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In vitro and *in vivo* Estrogenic Effects of Fluorotelomer Alcohols in Medaka (*Oryzias latipes*)

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Abstract—The present study demonstrates the estrogenic effects of two fluorotelomer alcohols, such as 1*H*,1*H*,2*H*,2*H*-perfluorooctan-1-ol (6:2 FTOH) and 1*H*,1*H*,2*H*,2*H*-perfluorodecan-1-ol (8:2 FTOH), in medaka (*Oryzias latipes*). An *in vitro* yeast two-hybrid assay indicated a significant, dose-dependent interaction between medaka estrogen receptor α (ERα) and coactivator TIF2 upon treatment with 6:2 FTOH or 8:2 FTOH. In contrast, no interaction with the ERα was observed upon treatment with perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorododecanoic acid (PFDA) or perfluoroundecanoic acid (PFUnDA). Expression analysis of hepatic vitellogenin (VTG) protein showed estrogenic potentials with, 6:2 FTOH and 8:2 FTOH, indicative of the induction of VTG synthesis in the livers of male medaka. We also investigated mRNA expression levels of two ER subtypes and two VTGs in the livers of male medaka following exposure to FTOHs. Quantitative real-time PCR analyses revealed that hepatic ERα, VTG I, and VTG II mRNA responded rapidly to FTOHs such as 6:2 FTOH and 8:2 FTOH after 8-h exposure, whereas no effects of these compounds on ERβ mRNA transcription were observed. These results strongly suggest that certain FTOHs, such as 6:2 FTOH and 8:2 FTOH, induce hepatic VTG through activation of ERα in male medaka.

Keywords: fluorotelomer alcohols, *Oryzias latipes*, Vitellogenin, Estrogen receptor α

INTRODUCTION

Fluorotelomer alcohols (FTOHs), such as 1*H*,1*H*,2*H*,2*H*-perfluorooctan-1-ol (6:2 FTOH) and 1*H*,1*H*,2*H*,2*H*-perfluoro-decan-1-ol (8:2 FTOH), are classes of compounds recently identified as potential sources of perfluoroalkylcarboxylate (PFCA) contaminants, including perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA), in the environment. These FTOHs are typically

used as precursor compounds in the manufacture of fluorinated polymers used in paper and carpet treatments; it is estimated that global production from 2000 to 2002 exceeded 5000 tons/year (Prevedouros *et al.*, 2006). A previous study demonstrated the occurrence of these compounds at concentrations ranging from 7 to 196 pg/m^3 or from 11 to 165 pg/m^3 in the troposphere (Martin *et al.*, 2003; Stock *et al.*, 2004). However, no information is available about FTOH contamination levels in other parts of the environment.

Limited data are currently available on the toxicological effects and risks of FTOHs in experimental animals such as mice and rats. Previous studies demonstrated that 8:2 FTOH is metabolized to PFOA and PFNA in the livers of rats, indicating that these compounds are associated with the induction of peroxisome proliferation and peroxisomal acyl-CoA oxidase (ACOX) activity (Kudo *et al.*, 2005). Exposure of mouse neonates to 8:2 FTOH caused developmental toxicity (Henderson and Smith, 2007). Interestingly, a recent study utilizing an E-screen assay of MCF-7 cell lines showed that 6:2 FTOH and 8:2 FTOH could promote proliferation, indicating the estrogenic potential of FTOHs *in vitro* (Maras *et al.*, 2006). An earlier study from our laboratory investigated the effects of FTOHs such as 6:2 FTOH and 8:2 FTOH on the transcriptional activity of human estrogen receptor (hER) subtypes hERα and hERβ *in vitro* (Ishibashi *et al.*, 2007). However, there is no information on the potential estrogenic effects of these FTOHs *in vivo*.

