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論文題目：Studies on toxicity assessment of emerging environmental pollutants and unraveling the underlying mechanisms of toxicity based on the morphological and transcriptomic approaches using developing zebrafish

（発達期ゼブラフィッシュを用いた形態観察およびトランスクリプトーム手法による新興環境汚染物質の毒性評価と毒性機序解明に関する研究）

要旨

The chemical industry is an industry that synthesizes and produces useful products to humankind and has continuously developed since the Industrial Revolution. However, many studies have reported that numerous chemicals that are frequently used in the chemical industry have been released to the environment and caused various physiological disorders such as endocrine disruption and developmental toxicity not only in wild birds and fish but also in humans. It is expected that investigating the toxicity and its mechanisms of such environmental pollutants could contribute to the investigation of unknown environmental pollutants or new analogs of them. Therefore, rapid and comprehensive methods to assess toxicity and explore various underlying mechanisms of various environmental pollutants are required. For this reason, this study aims to explore toxicity and its mechanisms affecting living organisms caused by emerging environmental pollutants by morphological assessment and transcriptomic approaches using zebrafish as a model animal.

Exposure experiments for morphological assessment of four organophosphorus flame retardants (OPFRs) (triphenyl phosphate (TPHP), tris (1,3-dichloro-2-propyl) phosphate (TDCIPP), tris (2-chloroethyl) phosphate (TCEP), and 2-ethylhexyl diphenyl phosphate (EHDPHP)) and their metabolites (3-hydroxylphenyl diphenyl phosphate (HO-*m*-TPHP), 4-hydroxylphenyl diphenyl phosphate (HO-*p*-TPHP), diphenyl phosphate (DPHP), bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), bis(2-chloroethyl) phosphate (BCEP), and 2-ethyl-5-hydroxyhexyl diphenyl phosphate (5-HO-EHDPHP)) were performed by exposing the chemicals to zebrafish embryos for 24 hours. Some of the chemicals that showed clear growth inhibition in morphological assessment was measured. In gene expression analysis using quantitative real time PCR (qPCR), the expression levels of endocrine disruption and growth-related genes were measured. In addition, transcriptomic analysis was performed using RNA sequencing (RNA-Seq) of embryos exposed

to EHDPHP and its two main metabolites, 5-HO-EHDPHP and EHPHP. Morphological assessment and RNA-Seq analysis were also performed to perfluorinated alkyl substances (PFAS) (perfluorooctane sulfonate (PFOS) and perfluorohexane sulfonate (PFHxS)) and polybrominated dibenzo-*p*-dioxins (PBDDs) (1,3,7-tribromodibenzo-*p*-dioxin (1,3,7-TrBDD), 1,3,8-tribromodibenzo-*p*-dioxin (1,3,8-TrBDD), 2,3,7,8-tetrabromodibenzo-*p*-dioxin (2,3,7,8-TeBDD)).

In Chapter Ⅰ, cardiovascular toxicity was observed not only by parent chemicals including TPHP, TDCIPP, and EHDPHP, but also some of their metabolites including HO-*m*-TPHP, HO-*p*-TPHP, and 5-HO-EHDPHP in zebrafish embryos at 72 hours post fertilization (hpf). On the other hand, no significant cardiovascular toxicity was found in embryos exposed to TCEP, DPHP, BDCIPP, BCEP, and EHPHP. Furthermore, it was considered that circulatory failure by OPFRs and their metabolites was enhanced with higher octanol-water partition coefficient (Log K_{ow}). These results may indicate that some of OPFR metabolites (HO-*m*-TPHP, HO-*p*-TPHP, and 5-HO-EHDPHP) have similar potent of cardiovascular toxicity to their parent chemicals and lipophilicity is one of factors responsible for toxic effects induced by OPFRs and their metabolites.

