Parallel Biotransformation of Tetrabromobisphenol A in Xenopus laevis and Mammals: Xenopus as a Model for Endocrine Perturbation Studies

Jean-Baptiste Fini,* Anne Riu,†‡ Laurent Debrauwer,†‡ Anne Hillenweck,†‡ Sébastien Le Mévé,* Sylvie Chevolleau,†‡ Abdelaye Boulahtouf,§ Karima Palmier,* Patrick Balague,§ Jean-Pierre Cravedi,†‡ Barbara A. Demeneix,* and Daniel Zalko†‡††1

*UMR CNRS 7221, Evolution des Régulations Endocriniennes Department of Regulations, Development and Molecular Diversity, Muséum National d’Histoire Naturelle, 75231 Paris Cedex 05, France; †INRA, UMR1331, Toxalim, Research Centre in Food Toxicology, F-31027 Toulouse, France; §Université de Toulouse, INP, ENV, EIP, UPS, UMR1331, Toxalim, F-31076 Toulouse, France; and §IRCM, Institut de Recherche en Cancérologie de Montpellier, INSERM, U896, Université Montpellier1, CRLC Val d’Aurelle Paul Lamarque, Montpellier 34298, France

1To whom correspondence should be addressed at INRA, UMR1331, 180 chemin de Tournefeuille, F-31027 Toulouse, France. Fax: +335-612-852-44.
E-mail: dzalko@toulouse.inra.fr.

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The flame retardant tetrabromobisphenol A (TBBPA) is a high production flame retardant that interferes with thyroid hormone (TH) signaling. Despite its rapid metabolism in mammals, TBBPA is found in significant amounts in different tissues. Such findings highlight first a need to better understand the effects of TBBPA and its metabolites and second the need to develop models to address these questions experimentally. We used Xenopus laevis tadpoles to follow radiolabeled 14C-TBBPA uptake and metabolism. Extensive and rapid uptake of radioactivity was observed, tadpoles metabolizing >94% of 14C-TBBPA within 8 h. Four metabolites were identified in water and tadpole extracts: TBBPA-glucuronide, TBBPA-glucuronide-sulfate, TBBPA-sulfate, and TBBPA-disulfate. These metabolites are identical to the TBBPA conjugates characterized in mammals, including humans. Most radioactivity (>75%) was associated with sulfated conjugates. The antithyroid effects of TBBPA and the metabolites were compared using two in vivo measures: tadpole morphology and an in vivo tadpole TH reporter gene assay. Only TBBPA, and not the sulfated metabolites, disrupted thyroid signaling. Moreover, TBBPA treatment did not affect expression of phase II enzymes involved in TH metabolism, suggesting that the antithyroid effects of TBBPA are not due to indirect effects on TH metabolism. Finally, we show that only the parent TBBPA inhibits T3-induced transactivation in cells expressing human, zebrafish, or X. laevis TH receptor, TRα1. We conclude, first, that perturbation of thyroid signaling by TBBPA is likely due to rapid direct action of the parent compound, and second, that Xenopus is an excellent vertebrate model for biotransformation studies, displaying homologous pathways to mammals.

Key Words: thyroid hormone; TBBPA; biotransformation; metabolism; endocrine disruption; phase II metabolism; vertebrate model.

Tetrabromobisphenol A (TBBPA) is the largest selling brominated flame retardant (BFR), with worldwide demand over 210,000 tons/year (Alaee et al., 2003), being used for production of epoxy resins, to flameproof electronic devices (Birnbaum and Staskal, 2004). TBBPA was first employed to replace polybrominated diphenylethers (PBDEs), the use of which caused concern because of their high environmental persistency (Eriksson et al., 2001). Substitution with TBBPA has been justified by the fact that estimates of its half-life in mammals are relatively short (<24 h) and the extrapolated risk for human exposure and toxicity thought to be low (Kuester et al., 2007). However, TBBPA has been found repeatedly in environmental and human samples (Law et al., 2006; Sjödin et al., 2003). TBBPA was found in blood (Sjödin et al., 1999) and breast milk (Shi et al., 2009). A recent and extensive human exposure study, carried out in France on pregnant women, found TBBPA to be the major BFR present in both maternal and cord serum samples (Cariou et al., 2008). This finding is particularly preoccupying, given the adverse effects of TBBPA as an endocrine disruptor and more particularly on thyroid signaling (for review, see Birnbaum and Staskal, 2004).

