There have been several reports on PFOS and PFOA concentrations in human serum (Fromme et al., 2009). Olsen et al. (2005) reported that the serum concentrations of PFOS and other fluorochemicals increased between 1974 and 1989, however did not change between 1989 and 2001 in the United States. Moreover, after 3M Company phased out PFOS and PFOA production, decreases in the PFOS and PFOA concentrations were observed in the United States (Olsen et al., 2008). However, analyses of systematically collected archived samples in the Human Specimen Bank of Kyoto University (HSB-Kyoto) (Koizumi et al., 2005) revealed that the serum concentrations of PFOS and PFOA in Miyagi, Japan increased from 1.1 ng mL^{-1} to 3.5 ng mL^{-1} and from 0.2 to 2.8, respectively, between 1977 and 2003 (Harada et al., 2004, 2007), while the corresponding levels in mainland China increased from 2.4 to 31.4 and from 0.78 to 4.9, respectively, between 1999 and 2002 (Jin et al., 2007). It should be assessed whether decreases similar to United States are observed in Asian countries because manufacture and importation of PFOS and PFOA remained continued even in 2006 (OECD, 2006).

In the present study, we investigated the current serum concentrations of PFOS and PFOA in three Asian countries, namely Japan, Korea and Vietnam. These compounds have been detected in coastal seawaters at comparable levels in Japan and Korea (Yamashita et al., 2005). As modeled by Pistocchi and Loos (2009), land-based PFOS and PFOA emissions can be reflected in coastal areas, therefore use of those chemicals in Asian countries are unlikely to be negligible. We selected Busan and Seoul in Korea, since they are urban and industrialized cities similar to Osaka in Japan. To confirm the consistency of the temporal trends in Asian countries other than Japan, we also used archived historical serum samples from Korean women stored in a biological specimen bank (Koizumi et al., 2005, 2009). Hanoi in Vietnam was selected to evaluate the effects of recent industrialization on PFOS and PFOA contamination.

2. Methods and materials

2.1. Experimental design and study population

To evaluate possible international differences, we compared 258 samples collected from Japan (Sendai, Takayama and Osaka), Korea (Busan and Seoul) and Vietnam (Hanoi) in 2007–2008. Since previous reports showed that Osaka had higher levels of PFOA than other areas in Japan, including suburban (Sendai) and rural (Takayama) areas (Harada et al., 2007; Kärrman et al., 2009), we also aimed to demonstrate the trends after the phase-out of PFOS and PFOA by 3M company and/or other manufacturers in both high- and low-exposed populations. Study population in Osaka was residents around a fluoropolymer manufacturer, Daikin Company. A total of 258 serum samples with information on the donor age, sex (female), body mass index and residential history (>5 years in each area) were selected from the archived samples in HSB-Kyoto (Koizumi et al., 2005, 2009) (Table 1).

In addition, to evaluate the long-term trends in serum PFOS and PFOA concentrations in Korea, historical serum samples were also obtained from HSB-Kyoto. Samples from Seoul and Busan were collected from healthy women (Table 1).

Although the mean ages in several areas differed significantly, these differences are unlikely to have biological effects on the serum levels based on previous reports (Harada et al., 2005, 2007). Since female population has lower PFOS and PFOA levels than in male as previously reported (Calafat et al., 2006; Harada et al., 2004, 2007), comparison with other reported data was limited to data from female population.

Serum was separated from red blood cells and other cellular components and then stored at −20 °C until analysis.

The research protocol for the present study was reviewed and approved by the Ethics Committee of the Kyoto University Graduate School of Medicine on 14 November 2003 (E25).

2.2. Reagents

Ammonium acetate (purity >99%, pa for HPLC) was purchased from Aldrich (Steinheim, Germany). Methanol (PCB and pesticide grade), acetonitrile (LC–MS grade) and water (distilled LC–MS grade) were obtained from Kanto Chemicals (Tokyo, Japan). Acetic acid was purchased from Nacalai Tesque (Kyoto, Japan). PFOS, heptadecafluorooctane sulfonic acid potassium salt (>98%) and PFOA, pentadecafluorooctanoic acid ammonium salt (>98%) were purchased from Fluka (Milwaukee, WI). $^{13}C_4$-labeled PFOS and $^{13}C_4$-labeled PFOA were obtained from Wellington Laboratories (Guelph, Ontario, Canada).

2.3. Determination of PFOS and PFOA in serum

Samples were subjected to a clean-up using a dispersive carbon method described by Powley et al. (2005). Briefly, the serum samples (0.5 mL) together with internal standards ($^{13}C_4$-PFOS and $^{13}C_4$-PFOA) were extracted with 5 mL of acetonitrile, followed by centrifugation at 1600 *g* for 15 min. The supernatants were transferred into new tubes with 25 mg of ENVI-Carb and 50 μL of acetic acid,

Table 1
Study area and study population.

Sampling site	Population (×10^3)	Latitude and longitude	Year	*n*	Age[a]	(Range)	HSD test[b]
Japan							
Sendai	1031	38°17′04″N140°55′46″E	2008	50	37.5 ± 9.44	(21–53)	CDE
Takayama	94	36°08′13″N137°15′16″E	2008	50	40.5 ± 4.78	(29–49)	BC
Osaka	2652	34°45′31″N135°31′52″E	2008	50	45.9 ± 8.92	(30–63)	A
Korea							
Busan	3711	35°14′39″N129°05′54″E	2008	35	40.1 ± 6.44	(18–49)	BCD
	3732	–	2000	30	35.4 ± 4.27	(28–45)	DEF
	3961	–	1994	39	42.3 ± 4.65	(34–52)	AB
Seoul	10 421	37°27′52″N127°01′56″E	2007	36	34.5 ± 8.24	(20–54)	EF
	10 798	–	1994	24	38.0 ± 7.41	(24–51)	BCDE
Vietnam							
Hanoi	6232	21°00′08″N105°49′50″E	2007–2008	37	30.2 ± 5.76	(20–40)	F

[a] Data are presented as mean ± standard deviation.
[b] Means of age with different letters differed significantly ($p < 0.05$ by Tukey's HSD test). For example, the letters A and B indicate that the corresponding values differ significantly at $p < 0.05$., while A and AB or AB and B indicated that the corresponding values do not differ significantly.

Table 2
Recoveries, accuracy, and detection limits for PFOS and PFOA analysis in human serum samples.

Compound	Transition quantification (confirmation)	Recovery and (reproducibility) % (CV%)[a] ($n = 5$)	Instrument detection limit[b] (ng mL^{-1})	Method detection limit (ng mL^{-1})
PFOS	498.9 > 80.0 (498.9 > 99.0, 498.9 > 129.9)	96.2 (8.41)	0.1	0.2
PFOA	412.9 > 369.0 (412.9 > 218.9, 412.9 > 168.9)	97.9 (15.6)	0.1	0.2

[a] CV = coefficient of variation.
[b] 10 μl Injection.

and the solutions were mixed by vortexing for 30 s. After centrifugation at 1600 *g* for 15 min, the extracts were reduced in volume and filtered through 0.2-μm GHP membranes (Pall, East Hills, NY).

Analyses were performed using an LC 20 system (Shimadzu, Tokyo, Japan) coupled to an MDS Sciex API 3200 MS/MS instrument (Applied Biosystems, Toronto, Canada) with an atmospheric electrospray interface operating in the negative ion mode. Separation was performed using an Inertsil ODS-SP column (150-mm length, 2.1-mm i.d., 3-μm particles, 120-Å pore size; GL Science, Tokyo, Japan). The column was kept at 40 °C. The injection volume was 10 μL and the flow rate was set to 200 μL min^{-1}. A gradient program was employed using two mobile phases consisting of 10 mM ammonium acetate in acetonitrile and 10 mM ammonium acetate in water. A multiple reaction monitoring program divided into two periods with optimized parameters for each analyte was used to measure the two product ions (Table 2).

2.4. Quality assurance

Quantification was performed using an internal standard method with the external standards dissolved in 35% methanol in water. $^{13}C_4$-PFOS was used as the internal standard for PFOS, while $^{13}C_4$-PFOA was used for PFOA. The performance of the method can be seen in Table 2. The recoveries were evaluated by five replicate fortifications (fortified by 5 ng mL^{-1}) of a human serum sample with low contamination (4.43 ± 0.187 and 2.10 ± 0.199 ng mL^{-1} for PFOS and PFOA, respectively). The procedural blank levels were evaluated in duplicate for 11 samples each.

Ten samples from Takayama analyzed in 2004 (Harada et al., 2007) and 10 samples from Osaka analyzed in 2007 (Kärrman et al., 2009) were reanalyzed using the above method in 2009. The reanalyzed serum samples from Takayama showed 3.94 ± 1.39 ng mL^{-1} and 27.6 ± 17.5 ng mL^{-1} for PFOS and PFOA, respectively, which are 98.3% and 100.7% of the levels obtained in 2004 (4.01 ± 1.04 ng mL^{-1} and 27.4 ± 16.9 ng mL^{-1}). The reanalyzed samples from Osaka showed 31.5 ± 11.5 ng mL^{-1} and 24.4 ± 3.31 ng mL^{-1} for PFOS and PFOA, respectively, which are 102.1% and 88.6% of the levels obtained in 2007 (30.9 ± 11.8 ng mL^{-1} and 27.6 ± 3.67 ng mL^{-1}).

2.5. Statistical analysis

All statistical analyses were carried out using the JMP software (Version 4; SAS Institute Inc., Cary, NC). Values of $p < 0.05$ were considered to indicate statistical significance. Concentrations of less than the detection limit were converted into half of detection limit for statistical analyses. Statistical analyses were conducted after logarithmic transformation of the serum concentrations because the serum levels of PFOS and PFOA in the samples displayed skewed patterns. After logarithmic transformation, concentrations of PFOS and PFOA distributed normally ($p = 0.633$ and $p = 0.0827$ by Shapiro–Wilk W-test, respectively). Differences between mean values were tested by Tukey–Kramer's honestly significant difference (HSD) test after ANOVA. Analysis of covariance (ANCOVA) was used to adjust for the differences in age. Age-adjusted means were calculated from a least square mean of ANCOVA model. Correlation was tested by Pearson's product moment correlation coefficient.

3. Results

3.1. PFOS and PFOA concentrations in 2007–2008

The descriptive statistics for PFOS and PFOA are presented in Table 3. Most samples contained PFOS and PFOA at concentrations above the method detection limit, except for four samples for PFOA from Hanoi. As shown in comparisons among three countries, the geometric means (geometric standard deviations) of the serum PFOS levels (ng mL^{-1}) was comparable in Japan, Korea and Vietnam (6.19 (1.65), 8.43 (1.39) and 6.78 (1.60), respectively). In contrast to PFOS, the geometric means (geometric standard deviations) of the serum PFOA levels were higher in Japan and Korea (4.10 (2.78) and 3.48 (1.49), respectively) than in Vietnam (0.575 (2.32)).

When participants were divided to six sampling sites, there were significant spatial differences in both the serum PFOS and PFOA concentrations (ANOVA: $p < 0.001$) (Table 3). Participants from Osaka and Busan exhibited significantly higher serum PFOS levels than in other areas, which were followed by participants from Seoul, Hanoi, Sendai and Takayama (Tukey–Kramer's HSD test: $p < 0.05$). On the other hand, the PFOA levels showed an apparent difference between Osaka (geometric mean, 14.2 ng mL^{-1}) and Hanoi (geometric mean, 0.6 ng mL^{-1}) (Tukey–Kramer's HSD test: $p < 0.05$).

Logarithm transformed PFOS and PFOA concentrations were significantly correlated with each other in Japan or Korea ($r = 0.67$, $p < 0.001$ and $r = 0.62$, $p < 0.001$, respectively), but not in Vietnam ($r = 0.16$, $p = 0.34$).

3.2. Temporal trends in the serum concentrations of PFOS and PFOA in Korea

The PFOS and PFOA concentrations in the serum samples collected in Busan and Seoul between 1994 and 2008 are shown in Table 3. The concentrations in all samples were greater than the detection limit. There were no significant differences in the PFOS levels among three time points in Busan and between 1994 and 2007 in Seoul ($p > 0.05$ by ANOVA). In contrast, there was a significant increase in the serum PFOA concentrations in Busan from 2000 to 2008 (1.24-fold, $p < 0.05$ by Tukey–Kramer's HSD test). The serum PFOA concentrations in Seoul also exhibited a 1.41-fold increase from 1994 to 2007 ($p = 0.0002$ by ANOVA).

