Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.4, No.4, 179-186, 2017

Original Article

Disposition of perfluorododecanoic acid in male rats after oral administration

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(Received June 21, 2017; Accepted June 29, 2017)

ABSTRACT — The disposition of perfluorododecanoic acid (PFDoA), a perfluorocarboxylic acid with 12 carbon atoms, was studied in male rats. Rats received an oral administration of PFDoA at a dose of 50 mg/kg. The body weights of PFDoA-treated rats were slightly less than those of vehicle-treated control rats. PFDoA administration resulted in an increase in liver weight; it was highest at 5 days after the treatment and gradually decreased thereafter. Higher liver weight was observed until 70 days after the treatment. Concentrations of PFDoA in plasma and various tissues were estimated up to 70 days after dosing. A large amount of PFDoA was found in the liver. The PFDoA concentration was 263.94 ± 32.94 μg/g in the liver; the value was 7.93 times higher than that of serum 5 days after treatment. The hepatic PFDoA amount was found to be 29.63% of the dose. A certain amount of PFDoA was found in the brain and adipose tissues where perfluorocarboxylic acids with less than 11 carbon atoms were sparsely distributed. The half-life of PFDoA was 55.3, 49.3, 52.4, 57.1, and 49.8 days for serum, liver, kidneys, brain, and adipose tissue, respectively. PFDoA increased hepatic levels of mRNA for *Cyp4A10*, *Acot1*, and *Acox1*, target genes of PPARα, suggesting that PFDoA can activate PPARα, as was observed with other PFCAs. Elevated levels of these 3 genes were observed 70 days after treatment, and the levels were less than those at 7 days. The differences between PFDoA and PFCAs with less than 11 carbon atoms were discussed.

Key words: Perfluorododecanoic acid, Tissue distribution, Biological half-life, PPARα activation

INTRODUCTION

Perfluoroalkyl and polyfluoroalkyl substances exhibit unique properties and have been widely used as polymers and surfactants for numerous industrial and commercial applications (Buck *et al*., 2011). These chemicals, including perfluoroalkyl acids, fluorotelomer substances, perfluoroalkane sulfonamide substances, and fluoropolymers, are emitted into the environment and converted by abiotic or biotic environmental processes into long-chain perfluoroalkyl acids (PFAAs) (Buck *et al*., 2011; Renner, 2001; Prevedouros *et al*., 2006; Wang *et al*., 2013, 2014). PFAAs are extremely stable, non-biodegradable, and persistent in the environment (Lau *et al*., 2007). Indeed, PFAAs are found widely throughout the environment including in the air, water, soil, sediment, and sludge from wastewater treatment plants (Calafat *et al*., 2007; Houde *et al*., 2006; Kim and Kannan, 2007; Lau *et al*., 2007; Yamashita *et al*., 2005). PFAAs have become a focus of public health concern due to their widespread presence in wildlife and humans, in addition to their ubiquitous presence in environmental media such as the air, water, soil, sediment, food, and drinking water (Houde *et al*., 2006; Calafat *et al*., 2007; Lau *et al*., 2007). Among PFAAs, perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) have been extensively studied for their toxicokinetics and toxicity, since these PFAAs have been shown to be the predominant PFAAs in humans and wildlife and are the most abundant in the environment (Giegy, 2010; Lau *et al*., 2007; Kato *et al*., 2011).

PFCAs with more than 9 carbon atoms have been also detected in fish (Stahl *et al*., 2014) and wildlife (Ahrens

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et al., 2009; Rubarth *et al*., 2011). These findings suggest that humans are also exposed to such PFCAs. In fact, both perfluoroundecanoic acid and perfluorododecanoic acid (PFDoA) were detected in human serum (Goralczyk *et al*., 2015) and human breast milk (Motas Guzman *et al*., 2016). In a study by Gebbik and Cousins (2015), human exposure to PFDoA was estimated to be 51 pg/kg/day (intermediate exposure scenario), with 12.4% of that PFOA, and 18.9% of that PFOS. In rodents, PFCAs with longer carbon chain lengths exhibits longer half-lives among PFCAs with 6-10 carbon atoms (Kudo, 2015). However, a toxicokinetic study of PFAAs with carbon chains longer than 10 remains to be conducted. Recently, we demonstrated that PFDoA accumulates in the brain and causes significant cognitive deficits in rats after a single oral dose of PFDoA (Kawabata *et al*., 2017). This was markedly different from PFCAs with 6-10 carbon atoms, which were sparsely distributed in the brain (Kudo, 2015). Therefore, information on the toxicokinetics of PFDoA is indispensable to estimate the toxicity of PFDoA. In the present study, the distribution of PFDoA was studied in male rats that received a single oral dose of PFDoA. The half-life of PFDoA in various tissues was also estimated.

