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The effects of perfluoro-octanoic acid on hepatic peroxisome proliferation and related parameters show no sex-related differences in mice

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Male and female C57Bl/6 mice were administered perfluoro-octanoic acid (PFOA; 0.02-0.05% w/w; 5–10 days) in their diet. This treatment resulted in a several-fold induction of hepatic peroxisomal fatty acid β-oxidation (monitored as increases in cyanide-insensitive palmitoyl-CoA oxidation, lauroyl-CoA oxidase and catalase activity) in all animals. The protein content of the hepatic mitochondrial fraction was also increased in all mice exposed to PFOA. Furthermore, studies on xenobiotic-metabolizing enzymes revealed no sex-related difference in the response to PFOA. All mice demonstrated a dramatic increase in ω-hydroxylation of lauric acid. Cytosolic epoxide hydrolase, glutathione transferase and DT-diaphorase activities were increased about 2–5-fold. These results with mice differ dramatically from previous studies and our own experiments here with Wistar rats, in which exposure to PFOA causes hepatic peroxisome proliferation in male animals, whereas females are unaffected.

INTRODUCTION

It is now well established that a number of hypolipidaemic agents cause an increase in the size and number of peroxisomes in rodent liver cells [1–3]. Some of these substances are components of clinical drugs, and others are widely used in industry.

Peroxisomes are involved primarily in catabolic pathways, such as β-oxidation of branched or very-long-chain fatty acids [4]. Proliferation of peroxisomes thus results in an increased capacity to catabolize fatty acids and can be monitored by measuring this activity. H_2O_2 is produced by peroxisomal fatty acid β-oxidation and subsequently converted into water by catalase. There is, however, no correlation between the extent of peroxisome proliferation and peroxisomal catalase activity [5]. Peroxisome proliferators cause a dramatic increase in catalase activity in the cytosolic fraction from mouse liver, whereas the increase in the corresponding peroxisomal activity is slight. The fact that prolonged proliferation of peroxisomes leads to carcinogenesis in rodents may be explained by genotoxic effects due to enhanced levels of H_2O_2 produced by peroxisomes.

Perfluoro-octanoic acid (PFOA) and other perfluorinated carboxylic acids are used commercially as wetting agents and corrosion inhibitors and to improve the resistance of paper to water and oil [6]. It has been reported previously that PFOA has a strong inducing effect on peroxisomal fatty acid β-oxidation in male Wistar rats, but no effect in females [7]. Hormonal manipulations in these rats revealed that this induction is dependent on testosterone. Moreover, it has been established that female rats excrete PFOA in their urine more rapidly than do male rats [8]. If testosterone plays a direct role in the proliferation of peroxisomes, this effect could be a useful tool in studies on the mechanism(s) underlying this process. On the other hand, it is possible that testosterone simply affects the excretion of PFOA and does not affect peroxisomes.

Our aim in the present study was to determine whether a sex-related difference in the response to PFOA is present in mice as well, i.e. to examine whether this is a general phenomenon. Mice were chosen, since this animal is often used in studies on peroxisome proliferation, also in our laboratory.

We therefore exposed C57Bl/6 mice of both sexes to PFOA and assayed catalase activity, cyanide-insensitive palmitoyl-CoA oxidation and lauroyl-CoA oxidase activity as markers for peroxisome proliferation. In addition, we studied certain xenobiotic-metabolizing enzymes known to be affected by all peroxisome proliferators. The microsomal cytochrome *P*-452 system (specialized for ω-hydroxylation of lauric acid) and cytosolic epoxide hydrolase are induced by these substances [9,10]. The activity of cytosolic DT-diaphorase is often increased (A.-K. Sohlenius, K. Andersson & J. W. DePierre, unpublished work), which might be explained by an enhanced oxidative stress in the cell. In contrast, hepatic cytosolic glutathione transferase activity is often depressed by peroxisome proliferators [11].

There are some reports that peroxisome proliferators also affect mitochondria. It has, for example, been demonstrated that mitochondrial size decreases in the livers of mice treated with clofibrate [12]. This decrease leads to a change in the distribution of mitochondria between hepatic subcellular fractions. For this reason, we studied the effect of PFOA on mitochondrial protein content.