3.3. Correlations between PFOS and PFOA levels and age

Analysis of covariance showed that age and location were significant factors for the serum PFOS levels in 1994–2008 ($p < 0.001$ for both variables). The estimated parameter of age

Table 3
Serum concentrations of PFOS and PFOA.

	Year	*n*	PFOS concentration (ng mL^{-1})						Spatial trend in 2007–2008[a]	Difference among sampling year[b]	Age-adjusted GM (GSEM)[c]
			GM(GSD)	AM	Range	Q1	Q2	Q3			
Comparisons among 3 countries											
Japan	2008	150	6.19 (1.65)	7.07	1.99–26.9	4.31	5.83	8.44	–	–	–
Korea	2007–8	71	8.43 (1.39)	8.89	3.21–19.1	6.73	8.16	10.9	–	–	–
Vietnam	2007–8	37	6.78 (1.60)	7.50	1.89–14.6	5.25	6.71	10.4	–	–	–
Comparisons among six sampling sites											
Sendai	2008	50	4.86 (1.45)	5.19	1.99–10.0	3.90	4.84	6.60	C	–	4.97 (1.06)
Takayama	2008	50	5.25 (1.47)	5.67	2.61–14.1	4.20	5.01	6.57	C	–	5.11 (1.06)
Osaka	2008	50	9.30 (1.59)	10.4	4.05–26.9	6.58	9.16	13.0	A	–	8.29 (1.06)
Busan	1994	39	10.8 (1.60)	12.1	4.81–31.4	8.03	10.6	14.8	–	n.s.	10.2 (1.07)
	2000	30	9.13 (1.55)	9.89	2.98–19.4	7.26	9.89	11.9	–	n.s.	9.65 (1.07)
	2008	35	9.36 (1.42)	9.93	3.91–19.1	7.49	8.85	11.5	A	n.s.	9.16 (1.07)
Seoul	1994	24	7.63 (1.40)	8.07	4.83–15.7	5.91	7.23	10.2	–	n.s.	7.73 (1.08)
	2007	36	7.61 (1.32)	7.88	3.21–12.2	6.18	7.69	9.30	AB	n.s.	8.14 (1.07)
Hanoi	2007–8	37	6.78 (1.60)	7.50	1.89–14.6	5.25	6.71	10.4	B	–	7.67 (1.07)
			PFOA concentration (ng mL^{-1})								
Comparisons among 3 countries											
Japan	2008	150	4.10 (2.78)	7.64	0.770–169	1.81	3.04	10.7	–	–	–
Korea	2007–2008	71	3.48 (1.49)	3.77	1.67–9.00	2.55	3.55	4.68	–	–	–
Vietnam	2007–2008	37	0.575 (2.32)	0.730	n.d.–1.57	0.488	0.749	1.00	–	–	–
Comparisons among six sampling sites											
Sendai	2008	50	2.22 (1.58)	2.47	0.838–5.71	1.61	2.14	2.90	C	–	2.25 (1.07)
Takayama	2008	50	2.19 (1.74)	2.58	0.770–9.24	1.42	2.04	3.20	C	–	2.15 (1.07)
Osaka	2008	50	14.2 (1.73)	17.9	5.84–169	10.4	13.2	16.7	A	–	13.1 (1.08)
Busan	1994	39	4.08 (1.34)	4.25	2.07–7.00	3.39	4.13	5.01	–	AB	3.92 (1.08)
	2000	30	3.66 (1.50)	3.93	1.22–6.64	2.67	4.23	4.82	–	A	3.80 (1.09)
	2008	35	4.54 (1.38)	4.77	2.57–9.00	3.53	4.68	5.93	B	B	4.47 (1.09)
Seoul	1994	24	1.91 (1.47)	2.04	0.784–3.74	1.48	2.10	2.41	–	A	1.93 (1.10)
	2007	36	2.70 (1.32)	2.80	1.67–4.38	2.16	2.57	3.58	C	B	2.83 (1.09)
Hanoi	2007–2008	37	0.575 (2.32)	0.730	n.d.–1.57	0.488	0.749	1.00	D	–	0.626 (1.09)

GM: geometric mean; GSD: geometric standard deviation; AM: arithmetic mean; Q1: 25th percentile; Q2: median; Q3: 75th percentile; GSEM, geometric standard error of the mean; n.s.: not significant.

[a] GMs with different letters differed significantly from each other site among six sampling sites in 2007–2008 ($p < 0.05$ by Tukey's HSD test). For example, the letters A and B indicate that the corresponding values differ significantly at $p < 0.05$., while A and AB or AB and B indicated that the corresponding values do not differ significantly.

[b] GMs with different letters differed significantly from each other year within the same sampling sites in Korea ($p < 0.05$ by Tukey's HSD test).

[c] GMs were adjusted for age of 38.3 years by analysis of covariance.

showed a coefficient of $10^{0.0081 \times (age - 38.8)}$ for the serum PFOS levels. Correlations between age and PFOS levels were also observed in Japan and Korea ($r = 0.41$, $p < 0.001$ and $r = 0.51$, $p < 0.001$, respectively), but not in Hanoi ($r = 0.21$, $p = 0.21$). This correlation in Korea was consistent in 1994 and 2007–2008 ($r = 0.44$, $p < 0.001$ and $r = 0.52$, $p < 0.001$, respectively).

The geometric mean of the serum PFOA levels in 1994–2008 was also affected by age and location ($p = 0.009$ for age and $p < 0.001$ for location). The effect of age on the serum PFOA levels, $10^{0.0052 \times (age - 38.8)}$, was relatively moderate compared with that of PFOS. PFOA levels correlated with age in Japan and Korea ($r = 0.41$, $p < 0.0001$ and $r = 0.39$, $p < 0.001$, respectively), but not in Hanoi ($r = 0.20$, $p = 0.23$). However, the effect of age was moderate in 1994 ($r = 0.27$, $p = 0.03$), compared with that in 2007–2008 ($r = 0.39$, $p < 0.001$).

Even after adjustment for age, the spatial trends remained unchanged for both PFOS and PFOA (Table 3).

4. Discussion

In the present study, we analyzed serum samples from three countries in East Asia. It was confirmed that the PFOS levels had not changed significantly from 1994 to 2008 in Korea, while the serum levels of PFOA had increased even after phase-out by 3M Company, suggesting that production and use by other manufacturers and industries might contribute to exposure to PFOS and PFOA in Korea. This is the first report on these historical trends in Korea.

In Japan, the PFOS levels in female serum samples seemed to decline from 2004 to 2008 (Fig. 1). The PFOS concentrations in 2008 decreased to 22.3–66.7% of the values obtained in 2003–2004 (Harada et al., 2007; Kärrman et al., 2009). These findings are consistent with a survey conducted in the United States (Olsen et al., 2008).

In contrast, PFOA did not show a clear decline from 2003 to 2008 in Japan. The levels in Sendai and Takayama slightly decreased by 14% and 10% compared with the levels in 2004, respectively, and these differences were not statistically significant. The PFOA levels in Osaka declined significantly from 2004 to 2008, suggesting large variations in the exposure sources, such as ambient air, drinking water and food, even in the same population (Saito et al., 2004; Harada et al., 2006; Kärrman et al., 2009). Indeed, survey population in 2004 and 2008 resided around a fluoropolymer manufacturer. Under the guidance of government of Osaka prefecture, the manufacture reduced emission of PFOA by 82% in 2006 and 89% in 2007 compared with emission in 2000 (Osaka Prefecture Government, 2008). These findings suggests declines in serum PFCs levels might largely differ from high- and low-exposed populations.

In Hanoi, the PFOS levels were comparable with those in other urban and industrial cities (Osaka, Seoul and Busan). Despite the occurrence of PFOS, the geometric mean of the PFOA concentrations in Hanoi (0.575 ng mL^{-1}) was far lower than those in other Asian countries (Kannan et al., 2004; Yang et al., 2004; Guruge et al., 2005; Kärrman et al., 2006; Yeung et al., 2006; Jin et al., 2007). The lack of correlation between PFOS and PFOA in Vietnam also suggested that PFOS may have a unique exposure route that differed from those in the other countries examined (reviews by Fromme et al. (2009) and Harada and Koizumi (2009)).

Declines in the serum PFOS levels were not consistently observed in Korea in the present study. Temporal changes in the exposure to PFOS and PFOA have not been evaluated. The phase-out of PFOS and PFOA production by 3M Company might have an effect in limited sources of exposure. Serum levels of PFOS have been detected at higher levels in North American populations

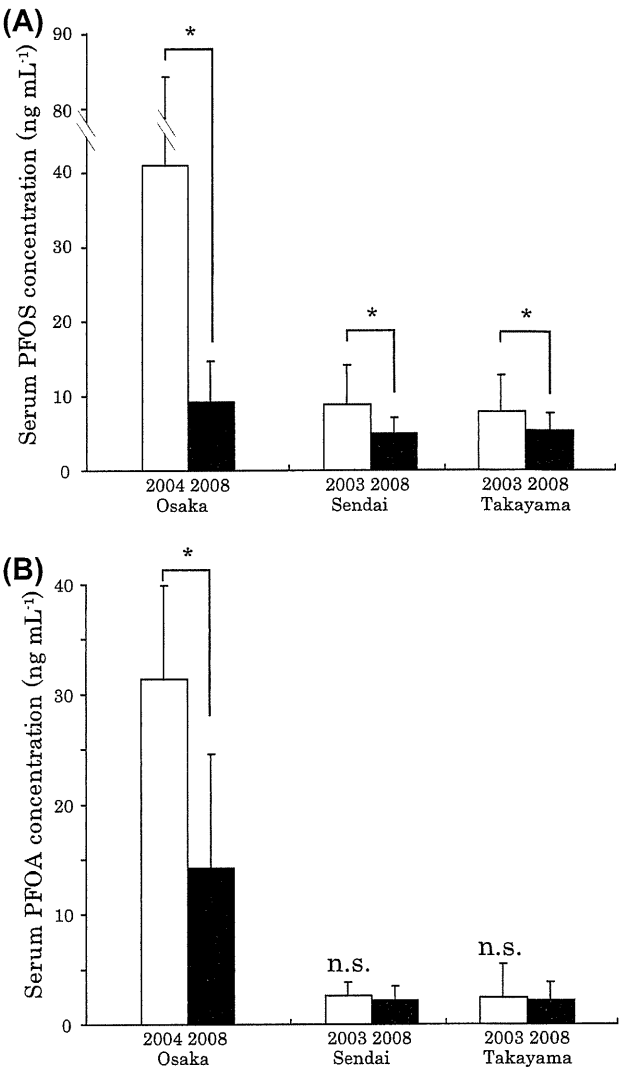

Fig. 1. Time trends in the serum PFOA (A) and PFOS (B) levels in Japanese females from Sendai, Takayama and Osaka. The filled bars represent the results obtained in this study and the open bars represent previously reported data in identical sampling areas by Harada et al. (2007) and Kärrman et al. (2009). Data are shown as geometric means and geometric standard deviations. The asterisks indicate statistically significant difference between 2 sampling years by ANOVA. n.s. indicates there was no significant difference ($p > 0.05$ by ANOVA).

compared with the populations examined in this study (Fromme et al., 2009), and may be more influenced by PFOS-related applications than by indirect exposure such as food intake (Trudel et al., 2008; Vestergren et al., 2008). Therefore, there remains a large amount of uncertainty in predicting the future transition of these serum levels based on the present knowledge of the fate and exposure of PFOS and PFOA.

The high human serum concentrations of PFOA in Osaka, which remain unchanged, raise an alarming signal. Recent epidemiological data have suggested effects on the birth weight in the general population and revealed inverse correlations with PFOA levels that were lower than the average level in Osaka (Apelberg et al., 2007; Fei et al., 2007, 2009; Washino et al., 2009). Further investigations will be required to specifically characterize the risks of exposure for residents.

In addition, the observed variations in the temporal and spatial trends warrant further investigations into the sources and exposure routes in the individual countries, which were not conducted

in the present study. An international study to investigate exposure sources, including air, food and water, is needed to adequately account for the regional differences and to control fluorinated pollutants.