MATERIALS AND METHODS

Materials

PFDoA (> 93%) was purchased from Tokyo Chemical Industry, Co. Ltd. (Tokyo, Japan). PFDA (> 96%) was from Sigma-Aldrich (St. Louis, MO, USA). 3-Bromoacetyl-7-methoxycoumarin was prepared as previously described (Ohya *et al*., 1998). Corn oil was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of analytical grade.

Animals and treatments

All animal procedures were approved by the Institutional Animal Care Committee of Josai University in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan). Male Wistar rats were purchased from SLC (Hamamatsu, Japan) and were allowed to acclimate to the housing facility for at least 1 week prior to treatment. Animals were given free access to water and food and were kept in a humidity- and temperature- (23 ± 2°C) controlled environment with a 12-hr light/dark cycle.

Rats, at the age of 8 weeks, were given a single oral administration of vehicle alone or PFDoA at a dose of 50 mg/kg. PFDoA was suspended in corn oil (4 mL/kg) and administered to animals. The animals were anesthetized with diethyl ether, and blood was withdrawn from the inferior vena cava 3, 5, 7, 10, 14, 21, 28, 42, 56, and 70 days after the administration of PFDoA, and 7, 28, and 70 days after the administration of vehicle. Then, the whole body was perfused with approximately 300 mL of ice-cold saline from the left ventricle to remove the blood. The brain, liver, kidneys, spleen, lung, testis, and adipose tissue (epididymal, mesenteric, and subcutaneous) were quickly removed, weighed, frozen in liquid nitrogen, and stored at -80°C until use. Serum was prepared by centrifugation and stored at -30°C until analysis. The tissues (liver, kidneys, lung, spleen, and the brain) were homogenized with 9 volumes of 0.25 M sucrose, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.4) in a Potter glass-Teflon homogenizer. For homogenization of the heart and testis, a Polytron homogenizer was used. A portion of the adipose tissue was homogenized with 10 volumes of *n*-hexane:ethyl acetate (1:1, v/v) in a Polytron homogenizer. A portion of the homogenate was subjected to PFCA analysis. Serum levels of alanine aminotransferase and aspartate aminotransferase (AST) were assayed using a Transaminase CII test Wako (Wako Pure Chemical Industries, Ltd.).

Analysis of PFCAs

The concentrations of PFCAs in serum and tissue samples were determined as previously described (Ohya *et al*., 1998). In brief, PFCAs were extracted with ethyl acetate:*n*-hexane (1:1, v/v) from an aliquot of serum or tissue homogenates as an ion pair with tetrabutylammonium after adding known amounts of PFDA as an internal standard. The extract was then incubated with 3-bromoacetyl-7-methoxycoumarin to yield acetylmethoxycoumarin derivatives of PFCAs, which were separated with high performance liquid chromatography using a fluorescence detector.

Toxicokinetic analysis

A 1-compartment, first order elimination model was used. Half-life values were calculated from the first-order rate constant associated with the observed terminal (log-linear) portion of the tissue concentration versus the time curve, as estimated by a linear regression.

Quantitative Real-Time PCR

Total RNA was isolated from liver tissues using QIAzol reagent and an RNeasy kit (QIAGEN, Hilden, Germany). cDNA was synthesized from 500 ng of total RNA with avian myeloblastosis virus reverse transcriptase (Takara Bio Inc., Otsu, Japan). PCR amplification was conducted using SYBR Premix EX Taq