Thyroid hormones (TH) are essential for the normal development, growth, and metabolism of all vertebrates (Zoeller et al., 2002), playing a major role in neurogenesis and brain function at all stages of development (Bernal, 2007). Circulating levels of TH peak during critical developmental phases, which include periods of rapid brain growth. Moreover, TH is a key developmental hormone, peaking at birth, paralleling amphibian metamorphosis that is totally dependent upon the more biologically active TH, tri-iodothyronine (T3) (Leloup and Buscaglia, 1977). TBBPA shares structural similarities with TH, namely tetra-iodothyronine (thyroxine or T4) and T3, immediately raising the question of its potential to disrupt TH signaling. Data on the endocrine-disrupting effects of BFRs, including TBBPA, have been reviewed (Darnerud, 2008). However, the available toxicity database
for TBBPA is still insufficient for human and ecological risk assessment.

TBBPA has been associated with disruption of TH signaling at different levels. TBBPA binds to transthyretin (TTR), the carrier protein for T₄, 10 times more effectively than T₄ (Meerts et al., 2000). Disruption of T₃ binding to rat TH receptors (TR) by TBBPA has been reported by Kitamura et al. (2005), and transient transfection studies in CV-1 cells described antagonistic effects of TBBPA (Sun et al., 2009). The in vivo effects of TBBPA on thyroid signaling have been mainly studied in amphibians (Fini et al., 2007; Jagynitsch et al., 2006; Shi et al., 2011; Veldhoen et al., 2006). Conversely, most studies on the metabolic fate of TBBPA have been performed in mammals, the main metabolites identified being sulfated and glucuronidated conjugates in rats (Hakk et al., 2000; Knudsen et al., 2007; Kuester et al., 2007; Schauer et al., 2006). Schauer et al. (2006) also reported a predominance of phase II biotransformation pathway products, concluding that TBBPA was completely metabolized in rats and humans into sulfates and glucuronides and then excreted in urine. Recently, we demonstrated that Xenopus laevis tadpoles, even at early developmental stages, expresses metabolic capacities enabling efficient biotransformation of endocrine disruptors such as bisphenol A (Fini et al., 2009). These metabolic capacities, coupled with the high conservation of TH signaling between amphibian and mammals, suggest that X. laevis tadpoles can be used to study TH disruption in the context of human risk assessment. To evaluate better the use of X. laevis as a vertebrate model for mammalian endocrine disruption, we assessed the fate of TBBPA in X. laevis tadpoles, also asking whether the antithyroid effects are due to the parent TBBPA or a metabolite. Using ¹⁴C-TBBPA, we examined the kinetics of TBBPA uptake from water, as well as its excretion. Metabolic profiles were studied and metabolites identified. The effects of TBBPA and its major metabolites (chemically synthesized) on TH signaling were assessed using two readouts: tadpole morphology and an in vivo TH reporter assay. We also assessed whether TBBPA or its metabolites exerted indirect actions through a deregulation of TH-metabolizing enzymes such as UDP-glucuronyl transferases (UGTs), sulfotransferases (SULTs), or deiodinases (for review, see Visser, 1996; Wu et al., 2005). Finally, we studied the ability of sulfate metabolites and TBBPA to exert direct actions on the TRα using a reporter cell assay. Taken together, the data on metabolites show that the X. laevis tadpole is an excellent vertebrate model for biotransformation studies, given its homologous pathways to mammals and second that only the parent TBBPA interferes with TH signaling.

MATERIALS AND METHODS

Chemicals

Radiolabeled TBBPA was synthesized from ring ¹⁴C-BPA (Moravek Biochemicals, CA) as previously described (Zalko et al., 2006). TBBPA radio-purity was checked by radio-high performance liquid chromatography (HPLC) and was over 99.8%. Its specific activity was 6.72 KBq/µg and its structure was confirmed by electrospray ionization (ESI) coupled with mass spectrometry (MS) and nuclear magnetic resonance (NMR). Mono- and disulfate TBBPA conjugates were synthesized from TBBPA and purified by solid-phase extraction as previously described (Riu et al., 2011b). The purity and chemical structure of sulfated conjugates were checked by HPLC, ESI-MS, and NMR. All solvents were of analytical grade and were purchased from Scharlau Chemie SA (Barcelona, Spain) and from Sigma-Aldrich (Saint-Quentin Fallavier, France). The complete list is available in Supplementary material.

