Cod: 2.3013

FACILITATION OF ADIPOCYTE DIFFERENTIATION OF 3T3-L1 CELLS BY DEBROMINATED TETRABROMOBISPHENOL A COMPOUNDS DETECTED IN JAPANESE BREAST MILK

T. Yuzuriha[1], H. Kakutani[1], T. Nakao[1], S. Ohta[1]

[1]*Faculty of Pharmaceutical Sciences, Setsunan University, 45-1, Nagaotoge-cho, Hirakata, Osaka, 573-0101, Japan*

Introduction

##Tetrabromobisphenol A (TeBBPA) is considered to be a 'safe flame retardant' because its contamination level in breast milk samples is low, its half-life in the body is short, and no marked toxicity has been reported; however, we herein investigated not only TeBBPA, but also its debrominated congeners in order to determine the influence of TeBBPA on human health because it is debrominated in the environment and body. Information on the in vivo kinetics of these debrominated congeners is limited because their reference materials are not commercially available.

##We newly synthesized materials for the debrominated congeners tribromobisphenol A (TriBBPA), dibromobisphenol A (2,2'-DiBBPA, 2,6-DiBBPA), and monobromobisphenol A (MoBBPA) evaluated the actual extent of contamination with BPA, TeBBPA, and its debrominated congeners in breast milk samples.

##In the present study, to elucidate the biological homeostasis-disrupting effects of TeBBPA and its debrominated congeners, such as their influences on carbohydrate and lipid metabolism through PPARγ, we investigated the promotion of differentiation to adipocytes using 3T3-L1 cells

Materials and methods

1). Standard chemicals

##TeBBPA congeners, MoBBPA, 2,2'-DiBBPA, 2,6-DiBBPA, and TriBBPA were synthesized. The structures of the synthesized chemicals were confirmed by ESI-MS and NMR. By analyzing each spectrum, each fractionated compound was identified as MoBBPA, 2,2'-DiBBPA, 2,6-DiBBPA, or TriBBPA. The purity of all compounds was higher than 99%.

2). Analytical procedure for TeBBPA and debrominated congeners in human breast milk

After Institutional Review Board approval and informed consent, human breast milk samples (total volume of 150 mL) were collected from 12 nursing women (age; 23–37 years) one month after delivery between 2011 and 2013. TeBBPA and debrominated congeners in human breast milk were analysed by GC/MS.

3). Adipocyte differentiation and determination of lipid droplets

##3T3-L1 cells were incubated in the presence of TeBBPA congeners at 10 mM for 14 days. Lipid droplets were stained with Oil Red O. Morphological changes were observed under a microscope. Quantitative measurement of lipid droplets stained with Oil Red O. Oil Red O in lipid droplets was eluted with isopropanol, and optical density was measured at 540 nm. Intracellular triglyceride contents were measured and normalized to protein concentrations.

4). Adipogenic gene mRNA level and protein expression

##3T3-L1 cells were incubated in the presence of TeBBPA congeners at 10 mM for 14 days. aP2, PPARγ, C/EBPα mRNA and aP2 protein expression levels were measured by quantitative real-time PCR and western blot analysis, respectively. The relative mRNA level was expressed as a fold induction from that in DMSO-treated cells.

5). Luciferase reporter gene assay

##Transactivation of PPARγ in TeBBPA congener-induced HepG2 cells. HepG2 cells were co-transfected with PGV-P2-ACO, phRL-TK, and pcDNA3-hPPAR. Transfected cells were incubated in the presence of TeBBPA congeners for 24 h and then used in reporter gene assays. Luciferase activities from reporter plasmids were normalized to Renilla luciferase activities.