To investigate estrogenic and androgenic effects of chemicals, various screening and testing systems have been established in vertebrates and invertebrates (U.S. Environmental Protection Agency, 1998; Organization for Economic Cooperation and Development, 1999). Teleost fish such as Japanese medaka (*Oryzias latipes*), fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*) have been proposed as model organisms. Among these organisms, medaka fish are suitable for assessing the estrogenic effects of chemicals. Natural estrogens, such as estradiol-17β (E2), regulate estrogen-responsive genes by binding to a specific ER; the estrogen-ER complex then interacts with the estrogen-responsive elements (EREs) of the target promoter genes to modulate their transcriptional activity. Among the estrogen-responsive genes, vitellogenin (VTG), an estrogen-inducible phosphoprotein and complex precursor protein of egg yolk, is a useful biomarker for detecting estrogenic contamination of aquatic environments. A number of environmental estrogens, such as alkylphenolic compounds and synthetic estrogens, induce VTG synthesis in male medaka (Ishibashi *et al.*, 2005; Yamaguchi *et al.*, 2005).

Perfluorochemicals (PFCs) such as PFOA and perfluorooctane sulfonate (PFOS) have been detected in wildlife, including fish (Kannan *et al.*, 2001; Nakata *et al.*, 2006). These findings suggest that contamination by these compounds, particularly PFOA, may be due to the degradation of FTOHs such as 8:2 FTOH in the environment, or to the metabolism of FTOHs in the biota. The results of a field study indicated that the metabolism of 8:2 FTOH in fish and experimental animals such as rats could be similar, since an increased concentration of PFOA, but no significant increase in the level of PFNA, was detected in fish

sampled from Lake Michigan (Furdui *et al*., 2007). These studies point to the importance of investigating the potential biological effects and risks of FTOHs in aquatic species such as fish. The present study demonstrates the estrogenic effects of FTOHs in an *in vitro* yeast-two-hybrid assay involving medaka ERα and coactivator TIF2. Furthermore, in order to evaluate the estrogenic potential of FTOHs *in vivo*, we investigated mRNA expression levels of estrogen-responsive genes such as ERs (ERα and β) and VTGs (VTG I and II), as well as the level of VTG protein, in the livers of male medaka exposed to FTOHs in water.

MATERIALS AND METHODS

Test chemicals

6:2 FTOH (Alfa Aesar, Ward Hill, MA, USA), 8:2 FTOH (Alfa Aesar), 2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-nonadecafluoro-1-decanol (NFDH, Wako Pure Chemical Industries, Ltd., Tokyo, Japan), PFOS (Avocado Research Chemicals Ltd., Lancashire, UK), PFOA (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), PFNA (Sigma, St. Louis, MO, USA), perfluorodecanoic acid (PFDA, Sigma), perfluoroundecanoic acid (PFUnDA, Sigma) and E2 (a positive control; Sigma) were used in this study. All of these compounds were over 97% purity. Test solutions were prepared by dissolving the reagents in DMSO (dimethyl sulfoxide, Wako Pure Chemical Industries, Ltd.).

Yeast two-hybrid assay

The assay for determining the estrogenic effects of the test chemicals was performed as previously described (Miyahara *et al*., 2003). Yeast cells (*Saccharomyces cervisiae* Y190) were modified by incorporation of medaka ERα, an expression plasmid of the coactivator TIF2, and a β-galactosidase expression reporter in a yeast two-hybrid assay (Nishikawa *et al*., 1999). The exposure concentrations of PFCs including FTOHs (concentration ranges tested: 0.01–1000 μM) and E2 (156–10000 pM) were selected on the basis of the results of our previous study (Ishibashi *et al*., 2007). All experiments were performed in triplicate for each compound (two replicates for each concentration).

Exposure of medaka to the test chemicals

The male medaka used in this study were from a stable colony maintained in glass tanks in our laboratory. In order to eliminate endogenous sex steroids such as estrogens and androgens produced in female fish, the male fish were kept in a 30-l glass tank under a 16:8 light:dark photoperiod at 25 ± 1°C for 2 weeks, and fed an estrogenic-free diet of *Artemia nauplii* (<24 h after hatching) (Inudo *et al*., 2004) twice daily.

