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Property of cytochrome P450 1A inducibility by polychlorinated/brominated biphenyls (Co-PXBs) detected in Japanese breast milk

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ABSTRACT

Coplanar polychlorinated/brominated biphenyls (Co-PXBs) belong to a class of structurally similar chemicals known as polyhalogenated aromatic hydrocarbons. We found that the milk of Japanese primiparous and multiparous mothers was similarly contaminated with Co-PXB congeners. Co-PXBs time- and dose-dependently increased ethoxyresorufin-O-deethoxylase (EROD activity) in HepG2 cells. The EROD activity of liver microsomes collected from C57BL/6 mice exposed to these congeners substituted with one or two, and with three or five bromine atoms time-dependently decreased and increased, respectively. These results indicate that introducing bromine into the chemical frame of a polychlorinated biphenyl tends to increase CYP1A activity *in vitro* and *in vivo* and that the number substituted bromine atoms alters the metabolism profiles. If Co-PXBs are more toxic than Co-PCBs, our findings suggest that the TEQ of Co-PXBs is important for human health risk.

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1. Introduction

The specific, highly hazardous xenobiotics known as "dioxins" comprising dibenzo-*p*-dioxins, dibenzofurans

and biphenyls are environmental and food contaminants. These coplanar compounds are long-lived and ubiquitous in the environment; and are detectable at picomolar levels in foods and human tissues.

Similar to polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDDs/DFs), polybrominated and polychlorinated/brominated dioxins, such as polybrominated dibenzo-*p*-dioxins and dibenzofurans (PBDDs/DFs) and polychlorinated/brominated dibenzo-*p*-dioxins and dibenzofurans (PXDDs/DFs), are generated as by-products of plastic materials containing precursors such as brominated flame retardants (BFRs) during combustion in municipal solid waste (MSW) incinerators [1,2]. Several studies have identified PBDDs/DFs and PXDDs/DFs derived from BFRs in fly ash and/or flue gas in various incinerators [3,4]. To generate PXDDs/DFs from a mixture of bromine and chlorine is easier than to generate PBDDs/DFs *de novo* from all available bromine inside incinerators [5,6].

Coplanar polychlorinated (Co-PCBs) and coplanar poly-brominated (Co-PBBs) biphenyls have been investigated in detail [7–9], but little is known about coplanar polychlorinated/brominated biphenyls (Co-PXBs). These compounds belong to a class of structurally similar chemicals known as polyhalogenated aromatic hydrocarbons that includes human endocrine disruptors such as Co-PCBs and they can be formed by *de novo* synthesis from precursor BFRs. We estimated that the generations of Co-PXBs and PXDDs/DFs in municipal waste incinerators are similar, and that Co-PXBs are easier to form from a mixture of bromine and chlorine than Co-PBBs from all bromine available in an incinerator. Because information about contamination levels and the toxic potency of Co-PXBs is scant, further investigation is required [10–12].

Cytochrome P450 1A (CYP1A) enzymes comprise a key factor for human health as they can detoxify xenobiotics such as pesticides and other environmental contaminants. The ethoxyresorufin-*O*-deethoxylase (EROD) activity of CYP1A isozymes is widely used to measure CYP induction by dioxin-like compounds [13]. Increased EROD activity is an established parameter for determining the potency of aryl hydrocarbon receptor (AhR) agonists such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [14]. The relative effect potency (REP) of individual dioxins and their related compounds is used to determine toxic and biological effects relative to those of a reference compound, usually TCDD. The total toxic equivalent (TEQ) is operationally defined as the sum of the products of the concentration of each compound multiplied by its toxic equivalency factor (TEF), and it can be estimated as the total TCDD-like activity of a congener mixture [15]. In the absence of robust data about Co-PXB toxicity, TEFs have not been determined, but the TEF/REP values of Co-PXBs are essential to estimate the toxicity of these compounds.

Here, we investigated the amount of Co-PXB contamination in Japanese breast milk and CYP1A inducibility in HepG2 cells and in C57BL/6 mice exposed to Co-PXBs.

