PPARγ AND ERα ARE NUCLEAR RECEPTORS TARGETS OF TBBPA, BPA AND RELATED HALOGENATED COMPOUNDS.

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Abstract

Tetrabromobisphenol A (TBBPA) and tetrachlorobisphenol A (TCBPA) are halogenated analogues of the model xeno-estrogen bisphenol A (BPA). In this study, we used bioluminescent cell lines transfected either with human estrogen receptors (HELN-ERs) or with human PPARs (HELN-PPARs), to investigate the respective estrogenic potencies and peroxysome proliferator-activated receptors (PPARs) activities of BPA, TCBPA, TBBPA, and other brominated BPAs (mono-, di-, and tri-bromo-BPA). The agonistic activity of these compounds towards human ER and PPARγ were shown to depend upon their halogenated degree. Brominated BPAs with the highest degree of bromination exhibited the lowest estrogenic potency. Conversely, BPAs with the highest degree of halogenation had the highest potency to activate PPARγ (EC_{50} values of 1 µM for TBPPA and TCBPA, respectively), while no agonistic activity was observed using HELN-PPARα or PPARδ. As expected, similar results were obtained using nuclear receptors-ligand binding domain (NR-LBD) based affinity columns (recombinant human ERα-LBD, aa 302-552, or native human PPARγ-LBD, aa 205-475). The higher the degree of BPA bromination, the greater the observed retention of tested compounds on PPARγ affinity columns, and the lower their retention on ERα-based columns. hERα-LBD columns, as well as native human PPARγ-LBD based affinity columns were able to capture both high and low affinity standard ligands. In addition, PPARγ-LBD affinity columns allowed to characterise new PPARγ ligands formed following *in vitro* biotransformation of TBBPA. These results suggest that this BFR, but also some of its *in vitro* biotransformation products, may be involved in the onset of NR-triggered effects on endocrine homeostasis.

Introduction

Bisphenol A (BPA) is widely used in the manufacture of plastics including polycarbonate plastics, food can resin linings and dental sealants. BPA is one of the highest-volume chemicals produced worldwide, with a global production capacity evaluated in 2003 around 3 millions tons[1]. BPA halogenated analogues (brominated or chlorinated) can be used to produce flame retardants. Tetrabromobisphenol A (TBBPA) is one of the most commonly used, with more than 120 000 tons produced annually[2]. Tetrachlorobisphenol A (TCBPA) was also reported to be used commercially as a flame retardant, but in much lower quantities[3]. Recent investigations have also shown that the chlorination of BPA in aqueous media can occur, resulting in the formation of TCBPA[4]. Contrary to BPA and TBBPA, which have been found in environment[5], wildlife[6] and human tissues[1,7], very few studies have to date reported the occurrence of TCBPA in the environment[3], and none in human. However, it was recently demonstrated that chlorinated BPAs (mono-, di- and tri-Cl-BPA) can be detected in human adipose tissue[8]. These chlorinated compounds have also been found in the environment, and may result either from the incomplete chlorination of BPA by a chemical process, or from TCBPA dechlorination. TBBPA readily debrominates into lesser brominated analogs (mono-, di- and tri-BBPA) under both aerobic and anaerobic conditions in soil and in river sediment[9]. All these halogenated analogues of BPA, brominated or chlorinated, could potentially have hazardous effects on human health.

Flame retardants as well as BPA can act as endocrine disrupters[6]. BPA has already been demonstrated to be a xenoestrogen able to exert its effects *in vivo* and *in vitro*[1]. Toxicity studies of the chlorinated derivates suggest that their estrogenic activity could be stronger than that of BPA[10]. It was also reported that with an increasing number of bromine atoms, brominated BPAs are decreasingly potent agonists of the human estrogen receptors[11]. Because of their structural similarities with thyroxine (T4), TBBPA and lesser brominated analogues could competitively bind human transthyretin (TTR)[6]. Likewise, TCBPA is a candidate thyroid hormone disrupting

chemical[12]. Little is known about the effects of these compounds on other hormonal systems. Recently, TBBPA has been reported to be an agonist of the peroxisome proliferor-activated receptor γ (PPARγ)[13]. PPARγ is a member of the nuclear receptor (NR) superfamily involved in the control of lipid storage and plays a critical role in the induction of adipogenesis. This NR could be involved in many human metabolic diseases such as obesity and type II diabetes[14].

