TETRABROMOBISPHENOL A REGULATES MRNA EXPRESSION INVOLVED IN ADIPOGENESIS IN 3T3-L1 ADIPOCYTES THROUGH PPARγ ACTIVATION

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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].

Tetrabromobisphenol A (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, andwashing 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, previous results demonstrated that TBBPA showed agonistic activity on specifically peroxisome proliferator-activated receptor (PPAR) γ1 and γ2, and tended to promote the PPARγ target gene expression in HepG2.

PPARγ is nuclear hormone receptor that function as transcription factors, regulating the expression of genes involved in the metabolism of fatty acids and carbohydrates. PPARγ is highly expressed in adipocytes and, to a lesser extent, in other tissues, including the liver. The evidence supporting a key role for PPARγ in adipogenesis is strong, but it is entirely based on "gain of function" experiments. For example, it has been shown that the ectopic expression and activation of PPARγ in undetermined fibroblasts are sufficient to induce an adipogenic response that includes morphological changes, lipid accumulation, and expression of most of the genes characteristic of this cell type.

In this study, we examined whether TBBPA had the ability to accelerate differentiation of 3T3-L1 mouse fibroblasts into adipocytes.

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

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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. One day after confluence (designated as day 0), cell differentiation was induced by adipogenesis initiation media (0.5 mM IBMX, 0.25 μM DEX, and 0.1 μM insulin), which were added to the culture medium. This medium was replaced by DMEM containing 10%FBS 48h after initiating differentiation and was subsequently replaced every other day. TBBPA was added as a supplement from day 0 to day 7 after induction of differentiation and also during every media change.

3) *Oil red O staining*

Day1, 3, 5, 7, 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) *RNA extraction and RT-PCR*

To determine the time-course effect of TBBPA n adipocyte differentiation-related gene and PPARγ target gene expression, cells were harvested at 1, 3, 5, and 7 days after initiating differentiation. 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 can trigger 3T3-L1 fibroblasts to differentiate into adipocytes, the confluent culture of 3T3-L1 fibroblasts were treated with or without 10 μM TBBPA for 1, 3, 5, 7 days. We investigated the effect of TBBPA on the expression of adipogenic genes such as aP2 and PPARγ by quantitative PCR. As shown in Fig. 1A, by the treatment with TBBPA increases aP2 mRNA expression on day 1, 3, 5, and 7 was 2.7, 4.9, 20, and 23 fold, respectively. Similarly, PPARγ mRNA expression were increased 1.4, 1.5, 2.7, 3.5 fold. We also examined whether TBBPA induced differentiation via activation of PPARγ. We pre-treated cells with 10 μM GW9662 during treatment of TBBPA for 7 days. Up-regulation of aP2 and PPARγ mRNA expression was significantly inhibited by GW9662 (Fig. 1B). These results suggest that TBBPA can trigger 3T3-L1 fibroblasts to differentiate into adipocytes via PPARγ activity.

Next, to investigate whether TBBPA can accelerate adipocyte conversion, the confluent culture of 3T3-L1 fibroblasts were treated with a combination of IBMX, DEX, and insulin in the presence of TBBPA during the first 2-day treatment period. We observed Oil red O stained intracellular lipid droplets on day 5, and 7 after initiating cell differentiation (Fig. 2A). Intracellular lipid accumulation was increased cell treated with TBBPA

(Fig. 2B). Furthermore, aP2 and PPARγ expression levels also increased every 2 days (Fig. 3A). Thus, TBBPA treatment enhanced adipocyte differentiation of 3T3-L1 preadipocytes.

PPARγ is highly expressed in adipocyte. To investigate the effects of TBBPA on the PPARγ activation, we measured mRNA expression of PPARγ target genes such as CD36 and fatty acid synthase (FAS) in 3T3-L1 adipocytes treated with or without TBBPA. The TBBPA treated 3T3-L1 adipocytes showed significant induction of CD36 and FAS mRNA expression in comparison to control cells (DMSO treated), as shown in Fig. 3B.

In this study, we showed that TBBPA resulted in stimulation of 3T3-L1 adipocyte differentiation via PPARγ activation. 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 TBBPA was able to enter fibroblasts in the differentiation process and enhance the adipocyte conversion with differentiation media, this suggests *in vivo* prolonged exposure to TBBPA might increase body fat mass and involve the development of obesity. Further study is needed to clarify toxic mechanism of TBBPA in adipose tissue liver *in vivo*.

