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

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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to induce adverse effects on human health, especially through the activation of endocrine pathways, it is about to be withdrawn from the European market and replaced by analogues such as bisphenol S (BPS).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.

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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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 pre-adipose 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,

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