The first objective of this study was to determine the effect of the halogenation level of chlorinated and brominated BPAs on both their affinity and agonistic activity towards ERs and PPARs. Estrogenic and PPARs activities of halogenated BPAs were first evaluated using ERs reporter cell lines (ERα and ERβ) and the three PPAR reporter cell lines (PPARα, PPARδ and PPARγ). Further, we explored the capability of ERα and PPARγ affinity columns to retain known high affinity radio-labelled ligands as well as low affinity radio-labelled flame retardants. This tool is based on NR ligand binding domains (LBD) immobilised on agarose columns and on the capability of NR to bind endocrine disrupter ligands. It has already been successfully applied to investigate biologically active compounds present in environmental and food samples, using recombinant human ERα-LBD affinity columns[15]. In a second step, the combination of PPARγ affinity columns with analytical methods (HPLC and mass spectrometry) was successfully adapted for the isolation and structural identification of new agonistic PPARγ ligands formed following the *in vitro* biotransformation of TBBPA.

Materials and Methods

Radio-labelled TBBPA [2,2-bis(3,5-dibromo-4-hydroxyphenyl) propane] was synthesised from ring-[^{14}C]-BPA (Moravek biochemichals, CA, USA, specific activity: 7.4 GBq mmol^{-1}) as described in Zalko et al.[16]. [^{14}C]-TBBPA purity was checked by radio-HPLC (> 99.8%). Its specific activity was 3080 Bq.nmol^{-1}. Radio-labelled brominated congeners of BPA (monobromo-, dibromo-, tribromo- and tetrabromo-bisphenol A) were synthesised from ring-[^{14}C]-BPA: 2.2 equivalents of bromine were added to 1.48 MBq [^{14}C]-BPA and 2 mg unlabelled BPA in 80 µl methanol/water (1:1 v/v); after 2h at room temperature, the reaction was quenched with sodium bisulphite, extracted with dichloromethane. Radio-labelled [^{14}C]-brominated-BPA congeners were individually isolated and purified by HPLC coupled to fraction collection (Gilson 202, Gilson France, Villiers-Le-Bel, France). The radiochemical purity of all brominated congeners was checked by radio-HPLC (> 99.8%). Their specific activity was 168 Bq.nmol^{-1}. Tetrachlorobisphenol A (TCBPA) was purchased from TCI Europe (Belgium). Radio-labelled TCBPA being not commercially available, only unlabelled TCBPA was used. A radio-labelled pharmaceutical agonist ligand of PPARγ was used as reference, as well as [^{14}C]-17β-estradiol (NEN) for ERα.

HPLC analyses were performed on a Spectra system P1000 (Thermo Electron) connected to a Flo-one/β A500 radioactivity detector (Radiomatic™ 610TR, Perkin Elmer). HPLC conditions were adapted from radio-HPLC System "II" previously described in Zalko et al.[16].

Rat liver sub-cellular fractions were prepared as described in Cabaton et al.[17]. *In vitro* biotransformations of TBBPA were performed as described in Zalko et al.[16]. Briefly, 2 hr sub-cellular fractions incubations were carried out at 37°C under shaking, in the presence of radio-labelled [^{14}C]-TBBPA (6700 Bq) fortified with unlabelled TBBPA (8 µM), with 2 mg microsomal protein 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.

