2) Cell culture

Human hepatocarcinoma HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nacalai tesque) containing 10% fetal bovine serum (FBS) (SIGMA-ALDRICH), 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. HepG2 cells (3×10^4 cells/well) were cultured in DMEM supplemented with 10% charcoal/dextran-treated FBS (Thermo Fisher Scientific) on a 96-well plate before plasmid transfection. After 16–20 h, the cells were transfected with 80 ng of PGV-P2-ACOx2, 20 ng of phRL-TK, and 10 ng of nuclear receptors expression plasmids using Lipofectamine 2000 (Invitrogen) 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) and a luminometer (Berthold technologies) according to the manufacturer's instructions. Rosiglitazone (PPARγ) and GW501516 (PPARδ) were used as positive controls.

4) Quantitative real-time RT-PCR

Total RNA was isolated from cultured cells using ISOGEN (Nippon gene) 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). 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). β-actin were used for normalizing each expression data set.

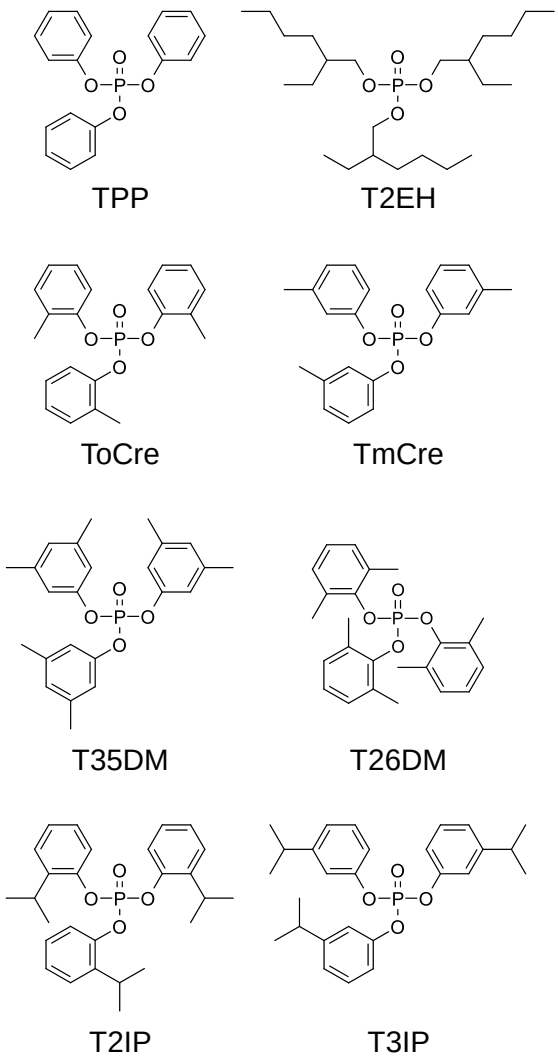

Figure 2 Structures of organophosphorus flame retardants used in this study.

5) Western blotting

HepG2 cells were lysed in RIPA buffer (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail) and standed on ice for 30 min. Cell lysates were centrifuged at 14,000 g for 15 min and resultant supernatants were stored at -80°C. After cell lysates had been prepared, protein contents were determined with a BCA protein assay kit (Thermo Fisher Scientific). The lysates were boiled in sample buffer and resolved by SDS-PAGE. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. Western blot analyses were performed using anti-human PPARγ (Santa Cruz Biotechnology), or anti-GAPDH (Santa Cruz Biotechnology) antibodies. After incubating with a horseradish peroxidase-conjugated detection antibody (MerckMillipore), the signal was visualized by chemiluminescence (Nacalai Tesque) using ChemiDoc (Bio-Rad Laboratories,Inc.).

6) 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), 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:

In order 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 10 μM, therefore, the maximum concentration was set at 10 μM in subsequent experiments.

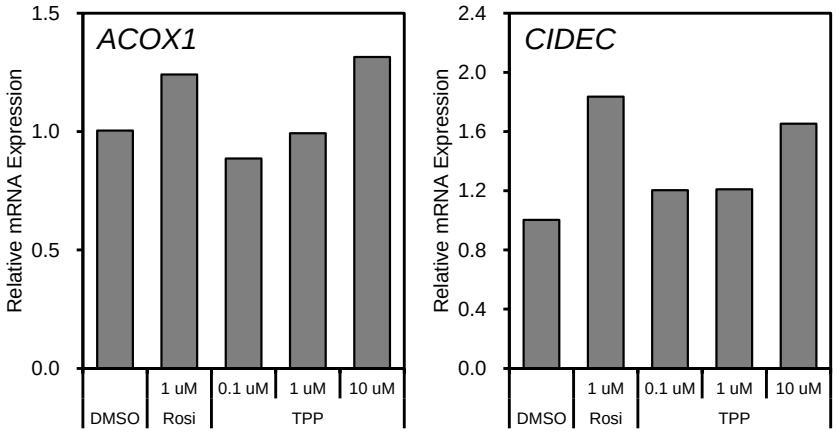

Figure 3 TPP increases the expression level of genes related glucose and lipid metabolism. (*ACOX1*; *acyl-coenzyme A oxidase 1, CIDEC; cell death inducing DFFA like effector C)*

HepG2 cells were stimulated with eight PFRs and mRNA expression levels were examined by quantitative real-time RT-PCR, in order to evaluate the influence on expression levels of genes related to glucose and lipid metabolism in the liver. TPP increased the expression level of these genes (*e.g. acyl-coenzyme A oxidase 1* (*ACOX1*), *cell death inducing DFFA like effector C* (*CIDEC*)) in a concentration-dependent manner (**Figure 3**). *ACOX1* gene is mainly transcriptionally regulated by PPARα, and the protein encoded by this gene catalyzes the desaturation of acyl-CoAs to 2-trans-enoyl-CoAs. *CIDEC* is mainly transcriptionally regulated by PPARγ, and the protein encoded by this gene promotes lipid droplet formation in adipocytes and may mediate adipocyte apotosis. This gene is not expressed in normal liver but only in fatty liver[7], suggesting that TPP may induce hepatic steatosis.