Animals

Wild-type animals were bred for use in the metabolic studies, quantitative polymerase chain reactions (qPCRs), and morphology experiments. For gene reporter assay, F₁ generation of X. laevis embryos, bearing the TH/βZIP-GFP construct, was obtained after breeding TH/βZIP-GFP males and wild-type females. Transgenic founders were obtained as previously described (Fini et al., 2007). All animals were raised in dechlorinated water/tap water (2.3, vol/vol) and were staged according to Nieuwkoop and Faber (1994). The care and treatment of animals were in accordance with institutional and national guidelines (Charte Nationale Portant sur l’Ethique de l’Expérimentation Animale, 2009).

Analytical Procedures

All experiments were carried out using low-binding tips (VWR International) and glass material silanized with dimethylchlorosilane/toluene (5:95, vol/vol). Radioactivity in liquid samples was determined by direct counting on a Packard liquid scintillation counter (Model TriCarb 2200CA; Packard Instruments, Meriden, CT) using Packard Ultima Gold as the scintillation cocktail. Tadpoles from each group were pooled and immediately frozen in liquid nitrogen until extraction. Pools of tadpoles was homogenized with a Polytron homogenizer (Kinematica AG, Lucern, Switzerland) in pH 7.4 phosphate buffer and centrifugation at 250 × g (10 min, 4°C). Then, a second extraction was carried out in the same conditions but using water-saturated ethyl acetate. The organic and aqueous phases were separated and their radioactivity was measured by direct counting of aliquots using the scintillation counter. Residual radioactivity in the tadpoles’ pellets (non-extractable radioactivity) was determined by complete combustion using a Packard Oxidizer 306 (Packard Instruments). TBBPA and metabolites were quantified by integrating the area of the radio-chromatographic peaks.

Metabolites Structural Characterization

Metabolite isolation was carried out as described (Zalko et al., 2006). Briefly, tadpoles’ supernatant extracts were concentrated under a nitrogen stream, rediluted in 8 ml acetonitrile (pH 3.2):acetic acid (80:20, vol/vol), and fractionated on 1 g Chromabond C18 ec glass cartridges (Macherey Nagel) previously washed with 4 ml acetonitrile and equilibrated with 8 ml acetic acid (pH 3.2):acetone (80:20, vol/vol). Elution was performed successively with 4 ml acetic acid (pH 3.2):acetone (80:20, vol/vol), 8 ml acetic acid (pH 3.2):acetone (60:40, vol/vol), and 8 ml acetone. Metabolite structural characterization was carried out by liquid chromatography (LC)-MS using negative ESI. All experiments were performed on an LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher, Les Ulis, France) and low-resolution MS/MS on an LCQ-quadrupole ion trap mass spectrometer (Thermo Fisher) were carried out. Details on the mass spectrometric operating parameters are given in Supplementary materials.

Treatment of Tadpoles

Kinetics. ¹³C-TBBPA (13,000 Bq) was added to unlabeled TBBPA, to a concentration of 10⁻⁶M, or was used alone (3020 Bq for 10⁻⁴M). For each dose, TBBPA, in ethanol, was added to dechlorinated water (1:1000, vol/vol final concentration) with or without 5.10⁻⁴M T₃. Each group of NF45 tadpoles (n = 15) was placed in a silanized glass tube and 8 ml of the required solution. One water sample from each group was taken at 0, 10, 30 min, 1, 2, 3, 6, and 23 h. TBBPA solutions were renewed at 24 and 48 h. After each TBBPA renewal (on days 2 and 3), water samples were taken at the same time points as on day 1, until the 72-h time point. At 72 h, all tadpoles (15) from each group were pooled.
and deep frozen on dry ice until extracted. Radioactivity in water samples was measured using a liquid scintillation counter Packard 2200CA (PerkinElmer Life Sciences, Courtaboeuf, France) and Packard Ultima Gold as the scintillation cocktail.