Generation of HELN ERα and HELN ERβ reporter cell lines, and HELN GAL4 PPARs reporter cell lines (hPPARα, hPPARδ, and hPPARγ) were performed in 2 steps, as decribed in Balaguer et al.[18], and in Le Maire et al.[19], respectively. Stable gene expression assays carried out with these reporter cell lines were quantified by luciferase activity using the diffusion of luciferin, as already described in Balaguer et al.[18]. Luciferase assays were performed with or without fetal calf serum (FCS).

The recombinant ERα-LBD (aa 302-552) encoding plasmid, as well as the native human PPARγ-LBD (aa 205-475) encoding plasmid, was produced in BL_{21} DE_3 electrocompetent *Escherichia coli* cells using the procedure already described in Riu et al.[15]. The concentration was around 2.5 µM and 25 µM for hERα-LBD and hPPARγ-LBD, respectively.

Affinity columns were performed as already described in Riu et al[15]. First, ERα affinity columns were validated using radio-labeled compounds, as well as PPARγ using a radio-labeled ligand of this NR. Halogenated BPA congeners (1 nmol) were next loaded on ERα (2.5 nmol) and PPARγ (5 nmol) immobilized on Ni-NTA phase, in order to determine the binding capacity of these molecules on these nuclear receptors.
In order to trap possible PPARγ ligands formed following TBBPA biotransformation, when incubated with rat liver sub-cellular fractions, 3 nmol of the supernatant of incubations, diluted in 500 µl WB (final volume 1 mL, with 5 % of organic solvent) were applied on 20 nmol of PPARγ, previously immobilized on 250 µL Ni-NTA phase. Ligands trapped on PPARγ and recovered in eluted fractions were concentrated using SPE C18 glass cartridges (Macherey Nagel), then analysed by HPLC coupled to a Flo-one/β A500 radioactivity detector.

Results and Discussion

Dose-response curves of BPA and halogenated congeners in HELN cells. All compounds were first tested on HELN-ERα, HELN-ERβ and HELN-PPARs cell lines. Hela cells are known to have lost metabolic capability and were used in this study in order to circumvent enzymatic biotransformation of tested compounds. As expected, BPA as well as all halogenated congeners except TBBPA showed an agonistic activity on ERα (figure 1A). Among the tested compounds, BPA had the highest potency to transactivate luciferase gene expression in ERα cells (EC_{50} 0,5 µM), followed by mBBPA and diBBPA (EC_{50} 2 µM), then triBBPA and TCBPA. The two latter compounds displayed a similar low agonistic activity on ERα with EC_{50} values > 10 µM. The same tendency was observed when using HELN-ERβ cell lines, except for triBBPA and TCBPA for which no agonistic activity was observed on ERβ. The results obtained with our stably transfected Hela cells for brominated BPAs are in good accordance with those reported by Meerts et al. using T47D breast cancer cells[11].

