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EFFECTS OF PERFLUORO FATTY ACIDS ON XENOBIOTIC-METABOLIZING ENZYMES, ENZYMES WHICH DETOXIFY REACTIVE FORMS OF OXYGEN AND LIPID PEROXIDATION IN MOUSE LIVER

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Abstract—Male mice were exposed via their diet to perfluoro fatty acids of various chain-lengths (2–10 carbon atoms) at different doses (0.02 and 0.1% weight) and for different periods of time (2–10 days). Thereafter, we monitored effects on liver and body weights and a number of hepatic parameters, including mitochondrial protein content, microsomal contents of cytochromes P450 and b_5, NADPH-cytochrome P450 reductase activity [measured as NADPH-cytochrome *c* reductase (EC 1.6.2.3)], microsomal and cytosolic epoxide hydrolase (EC 3.3.2.3) activities, cytosolic DT-diaphorase (EC 1.6.99.2), glutathione transferase (EC 2.5.1.18), glutathione peroxidase (EC 1.11.1.9) and superoxide dismutase (EC 1.15.1.1) activities, and levels of thiobarbituric acid-reactive material (as an indicator of lipid peroxidation) in the mitochondrial subfraction. The most dramatic changes observed were a 5–9-fold increase in mitochondrial protein, a 3–6-fold increase in the microsomal content of cytochrome P450, a 3–10-fold increase in cytosolic DT-diaphorase activity, an approximately 2-fold increase in cytosolic epoxide hydrolase activity and as much as a 60% decrease in the level of thiobarbituric acid-reactive compounds in the mitochondrial fraction. Smaller increases in microsomal epoxide hydrolase activity and decreases in cytosolic glutathione peroxidase activity were also observed. Of the perfluoro fatty acids tested, perfluorooctanoic acid caused the largest changes in the parameters examined here. Dietary exposure of mice to a 0.02% dose of this substance for 10 days results in a maximal or near-maximal effect in most cases.

In 1976 Lazarow and deDuve [1] discovered that peroxisomes contain a distinct system for the β-oxidation of fatty acids. In addition, during the past decade it has become clear that exposure to a large number of structurally different xenobiotics can result in an increase in the number and size of hepatic peroxisomes in rodents, as well as in a selective induction of the capacity of these organelles for fatty acid β-oxidation [2–4]. The growing list of so-called peroxisome proliferators includes industrial chemicals such as phthalates, phenoxyacetic acids and perfluoro fatty acids, as well as various hypolipidemic clinical drugs.

In general, long-term treatment of rodents with peroxisome proliferators has been found to give rise to liver tumours [2–4]. Interestingly, thorough investigation of several representatives of this class of substances, including di(2-ethylhexyl)phthalate, has revealed only minor effects on nuclear DNA [4, 5]. This has led to the belief that peroxisome proliferators may constitute a new class of chemical carcinogens, which transform cells by epigenetic mechanisms. Alternatively, these compounds may act as promoters. In either case, the observation that peroxisome proliferators increase the level of peroxisomal fatty acid β-oxidation (which produces H_2O_2) to a greater degree than the cellular level of catalase (EC 1.11.1.6) (which dismutates H_2O_2 to water and molecular oxygen) [2–4] may be relevant. Such a combination of changes is expected to increase intracellular oxidative stress and this stress may be involved in the processes of transformation, promotion and/or progression.

In addition, exposure to peroxisome proliferators affects many of the xenobiotic-metabolizing enzymes in rodent liver. Microsomal cytochrome P450 IVA1 (P452), which is specialized for the ω-hydroxylation of lauric acid, is induced many-fold [6]. Cytosolic epoxide hydrolase (EC 3.3.2.3) and, usually, the corresponding microsomal enzyme are also induced [7]. On the other hand, cytosolic glutathione transferase (EC 2.5.1.18) activity in rodent liver is often decreased upon exposure to peroxisome proliferators [8–10].

It is still unclear whether such treatment also affects other xenobiotic-metabolizing systems or enzymes involved in cellular defenses against reactive oxygen. This question is of great importance, since reactive intermediates of xenobiotic metabolism and reactive species of oxygen are thought to be involved in the development of many toxic and genotoxic effects. In addition, many so-called xenobiotic-metabolizing enzymes can also metabolize certain hydrophobic endobiotics.

