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Comparative studies on sex-related difference in biochemical responses of livers to perfluorooctanoic acid between rats and mice

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Exposure to perfluorinated carboxylic acids, such as perfluorooctanoic acid (PFOA*) and perfluorodecanoic acid, occurs primarily through their industrial and commercial applications. Although perfluorinated compounds are originally considered to be metabolically inert [1], perfluorinated carboxylic acids were found to induce peroxisome proliferation [2]. Our previous study [3] demonstrated that PFOA caused the marked increases in the activities of several hepatic enzymes which are located in different subcellular organelles in rat liver and that these inductions were strongly dependent on sex hormones. It appears, moreover, that this marked sex-related difference, which is not so apparent in other peroxisome proliferators, suggests that properties of PFOA are very different from other peroxisome proliferators. These findings raised the questions of whether there is a marked sex-related difference in the response of other species of animals to PFOA as well and of whether the marked sex-related difference seen with rats in response to short-term treatment with PFOA is still apparent after a prolonged treatment with PFOA. This kind of information may be of imporance for the hazard assessment of PFOA to human. In this context, we studied the effects of PFOA on biochemical parameters in both sexes of rats and mice and the responses of male and female rats to the 26-week-feeding of dietary PFOA.

* Abbreviation: PFOA, perfluorooctanoic acid.

Materials and methods

Materials. PFOA was purchased from Aldrich (Mil-

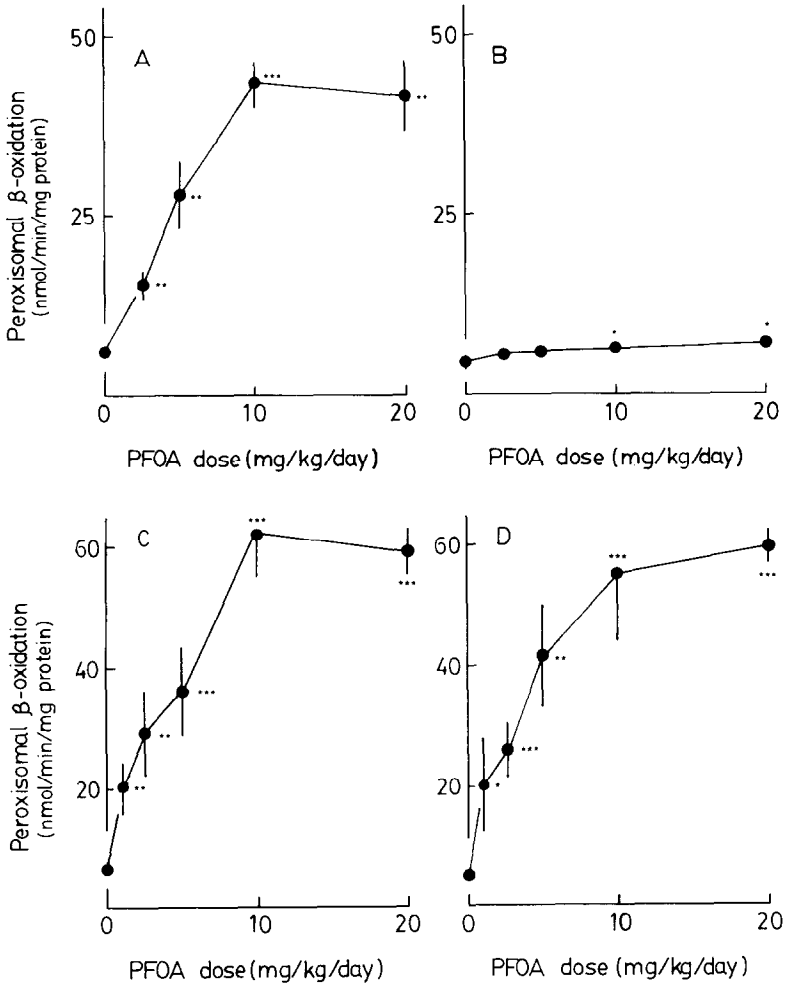

Fig. 1. Dose–response effect of PFOA on peroxisomal β-oxidation in livers of rats and mice. Animals were intraperitoneally injected with various doses of PFOA once a day for a week. Each value represents mean ± SD for 3–5 animals. P values for results significantly differed from control data at *$P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. (A) Male rats; (B) female rats; (C) male mice; (D) female mice.

waukee, WI) palmitoyl-CoA, acetyl-CoA, L-carnitine and bovine serum albumin were obtained from the Sigma Chemical Co. (St Louis, MO); CoA and NAD^+ were from Oriental Yeast Co. (Tokyo, Japan). All other reagents were of analytical grade.