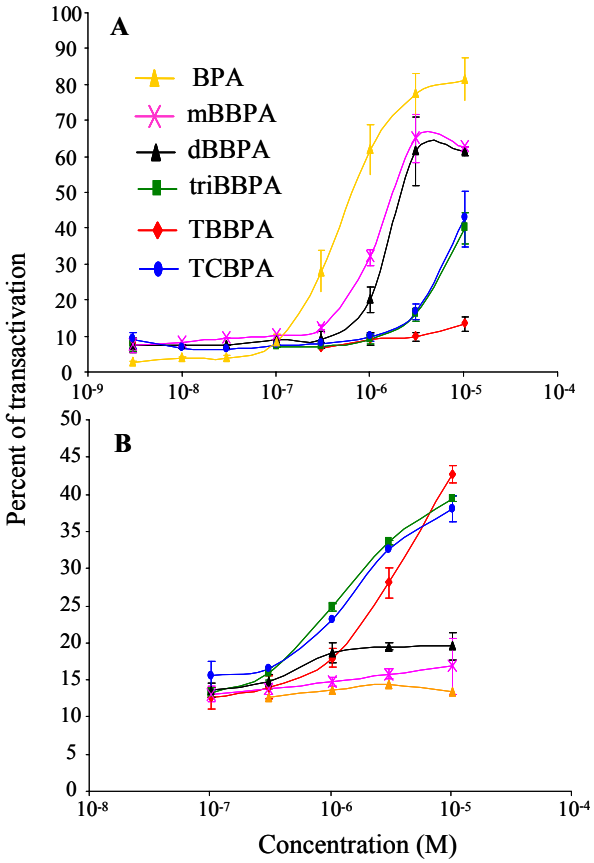

Figure 1. HELN-ERα (A) and HELN-PPARγ (B) cell luciferase assays for halogenated BPAs. Results are expressed as a percentage for luciferase activity measured per well (mean ± SEM, n=4). Values obtained in the presence of 10 nM reference compounds were taken as 100.

In the HELN-PPARγ cell line, the potency of the tested bisphenols to transactivate luciferase gene expression was reversed, compared to ER (figure 1B), with the highest agonistic activity observed for TBBPA and TCBPA (EC_{50} values of 1 µM) followed by triBBPA (EC_{50}: 3 µM). Compared to the reference pharmaceutical high affinity ligand (EC_{50}: 1 nM), these halogenated BPAs can be considered as low affinity ligands of PPARγ. However in foetal calf serum free cultures using the HELN-PPARγ cell line, the respective binding affinities and agonistic activities of TBBPA, triBPPA and TCBPA were higher than observed when incubations were carried out in the presence of FCS (data not shown). EC_{50} values in serum free culture were more than a 10-fold higher. This could be due to the chemical properties of halogenated BPAs. Indeed, solubility in water decreased with the increasing number of bromine atoms on BPA. Hydrophobic compounds such as TBBPA could be sequestered by FCS proteins. As already reported in few studies, the effects of many chemicals on cellular processes are governed by their ability to enter the cell, which is in turn a function of the composition of the cell's environment[20]. In our case the bioavailability of polyhalogenated BPAs bound to protein was lower

than that of BPA. The observed effects were consequently underestimated in FCS medium assays. Halogenated BPAs were also tested on HELN PPARα and HELN PPARδ cell lines; no agonistic activity was found using these PPAR subtypes (data not shown). Poly-halogenated BPAs are thus specifically agonist ligands of PPARγ. Our results show that the higher is the halogenation level of BPA, the lower the agonistic activity of halogenated BPAs towards human estrogen receptors, and the higher their agonistic activities towards human PPARγ.

NR-based affinity columns assays using high and low affinity ligands. NR-based affinity columns were validated using radio-labelled high-affinity ligands. Radio-labelled standards allowed a rapid control of the binding capacity of ligands on the immobilised nuclear receptors. The binding capacity of [^{14}C]-17β-Estradiol ([^{14}C]-E2) on immobilised hERα-LBD was confirmed, with more than 91% of the radioactivity loaded on column recovered in eluted fractions. The PPARγ pharmaceutical high affinity ligand was used in order to test the binding capacity of hPPARγ-LBD immobilised on Ni-NTA phase. As observed in figure 2, more than 95 % of the radioactivity was trapped on immobilised PPARγ for this reference compound.

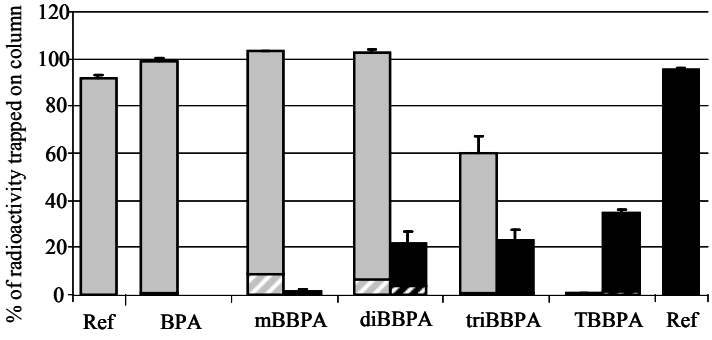

Figure 2. Determination of binding capability of recombinant ERα-LBD (grey bars) and native PPARγ-LBD (black bars) immobilised on agarose columns to capture standard radioactive ligands (mean ± SD, n = 3), and their binding specificity controlled with unlabelled high affinity ligands of reference in excess loaded on columns before tested chemicals (hatched bars).