The relatively recent discovery that perfluoro fatty acids also cause peroxisome proliferation [e.g. 11–13] is of great interest to us for a number of reasons. In the first place, these compounds are structural

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analogues of naturally occurring fatty acids and at the same time are very potent as peroxisome proliferators. This combination of characteristics may make them highly useful in studies designed to reveal the mechanism underlying peroxisome proliferation. In addition, the effects of perfluoro fatty acids appear to be persistent [e.g. 14] and industrial usage of these substances is increasing. Finally, exposure of rodents to perfluoro fatty acids can give rise to a peculiar "wasting" syndrome involving severe weight loss and hypothermia [e.g. 15].

In the present study we fed mice a diet containing perfluoro fatty acids of varying chain-lengths (2–10 carbon atoms) at different doses (0.02 or 0.1% weight) for different periods of time (2–10 days). Thereafter, we monitored the effects on various enzymes involved in xenobiotic metabolism and/or in deactivation of reactive species of oxygen, as well as on lipid peroxidation.

MATERIALS AND METHODS

Chemicals. Trifluoroacetic acid (98%, sodium salt, powder), heptafluorobutyric acid (98%, liquid), pentadecafluorooctanoic acid (98%, powder) and nonadecafluorodecanoic acid (98%, powder) were all purchased from Aldrich-Chemie (Steinheim, Germany) or Merck (Darmstadt, Germany). Dithionite, NADPH, cytochrome *c*, menadione, dicumarol, 1-chloro-2,4-dinitrobenzene, H_2O_2 (30%), xanthine, xanthine oxidase and thiobarbituric acid were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

trans-Stilbene oxide (EGA-Chemie, Steinheim, Germany) and *cis*-stilbene (Merck) were bought from the companies indicated. *cis*-Stilbene oxide was synthesized by *m*-chloroperoxybenzoic acid oxidation of *cis*-stilbene and purified by silica gel column chromatography (using hexane:ethyl acetate, 95:5, as the eluant) prior to use. [7, 8-^{3}H]*cis*- and -*trans*-stilbene oxide (2 Ci/mmol) were synthesized according to Gill *et al.* [16] and purified by TLC using hexane:ethyl acetate (95:5, v/v) with two developments. The bands containing these compounds were scraped off the plate and eluted twice with 2 mL ethanol, followed by filtration through glass-wool. The final purities were estimated to be 99.9%.

All other chemicals were at least of analytical grade and obtained from common commercial sources.

Treatment of animals. Male C57Bl/6 mice (ALAB, Sollentuna, Sweden) weighing about 20 g were used throughout this study. The animals were housed in steel cages in groups of three or four with a 12 hr light/dark cycle at 25°. They were provided with commercial food (R_3, containing 5% fat, 24% protein and 49% carbohydrate: Astra Ewos AB, Södertälje, Sweden) in powdered form, with or without perfluoro fatty acid, and tap water *ad lib.* for the time periods indicated, at the end of which the animals were killed.

All perfluoro fatty acids were administered in the diet, which was prepared as follows: solid substances were finely ground, if necessary, and mixed thoroughly with the powdered chow. Liquid substances were dissolved in 20 mL acetone and then mixed with 100 g powdered chow. This food was then dried in a ventilated hood for at least 16 hr, after which no smell of acetone could be detected. The control diet for each substance was prepared in an identical manner, but without addition of the perfluoro fatty acid. For practical reasons, diets were stored in the coldroom for up to a week before use. All dosages are given as weight percentages in the diet.

Preparation of subcellular fractions. At the end of the treatment period, the animals were killed by cervical dislocation. The liver was dissected out and, after removal of the gallbladder, placed in ice-cold 0.25 M sucrose and weighed. After dicing with scissors, the tissue was homogenized in 2 vol. of ice-cold 0.25 M sucrose using four up-and-down strokes of a Potter–Elvehjem homogenizer at 440 RPM. After dilution to 1 g liver/5 mL suspension, an aliquot of this homogenate was saved and the rest centrifuged at 600 g_{av} for 10 min to give the nuclear pellet which was resuspended in 0.25 M sucrose to a volume equivalent to 1 g liver/5 mL. The resulting supernatant was then centrifuged at 10,000 g_{av} for 10 min. This second pellet, the mitochondrial fraction, was resuspended, washed twice in sucrose by centrifugation and finally resuspended in 0.25 M sucrose to a volume of 2.0 mL. The 10,000 g_{av} supernatant was centrifuged at 133,000 g_{av} for 60 min, and the resulting microsomal pellet was washed once in 0.15 M Tris–Cl, pH 8.0 and thereafter resuspended in 0.25 M sucrose to a final volume of 2.0 mL. The 133,000 g_{av} supernatant was designated cytosol and had a volume of about 3 mL. The subcellular fractions obtained using this procedure have been characterized thoroughly in an earlier report [17].