In addition to the experiments on mice, we performed a parallel study on Wistar rats, in order to confirm reported sex differences in this animal's response to PFOA.

EXPERIMENTAL

Chemicals

PFOA was purchased from Aldrich Chemie (Steinheim, Germany); lauroyl-CoA, CoA, FAD, β-NAD^+, cytochrome *c*, dicoumarol, Triton X-100, Trizma base and BSA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); s-palmitoyl-CoA, peroxidase, dithiothreitol and NADPH were from Boehringer Mannheim (Bromma, Sweden); H_2O_2 (30%), KCN, Folin–Ciocalteu's phenol reagent, 1-chloro-2,4-dinitrobenzene

Abbreviation used: PFOA, perfluoro-octanoic acid.
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and menadione were from E. Merck (Darmstadt, Germany); [1-^{14}C]lauric acid was from Amersham (Solna, Sweden); and sucrose was from BDH (Poole, Dorset, U.K.).

[^{3}H]-*trans*-Stilbene oxide (2 Ci/mmol) was synthesized by Dr. Åke Pilotti and co-workers at the Department of Organic Chemistry, University of Stockholm, Sweden, as described by Gill *et al.* [13].

Animals and treatment

Expt. A. C57Bl/6 female and male mice (ALAB, Sollentuna, Sweden), weighing about 20 g and 20–30 g respectively, were housed and treated in steel cages in groups of three. They were kept under a 12 h-light/12 h-dark cycle at 25°C and given free access to laboratory chow R3 containing 5% fat, 24% protein and 49% carbohydrate (Astra Ewos AB, Södertälje, Sweden). PFOA was dissolved in 20 ml of acetone and mixed with 100 g of powdered food, in order to prepare the PFOA-containing diet. This chow was dried in a ventilated hood until no smell of acetone was detectable (12–24 h). Control chow was mixed with acetone alone.

The animals were exposed to 0.02% (w/w) PFOA for 7 days. In addition, groups of female mice were treated with 0.05% PFOA for 5 and 10 days.

Expt. B. Female and male Wistar rats (ALAB), weighing about 145 g and 200 g respectively, were housed and treated in steel cages in groups of three. The animals were kept under a 12 h-light/12 h-dark cycle at 25°C and given free access to the commercial rat diet R3 (Astra Ewos AB). Three rats of each sex were exposed to 0.02% PFOA in the diet for 7 days.

Subcellular fractionation

At the end of the experiment, the animals were killed and the livers removed (mouse liver was freed from the gall-bladder) and thereafter rinsed in ice-cold 0.25 M-sucrose. The livers were weighed and individually homogenized at 440 rev./min. in ice-cold 0.25 M-sucrose by using four up-and-down strokes of a Potter/Elvehjem homogenizer. More sucrose solution was then added to give a 20% (w/v) homogenate.

Centrifugation at 600 $g_{av.}$ for 10 min gave the nuclear pellet, which was subsequently resuspended in sucrose to the same volume as the original 20% homogenate. The supernatant was centrifuged at 10000 $g_{av.}$ for 10 min to give a pellet containing the mitochondrial fraction (consisting primarily of mitochondria, peroxisomes and lysosomes). This pellet was resuspended and washed twice by centrifugation (10 min/10000 g) in sucrose. Finally, the mitochondrial fraction was resuspended in sucrose to give a volume of 2.0 ml for mouse and 6.0 ml for rat. The supernatant from the 10000 $g_{av.}$ centrifugation was further centrifuged at 105000 $g_{av.}$ for 60 min. After measurement of the volume of the high-speed supernatant (cytosol; about 4 ml for mouse and 20 ml for rat), this fraction was saved. The pellet (the microsomal fraction) was washed once by centrifugation (60 min/105000 g) in 0.15 M-Tris/HCl (pH 8.0) and then resuspended in sucrose to give a final volume of 2.0 ml for mouse and 6.0 ml for rat. The subcellular fractions prepared in this manner were carefully analysed for cross-contamination in a previous study [14].