As the mechanism by which TPP increase the expression level of PPAR target gene, there are mainly two possibilities. The one is that TPP may be agonists of PPAR, and the second is that TPP may increase the expression level of PPAR. Therefore, first, a reporter gene assay was performed to evaluate PPAR (α, γ, δ) activity of PFRs. As a result, none of PFRs showed its activation effect on PPARγ and δ (**Figure 4**). Next, changes in gene expression levels of PPAR upon stimulation with PFRs were examined. As a result, it was observed that TPP in which the expression levels of metabolism related genes were varied increased the expression levels of PPARα and PPARγ genes in a concentration-dependent manner (**Figure 5**). It was suggested that PFRs promotes transcription of a target gene by increasing the expression level of PPAR gene in liver, not as an agonist of PPAR.

In this study, we examined the effects of nuclear receptors involved in metabolic diseases on eight types of PFRs. As a result, it was suggested that TPP disrupt the expression of metabolic related genes by increasing the expression level of PPAR, which is a key regulator of energy metabolism in the liver. Excessive activation of PPAR induces hepatic disorder and fat accumulation in the liver, suggesting that PFRs unintentionally exposed may contribute to the aggravation of fatty liver and other metabolic diseases. From the above, it seems that a more detailed analysis combining exposure actual conditions and toxic effects is necessary for the relevance between PFRs and metabolic diseases.

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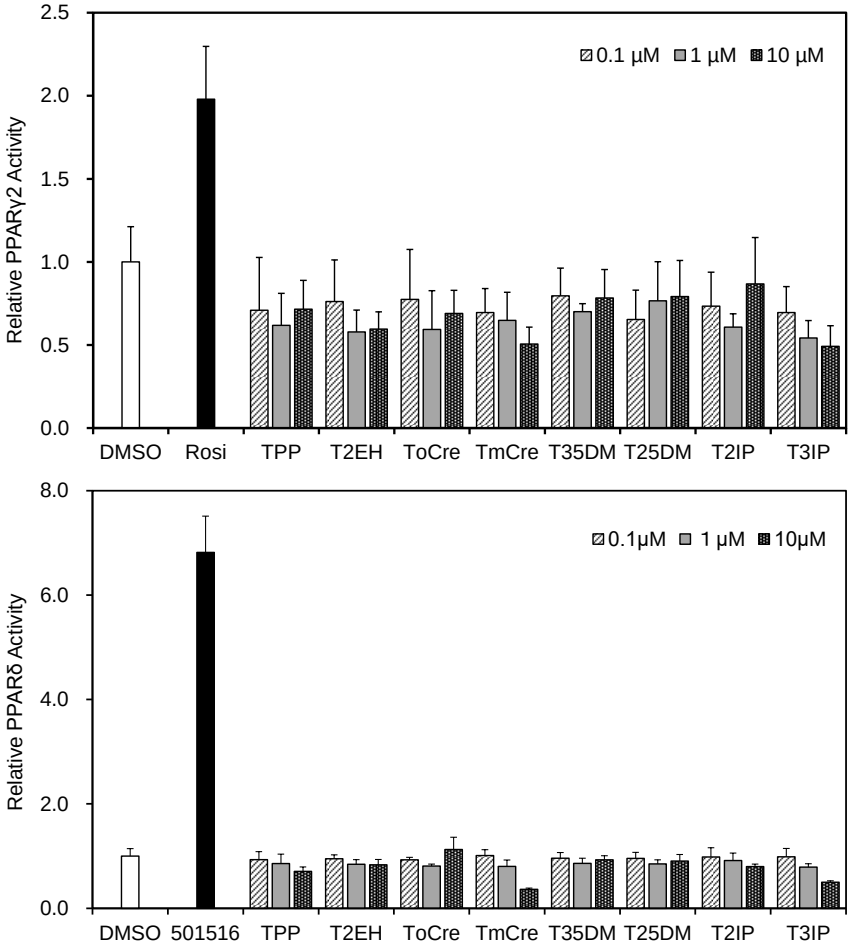

Figure 4 Investigation of transcriptional activitation of PPARγ and δ by PFRs.

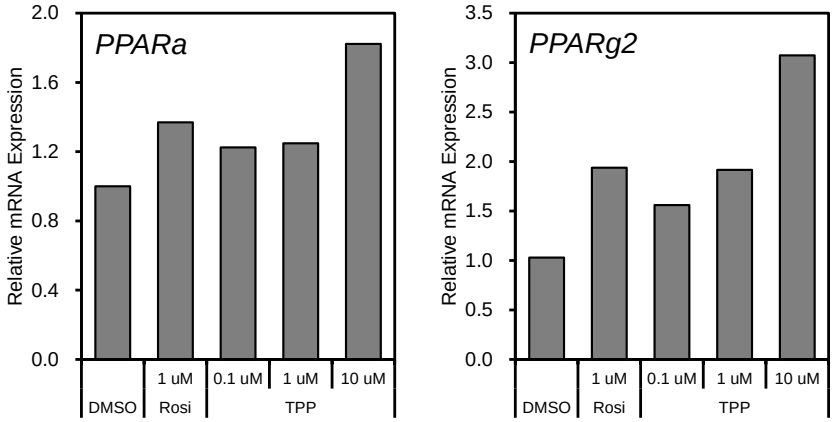

Figure 5 TPP increases the expression level of PPARs.