The male medaka were exposed to the test compounds according to the method previously reported (Ishibashi *et al*., 2005; Yamaguchi *et al*., 2005); the concentrations used in these exposure experiments were based on the results from the yeast two-hybrid assay for estrogenic activity towards medaka ERα. For

measurement of hepatic VTG protein, six adult male medaka (about 3 months after hatching; approximate mean body weight 213 mg; approximate mean total length 30 mm) were exposed to 6:2 FTOH (nominal concentration of 0.01, 0.1, 1 and 10 μM), 8:2 FTOH (nominal concentration of 0.01, 0.1, 1, 10 and 100 μM) or NFDH (nominal concentration of 0.01, 0.1, 1, 10 and 100 μM) in 1000-ml glass beakers for 3 d at 25 ± 1°C (n = 6/dose). Test solutions were changed every 24 h, and the fish were subjected to a 16:8-h light:dark photoperiod. The fish were fed a diet of *Artemia nauplii* (<24 h after hatching) twice daily for 3 d. For expression analysis of estrogen-responsive genes in the liver, three adult male medaka (about 3 months after hatching; approximate mean body weight 210 mg; approximate mean total length 29 mm) were exposed to 6:2 FTOH (nominal concentration of 10 and 100 μM), 8:2 FTOH (nominal concentration of 100 and 1000 μM) or NFDH (nominal concentration of 100 and 1000 μM) in 500-ml glass beakers at 25 ± 1°C (n = 3/dose). The fish were exposed for 8-h during the light photoperiod, and were not fed during the exposure period. In both exposure experiments, the control group was only exposed to the carrier solvent (0.01% DMSO). At the end of the exposure period, the livers were sampled, rapidly frozen in liquid nitrogen, and stored at –80°C until analysis.

Measurement of hepatic VTG protein

An enzyme-linked immunosorbent assay was used to specifically measure hepatic VTG levels in medaka (EnBio Tec Laboratories Co., Ltd., Tokyo, Japan). Measurements were performed according to the manufacture's instructions. Liver samples were individually homogenized in 200 μl of ice-cold assay buffer using a handheld homogenizer while the samples were kept on ice. The homogenized samples were centrifuged at 8000 × g for 10 min at 4°C, and the supernatants were collected and frozen at –80°C. Purified medaka VTG (10, 20, 50 and 100 ng ml^{-1}) were used as standards, and VTG levels in samples diluted with cold assay buffer were measured in duplicate. The assays were performed at room temperature. Concentrations of VTG in the hepatic samples were calculated from the linear portion of the medaka VTG standard curve.

RNA preparation and quantitative real-time PCR (Q-PCR)

Total RNA was isolated from the liver tissue of male fish by the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method using TRIzol reagent (Invitrogen Co., Ltd., Japan) according to the manufacturer's instructions. The RT reaction mixture contained 5 μg of total RNA, 1 μl of oligo (dT) primer and diethylpyrocarbonate (DEPC)-treated water. The reaction mixture was heated to 70°C for 10 min and quickly chilled on ice. After cooling, 4 μl of 5 x reaction buffer containing 25 mM $MgCl_2$, 2 μl of deoxynucleotide triphosphates (dNTPs; 10 mM each), 1 μl of RNase inhibitor, and 1 μl of ReverTra Ace (Toyobo, Co., Ltd., Osaka, Japan) were added to a total volume of 20 μl, and the reaction mixture was incubated for 60 min at 42°C. The reaction mixture was then heated to 90°C for 5 min to stop the RT. One microliter of the cDNA preparation was amplified

Table 1. Sequence of primer pairs used in the Q-PCR experiments.