(Takara). Amplification and detection were performed with the Step One Plus real-time PCR system (Life Technologies Corp., Carlsbad, CA, USA). The thermal cycling program was as follows: a 10-s denaturation step at 95°C, followed by 50 cycles of 5-s denaturation steps at 95°C, and a 34-s annealing step at 60°C. After the reaction, dissociation curve analyses were performed in order to confirm the amplification of a single PCR product. Changes in gene expression were calculated using the comparative threshold cycle (Ct) method. Ct values were first normalized by subtracting the Ct value obtained from β-actin (control). Three target genes were chosen for estimating peroxisome-proliferator-activated receptor α, namely, *Cyp4A10, Acot1,* and *Acox1*. Primer pairs used for quantification of the gene were as follows: *Cyp4A10* (NM_153307) forward 5'-GAGGAACTGCATTGGGAAAC-3', and reverse 5'-GTGGGATCTGGCAGTAGCTCA-3'; *Acot1* (NM_031315.1) forward 5'-ACTACGACGACCTCCCCAAGA, and reverse 5'-TGGCCACGCAGGTAGTTCA-3'; *Acox1* (NM_017340) forward 5'-TTCGTGCAGCCAGATTGGTAG-3', and reverse 5'- CGGCTTTGTCTTGAATCTTGG-3'; *β-actin* forward 5'- TGCAGAAGGAGATTACTGCC-3', and reverse 5'- CGCAGCTCAGTAACAGTCC-3'.

Statistical analysis

All data are expressed as the means ± S.D. Statistical significance between 2 means was estimated by the Student's *t*-test. The threshold for assessing significance was $P < 0.05$, 2-tailed.

RESULTS

Effects of PFDoA on body weight and tissue weights

A difference in the body weight was observed between vehicle- and PFDoA-treated rats (Fig. 1A). A slight but significant difference was observed within 28 days after the treatment, and thereafter, observed at 42 days and 56 days after the treatment (Fig. 1A). A significant increase in liver weight was observed in the PFDoA-treated group up to 70 days after the treatment (Fig. 1B); the liver weight relative to body weight was highest at 5 days and then gradually declined. The weights of the brain, heart, kidneys, spleen, and epididymal fat did not differ between vehicle- and PFDoA-treated rats (data not shown). Lung weight could not be correctly estimated because tissues were swollen during perfusion. Serum levels of ALT and AST in PFDoA-treated rats (10.32 ± 3.31 and 26.31 ± 3.98 Karmen unit/mL, respectively) did not differ from those in control rats (8.58 ± 2.19 and 23.01 ± 3.63 Karmen unit/mL, respectively).

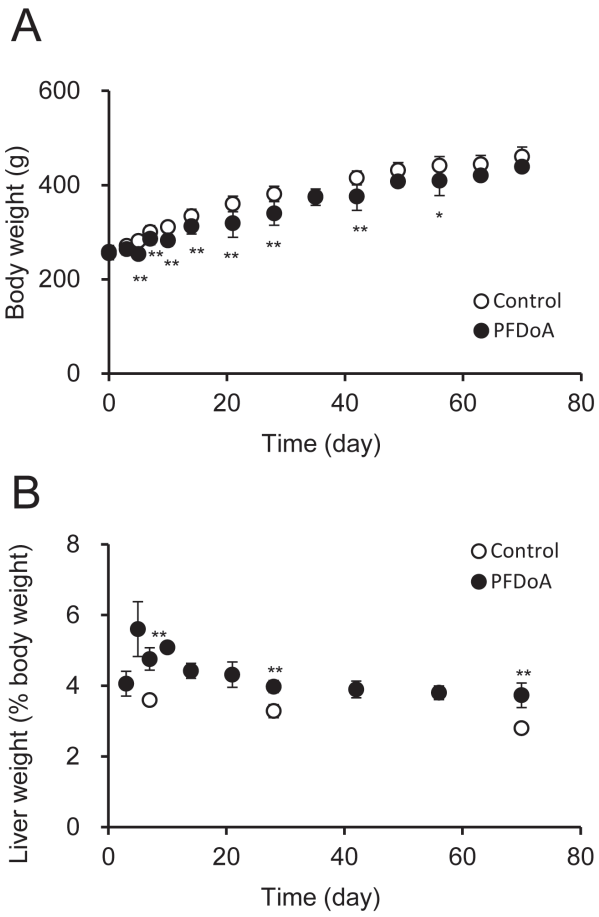

Fig. 1. Changes in body weight and liver weight after oral administration of PFDoA. Rats received an oral gavage of PFDoA at a dose of 50 mg/kg. Body weight (A) and liver weight (B) were monitored up to 70 days after dosing. Values indicate the means ± S.D. for 4-5 animals for each time point. Significant differences between the PFDoA-treated group and the vehicle-treated control group are indicated by $^{*}P < 0.05$ and $^{**}P < 0.01$.