Assays

All enzyme assays were performed with saturating substrate concentrations and conditions of linearity with time and protein. The activity of catalase was monitored spectrophotometrically at 240 nm with H_2O_2 as substrate [15]. Peroxisomal lauroyl-CoA oxidase activity was measured by assaying H_2O_2 production by a fluorimetric method based on the peroxidase-coupled oxidation of 4-hydroxyphenylacetic acid [16]. Palmitoyl-CoA oxidation was measured spectrophotometrically by monitoring the reduction of NAD^+ at 340 nm and with KCN as an inhibitor of mitochondrial β-oxidation [17,18]. DT-diaphorase was measured spectrophotometrically at 550 nm by monitoring the reduction of cytochrome *c*, which continually reoxidizes menadione [19]. Cytosolic epoxide hydrolase was monitored with [^{3}H]-*trans*-stilbene oxide as substrate essentially as described by Gill *et al.* [13]. Glutathione transferase activity was measured spectrophotometrically at 240 nm with 1-chloro-2,4-dinitrobenzene as substrate [20]. ω-Hydroxylation of lauric acid was determined by a radiometric method with [^{14}C]lauric acid as substrate [21]. Protein concentrations were determined by the method of Lowry *et al.* [22], with BSA as standard.

Statistical analysis

Data are given as means ± S.D. and the results of Student's *t* test are presented where appropriate.

RESULTS

Effects of PFOA on liver somatic index and mitochondrial, cytosolic and microsomal protein contents

After dietary treatment with 0.02% PFOA for 1 week, each male mouse lost approx. 3.5 g and each female mice about 2.1 g in body wt. As shown in Table 1, all treated mice demonstrated a dramatic increase in liver weight. This increase was about 65% for male and approx. 83% for female mice. Owing to the changes in body and liver weights, all treated animals showed an increase in liver somatic index (liver wt./body wt.).

The effects of PFOA on mouse liver mitochondrial, cytosolic and microsomal protein contents are documented in Table 2. A 4.5-fold increase in the protein content of the mitochondrial fraction from male mice was found after 7 days of PFOA treatment. Female mice showed approx. 5.3-fold increase in this same parameter. PFOA also had a small effect on the protein content of the microsomal fraction from male mice, but no other significant changes were observed.

Effects of PFOA on peroxisomal fatty acid β-oxidation in mouse liver

Peroxisomal fatty acid β-oxidation in both female and male mice was dramatically affected by PFOA. Table 3 shows the effect on palmitoyl CoA-oxidation in the mitochondrial fraction. In all mice this activity was increased about 16–17-fold after 7 days of exposure to 0.02% PFOA. The fatty acyl-CoA oxidase activity (also shown in Table 3) demonstrated about a 14-fold increase in both sexes after similar treatment.

Effects of PFOA on catalase activity in mouse liver

The effect of PFOA on catalase activities was also determined. The control activities in the nuclear, mitochondrial and cytosolic fractions from female mice were 24.0 ± 6.3, 10.6 ± 1.1 and 2.4 ± 0.5 mmol/min per g of liver respectively. As shown in Table 4, a 14-fold increase in catalase activity was observed in the cytosolic fraction from female mice after 7 days of treatment with 0.02% PFOA. The increases in the two other fractions were slight (results not shown). In male mice, the corresponding increase in the cytosolic fraction was 10-fold (also shown in Table 4).

Effects of PFOA on lauric acid ω-hydroxylation and cytosolic epoxide hydrolase, glutathione transferase and DT-diaphorase activities in mouse liver

Female mice demonstrated a 26-fold increase in ω-hydroxylation of lauric acid (measured in the microsomal fraction) after

Table 1. Effects of PFOA treatment on mouse liver weight and liver somatic index

All values are means ± S.D. for three animals, except those for the female control group, which are means ± S.D. for six animals: ***$P < 0.001$ compared with the control group.