Gene name (accession number)	Primer sequence (upper: forward primer; lower: reverse primer)
ERα	5′-GTCAGTCGGGTTACTTGGCC-3′
(AB033491)	5′-CATCACCTTGTCCCAACCTG-3′
ERβ	5′-GTGGACTCAACTTTCGGC-3′
(AB070901)	5′-CACGTCGCAGCAGGATCTT-3′
VTG I	5′-TGGAAAGGCTGATGGGGAAG-3′
(AB064320)	5′-AACTGCAGGCATGGTGAGCC-3′
VTG II	5′-GTCTTCAGGAGGTCTTCTTC-3′
(AB074891)	5′-GGTAGACAATGGTATCCGAC-3′
β-actin	5′-AGACCACCTACAGCATC-3′
(S74868)	5′-TCTCCTTCTGCATTCTGTCT-3′

on a Stratagene Mx3000P™ Real-Time PCR instrument using FullVelocity® SYBR® Green QPCR Master Mix (Stratagene, La Jolla, CA, USA) in the presence of 10 μM of the primers listed in Table 1. Reaction mixtures were denatured for 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 62°C, and 1 min at 72°C. The expression levels of mRNA of estrogen-responsive genes were normalized to the expression level of β-actin mRNA. All experiments were performed in triplicate for each sample.

Statistical analysis

All the statistical analyses were performed using StatView J 5.0 (SAS Institute Inc., Cary, NC, USA). In order to determine the estrogenic effect of test compounds on medaka ERα, their *in vitro* estrogenicity was recorded as the 10% effective concentration (EC_{x10}), defined as the concentration of test solution producing a chemiluminescent signal 10x that of the blank control. The EC_{x10} of each chemical examined was calculated using SigmaPlot (version 9.0; Systat, Inc., Richmond, CA, USA). The inverse of the EC_{x10} value for E2 was set to 100. Similar procedures were performed with the other samples to calculate their E2 relative activity. For the *in vivo* assay, the experimental data were checked for assumptions of homogeneity of variance across treatments using a Bartlett test. When the assumptions were met, significant differences in hepatic VTG mRNA/protein levels between the treated groups and controls were analyzed by a one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test. Where no homogeneity was observed in the data, the nonparametric Kruskal-Wallis test was used, followed by a Mann-Whitney *U* test with Bonferroni's adjustment. The difference was considered significant at $P < 0.05$.

RESULTS

To evaluate the estrogenic effects of FTOHs *in vitro*, we investigated

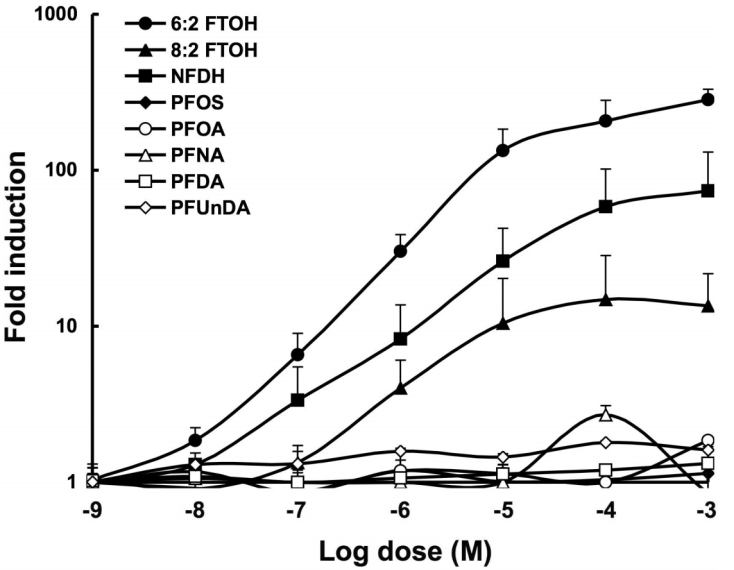

Fig. 1. Estrogenic effects of 6:2 FTOH, 8:2 FTOH, NFDH, PFOS, PFOA, PFNA, PFDA and PFUnDA on medaka ERα using the yeast two-hybrid assay. Data are presented as the mean, and vertical error bars represent the standard deviation (n = 6).