Tissue distribution of PFDoA

PFDoA concentrations in various tissues and serum were determined 5 days after an oral dose of PFDoA at a dose of 50 mg/kg (Table 1). PFDoA was found to be distributed mainly in the liver, in which the PFDoA concentration was 7.93 times higher than that in serum. Approximately 30% of the dosed PFDoA was found to be distributed in the liver. PFDoA was also found in the kidneys, lung, and spleen, in which PFDoA concentrations were higher than that in serum. It is noteworthy that PFDoA was also found in the brain and adipose tis-

Table 1. PFDoA concentrations in various tissues in rats 5 days after oral administration of PFDoA.

	Tissue weight (g)	Tissue weight (% body weight)	PFDoA conc. (μg/g or mL)	Ratio to serum	PFDoA (% dose)
Serum	7.71 ± 0.32[a]	-	33.30 ± 2.77	1.000	2.06 ± 0.17
Liver	14.23 ± 1.99	5.61 ± 0.78	263.94 ± 32.94**	7.925	29.63 ± 5.47
Kidney	2.64 ± 0.21	1.04 ± 0.08	55.92 ± 9.43**	1.679	1.16 ± 0.17
Lung	2.00 ± 0.12[b]	0.79 ± 0.05	39.79 ± 4.75*[c]	1.166	0.63 ± 0.09
Heart	0.86 ± 0.07	0.34 ± 0.03	17.37 ± 2.54**	0.522	0.12 ± 0.01
Spleen	0.44 ± 0.04	0.18 ± 0.02	43.96 ± 4.51**	1.320	0.15 ± 0.02
Brain	1.92 ± 0.03	0.76 ± 0.02	33.85 ± 3.40	1.016	0.51 ± 0.04
Testis	2.51 ± 0.08	0.99 ± 0.06	19.51 ± 1.12**	0.587	0.39 ± 0.04
Adipose tissue					
epididymal	2.25 ± 0.30	0.89 ± 0.12	9.52 ± 1.19**	0.286	0.17 ± 0.03
mesentric	1.22 ± 0.23	0.48 ± 0.08	20.98 ± 4.26**	0.630	0.20 ± 0.01
subcutaneous	-	-	8.80 ± 0.62**	0.264	-

[a], Serum volume was calculated as 3.1% of body weight.
[b], Tissue weight is higher than correct values due to perfusion.
[c], Tissue concentration was underestimated due to swelling during perfusion.
Significant differences between the tissue concentration and serum concentration are indicated by *$P < 0.05$, **$P < 0.01$.

sues. PFDoA concentration was highest in mesenteric fat among the 3 different types of adipose tissues.

Disappearance of PFDoA

The concentrations of PFDoA in serum, liver, kidneys, adipose tissue (epididymal fat), and the brain were determined up to 70 days after treatment (Fig. 2). PFDoA reached its maximum concentration 5 days after treatment in serum, liver, and kidneys, 7 days after treatment in the brain, and 10 days after treatment in adipose tissue, and thereafter gradually declined (Fig. 2, Table 2). The biological half-life was calculated to be 55.3, 49.3, 52.4, 57.1, and 49.8 days in serum, liver, kidney, brain, and adipose tissue, respectively. The time course of tissue PFDoA concentrations relative to serum is shown in Fig. 3. The ratio was not altered in the liver and kidneys, while it initially increased and reached a steady value in the brain and adipose tissue.

PPARα activation

PFCAs have been shown to activate PPARα. To evaluate whether PFDoA activates PPARα in the liver, mRNA levels of *Acot1, Cyp4A10,* and *Acox1* were determined 7 and 70 days after administration of PFDoA. mRNA levels of these genes were higher in PFDoA-administered mRNA rats compared to those in control rats (Fig. 4A-C). The levels of these genes in PFDoA-treated rats declined with time.