Animals and treatment. Rats of Wistar strain and ddY strain were fed on a standard diet (Clea CE-2, obtained from Clea Japan, Tokyo, Japan). For short-term experiments, male (170–180 g) and female rats (120–130 g) aged 6 weeks were intraperitoneally administered with PFOA (as sodium salt in 0.9% NaCl) at doses of 1, 2.5, 5, 10 and 20 mg/kg once a day for 1 week. Male (30–35 g) and female (20–25 g) mice aged 6 weeks were treated with PFOA as the same manner for rats. For long-term treatments with PFOA, male and female rats aged 5 weeks at the initiation of the treatment were fed on a diet containing 0.01% PFOA (supplied by Clea Japan) for 2 or 26 weeks. Non-treated control male and female rats were fed on a control diet for 2 weeks (as young control rats) or for 26 weeks (as age-matched control rats). At the end of the treatments, the animals were killed and post-nuclear supernatant of liver was prepared as described previously [4].

Analytical procedures. Cyanide-insensitive palmitoyl-CoA oxidation [4] and carnitine acetyltransferase [5] were assayed by using post-nuclear supernatant as enzyme sources. Protein concentration was measured by the method of Lowry *et al.* [6] with bovine serum albumin as standard. Statistical analyses were performed by Student's *t*-test for two means.

Results and discussion

Species difference in the response to PFOA. PFOA was given intraperitoneally to cancel the difference, if any, of the absorption of PFOA through the intestine among species and sexes of animals. The administration of PFOA at various doses to male rats for 1 week led to a dose-dependent increase in the activity of peroxisomal β-oxidation in male rats (Fig. 1A), with a slight, but significant, induction being observed in female rats at doses of 10 and 20 mg/kg (Fig. 1B). The activity was enhanced in a dose-dependent manner by the treatment of male mice with PFOA, as was seen with male rats (Fig. 1C). In contrast to female rats, however, the activity of peroxisomal β-oxidation in female mice was markedly induced (Fig. 1D). The results obtained for carnitine acetyltransferase were similar to those of peroxisomal β-oxidation (Fig. 2). In accordance with the responses of hepatic enzymes to PFOA, a dose-dependent enlargement of liver was brought about in male rats and mice of both sexes, but PFOA produced no hepatomegaly in female rats (data not shown). The present results show that there is a marked difference in the response of female animals to PFOA even within rodents. It has been reported that female rats excreted PFOA much faster than male rats [7]. This may conceivably be attributable, in part, for the marked sex-related difference observed in rats. At present, however, less information is available about the comparison of the response to peroxisome proliferators between male and female mice.

Effects of prolonged administration of PFOA. The findings of slow excretion of PFOA from male rats [7] led to the inference that the prolonged administration of PFOA to rats may render this chemical accumulative in rats, so that more marked or more toxic effects on livers of male rats may be observed; the chronic long-term administration might produce more marked induction of hepatic enzymes responsive to peroxisome proliferators in female rats as