2. Material and methods

2.1. Dioxin standards

$^{13}C_{12}$-labelled and unlabeled PCDDs/DFs, Co-PCBs, PXDDs/DFs and Co-PXBs used in this study were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). The congeners comprised seven PCDDs, 10 PCDFs and 14 Co-PCBs with TEFs assigned by the WHO in 2006, as well as three PXDDs and five PXDFs as described [16]. The eight Co-PXBs were 3,3′,4,4′,5-pentachlorobiphenyl (PCB #126; PCB), 4′-monobromo-3,3′,4,5-tetrachlorobiphenyl (structurally similar to PCB #126; #126-Br), 4′-monobromo-2,3,3′,4-tetrachlorobiphenyl (structurally similar to PCB #105; #105-Br), 4′-monobromo-2,3′,4,5-tetrachlorobiphenyl (structurally similar to PCB #118; #118-Br), 4′-monobromo-3,3′,4,5,5′-pentachlorobiphenyl (structurally similar to PCB #169; #169-Br), 3,4-dibromo-3′,4′,5′-trichlorobiphenyl (structurally similar to PCB #126; #126-2Br), 3′4′5′-tribromo-3,4-dichlorobiphenyl (structurally similar to PCB #126; #126-3Br) and

3,3′,4,4′,5′-pentabromobiphenyl (structurally similar to PCB #126; PBB). Six congeners (PCB, #126-Br, #126-3Br, #105-Br, #118-Br and #169-Br) and five congeners (PCB, #126-Br, #126-2Br, #126-3Br and PBB) were used for the determination of contamination levels in breast milk and assays *in vitro* and *in vivo*, respectively.

2.2. Analysis of Co-PXBs in human breast milk

Our Institutional Review Board approved the study and four primiparous (age, 25–28 y) and six multiparous (age, 22–37 y) women provided written, informed consent to donate breast milk at 5 and 30 days after delivery of their newborns between 2010 and 2011 in Osaka Prefecture, Japan. Samples (50 mL) of breast milk collected three times on the same day and shortly after eating were stored at $-40\,^\circ$C. We used isotope dilution to analyze the amounts of PCDDs/DFs, PXDDs/DFs, Co-PCBs and Co-PXBs in breast milk samples as described previous our reports [11,12,16].

2.3. Methods for QA/QC and toxic evaluation of dioxin congeners

We compared the retention times and mass spectra of duplicate samples containing PCDDs/DFs, Co-PCBs, PXDDs/DFs and Co-PXBs with those of authentic standards. Peak area ratios for $(M)^+/(M+2)^+$ were determined on SIM chromatograms. Acceptance criteria were established from rates of -30% to 30% determined from authentic standards. The concentrations of pairs of congeners were corrected by comparisons with the recovery of their respective ^{13}C-internal standards. Data were collected from samples with recovery rates ranging from 60% to 120% of their respective internal standards. Assuming that the toxicity of PXDD/DF and Co-PXB congeners was equal to that of the corresponding PCDDs/DFs and Co-PCBs [15], we calculated the contribution ratios of PCDDs/DFs, PXDFs/DFs, Co-PCBs and Co-PXBs to the total TEQ using 2,3,7,8-tetrachlorobibenzo-*p*-dioxin (TCDD) equivalency factors (WHO-2006 TEF). Infant's daily intake was calculated based on the average infant's consumption and the average infant's body weight to be 600 mL/day and 4 kg, respectively, from mother's questionnaire using average TEQ concentration.

2.4. Cell culture

The human hepatocarcinoma cell line HepG2 (American Type Culture Collection, Rockville, MD, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C and used between passage numbers 25 and 30. We dissolved Co-PXB congeners in dimethyl sulfoxide (DMSO; final concentration in culture medium, 0.2% v/v) for assays *in vitro*. The cytotoxicity of the Co-PXB congeners and DMSO was determined using tetrazolium-based colorimetric WST-8 kits (Nacalai Tesque, Kyoto, Japan) and CytoTox96 NonRadioactive Cytotoxicity assays (Promega, Madison, WI, USA) of lactate dehydrogenase (LDH) according to the respective manufacturer's protocols.