Radio-labelled BPA and each of the brominated congeners (1 nmol) were loaded on columns after the immobilisation of 2 nmol ERα or 5 nmol PPARγ on the Ni-NTA phase. In ERα-based affinity columns assays, most of the radioactivity was recovered in the eluted fractions (98.0 ± 1.9%, 94.6 ± 0.2%, 96.2 ± 1.1% and 59.1 ± 7.3% for BPA, mBBPA, diBBPA and triBBPA, respectively). Like in cell lines assays, TBBPA did not bind the ERα-LBD immobilised on phase, with most of the radioactivity loaded on column recovered in washing fractions (data not shown). Conversely, when PPARγ-LBD was immobilised on Ni-NTA phase, most of the radioactivity loaded was recovered in eluted fractions, in descending order, for TBBPA, triBBPA and diBBPA (32.7 ± 1.7%, 22.6 ± 3.7% and 18.4 ± 4.8%, respectively). Neither mBBPA, nor BPA were trapped on PPARγ-LBD affinity columns. Binding specificity to the NR-LBD was evaluated for each tested compound using a large amount of non radio-labelled high affinity ligand trapped on columns before loading the radio-labelled compound of interest (hatched bars, figure 2). Moreover, none of the tested compounds was retained on columns only loaded with the phase, without NR immobilised on it. As expected, the higher was the degree of BPA bromination, the greater was the observed retention on PPARγ affinity columns and the lower the retention on ERα-based columns. Like hERα-LBD columns, native human PPARγ-LBD based affinity columns were able to capture both standard high affinity and low affinity ligands. In that way, these affinity columns appear to be promising tools that could be used to isolate new PPARγ ligands from complex matrices.

Highlight of new ligands of PPARγ from in vitro biotransformations of TBBPA. Three major metabolites (T_1, T_2 and T_3) were observed on the radio-chromatograms obtained from 2h incubations of TBBPA (8 µM) with rat liver microsomes, in accordance with previously published results[16] (figure 3A). Metabolites, T_1, T_2 and T_3, were found to co-elute with the authentic standards of 3-hydroxy-2,6-4-isopropyl-phenol, 2,6-dibromo-4-(2',6'-dibromo-1'-hydroxycumyl)-phenoxy-3",5"-dibromo-4"-hydroxybenzene and, 2,6-dibromo-4-(2',6'-dibromo-1'-hydroxycumyl)-phenoxy-2"-hydroxy-3"-bromo-5"-(2',6'-dibromo-1'-hydroxycumyl)benzene, respectively. Radio-HPLC analyses showed that most of the radioactivity recovered in microsomal incubation supernatant was associated with unchanged TBBPA, which was eluted at a retention time (R_T) of 18 min (figure 3A).

Supernatants of TBBPA incubations carried out with rat liver microsomes were processed and loaded on recombinant PPARγ-based columns. Radioactivity present in each fraction was directly determined by counting. As shown in Figure 3B, more than 40 % of the radioactivity was recovered in the eluted fractions. Proteins were precipitated with three volumes of methanol, and an aliquot of the eluted fractions was analysed by radio-HPLC

(Figure 3C). The radio-chromatogram showed 3 major compounds eluted at 18, 38 and 40.6 min, corresponding to TBBPA, T_2 and T_3, respectively. Most of the PPAR bound radioactivity was associated with unchanged TBBPA (82 %). The remainder of the radioactivity was associated with metabolites. T_1 was not bound to PPARγ-based columns (it was totally recovered in washing fractions), but TBBPA, T2 and T3 were bound to these columns. Accordingly, these 2 TBBPA metabolites were identified as new PPARγ ligands with a low-binding affinity. Their agonistic or antagonistic activities for this nuclear receptor are under investigation.