Enzyme and protein assays. Microsomal cytochrome P450 content was determined from the difference spectrum between the reduced cytochrome and its carbon monoxide complex, according to Omura and Sato [18].

NADPH-cytochrome P450 reductase activity was assayed spectrophotometrically as NADPH-cytochrome *c* reductase (EC 1.6.2.3) [19].

Cytochrome b_5 was quantitated on the basis of the difference spectrum between its oxidized and reduced forms [18].

Microsomal and cytosolic epoxide hydrolase activities were assayed using *cis*- and *trans*-[^{3}H]-stilbene oxide, respectively, as substrate [16]. After incubation, the diol product and remaining substrate were separated by a simple extraction procedure and quantitated by scintillation counting.

DT-diaphorase (EC 1.6.99.2) activity was determined by following the dicumarol-insensitive reduction of cytochrome *c* by menadione at 550 nm [20].

Glutathione transferase activity was quantitated spectrophotometrically at 340 nm using 1-chloro-2,4-dinitrobenzene as the second substrate [21, 22].

Glutathione peroxidase (EC 1.11.1.9) activity was assayed spectrophotometrically by following the rate of disappearance of NADPH at 340 nm, according to Gunzler *et al.* [23].

Superoxide dismutase (EC 1.15.1.1) activity was

Table 1. Effects of dietary treatment of mice with two different concentrations of perfluorooctanoic acid for different time periods on body and liver weights and liver somatic index

Weight % perfluorooctanoic acid in the diet	Period of treatment (days)	Body weight (g) Before treatment	Body weight (g) After treatment	Liver weight (g)	Liver somatic index§
None (control)	2	21.2 ± 1.7	21.0 ± 1.3	1.12 ± 0.12	5.16 ± 0.5
	5	21.2 ± 1.7	20.6 ± 1.5	1.07 ± 0.15	5.10 ± 0.5
	10	22.1 ± 1.2	22.6 ± 0.8	1.17 ± 0.06	5.10 ± 0.5
0.02	5	19.6 ± 0.9	19.3 ± 0.6	1.87 ± 0.12†	10.8 ± 0.8‡
	10	22.2 ± 2.1	17.0 ± 1.5*	2.13 ± 0.23†	12.5 ± 0.9‡
0.1	2	21.9 ± 1.0	18.3 ± 0.7*	1.37 ± 0.21	7.4 ± 0.6†
	5	20.5 ± 0.5	15.1 ± 0.4†	1.53 ± 0.06†	10.2 ± 0.9†

Animals were killed after the time periods indicated.

The values are means ± SD for three animals.

* $P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ compared with the control group

§ Liver somatic index = (liver weight after treatment/body weight after treatment) ×100.

determined spectrophotometrically by the procedure of Crapo *et al.* [24], in which xanthine/xanthine oxidase is used as the source of superoxide and cytochrome *c* as the indicating scavenger for this radical. Superoxide dismutase competes for the superoxide radical and thus inhibits reduction of cytochrome *c*.

Lipid peroxidation was quantitated by assaying thiobarbituric acid-reactive compounds [25] and by measuring uptake of O_2 in the presence of ADP-Fe^{2+} and ascorbate using an oxygen electrode [26]. Before determining thiobarbituric acid-reactive compounds, the mitochondrial fraction was washed with buffer to remove sucrose, in order to eliminate the interference caused by high concentrations of this disaccharide. Two identical, but separate experiments are documented in Table 9.

Protein was measured by the method of Lowry *et al.* [27] with bovine serum albumin as standard.

Statistical analysis. Each experimental group contained three or four animals, as indicated. Values are presented as means ± SD and statistical significance was tested for using Student's *t*-test.

RESULTS

Effects of dose, period of administration and chain-length on body and liver weights and protein contents of the hepatic subcellular fractions

The effects of dietary exposure of mice to 0.02 or 0.1 weight % perfluorooctanoic acid for 2, 5 or 10 days on body and liver weights are documented in Table 1 and the corresponding effects on the protein contents of hepatic subcellular fractions in Table 2. The effects of dietary exposure of mice to 0.02% perfluoroacetic, perfluorobutyric, perfluorooctanoic or perfluorodecanoic acid for 10 days on body and liver weights are presented in Table 3 and the corresponding effects of the protein contents of hepatic subcellular fractions in Table 4. (Treatment with perfluoro fatty acids for 10 days at the higher dose, i.e. 0.1%, was avoided in order to minimize the risk of toxicity).