Results and discussion

##TriBBPA was detected at higher levels than that of TeBBPA, while DiBBPA and MoBBPA were detected at lower levels than that of TeBBPA in milk samples collected from 12 samples (Table 1), suggesting that most of the debrominated congeners, excluding BPA were derived from TeBBPA and excreted from the body through breast milk. Shi et al. and Cariou et al. previously investigated TeBBPA levels in breast milk samples from donors in China (N.D.–5.12 ng/g lipid) and France (0.062-37.3 ng/

g lipid, mean: 4.11 ng/g lipid). These values are comparable with our results (0.00674–0.344 ng/mL, mean: 0.0644 ng/mL, lipid 4.43 g/100 mL). Schauer et al. (2006) analyzed the metabolites of orally administered TeBBPA in humans and rats, and found that TeBBPA was not detected in human blood or urine; only its glucuronic acid conjugate was detected. In contrast, the glucuronic acid and sulfate conjugates of TeBBPA were both detected in the blood and urine of rats, whereas the unchanged form, TeBBPA, was only detected in blood. In previous studies on the metabolism of TeBBPA in the body, the TeBBPA administered was mostly conjugated with glucuronic acid and sulfate; however, the results of the present study suggested that TeBBPA was metabolized to TriBBPA through competitive metabolism by debromination and glucuronic acid conjugation, and then rapidly transferred to breast milk or excreted from the body through metabolism, such as glucuronic acid and sulfate conjugation.

##Riu et al. (2011) demonstrated that when TeBBPA was added to NIH-3T3 cells with 3 inducers: insulin, dexamethazone, and 3-isobuthyl-1-methylxanthine, the accumulation of fat droplets was promoted in cells and aP2 gene expression levels were increased 24 h after this addition. Masuno et al. (2002) also reported that BPA promoted the differentiation of 3T3-L1 cells to adipocytes. We investigated lipid droplet accumulation and aP2 gene expression induced by BPA, TeBBPA, and the 4 debrominated congeners using 3T3-L1 cells under the same conditions as above in order to investigate the actions of the synthesized debrominated congeners; however, no significant difference was noted among the 6 compounds in the presence of the 3 inducers after 24 h and thereafter, although aP2 gene expression levels were slightly different at 24 h. The differentiation of 3T3-L1 cells to adipocytes induced by the 3 inducers has beendivided into early (Day 0–2) and late (Day 2–4) phases. Based on previous findings and our experimental results (Fig. 1), we assumed that exposure to TeBBPA in the early phase of differentiation influenced the later differentiation and maturation of adipocytes.

##PPARγ is the master regulator of adipocyte differentiation. All debrominated congeners exhibited PPARγ activity, whereas only TeBBPA, TriBBPA, and 2,6-DiBBPA promoted adipocyte differentiation. A comparison between 2,2'-DiBBPA and 2,6-DiBBPA revealed that only 2,6-DiBBPA promoted differentiation, and the PPARγ activity level of 2,6-DiBBPA was slightly higher. Riu et al. (2011) showed that the 2 phenol groups of each benzene ring constituting TeBBPA formed hydrogen bonds with Ser387 and Ser342 in the PPARγ ligand-binding pocket, and 4 bromine atoms interacted with the ligand-binding pocket through van der Waals forces. Based on these findings, we assumed that retention of the chemical structure sandwiched by the 2 bromine atoms by at least one of the phenolic hydroxyl groups was necessary to maintain affinity to the ligand-binding pocket of PPARγ.

##Since PPARγ controls lipid and carbohydrate metabolism, substances that exhibit PPARγ activity may disrupt the energy balance in the body. Our study clarified that TeBBPA and its debrominated congener, TriBBPA, accumulated at high levels in breast milk and the debrominated congeners promoted adipocyte differentiation, showing that a comprehensive evaluation of the influences of these compounds including the debrominated congeners of TeBBPA on health in infants is necessary, in addition to the current evaluation of human contamination with BPA and TeBBPA through foods and baby bottles as well as their toxicities.

Acknowledgements

This study was supported by the Health and Labour Sciences Research Grant of Japan (H23-Food-Young Scientist-017, Grant no. 11103282) and by a Grant-in-Aid (Grant no. 25281032) from the Japan Society for the Promotion of Science.

