Investigation on effect of organophosphorus flame retardants (PFRs) on metabolic disease related receptors

Tomohiro Yuzuriha*, Hideki Kakutani, Teruyuki Nakao, and Souichi Ohta

Faculty of Pharmaceutical Sciences, Setsunan University, 45-1, Nagaotoge-cho, Hirakata, Osaka, 573-0101, Japan. *: tomohiro.yuzuriha@pharm.setsunan.ac.jp

Introduction:

Flame retardants (FRs) are used in our daily necessities and home appliances such as plastics, rubber and fibers for the purpose of prevention spreading fire in use. Brominated flame retardants (BFRs) have been mainly used in Japan. However, polybrominated diphenyl ethers (PBDEs), which is one of BFRs, has been regulated by the Stockholm Convention and Act on the Evaluation of Chemical Substances and Regulation of Their Manufacture, etc in Japan due to high accumulation to living bodies, thyroid hormone disturbance action, and so on. For this reason, organophosphorus flame retardants (PFRs) have been widely used as substitutes for BFRs in recent years (**Figure 1**). The demand for PFRs has increased sharply since around 1998, and now 21,500 t is used. However, some PFRs, e.g. tris(1,3-dichloro-2-propyl) phosphate (TDCPP) are regulated by laws on household products regulation for textiles, beds, curtains, and floor coverings in Japan. Furthermore, restrictions on PFRs such as Reach regulation in EU and prohibition of TDCPP and tris(2-ethylhexyl) phosphate (T(2EH)P) in Washington State etc. in North America are under way. Regarding the toxicity of PFRs, triphenyl phosphate (TPP) shows antiandrogen action in in vitro studies[1], TDCPP shows thyroid hormone secretion and disturbance action[2], and so on. Therefore, there is concern about health effects on humans.

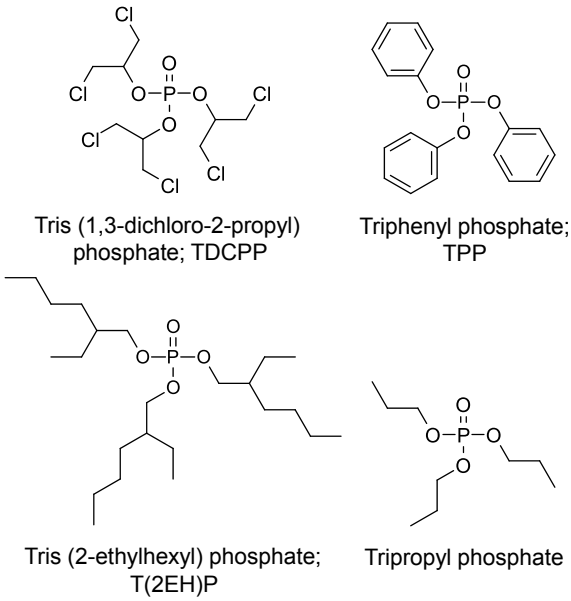

Figure 1 Structures of PFRs.

On the other hand, in our laboratory, human breast milk was analyzed, and various types of PFRs including TPP and Tripropyl phosphate were detected. We also found that TPP has cholinesterase activity. However, there are few reports on toxicity of these PFRs, and it is urgent to study more various toxicity for various types of PFRs. It has been reported that TPP detected at a relatively high concentration in human breast milk has a peroxisome proliferator-related receptor gamma (PPARγ) activating effects[3,4]. PPARγ is a nuclear receptor that controls the transcription of genes related to energy metabolism such as lipid and sugar metabolism and is involved in the onset and exacerbation of metabolic diseases typified by obesity and diabetes[5]. The authors group revealed that tetrabromobisphenol A (TeBBPA) which is one of BFRs

and its debrominated congeners have high affinity for PPARγ and affect adipocyte differentiation from mouse fibroblast cell line 3T3-L1 cells[6].

The authors speculated that other PFRs similarly show nuclear receptors including PPARγ activating action and eventually energy metabolism disrupting action. In this study, we aim to elucidate the influence of PFRs on the energy metabolism system, and as its first approach, we evaluated nuclear receptors activity using reporter gene assays for eight kinds of PFRs including TPP. In addition, we examined the effect on the expression level of metabolism-related genes.