In Chapter Ⅱ, the expression of genes that are related to endocrine disruption and growth inhibition was measured to verify significant growth inhibition caused by TPHP and TDCIPP exposure that was shown in Chapter Ⅰ. HO-*p*-TPHP, a metabolite of TPHP upregulated the expression level of *CYP19A1b*, a marker gene for estrogenic potency. The induction of *CYP19A1b* by HO-*p*-TPHP was decreased to the control level by co-exposure with ICI, an estrogen receptor antagonist, showing that its effect was mediated by estrogen receptor. The decrease in body length was significant in embryos exposed to TPHP and TDCIPP but slight and not significant in HO-*m*-TPHP and HO-*p*-TPHP. Furthermore, exposure to TPHP and its metabolites caused changes in the expression levels of genes (*dio1*, *dio2*, *trα*, and *ttr*) that control the synthesis and activity of thyroid hormones, which are closely related to development and growth of embryo. Unlike TPHP and their metabolites, TDCIPP exposure significantly inhibited the expression of growth hormone (*gh*) and prolactin (*prl*) genes. These results might indicate that the growth inhibitory mechanisms of TPHP and TDCIPP are different.

In Chapter Ⅲ, analysis of toxicity mechanism of EHDPHP and its metabolites, 5-HO-EHDPHP and EHPHP using RNA-Seq. To avoid secondary alteration of gene expression level caused by cardiovascular toxicity, exposure for RNA-Seq was conducted at the highest concentrations that did not induce cardiovascular toxicity. As a result, EHDPHP, 5-HO-EHDPHP, and EHPHP elicited various gene expression and pathway changes at the mRNA level. EHDPHP and 5-HO-EHDPHP showed significant alterations in genes related to glucose homeostasis, retinol metabolism, guanylate cyclase (GUCY)- cyclic guanosine monophosphate (cGMP), and calcineurin signaling. On the other hand, EHPHP exposure caused significant changes in immunity-related genes. These results may

exhibit that EHDPHP and 5-HO-EHDPHP have a common mechanism of toxicity through alteration of energy metabolism, whereas EHPHP may result in disruption of immune system.

In Chapter Ⅳ and Ⅴ, developmental toxicities of PFAS (PFOS and PFHxS) and PBDDs (1, 3, 7-TrBDD, 1, 3, 8-TrBDD, and 2, 3, 7, 8-TeBDD) and their mechanisms were assessed using morphological assessment and RNA-Seq was performed as indicated in Chapter Ⅰ and Ⅲ. The two selected PFAS, both PFOS and PFHxS induced significant developmental toxicity, showing higher dose effect by PFOS than PFHxS. RNA-Seq analysis showed that PFHxS exposure resulted in alteration of genes related to lipid metabolism including peroxisome proliferator-activated receptor (PPAR). On the other hand, the expression levels of genes related to mitogen activated protein kinase (MAPK), fibroblast growth factor (FGF), and neural and behavioral pathways, especially calcium ion concentration homeostasis were significantly altered by PFOS and PFHxS. Excessive intracellular calcium ion influx through N-methyl-D-aspartate receptor (NMDAR), which is a member of glutamate-gated channel with high permeability to calcium ion could induce apoptosis. In addition, disruption of PPARs can induce disruption of energy metabolism through mitochondrial fatty acid β-oxidation. Thus, it may be indicated that the disruption of homeostasis of calcium ion concentration is one factor of toxic effects induced by PFOS and PFHxS, whereas PFHxS could induce cardiovascular toxicity through PPAR-related pathways.

Among the selected PBDDs, 2, 3, 7, 8-TeBDD showed the highest dose effect, followed by 1, 3, 7-TrBDD. However, 1, 3, 8-TrBDD did not show any significant toxicity in the current study. As a result of RNA-Seq analysis, changes of genes related to lipid metabolism including solute carrier (SLC) family and innate immunity including chemokine and complement were observed in embryos exposed to 2, 3, 7, 8-TeBDD. On the other hand, genes related circadian rhythm were significantly changed by 1, 3, 7-TrBDD and 1, 3, 8-TrBDD exposure. Since the circadian rhythm pathway can elicit metabolic changes by interacting with aryl hydrocarbon receptor (AHR) signaling, the developmental toxicity induced by PBDDs might considered to be caused by mechanisms that disrupt lipid, immune system or nutrient metabolism.

From the results of the transcriptome analysis in Chapters 3 to 5, it was possible to explore various candidate mechanisms that were considered to be related to the toxic effects of emerging environmental pollutants. Although additional studies such as oxidative stress, apoptosis, or lipid and calcium ion influx are required to elucidate the mechanism, candidate mechanisms that could not be obtained by low-throughput methods were obtained and showed that the morphological and transcriptomic approaches using developing zebrafish are useful for assessing toxicity and exploring toxic mechanisms of emerging environmental pollutants.