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Biotransformation of bisphenol F by human and rat liver subcellular fractions

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ABSTRACT

Bisphenol F [4,4′-dihydroxydiphenyl-methane] (BPF) has a broad range of applications in industry (liners lacquers, adhesives, plastics, coating of drinks and food cans). Free monomers of this bisphenol can be released into the environment and enter the food chain, very likely resulting in the exposure of humans to low doses of BPF. This synthetic compound has been reported to be estrogenic. A study of BPF distribution and metabolism in rats has demonstrated the formation of many metabolites, with multiple biotransformation pathways. In the present work we investigated the *in vitro* biotransformation of radiolabelled BPF using rat and human liver subcellular fractions. BPF metabolites were separated, isolated by high-performance liquid chromatography (HPLC), and analysed by mass spectrometry (MS), MS^n, and nuclear magnetic resonance (NMR). Many of these metabolites were characterized for the first time in mammals and in humans. BPF is metabolised into the corresponding glucuronide and sulfate (liver S9 fractions). In addition to these phase II biotransformation products, various hydroxylated metabolites are formed, as well as structurally related apolar metabolites. These phase I metabolic pathways are dominant for incubations carried out with liver microsomes and also present for incubations carried out with liver S9 fractions. The formation of the main metabolites, namely *meta*-hydroxylated BPF and *ortho*-hydroxylated BPF (catechol BPF) is P450 dependent, as is the formation of the less polar metabolites characterized as BPF dimers. Both the formation of a catechol and of dimeric metabolites correspond to biotransformation pathways shared by BPF, other bisphenols and estradiol.

1. Introduction

Man-made chemicals used for agricultural, industrial or domestic purposes can be released into the environment, enter the food chain, and produce a number of disorders in animals, and possibly in human (Colborn et al., 1993; Rasier et al., 2006; Calafat et al., 2006). Among these chemicals, bisphenols and related compounds form a large family of molecules. They are used in the production of epoxy resins and polycarbonates which are widely employed in industry, in the manufacture of lacquers, liners, adhesives plastics and water pipes (Jana et al., 2005; Crathorne et al., 1986). They are also used in dental materials, restorative materials, oral prosthetic devices, tissue substitutes and coatings for food packaging (Hashimoto and Nakamura, 2000; Inoue et al., 2003; Perez et al., 1998). The most popular coating varnishes and lacquers used in drink and food cans are those based on vinilic organosols (novolacs), which include in their composition epoxy resins obtained from BADGE (Bisphenol A Diglycidyl Ether) or from BFDGE (Bisphenol F Diglycidyl Ether) (Nerin et al., 2002). Incomplete polymerization or coating varnishes deterioration can result in leakage of bisphenols. Bisphenol F [4,4′-dihydroxydiphenyl-methane] (BPF, Fig. 1) is a diphenylalkane. Its structure is very similar to that of Bisphenol A (BPA). Both molecules can be released from packaging material and migrate into beverages and foods, the rate of migration being enhanced by treatments such as heat processing. BPF residues have been identified in food in contact with epoxy coatings such as novolac glycidyl ethers (NOGE) (Grob et al., 1999) as well as in drinking water from water pipes renovated with BFDGE (Crathorne et al., 1986). BPF monomers have also been detected in canned foods and can lids when acetonitrile was used as extraction solvent (3.4×10^{-3} mg/dm^2 to 7.7×10^{-3} mg/dm^2 of can or can lid) and these amounts were 3 time higher than those detected for BPA in the same conditions (Jordakova et al., 2003). Such leaching process could result in a daily exposure of humans to low amounts of BPF.

BPF and BPA have been reported to have estrogen agonistic properties (Yamasaki et al., 2002). Bisphenols have been shown to alter the development and/or disrupt reproductive physiology in many animal species even at low doses, namely those that

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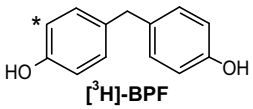

Fig. 1. Chemical structure of Bisphenol F (BPF) and position of ^{3}H (*) labelling.

may be relevant to actual human exposure (Maffini et al., 2006; Rasier et al., 2006). However, the issue of the low doses effects of compounds such as bisphenol A remains very controversial (Ashby et al., 2004; Goodman et al., 2006; Richter et al., 2007; vom Saal and Hughes, 2005). Exposure to endocrine disrupter chemicals *in utero* during critical development stages and, more generally, disturbance of the early life environment may have an impact on child and on adult health (Cummings and Kavlock, 2004; Plagemann, 2005). Endocrine disrupters exhibit distinct biological activities including (anti-) estrogenic, or (anti-) androgenic effects (Gray et al., 1997). These effects can be triggered by a direct binding to steroid hormone receptors but also by indirect mechanisms such as the disruption of the biosynthesis or the catabolism of steroids (Fischer, 2004; Sharpe and Irvine, 2004). BPA has been shown to be an endocrine disrupter *in vivo* and *in vitro*, possessing an estrogenic activity (Dodds and Lawson, 1936; Kang et al., 2006; Olea et al., 1996). *In vivo*, the estrogenic potency of BPF was demonstrated in ovariectomized rats resulting in a full vaginal cornification with complete absence of leukocyte, indicating a positive estrus response (Dodds and Lawson, 1936). In contrast, no clear endocrine-mediated changes were detected by Higashihara et al. (2007) in young adult rats exposed during at least 28 days to 20, 100 and 500 mg BPF/kg diet. Nevertheless, based on clinical biochemical parameters, these authors concluded that the main effect of BPF *in vivo* was liver toxicity. *In vitro*, using a yeast two-hybrid system, BPF was identified as the most estrogenic compound among BPA-related chemicals present in food packaging material or used in dentistry (Hashimoto and Nakamura, 2000; Hashimoto et al., 2001). In human cells, the proliferative response of MCF-7 cells (E-screen assay) increases when cells are exposed to BPA or to BPF, in a concentration-dependent manner (Perez et al., 1998; Stroheker et al., 2004). The latter authors showed that, according to EC50 values (84.8 nM and 410 nM, for BPF and BPA, respectively), BPF activity in the E-screen test was higher that of BPA, although the two molecules exhibited the same affinity for the estrogen receptor (ER).