2.5. Assays of EROD activity in vitro

HepG2 cells were seeded at a density of 3×10^4/well of 96-well microplates and incubated for 1 h at 37 °C with culture medium containing 5 μM 7-ethoxyresorufin (Sigma–Aldrich, St. Louis, MO, USA). Resorufin-associated fluorescence was measured at 550 nm excitation and 595 nm emission using a SpectraFluor microplate reader (TECAN, Männedorf, Switzerland). The EROD activity was normalized to cellular protein contents determined using BCA protein assay kits (Thermo Scientific, Rockford, IL, USA).

The dose–responses for EROD activity were modeled using the variable slope, sigmoid, Hill equation. The EROD EC_{50} is the concentration at which the induced enzyme activity was halfway between the bottom and top of each dose–response curve calculated using Prism software (Graph Pad Software). The relative effect potencies (REPs) of Co-PXBs were calculated based on EC_{50} values determined from EROD assays when the EC_{50} of TCDD was 1.0.

2.6. Assay of EROD activity in vivo

Female C57BL/6 mice (SLC Inc., Shizuoka, Japan) were housed at 23 ± 1.5 °C with a 12-h light/dark cycle and free access to standard rodent chow and water. The mice were allowed to adapt to their environment for at least 1 week and then experiments proceeded when they reached the age of 6 weeks according to the guidelines of Setsunan University.

Liver microsomes were prepared as described in ref. [17]. Briefly, the mice were given a single dose of 5, 10 and 50 nmol/kg of Co-PXBs dissolved in saline containing 1% ethanol and 10% Tween20 by oral gavage and then sacrificed with chloral hydrate 1, 2 or 3 days later. Excised livers were homogenized in 100 mM Tris–acetate buffer (pH 7.4), separated by centrifugation at 9,000 × g for 15 min at 4 °C, then the supernatant was separated by centrifugation at 105,000 × g for 60 min at 4 °C. The resulting pellet was homogenized in 100 mM potassium pyrophosphate buffer (pH 7.4) and separated by centrifugation at 105,000 × g for 60 min at 4 °C. The microsomal pellet was finally resuspended in 10 mM Tris–acetate buffer (pH 7.4) and stored at −80 °C. The protein concentration in the microsomes was determined according to the Lowry method using bovine serum albumin as the standard.

Diluted liver microsomes were incubated with the reaction mixture (50 mM phosphate buffer, 5 mM MgCl₂, 0.5 mM NADP⁺, 1 IU/mL G-6-P dehydrogenase and 2.4 μM 7-ethoxyresorufin) for 5 min at 37 °C, then the reaction started by adding 5 mM glucose-6-phosphate proceeded for 10 min at 37 °C. Resorufin production was measured using SPECTRA FLUOR.

2.7. Statistical analysis

The statistical significance of differences in means was determined using Student's *t*-test or a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. *P* values < 0.05 were considered significant. All data were statistically analyzed using Prism software.

3. Results

3.1. Contamination of breast milk with ∑PCDDs/DFs, ∑PXDDs/DFs, ∑Co-PCBs and ∑Co-PXBs

We determined the contamination levels of the congeners, ∑PCDDs/DFs (*n* = 17), ∑PXDDs/DFs (*n* = 8), ∑Co-PCBs (*n* = 14) and ∑Co-PXBs (*n* = 5) in mother's milk. The baseline characteristics of the 10 donors of breast milk did not significantly differ. The average lipid content of the milk was 3.6% (range, 2.3–4.6%). Tables 1 and 2 show the actual and TEQ concentrations of ∑PCDDs/DFs, ∑PXDDs/DFs, ∑Co-PCBs and ∑Co-PXBs at 5 and 30 days after neonatal delivery, respectively. Fig. 1A summarizes the contribution ratio to the total TEQ concentrations of the dioxin analogs, PCDDs/DFs, PXDDs/DFs, Co-PCBs and Co-PXBs. The major components were PCDDs/DFs, Co-PCBs and Co-PXBs, whereas the amount of PXDDs/DFs was negligible. This contribution of Co-PXBs cannot be ignored because they account for 10% of the total TEQ concentration and yet only five congeners were selected for analysis. The ratios of ∑PCDDs/DFs, ∑PXDDs/DFs, ∑Co-PCBs and

Table 1
Levels of PCDDs/DFs, PXDDs/DFs, Co-PCBs and Co-PXBs contamination in breast milk at 5 days after neonatal delivery in Japan.

