dong et al., 2011) involved in the biological responses of both normal and neoplastic cells. BPA has been shown to induce a rapid activation of Erk1/2 through GPR30 in human breast cancer cells (Dong et al., 2011; Pupo et al., 2012) and to boost mouse spermatogonial cell proliferation by inducing GPR30 expression. Neither BPA nor BPS was able to modify the GPR30 transcript. It is possible that BPA action in the GPR30 pathway happens only in hormone-sensitive cells (ovarian cancer cells, spermatogonial cells) and that GPR30 is not implicated at low concentrations of BPA in non hormonal-responsive cells such as adipocytes.

Estrogen-related receptors (ERRs) are orphan nuclear receptors that may regulate transcription of metabolic genes (Giguere et al., 1988). Among them, ERRα and ERRγ are particularly expressed in mitochondria-rich tissues and ERRα stimulates gene expression associated with mitochondrial biogenesis and energy production (Deblois and Giguere, 2011). ERRα-deficient mice show a reduction in fat mass and resistance to high-fat diet-induced obesity (Luo et al., 2003), indicating that ERRα can participate in the development of white adipose tissue. The PPARγ coactivator-1α and β (PGC-1α and PGC-1β) are also considered as key regulators in the energy production pathways (Lin et al., 2005; Ijichi et al., 2007). Moreover BPA has been reported to bind with high affinity to ERRγ (Matsushima et al., 2007).

Besides these previous findings, the contribution of ERRs to adipogenesis remains to be investigated in the context of bisphenol treatment. In our conditions, BPA treatment increased the expression of ERRγ mRNA; the same result was obtained with the ERRα mRNA. Stable expression of ERRα in 3T3-L1 cells was shown to up-regulate adipogenic marker genes and promote triglyceride accumulation during 3T3-L1 differentiation (Ijichi et al., 2007). Therefore ERRα could play a critical role in adipocyte differentiation by modulating the expression of various adipogenesis-related genes. Thus BPA could activate adipocyte differentiation through its interaction with the orphan receptors ERRα and γ. Conversely, BPS had no effect on ERRα mRNA levels and reduced the expression of ERRγ gene with, in parallel, an increase in PGC-1α mRNA levels. PGC-1 coactivators play a critical role in the maintenance of glucose, lipid, and energy homeostasis and are likely involved in the pathogenic conditions such as obesity, diabetes, neurodegeneration, and cardiomyopathy (Lin et al., 2005). Increased PGC-1α activity clearly contributes to elevate hepatic glucose output and the development of hyperglycemia (Kim et al., 2012). Thus BPS induced up-regulation of PGC-1α could be related to the induction of the uptake of glucose that we observed. Moreover, BPS had no effect on the expression of the ERRα gene, which could result in an absence of the negative control of PGC-1α. PGC-1α has been linked to glucose uptake (Cao et al., 2014), as inhibition of PGC-1α by siRNA resulted in the down-regulation of GLUT-4. Hence insulin resistance *per se* may lead to reduced PGC-1α expression and mitochondrial dysfunction, which may in turn aggravate insulin resistance, resulting in a deleterious spiral. It is tempting to speculate that elevating the activity of PGC-1α might provide beneficial effects on insulin sensitivity (Lin et al., 2005). Insulin resistance is a critical pathogenic process linking obesity to type 2 diabetes and cardiovascular diseases. A better glucose uptake in fat cells may participate in the amelioration of insulin sensitivity at the whole body level.

Diabetes is related to NAFLD (non alcoholic fatty liver disease) and BPA implication in NAFLD had been shown (Huc et al., 2012; Marmugi et al., 2012; Grasselli et al., 2013). In this study we used HepG2 cells as a cellular model of human hepatocytes. Interestingly, the results obtained were similar in this human cell line as in the mouse adipose cell line: both bisphenols significantly increased the triglyceride cellular content in the same order of magnitude in both cell lines. Moreover, the significant increase in glucose uptake with only BPS was identified in HepG2 cell line, suggesting that BPS could induce a decrease in circulating glucose. These effects had been found with natural molecules that have been proposed as anti-diabetic compounds (Alonso-Castro and Salazar-Olivo, 2008; Zhang et al., 2011, 2012).

In a previous study we carried out a comparison between BPA and BPS in human hepatic cell models to evaluate the potential involvement in NAFLD (Peyre et al., 2014). Bisphenol A was shown to activate the pregnane X receptor while BPS did not (Sui et al., 2012a; Kuzbari et al., 2013; Peyre et al., 2014). This again highlights the difference in metabolic processes between the two molecules. We also showed that BPS, at the used concentrations, does not appear to induce metabolic syndrome as it was unable to induce lipid bio-accumulation in the hepatoma cell lines tested, and had no effect on the modulation of metabolic genes tested. The phenolic compounds, especially bisphenols, have notoriously shown non-monotonic dose–response functions and very different effects at environmentally relevant doses as compared to higher exposures (Vinas et al., 2012; Vandenberg et al., 2013; Vandenberg, 2013). Many studies reported that low doses of BPA induced lipid accumulation in HepG2 cells (Huc et al., 2012), in other hepatocytes (Lakhal L, personal communication) or in rodents (Marmugi et al., 2012; Jiang et al., 2014) and that BPS may have non-genomic mechanisms of action at the low concentration range likely to be present in environmental samples (Vinas and Watson, 2013). The results presented in this current study, that complement those obtained with higher doses (Peyre et al., 2014), are in agreement with the non-monotonic effects of bisphenols (Vandenberg et al., 2013).

In the current study, none of the results obtained with either BPA or the BPS could be reproduced with DES used as estrogenic reference, suggesting that the xeno-estrogenic property of these compounds is probably not involved in the processes that we have observed. Further studies could be designed adding BPA or BPS in association with an estrogenic compound to test the E-enhancers hypothesis of bisphenols (Barrett, 2013).

In summary, we have shown that BPS, a substitute for BPA, at concentrations relevant to environmental doses, shows molecular and biological effects that are quite different from BPA, suggesting a compound-specific mechanism. We have also highlighted the non-monotonic effects of BPS with different cellular effects regarding the concentration used (very low *versus* higher doses). Both could participate in metabolic deregulations that may contribute to processes involved in overweight, obesity, metabolic syndrome or NAFLD. A substitution of BPA by its structural analogue BPS should be carried out with caution, even if this environmental contaminant could be less noxious than BPA, especially in terms of energy balance, as it seems that its effects, if confirmed *in vivo*, might resemble anti-diabetic effects. These results will be strengthened by the results of our *in vivo* study already in progress, using mice exposed chronically to BPA or BPS, fed or not with a high fat diet.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2014.07.025.

Conflict of interest

The authors declare that there are no conflicts of interest

Acknowledgments

The MELBA project received Public Institutional Funding from INRA and from the French Program on Endocrine Disruption, PNRPE 2011. This work was also funded by the French Ministry of Ecology (in the framework of the ACTIVISME project 21000315) and by the “Conseil Général des Alpes Maritimes” (in the framework of the HCSTOX project 21000433).

We would like to thank Dr L. Huc for reviewing the manuscript.

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