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Original Article

Role of Peroxisome Proliferator-activated Receptor-α in Hepatobiliary Injury Induced by Ammonium Perfluorooctanoate in Mouse Liver

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Abstract: Peroxisome proliferator-activated receptor-α (PPARα) has been suggested to protect against chemically induced hepatobiliary injuries in rodents. This function could mask the potential toxicities of perfluorooctanoic acid (PFOA) that is an emerging environmental contaminant and a weak ligand of PPARα. However its function has not been clarified. In this study, PFOA was found to elicit hepatocyte and bile duct injuries in *Pparα*-null mice after 4 wk treatment with PFOA ammonium salt (0, 12.5, 25, 50 μmol/kg/d, gavage). In wild-type mice, PFOA caused major hepatocellular damage dose-dependently and minor cholangiopathy observed only at 25 and 50 μmol/kg. In treated *Pparα*-null mice, PFOA produced marked fat accumulation, severe cholangiopathy, hepatocellular damage and apoptotic cells especially in bile ducts. Oxidative stress was also increased 4-fold at 50 μmol/kg and *TNF-α* mRNA was upregulated more than 3-fold at 25 μmol/kg in *Pparα*-null mice. Biliary bile acid/phospholipid ratios were higher in *Pparα*-null mice than in wild-type mice. Results from these studies suggest that PPARα is protective against PFOA and have a critical role in drug induced hepatobiliary injury.

Key words: Peroxisome proliferator-activated receptor-α, Perfluorooctanoic acid, Hepatobiliary injury, Bile acid transporter, Histopathology

Introduction

Peroxisome proliferator-activated receptor-α (PPARα) is a ligand-activated receptor that mediates critical transcriptional regulation of genes associated with lipid homeostasis. PPARα is also suggested to have important roles in inflammation, immune response and hepatocarcinogenesis, however the mechanism has not been clarified. Perfluorooctanoic acid (PFOA) is a fluorinated eight-carbon member of the perfluoroalkyl acid family that is amphiphilic and is used in the preparation of surfactants and fabricants[1]. The potential health risk for PFOA arises from its ubiquitous distribution and persistence in the environment, and its presence in humans and wildlife[2, 3]. PFOA is assumed to be a weak PPARα ligand because of its low degree PPARα transcriptional activations among PPARα ligands[4] and is carcinogenic to rodents[5, 6].

The pathophysiological roles of PPARα in toxicity caused by PFOA is well delineated by *Pparα*-null mice[7, 8]. Rosen *et al.* (2008) demonstrated that ablation of PPARα changes profiles of transcripts related to fatty acid metabolisms, inflammation, xenobiotic metabolism

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and cell cycle regulation[7]. Qualitative changes in transcripts modified hepatortoxicity significantly in *Pparα*-null mice, leading a conclusion that PPARα is required for PFOA-induced cellular alterations in mouse hepatocytes.

Recently, Hays *et al.* demonstrated that a weak PPARα ligand, bezafibrate, induces cholestasis without neoplastic changes in *Pparα*-null mice, and have concluded that PPARα protects against potential cholestasis, while it facilitates tumor promotion[9]. They have also demonstrated that a very specific PPARα ligends, Wy-14,643, does not induce cholestastis[9]. Thus, the toxicity profile of a chemical that up- or down-regulates via PPARα-dependent and independent pathways may be modified depending on its affinity to PPARα and its dose.

A reasonable conjecture would be that PFOA, which is known as a PPARα weak ligand, might also induce cholestatic disease in *Pparα*-null mice. No study on PFOA has ever investigated so far biliary duct toxicity. This study examined whether PFOA has the potential for inducing cholestatic disease and PPARα has a role in protecting against chemical induced choestasis. We investigated whether PFOA induces cholestasis in *Pparα*-null mice and the dose-response relationship between PFOA and toxicological responses in *Pparα* wild and null mice. It is well known that cholestasis is not a common response in mice, although it is a very common response to xenobiotics including therapeutic drugs in human[10]. Thus, the null genotype of PPARα might reveal bile duct toxicity of many PPARα inducers otherwise overlooked, although they may be recognized only at very high doses[11].

Subjects and Methods

Animals and treatment

Wild-type mice (129S4/SvlmJ) and *Pparα*-null mice (129S4/SvJae-Pparα^{tm1Gonz}/J) were originally provided from Dr. Frank J. Gonzalez (National Cancer Institute, Bethesda, MD) and housed in Kyoto University Institute of Laboratory Animals. All experiments were performed with male mice aged 8–10 wk (22–25 g). 39 wild-type mice and 40 *Pparα*-null mice were randomly assigned to four groups in accordance with the administered doses of PFOA (0, 12.5, 25, 50 μmol/kg/d). PFOA ammonium salt (>98% purity) was purchased from Fluka Chemical (Steinheim, Switzerland) and dissolved in deionized water. Mice were treated by oral gavage (8 ml/kg) daily for 4 wk and killed by euthanasia at the end of 4 wk, at which time, blood, liver and bile were collected.

Livers were weighed, and the tissue was fixed in 10% neutral-buffered formalin for light microscopic examination or 1% glutaraldehyde/1.44% paraformaldehyde solution for transmission electron microscopy for ultrastructural examination. The remaining portion was flash-frozen in liquid nitrogen and stored at –80°C.

Biochemical measurements

Biochemical analyses were performed on plasma samples. These analyses were examined by Nagahama Life Science Laboratory, Oriental Yeast Co. Ltd. (Shiga, Japan), and included aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as hepatocellular damage markers, total bilirubin (T-Bil) and total bile acid (TBA) as cholestatic markers, and total cholesterol (T-Cho) and triglyceride (TG) as fat metabolism markers.

Histology

For light microscopy, livers were processed by routine paraffin sectioning and staining with hematoxylin and eosin (HE). For the determination of apoptosis, a terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling, TUNEL, test was performed. An Apop Tag kit (Oncor, Gaithersburg, MD) was used according to the manufacturer's recommendations.

For ultrastructural studies, livers were post-fixed in 1% osmium tetroxide in 0.2 M phosphate buffer, routinely dehydrated through a graded ethanol series, and embedded in Epon using the Luft method[12]. Sections were cut in 80 μm on a Leica EM UC6 ultramicritome (Hitachi, Tokyo, Japan) with a diamond knife, and stained by the Reynolds method[13]. The grids were examined under a Hitachi 7650 transmission electron microscope (Hitachi).

Analysis of PFOA

Determination of PFOA in whole blood, bile and liver was performed using a modification of a method originally developed by Yline *et al*[14]. Diluted blood, bile or homogenized liver was combined with 10 μl of a 1 μg/ml solution of $^{13}C_2$-PFOA as an internal standard. One milliliter of tetrabutylammonium hydrogen sulfate and 2 ml 0.5 M sodium carbonate buffer solution (pH adjusted to 10) were combined and vortexed, then 2 ml methyl *tert*-butyl ether (MTBE) was added and vortexed. The tube was centrifuged to separate the aqueous and organic phases, and 1 ml of the MTBE layer was extracted, transferred to a glass tube, and evaporated to dryness at 38°C under a gentle stream of dry nitrogen. The residue was then redissolved in 100 μl of 100 mM benzyl bromide acetone for 1 h at 80°C and transferred to an autosampler vial. Extracts were analyzed using gas chromatography-mass spectrometry (Agilent 6890GC/5973MSD, Agilent Technologies Japan, Ltd., Tokyo, Japan) in electron impact ionization mode. PFOA was separated on an HP-5MS column (30 m length, 0.25 mm i.d., 0.25 μm film thickness) with a helium carrier gas. Splitless injections (2 μl) were performed with the injector set at 220°C, and

the split was opened after 1.5 min. The initial oven temperature was 60°C for 1.5 min, ramped at 15°C min^{-1} to 100°C, and then at 40°C min^{-1} to 240°C. Recoveries of $^{13}C_2$ PFOA from biological samples (n=3) were 94 ± 2.6% for blood, 97 ± 4.4% for bile and 94.7 ± 4.9% for liver, respectively.

Measurement of 8-hydroxydeoxyguanosine (8-OHdG) in the liver

8-OHdG/dG levels were measured as an indicator of oxidative DNA damage. The frozen livers were minced and gently homogenized in a homogenizer by 5 strokes in lysis solution (Qiagen, Tokyo, Japan), DNA was extracted from mice frozen liver using a DNA Extractor WB kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan)[15]. DNA was digested completely to nucleotides by combined treatment with Nuclease P1 (Wako Pure Chemical Industries, Ltd.) and alkaline phosphatase (Sigma Chemical Co., St. Louis, MO). Then the resulting deoxynucleoside mixture was injected into a high performance liquid chromatography apparatus (LC-10ADvp, Shimadzu, Kyoto, Japan) equipped with both a UV detector (SPD-10AVvp, Shimadzu) and an electrochemical detector (Coulochem model-5200-2, ESA, MA)[16]. Each liver was examined in duplicate and the means were reported.

Quantitative RT-PCR analysis for multidrug resistance protein 2 (Mdr2) and tumor necrosis factorα (TNF-α)

Quantitative real-time (RT)-PCR was used to study *Mdr2* and *Tnf* mRNA expression in the liver. Total RNA was extracted from the liver using RNeasy Lipid Tissue Mini Kit (Qiagen). Aliquots (10 ng) were amplified using QuantiTect® SYBR® Green RT-PCR (Qiagen). Quantification of the amplified products was performed on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems Japan, Tokyo, Japan). All expression data were normalized to glyceraldehyde 3-phophate dehydrogenase (*GAPDH*) mRNA from the same individual sample, to correct for differences in efficiency of RNA extraction and quality.

The following primers were used for RT-PCR: *GAPDH*: forward, 5'-ATGGTGAAGGTCGGTGTGAA-3'; reverse, 5'-GAGTGGAGTCATACTGGAAC-3'[17], corresponding to GenBank accession number M32599; *Mdr2*: forward, 5'-ATCCTATGCACTGGCCTTCTGGT-3'; reverse, 5'-GAAAGCATCAATACAGGGGGCAG-3'[18], corresponding to GenBank accession number NM_008830; *Tnf* forward, 5'-TCTTCTCAAAATTCGAGTGACAAG-3'; reverse, 5'-GAGAACCTGGGAGTAGACAAGGTA-3', (note: designed in our lab) corresponding to GenBank accession number NM_013693.

Determination of bile acid/phospholipid ratio (BA/PL) in bile

Commercially available kit was used for determination of bile acid and phospholipid contents in bile (Wako Pure Chemical Industries). For bile acid determination, 0.1 μl bile was diluted in 200 μl double distilled H_2O, that was added to 500 μl 3-α-hydroxysteroid dehydrogenase, incubated at 37°C for 10 min, added to 500 μl response fixing solution, and absorbance was read at 560 nm using a Hitachi U-2000A spectrophotometer (Hitachi). For phospholipid determination, 0.4 μl bile was diluted in 20 μl double distilled H_2O, that was added to 3.0 ml color reagent (Phospholipid-C Test Wako, Wako Pure Chemical Industries), incubated at 37°C for 5 min, and absorbance was read at 600 nm against a color reagent blank.

Western blot analysis of BSEP and MRP2

Western blot analysis was carried out for quantification of the protein levels of the canalicular bile salt export pump (BSEP) and the canalicular multidrug resistance-associated protein 2 (MRP2). Membrane protein samples mixed with sample loading buffer (15 μg protein/lane) were loaded after heating for 10 min at 70°C onto a 3–8% Tris-acetate gel. Following electrophoresis, proteins in the gel were electrotransferred to PVDF-plus membranes (Immobilon-P Transfer Membrane; Millipore) for 1 h at 30 V at 4°C. Membranes were blocked for 1 h at room temperature with 5% non-fat dry milk in Tris-buffered saline that contained 0.05% Tween-20 (TBS-T). Blots were then incubated for 1 h at room temperature with the primary polyclonal antibody of rabbit BSEP, which was kindly provided by Rexue Wang, (British Columbia Cancer Research Center, Vancouver, BC, Canada) and rat MRP2, which was kindly provided by Bruno Stieger (University Hospital, Zurich, Switzerland). GAPDH antibody was used as a loading control. Each primary antibody was diluted in blocking buffer (1:5,000 for BSEP, 1:4,000 for MRP2, 1:1,000 for GAPDH). After thorough washing, blots were incubated with donkey anti-rabbit IgG horseradish peroxidase-linked secondary antibody (1:4,000 dilution with 5% non-fat milk in TBS-T) for 1 h. Immunoreactive bands were detected with an enhanced chemical luminescence (ECL) kit (Immobilon Western; Millipore). BSEP and MRP2 proteins were visualized by exposure to Fuji Medical X-Ray film (FUJIFILM Medical Co., Ltd, Tokyo, Japan).