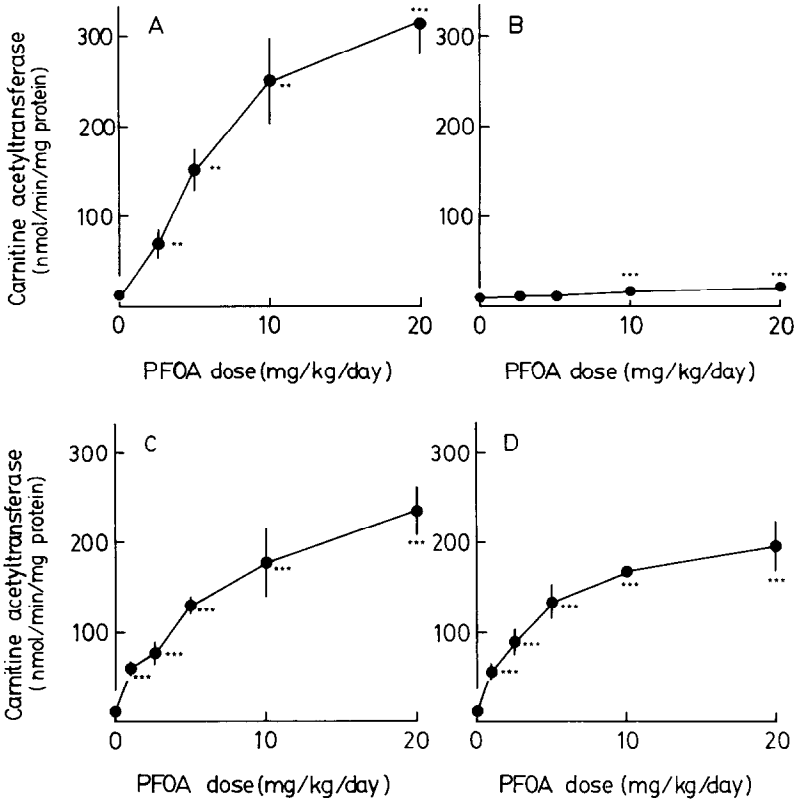

Fig. 2. Dose–response effect of PFOA on carnitine acetyltransferase in livers of rats and mice. Animals were treated as described in Fig. 1. Each value represents mean ± SD for 3–5 animals. P values for results significantly differed from control data at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. (A) Male rats; (B) female rats; (C) male mice; (D) female mice.

Table 1. Effects of prolonged administration of PFOA on the activities of peroxisomal β-oxidation and carnitine acetyltransferase in livers of male and female rats

Treatment of animals	Peroxisomal β-oxidation (nmol/min/mg protein)	Carnitine acetyltransferase (nmol/min/mg protein)
Young male rats		
Control	4.4 ± 0.9	11.2 ± 1.6
0.01% PFOA 2 weeks	24.0 ± 2.1*	162.0 ± 18.6*
0.01% PFOA 26 weeks	16.0 ± 2.2*‡	188.8 ± 30.5*‡
Age-matched male control	2.9 ± 0.5	10.4 ± 1.0
Young female rats		
Control	5.5 ± 0.6	9.6 ± 1.2
0.01% PFOA 2 weeks	6.5 ± 1.1	26.2 ± 2.8*
0.01% PFOA 26 weeks	5.3 ± 0.7†	36.0 ± 6.0*‡
Age-matched female control	2.9 ± 0.6	12.9 ± 2.1

Male and female rats were fed on a diet containing 0.01% (w/w) PFOA for 2 and 26 weeks. Values are means ± SD for four animals. P values for results significantly differed from young control data at * $P < 0.001$ and from age-matched control data at † $P < 0.01$; ‡ $P < 0.001$.

well. In contrast to the expectation, although the increased activities of peroxisomal β-oxidation and carnitine acetyltransferase were unchanging throughout the period of the treatment with PFOA for 26 weeks (Table 1). Moreover, the administration of PFOA for 26 weeks did not affect accumulatively to female rats with regard to hepatic enzyme inductions. None of the greater toxic effect was seen in male rats treated with PFOA for the long-term. Consequently, the sex-related difference in the response of rats to PFOA is still striking after the chronic long-term treatment.

Concluding discussion. Many peroxisome proliferators were shown to increase the incidence of hepatocellular carcinomas in rodents after their long-term administrations [8–10]. Peroxisome proliferators are employed for many different kinds of purposes, such as hypolipidemic drugs (clofibrate and related compounds), plasticizers (di-(2-ethylhexyl)phthalate and related compounds) and herbicides (2,4-dichlorophenoxyacetic acid and related compounds). Although hypolipidemic fibrates are potent peroxisome proliferators [8, 9], the utilization is restricted to patients. On the other hand, there is a general risk for public to be exposed to plasticizers and herbicides; nevertheless these chemicals are relatively weak peroxisome proliferators [11]. PFOA has been recently confirmed to proliferate peroxisome greatly [12]. The utilization of PFOA is much wider compared with other known peroxisome proliferators. The production of perfluorocarboxylic acids are increasing recently, so that the risk is probably increasing even for people, who are not industrial workers, to be exposed to these compounds. In this context, PFOA is very different from other known peroxisome proliferators. The excretion of PFOA from man seems to be very slow [13], whereas little information is available about sex-related difference in the response of human to PFOA. The toxico-kinetic data from rhesus monkey showed no sex-related difference in the concentrations of PFOA in serum and liver [14]. Our present study showed clearly that the effects of PFOA on animals were very different among species and sexes even within rodents and that the sex-related difference in the effects of PFOA was still evident even after long-term administration of PFOA to rats. Accordingly, biochemical information should be accumulated to extrapolate the hazard of perfluorocarboxylic acids to human and the information should be based on the experiments using animals of both sexes of many species.