Treatment with perfluoro fatty acids decreased the body weights of mice (Tables 1 and 3), increased their liver weights (Tables 1 and 3) and dramatically increased the protein content of the mitochondrial fraction (Tables 2 and 4). These effects were expected, since they usually accompany peroxisome proliferation [28]. The increase in mitochondrial protein is due primarily to a redistribution of mitochondria from the nuclear to the mitochondrial fraction, apparently caused by a decrease in their average size [29]. The largest effects were obtained with perfluorooctanoic acid and perfluorodecanoic acid, whereas perfluoroacetic acid was essentially inactive.

The changes in protein contents of the mitochondrial and, in certain cases, microsomal and cytosolic fractions documented in Tables 2 and 4 should be kept in mind when examining the data documented in the other tables. If one wishes to compare total activities, then the specific activities presented in the following tables must be multiplied by the protein contents documented in Tables 2 and 4.

Effects of dose, period of administration and chain-length on various xenobiotic-metabolizing enzymes

Dietary treatment of mice with perfluorooctanoic acid caused a dramatic increase in the hepatic microsomal content of cytochrome P450 (Tables 5 and 6). This increase was 6.5-fold when the animals received 0.02% perfluorooctanoic acid for 5 days. The effects of 0.02% perfluorooctanoic acid for 5 days were as great as those seen at a dose of 0.1% for the same length of time. None of the other perfluoro fatty acids tested, i.e. perfluoroacetic, perfluorobutyric and perfluorodecanoic acid, affected the total microsomal content of cytochrome P450 (Table 6). On the other hand, all of these substances caused increases in NADPH-cytochrome P450 reductase activity (measured as NADPH-cytochrome c reductase). None of the substances tested had any noteworthy effect on microsomal cytochrome b_5 content (Table 6).

As is invariably the case with peroxisome proliferators [7], exposure of mice to perfluoro fatty

Table 2. Effects of dietary treatment of mice with two different concentrations of perfluorooctanoic acid for different time periods on the protein contents of hepatic mitochondrial, microsomal and cytosolic subfractions

Weight % perfluorooctanoic acid in the diet	Period of treatment (days)	Protein content		
		Mitochondrial fraction	Microsomal fraction (mg/g liver, wet weight)	Cytosolic fraction
None (control)	10	5.5 ± 2.2	10.3 ± 2.5	28.6 ± 5.2
0.02	5	47.7 ± 8.9†	16.6 ± 3.4	31.6 ± 3.6
	10	27.2 ± 6.8†	16.6 ± 1.6*	26.2 ± 2.8
0.1	2	22.5 ± 1.3‡	13.8 ± 2.4	26.4 ± 3.2
	5	31.7 ± 2.5‡	16.0 ± 0.9*	27.4 ± 3.2

Animals were killed after the time periods indicated.
The values are means ± SD for three animals.
* $P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ compared with the control group.

Table 3. Effects of dietary treatment of mice with different perfluoro fatty acids for 10 days on body and liver weights and liver somatic index

Dietary additive (0.02 weight %)	Body weight (g)		Liver weight (g)	Liver somatic index‡
	Before treatment	After treatment		
None (control)	20.7 ± 0.5	21.6 ± 0.8	1.1 ± 0.2	5.2 ± 0.7
Perfluoroacetic acid	22.0 ± 0.8*	22.4 ± 0.7	1.3 ± 0.1	5.9 ± 0.4
Perfluorobutyric acid	23.5 ± 0.7†	25.0 ± 0.7†	1.8 ± 0.4*	7.2 ± 1.3*
Perfluorooctanoic acid	21.0 ± 0.4	18.2 ± 0.3†	2.1 ± 0.2†	11.4 ± 0.8†
Perfluorodecanoic acid	22.1 ± 1.1	14.7 ± 0.9†	1.5 ± 0.2*	10.4 ± 1.0†

The values are means ± SD for four animals.
* $P < 0.05$, †$P < 0.001$ compared with the control group.
‡ Liver somatic index = (liver weight after treatment/body weight after treatment) ×100.