Statistical analysis

Nine or 10 animals were studied in each group. All results were expressed as mean ± SD. Comparisons between two groups were performed using an unpaired Student's *t* test, and Dunnett's test for dose-response experiments. Levene's test was used to assess the equal-

ity of variance. Trend test was performed using Jonckheere's test. $p<0.05$ was considered to be statistically significant. Statistical analyses were done on SAS software (ver.8.2).

Results and Discussion

Body and liver weights (Table 1)

Body and liver weight changes after exposure to PFOA in both groups of mice are shown in Table 1. Absolute and relative liver weights (% body weight) were increased approximately three fold in wild-type or *Pparα*-null mice, and induction of hepatomegaly reached plateau levels in both genetic backgrounds at doses higher or equal to 12.5 μmol/kg. These results demonstrated that PFOA induced hepatomegaly through non-PPARα-mediated pathways as previously reported[19].

Biochemical analysis (Table 2)

In wild-type mice, judging from the plasma AST and plasma ALT values, 12.5 or 25 μmol/kg PFOA caused hepatocellular damages with slight changes in T-Bil and TBA. The hepatocellular damages seemed to increase with increase in dose. Mild cholestasis was apparent at 50 μmol/kg, at which dose mild increases in T-Bil and TBA were observed. T-Cho was decreased after treatment with PFOA at 25 and 50 μmol/kg. TG was increased after treatment with PFOA at 12.5 and 25 μmol/kg, but was the same level at 50 μmol/kg.

In *Pparα*-null mice, 12.5 or 25 μmol/kg PFOA treatment induced mild hepatocellular damages indicated by ALT but those changes were not accompanied by elevation of T-Bil or TBA. At 50 μmol/kg, PFOA, however, induced extensive hepatocellular damages and cholestasis simultaneously with a sharp contrast with wild cholestasis in wild mice. TG metabolism was significantly disturbed, even at 12.5 μmol/kg, while cholesterol metabolism was disturbed only at the highest dose of 50 μmol/kg.

Biochemical analysis suggested a significant modification of liver toxicity of PFOA by PPARα. Hepatocytes were more vulnerable than bile duct cells to PFOA in wild-type mice. In contrast, ablation of PPARα rendered the hepatocytes tolerable to PFOA-induced damage, at doses lower than 50 μmol/kg, while extensive hepatic and bile duct injuries occurred at 50 μmol/kg as shown in next section. In addition, metabolism of both T-Cho and TG was impaired more extensively in *Pparα* null than wild-type mice.

Histology

In PFOA wild-type mice, PFOA induced hepatocellular hypertrophy. The liver parenchyma showed dose-dependent eosinophilic cytoplasmic changes that were morphologically consistent with peroxisome proliferation (Fig. 1A–D). However, no fat droplets or focal necrosis were observed in control or treated mice at any doses. Bile duct epithelium showed a slight increase in thickness, which suggested that slight cholangiopathy occurred at 25 and 50 μmol/kg (Fig. 1C, D).

The histological appearance in control *Pparα*-null mice showed greater occurrence of microvesicular steatosis than in wild control mice (Fig. 1E). In PFOA-treated *Pparα*-null mice, the hepatocytes showed not only hepatocellular hypertrophy, but also cytoplasmic vacuolation and an increase in microvesicular steatosis (Fig. 1F–H). Focal necrosis was detectable at 50 μmol/kg (Fig. 1I). The most characteristic change was cholangiopathy. Although it was found in both wild and *Pparα*-null mice

Table 1. Body weight and liver weight changes after exposure to PFOA in wild-type and *Pparα*-null mice

PFOA dose levels (μmol/kg)	At the start of the experiment		At sacrifice after 4 wk dosing				
	Gross body weight (g)	Body weight - Liver weight (g)[a]	Gross body weight (g)	Liver weight (g)	Relative liver weight (%)	Body weight - Liver weight (g)	Body weight gain excluded liver
Wild-type							
0 (n=9)	23.9 ± 1.97	23.0 ± 1.89	26.6 ± 2.13	1.0 ± 0.08	3.7 ± 0.4	25.7 ± 2.22	2.7 ± 1.36
12.5 (n=10)	23.8 ± 0.79	22.9 ± 0.76	27.5 ± 2.07	3.2 ± 0.20***	11.3 ± 0.6***	24.7 ± 1.98	1.8 ± 1.95
25 (n=10)	24.2 ± 1.98	23.3 ± 1.90	25.5 ± 1.94	3.3 ± 0.30***	12.9 ± 0.8***	22.5 ± 1.71	–0.9 ± 1.64***
50 (n=10)	24.5 ± 1.67	23.6 ± 1.61	23.0 ± 2.90**	3.3 ± 0.45***	13.1 ± 0.9***	20.5 ± 2.50	–3.1 ± 2.09***
Pparα (−/−)							
0 (n=10)	22.7 ± 1.53	21.6 ± 1.46	25.0 ± 1.56	1.0 ± 0.12	4.7 ± 2.1	24.1 ± 1.37	2.5 ± 0.58
12.5 (n=10)	23.2 ± 1.87	22.1 ± 1.78	27.9 ± 1.99**	3.3 ± 0.45***	11.6 ± 1.7***	25.1 ± 1.78	3.0 ± 1.44
25 (n=10)	23.5 ± 1.54	22.4 ± 1.47	27.4 ± 0.93*	3.4 ± 0.23***	11.9 ± 1.2***	24.5 ± 1.16	2.1 ± 1.37
50 (n=10)	23.4 ± 1.88	22.3 ± 1.80	26.4 ± 2.07	3.4 ± 0.51***	13.0 ± 1.6***	23.7 ± 2.64	1.4 ± 1.98

Note: Values are expressed as mean ± SD.

*$p<0.05$, **$p<0.01$, ***$p<0.001$ by Dunnett's test compared with 0 μmol/kg.

[a]Liver weight was assumed as 3.7% in wild-type and 4.7% in *Pparα*-null mice of gross body weight.

Table 2. Plasma analysis of PFOA-treated wild-type and *Pparα*-null mice

PFOA dose levels (μmol/kg)	AST (IU/l)		ALT (IU/l)	
	Wild-type	*Pparα* (−/−)	Wild-type	*Pparα* (−/−)
0 (n=9, 10)	145 ± 71.1	137 ± 25.9	26 ± 7.1	23 ± 7.2
12.5 (n=10)	175 ± 29.2	145 ± 31.5†	176 ± 62.4^{+++}	136 ± 45.3^{+++}
25 (n=10)	265 ± 146.2*	152 ± 20.2†	284 ± 158.9^{+++}	176 ± 42.8^{+++}
50 (n=10)	365 ± 106.0***	870 ± 523.5***†††	328 ± 128.9^{+++}	1356 ± 744$^{+++\dagger\dagger\dagger}$
	T-Bil (mg/dl)		TBA (mmol/l)	
	Wild-type	*Pparα* (−/−)	Wild-type	*Pparα* (−/−)
0 (n=9, 10)	0.09 ± 0.05	0.06 ± 0.02	4.5 ± 7.2	2.4 ± 2.6
12.5 (n=10)	0.05 ± 0.01*	0.02 ± 0.01†††	4.5 ± 1.6	1.0 ± 0†††
25 (n=10)	0.09 ± 0.03	0.03 ± 0.01†††	9.0 ± 4.6	1.4 ± 0.6††
50 (n=10)	0.15 ± 0.04**	0.47 ± 0.39***†	12.5 ± 9.9	34.8 ± 9.1***††
	T-Cho (mg/dl)		TG (mg/dl)	
	Wild-type	*Pparα* (−/−)	Wild-type	*Pparα* (−/−)
0 (n=9, 10)	115 ± 9.7	136 ± 26.8†††	59 ± 17.6	45 ± 17.3
12.5 (n=10)	109 ± 17.7	84 ± 21.9***†	87 ± 15.7**	91 ± 35.5**
25 (n=10)	95 ± 15.4**	87 ± 13.7***	89 ± 28.4**	105 ± 23.8***
50 (n=10)	86 ± 11.6***	226 ± 23.0***†††	51 ± 18.4	114 ± 32.1***†††

AST, aspartate aminotransferase; ALT, alanine aminotransferase; T-Bil, total bilirubin.
TBA, total bile acid; T-Cho, total cholesterol; TG, triglyceride.
Data are expressed as mean ± SD.
*p<0.05, **p<0.01, ***p<0.001 by Dunnett's test compared with 0 μmol/kg.
^{+}p<0.05, ^{++}p<0.01, ^{+++}p<0.001 by Dunnett's test after log-transformation due to heteroscedasticity (Levene's test p<0.05).
$^{\dagger}p$<0.05, $^{\dagger\dagger}p$<0.01, $^{\dagger\dagger\dagger}p$<0.001 by t-test compared with wild-type and *Pparα*-null mice at same PFOA-dose level.

treated with PFOA at 25 (Fig. 1C and G) and 50 μmol/kg (Fig. 1D and H), it was more intensive in the latter than in the former (Fig. 1C, D, G, H). In particular, it was shown in *Pparα*-null mice that bile ducts were surrounded by a few inflammatory cells and areas of fibrosis and bile plaque (Fig. 1H).

TUNEL staining demonstrated increased apoptosis in hepatic cells, hepatic arterial walls and bile-duct epithelium in wild-type mice treated with PFOA at 25 and 50 μmol/kg (Fig. 2A, B, E, F). On the other hand, in *Pparα*-null mice, positive staining was observed mainly in bile duct epithelium at 25 and 50 μmol/kg (Fig. 2C, D, G, H).

The ultrastructure of livers from control wild-type mice (Fig. 3A) exhibited numerous glycogen granules, normal lamellar arrangement of the rough endoplasmic reticulum (RER), a few normal dense peroxisomes and mitochondria. In contrast to control livers, treated wild-type mice (Fig. 3B–D) displayed dose-dependent, hepatocyte hypertrophy, reduction or disappearance of glycogen granules, degranulation and disruption of the RER, nuclear vacuoles, extensive peroxisome proliferation, and slight proliferation of mitochondria. There were larger numbers and sizes of dark-staining peroxisomes and increased small, round-shaped mitochondria (Fig. 3B–D, I).

In control *Pparα*-null mice (Fig. 3E) there were discernible amounts of small fat deposits in the cytoplasm. In treated *Pparα*-null mice (Fig. 3F–H) there were dose-dependent hepatocyte hypertrophy, decreased amounts of glycogen granules, degranulation and disruption of the RER, and increased numbers of mitochondria. There is increased cytoplasmic lipid accumulation to varying extents, extensive mitochondrial changes that consisted of slight swelling, decreased matrix density and inconspicuous criste, but no peroxisome proliferation (Fig. 3F–H, J). In addition, bile duct epithelium showed degradation of cytoplasmic structure, vacuolization, and disintegration of nuclei and organelles. Severe bile duct epithelium injury was observed, with periductal infiltration of fibroblasts and macrophages, and fibrosis (Fig. 3K).