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REFERENCES

1. Clark LC Jr, Becattini F, Kaplan S, Obrock V, Cohen D and Becker C, Perfluorocarbons having a short dwell time in the liver. *Science* **181**: 680–682, 1973.
2. Ikeda T, Aiba K, Fukuda K and Tanaka M, The induction of peroxisome proliferation in rat liver by perfluorinated fatty acids, metabolically inert derivatives of fatty acids *J Biochem* **98**: 475–482, 1985.
3. Kawashima Y, Uy-Yu N and Kozuka H, Sex-related difference in the inductions by perfluoro-octanoic acid of peroxisomal β-oxidation, microsomal 1-acylglycerophosphocholine acyltransferase and cytosolic long-chain acyl-CoA hydrolase in rat liver. *Biochem J* **261**: 595–600, 1989.
4. Kawashima Y, Katoh H and Kozuka H, Differential effects of altered hormonal state on the induction of acyl-CoA hydrolases and peroxisomal β-oxidation by clofibric acid. *Biochim Biophys Acta* **750**: 365–372, 1983.
5. Kähönen MT, Effect of clofibrate treatment on carnitine acyltransferases in different subcellular fractions of rat liver. *Biochim Biophys Acta* **428**: 690–701, 1976.
6. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.

7. Hanhijärvi H, Ylinen M, Kojo A and Kosma V-M, Elimination and toxicity of perfluorooctanoic acid during subchronic administration in the Wistar rat. *Pharmacol Toxicol* **61**: 66–68, 1987.
8. Reddy JK and Lalwani ND, Carcinogenesis by hepatic peroxisome proliferators: evaluation of the risk of hypolipidemic drugs and industrial plasticizers to humans. *CRC Crit Rev Toxicol* **12**: 1–58, 1983.
9. Hawkins JM, Jones WE, Bonner FW and Gibson GG, The effect of peroxisome proliferators on microsomal, peroxisomal, and mitochondrial enzyme activities in the liver and kidney. *Drug Metab Rev* **18**: 441–515, 1987.
10. Rao MS and Reddy JK, Peroxisome proliferation and hepatocarcinogenesis. *Carcinogenesis* **8**: 631–636, 1987.
11. Kawashima Y, Katoh H, Nakajima S, Kozuka H and Uchiyama M, Effects of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid on peroxisomal enzymes in rat liver. *Biochem Pharmacol* **33**: 241–245, 1984.
12. Kawashima Y, Horii S, Matsunaga T, Hirose A, Adachi T and Kozuka H, Co-induction by peroxisome proliferators of microsomal 1-acylglycerophosphocholine acyltransferase with peroxisomal β-oxidation in rat liver. *Biochim Biophys Acta* **1005**: 123–129, 1989.
13. Ubel FA, Sorenson SD and Roach DE, Health status of plant workers exposed to fluorochemicals—a preliminary report. *Am Ind Hyg Assoc J* **41**: 584–589, 1980.
14. Griffith FD and Long JE, Animal toxicity studies with ammonium perfluorooctanoate. *Am Ind Hyg Assoc J* **41**: 576–583, 1980.