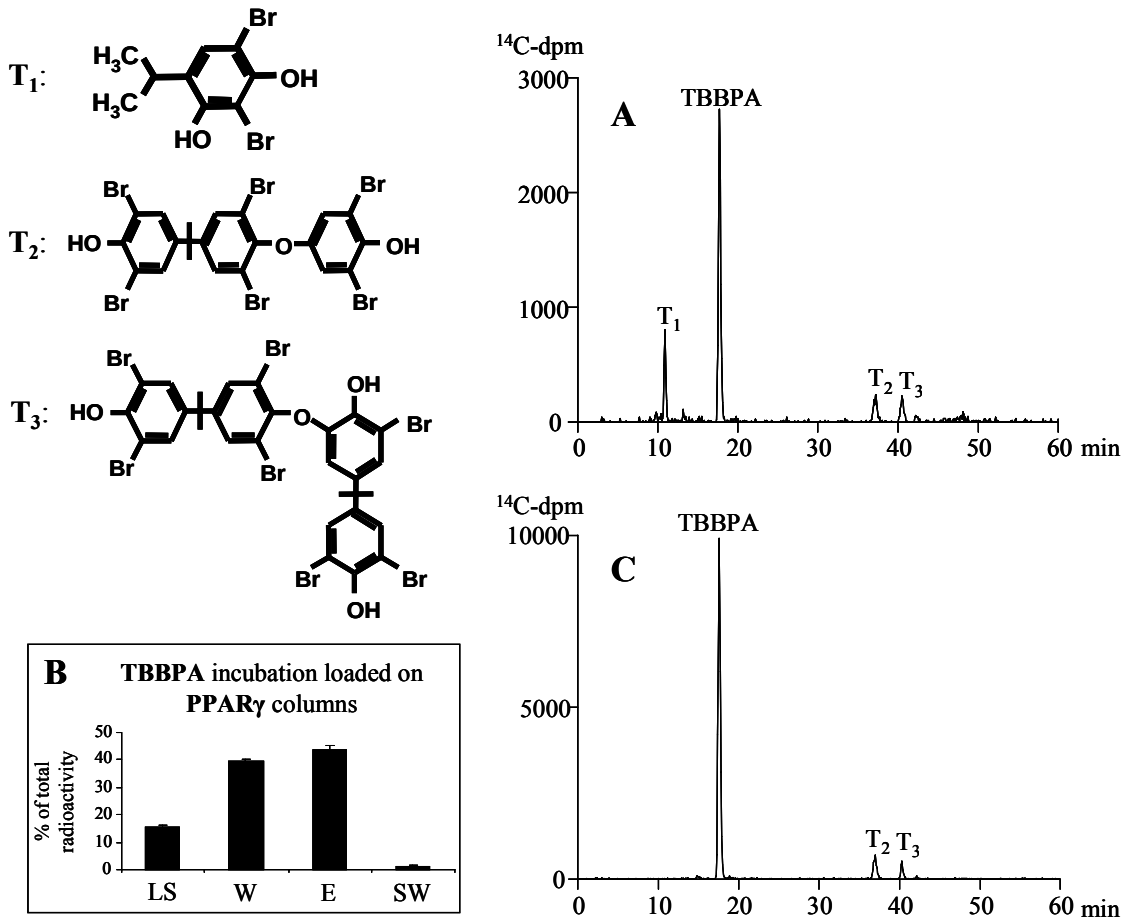

Figure 3. Typical radio-HPLC metabolic profile obtained from incubation of radio-labelled TBBPA (8 µM) with rat liver microsomes (A) and structure of the major metabolites observed. Radioactivity distribution in collected fractions (mean ± SD, n = 3) (B), and radio-chromatogram corresponding to eluted fractions (C) obtained from incubation supernatants loaded on PPARγ-based affinity columns.

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