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Hepatic Peroxisome Proliferation in Vitamin A-Deficient Mice Without a Simultaneous Increase in Peroxisomal Acyl-CoA Oxidase Activity

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ABSTRACT. Vitamin A-adequate and vitamin A-deficient C57Bl/6 mice were treated for ten days with 0.02% (w/w) perfluorooctanoic acid (PFOA) in their diet. Treated vitamin A-adequate and -deficient mice demonstrated approximately the same increases in liver somatic index (g liver/g body weight) (somewhat more than 2-fold) and mitochondrial protein content (5-fold). PFOA treatment resulted in a 26-fold increase in hepatic peroxisomal lauroyl-CoA oxidase activity in vitamin A-adequate mice, whereas the same activity was unchanged in vitamin A-deficient mice. Vitamin A deficiency itself caused a 3- to 4-fold increase in cytosolic catalase activity and a smaller increase in the activity of microsomal cytochrome P-450 IVA (lauric acid ω- and ω-1 hydroxylase) in this same organ. The induction of the activities of these enzymes was less prominent in vitamin A-deficient mice compared with the effect caused by PFOA in vitamin A-adequate mice, resulting in approximately the same maximal values for these parameters in both groups (i.e. approx. 21 mmol/g liver · min and 350 nmol/g liver · min, respectively). A 70 kDa protein, presumably the multifunctional protein, was shown by Commassie blue staining of SDS-polyacrylamide gels and by immunoblotting (with antibodies towards the multifunctional protein) to be induced to approximately the same degree in vitamin A-adequate and -deficient mice. A morphometric study revealed that PFOA causes the same extent of hepatic peroxisome proliferation in vitamin A-deficient as in vitamin A-adequate mice. The possibility that PFOA exerts its effect *in vivo* through at least two different mechanisms is discussed. BIOCHEM PHARMACOL 51;6:821–827, 1996.

KEY WORDS. vitamin A deficiency; peroxisome proliferation; perfluorooctanoic acid; mouse

Treatment of rodents with hypolipidemic drugs and numerous other xenobiotics results in hepatic peroxisome proliferation and induction of peroxisomal fatty acid β-oxidation [1–3]. The first and rate-limiting step of peroxisomal fatty acid β-oxidation is catalyzed by acyl-CoA oxidase. It is known that a member of the steroid receptor family is activated by peroxisome proliferators [4], and that this PPAR§ binds to a PPRE upstream from the structural gene for acyl-CoA oxidase [5]. More recently, it was shown that another nuclear receptor, the RXR, forms a heterodimer with PPAR, and that this heterodimer binds to PPRE and acts as a transcriptor factor for the gene [6, 7]. The major ligand for RXR is 9-*cis*-retinoic acid, a vitamin A metabolite [8].

Until now, almost all studies on the involvement of PPAR, RXR, vitamin A, and/or 9-*cis*-retinoic acid in peroxisome proliferation have been performed using molecular biological and other *in vitro* systems. It is therefore of great interest to study these mechanisms *in vivo*. To achieve a more detailed understanding of the involvement of vitamin A in peroxisome proliferation and associated biological effects, we rendered male C57Bl/6 mice vitamin A-deficient and then used them for such *in vivo* studies. The effects of perfluorooctanoic acid on parameters known to be affected during peroxisome proliferation were investigated in these animals and compared to the corresponding effects in vitamin A-adequate mice.

Perfluoro fatty acids are used industrially as wetting agents and corrosion inhibitors as well as to improve the resistance of paper to water and oil [9]. Previous studies have shown that perfluorooctanoic acid is a highly potent peroxisome proliferator in mouse liver [10, 11]. Some of the results reported here have appeared previously in preliminary form [12].

MATERIALS AND METHODS

Chemicals

Perfluorooctanoic acid (98%) was purchased from Aldrich Chemie (Steinheim, Germany); lauroyl-CoA, 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); s-palmitoyl-CoA and horseradish peroxidase were from Boe-

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§ *Abbreviations:* PFOA, perfluorooctanoic acid; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-responsive element; and RXR, retinoid X receptor.

hringer Mannheim (Bromma, Sweden); hydrogen peroxide (30%), KCN, Folin-Ciocalteus Phenolreagenz, and lauric acid from E. Merck (Darmstadt, FRG); sucrose from BDH Limited (Poole, U.K.); and [1-C^{14}] lauric acid from Amersham (Solna, Sweden). Antibodies towards the multifunctional protein were kindly provided by Prof. Kalervo Hiltunen, University of Oulu, Finland. All other chemicals were of reagent grade and were also procurred from common commercial sources.