Dioxin analog	Actual concentration (pg/g lipid)			TEQ concentration (pg-TEQ/g lipid)			Daily intake (pg-TEQ/kg b.w./day)
	Range	Median	Average	Range	Median	Average	
Primiparous donors							
∑PCDDs/DFs	42–172	42	84	2.4–12	5.1	6.3	28.4
∑PXDDs/DFs	0–22	0	8.8	0–0.012	0.0000085	0.003	0.014
∑Co-PCBs	12,866–54,863	12,866	30,539	1.1–7.7	3.8	4.1	18.5
∑Co-PXBs	12–33	12	22	0.46–1.1	0.78	0.8	3.6
Multiparous donors							
∑PCDDs/DFs	26–179	81	92	0.42–4.6	1.4	1.9	8.6
∑PXDDs/DFs	0–11	0	2.9	0–0.005	0	0.001	0.005
∑Co-PCBs	2138–48,999	10,882	16,516	0.29–7.2	2.9	3.0	13.5
∑Co-PXBs	9.5–54	22	27	0.42–1.4	0.73	0.81	3.6

Range represents minimum to maximum concentration of dioxin analogs. Data are shown as means of duplicate samples from primiparous (*n* = 4) and multiparous (*n* = 6) donors.

Table 2
Levels of PCDDs/DFs, PXDDs/DFs, Co-PCBs and Co-PXBs contamination of breast milk at 30 days after neonatal delivery in Japan.

Dioxin analog	Actual concentration (pg/g lipid)			TEQ concentration (pg-TEQ/g lipid)			Daily intake (pg-TEQ/kg b.w./day)
	Range	Median	Average	Range	Median	Average	
Primiparous donors							
∑PCDDs/DFs	38–161	144	114	1.9–11	3.0	5.4	24.3
∑PXDDs/DFs	0–8.9	4.8	4.6	0–0.002	0	0.001	0.005
∑Co-PCBs	14,261–59,860	29,218	34,446	1.8–7.1	4.3	4.4	19.8
∑Co-PXBs	19–35	25	26	0.51–0.84	0.76	0.70	3.2
Multiparous donors							
∑PCDDs/DFs	21–205	57	85	0.36–4.2	1.8	1.7	7.7
∑PXDDs/DFs	0–4.4	0.2	1.2	0–0.004	0.001	0.002	0.009
∑Co-PCBs	2749–15,441	11,418	10,257	0.56–3.2	1.1	1.9	8.6
∑Co-PXBs	13–28	21	21	0.45–1.2	0.68	0.75	3.4

Range represents minimum to maximum concentration of dioxin analogs. Data are shown as means of duplicate samples from primiparous ($n = 4$) and multiparous ($n = 6$) donors.

∑Co-PXBs to the total TEQ concentration did not significantly differ between primiparous and multiparous donors at 5 and 30 days after neonatal delivery.

We then compared the actual and TEQ concentrations of each Co-PXB congener in more detail. Tables 3 and 4 show the actual and TEQ concentrations of #105-Br, #118-Br, #126-Br, #126-3Br and #169-Br at 5 and 30 days after delivery, respectively. Fig. 1B and C summarizes the contribution of Co-PXB congeners to the total actual and total TEQ concentrations. The actual concentrations of #105-Br and #169-Br were high in all milk samples and those of #118-Br, #126-Br and #126-3Br were similar. On

Table 3
Levels of contamination with Co-PXB congeners in breast milk at 5 days after neonatal delivery in Japan.