Materials and methods:

1) Chemicals

We used eight kinds of PFRs as below: Triphenyl phosphate; TPP, Tris(2-ethylhexyl) phosphate; T(2EH)P, Tri-*o*-cresyl phosphate; ToCreP, Tri-*m*-cresyl phosphate; TmCreP, Tris(3,5-dimethylphenyl) phosphate; T35DMPP, Tris(2,6-dimethylphenyl) phosphate; T26DMPP, Tris(2-isopropylphenyl) phosphate; T3IPPP, Tris(3-isopropylphenyl) phosphate; T2IPPP.

2) Cell culture

Human hepatocarcinoma HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nacalai tesque, Kyoto, Japan) containing 10% fetal bovine serum (FBS) (SIGMA-ALDRICH, MO, USA), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Nacalai tesque) at 37°C. The cytotoxicity of the chemicals and DMSO was determined using a tetrazolium-based colorimetric assay, the WST-8 kit (Nacalai tesque), according to the manufacturer's protocol.

3) Transient transfections and luciferase assays

Luciferase assays were performed as described previously[7]. HepG2 cells (3×10^4 cells/well) were cultured in DMEM supplemented with 10% HyClone charcoal/dextran-treated FBS (Thermo Scientific, MA, USA) on a 96-well plate before plasmid transfection. After 16–20 h, the cells were transfected with 80 ng of PGV-P2-ACO, 20 ng of phRL-TK, and 10 ng of nuclear receptors expression plasmids using Lipofectamine 2000 (Invitrogen, CA, USA) for 24 h. After transfection, the cells were incubated with the chemicals for 24 h, and then firefly and Renilla luciferase activities were both quantified using a Dual-Luciferase Reporter Assay System (Promega, WI, USA) and a luminometer (Berthold technologies, Bad Wildbad, Germany) according to the manufacturer's instructions.

4) Quantitative real-time RT-PCR

Total RNA was isolated from cultured cells using ISOGEN (Nippongene, Tokyo, Japan) according to the manufacturer's instructions. The first strand cDNA was synthesized from total RNA of each sample using the PrimeScript™ RT reagent Kit (TaKaRa Bio, Kyoto, Japan). The cDNAs were used as templates for individual PCR reactions using specific primer sets (Invitrogen). PCR reactions were carried out using KAPA SYBR FAST Universal qPCR kit (Kappa Biosystems, MA, USA). β-actin were used for normalizing each expression data set.

5) Statistical analysis

Statistical analyses were performed by two sample t-test or Dunnett's multiple comparison test using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan)[8], which is a graphical user

interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics.

Results and discussion:

To determine the concentration of PFRs to be used in subsequent experiments, the cytotoxicity of eight kinds of PFRs to HepG2 cells was examined using tetrazolium-based colorimetric assay. As a result, no significant cytotoxicity was observed in all PFRs at 1-100 μM, sand therefore, the maximum concentration was set at 100 μM in subsequent experiments.

Next, we performed a luciferase assay using HepG2 cells and investigated the nuclear receptors (PPARα, PPARδ, PPARγ, farnesoid X receptor (FXR), etc.) activating effect of eight kinds of PFRs. As a result, several PFRs were observed to enhance or attenuate luciferase activity in various nuclear receptor activity evaluation systems. Depend on PFRs concentration, their PPARγ activity was also increased (**Figure 2**). It is suggesting that these PFRs disrupt nuclear receptor activity.

Furthermore, in order to investigate the influence of PFRs on the energy metabolism system via nuclear receptors, it was examined that the effect of PFRs on the expression level of energy metabolism-related genes in HepG2 cells. As a result of investigating mRNA expression level of nuclear receptor target gene using quantitative RT-PCR method, PFRs varied the expression levels of several metabolism-related genes (**Figure 3**). These results suggest that PFRs disrupt energy metabolism via nuclear receptors including PPARγ.