Morphological and reporter gene assays. NF45 tadpoles were placed 15 per well in transparent flat six-well plates from TPP (Switzerland) at 23°C (±0.5°C). Each well was filled with 8 ml of partially dechlorinated water. Treatments were performed with daily renewal, during 6 days for morphology assay and 3 days for gene reporter assay. Tadpoles were anesthetized in MS 222 0.01%. Photographs were taken using an M216 Leica Microsystem stereomicroscope (Rueil Malmaison, France) equipped with a Retiga SRV camera (Qimaging, Canada). Tadpoles’ head areas were determined using ImageJ free software (scale 80267 pixel/mm).

Transient Transfection Experiments

Human, zebrafish, and X. laevis TRα activity was monitored on (GAL4RE)5-β-globin-luciferase construct using species-specific ligand-binding domain (LBD) inserted in pSG5-GAL4-puro plasmid. pSG5-GAL4-puro and (GAL4RE)5-β-globin-luciferase were already described (le Maire et al., 2009).

Zebrafish TRα-LBD was synthesized by Eurofins MWG Operon (Les Ulis, France) and cloned between BamHI and XhoI restriction sites in pSG5-GAL4-puro. hTRα-LBD was cloned from entire human TRα, and xTRα-LBD was cloned from entire X. laevis TRα (for details, see Supplemental materials). Transient transfection assays were performed in HeLa cells using Jet-PEI (Ozyme, Saint-Quentin en Yvelines, France) according to the manufacturer’s instructions. Luciferase assays were performed with the Promega dual-reporter kit, according to the manufacturer’s instructions. Renilla luciferase encoded by the normalization vector pRLTK (Promega, Charbonnieres-les-Bains, France) was used as an internal control for firefly luciferase normalization. Tests were performed in triplicate in at least two independent experiments and data were expressed as mean ± SD.

Statistical Analysis of Results

Kinetic data, radioactivity measurements, and reports are expressed as mean ± SD. Morphological, GFP assay, and qPCRs results are expressed as mean ± SEM. GraphPad Prism 4 software was used for statistical analysis. Differences between means were analyzed using one-way ANOVA (nonparametric Kruskal-Wallis test) followed by Dunn’s test. Differences were considered significant at p ≤ 0.05. Statistical significance is indicated as follows: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

RESULTS

Rapid Uptake and Extensive Metabolization of TBBPA by Tadpoles

Xenopus laevis tadpoles were waterborne exposed for 72 h to 14C-TBBPA (10⁻⁶M or 10⁻⁷M, with daily renewal) with or without exogenously supplied T₃ in aquarium water. Water samples were taken at different time points to establish TBBPA uptake kinetics. Figure 1 summarizes radioactivity levels in water over the 3-day experiment. The whole data set is presented in Supplementary table 1. TBBPA was rapidly taken up. After 2 h of exposure, only 22.7 ± 1.3% or 25.0 ± 1.7% of radioactivity was found in water (10⁻⁶M, with or without T₃, respectively). Using 10⁻⁷M TBBPA, maximal absorption was observed at 1 h. A progressive release of radioactivity back into water was observed in all groups, reaching 62.8 ± 2.1% and 75.4 ± 3.5% of the initial radioactivity applied after 23 h for the 10⁻⁶M and 10⁻⁷M groups, respectively. Similar absorption kinetics were observed after the renewal of media at 24 h. TBBPA uptake was lower for this second day of exposure but still high, with at least 57.0 ± 4.1% and 43.8 ± 1.5% for the 10⁻⁶M and 10⁻⁷M groups, respectively. T₃ addition into the aquarium water did not significantly affect the uptake or release of radioactivity. Following the renewal of media at 48 h, a similar pattern was observed, with no significant difference in the uptake depending on TBBPA concentration. Taken together, these data demonstrated rapid absorption of 14C-TBBPA by tadpoles, followed by
a gradual release of the parent TBBPA or metabolites into the water. For all groups, the 3-day radioactivity recovery ranged between 91.0 and 97.8%, indicating negligible loss (Supplementary fig. 1). Around 15% of the total radioactivity administered was found in tadpoles (T) at the end of the experiment, regardless of the TBBPA concentration or T₃ supply.