protein-protein interactions between medaka ERα and coactivator TIF2 using a yeast two-hybrid assay. Treatment with 6:2 FTOH, 8:2 FTOH or NFDH induced medaka ERα-mediated transcriptional activity in a dose-dependent manner (Fig. 1); the EC_{x10}s of 6:2 FTOH, 8:2 FTOH and NFDH for activation of medaka ERα were estimated to be 0.26, 9.3 and 2.5 μM, respectively. No estrogenic effects of PFOS, PFOA, PFNA, PFDA or PFUnDA on medaka ERα were observed in the concentration range tested in this study (0.01 to 1000 μM) (Fig. 1). Since the EC_{x10} value of the E2 positive control was calculated to be 410 pM, the relative ranking of the chemicals tested for activating medaka ERα was E2 (100) >>> 6:2 FTOH (0.16) > NFDH (0.016) > 8:2 FTOH (0.0044).

To assess the potential estrogenic effects of FTOHs *in vivo*, we investigated the induction of hepatic VTG protein synthesis in males following exposure to 6:2 FTOH, 8:2 FTOH or NFDH for 3 d. Levels of hepatic VTG production in male medaka exposed to 1 or 10 μM 6:2 FTOH, 10 or 100 μM 8:2 FTOH, and 1, 10 or 100 μM NFDH for 3 d were significantly higher as compared to the control group ($P < 0.05$) (Fig. 2). Although higher hepatic VTG concentrations following exposure to 1 μM 8:2 FTOH was observed compared to the control, no statistically significant difference was determined because of large variations among the VTG concentrations (Fig. 2). The hepatosomatic index (HSI) levels in adult male medaka exposed to >10 μM 6:2 FTOH, 8:2 FTOH or NFDH for 3 d were significantly higher than in the control group ($P < 0.05$) (data not shown).

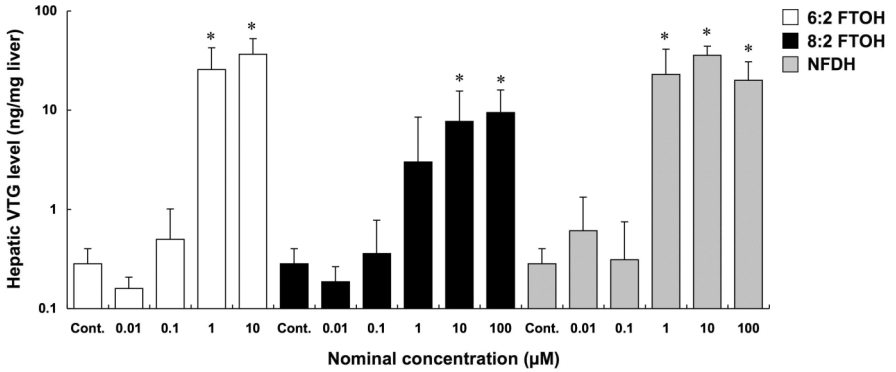

Fig. 2. Concentrations of hepatic VTG protein in male medaka after exposure to 6:2 FTOH, 8:2 FTOH or NFDH for 3 days. The control fish were only exposed to the solvent carrier (0.01% DMSO). Values shown are the mean VTG concentration in male fish (n = 6/dose). Error bars represent the standard deviation of the mean. The asterisk symbol (*) denotes significant differences as compared to the control group ($P < 0.05$).