Group and treatment	Liver wt. (g)	Liver somatic index (g of liver/g body wt.)
Male mice		
None (control)	1.16 ± 0.04	0.059 ± 0.002
0.02% PFOA for 7 days	1.91 ± 0.10***	0.133 ± 0.011***
Female mice		
None (control)	1.01 ± 0.10	0.054 ± 0.004
0.02% PFOA for 7 days	1.84 ± 0.35***	0.138 ± 0.008***
0.05% PFOA for 5 days	1.92 ± 0.18***	0.114 ± 0.008***
0.05% PFOA for 10 days	1.63 ± 0.06***	0.111 ± 0.003***

7 days of PFOA treatment (Table 4). This same activity was induced 34-fold in male mice.

The cytosolic DT-diaphorase activity in female and male mice after the treatment was increased 2- and 5-fold respectively (Table 4). The activities of glutathione transferase and epoxide hydrolase in the cytosolic fraction were increased about 2–3-fold in all mice.

Corresponding effects of PFOA on male and female Wistar rats

Male rats demonstrated an almost 30% increase in liver weight and a 45% increase in liver somatic index after 7 days of exposure to PFOA. The protein content of the mitochondrial fraction was increased 2.7-fold by PFOA. No effect at all was observed on the female liver weight, and the mitochondrial protein showed no increase in this case.

PFOA had no effect on cytosolic catalase activity in females, whereas male rats demonstrated a small increase. The catalase activity recovered in the mitochondrial fraction was not affected by PFOA in male or female rats. Palmitoyl CoA-oxidation in the mitochondrial fraction from male rats was increased 12-fold, but was unaffected in female animals.

DISCUSSION

This study demonstrates that male and female mice are affected by dietary PFOA to about the same extent. However, it has been reported previously that Wistar rats exhibit a sex-related difference in the response to PFOA [7]. Exposure to PFOA causes hepatic peroxisome proliferation in male rats, whereas females are unaffected. These observations were also confirmed in the present study.

The increased liver weights of the mice (Table 1) were interpreted as a result of an increase in the number and/or size of the cells. It is not clear whether the decreased body weights of the mice are an effect of decreased food intake, owing to the 'bad' taste of PFOA, or an effect of alterations in metabolic processes. A study on the response of rats to perfluorodecanoic acid, another perfluorinated carboxylic acid, has revealed that this compound may act as an uncoupler of oxidative phosphorylation and at the same time as an inhibitor of electron transport [23]. It was suggested that these phenomena could explain the severe loss of body weight in exposed rats. Since PFOA and perfluorodecanoic acid are structurally related to

Table 2. Effects of PFOA treatment on mouse liver mitochondrial, microsomal and cytosolic protein contents

All values (mg/g liver wet wt.) are means ± S.D. for three animals, except those for the female cytosolic and microsomal control groups, which are means ± S.D. for six animals: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the control group.

Group and treatment	Mitochondrial protein	Microsomal protein	Cytosolic protein
Male mice			
None (control)	9.4 ± 2.1	6.2 ± 2.0	40.6 ± 2.6
0.02% PFOA for 7 days	41.9 ± 2.6***	10.9 ± 0.8*	43.8 ± 1.7
Female mice			
None (control)	7.8 ± 1.7	8.0 ± 2.2	35.9 ± 7.8
0.02% PFOA for 7 days	41.5 ± 7.2**	9.3 ± 0.4	37.4 ± 0.9
0.05% PFOA for 5 days	35.8 ± 3.1***	7.5 ± 0.9	46.2 ± 4.9
0.05% PFOA for 10 days	31.1 ± 0.4***	9.2 ± 2.1	38.6 ± 4.1

Table 3. Effects of PFOA treatment on peroxisomal fatty acid β-oxidation in the hepatic mitochondrial fraction from mice

All values are means ± S.D. for three animals, except those for the female control group, which are means ± S.D. for six animals: ***$P < 0.001$ compared with the control group.