Co-PXB congener	Actual concentration (pg/g lipid)			TEQ concentration (pg-TEQ/g lipid)			Daily intake (pg-TEQ/kg b.w./day)
	Range	Median	Average	Range	Median	Average	
Primiparous donors							
#105-Br	2.3–7.9	4.4	4.8	0.00007–0.0002	0.00013	0.00014	0.0006
#118-Br	0–5.2	2.0	2.3	0–0.00016	0.000062	0.000071	0.0003
#126-Br	1.3–4.8	2.6	2.8	0.13–0.48	0.26	0.28	1.3
#126-3Br	1.1–4.1	1.4	2.0	0.11–0.41	0.14	0.20	0.89
#169-Br	5.8–18	8.6	10	0.17–0.54	0.26	0.31	1.4
Multiparous donors							
#105-Br	0–7.6	5	7.2	0.00006–0.001	0.0001	0.0002	0.00097
#118-Br	2–18	2.9	3.4	0–0.00023	0.00009	0.0001	0.0005
#126-Br	0.8–5.6	2.2	2.5	0.08–0.56	0.22	0.25	1.1
#126-3Br	0.91–3.9	2.2	2.0	0.091–0.39	0.22	0.20	0.92
#169-Br	4.5–21	13	12	0.14–0.62	0.39	0.36	1.6

Range represents minimum to maximum concentration of Co-PXB congeners. Data are shown as means of duplicate samples from primiparous ($n = 4$) and multiparous ($n = 6$) donors.

Table 4
Levels of contamination with Co-PXB congeners in breast milk at 30 days after neonatal delivery in Japan.

Co-PXB congener	Actual concentration (pg/g lipid)			TEQ concentration (pg-TEQ/g lipid)			Daily intake (pg-TEQ/kg b.w./day)
	Range	Median	Average	Range	Median	Average	
Primiparous donors							
#105-Br	2.1–12	3.1	5.7	0.00006–0.0004	0.000093	0.000172	0.0008
#118-Br	6.4–8.5	7.9	7.6	0.0002–0.0003	0.00024	0.00023	0.0010
#126-Br	2.1–3.7	2.8	2.9	0.21–0.37	0.28	0.29	1.3
#126-3Br	0.47–2.7	1.8	1.7	0.047–0.27	0.18	0.17	0.75
#169-Br	6.1–9.6	9.3	8.3	0.18–0.29	0.28	0.25	1.1
Multiparous donors							
#105-Br	2.3–7.2	3.4	4.1	0.00007–0.0002	0.0001	0.0001	0.0006
#118-Br	1.9–5.9	3.4	3.6	0.00006–0.0002	0.0001	0.0001	0.0005
#126-Br	1.4–6.6	3.4	3.7	0.14–0.66	0.34	0.37	1.7
#126-3Br	0.41–2.4	1.1	1.3	0.041–0.24	0.11	0.13	0.56
#169-Br	3.3–13	8.6	8.4	0.1–0.39	0.26	0.25	1.1

Range represents minimum to maximum concentration of Co-PXB congeners. Data are shown as means of duplicate samples from primiparous ($n = 4$) and multiparous ($n = 6$) donors.
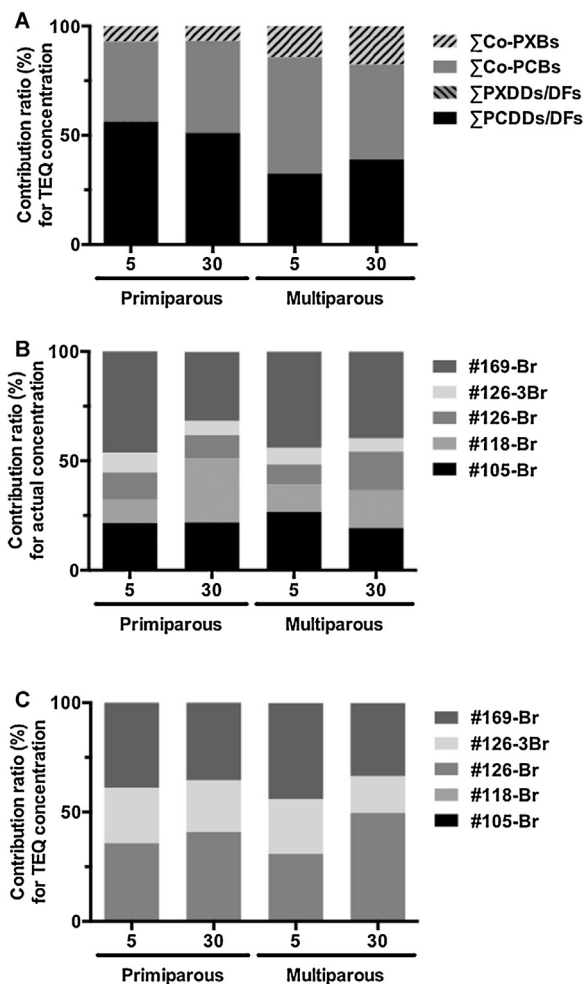
Fig. 1. Contribution rates of PCDDs/DFs, PXDDs/DFs, Co-PCBs and Co-PXB congeners to total TEQ and actual concentrations of dioxin analogs in breast milk from Japanese donors. Contribution indicated as ratios (%) of (A) total TEQ concentrations of four dioxin analogs (PCDDs/DFs, PXDDs/DFs, Co-PCBs and Co-PXBs), (B) total actual concentration of five Co-PXB congeners and (C) total TEQ concentrations of five Co-PXB congeners. TEQ concentrations of Co-PXBs were calculated based on assumption of similar toxicity between corresponding Co-PXB and Co-PCB congeners. Data are shown as means (primiparous, $n = 4$; multiparous, $n = 6$).