DISCUSSION

In the present study, we revealed the tissue distribution of PFDoA in male rats. PFDoA was distributed in the brain, of which concentrations of PFDoA were comparable to that in serum. PFDoA was also distributed in adipose tissues. The biological half-life was estimated to be 50-57 days in serum and various tissues. In addition, PFDoA was shown to activate PPARα in the liver, as was observed with other PFCAs.

It has been recognized that distribution of PFAAs to the brain is limited (Kudo, 2015; Mariussen, 2012). In adult rats, the levels of PFOA and perfluorononanoic acid (PFNA) in the brain were only 1-3% of those in the liver and serum (Austin *et al*., 2003; Benskin *et al*., 2009). Our previous study showed that PFOA concentrations in plasma, liver, and the brain were 139, 254, and 3.3 nmol/g, respectively, 2 hr after an intravenous administration of PFOA at a dose of 40 μmol/kg (Kudo *et al*., 2007). Recently, we have reported that cognitive deficits were found in PFDoA-administered rats (Kawabata *et al*., 2017). Surprisingly, PFDoA was found to accumulate in the brain at a concentration higher than that in serum 9 days after dosing. In general, the toxicity of PFAAs in tissues is considered to be highly associated with their concentrations in the tissue. Therefore, it is necessary to evaluate the toxicokinetics of PFDoA. The present study revealed 2 features of PFDoA in tissue distribution, namely, 1) PFDoA is highly distributed in the liver compared to other organs including serum, and

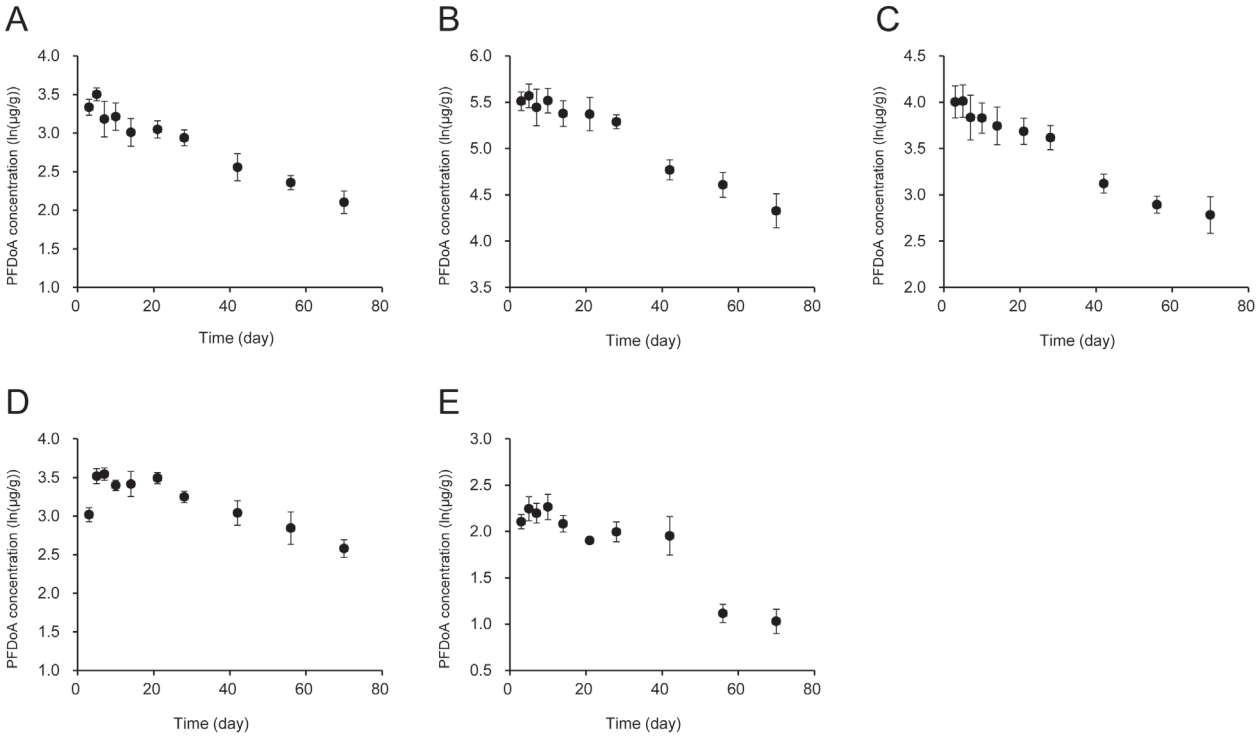

Fig. 2. Time course of the PFDoA concentration in serum, liver, kidney, brain, and adipose tissue. Rats received an oral gavage of PFDoA at a dose of 50 mg/kg. PFDoA concentrations in serum (A), liver (B), kidney (C), brain (D), and epididymal adipose tissue (E) were monitored. Values indicate the means ± S.D. for 4-5 animals for each time point.