Animals and Treatment

Male C57Bl/6 mice (ALAB, Sollentuna, Sweden) were rendered vitamin A-deficient according to Smith and coworkers [13] by supplying pregnant mice with a vitamin A-deficient diet during the last two weeks of pregnancy and feeding the offspring this same diet after weaning. The vitamin A-deficient offspring were utilized for studies at 10–12 weeks of age, at which time the serum level of vitamin A is <10% of the normal value [13]. After longer periods of vitamin A deficiency, the mice begin to show signs of toxicity [13].

All mice were housed in steel cages with a 12 hour light-dark cycle at 25°C. They were given free access to a laboratory vitamin A-deficient (Vitamin A test diet) from USB (Cleveland, Ohio, U.S.A.) or a vitamin A-adequate diet (Beekay feeds rat and mouse standard diet, ALAB, Sollentuna, Sweden). To prepare the PFOA-containing diet, this compound was dissolved in 20 mL acetone and mixed with 100 g powdered food, and the chow dried in a ventilated hood until no smell of acetone was detectable (12–24 hr). Control chow was treated in the same manner with acetone, but without PFOA. All diets were supplied as pellets (the chow was mixed with water, baked, and thereafter dried overnight in a ventilated hood).

Vitamin A-deficient and vitamin A-adequate mice were treated for ten days with 0.02% PFOA in their diet.

Subcellular Fractionation

At the end of the experiment, the mice were killed by cervical dislocation and the livers removed, freed from the gallbladder, and thereafter rinsed in ice-cold 0.25 M sucrose. The livers were weighed and homogenized individually at 440 rpm in ice-cold 0.25 M sucrose using 4 up-and-down strokes of a Potter-Elvehjem homogenizer. More sucrose was added to give a 20% homogenate, which was subsequently centrifuged at 600 g_{av} for 10 min. The resulting supernatant was centrifuged at 10,000 g_{av} for 10 min to give a "mitochondrial" pellet containing primarily mitochondria, peroxisomes, and lysosomes. This pellet was resuspended and washed twice by centrifugation in sucrose. Finally, this fraction (referred to as the mitochondrial fraction) was resuspended in sucrose to give a volume of 2.0 mL. The supernatant from the original 10,000 g_{av} centrifugation was further centrifuged at 105,000 g_{av} for 60 min and the high-speed supernatant (cytosol, approx. 4 mL) saved. Studies on marker enzymes have shown that the mitochondrial fraction prepared in this manner contains 50% of the peroxisomes, the microsomal fraction 67% of the endoplasmatic reticulum, and the high-speed supernatant 92% of the cytosol [14].

Hepatic Levels of Vitamin A

Liver samples were sent to the Swedish Bureau of Veterinary Medicine (SVA), Uppsala, Sweden, for vitamin A analysis. After alkaline hydrolysis followed by extraction with hexane, retinoids were separated by high-performance liquid chromatography (HPLC) on a straight phase silica column. The mobile phase used was hexane: 1,4-dioxane, 49:1 (v/v). Retinol served as an external standard.

Assays

The enzyme assays were performed with saturating substrate concentrations and conditions of linearity with time and protein (with the exception of catalase, which cannot be saturated under reasonable conditions [15]). Peroxisomal acyl-CoA oxidase activity was measured with lauroyl-CoA as substrate by assaying hydrogen peroxide production with a fluorimetric method based on the peroxidase-coupled oxidation of 4-hydroxyphenylacetic acid [16, 17]. Cytosolic palmitoyl-CoA hydrolase was measured spectrophotometrically at 412 nm with 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) as reagent [18]. The activity of catalase was monitored spectrophotometrically at 240 nm with hydrogen peroxide as substrate [19]. Omega-hydroxylation of lauric acid was determined using a radiometric method with ^{14}C-lauric acid as substrate [20]. Protein concentrations were determined by the method of Lowry *et al.* [21] with bovine serum albumin as standard.

SDS-Polyacrylamide Gel Electrophoresis

Proteins were separated by electrophoresis using a 7.5% (w/v) SDS polyacrylamide-gel (SDS-PAGE) according to Laemmli [22]. Each well was loaded with 10 μg protein. The protein bands were detected by staining with Coomassie brilliant blue.

Electron Microscopy

Liver slices were rinsed in 1.5% glutaraldehyde (in sodium cacodylate buffer) and fixed in this solution for 24 hours at 4°C. The slices were thereafter rinsed twice in cold cacodylate buffer and post-fixed in 1% osmium tetroxide (in sodium phosphate buffer) for 12 hours. The slices were rinsed as above and thereafter dehydrated in graded ethanol and embedded in LX 112.

Each of the four groups (vitamin A-adequate and vitamin A-deficient with or without PFOA treatment) numbered five animals, except for the vitamin A-deficient group treated with PFOA, which consisted of four animals. Five blocks were selected at random from each animal and nine prints from each

TABLE 1. Hepatic vitamin A levels in mice fed a normal or vitamin A-deficient diet

Diets	Hepatic vitamin A (μg/g liver)
Normal (24,000 IU vitamin A acetate/kg)	307 ± 33*
Vitamin A-deficient	<5

* Mean value ± standard deviation for four animals.

group were analyzed. The number of peroxisomes was counted and the volume density determined using a Zeiss Videoplan instrument at a magnification of 40,000×.