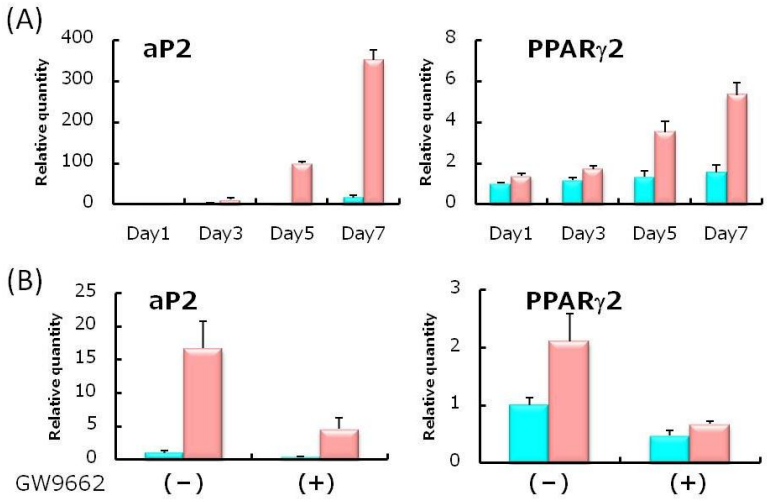

Fig. 1 Effect of TBBPA on adipocyte-specific gene expression in 3T3-L1 preadipocytes

(A) The confluent culture of 3T3-L1 cells were treated with or without TBBPA up to 7. The time course of adipocyte specific gene expression..

(B) The confluent culture of 3T3-L1 cells were treated with or without GW9662 during treatment TBBPA for 7 days. 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.

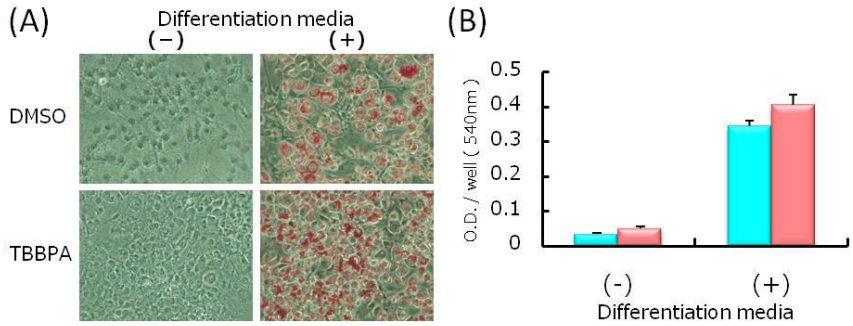

Fig. 2 Lipid accumulation in 3T3-L1 adipocytes was assessed by Oil red O staining

The confluent cultures were treated with a combination of adipogenesis initiation media with or without TBBPA for two days, and subsequently treated with TBBPA up to day 7. At the end of the experiments, the cells were fixed with 4% paraformaldehyde and then stained with Oil red O. (A) The morphological changes were photographed based on staining lipid accumulation.

(B) Quantitative measurement of lipid accumulation . Cells were destained with isopropanol an staining was quantified by measuring the optical density (O. D.) at 540 nm. Values are shown as mean ± S.D. (n=3).

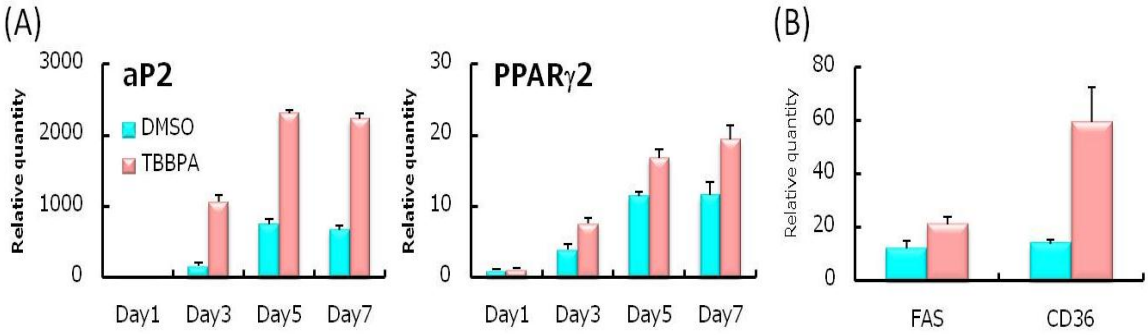

Fig. 3 Effect of TBBPA on adipocyte-specific gene and PPARγ target gene expression in 3T3-L1 adipocytes

The confluent cultures were treated with a combination of adipogenesis initiation media with or without TBBPA for two days, and subsequently treated with TBBPA up to day 7. 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) adipocyte specific gene (aP2, PPARγ) (B) PPARγ target gene (CD36, FAS)

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