Table 4. Effects of dietary treatment of mice with different perfluoro fatty acids for 10 days on the protein contents of hepatic mitochondrial, microsomal and cytosolic subfractions

Dietary additive (0.02 weight %)	Protein content		
	Mitochondrial fraction	Microsomal fraction (mg/g liver, wet weight)	Cytosolic fraction
None (control)	6.6 ± 1.3	6.2 ± 2.1	26.4 ± 1.6
Perfluoroacetic acid	9.9 ± 1.5*	7.7 ± 1.3	26.8 ± 2.0
Perfluorobutyric acid	19.9 ± 4.0‡	11.1 ± 1.7*	25.4 ± 0.6
Perfluorooctanoic acid	32.5 ± 3.7‡	11.0 ± 1.3†	30.0 ± 3.4
Perfluorodecanoic acid	26.3 ± 3.0‡	8.7 ± 2.1	18.8 ± 2.0†

The values are means ± SD for four animals.
* $P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ compared with the control group.

acids induced the level of hepatic cytosolic epoxide hydrolase activity about 2-fold (Tables 7 and 8). In this case all of the compounds tested had a significant effect. Again, the effects of 0.02% perfluorooctanoic acid were as great as those seen with a dose of 0.1% for the same period. Some induction of microsomal epoxide hydrolase activity was also obtained upon dietary exposure to perfluorooctanoic or perfluorodecanoic acid (Tables 5 and 6).

Exposure to all of the perfluoro fatty acids tested

Table 5. Effects of dietary treatment of mice with two different concentrations of perfluorooctanoic acid for different time periods on hepatic microsomal cytochrome P450 and cytochrome b_5 contents, and activities of cytochrome P450 reductase and epoxide hydrolase

Weight % perfluorooctanoic acid in the diet	Period of treatment (days)	Cytochrome P450‡	Cytochrome P450 reductase§	Cytochrome b_5‡	Epoxide hydrolase‖
None (control)	10	0.27 ± 0.09	29 ± 14	0.15 ± 0.07	2.00 ± 0.43
0.02	5	1.76 ± 0.31†	63 ± 17	0.16 ± 0.04	3.26 ± 0.62*
	10	1.06 ± 0.27†	58 ± 2*	0.13 ± 0.04	4.77 ± 0.57†
0.1	2	1.07 ± 0.23†	45 ± 3	0.07 ± 0.02	1.97 ± 0.41
	5	1.57 ± 0.30†	80 ± 3†	0.17 ± 0.03	3.35 ± 0.53*

Animals were killed after the time periods indicated.
The values are means ± SD for three animals.
* $P < 0.05$, †$P < 0.01$ compared with the control group.
‡ nmol/mg microsomal protein.
§ nmol cytochrome *c* reduced/min/mg microsomal protein.
‖ nmol *cis*-stilbene oxide metabolized/min/mg microsomal protein.

Table 6. Effects of dietary treatment of mice with different perfluoro fatty acids for 10 days on microsomal contents of cytochrome P450 and cytochrome b_5, and activities of cytochrome P450 reductase and epoxide hydrolase

Dietary additive (0.02 weight %)	Cytochrome P450§	Cytochrome P450 reductase‖	Cytochrome b_5§	Epoxide hydrolase¶
None (control)	0.41 ± 0.21	18.2 ± 3.6	0.20 ± 0.06	1.75 ± 0.39
Perfluoroacetic acid	0.39 ± 0.16	26.9 ± 4.5*	0.19 ± 0.01	1.65 ± 0.21
Perfluorobutyric acid	0.60 ± 0.16	36.5 ± 5.8†	0.23 ± 0.04	1.58 ± 0.21
Perfluorooctanoic acid	1.23 ± 0.16‡	70.1 ± 10.9‡	0.34 ± 0.06*	5.10 ± 0.29‡
Perfluorodecanoic acid	0.45 ± 0.04	63.9 ± 3.9‡	0.17 ± 0.01	3.93 ± 1.07†

The values are means ± SD for four animals.
* $P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ compared with the control group.
§ nmol/mg microsomal protein.
‖ nmol cytochrome *c* reduced/min/mg microsomal protein.
¶ nmol *cis*-stilbene oxide metabolized/min/mg microsomal protein.