**Metabolite Identification by LC-ESI-MS**

Radio-HPLC and LC-MS were used to investigate the metabolism of TBBPA in exposed tadpoles. Metabolites were extracted from water at all time points and from tadpoles at 72 h. A typical profile for water is shown in Figure 2A (10⁻⁶M TBBPA, 24 h). At this point, virtually all the TBBPA administered was biotransformed into four metabolites (M1–M4) that eluted at 11, 22, 29.5, and 46 min, respectively, under our analytical conditions. LC-ESI-MS analysis: For M1, the MS spectrum displayed a [M-H]⁻ ion cluster centered on m/z 719 with an isotopic pattern consistent with a fourfold brominated compound (Supplementary fig. 3A). A loss of 176 u.m.a. (m/z 543 production) characteristic of a glucuronide conjugate was observed on the MS/MS spectrum of the m/z 719 precursor ion (data not shown), suggesting the identity of M1 as the TBBPA-glucuronide (Fig. 2B). In the same way, M2, as well as the major metabolite M3, displayed [M-H]⁻ ion clusters centered on m/z 799 (Supplementary fig. 3B) and m/z 623 (Supplementary fig. 3C), respectively. The MS/MS spectrum of the m/z 799 precursor ion of M2 exhibited three main diagnostic product ions at m/z 719 (loss of 80 u.m.a. characteristic of a sulfate conjugate), m/z 623 (loss of 176 u.m.a.), and m/z 543 (loss of 176 + 80 u.m.a.), whereas M3 (m/z 623 precursor ion) yielded one diagnostic product ion at m/z 543. M2 and M3 were thus identified as the TBBPA-glucuronide-sulfate and as TBBPA-sulfate, respectively (Fig. 2B). The MS spectrum of M4 displayed a base peak at m/z 725 (Supplementary fig. 3D),
which could be attributed to a [M-2H+Na]⁻ ion corresponding to TBBPA-disulfate. This finding was confirmed by the MS/MS analysis performed on the selected m/z 725 precursor ion, which showed a characteristic elimination of 80 a.m.u. (SO₃-2 group) yielding the m/z 645 product ion. All these data were further confirmed by accurate mass measurements that fitted each metabolite elemental precise mass measurements to within 3 ppm (data not shown).

**TBBPA-Monosulfate Is the Major Metabolite**

The relative proportions of metabolites, in aquarium water (24, 48, and 72 h) and in tadpoles (at 72 h), were calculated by integrating radio-HPLC peaks. T₃ addition did not significantly affect metabolic profiles except for the disulfate measurement in tadpoles at 72 h (17.52 ± 1.19% vs. 41.53 ± 1.80% without or with T₃). Hence, only results from TBBPA treatments without T₃ are presented in Figures 2C and 2D (complete data in Supplementary table 2). In water, regardless of TBBPA concentration, almost all the radioactivity was recovered as metabolites, at 24, 48, and 72 h, respectively.

At 24 h, at least 75% of the radioactivity in water samples corresponded to purely sulfated conjugates (TBBPA-monosulfate, M₃, and TBBPA-disulfate, M₄) for TBBPA (10⁻⁶M and 10⁻⁷M) ± T₃. Two other conjugates were identified, namely TBBPA-glucuronide, M₁, and a mixed glucuronic acid/sulfate double conjugate, M₂, demonstrating functional UDP-glucuronyl transferase (UGT) activity. The TBBPA-glucuronide accounted for 12.30 ± 2.72% to 3.82 ± 4.59%, in groups with or without T₃. At 48 and 72 h, the proportion of the different TBBPA metabolites excreted in water was nearly identical for each dose tested and were not significantly affected by T₃. Around 98% of the radioactivity was detected as sulfated conjugates of TBBPA, M₃ being the major metabolite. Interestingly, at 72 h, a larger proportion of unchanged (parent) TBBPA was observed for the lower dose treatment groups.

