TETRABROMOBISPHENOL A AND ITS DEBROMINATED COMPOUNDS REGULATE ADIPOGENESIS IN 3T3-L1 ADIPOCYTES

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Introduction

The prevalence of ovesity has increased at an alarming rate. The etiology of obesity is complex and multifactorial. In 1997, Barker hypothesized that fetal nutrition contributes to the development of metabolic syndrome diseases later in life. Since then it has been shown that pre- and postnatal genotype–environment interactions contribute to the high prevalence of obesity. The increased incidence of metabolic diseases correlates with substantial changes in the chemical environment over the past 40 years. Endocrine disrupting chemicals (EDC) like bisphenol A (BPA), bis(2-ethylhexyl) phthalate (DEHP) and tetrabromobisphenol A (TBBPA) are known to affect endocrine mediated pathways by binding to (nuclear) hormone receptors [1–3], which is specifically critical during development [4].

TBBPA is a highly lipophilic halogenated aromatic molecule and currently the most widely used type of brominated flame retardant (BFR). TBBPA and other BFRs are employed as additives in the manufacturing of office and home electronic equipment, such as computer boards, printers, mobile phones, televisions, and washing machines. It is released into the environment from both additive- and reactive-treated products, and measurable amounts were detected in air, soils, and sediments [5]. TBBPA have also been detected in pooled human serum[6], human milk[7] and umbilical cords from mother-infant pairs[8]. It has been suggested that the primary toxic effect of TBBPA, as of other brominated flame retardants, is the disruption of thyroid homeostasis. Moreover, TeBBPA can be degraded to follow a path of stepwise dehalogenation until becoming BPA, which is estrogenic endocrine disrupter, in environment and in living body. However, besides the toxicity of TeBBPA, it is unclear that of MoBBPA, DiBBPA and TriBBPA. Our previous results demonstrated that TBBPA showed agonistic activity on specifically peroxisome proliferator-activated receptor (PPAR) γ1 and γ2, tended to promote the PPARγ target gene expression in HepG2, and had the ability to accelerate differentiation of 3T3-L1 mouse fibroblasts into adipocytes.

Adipocyte differentiation (adipogenesis) is a complex process that includes coordinated changes in hormone sensitivity and gene expression in response to various stimuli including a number of transcription factors and lipid mediators. CCAAT/Enhancer-binding proteins (C/EBP) and PPARγ are important transcription factors involved in the regulation of adipogenesis. However, this familiar landscape has been considerably broadened in recent years by the dentification of novel factors that participate in the regulation of adipogenesis, either favoring or inhibiting it, through their effects on chromatin. Epigenetic determinants control the accessibility of promoter chromatin and establish lineage-specific heritable states of gene expression through the modulation of DNA methylation and posttranslational modification of core histones. Therefore, the expression and activities of histone-modifying enzymes should be distinctly regulated during adipocyte differentiation. The methylation of lysine residues in histones is an important epigenetic event that correlates with functionally distinct regions of chromatin. Setdb1 and Setd8 are the histone lysine methyltransferases (HKMTs) that trimethylate histone H3K9 and monomethylate histone H4K20, respectively.

In this study, we examined whether dehalogenated compounds of TBBPA had also the ability to promoted differentiation of 3T3-L1 mouse fibroblasts into adipocytes and, adipogenesis by the chemicals was interacted with epigenetic signals .

Materials and methods

1) *Reagents and Chemicals*

Tetrabromobisphenol A (TBBPA), dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), and insulin were purchased from Sigma (MO, USA). Chemical were dissolved as stock solutions in dimethyl sulfoxide (DMSO), and then the final concentration of the solvent in the culture medium was 0.1% v/v. Dulbecco’s modified Eagles medium (DMEM), penicillin and streptomycin mixture, and trypsin-EDTA were purchased

from Nacalai Tesque (Kyoto, Japan). Other reagents were obtained from Wako Pure Chemicals (Osaka, Japan) and Nacalai Tesque.

2) *Cell cultures*

Mouse 3T3-L1 fibroblast cells, obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan), were seeded in twelve-well tissue culture plates. The cells were cultured at 37℃ in a humidified atmosphere with 5% CO_2 in DMEM supplemented with 10% Fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Two day after confluence (designated as day 0), cells were treated with chemicals, which were added to the culture medium. This medium was subsequently replaced every other day for two weeks. The chemicals were dissolved in DMSO with a final maximal concentration of 0.1%. Therefore, a solvent control (0.1% DMSO) was included in each experiment.