Table 2. Toxicokinetic parameters for PFDoA.

Tissue	Half-life (day)	Cmax (μg/g or mL)	Tmax (day)
Serum	55.3	54.24 ± 4.50	5
Liver	49.3	429.86 ± 53.65	5
Kidney	52.4	91.07 ± 15.36	5
Brain	57.1	56.56 ± 4.49	7
Adipose tissue (epididymal)	49.8	15.81 ± 2.15	10

2) PFDoA is distributed in the brain and adipose tissue. The rates of PFDoA distribution to these tissues were slower compared to those to the liver and kidneys (Fig. 3). Tissue distribution of PFDoA has been reported in rainbow trout, but brain and adipose tissue concentrations were not investigated in the study (Martin *et al*., 2003b). Lipophilicity of chemicals is considered to be responsible for tissue distribution. In this context, the values of $LogK_{OW}$ were predicted to be 6.30, 8.23, and 10.16 for PFOA, perfluorodecanoic acid (PFDA), and PFDoA, respectively (Bhatarai and Gramatica,

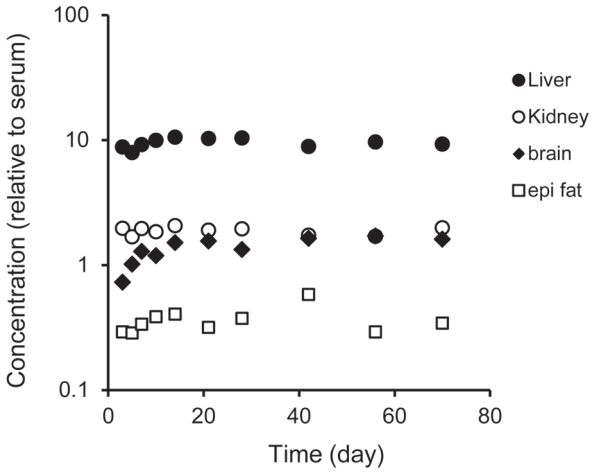

Fig. 3. Time course of the PFDoA concentration relative to serum in various tissues. Rats received an oral gavage of PFDoA at a dose of 50 mg/kg. The PFDoA concentration relative to serum was calculated by dividing the mean concentration of tissue by the mean concentration of serum. Values indicate the means of 4-5 animals for each time point.