In tadpole extracts, examined at the end of the experiment (72 h), the major compound detected was the monosulfate conjugate M₃, just as in water. However, M₁ accounted for roughly one-fourth of the total radioactivity. Less than 6% of the radioactivity was recovered as the parent TBBPA after 10⁻⁶M exposure in tadpole tissues (Fig. 2D).

**TBBPA, and Not the Metabolites, Disrupt TH Signaling**

We next addressed whether the antithyroidal effects observed in *X. laevis* (Fini et al., 2007; Jagnytsch et al., 2006) were due to the parent TBBPA or its major metabolites. Both mono- and disulfates were synthesized and purified. Two different assays were carried out (Fig. 3). First, effects on T₃-induced morphological changes were addressed using a test based on T₃-induced gill regression (Figs. 3A and 3B) as described by Tata (1968). T₃ treatment significantly reduced tadpole head areas, due to gill regression and Meckel cartilage transformation, producing a triangular shape, with no effect in controls (Fig. 3B). Head areas were measured after 6 days in tadpoles exposed to T₃ (5nM), T₃ + TBBPA, T₃ + M₃, or T₃ + M₄ (Figs. 3B and 3C). Next, we used transgenic TH-responsive reporter tadpoles (Fini et al., 2007). TBBPA at 10⁻⁶M, but none of its sulfate conjugates, repressed T₃-induced GFP expression in vivo signaling (Fig. 3D). Again, as for the in vivo morphological gene assay, only TBBPA and not the metabolites had an antithyroidal effect (Figs. 3C and 3D).

**TBBPA Exposure Does Not Alter Expression of TH-Metabolizing Enzymes**

Certain xenobiotics, such as polychlorobiphenyls (PCBs) and bromodiphenylethers (BDEs), induce TH-metabolizing enzymes (Richardson et al., 2008). Given the high levels of TBBPA-sulfates found both in water and in tadpoles, we hypothesized that the phase II enzymes involved in TH sulfonation could be induced by TBBPA, thereby decreasing TH levels. Wild-type tadpoles were exposed to 10⁻⁶M TBBPA (± T₃). Total messenger RNA (mRNA) was extracted, and levels of key UGTs and SULTs enzymes involved in TH metabolism were measured by qPCR. T₃ treatment significantly downregulated UGT1A1 and UGT1A6, but TBBPA exposure was without effect. SULT1A1 transcription was not altered by T₃ or TBBPA (Supplementary fig. 4, upper panel). Other enzymes implied in TH homeostasis, namely the three deiodinases, were examined. TBBPA exposure had no significant effect on the mRNA levels of any of these enzymes (Supplementary fig. 4, lower panel).

**TBBPA Displaces T3 From TRs**

TBBPA binds to rat TR (Kitamura et al., 2005); we assessed binding and transcriptional activity of TBBPA and its major metabolites using a reporter system based on fusion of the LBD from human, *X. laevis*, or zebrafish TRα to a GAL4 DNA-binding domain. TBBPA, but none of its sulfated conjugates, displaced T3 from TRα in any species (Fig. 4). TBBPA alone also bound to the human TRα-LBD, activating transcription when applied alone at 3 and 10μM, whereas increased binding of TBBPA alone did not reach significance on *X. laevis* or zebrafish TR using nonparametric ANOVA (Fig. 4).

**DISCUSSION**

Three original findings arise from this work. First, we show that TBBPA and not its metabolites interfere with thyroid signaling in amphibians. Second, comparative TR-binding assays on three species, including humans, show the effects of TBBPA on thyroid signaling are direct. Third, the results on metabolites formed reveal strong parallels for TBBPA metabolism in mammals and *X. laevis*, providing an easier and cheaper way to infer potential effects of endocrine-disrupting compounds in human risk assessment studies.

**TBBPA but Not the Sulfated Metabolites Act on TH Signaling**

The Organization of Economic Cooperation and Development (OECD, 2009) has ratified an Amphibian Metamorphosis Assay
(OECD 231) that requires a 5-week testing protocol and measurement of many morphological endpoints. A more rapid (72 h) X. laevis transcriptional reporter assay test has been optimized for screening TH disrupters. This test detects thyroid agonists or antagonists and has revealed antithyroid effects of TBBPA (Fini et al., 2007). With the aim of clarifying mode of action underlying the endocrine-disrupting effect of TBBPA, we isolated and examined the physiological actions of its metabolites.