Pharmacokinetics of PFOA in whole blood, bile and liver (Table 3)

In order to investigate whether the absence of PPARα changed the pharmacokinetics of PFOA, the concentration of PFOA was determined in whole blood, bile and liver after dosing for 4 wk. The concentrations of PFOA in whole blood increased in proportion to dose, in both

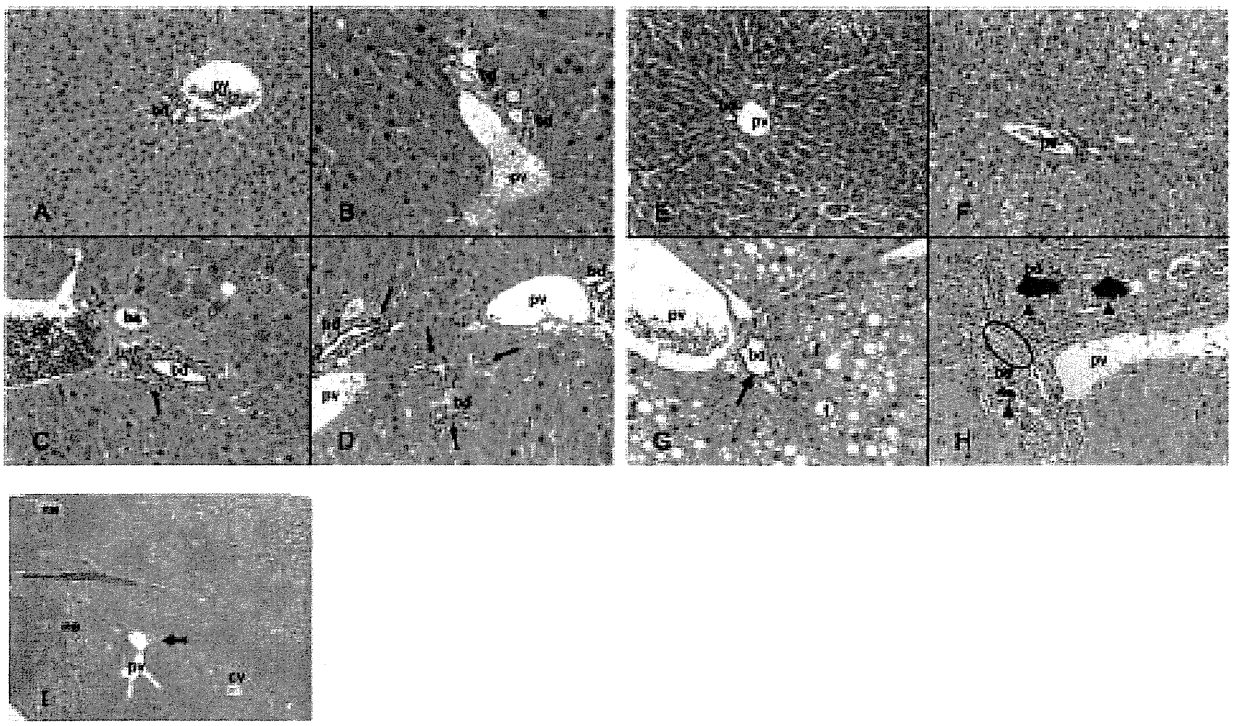

Fig. 1. Effects of PFOA on the mouse liver by oral gavage for 4 wk.
Hematoxylin-eosin stained sections of liver from control wild-type mice (A), wild-type mice treated with PFOA at 12.5 μmol/kg (B), 25 μmol/kg (C), 50 μmol/kg (D), control *Pparα*-null mice (E), *Pparα*-null mice treated with PFOA at 12.5 μmol/kg (F), 25 μmol/kg (G), 50 μmol/kg (H, I). Original magnification, × 200 (A–H), × 40 (I). Wild-type mice treated with PFOA (B–D) have diffuse hepatocyte hypertrophy with numerous eosinophilic cytoplasmic granules. Control *Pparα*-null mice (E) has scattered small fat vacuoles. Centrilobular fat accumulations were increased dose-independently in *Pparα*-null mice treated with PFOA at 12.5 μmol/kg (F), 25 μmol/kg (G), 50 μmol/kg (H, I). Focal necrosises are scattered with fat accumulation and proliferation of bile ductules is prominent in the portal tracts in *Pparα*-null mice treated with PFOA at 50 μmol/kg (I). Diffuse hepatocyte hypertrophy was observed in both mouse lines treated (B–D, F–H). Bile duct epithelial thickness (arrow) was observed in both mouse lines treated at 25 μmol/kg (C, G) and 50 μmol/kg (D, H). Diffusely distributed, fine, fatty droplets and ground-glass appearance is showed at 12.5 μmol/kg (F) and 25 μmol/kg (G) in *Pparα*-null mice. Note hyperplastic changes in the biliary duct epithelium with bile plaque (arrow head) and fibrosis (open circle) as evidenced by proliferation of bile ductules (arrow) in *Pparα*-null mice treated with PFOA at 50 μmol/kg (H). cv, central vein; pv, portal vein; ha, hepatic artery; bd, bile duct; f, fat droplet; ne, necrosis.

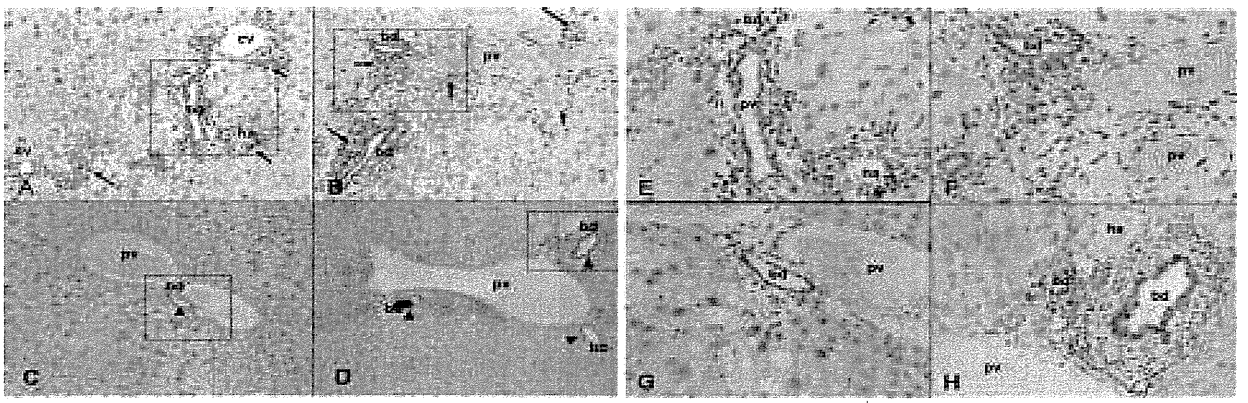

Fig. 2. Distribution of apoptotic cells in liver PFOA treated by oral gavage for 4 wk by immunohistochemistry for TUNEL.
Wild-type mice treated with PFOA at 25 μmol/kg (A, E) and 50 μmol/kg (B, F), *Pparα*-null mice treated with PFOA at 25 μmol/kg (C, G) and 50 μmol/kg (D, H). Original magnification, × 100 (A–D), × 400 (E–H) the extended a part surrounded with a square in A–D, respectively. Wild-type mice treated with PFOA at 25 μmol/kg (A, E) and 50 μmol/kg (B, F) show diffuse positive stains in hepatocyte, vessel wall, and bile duct epithelium (arrow). *Pparα*-null mice treated with PFOA at 25 μmol/kg (C, G) and 50 μmol/kg (D, H) show positive stains mainly in bile duct epithelium (arrow head). cv, central vein; pv, portal vein; ha, hepatic artery; bd, bile duct.

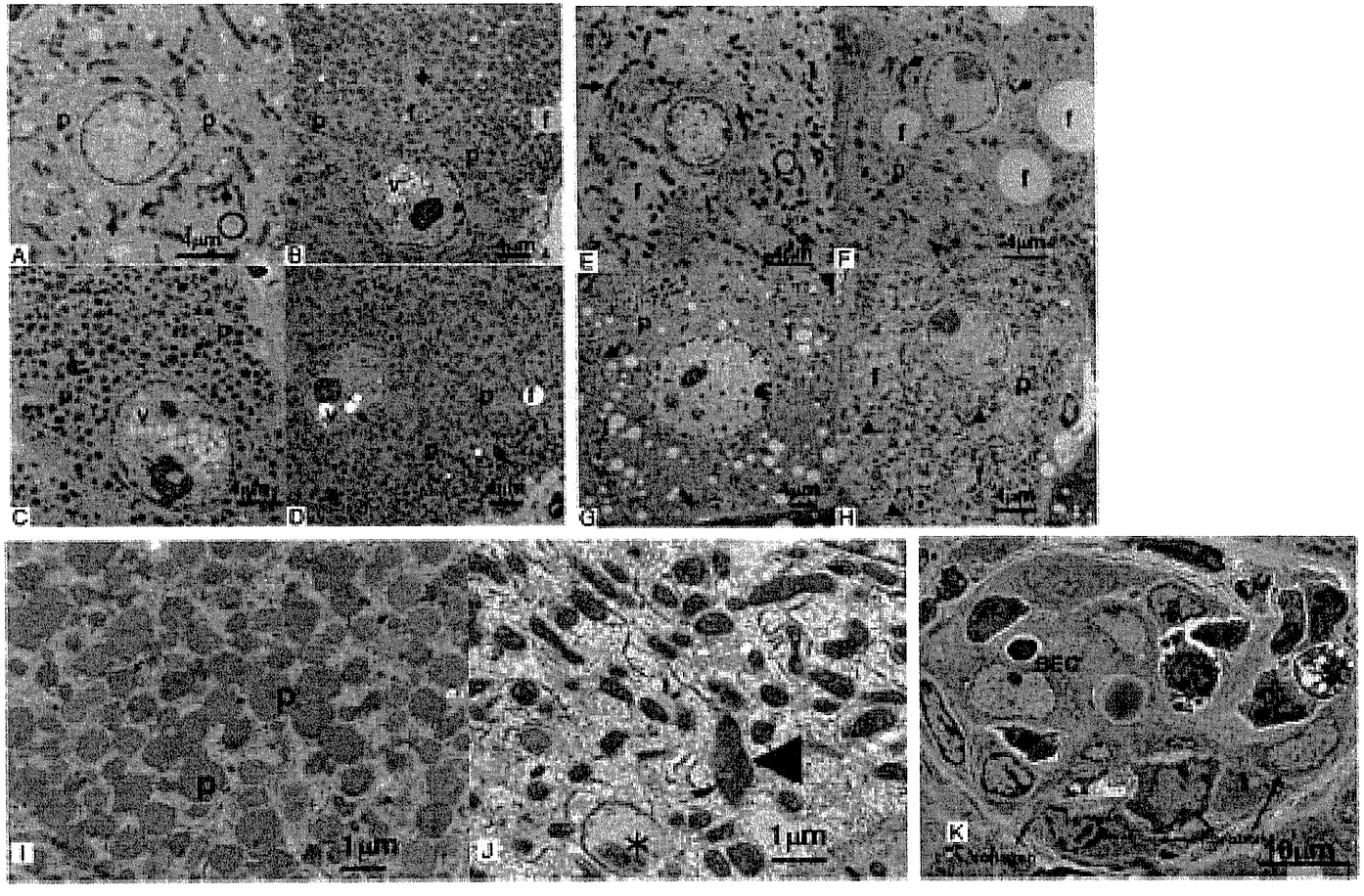

Fig. 3. Ultrastructure of hepatocyte and bile duct epithelium cells in control and after treatments of wild-type mice and *Pparα*-null mice with PFOA by oral gavage for 4 wk.

Hepatocytes from control wild-type mice (A), wild-type mice treated with PFOA at 12.5 μmol/kg (B), 25 μmol/kg (C), 50 μmol/kg (D, I), Control *Pparα*-null mice (E), *Pparα*-null mice treated with PFOA at 12.5 μmol/kg (F), 25 μmol/kg (G), 50 μmol/kg (H, J), Bile duct epithelial cell (BEC) of *Pparα*-null mice treated with PFOA at 50 μmol/kg (K).

Numerous glycogen granules (circle) are observed in control wild-type mice (A). The increased number and size of dark staining peroxisomes were shown in treated wild-type mice (B–D, I). Hepatocytes from control *Pparα*-null mice (E) are similar to control wild-type mice with fewer fat droplets (f) in cytoplasm. In contrast to controls, treated *Pparα*-null mice (F–H, J) also display hepatocyte hypertrophy, decreased glycogen granules, degranulation and disruption of the rough endoplasmic reticulum, and increased mitochondria in dose-dependently. The marked different points contrasts to wild-type mice treated with PFOA are increased fat droplets in cytoplasm, a few peroxisomes, and a variable size and shape of mitochondria (F–H, J). Note that peroxisomes are markedly increased and slightly enlarged in size in wild-type mice treated with PFOA at 50 μmol/kg (I), and mitochondria are pleomorphic, enlarged (*), and disorganization of cristae (arrowhead) in *Pparα*-null mice treated with PFOA at 50 μmol/kg (J). BECs (K) showed degradation of cytoplasmic structure, vacuolization, disintegration of nuclei and organelles, and were surrounded with fibroblasts and collagen. p, peroxisome; f, fat droplet; v, vacuole. (A–H) Bar=4 μm, (I, J) Bar=1 μm, (K) Bar=10 μm.