The metabolism of bisphenols is of great importance for the assessment of their toxicity at the cellular level and for understanding their bioavailability and biological properties. Several studies on the biotransformation of BPA have been carried out *in vivo* and *in vitro*. In pregnant mice, the formation of the glucuronic acid conjugate of BPA, of several double conjugates, and of conjugated methoxylated compounds excreted in urine has been demonstrated (Zalko et al., 2003). The latter finding strongly suggests the production of potentially reactive metabolites, namely catechol BPA, *in vivo*. Other studies showed that BPA is oxidized by mice liver microsomes and S9 fractions, and demonstrated the *in vitro* P450-dependent formation of reactive intermediates (Jaeg et al., 2004) which have been reported to form adducts with DNA (Atkinson and Roy, 1995a,b). BPA was also shown to cause the inhibition of certain cytochrome P450 isoforms and to interfere with the conjugation of other molecules, such as testosterone and umbelliferone (Pfeiffer and Metzler, 2004). For BPF, although the liver toxicity of the molecule has recently been documented in rats (Higashihara et al., 2007), data about the metabolic fate of this compound in animal models are limited. A study of BPF distribution and metabolism in pregnant and non pregnant rats has shown the formation of several metabolites demonstrating that the biotransformation of BPF is complex and that BPF is metabolized into a large number of metabolites (Cabaton et al., 2006). The present work investigates the metabolic pathways of BPF, using radio-labelled BPF and rat and human liver subcellular fractions. BPF metabolites were isolated using high-performance liquid chromatography (HPLC) and their structures were investigated using mass spectrometry (MS), MS^n, and nuclear magnetic resonance (NMR).

2. Materials and methods

2.1. Chemicals

[^{3}H]-BPF (4,4′-dihydroxydiphenyl-methane) (Fig. 1), with a specific activity of 300.36 MBq/mmol, was purchased from Izotop (Budapest, Hungary) and stored in ethanol at −20 °C. Prior to the experiments, the solution was evaporated to dryness under a nitrogen stream and was re-suspended in acetonitrile. Its purity was verified by radio-HPLC and was higher than 99.3%. Unlabelled BPF (>98% pure, CAS #620-92-8), NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, ATP, UDP-glucuronic acid and β-glucuronidase were obtained from Sigma Aldrich (L'Isle d'Abeau Chesnes, France). All solvents (analytical grade) were purchased from Scharlau Chemie SA (Barcelona, Spain).

Ultrapure water from Milli-Q system (Millipore, Saint Quentin en Yvelines, France) was used for *in vitro* preparations and for HPLC mobile phases.

2.2. Subcellular fractions and in vitro incubations

2.2.1. Human and rat liver sub-cellular fractions

S9 and microsomes from human male and female were purchased from TEBU (Le-Perray-en-Yvelines, France) and obtained from pools of 10 donors. Three female and three male Wistar rats weighing 200–250 g and fed standard commercial diets (UAR, Villemoisson-Sur-Orge, France) were killed by cervical dislocation followed by exsanguination and livers were perfused immediately using 0.1 M sodium phosphate buffer (pH 7.4). Livers were weighed and homogenized using a Potter-Elvehjem Teflon glass homogeniser in 4 volumes g^{-1} of ice-cold phosphate buffer. S9 fractions were obtained by centrifugation at 10,000*g* for 20 min at 5 °C. Microsomal pellets were obtained by centrifugation of S9 fractions at 105,000*g* for 60 min. Microsomes were resuspended with gentle homogenization in 1 volume g^{-1} of 0.1 M phosphate buffer containing 20% glycerol. S9 fractions and microsomes were stored at −80 °C until use. The protein contents of sub-cellular fractions were determined by the method of Lowry et al. (1951).

2.2.2. Metabolism of [^{3}H-BPF] by rat and human liver S9 fractions and microsomes

In vitro biotransformations of BPF were studied by incubating 2 h at 37 °C under shaking, radio-labelled [^{3}H]-BPF (3000 Bq) fortified with unlabelled BPF at different concentrations (10, 25, 50, 75, 100 and 200 μM) with 2 mg human or rat microsomal protein or with 7 mg human or rat S9 protein corresponding to 100 mg liver (10 and 50 μM BPF). Incubations were performed in a final volume of 1 mL 0.1 M sodium/potassium buffer, 5 mM $MgCl_2$ (pH 7.4) containing a NADPH generating system (1.3 mM NADP, 5 mM glucose-6-phosphate, 2 IU glucose-6-phosphate dehydrogenase) and ascorbic acid 5 mM. All incubations were carried out in triplicate. At the end of incubations, radioactivity measurements were performed for each concentration assay to control the BPF concentration in media. Whatever the *in vitro* system considered, more than 95% of the radioactivity put in incubations was recovered, with no significant difference between rats and humans, nor between females and males.