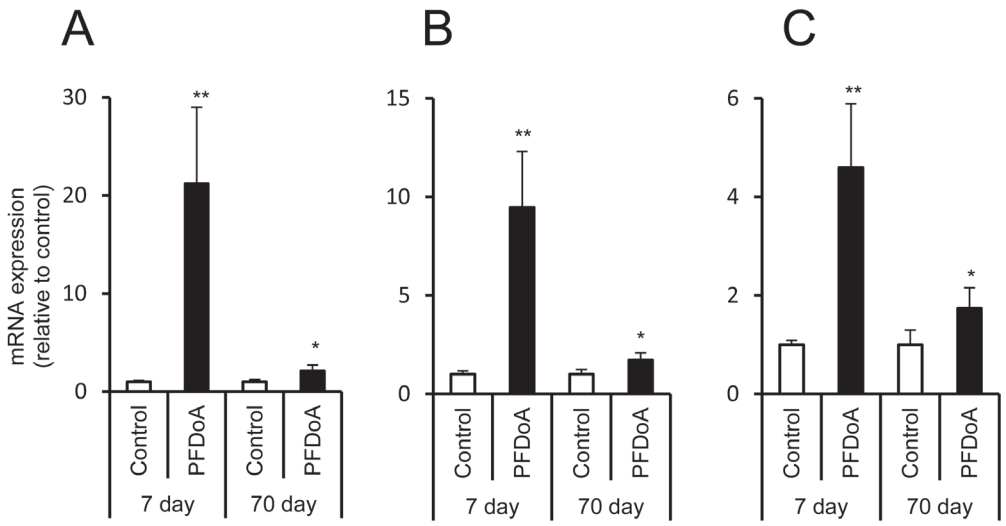

Fig. 4. mRNA levels of PPARα-target genes in the liver of PFDoA-administered rats. Rats received an oral gavage of PFDoA at a dose of 50 mg/kg. mRNA levels for *Acot1* (A)*, Cyp4A10* (B), and *Acox1* (C) were determined at 7 and 70 days after administration. Values indicate the means ± S.D. for 4-5 animals for each time point. Significant differences between the PFDoA-treated group and the vehicle-treated control group are indicated by $^{*}P < 0.05$ and $^{**}P < 0.01$.

2011). The distribution of PFDoA in the brain and adipose tissue appears to be due to the high lipophilicity of PFDoA; moreover, other mechanisms, such as binding to serum albumin, may be responsible for the distribution of PFDoA since tissue PFDoA concentrations relative to serum were higher compared to PFOA.

The biological half-life of PFDoA in serum, liver, kidney, brain, and adipose tissue was estimated to be 50-57 days (Table 2). In male rats, the half-life of perfluorohexanoic acid, perfluoroheptanoic acid, PFOA, PFNA, and PFDA has been shown to be 1 hr, 2.4 hr, 5.6 days, 30 days, and 40 days, respectively (Chengelis *et al*., 2009; Ohmori *et al*., 2003). Therefore, PFCAs with longer carbon chain lengths exhibit a longer half-life among PFCAs with a 6-10 carbon chain length. The half-life of PFDoA in serum, estimated to be 55.3 day in the present study, is in accordance with this trend. According to Martin *et al.* (2003a), a similar trend has been observed in juvenile rainbow trout among PFCAs with a 6-12 carbon chain length. According to the present findings, the half-life of PFDoA does not appear to differ significantly between tissues, although standard deviation of the estimated half-lives was not performed because animals were sacrificed at different time points.

Usually, transcriptional activation of PPARα-related genes is used as a sensitive detection method for PFCA exposure. In this context, 3 PPARα-related genes were estimated at 7 and 70 days after the administration (Fig. 4). As expected, mRNA levels of *Acot1*, *Cyp4A10,* and *Acox1* were elevated, and the levels declined with decreasing hepatic PFDoA concentrations. PFDoA was shown to be a less potent activator of PPARα compared to PFCAs with carbon chain lengths of 6-10 in various animal cells (Buhrke *et al*., 2013; Ishibashi *et al*., 2011; Rosen *et al*., 2013; Wolf *et al*., 2012). In the present study, induction of these genes was mild in spite of the high concentration of PFDoA in the liver.

Kato *et al.* (2015) have reported that hypertrophy, necrosis, and inflammatory cholestasis were observed in the liver after chronic administration of PFDoA at a dose of 0.5 and 2.5 mg/kg for 42-47 days in male rats. Hepatotoxicity of PFDoA has been shown to be due to production of reactive oxygen species in rats (Liu *et al*., 2016). Our present study showed high accumulation of PFDoA in the liver. However, the PFDoA concentration was not estimated in their study. Thus, the hepatotoxic effects of PFDoA seem to be due to the high concentration of this chemical, therefore, estimation of the tissue concentration may be helpful in evaluating the toxicity of PFDoA. In fact, cognitive deficit has been observed with PFDoA, but not PFOA and PFDA, and only PFDoA was accumulated in the brain (Kawabata *et al*., 2017). In this context, PFDoA may exhibit toxic effects in tissues where PFDoA accumulates, such as adipose tissue.

In conclusion, the present study demonstrated that the features of PFDoA distribution are different from those of

other PFCAs that have been reported; PFDoA prefers to distribute in the brain and adipose tissue compared to other PFCAs with a shorter carbon chain length. The half-life of PFDoA in serum was longer than those of PFCAs with carbon chain lengths shorter than 11. The information provided by the present study may be helpful in evaluating the toxicity of PFDoA, because investigation of the effects of PFDoA in tissues where PFDoA is distributed remains to be conducted.

Conflict of interest---- The authors declare that there is no conflict of interest.

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