Table 3. Whole blood, bile and liver concentrations of PFOA in wild-type and *Pparα*-null mice

PFOA dose levels (μmol/kg)	PFOA concentration (μg/ml)					
	Whole blood		Bile		Liver	
	Wild-type	*Pparα* (−/−)	Wild-type	*Pparα* (−/−)	Wild-type	*Pparα* (−/−)
0 (n=9, 10)	nd	nd	nd	nd	nd	nd
12.5 (n=10)	20.6 ± 2.4	19.3 ± 2.2	56.8 ± 26.9	19.6 ± 2.2	181.2 ± 6.3	172.3 ± 8.9
25 (n=10)	46.9 ± 3.2	36.4 ± 2.7*	784.0 ± 137.6	62.9 ± 16.7**	198.8 ± 15.4	218.3 ± 14.5
50 (n=10)	64.2 ± 6.5	71.2 ± 8.0	2174.0 ± 322.4	383.0 ± 109.9**	211.6 ± 13.3	239.7 ± 25.0

Data are expressed as mean ± SD.
*p<0.05, **p<0.01, ***p<0.001 by t-test compared between wild-type and *Pparα*-null mice.
nd; not detected (less than 0.001 μg/ml).

wild-type and *Pparα*-null mice (Table 3). On the other hand, the concentrations in liver reached similar saturation levels at 12.5 μmol/kg in wild-type and *Pparα*-null mice.

The concentrations of PFOA in bile increased with dose; it increased by 13.8 times from 56.8 μg/ml at 12.5 μmol/kg to 784 μg/ml at 25 μmol/kg, and 38 times to 2,174 μg/ml at 50 μmol/kg in wild-type mice. Enhanced PFOA excretion indicates that the liver has a PFOA transport capacity from hepatocytes to bile duct that can be mediated at least partly by PPARα. In contrast, much lower increases were observed in *Pparα*-null mice. PFOA concentrations increased by 3.2 times from 19.6 μg/ml at 12.5 μmol/kg to 62.9 μg/ml at 25 μmol/kg, and by 19.5 times to 383.0 μg/ml at 50 μmol/kg, demonstrating existence of capacity-limited and PPARα-independent PFOA transport.

8-OHdG levels in liver and quantitative RT-PCR of TNF-α mRNA

In wild-type mice, PFOA did not elevate the levels of 8-OHdG in liver significantly at any dose (Fig. 4A). In contrast, in *Pparα*-null mice, the levels of 8-OHdG tended to increase dose-dependently (p<0.05), which was significantly increased at 50 μmol/kg (p<0.05) (Fig. 4A). PFOA did not alter the levels of *TNF-α* mRNA in wild-type mice (Fig. 4B). However, PFOA upregulated *TNF-α* mRNA significantly at doses of 25 and 50 μmol/kg in *Pparα*-null mice (p<0.01 and p<0.05, respectively) (Fig. 4B).

These data demonstrated that ablation of PPARα exacerbated oxidative damage and enhanced production of inflammatory cytokines after PFOA administration.

Quantitative RT-PCR for Mdr2, bile acids/phospholipids ratio in bile

We investigated three prototypical hepatobiliary transporters. MDR2 transports biliary phospholipids from hepatocytes to bile via the canalicular phospholipid flippase, which alleviates bile acid toxicity in cholangiocytes[20]. BSEP transports bile acid from hepatocytes to bile via the canaliculi to keep bile acid concentrations constant in bile[21, 22], and confers resistance to canalicular damages in humans[23]. MRP2 is a transporter of bile acid and is a sensitive indicator of canalicular damages[24]. Recently, PFOA has been reported to regulate liver transporters, organic anion transporting polypeptides (OATPs) and multidrug resistance-associated proteins responsible for uptake of bile acids (BAs) and other organic compounds into liver, primarily via activation of PPARα[25, 26]. For investigating expression of MDR2, we performed quantitative RT-PCR instead of Western blotting because the MDR2 antibody was not specific for mouse MDR2 (data not shown). In wild-type mice, the expression of *Mdr2* mRNA was significantly upregulated by PFOA at 12.5, 25 and 50 μmol/kg (p<0.05, p<0.01 and p<0.01, respectively) (Fig. 4C). In *Pparα*-null mice, *Mdr2* mRNA was not induced by PFOA at 12.5 μmol/kg, however, it was induced significantly at 25 and 50 μmol/kg (p<0.05 and p<0.01, respectively) (Fig. 4C). This non-PPARα-mediated increase in *Mdr2* mRNA may likely be attributable to the increase in bile acid at high doses[27] or other nuclear receptors[28]. To confirm adaptive phospholipid transport, we examined the biliary bile acid to phospholipid (BA/PL) ratio (Fig. 4D). As expected, BA/PL ratio decreased significantly in a dose-dependent manner in PFOA-treated wild-type mice (p<0.01). However, no such significant adaptation was observed in PFOA-treated *Pparα*-null mice, suggesting that bile duct protective mechanism characterized by increasing phospholipid transport into bile did not work in the null mice.

Western blotting for BSEP and MRP2

Protein levels of BSEP were downregulated in treated wild-type mice significantly at 50 μmol/kg (p<0.01). In contrast, in *Pparα*-null mice, protein level of BSEP was increased significantly at 12.5 μmol/kg (p<0.01), however decreased significantly at 50 μmol/kg (p<0.05) (Fig. 5).

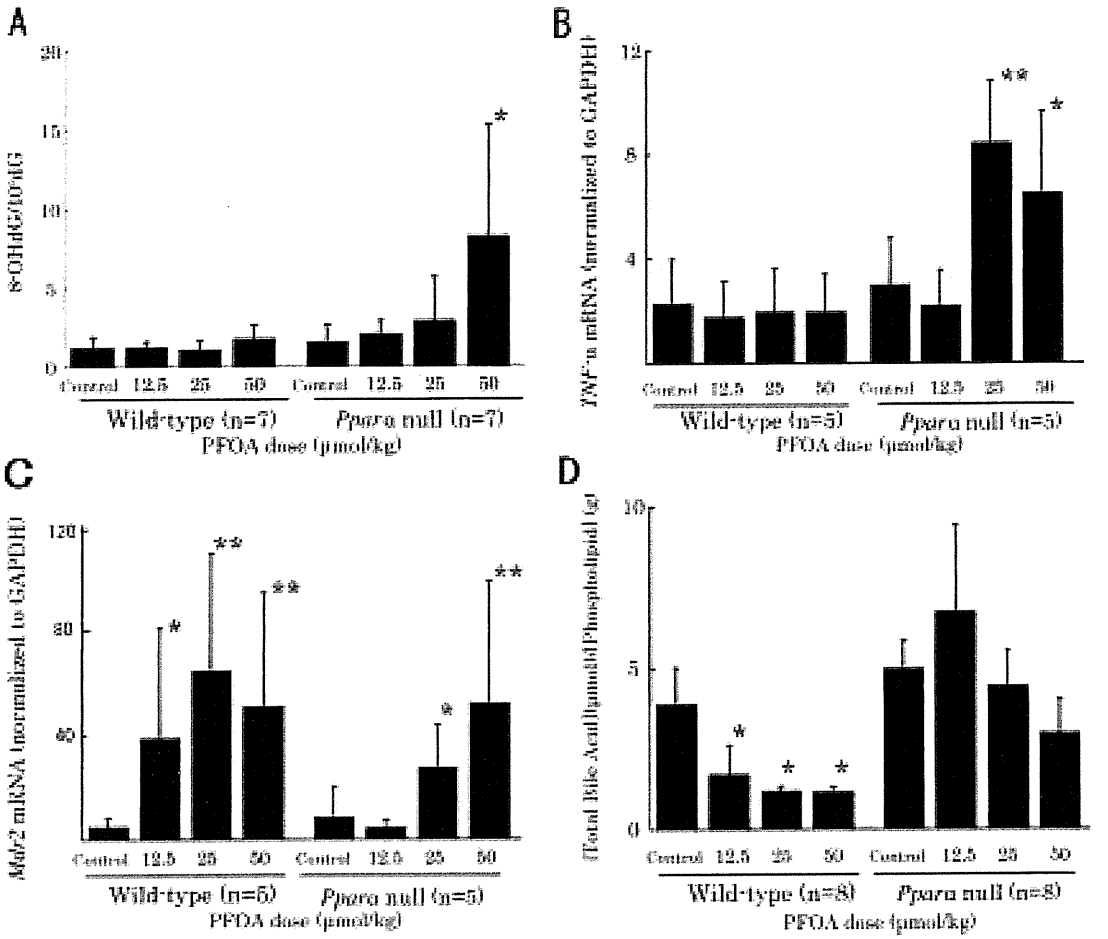

Fig. 4. Effects of PFOA on biomarkers associated with liver injury.

(A) Effects of PFOA on 8-hydroxydeoxyguanosine from unfractionated livers of wild-type and *Pparα*-null mice.

This figure reveals that the levels of 8-OHdG tend to increase dose-dependently in *Pparα*-null mice (Jonckheere's test, $p<0.05$), in which the levels are increased significantly at 50 μmol/kg ($p<0.05$).

(B) The expressions of *TNF-α* mRNA are significantly increased in *Pparα*-null mice treated with PFOA at 25 ($p<0.01$) and 50 μmol/kg ($p<0.05$). (C) The expressions of *Mdr2* mRNA are significantly up-regulated in wild-type mice treated with PFOA at all doses (at 12.5 μmol/kg, 25 μmol/kg and 50 μmol/kg, $p<0.05$, $p<0.01$, respectively). In *Pparα*-null mice treated with PFOA, the expressions of *Mdr2* mRNA are not induced at 12.5 μmol/kg, however induced at 25 μmol/kg ($p<0.05$) and 50 μmol/kg ($p<0.01$) significantly.

(D) Effects of PFOA on biliary total bile acid/phospholipid (BA/PL) ratio.

Biliary BA/PL ratios show significant decrease in wild-type mice treated with PFOA dose-dependently ($p<0.05$). However, no such significant adaptation is observed in *Pparα*-null mice treated with PFOA. Data are presented as mean ± SD. Trend test is Jonckheere's test. *$p<0.05$, **$p<0.01$ versus control controls in each group. Log-transformation was performed for expressions of *Mdr2* mRNA levels due to heteroscedusticity.

The decreased levels of BSEP in *Pparα*-null mice were very likely induced by severe injury of the hepatobiliary system and inflammation[29, 30]. Protein levels of MRP2 decreased in both wild-type mice and *Pparα*-null mice at 50 μmol/kg ($p<0.05$ for both types) (Fig. 5).

Bile duct transporters demonstrated that there were several PPARα-mediated adaptive responses in wild-type mice to alleviate toxicity of PFOA, such as up-regulation of *Mdr2* mRNA and down-regulation of BSEP. In contrast, these responses were not mobilized in concert in PFOA-treated *Pparα*-null mice. Ablation of PPARα made mice highly susceptible to bile duct injury. MRP2 protein levels decreased in both wild-type and *Pparα*-null mice, which might be independent to PPARα.