PFR is widely used all over the world as a relatively safe flame retardant. In our previous studies, various types of PFRs have been detected from environmental samples and biological samples, thus there is a possibility that they may disturb the energy homeostasis of the living body. However, detailed biotoxic effects have not been elucidated.

Our study clarified that PFRs regulate the expression of metabolism-related genes via nuclear receptors and disturb energy metabolism. Further study is needed to reveal the energy metabolism disturbance action of PFRs and its mechanism.

References:

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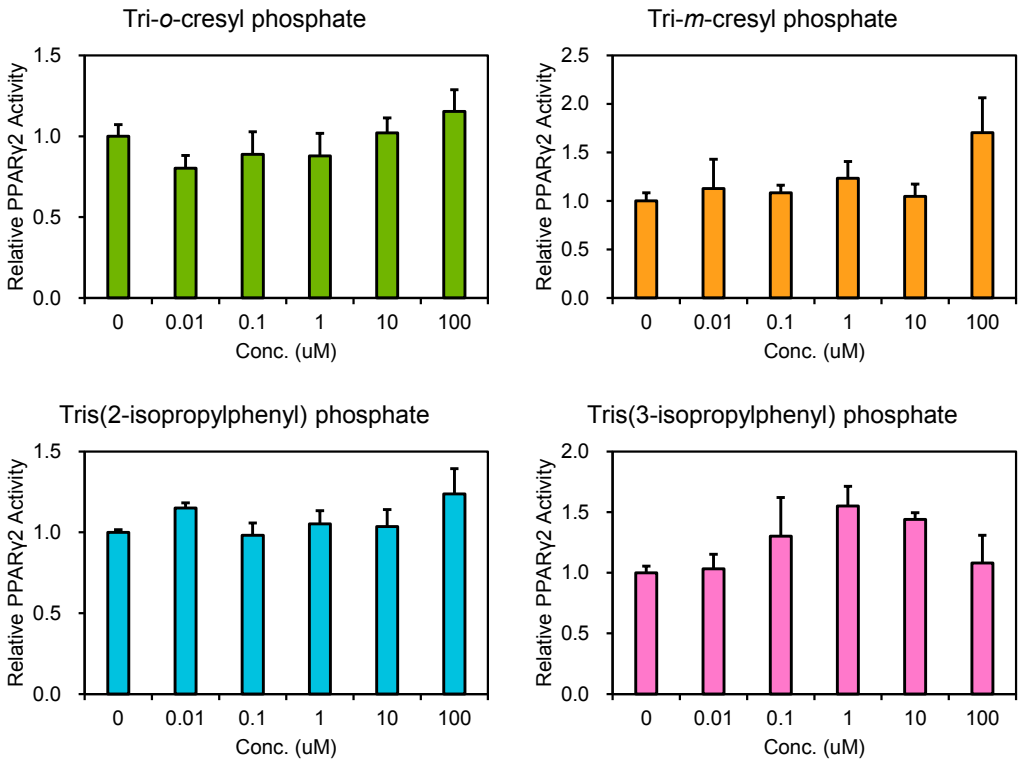

Figure 2 Transcriptional activation of PPARγ2 by PFRs.

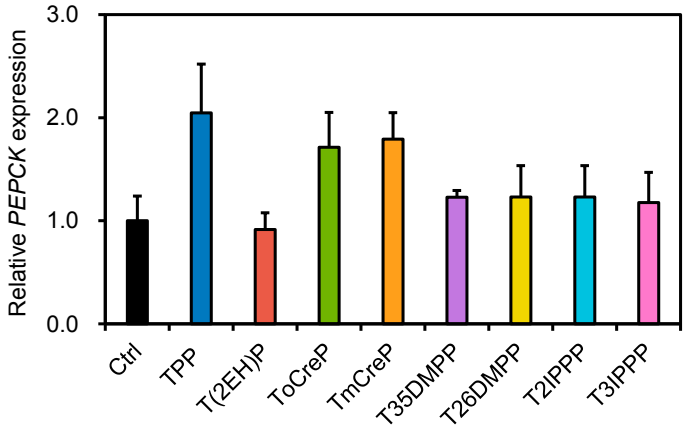

Figure 3 PFRs increase PPARγ target genes.