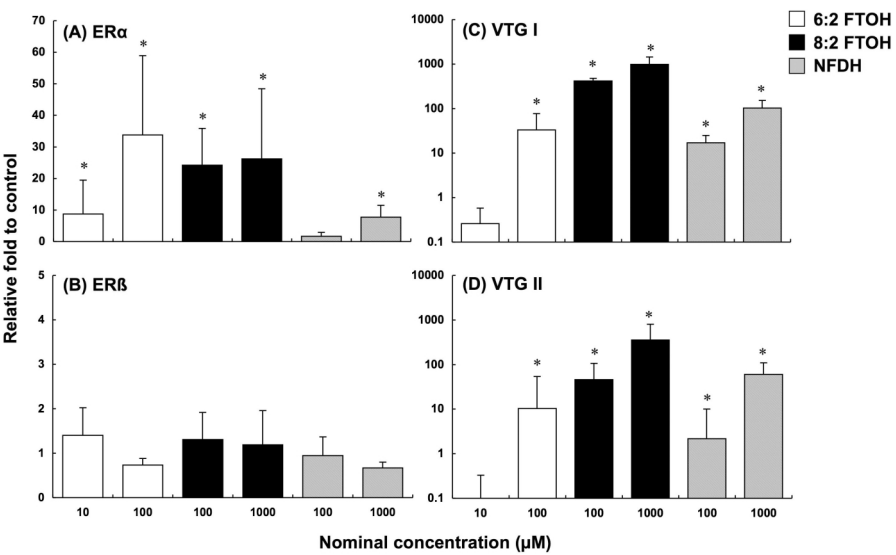

Fig. 3. Expression levels of ERα (A), ERβ (B), VTG I (C) and VTG II (D) mRNA in the livers of male medaka exposed to 6:2 FTOH, 8:2 FTOH or NFDH for 8 h. The control fish were only exposed to solvent carrier (0.01% DMSO). Hepatic mRNA expression levels were quantified using a Q-PCR method. Data bars represent mean mRNA expression values ± S.D. (relative to β-actin mRNA expression) of n = 3 fish. The asterisk symbol (*) denotes significant differences as compared to the control group ($P < 0.05$).

To better understand the molecular mechanism by which hepatic VTG protein is induced by treatment with 6:2 FTOH, 8:2 FTOH or NFDH, we assessed expression levels of the mRNA of estrogen-responsive genes such as ERs (ERα and ERβ) and VTGs (VTG I and VTG II) in the livers of male medaka following exposure to these compounds using Q-PCR techniques. Up-regulation of hepatic ERα mRNA was observed in male medaka exposed to 10 or 100 μM 6:2 FTOH, 100 or 1000 μM 8:2 FTOH, and 1000 μM NFDH for 8 h (Fig. 3A), whereas no effects of these compounds on the expression level of hepatic ERβ mRNA were observed (Fig. 3B). The induction of expression levels of hepatic VTG I and VTG II mRNA was observed in male medaka exposed to 100 μM 6:2 FTOH, 100 or 1000 μM 8:2 FTOH, and 100 or 1000 μM NFDH for 8 h (Figs. 3C and D). In order to investigate the estrogenic potential of PFNA and PFDA, male medaka were exposed to these compounds at nominal concentrations of 10, 100 or 1000 μM for 8 h. During exposure, all the males treated with 1000 μM PFNA, and 100 or 1000 μM PFDA, died as a result of swelling of the abdomen. Neither PFNA nor PFDA affected the expression level of estrogen-responsive genes at the concentrations tested in this study (data not shown).

DISCUSSION

The present study demonstrates the induction of VTG protein synthesis and up-regulation of estrogen-responsive genes such as ERs and VTGs in the livers of male medaka treated with 6:2 FTOH, 8:2 FTOH or NFDH. To our knowledge, this is the first report of the estrogenic potential of certain FTOHs such as 6:2 FTOH and 8:2 FTOH *in vivo*. These findings support the results of *in vitro* yeast two-hybrid assays showing protein-protein interactions of medaka ERα and coactivator TIF2 following treatment with these compounds.