Table 7. Effects of dietary treatment of mice with two different concentrations of perfluorooctanoic acid for different time periods on hepatic cytosolic DT-diaphorase, glutathione transferase, glutathione peroxidase, superoxide dismutase and epoxide hydrolase activities

Weight % perfluorooctanoic acid in the diet	Period of treatment (days)	DT-diaphorase§	Glutathione transferase‖	Glutathione peroxidase¶	Superoxide dismutase**	Epoxide hydrolase††
None (control)	10	277 ± 55	7.73 ± 1.39	1.84 ± 0.65	146 ± 44	7.42 ± 0.87
0.02	5	733 ± 220*	8.46 ± 0.45	1.19 ± 0.04	129 ± 24	17.1 ± 5.8*
	10	1360 ± 85‡	9.84 ± 1.62	1.27 ± 0.06	142 ± 43	15.2 ± 0.8‡
0.1	2	599 ± 220	7.82 ± 1.48	1.44 ± 0.08	129 ± 18	14.9 ± 2.5†
	5	1050 ± 280†	11.6 ± 2.9	1.44 ± 0.09	178 ± 11	16.9 ± 3.2†

Animals were killed after the time periods indicates.
The values are means ± SD for three animals.
* $P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ compared with the control group.
§ nmol NADPH oxidized/min/mg cytosolic protein.
‖ nmol 1 chloro-2,4 dinitrobenzene conjugated/min/mg cytosolic protein.
¶ μmol NADPH oxidized/min/mg cytosolic protein.
** U/min/mg cytosolic protein (one unit = quantity of SOD required to produce 50% inhibition of the rate of reduction of cytochrome *c*).
†† nmol *trans*-stilbene oxide metabolized/min/mg cytosolic protein.

Table 8. Effects of dietary treatment of mice with different perfluoro fatty acids for 10 days on hepatic cytosolic DT-diaphorase, glutathione transferase, glutathione peroxidase, superoxide dismutase and epoxide hydrolase activities

Dietary additive (0.02 weight %)	DT-diaphorase§	Glutathione transferase‖	Glutathione peroxidase¶	Superioxide dismutase**	Epoxide hydrolase††
None (control)	208 ± 20	6.83 ± 1.73	1.12 ± 0.13	89.3 ± 15.6	3.97 ± 0.34
Perfluoroacetic acid	141 ± 26	7.17 ± 1.02	1.20 ± 0.15	146 ± 14†	7.20 ± 1.60†
Perfluorobutyric acid	259 ± 44	5.32 ± 0.46	1.15 ± 0.09	155 ± 7‡	8.40 ± 1.80†
Perfluorooctanoic acid	1120 ± 355†	8.76 ± 2.20	0.80 ± 0.11†	137 ± 20†	8.20 ± 0.70‡
Perfluorodecanoic acid	726 ± 347*	5.59 ± 0.88	0.72 ± 0.11†	142 ± 4‡	9.70 ± 0.90‡

The values are means ± SD for three animals.

* $P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ compared with the control group.

§ nmol NADPH oxidized/min/mg cytosolic protein.

‖ nmol 1 chloro-2,4 dinitrobenzene conjugated/min/mg cytosolic protein.

¶ μmol NADPH oxidized/min/mg cytosolic protein.

** U/min/mg cytosolic protein (one unit = quantity of SOD required to produce 50% inhibition of the rate of reduction of cytochrome *c*).

†† nmol *trans*-stilbene oxide metabolized/min/mg cytosolic protein.

Table 9. Effects of dietary treatment with 0.02% perfluorooctanoic acid for 10 days on lipid peroxidation in the mitochondrial fraction from mouse liver

Experiment	Group	Thiobarbituric acid-reactive material†	O_2 consumption in the presence of ADP-Fe^{2+} and ascorbate‡
1	Control	12.4 ± 1.9 (100%)	286 ± 20.6 (100%)
	Exposed	8.22 ± 0.66 (66%)*	131 ± 12.5 (46%)*
2	Control	14.8 ± 0.50 (100%)	277 ± 17.1 (100%)
	Exposed	6.16 ± 0.41 (42%)*	124 ± 3.8 (45%)*

The values are means ± SD (percentage of the corresponding control value) for four animals.

* $P < 0.001$ compared with the corresponding control value.

† nmol malondialdehyde/mg mitochondrial protein.

‡ nmol O_2/min/mg mitochondrial protein.

here resulted in little or no effect on total cytosolic glutathione transferase activity (measured using 1-chloro-2,4-dinitrobenzene as the second substrate) (Tables 7 and 8).