3) *Oil red O staining*

The cells were stained with Oil-Red-O to detect oil droplets in adipocytes. After washing twice with phosphate buffered saline (PBS), cells were fixed with 4% paraformaldehyde in PBS at room temperature for 16 hours and then stained with 3.3 mg/ml Oil-red-O in 60% isopropanol for an hour. Cells were washed with PBS twice and observed under a microscope. Cells were considered lipid-positive when droplets were stained red. For quantitative analysis, stained Oil Red O was eluted with isopropanol and the optical absorbance was measured at a wavelength of 540 nm.!

4) *Measurement of triglycerides*

The cells were lysed with lysis buffer and measured for triglyceride content on day 14. The triglyceride content in the cell lysates were quantified using the Triglyceride E test WAKO. Intracellular triglycerides were normalized by protein conentrations.!

5) *RNA extraction and RT-PCR*

To determine the time-course effect of chemicals n adipocyte differentiation-related gene and PPARγ target gene expression. Total RNA was isolated from 3T3-L1 cells using ISOGEN reagent (Wako), following manufacturer's recommendations. Concentration of the isolated total RNA was determined by measuring the absorbance at 260 nm using a Nanodrop 2000 spectrophotometer (Thermo Scientific). RNA was reverse transcribed to complementary DNA (cDNA) using PrimeScript RT reagent Kit (TAKARA). Quantitative real-time PCR analyses were performed on a Thermal Cycler Dice Real Time System (TAKARA). The PCR master mixture contained SYBR Green supermix, 0.8 μM forward and reverse primer, and cDNA in a total volume of 20 μl. The following program was used for denaturation and amplification of the cDNA: 30 sec at 95 °C, followed by 40 cycles of 5 sec at 95 °C and 30 sec at 60 °C. The expression levels of target genes were normalized using β-actin as an internal control.

Results and discussion

To determine whether TBBPA related compounds can trigger 3T3-L1 fibroblasts to differentiate into adipocytes, the confluent culture of 3T3-L1 fibroblasts were treated with or without 10 μM BPA related compounds for 2 weeks. We investigated the effect of the chemicals on the expression of adipogenic genes such as aP2 and PPARγ by quantitative PCR. As shown in Fig. 1, by the treatment with TBBPA increases aP2 and PPARγ mRNA expression on day 14. Similarly, these expressions were increased following the number of bromine of the chemicals. Moreover, we observed Oil red O stained intracellular lipid droplets on day 14 after initiating cell differentiation (Fig. 2A, B). Intracellular lipid accumulation was increased in the cell treated with TBBPA, TriBBPA and 3,5-DiBBPA (Fig. 2). These results suggest that TBBPA, TriBBPA and 3,5-DiBBPA can trigger 3T3-L1 fibroblasts to differentiate into adipocytes.

Then, to investigate whether adipogenesis by the chemicals exposure was correlated with epigenetic signals, the confluent culture of 3T3-L1 fibroblasts were treated with control (DMSO 0.1%), BPA (10 μM) and TBBPA (10 μM) for 2 weeks, and collected for RNA samples every 4 or 5 days. At first, we analysed the expression of transcription factors, C/EBPs and PPARγ. It is reported that C/EBPβ is induced very early during differentiation, and these in turn activate two critical proadipogenic transcription factors, PPARγ and C/EBPα. PPARγ and

C/EBPα mutually stimuliate each other and mediate the transition to the adipocyte phenotype. The expressions of the factors did not change by BPA exposure. On the other hands, C/EBPα and PPARγ expressions by TBBPA exposure were increased gradually during adipogenesis. In addition, C/EBPβ expression by TBBPA exposure trended to be slightly increased on day 9 (Fig. 3A). Next, to examine if these SET domain proteins (Setdb1 and Setd8) expression show altered by the chemicals. As shown in Fig. 3B, Setdb1 mRNA equally degraded either in the absence or presence of the chemicals. In comparison, we observed that Setd8 mRNA was increased gradually during adipogenesis by TBBPA exposure. This increase curve was similar to that of PPARγ. This result suggested that adipocyte differentiation induced by TBBPA was interacted with histone methylation.

In this study, we showed that TBBPA and the other brominated BPA resulted in stimulation of 3T3-L1 adipocyte differentiation. Obesity is one of the greatest concerns in public health. Obesity is the result of an increase in body fat mass produced by either an enlargement of fat cells or number of these cells, or both. Since the brominated BPA were able to enter fibroblasts in the differentiation process and enhance the adipocyte conversion, this suggests *in vivo* prolonged exposure to the chemicals might increase body fat mass and involve the development of obesity. Moreover adipocyte differentiation induced by TBBPA was interacted with histone methylation. Further study is needed to clarify toxic mechanism of the chemicals in adipose tissue.