	Palmitoyl-CoA oxidation		Lauroyl-CoA oxidase	
Group and treatment	(nmol/min per g of liver)	(% of control)	(nmol/min per g of liver)	(% of control)
Male mice				
None (control)	252 ± 16	100	217 ± 16	100
0.02% PFOA for 7 days	4230 ± 10	1680***	2920 ± 390	1350***
Female mice				
None (control)	241 ± 95	100	247 ± 50	100
0.02% PFOA for 7 days	3860 ± 1440	1600***	3460 ± 240	1400***
0.05% PFOA for 5 days	4670 ± 560	1940***	3850 ± 420	1560***
0.05% PFOA for 10 days	5360 ± 70	2220***	4710 ± 650	1910***

Table 4. Effects of PFOA treatment on microsomal ω-hydroxylation of lauric acid and on cytosolic catalase, DT-diaphorase, glutathione transferase and epoxide hydrolase activities in the livers of mice

All values are means ± S.D. for three animals, except those for the female control group, which are means ± S.D. for six animals: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the control group.

Group and treatment	ω-Hydroxylation		Catalase		DT-diaphorase		Glutathione transferase		Epoxide hydrolase	
	(nmol/min per g of liver)	(%)	(mmol/min per g of liver)	(%)	(μmol/min per g of liver)	(%)	(μmol/min per g of liver)	(%)	(nmol/min per g of liver)	(%)
Male mice										
None (control)	13.2 ± 4.1	100	3.6 ± 0.5	100	4.1 ± 0.9	100	190 ± 40	100	188 ± 20	100
0.02% PFOA for 7 days	446.6 ± 50.8	3380***	36.6 ± 1.3	1020***	20.3 ± 6.7	495*	320 ± 0.0	168**	615 ± 45	327***
Female mice										
None (control)	22.6 ± 14.8	100	2.4 ± 0.5	100	11.8 ± 3.0	100	90 ± 20	100	146 ± 27	100
0.02% PFOA for 7 days	598.2 ± 1.0	2650***	33.4 ± 7.1	1390***	20.0 ± 0.0	169*	310 ± 70	344**	458 ± 142	314*

each other, it is likely that they affect animals in a similar manner. Thus it is possible that the decrease in the body weights of mice after PFOA treatment may be explained by alterations in mitochondrial respiration.

It has been reported previously that peroxisome proliferators cause an increase in the protein content of the mitochondrial fraction [12]. Accordingly, this protein content was increased several-fold after treatment with PFOA (Table 2). These findings can be explained by a decreased mitochondrial size and thus a redistribution of mitochondria from the nuclear to the mitochondrial fraction [12]. The number of mitochondria is increased simultaneously with the decrease in size, but the total mitochondrial volume is evidently constant during this process.

The greatest increase in catalase activity in the livers of mice treated with PFOA was found in the cytosolic fraction (Table 4). Similar results have previously been obtained after treatment with different peroxisome proliferators. The production of H_2O_2 is enhanced by peroxisome proliferators, and it is therefore astonishing that little or no increase in catalase activity can be demonstrated in the peroxisomes. One hypothesis is that two different forms of catalase are present, one peroxisomal and one cytosolic [24]. Only the latter of these forms is thought to increase after treatment with peroxisome proliferators. Another hypothesis is that catalase may be released into the cytosol after administration of peroxisome proliferators [5].

Both female and male mice demonstrated a large increase in peroxisomal fatty acid β-oxidation after treatment with PFOA (Table 3). ω-Hydroxylation of lauric acid was also dramatically affected by PFOA (Table 4). DT-diaphorase activity was significantly lower in untreated male mice than in untreated female mice (Table 4). After PFOA treatment, male and female mice demonstrated similar levels of DT-diaphorase. The activity of cytosolic glutathione tranferase is in general decreased by peroxisome proliferators [11]. However, PFOA has an inducing effect on glutathione transferase in the cytosolic fraction from both male and female mice (Table 4). As expected for cytosolic epoxide hydrolase, this enzyme was induced by PFOA (Table 4).

It has been established that the sex-related difference in the response of rats to PFOA depends on testosterone [7]. Accordingly, female rats exhibit no enhanced peroxisomal fatty acid β-oxidation because of their lack of testosterone. It is, however, possible that testosterone only affects the excretion of PFOA and not the proliferation of peroxisomes. In mice, it is clear that testosterone does not affect the response to PFOA, since this compound causes proliferation and related effects in female as well as in male mice.

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