Fig. 2. Induction of cytochrome P450 1A activity in HepG2 cells incubated with Co-PXBs. HepG2 cells were incubated with 10 nM Co-PXBs for various lengths of time (A) and at various concentrations for 8 h (B), and then EROD activity was measured. Relative activity is expressed as fold induction compared with vehicle. Data are shown as means ± SD ($n = 5$). Abbreviations: #126-Br, 4′-monobromo-3,3′,4,5-tetrachlorobiphenyl; #126-2Br, 3,4-dibromo-3′,4′,5′-trichlorobiphenyl; #126-3Br, 3′4′5′-tribromo-3,4-dichlorobiphenyl; PBB: 3,3′,4,4′,5′-pentabromobiphenyl; PCB, 3,3′,4,4′,5-pentachlorobiphenyl.

the other hand, #126-Br, #126-3Br and #169-Br accounted for over 99% of the total TEQ concentration. Therefore, the toxicity of Co-PXB congener type #126 required evaluation as a human contaminant.

3.2. Cytochrome P450 1A activity induced by Co-PXBs determined as activity of EROD in vitro

Because PCB #126 was the most toxic Co-PCB and had dioxin-like activities, we focused on its congeners in which bromine was substituted with chlorine. We assayed EROD activity in HepG2 cells to determine whether Co-PXBs induced P450 1A activity. Fig. 2A shows that Co-PXBs time-dependently increased intracellular EROD activity and that it peaked at 6 h; thereafter, the peak activity was sustained
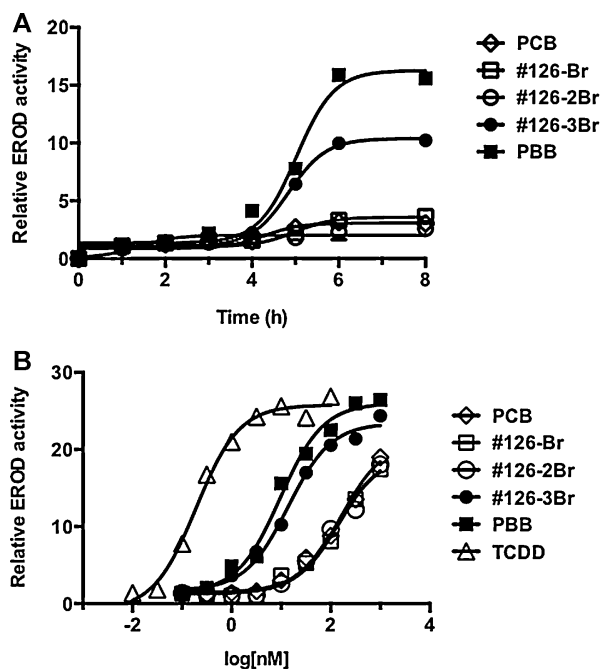
for 24 h (data not shown). The initial response times on time-response curves were similar among Co-PXB congeners. The activity of EROD dose-dependently increased at 8 h (Fig. 2B). However, the dose–response curves for #126-3Br and PBB obviously differed from those of PCB, #126-Br and #126-2Br. The EC_{50} values for EROD activity with TCDD, PCB, #126-Br, #126-2Br, #126-3Br and PBB were 0.19, 163.1, 149.4, 128.9, 13.78 and 9.51 nM, respectively. We calculated the REPs of Co-PXBs to determine the relative toxicity of Co-PXBs based on their EC_{50} values. The REPs for PCB, #126-Br, #126-2Br, #126-3Br and PBB were 0.0010, 0.0011, 0.0012, 0.0171 and 0.022, respectively. On the other hand, 1 μM #105-Br and #118-Br did not affect EROD activities, and the cytotoxicity was shown at over 1 μM (data not shown).