VTG has been widely used as a biomarker for screening estrogenic effects of compounds on aquatic organisms (Sumpter and Jobling, 1995). Our previous studies demonstrated that environmental estrogens, such as alkylphenolic compounds and synthetic estrogens, induce VTG synthesis in the livers of male medaka (Ishibashi *et al.*, 2005; Yamaguchi *et al.*, 2005). These estrogenic compounds regulate estrogen-responsive genes such as the VTG gene by binding to a specific ER, then the estrogen-ER complex interacts with the EREs of the target promoter genes to modulate their transcriptional activity. It has been reported that ER subtype ERα is auto-regulated by the presence of EREs in the promoter of the ERα gene in rainbow trout (*Oncorhynchus mykiss*) (Le Drean *et al.*, 1995). In a separate study, the pattern of induction of VTG mRNA in largemouth bass was similar to that observed for ERα following exposure to E2: there was significant dose dependent up-regulation of VTG mRNA, whereas the levels of ERβ were unchanged (Sabo-Attwood *et al.*, 2004). In line with these earlier studies, the present study demonstrates that the induction of expression levels of hepatic VTG I and II mRNA following exposure to 6:2 FTOH, 8:2 FTOH or NFDH requires ERα-mediated transcription, whereas no effect of these chemicals on the expression levels of ERβ mRNA were observed. These results

suggest that certain FTOHs have an estrogenic effect and can induce both VTG I and II mRNAs mediated by the ERα signaling pathway in the livers of male medaka.

Several studies have reported the induction of VTG protein in the liver/serum of male medaka exposed to estrogenic compounds. For example, Kang *et al*. (2003) demonstrated that hepatic VTG levels were significantly increased in male medaka exposed to 50.9 μg l^{-1} nonylphenol (NP) for 21 d. Our previous study revealed marked increases in hepatic VTG concentrations in mature male medaka after 21 d exposure to 1000 μg l^{-1} (4.4 μM) bisphenol A (BPA) (Ishibashi *et al*., 2005). In the present study, the production of hepatic VTG protein in male medaka exposed to 6:2 FTOH, 8:2 FTOH or NFDH for 3 d was significantly higher compared to the control group, with the lowest-observed-effect concentrations (LOECs) of these compounds estimated to be 1 μM. These results indicate that the estrogenic effects of these compounds for the induction of hepatic VTG synthesis in male medaka are greater than that of BPA, which is known to act as an estrogenic compound.

The present study suggests that certain FTOHs such as 6:2 FTOH and 8:2 FTOH induce hepatic VTG mRNA and protein via ERα signaling in male medaka, although the toxicological consequences of this response remain unclear. Several studies on medaka and fathead minnow reported a reduction of fecundity and/or fertility, as well as the induction of VTG production in fish exposed to estrogenic compounds such as E2 and 4-NP (Giesy *et al*., 2000; Kang *et al*., 2002, 2003). Previous studies also indicated that excessive induction of VTG possibly results in pathological abnormalities such as hypertrophy of hepatocytes or an increase in HSI values in male fish treated with estrogenic compounds (Schwaiger *et al*., 2000; Kang *et al*., 2002). The present study revealed that HSI levels in adult male medaka exposed to 6:2 FTOH, 8:2 FTOH or NFDH for 3 d were significantly higher than in the control group. This increase may be related to abnormal VTG synthesis in the livers of male medaka. In mammals, 8:2 FTOH is metabolized to PFOA and PFNA in rat liver, and these metabolites are associated with the induction of peroxisome proliferation and peroxisomal ACOX activity in the liver (Kudo *et al*., 2005). It has been shown that exposure of fathead minnow to PFOA may cause an alternation in peroxisome proliferation in the liver, as quantified by fatty ACOX activity (Oakes *et al*., 2004). Although this study demonstrated increased HSI in male medaka exposed to FTOHs such as 8:2 FTOH, no information is currently available on the combined effects and risks of FTOHs and/or their metabolites, including PFOA and PFNA, in fish. Furthermore, no information is available about FTOH contamination levels in the aquatic environment, although the previous study demonstrated the occurrence of these compounds in the troposphere (Martin *et al*., 2003; Stock *et al*., 2004). Of particular interest are effects and risks associated with population-related responses including impairment of reproduction and recruitment. Further research is required to clarify these aspects, as well as the occurrence of FTOHs in natural aquatic environments.

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