References

1. Masuno, H. et al. J. Lipid Res. 43, 676–84 (2002).
2. Riu, A. et al. Environ. Health Perspect. 119, 1227–32 (2011).
3. Schauer, U. M. D., Völkel, W. & Dekant, W. Toxicol. Sci. 91, 49–58 (2006).

Table 1. Contamination levels (ng/mL) of BPA, MoBBPA, 2,2'-DiBBPA, 2,6-DiBBPA, TriBBPA and TeBBPA in 12 breast milk samples.

Sample	BPA	MoBBPA	2,2′-DiBBPA	2,6-DiBBPA	TriBBPA	TeBBPA
A	0.249	N.D.	N.D.	N.D.	0.0170	0.0612
B	N.D.	N.D.	N.D.	N.D.	0.253	0.0526
C	0.0808	0.00913	0.00148	0.00106	0.0293	0.0715
D	0.165	N.D.	N.D.	N.D.	0.145	0.0280
E	0.00514	N.D.	N.D.	N.D.	0.000936	0.0386
F	0.389	N.D.	0.00841	0.00389	0.0835	0.0138
G	0.255	0.0470	N.D.	N.D.	0.0161	0.00930
H	0.0295	0.0493	N.D.	N.D.	0.0412	0.101
I	3.17	0.0854	N.D.	N.D.	0.968	0.344
J	0.658	0.0104	N.D.	0.00983	0.0115	0.0255
K	6.72	N. D.	N.D.	N.D.	0.205	0.00674
L	2.36	0.0718	0.0168	0.0125	0.0483	0.0198
Mean	1.28	0.0455	0.01	0.01	0.152	0.0644

N.D. was below the LOQ. LOQ: Limit of quantitation. BPA; 0.04 pg/mL, MoBBPA; 0.2 pg/mL, 2,2'-DiBBPA; 0.2 pg/mL, 2,6-DiBBPA; 0.2 pg/mL, TriBBPA; 0.2 pg/mL, and TeBBPA; 0.36 pg/mL. LOQ was defined as 10 times (S/N=10) for the noise level.

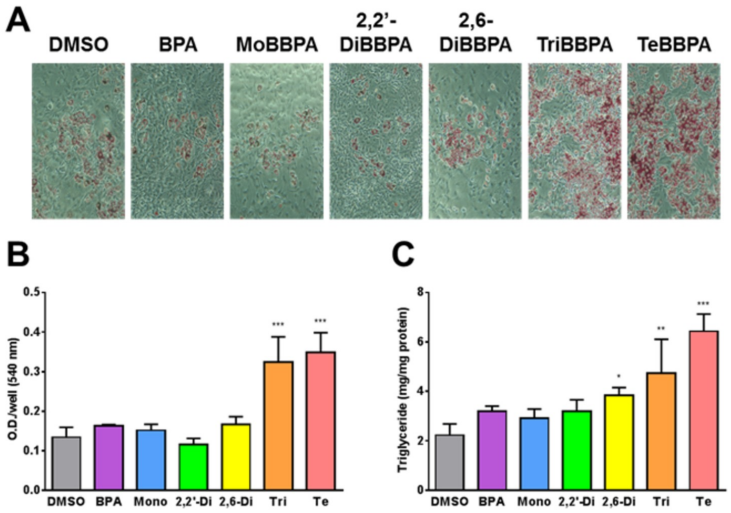

Fig. 1. Lipid accumulation in 3T3-L1 adipocytes. 3T3-L1. (A) Lipid droplets were stained with Oil Red O. (B) Quantitative measurement of lipid droplets stained with Oil Red O. (C) Intracellular triglyceride contents were measured. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from DMSO-treated cells. Data are presented as means ± SD (n=5). Mono; MoBBPA, 2,2'-Di; 2,2'-DiBBPA, 2,6-Di; 2,6-DiBBPA, Tri; TriBBPA, Te; TeBBPA.