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Bioactivation of bisphenol A and its analogs (BPF, BPAF, BPZ and DMBPA) in human liver microsomes

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ABSTRACT

Bisphenol A analogs are a class of chemicals known as diphenylmethanes, which contain two benzene rings separated by one central carbon atom, usually with a *para*-hydroxy group on both benzene rings. Bisphenol A (BPA) can induce an uterotrophic response in immature CD-1 mice and elicits estrogenic responses in many other experimental systems. Besides highlighting endocrine effects, a number of metabolic studies provide strong support for the idea that reactive species of BPA are formed *in vitro* and *in vivo* that can form covalent adducts with nucleophilic macromolecules and/or produce oxidative stress. We used a liquid chromatography with a triple quadrupole tandem mass spectrometry (LC–MS/MS) for the detection of metabolites and glutathione conjugates of BPA and its analogs (BPF, BPAF, BPZ and DMBPA) in human liver microsomes (HLM) or with recombinant CYP isozymes in the presence of NADPH and GSH as a trapping agent. We have confirmed that BPA and its structural analogs form hydroxylated metabolites and electrophilic species during bioactivation in HLM and CYP isozymes. These results provided important mechanistic insight into the metabolic fate of BPA structural analogs *in vitro*.

1. Introduction

Bisphenol A (BPA, 2,2-bis(4-hydroxyphenyl)propane) is a widely used industrial chemical that can be find as a monomer in polycarbonate and epoxy plastics, as well as an antioxidant in polyvinyl chloride plastics. BPA is a weak estrogen and is therefore regarded as one of the endocrine disrupting chemicals (EDCs) (Yoshihara et al., 2004). 2011 saw restrictions applied to the use of BPA monomers in plastic materials (EU ban on BPA in baby bottles) (Commission Directive 2011/8/EU, 2011). As a consequence, several other compounds structurally similar to BPA are also being utilized in the manufacture of resin and plastics. It is assumed that the production of these BPA analogs will increase annually, resulting in environmental contamination (Danzl et al., 2009).

BPA analogs are class of chemicals known as diphenylmethanes, which contain two benzene rings separated by one central carbon atom, with a *para*-hydroxy group on both benzene rings that is critical for binding to estrogen receptors (ERs) (Fang et al., 2001). The estrogenic activity of BPA was found by coincidence when polycarbonate flasks were autoclaved (Feldman et al., 1984). It was showed that BPA can induce an uterotrophic response in immature CD-1 mice 3 days after exposure at high doses such as 100 mg/kg, while the estimated human daily intake is approximately 1 μg/kg (Vandenberg et al., 2009; Markey et al., 2001). The estrogenic potency of BPF was confirmed in ovariectomized rats with a positive estrogenic response (Cabaton et al., 2008). Incubation of BPA with rat liver S9 fraction increased estrogenicity of BPA for several times and the active estrogenic metabolite was determined to be 4-methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene (MBP) (Yoshihara et al., 2004, 2001). Nakamura et al. (2011) observed an enhancement of estrogenic activity of the BPA metabolite hydroxycumyl alcohol (HCA) formed via *ipso*-substitution catalysed by cytochrome P450 (P450). In contrast, BPA-monoglucuronide is almost completely devoid of estrogenic activity (Matthews et al., 2001).

3-Hydroxy-BPA was reported as being one of the hydroxylated products formed by pregnant mice after the subcutaneous injection of BPA. An oxidation product of 3-hydroxy-BPA is *ortho*-quinone BPA, which has been reported to form adducts with DNA *in vitro* and *in vivo* by 1,4-Michael-type addition (Edmonds et al., 2004; Zalko et al., 2003). A study carried out by Jaeg et al. (2004) demonstrated that BPA is oxidized by CD-1 mice liver microsomes

Abbreviations: BP, bisphenol; BPA, bisphenol A; BPF, bisphenol F; BPAF, bisphenol AF; BPZ, bisphenol Z; DMBPA, 3,3′-dimethyl BPA; ER, estrogen receptor; ESI, electrospray ionization; GSH, reduced glutathione; HCA, hydroxycumyl alcohol; LC–MS/MS, liquid chromatography with tandem mass spectrometry; MBP, 4-methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene; MRM, multiple reaction monitoring; P450, cytochrome P450; PCP, phencyclidine; PIS, precursor ion scan.