3.3. Changes in EROD activity in mice exposed to Co-PXBs

We assayed EROD activity *in vivo* using liver microsomes collected from C57BL/6 mice administered with Co-PXBs to confirm differences in EROD activity elicited by Co-PXBs *in vitro*. The activity of EROD induced by Co-PXBs dose-dependently increased (Fig. 3A). Activity did not significantly differ among the congeners at 50 nmol/kg, whereas at 5 and 10 nmol/kg, #126-3Br and PBB increased EROD activity more significantly than the other Co-PXB congeners, providing a 5.2- and 4.9-fold relative increase in the activity of PCB-treated mouse microsomes at
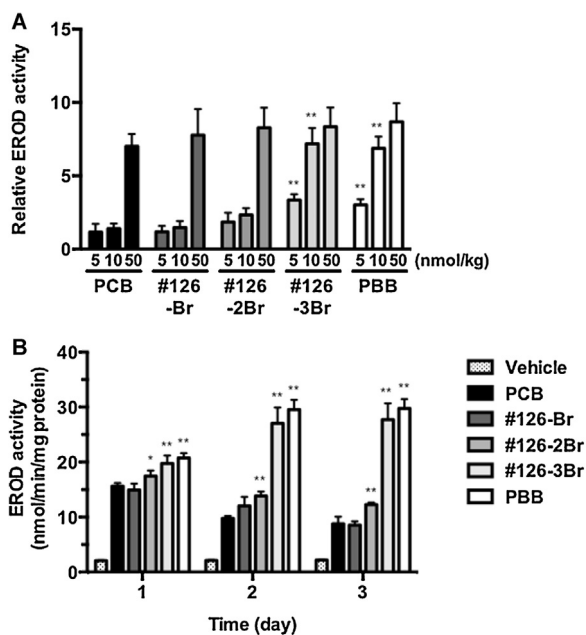
Fig. 3. CYP1A activity is enhanced in mouse liver microsomes incubated with Co-PXB. Mice were orally administered with Co-PXBs at 5, 10 and 50 nmol/kg and then EROD activity was measured in liver microsomes 2 days later (A). Mice were orally administered with Co-PXBs at 10 nmol/kg and then EROD activity was measured in liver microsomes at 1, 2 and 3 days later (B). Relative activity is expressed as fold induction compared with vehicle. Data are presented as means ± SE ($n = 10$). *$P < 0.05$ and **$P < 0.01$ compared with same concentration or same length of incubation with PCB. Abbreviations: #126-Br, 4′-monobromo-3,3′,4,5-tetrachlorobiphenyl; #126-2Br, 3,4-dibromo-3′,4′,5′-trichlorobiphenyl; #126-3Br, 3′4′5′-tribromo-3,4-dichlorobiphenyl; PBB, 3,3′,4,4′,5′-pentabromobiphenyl; PCB, 3,3′,4,4′,5-pentachlorobiphenyl.

10 nmol/kg, respectively. Fig. 3B shows that PCB, #126-Br and #126-2Br time-dependently decreased EROD activity in mouse microsomes. On the other hand, #126-3Br and PBB time-dependently increased the EROD activity, which remained the same between Days 2 and 3. These results indicated that introducing bromine into the chemical frame of a polychlorinated biphenyl tends to increase the expression of CYP1A activity *in vitro* and *in vivo*.