To embark this study, we have hypothesized that PFOA has a potential toxicity for bile duct as Bezafibrate does[9]. As expected, PFOA was shown to induce cholestatic lesions more intensively in *Pparα* null mice than in wild mice as demonstrated by clinical and pathological inves-

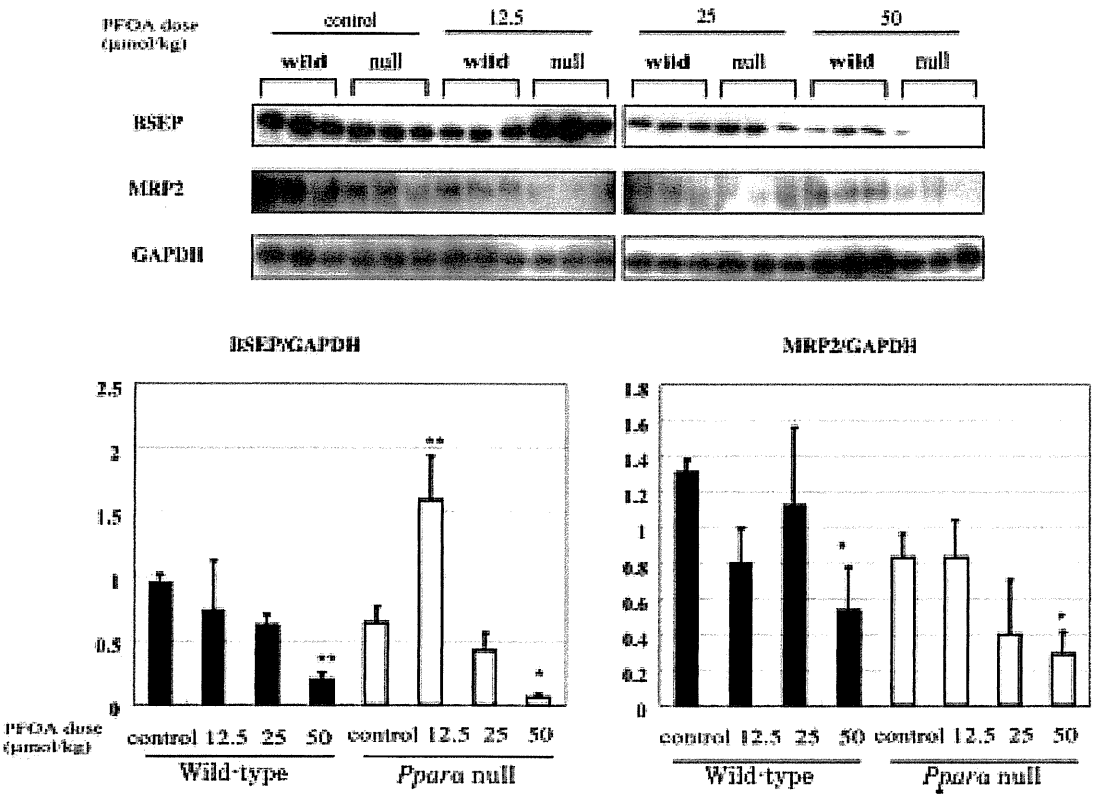

Fig. 5. Effects of PFOA on Hepatic BSEP and MRP2 protein levels.

Each panel represents an individual experiment. There is a significant decrease in BSEP protein level in wild-type mice treated with PFOA at 50 μmol/kg ($p<0.01$). In *Pparα*-null mice treated with PFOA, the levels are increased significantly at 12.5 μmol/kg ($p<0.01$), however decreased significantly at 50 μmol/kg ($p<0.05$). There is a significant decrease in MRP2 protein levels in both wild-type and *Pparα*-null mice treated with PFOA at 50 μmol/kg ($p<0.05$). Control wild-type mice, w0; wild-type mice PFOA treated with 12.5 μmol/kg, w12.5; 25 μmol/kg, w25, 50 μmol/kg, w50; control *Pparα*-null mice, n0; *Pparα*-null mice treated with PFOA at 12.5 μmol/kg, n12.5; 25 μmol/kg, n25, 50 μmol/kg, n50. Black bars, wild-type mice; white bars, *Pparα*-null mice. Densitometric values are presented as mean ± SD of 3 animals in each group. *$p<0.05$, **$p<0.01$ versus control in each group. Trend test is Jonckheere's test.

tigations. Simultaneously, we could demonstrate clear differences in dose dependent mobilization of transporters, *Mdr2* mRNA and BSEP, between wild and null mice. Furthermore, there were differences in inducing 8-OHdG, TNF-α induction and BA/PL ratios in bile between wild and null mice. This is the first study to demonstrate a potential toxicity of PFOA associated with cholestatic disease and PPARα dependent and independent responses.

Although hepatomegaly and increases in AST and ALT were observed in both wild-type and *Pparα* null mice, microscopic appearance and ultrastructure of liver indicated different modes of toxicity as evidenced by biomarkers investigated in this study.

In terms of the mechanism of bile duct injury, we focused on the changes in bile compositions and expression levels of hepatobilliary transporters. BA/PL ratio was decreased immediately in PFOA-treated wild-type mice. On the contrary it was increased at 12.5 μmol/kg and decreased gradually at higher PFOA dose in *Pparα*-null mice. In wild-type mice, the decreased BA/PL ratio may protect against bile duct-injury. Expression of *Mdr2* mRNA was clearly upregulated in all treated wild-type mice, whereas it was less upregulated in *Pparα*-null mice, which was consistent with BA/PL ratio in both groups of mice. The bile acid transporter, BSEP, also showed different responses between wild and null mice. Decreased BSEP levels were observed in both genetic background mice at higher doses, while *Pparα*-null mice showed a transient increase in BSEP protein levels at lowest dose, 12.5 μmol/kg. Although the entire signal transduction for eliciting responses remains unknown, several other factors such as farnesoid X receptor-α (FXRα), which is known to downregulate BSEP[31–33] and CAR[28] may also be involved.

In conclusion, this study revealed the new insights that PPARα is protective against cholestastis induced by the weak PPARα ligand PFOA in using mouse model. PFOA mobilized adaptive processes regulated by PPARα— fat metabolism by mitochondria and peroxisomes, oxidative stress, elevation of TNF-α and hepatobiliary transport systems. So we propose that PPARα activators may induce either hepatocellular or bile duct injury, depending on their affinity to PPARα and dose level. If so, cholestasis and its associated morbidities may also be taken into account for risk assessment of PFOA in humans since species differences is well characterized in PPARα-associated signal transduction[34]. Further studies are needed to clarify this hypothesis.

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Long-Term Simulation of Human Exposure to Atmospheric Perfluorooctanoic Acid (PFOA) and Perfluorooctanoate (PFO) in the Osaka Urban Area, Japan

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A publicly available atmospheric transport model, the Weather Research and Forecasting Chemistry Model (http://ruc.noaa.gov/wrf/WG11/), was used to simulate atmospheric perfluorooctanoic acid (PFOA) and perfluorooctanoate (PFO) emitted from a point source in the Osaka urban area (also known as Keihanshin), Japan. The time period of the simulation was from 1983 to 2008. The modeled air concentrations were highly correlated ($r = 0.91$) with the observed air concentrations. Intake levels by inhalation of simulated air concentrations and through the gastrointestinal tract as estimated by the food duplicate method were input to a pharmacokinetic model of the human body to simulate serum concentrations of PFOA and PFO (PFO(A)). For validation of the atmospheric model, simulated values were compared with those observed in serum samples. The simulated values generally agreed with those observed in serum samples from residents of the Keihanshin area ($r = 0.93$). It was confirmed that the atmospheric model was generally capable of projecting features of atmospheric PFO(A) as well as serum concentrations of PFO(A) in this case. The results indicated a dominant contribution of the atmospheric component to serum PFO(A) in humans near the point source in the Keihanshin area. In 2008, that contribution was about 70%.

Introduction

Perfluorooctanoic acid (PFOA) and its conjugate base, perfluorooctanoate (PFO), are artificial carboxylic acid and carboxylate used for industrial and commercial purposes such as lubricants, paints, surfactants, and additives for fluoropolymer (FP) production. PFOA and PFO are collectively referred to as PFO(A). PFO(A) has been produced and widely used since the 1940s and has accumulated in the environment because of its persistent character. In recent years, PFO(A) has become recognized as a hazardous substance owing to its toxicity and carcinogenic potential in animals (*1*).

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The sources of human exposure to PFO(A) are still unknown. PFO(A) concentrations in human serum collected in the Keihanshin area of Japan have been reported to be much higher than those collected in other regions (*2, 3*). Contamination levels in wastewater and ecological samples strongly suggest that the Daikin Industries plant in Settsu city in Osaka prefecture is an emission source of PFO(A) (*4, 5*). Assessment of the gastrointestinal tract (GIT) intake of PFO(A) by females residing within 4.5 km of the Settsu plant and females living in Karakuwa, a small town in northern Japan (Figure 1A), in 2004 strongly suggests a large contribution of inhaled PFO(A) to the total body burden of PFO(A) (*3*). Although there have been several air monitoring campaigns in the Keihanshin area (*6–8*), understanding of the contribution of exposure through inhalation is incomplete. To obtain a more comprehensive understanding, we propose a novel approach that combines atmospheric modeling and pharmacokinetic (PK) modeling.

In this work, we applied an atmospheric transport model to simulate long-term inhalation exposure to PFO(A) in the Keihanshin area from 1983 to 2008. The modeled surface air concentrations of PFO(A) served as inputs to a PK model to calculate PFO(A) concentrations in human serum. The current study demonstrated that a unified model could simulate effects of exposure by inhalation under the scenario in which a point emission source and its industrial activity are reasonably well-characterized.

Materials and Methods

Atmospheric Transport Model. We applied version 3.0 of the fully coupled Weather Research and Forecasting/Chemistry (WRF-Chem) model (*9*) (http://ruc.noaa.gov/wrf/WG11/). WRF is a nonhydrostatic mesoscale meteorological forecasting model developed by the collaboration of several atmospheric research institutes (*10*). Various chemical modules have been implemented in the WRF framework, creating WRF-Chem. Meteorological fields such as wind and precipitation are predicted simultaneously by the same model. In WRF-Chem, air constituents are transported by the predicted meteorological fields using the same grid, the same time step, and the same physical schemes without interpolation. The target domain was square (450 km wide) and centered on Settsu (Figure 1A). The horizontal resolution was 9 km. The vertical structure consisted of 18 layers from the surface to 300 hPa. Typically, the depth of the lowest layer was about 50 m. The boundary and initial conditions for meteorological prediction were derived from 6-h meteorological fields determined from JRA-25 reanalysis data sets provided by the Japan Meteorological Agency and the Central Research Institute of Electric Power Industry (*11*). Model options are described further in the Supporting Information (section S1).

In WRF-Chem, PFO(A) is transported by grid-resolved wind fields and subgrid scale mixing both as soluble gas and as particulate matter without any chemical production or degradation. The residence time of the chemical related to advection (out of model domain) probably dominates at the spatial scale of the model. The removal processes are wet deposition and dry deposition. Model formulations for wet deposition, including in-cloud and subcloud scavenging, were modified as described in the Supporting Information (section S2). Gravitational settling was considered in the transport and dry deposition of particles.