Effects of dose, period of administration and chain-length on enzymes which detoxify reactive forms of oxygen and on lipid peroxidation

Upon dietary exposure of mice to 0.02 weight % perfluorobutyric, perfluorooctanoic or perfluorodecanoic acid for 10 days, dramatic increases in hepatic cytosolic DT-diaphorase activity were observed (Table 8). The increase with perfluorooctanoic acid was about 5-fold, whereas perfluoroacetic and perfluorobutyric acids were inactive in this respect. In this case again the effect of 0.02% perfluorooctanoic acid for 5 days was approximately as large as the effect of 0.1% of this substance for the same period of time (Table 7). Small effects on hepatic cytosolic glutathione peroxidase and superoxide dismutase activities were also observed in some cases (Tables 7 and 8).

Highly striking was the effect of exposure to perfluorooctanoic acid (0.02% for 10 days) on the content of thiobarbituric acid-reactive material (used as a measure of lipid peroxidation) in the mitochondrial fraction from mouse liver (Table 9). This content was reduced by up to 60%. At the same time, non-enzymatic oxygen consumption by the mitochondrial fraction in the presence of ADP-Fe^{2+} and ascorbate (a measure of the capacity of the membranes to undergo lipid peroxidation) was reduced in a similar fashion. With the other perfluoro fatty acids tested, no significant effects on these two parameters could be observed (not shown).

DISCUSSION

It is quite clear that perfluoro fatty acids are not only potent peroxisome proliferators, but also cause a number of other dramatic changes in mouse liver, including hepatomegaly; a decrease in the average size of hepatic mitochondria (as reflected in an increased protein content of the mitochondrial fraction); changes in so-called xenobiotic-metabolizing enzymes; changes in cellular defenses against reactive oxygen; and a decrease in the level of products of lipid peroxidation recovered in the mitochondrial fraction, as well as in the capacity of this same fraction to undergo lipid peroxidation *in vitro*. In addition, perfluorodecanoic acid has been reported to uncouple rat liver mitochondria [15]. It may also be mentioned that perfluorooctanoic acid is a much better protein precipitant than is trichloroacetic acid [30], an observation which may have a bearing on the physiological effects of this compound.

It is well known that peroxisome proliferators in general induce cytochrome P450 and, in particular, cytochrome P450 IVA1 (P452), which is specialized for the ω-hydroxylation of lauric acid [6]. Indeed, to our knowledge, peroxisome proliferation is always accompanied by induction of ω-hydroxylation, which suggests that there may be some causal relationship between these two events [see 31]. Among peroxisome proliferators, perfluorooctanoic acid is one of the most powerful inducers of total microsomal cytochrome P450 in mouse liver [10]. However, the extent of such induction cannot be interpreted unambiguously, since there are numerous isozymes of cytochrome P450 in liver microsomes and induction of one of these may be accompanied by the loss of others [32]. Thus, the lack of induction of total microsomal cytochrome P450 by perfluoroacetic, perfluorobutyric and perfluorodecanoic acids does not necessarily mean that these substances do not induce cytochrome P450 IVA1. In addition, induction might have been obtained with these compounds using a different dose, period of exposure, route of administration or species. It would be of interest to determine whether the large increase in total hepatic microsomal cytochrome P450 content which occurs after exposure of mice to perfluorooctanoic acid is due entirely to an increase in this particular isozyme.

As far as we know, induction of cytosolic epoxide hydrolase in rodent liver also invariably associated with peroxisome proliferation [7, 10]. Thus, it is not surprising that perfluoro fatty acids also induce this enzyme in mouse liver. One possible explanation for this coupling is that cytosolic epoxide hydrolase is actually a peroxisomal enzyme. Immunochemical analysis supports this hypothesis [33], whereas subcellular fraction studies [34] and studies involving digitonin permeabilization of isolated mouse hepatocytes [35] suggest that this enzyme is present in both peroxisomes and the cytoplasm. In any case, it is quite clear that there is no strict quantitative correlation between the extents of peroxisome proliferation and induction of cytosolic epoxide hydrolase [10]. This is also illustrated by the perfluoro fatty acids: as seen here, perfluoroacetic, perfluorobutyric, perfluorooctanoic and perfluorodecanoic acids are all equally good inducers of cytosolic epoxide hydrolase, but, as shown elsewhere, perfluorooctanoic and perfluorodecanoic acids are considerably more potent as peroxisome proliferators [28].