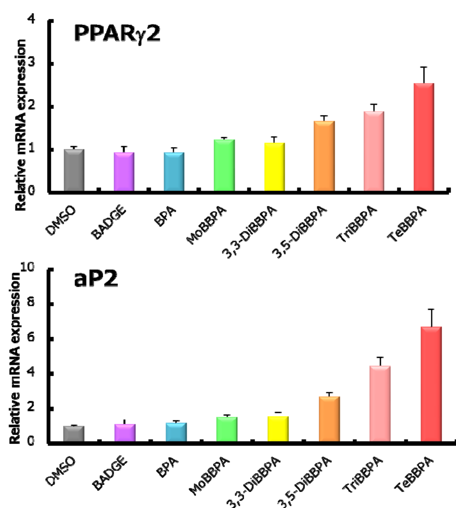

Fig. 1 Effect of BPA related compounds on adipocyte-specific gene expression in 3T3-L1 preadipocytes

The confluent culture of 3T3-L1 cells were treated with or without BPA related compounds up to 14. The levels of mRNA were corrected relative to that of β-actin mRNA. Values given for these relative mRNA levels are the mean ± S.D. for three experiments.

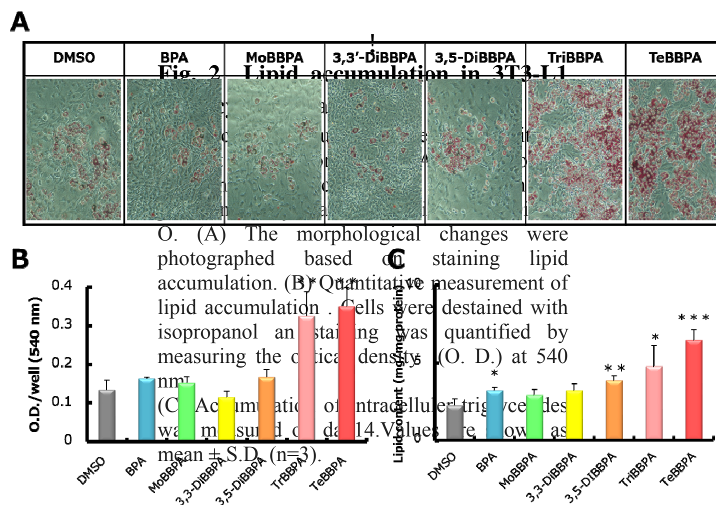

Fig. 2 Lipid accumulation in 3T3-L1 [illegible]

[illegible] O. (A) The morphological changes were photographed based on staining lipid accumulation. (B) Quantitative measurement of lipid accumulation . Cells were destained with isopropanol an[illegible] stai[illegible]ng was quantified by measuring the o[illegible]tica[illegible] density (O. D.) at 540 nm.
(C) Ac[illegible]mu[illegible]atio[illegible] of intracelluler trig[illegible]ce[illegible]des wa[illegible] m[illegible]su[illegible]d o[illegible] da[illegible] 14.Values [illegible]re [illegible]ow[illegible] as mean ± S.D. (n=3).

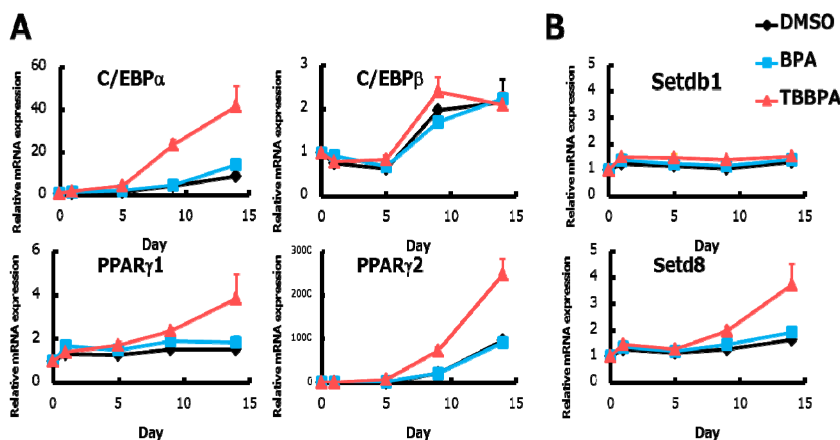

Fig. 3 Effect of TBBPA and BPA on adipocyte-specific transcription factors and histone methyltransferase mRNA expressions in 3T3-L1 preadipocytes

The confluent culture of 3T3-L1 cells were treated with or without the chemicals up to 14. The time course of these genes. The levels of mRNA were corrected relative to that of β-actin mRNA. Values given for these relative mRNA levels are the mean ± S.D. for three experiments. (A) Transcription factors (B) histone methyltransferase

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