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and S9 fractions into nine metabolites of BPA such as isopropylhydroxyphenol, a glutathione conjugate of BPA, glutathionyl-phenol, glutathionyl 4-isopropylphenol and BPA dimers. Most of the biotransformation studies thus far carried out have been done on BPA, while for other BPs only a relatively small number of studies with bisphenol F (BPF, bis(4-hydroxyphenyl)methane) have been performed. Cabaton et al. (2008) investigated the *in vitro* biotransformation of radiolabelled BPF using rat and human liver subcellular fractions and characterised the following metabolites: dihydroxylated BPF, *meta*-hydroxylated BPF (m-OH–BPF), *ortho*-hydroxylated BPF (o-OH–BPF) and BPF dimer. In another study, Cabaton et al. (2009) examined the genotoxic and endocrine activities of BPF and its metabolites and found that the most toxic compound was BPF compared to its other metabolites identified in rat urine: 4,4′-dihydroxybenzophenone, m-OH–BPF and o-OH–BPF. However, no biotransformation studies have been performed for other BPA analogs such as bisphenol AF (BPAF), bisphenol Z (BPZ) and 3,3′-dimethyl BPA (DMBPA).

In the current study, we have examined the biotransformations of BPA and its selected analogs (BPF, BPAF, BPZ and DMBPA) in human liver microsomes and with different human recombinant CYP450 isozymes (CYP3A4, CYP1A2, CYP2C8, CYP2C9, CYP2C19 and CYP2E1). On the basis of growing evidence that BPA can be chemically converted into reactive species, mostly via the P450 pathway, we decided to study the metabolic behavior of four BPA analogs in human liver microsomes and with different CYP450 isozymes using reduced glutathione (GSH) as a trapping agent. GSH conjugates were detected by LC–MS/MS using three modes of scanning: a full scan, multiple reaction monitoring (MRM) and precursor ion scan (PIS) of $m/z = 272$ with negative ionization (Dieckhaus et al., 2005; Ma and Zhu, 2009; Evans et al., 2004). To investigate P450 oxidative reactions, incubations were treated with NADPH as a cofactor and GSH as a trapping agent. GSH usage was also preferred due to the fact that it does not inhibit P450s. This configuration represents a classical testing system that is widely employed in detection of reactive metabolites (Peterson et al., 2005). The study focuses on the evaluation of metabolites that are formed in human liver microsomes as a consequence of carbon bridge cleavage and as a consequence of the hydroxylation of the bisphenol phenyl ring. On the basis of numerous metabolic studies for BPA (Ginsberg and Rice, 2009; Nakagawa and Tayama, 2000; Qiu et al., 2004; Pritchett et al., 2002; Kovacic, 2010) and obtained experimental results, the bioactivation pathways of BPA analogs in human liver microsomes have been proposed.