On the other hand, there is no strict coupling between peroxisome proliferation and the induction of microsomal epoxide hydrolase [10]. The effects of perfluorooctanoic and perfluorodecanoic acids on this enzyme were found here to be relatively small, while perfluoroacetic and perfluorobutyric acids had no effect at all. Thus, these two effects would seem to involve independent mechanisms.

By the same token, inhibition of hepatic cytosolic glutathione transferase activity is only caused by certain peroxisome proliferators [10]. The present results indicate that perfluoro fatty acids belong to the sub-group of peroxisome proliferators which do not affect the level of this activity in mouse liver. On the other hand, Schramm *et al.* [36] have reported that treatment of rats with perfluorodecanoic acid decreases the levels of glutathione transferase activities, proteins and mRNAs. This discrepancy may, of course, reflect a species difference and/or the different doses, periods of exposure and routes of administration employed by Schramm *et al.* [36]. However, Pastoor *et al.* [12] have reported that treatment of rats with ammonium perfluorooctanoate does not affect hepatic cytosolic glutathione transferase activity.

Not much is known about the increases in hepatic DT-diaphorase activity caused by exposure of rodents to peroxisome proliferators. We have observed such increases with certain of these substances previously,* but even in this case the two phenomena did not always occur together, suggesting that they are independent. In any case, perfluorobutyric, perfluorodecanoic and, especially, perfluorooctanoic acids are relatively potent inducers of DT-diaphorase. It will be of interest to determine whether this process is, indeed, a true induction (i.e. whether the increase in activity reflects an increased level of enzyme protein) and whether any particular isoform is induced selectively. DT-diaphorase reduces quinones, thereby allowing their conjugation and preventing their oxidative–reductive cycling with concomitant production of reactive oxygen species [37]. However, there would seem to be no obvious reason why quinones should arise to a larger extent in the livers of perfluorooctanoic acid-treated mice than in normal mice or mice treated with perixosome proliferators which do not induce DT-diaphorase.

The moderate reduction of cytosolic glutathione peroxidase activity in the livers of mice exposed here to perfluorooctanoic or perfluorodecanoic acid is also something of a mystery, especially considering the likelihood that hepatocytes with a large capacity for peroxisomal fatty acid β-oxidation detoxify larger amounts of H_2O_2 than normal hepatocytes. We have seen a similar effect in the livers of rats exposed to clofibrate†. Of relevance in this respect is the fact that Chen *et al.* [38] found that treatment of rats with perfluorodecanoic acid significantly increased the selenium content and selenium-dependent glutathione peroxidase of the hepatic cytosol.

Even less understandable at present is the decreased level of thiobarbituric acid-reactive compounds present in the mitochondrial fraction prepared from the livers of mice exposed to perfluorooctanoic acid, as well as the decreased capacity of this fraction to undergo lipid peroxidation initiated by ADP-Fe^{2+}-ascorbate. One possible cause might be a change in the fatty acid composition of this fraction, i.e. an increase in the degree of saturation. However, treatment of rats with perfluorooctanoic acid has been reported to have the opposite effect on hepatic fatty acids, i.e. the relative proportions of C18:1 and C20:3 are increased [39].

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* Lundgren B, Sohlenius A-K, Permadi H, Andersson K and DePierre JW, Effects of dietary treatment of mice with peroxisome proliferators on hepatic DT-diaphorase activity, with special emphasis on perfluoro fatty acids, submitted.

† Olsson U, Garberg P, Lundgren B, Andersson K, Hultby K, Bergstrand A, Högberg J and DePierre JW, Dietary treatment with clofibrate does not cause peroxisome proliferation in the livers of selenium-deficient rats, submitted.

When studying the effects of perfluoro fatty acids on these various parameters in mouse liver, it would seem most appropriate to use perfluorooctanoic acid as the model substance, since this substance generally causes the greatest changes. In addition, 0.02% perfluorooctanoic acid causes changes which are at least as extensive as those caused by a 0.1% dose, so that the lower dose should be utilized to minimize toxicity. A 5-day treatment period is adequate in most cases, although the effects on cytosolic DT-diaphorase activity are considerably higher after 10 days.

Finally, the changes in so-called xenobiotic-metabolizing enzymes and enzymes involved in cellular defenses against reactive oxygen caused by exposure to perfluoro fatty acids many have important consequences for the metabolism of both xeno- and endobiotics. In the future, possible synergistic effects as well as changes in endogenous homeostasis should be considered.

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