Few observational data have been collected on the state of atmospheric PFO(A). In 2005, we observed a bimodal size distribution of atmospheric PFO(A) with peaks around

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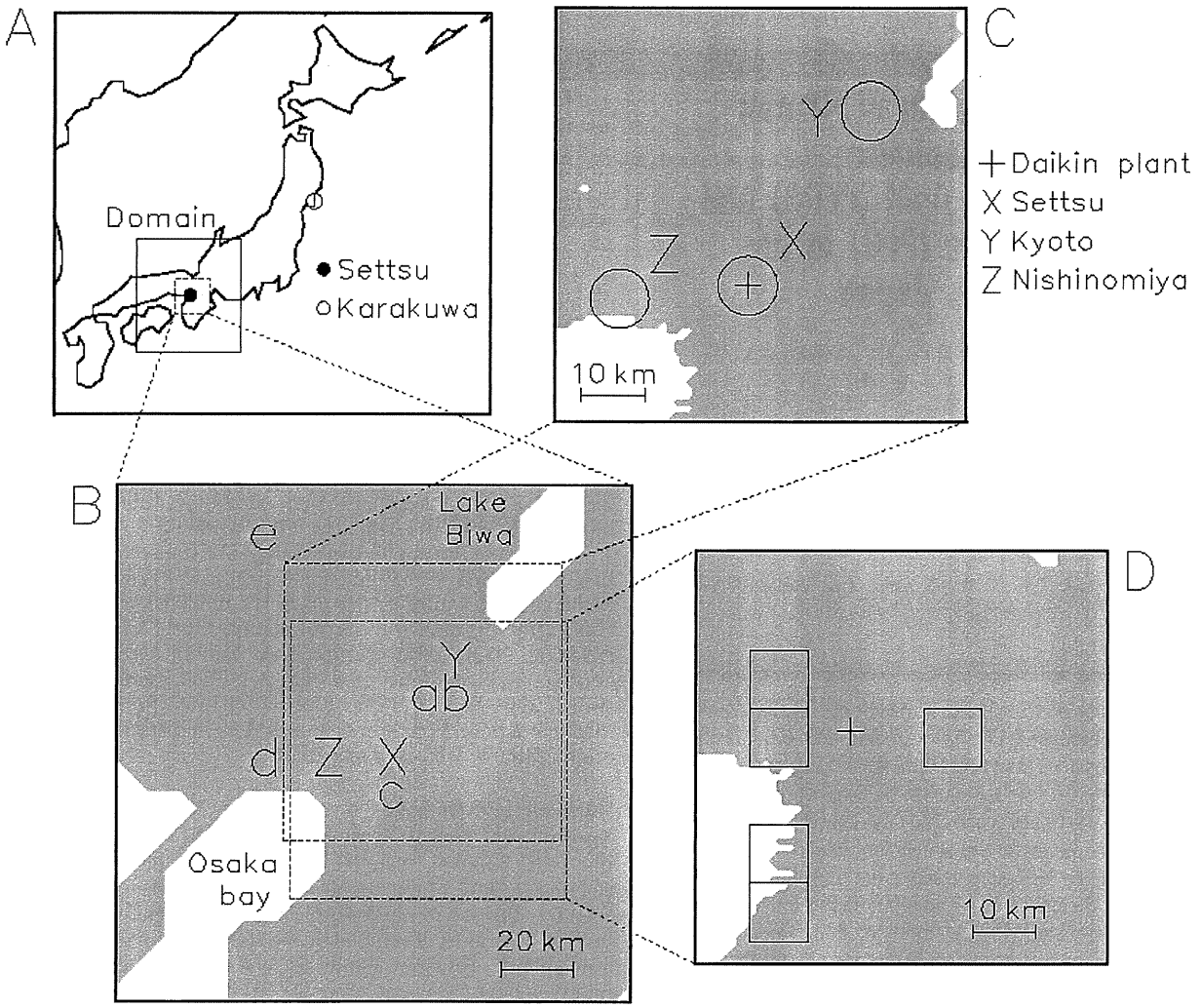

FIGURE 1. (A) Geographical locations of Settsu, Karakuwa, and the target domain. (B) An enlargement of the central region of the target domain. The characters represent the model grids corresponding to the observation sites (a-e for air and X-Z for serum) listed in Tables S3 and S4 (*2, 3, 6–8, 23, 24*). (C) Location of the Daikin plant and the three target population areas in the serum PFO(A) simulation of the Osaka urban area. Target areas have radii of 4.5 km and are centered at 34.761°N, 135.560°E (the Daikin plant location) for Settsu, 35.011°N, 135.768°E for Kyoto, and 34.738°N, 135.342°E for Nishinomiya. (D) Locations of the five grid elements of the atmospheric model used in the serum PFO(A) simulation for Osaka residents. The five grid elements are centered at 34.560°N, 135.470°E; 34.728°N, 135.470°E; 34.806°N, 135.470°E; 34.728°N, 135.770°E; and 34.482°N, 135.470°E. The position of the Daikin plant (+) is shown.

diameters of 0.7 and 3.3 μm in the town of Oyamazaki, which is about 20 km northeast of the Daikin plant (*6*), as shown in Figure S1. Although we did not measure gaseous PFOA directly, we trapped both gaseous PFOA and PFO(A) on particulate matter using quartz fiber filters (QFFs), on which gaseous PFOA has been reported to be absorbed irreversibly (*12*). Therefore, gaseous PFOA was included in the observed concentrations of atmospheric PFO(A). The air was collected with an Andersen cascade impactor sampler (AN-200, Tokyo Dylec Co., Tokyo, Japan). This sampler could divide atmospheric substances into nine size bins according to their aerodynamic size. Molecules of gaseous constituents were grouped as the smallest particles in the atmosphere. Accordingly, we recognized that gaseous PFOA belonged in the smallest size bin, which contained less than 10% of the total mass. In the present study, we assumed that 10% of PFO(A) is gaseous PFOA and the rest is particulate PFO(A), for which a fixed diameter of 5.0 μm was used. The effects of the fraction of gas and particle diameter on atmospheric concentrations were explored using sensitivity tests as described in the Supporting Information (section S6). The simulation period was from 1983 to 2008. An initial atmospheric concentration of zero was assumed.

PFO(A) Emission from the Daikin Plant in Settsu. The Daikin plant (34.761°N, 135.560°E) is the only major chemical plant manufacturing FP in Settsu. We estimated the annual flux of PFO(A) in discharged water at 6.6 t yr^{-1} in 2003 on the basis of an investigation of wastewater at the Aikawa Ryuiki disposal site (*4*). We derived the PFO(A) emission flux to the air as 2.3 t yr^{-1} in 2003 using an estimated ratio of the discharged portion in water to air of 65%:23% (*13*). The remaining 12% was discharged into the soil. The volatilization of gas from the surface water and soil was assumed to be negligible relative to the direct emission from the plant. The magnitude of the estimated emission flux was evaluated in relation to the national FP production, as described in the Supporting Information (section S3).

Annual emission rates were estimated from those in 2003 using the scale factors listed in Table S1. The scale factors were equal to the ratio of yearly national FP production to that for 2003 (*14*) before 2004; thereafter, they decreased following the values given in a press release from Daikin

(*15*). Estimated emission rates from the Daikin plant in Settsu peaked in 2000 and decreased after 2005 (Figure S2). Emission was assumed to occur exclusively in the lowest layer at the point corresponding to Settsu.

PFO(A) may originate from the degradation of fluorotelomer-based precursors. However, these indirect sources were considered to be negligible near such a strong point source in the Keihanshin area because they contribute very little even at the global scale (*13*). Wallington et al. used a three-dimensional global model to simulate PFO(A) production in the atmosphere from the oxidation of 8:2 fluorotelomer alcohols (*16*). The highest simulated PFO(A) concentration at 50 m was approximately 3×10^3 molecules cm^{-3}, which corresponds to 2×10^{-2} pg m^{-3} and is thus negligible in the context of the present study.

Air Monitoring Data Sets. We conducted air sampling to observe atmospheric PFO(A) concentrations in the town of Oyamazaki in 2001 and 2005, and in Fukuchiyama city (northern Kyoto prefecture) in 2005 (Figure 1B) (*6, 7*). We also used atmospheric concentrations observed in the Keihanshin area during an environmental survey sponsored by the Ministry of Environment of Japan in 2004 (*8*) (Table S3). Since those air samples were collected on QFFs, the data represent the sum of gaseous PFOA and particulate PFO(A). The total amounts of gaseous PFOA and particulate PFO(A) simulated by the current model were compared to those data.

Target Population and the PK Model. We simulated serum levels of PFO(A) in residents of three urban areas having radii of 4.5 km in the Keihanshin area. The three urban areas were Settsu, Kyoto, and Nishinomiya as shown in Figure 1C. The urban areas were centered at 34.761°N, 135.560°E (the location of the Daikin plant) for Settsu, 35.011°N, 135.768°E for Kyoto, and 34.738°N, 135.342°E for Nishinomiya. In addition, we simulated inhaled amounts of PFO(A) for Osaka residents, for which the geometric mean (GM) serum concentration of PFO(A) in 2003 was known (*2*). Inhaled amounts for Osaka residents were derived from surface air concentrations averaged over five grid elements determined from their geographical addresses (Figure 1D).

The serum level of PFO(A) was predicted using a one-compartment PK model (*17*)

$$V\frac{dC(t)}{dt} = \frac{E}{W} - (k_t + k_m) \cdot C(t) \quad (1)$$

$$k_t = \frac{\ln(2)}{T(1/2)}V,\ k_m = \frac{V_m}{W \cdot N_d} \quad (2)$$

where V is the volume distribution (300 mL kg^{-1} (*17*)), and C is the serum concentration (ng mL^{-1}) of PFO(A). E is the PFO(A) intake (ng day^{-1}), and W is the body weight (50 kg (*17*)). k_t and k_m represent the total clearance of males and menstrual serum loss (mL kg^{-1} day^{-1}), respectively. $T(1/2)$ is the biological half-life (1273 day (*18*)) of PFO(A). V_m is the menstrual serum loss volume (42.0 mL (*19*)), and N_d is the total number of days in the month.

In some previous studies, much greater values of V were applied (*20*). The rationale for selecting the current value (300 mL kg^{-1}) for the volume distribution was based on the total mass balance observed in our previous study (*21*) (see discussion in the Supporting Information; section S7). The Euler forward time step was set to 1 day. The initial concentrations were set at 2.6 ng mL^{-1} based on a linear regression of serum sample observations (*2*). In preliminary tests, we confirmed that the effects of initial concentrations persisted for only a few years.

The current PK model has two routes of exposure to PFO(A): inhalation and GIT intake. The exposure concentration of PFO(A) in inhaled air was derived from the surface air concentration calculated by the atmospheric model. The inhaled volume was assumed to be 20 m^3 day^{-1} (*22*). PFO(A) in the lungs was assumed to be absorbed completely by the respiratory tract. The absorption rates through respiration did not have significant effects as discussed in the Supporting Information (section S6). Intake, which represents GIT intake including PFO(A) intake from both diet and drinking water (*3*), was considered to be a function of time and proportional to the emission rate of PFO(A) from the plant until 2004. We assumed the lower and upper limits of intake for 2004 to be the 25th (43.4 ng day^{-1}) and the 75th (98.0 ng day^{-1}) percentiles of the GIT PFO(A) intake observations in food duplicate samples collected in Settsu in 2004 (*3*). Intake was assumed to be constant after 2004.

Diet, Drinking Water, and Serum Samples. To estimate intake of PFO(A) by the GIT, we used concentrations in food and drinking water consumed during 24-h periods by participants in the Settsu area by the food duplicate method (*23*). Those participants also donated serum samples. Daily intake of PFO(A) estimated from those samples was 61.4 ng day^{-1} [GM] with a standard deviation of 1.62 [geometric standard deviation (GSD)] (*3*). Serum samples for adult females living in the Settsu area, Kyoto area, Nishinomiya area, and Osaka areas (*2, 3, 24, 25*) (Table S4) were used for comparison with serum levels simulated by the PK model. The samples were donated by the Kyoto University Human Specimen Bank (*23*).

Evaluation of Model Fit. The fit of the simulated values to the observed values was evaluated by the fractional difference f (*26*) averaged over all samples

$$f = \frac{V_{mdl} - V_{obs}}{V_{mdl} + V_{obs}} \quad (3)$$

where V_{mdl} and V_{obs} are the modeled value and observed value, respectively. $|f| < 0.33$ indicates an error factor of less than 2.

Results and Discussion

Surface Air Concentration. Since PFO(A) has one strong point source and it is not produced in the atmosphere in these simulations, modeled air concentrations were quite variable and dependent on the wind field. The predicted winds agreed well with observed data in all seasons as described in the Supporting Information (section S4). The monthly average concentrations of PFO(A) modeled reflected wind directions, which were mainly northerly in January and southerly in July as shown in Figure S3, demonstrating the seasonality of modeled surface air concentrations in 2001. The monthly average concentration modeled in the town of Oyamazaki, which is grid point "a" in Figure S3, was 168 pg m^{-3} in January and 677 pg m^{-3} in July, whereas the instantaneous modeled concentrations in the 2-h interval changed markedly from greater than 2 ng m^{-3} to less than 10 pg m^{-3} depending on whether the location was downwind or upwind of the point source. The annual average distribution simulated showed no particular horizontal tendency. The modeled concentrations were greatest (>1 ng m^{-3}) near the source and were more than 100 pg m^{-3} throughout the Keihanshin area (Figure S3). Almost half (48%) of the PFO(A) emitted to the atmosphere was transported out of the domain area by air flow. The percentage of PFO(A) removed within the domain by dry and wet deposition were 46% and 6%, respectively (Supporting Information; section S5).

Modeled surface air concentrations of PFO(A) were compared with observations in the target domain listed in Table S3 (*6–8*). Predicted concentrations were averaged over the same periods as the observations. Observed PFO(A) concentrations ranged widely from 15.2 pg m^{-3} in Fukuchiyama city to 1261 pg m^{-3} in Osaka city (Table S3). The