Using 14C-TBBPA, extremely high TBBPA uptake rates and extensive metabolism were observed. After 8 h, most of 14C-TBBPA disappeared from the water then reappeared, in very low amounts in water samples, strongly suggesting metabolism and deconjugation by tadpoles (Supplementary fig. 2E). Similarly, in human volunteers, Schauer et al. (2006) reported that 8 h after a 100 μg/kg oral administration of TBBPA, the parent compound was undetectable in blood. Here, using LC-MS, we identified the main TBBPA metabolites as sulfated and glucuronidated conjugates, showing strong similarities with studies in mammals, including humans, that demonstrated the in vivo predominance of phase II metabolic pathways (Hakk et al., 2000; Schauer et al., 2006). These pathways appear fully functional in tadpoles, at least for phenolic compounds (this

FIG. 3. (A) Protocol for assessing biological activity of metabolites. For each assay, 15 tadpoles (transgenics for the GFP assay, wild-type for the morphological assay) were placed in contact with TBBPA, TBBPA-sulfate, and TBBPA-disulfate, each at 10^{-6}M ± T_3 5.10^{-9}M. Treatments were renewed every day. TBBPA solution was used as a positive control for inhibition of TH induced, fluorescence (GFP), or gill regression morphological assay. (B) Representative pictures of head morphology observed after six treatments with TBBPA (10^{-6}M) or its metabolites: TBBPA-monosulfate (Mono) and TBBPA-disulfate ± T_3 (5.10^{-9}M). Note that TBBPA reduces T_3-induced reduction of head area; scale bar 100 μm. (C) Quantification of morphological assay. Head areas of 15 tadpoles per group were measured using ImageJ software. T_3 reduces head size. TBBPA significantly inhibits this regression. No metabolites mimic TBBPA’s effect. Experiments were performed twice, providing similar results. (D) THbZIP eGFP transgenic tadpoles assay. Tadpoles (15 per group) were exposed to same products as for morphological assay. Graphs show fluorescence mean quantification for each group. For (C) and (D), typical experiments are shown and have been done three times providing same results. Statistics were done using one-way ANOVA followed by Dunn’s test, performed on results either with or without T_3: *p < 0.05, **p < 0.01, ***p < 0.001.
work and Fini et al., 2009). In X. laevis, as in mammal studies, the predominant TBBPA biotransformation pathway is sulfonation. TBBPA-sulfates accounted for the largest part of metabolites detected in water and tadpole samples at all time points. Extensive work on conventional and bile duct–cannulated rats by Hakk et al. (2000) using 14C-TBBPA demonstrated extensive metabolism of TBBPA. Interestingly, both TBBPA-glucuronide and a double conjugate, glucuronide-sulfate-TBBPA, were identified as minor metabolites, exactly as in X. laevis.

The predominance of TBBPA-sulfates, and the previously demonstrated TH-disrupting effects of TBBPA in X. laevis (Fini et al., 2007), led us to explore the involvement of these metabolites in the antithyroid effects. Though phase II metabolites are usually considered detoxification products, a number of biologically active conjugates are known (Olson et al., 1992). Moreover, in the recent studies, we demonstrated that TBBPA-sulfate could bind peroxisome proliferator-activated receptor (PPAR)γ as intensively as the TBBPA parent compound (Riu et al., 2011a, b). We examined the effects of TBBPA-mono-sulfate and TBBPA-di-sulfate using two TH response assays: a morphological test and a transgenic reporter gene assay. Neither metabolite had any effect in either test, corroborating the hypothesis that only the parent TBBPA antagonizes TH signaling. However, we cannot completely exclude effects of glucuronide conjugates, even if they are only present in low proportions compared with sulfated conjugates.