2. Methods

2.1. Materials

BPA [2,2-bis(4-hydroxyphenyl)propane, CAS Registry No. 80-05-7] (No. 239658 – analytical standard, for drug analysis), BPF [Bis(4-hydroxyphenyl)methane, CAS Registry No. 620-92-8] (No. B47006 – 98%), BPAF [4,4′-(Hexafluoroisopropylidene)diphenol, CAS Registry No. 1478-61-1] (No. 257591 – 97%), BPZ [4,4′-Cyclohexylidenebisphenol, CAS Registry No. 843-55-0] (No. 450421 – 98%), DMBPA [2,2-Bis(4-hydroxy-3-methylphenyl)propane, CAS Registry No. 79-97-0] (No. B45712 – 97%) were purchased from Sigma (Steinheim, Germany). 4-(Hydroxymethyl)phenol [CAS Registry No. 623-05-2] (No. 121030250 – 97%) was purchased from AcrosOrganics (Geel, Belgium) and 4-hexafluorohydroxyisopropylidene-phenol [CAS Registry No. 836-79-3] (No. Z235452368 – 97%) was purchased from SynQuest (Alachua, Florida). Sodium phosphate monobasic, anhydrous, purity >98% (CAS Registry No. 7558-80-7) was purchased from Sigma (Missouri, USA) with Catalogue number S3139. Glutathione, Reduced was purchased from Novagen (EMD Chemicals, Darmstadt, Germany) with Catalogue number 71075. Human male liver microsomes, pooled from CMV negative human liver, were purchased from Sigma (Missouri, USA), Product No. M 0442 with average CYP450 content of 400 pmol/mg protein. Cytochrome P450 enzymes, recombinant CYP1A2 (No. C3999) with CYP450 content of 60–80 pmol/mg protein, CYP2C8 (No. C6999) with CYP450 content of 55–75 pmol/mg protein, CYP2C9 (No. C3874) with CYP450 content of 50–65 pmol/mg protein, CYP2C19 (No. C3749) with CYP450 content of 50–65 pmol/mg protein, CYP2E1 (No. C3499) with CYP450 content of 35–50 pmol/mg protein and CYP3A4 (No. C3374) with CYP450 content of 40–60 pmol/mg protein expressed in Saccharomyces cerevisiae, coexpressed with human NADPH reductase were also obtained from Sigma (Missouri, USA). β-Nicotinamide adenine dinucleotide phosphate, reduced tetra(cyclohexylammonium) salt, CAS Registry No. 100929-71-3, was purchased from Sigma (Steinheim, Germany, N5130, ⩾95%). Methanol, bidistilled water, acetonitrile and formic acid were all LC–MS grade and were purchased from JT Baker.

2.2. Incubations with HLM or recombinant CYP450 isozymes

All incubations (total volume, 100 μL) were conducted at 37 °C for 60 min in 50 mM phosphate buffer (pH 7.4) of human liver microsomes or recombinant CYP450 isozymes containing 1 mg/mL, 5 mM $MgCl_2$, 5 mM GSH and 1 mM NADPH. First, microsomes or recombinant cytochrome P450 isozymes were suspended in a mixture of buffer, $MgCl_2$ and GSH. Bisphenol (BP) in methanol was added to reach a final concentration of 50 μM. The final concentration of methanol in the incubation media was 1%. The reaction mixture was pre-incubated at 37 °C for 5 min. In order to initiate the reaction, 20 μL of 5 mM NADPH was added. The reaction was quenched by adding 25 μL of ice cold acetonitrile. The incubation mixture was left at – 20 °C for 24 h and then centrifuged at 1300 g for 100 min at 4 °C. The supernatants were transferred into a 96-well plate for subsequent LC–MS/MS analysis. Control samples without NADPH or GSH were also prepared. Incubations prepared without NADPH were used as controls for non-NADPH-mediated bisphenol transformations, while the second group was prepared without GSH to demonstrate the functioning of NADPH-dependent microsomes or cytochrome P450 isozymes. All incubations were carried out in triplicates.

2.3. LC–MS/MS analysis of detected metabolites and glutathione conjugates

LC–MS/MS analyses were performed using an Agilent 1290 Infinity HPLC system coupled to a Triple Quad MS detector Agilent 6460 (Agilent Technologies, Santa Clara, USA) equipped with a Jet-Stream™ electrospray ionization (ESI) source operating in negative ionization mode. Chromatographic separation was performed on a C18 50 mm × 2.1 mm Kinetex column with 2.6 μm particles, protected by a 4 × 2 mm C18 cartridge column (Phenomenex, Torrance, USA) at 50 °C. The injection volume was 0.5 μL. Gradient elution at a flow rate of 0.65 mL/min consisted of the following steps: 95% A (bidistilled water) and 5% B (methanol) to 5% A and 95% B from 0 to 5 min, 5% A and 95% B from 5 to 6.5 min, and then to 95% A and 5% B from 6.5 to 6.7 min. Total run time was 7.2 min. The ion source parameters were set as follows: drying gas temperature: 300 °C, drying gas flow rate: 5 L/min, nebulizer pressure: 45 PSI (3.1×10^5 Pa), sheath gas temperature: 250 °C, sheath gas flow: 11 L/min, capillary entrance voltage: 3500 V, nozzle voltage: 500 V, fragmentor: 130 V, dwell time: 50 ms. For most accurate and sensitive quantification of metabolites, MRM mode was used. The presence of each GSH conjugate was additionally confirmed by a negative precursor ion scan (PIS) experiment searching for the pre-