4. Discussion

Brominated flame retardants currently occupy the largest market share among such compounds because they are inexpensive and very efficient. However, although regulations and restrictions have recently increased regarding the production and use of BFRs due to safety concerns [18,19], an estimated 48,000 tons of BFRs were used in Japan during 2009 [20]. Schäfer and Ballschmiter reported that PBDDs/DFs and PXDDs/DFs are generated as undesirable by-products of plastics containing BFRs that are burned in MSW incinerators [8], and 1,243 incinerators burn about 34.5 million tons/year of MSW in Japan [21]. Similar to PBDDs/DFs and PXDDs/DFs formed in MSW incinerators, Co-PXBs can be also formed by *de novo* synthesis from precursor BFRs. There were only low levels of Co-PXB contamination in breast milk of donors in Spain

with a little amount of waste incinerated and BFRs compared in Japan [10]. Therefore, the contamination of breast milk with Co-PXBs in Japan might reflect BFR combustion in MSW incinerators, and it may be arose from regional association. A higher ratio of PXDDs/DFs is easier to generate with a mixture of bromine and chlorine than of PBDDs/DFs using all available bromine in an incinerator [1,2]. We estimated that the same is true for Co-PXBs compared with Co-PBBs during incineration under these respective conditions, and therefore focused on Co-PXBs. The level of pollution caused by these Co-PXBs as well as their toxicity in the environment and in humans is important to understand.

The present study was based on a previous investigation that found high toxicity levels and high concentrations of Co-PCB #126 in biological specimens such as breast milk [22–24]. We evaluated five Co-PXB congeners and also found high levels of Co-PXB contamination in breast milk from Japanese donors. Because Co-PXBs account for 10% of the total TEQ concentration and we evaluated only five congeners, their contribution cannot be ignored. Although the levels of Co-PCB contamination did not significantly differ between primiparous and multiparous donors, more Co-PCB was found in breast milk at 5, than at 30 days after birth, whereas this was not consistently true of Co-PXBs. These results suggested that the metabolism or excretion of Co-PXBs and Co-PCBs differs. Notably, levels of Co-PXBs are relatively high in infants due to intake through breast milk. However, only a few studies have investigated Co-PXB contamination and toxicity [10–12].

The EROD activity of CYP1A is widely used to determine the induction of CYPs by dioxin-like compounds [13]. We clarified that the amount of EROD activity induced by Co-PXBs and Co-PCBs was similar, although most likely via different pathways. Previous findings indicate that PCB is metabolized to 4′-hydroxy-3,3′,4,5,5′-pentachlorobiphenyl (OH-PCB) via 4′,5′-epoxide formation and a subsequent NIH-shift of the 4′-chlorine to the 5′-position [25]. Halogen atoms have a negative inductive effect and since this effect of bromine (3,4,5-tribromine) is lower than that of chlorine (3,4,5-trichlorine), #126-3Br was less reactive than PCB. If Co-PXBs were also metabolized via an NIH-shift, #126-3Br would be more difficult to metabolize than PCB. In addition, Dannan et al. reported that the bromination of both *para* positions seems to render PBB resistant to microsomal metabolism [26]. Based on these and the present findings, we assumed that the substitution of bromine to the *para* area (3,4,5-positions) of chlorinated biphenyl is necessary to resist microsomal metabolism. The endocrine system is targeted by OH-PCBs [27,28] that can be more toxic than their precursor PCBs [29,30]. Consequently, further studies are required to clarify Co-PXB metabolism and the disruptive effects of Co-PXBs and their metabolites on the endocrine system.

In conclusion, we showed that only five Co-PXB congeners significantly and similarly contributed to the amount of toxins in breast milk collected from primiparous and multiparous donors in Japan. We also showed that the ability of Co-PCBs and Co-PXBs, especially #126-3Br to induce CYP1A differ. If Co-PXBs are more toxic than Co-PCBs, our findings suggest that the TEQ levels of Co-PXBs

pose a risk to human health, especially for infants with an immature or compromised immune system.

Conflict of interest

The authors have no conflicts of interest declare.

Acknowledgements

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