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Plasma protein binding and blood cell distribution of propranolol enantiomers in rats

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Although propranolol (PL*) is commercially available as a racemic mixture, (−)-PL is about 100 times more potent than (+)-PL as a β-blocker [1]. It is, therefore, clinically important to clarify any enantiomeric differences in the disposition of PL. As factors which contribute to the stereochemistry of the delivery and actions of β-blocking drugs, Walle *et al.* [2] have stressed the processes involving hepatic metabolism, plasma protein binding, and the storage and release of β-antagonists by adrenergic nerve endings. A number of studies have demonstrated stereoselectivity in the hepatic metabolism of PL [3–5], but limited information is available on the distribution of the two PL enantiomers. As the volume of distribution (V_d) of PL depends on the binding of PL to plasma protein [6], the difference in plasma protein binding between the two PL enantiomers may produce a distinct difference in their distribution patterns. Several reports have already demonstrated the stereoselective plasma protein binding of PL in humans as well as dogs [7–9]. However, all of the studies investigated stereoselectivity at only one constant concentration (around 100–150 ng/mL). Therefore, it is not clear whether the difference between the PL enantiomers in plasma binding is due to the difference in binding affinity, to the capacity, or to both parameters, and also whether or not enantiomer–enantiomer interaction exists at the binding site(s) in the plasma. The purpose of the present study was to clarify the characteristics of the stereoselective plasma protein binding observed in rats by applying a competitive inhibition model and to investigate the effect of stereoselective plasma binding of PL enantiomers on their distribution into blood cells.

Methods

Materials. (±)-PL hydrochloride and (−)-penbutolol sulfate were obtained from I.C.I.-Pharma Ltd. (Osaka, Japan) and Hoechst Japan Ltd. (Tokyo, Japan) respectively. Free (±)-PL base was resolved into its enantiomers by fractional crystallization of its diastereomer, which was obtained by reaction with di-*p*-toluoyl-L-(+)tartaric acid [10]. The hydrochloride salts of both enantiomers were then prepared. The purities of the enantiomers, as confirmed by optical density, melting point and HPLC resolution, were over 92 and 95% for (+)- and (−)-PL respectively.

Plasma protein binding. ^{3}H-Labeled (±)-PL (sp. act. 26.6 Ci/mmol; Amersham International Ltd.) was resolved into (+)-[^{3}H]PL and (−)-[^{3}H]PL (radiochemical purity 98%) by adopting a procedure similar to that used for measuring PL enantiomers in biological fluids and tissues as reported previously [11], employing a Chiralcel OD analytical column (Daicel Chemical Industries, Tokyo, Japan) with variable-wavelength UV (280 nm, Shimadzu SPD-2A) and radioisotope (Beckman 171) detectors. Binding of PL to rat plasma was measured by ultrafiltration (Ultrafree C3-LGC, Nihon Millipore Ltd., Tokyo, Japan). The influence of the column separation of (±)-[^{3}H]PL and the evaporation of the eluent on the binding properties of PL was assessed by comparing the binding of racemic [^{3}H]PL with that of a pseudoracemate, which was prepared by combining equal amounts of each radiolabeled enantiomer resolved [12]. The difference between f_p obtained with racemic [^{3}H]PL and that with a pseudoracemate was not statistically significant at a concentration of 15.4 μM (N = 4). Pooled heparinized plasma was obtained from male Wistar rats (body weight 220–320 g). A tracer amount of (+)-^{3}H-labeled PL enantiomer and the unlabeled (+)-PL were added to 0.5 mL of plasma to yield a final plasma (+)-PL concentration ranging from 0.386 to 38.6 μM. Following incubation at 37° for 5 min, the plasma was centri-

* Abbreviations: PL, propranolol; C_p, plasma total concentration of PL enantiomer; C_f, plasma free concentration of PL enantiomer; C_b, plasma bound concentration of PL enantiomer; f_p, plasma free fraction of PL enantiomer; N_p, binding capacity of plasma protein; K_d, dissociation constant of PL enantiomer–plasma protein complex; α, proportionality constant for non-specific binding of PL enantiomer; I_t, plasma total concentration of inhibitor (the opposite PL enantiomer); I_f, plasma free concentration of inhibitor; I_b, plasma bound concentration of inhibitor; K_i, dissociation constant of inhibitor–plasma protein complex; β, proportionality constant for non-specific binding of inhibitor; C_B, blood concentration of PL enantiomer; C_{bc}, blood cell concentration of PL enantiomer; Hc, hematocrit; R_b blood-to-plasma concentration ratio of PL enantiomer; and K_p^{bc}, blood cell-to-plasma free concentration ratio of PL enantiomer.