An alternative hypothesis to direct receptor-based effects of TBBPA (or its metabolites) is modulation of expression of enzymes involved in TH metabolism because such routes have been demonstrated for BDE-47 (Richardson et al., 2008). Sulfonation is an important step in the irreversible inactivation of TH (Visser, 1996). Organohalogenated compounds inhibited this process (Brouwer et al., 1998). As mRNA levels and enzyme activity are well correlated (Kester et al., 1999), we examined the levels of mRNA encoding enzymes. TBBPA did not modulate the levels of mRNA encoding SULT1A1, one of the major sulfotransferases involved in TH metabolism in humans and in amphibians (Kester et al., 1999; Rahman and Yamauchi, 2010). No effects of TBBPA were observed on UDP-glucuronyl transferases (UGT1A1 and UGT1A6) nor on the three deiodinases (D1, D2, and D3). However, in the presence of T3, TBBPA diminished D1 transcription, the deiodinase with the highest affinity for sulfated iodothyronine (Kester et al., 1999). It should be stressed that in agreement with previous studies using 14C-TBBPA (Hakk et al., 2000; Zalko et al., 2006), no evidence for TBBPA debromination was observed in our study.

**TBBPA Displaces T3 From TRs**

These data strongly suggest a direct action of TBBPA despite the active metabolism observed in X. laevis tadpoles, raising the question of the TH-disrupting mode of action. TBBPA displaces physiological concentrations of T3 from human, X. laevis, and zebrafish TRα. This displacement could well account for many of the in vivo antithyroid effects of TBBPA. Other actions could include crosstalk with other nuclear receptors. Recently, TBBPA has been found to be an activator of PPARγ (Riu et al., 2011b), a receptor that shares both a common heterodimeric partner with

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**FIG. 4.** Human, zebrafish, and Xenopus laevis TRα activity was monitored on (GAL4RE)-β-globin-luciferase construct. Cells were transfected with human LBD-TRα. (A) or X. laevis LBD-TRα. (B) or zebrafish LBD-TRα. (C) Transfections have been done twice in triplicates in presence or absence of 2nM T3 with either TBBPA or TBBPA-monosulfate at 1, 3, and 10μM concentrations. Statistical differences were determined using nonparametric ANOVA Kruskal-Wallis test followed by Dunn’s test; *p < 0.05, **p < 0.01.
TR and controls a number of common target genes involved in metabolic control (Kouidhi et al., 2010). Although X. laevis tadpoles rapidly metabolize TBBPA, it is present in its parent form at least for 2 h. During this time, TBBPA could act by modifying TH transport (Meerts et al., 2000) and at the receptor level, directly or indirectly through modulation of PPAR crosstalk.

Results from human studies (TBBPA residues have been found in many of the samples examined) and toxicokinetic data (rapid elimination) strongly suggest continuous human exposure to TBBPA. Numerous studies have demonstrated the presence of TBBPA in breast milk as well as umbilical cord serum, the latter finding demonstrating fetal exposure (Cariou et al., 2008; Shi et al., 2009). Despite these findings, TBBPA is commonly considered to be rapidly eliminated (Hagmar et al., 2001). Geyer et al. (2004) confirmed these data and found differences between half-life in blood (around 3 days) and adipose tissue (64 days in adult humans). In our study, after a 3-day exposure, 15% of the radioactivity administered in aqueous media for the 3 days persisted in tadpoles, TBBPA proportion being around 6% for the higher dose exposure and around 10% for the lower dose used. This finding raises the question of whether the TBBPA levels in human fluids (TBBPA and conjugates) result from higher exposure or unsuspected persistence.

**TBBPA Metabolism in X. laevis Parallels That in Mammals**

Our results highlight strong similarities between the metabolic capabilities of X. laevis and that of mammals. Furthermore, TR-binding data correlate well with human data, thus re-emphasizing the usefulness of the amphibian model for endocrine disruption studies (Kloas et al., 2009). This pertinence is particularly strong for thyroid disruption, given that amphibian metamorphosis is totally T3 dependent (Leloup and Bucaglia, 1977) and that metamorphosis parallels the perinatal period in humans and other mammals. Another advantage is that embryonic X. laevis is free living with external embryonic development, facilitating screening.

In conclusion, our results show that the antithyroid effects of exposure to TBBPA are due to the parent compound despite its rapid metabolism. Because THs are essential for neurodevelopment and TBBPA is found in significant quantities in human cord serum, these studies accentuate the need to better understand how the BFRs could impact both human development and environmental targets.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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