Statistical Analysis

Data are given as means ± standard deviations for four or five animals (as indicated), and the results of the Student's *t*-test are presented where appropriate.

RESULTS

Hepatic Vitamin A Levels

The vitamin A level in the livers of mice receiving a vitamin A-deficient diet as their single food source (11 weeks old) was less than 1.6% of the control value (Table 1).

Effects of PFOA on Liver-Somatic Index and Mitochondrial Protein Contents in Vitamin A-Adequate and -Deficient Mice

Treatment with PFOA resulted in a more than 2-fold increase in liver-somatic index (liver weight/body weight) in both vitamin A-adequate and -deficient mice (Table 2) as a result of a decrease in body weight (approx. 2–3 g) and an increase in liver weight (almost doubled). The protein content of the mitochondrial fraction was increased approximately 5-fold in all mice by PFOA (Table 2).

Effects of PFOA on Hepatic Peroxisomal Lauroyl-CoA Oxidase and Catalase activities in Vitamin A-Adequate and -Deficient Mice

Treatment of vitamin A-deficient mice with PFOA (0.02%, 10 days) had no effect on the activity of lauroyl-CoA oxidase, whereas this same activity was increased 26-fold in vitamin A-adequate mice. The activity of cytosolic catalase was increased 12-fold in vitamin A-adequate mice. Vitamin A deficiency itself caused a 3- to 4-fold increase in cytosolic catalase activity, and treatment with PFOA further increased this level 4-fold, so that the maximal values were approximately the same in both vitamin A-adequate and -deficient mice after treatment with PFOA (Table 2). The experiment documented in Table 2 involved 4 animals in each group, and is the same experiment documented in other tables and figures.

This was subsequently repeated twice, again with 4 mice in each group, and the results obtained were essentially the same, with two exceptions:

1. The induction of peroxisomal lauroyl-CoA oxidase in vitamin A-deficient animals in these other experiments was 4.7- and 8.8-fold; and
2. the increase in hepatic cytosolic catalase activity caused by vitamin A deficiency itself varied from 2- to 8-fold.

The significance of these findings is considered below in the Discussion.

Other experiments employing female instead of male mice demonstrated that there were no sex differences in any of the responses examined here (not shown).

TABLE 2. Effects of PFOA (0.02%, 10 days) on various hepatic parameters in vitamin A-adequate and -deficient mice

Parameter	Vitamin A-adequate			Vitamin A-deficient		
	Control	PFOA (% of control)		Control	PFOA (% of control)	
Liver somatic index (g liver/g body weight)	0.058 ± 0.003	0.129 ± 0.034	(222)*	0.053 ± 0.007	0.124 ± 0.005	(233)†
Mitochondrial protein (mg/g liver)	8.0 ± 0.41	40.5 ± 2.2	(506)†	9.4 ± 1.8	45.8 ± 4.5	(487)†
Peroxisomal enzymes						
Lauroyl-CoA oxidase (μmol/g liver · min)	0.430 ± 0.120	11.2 ± 0.84	(2610)†	0.232 ± 0.119	0.270 ± 0.076	(116)
Catalase (mmol/g liver · min)	15.3 ± 1.6	34.4 ± 2.2	(226)†	25.7 ± 4.3§	45.5 ± 2.4	(177)†
Cytosolic enzymes						
Catalase (mmol/g liver · min)	1.68 ± 0.5	20.1 ± 2.6	(1200)†	5.89 ± 1.0‖	22.6 ± 7.2	(384)*
Palmitoyl CoA hydrolase (nmol/g liver · min)	109 ± 16	1190 ± 27	(1090)†	131 ± 9.0	1400 ± 92	(1070)†
Microsomal enzyme						
Lauric acid ω and ω–1 hydroxylation (nmol/g liver · min)	26.8 ± 9.4	371 ± 81	(1380)†	46.1 ± 5.9‡	320 ± 93	(694)*

All values are means ± standard deviations for 4 animals.

* $P < 0.01$ compared to the corresponding control value.

† $P < 0.001$ compared to the corresponding control value.

‡ $P < 0.05$ compared to the control value for vitamin A-adequate mice.

§ $P < 0.01$ compared to the control value for vitamin A-adequate mice.

‖ $P < 0.001$ compared to the control value for vitamin A-adequate mice.

Effect of PFOA on the Level of a 70 kDa Protein and on the Multifunctional Protein in the Hepatic 'Mitochondrial' Fraction from Vitamin A-Adequate and -Deficient Mice

Samples from the hepatic 'mitochondrial' fraction from mice were applied to a 7.5% SDS/polyacrylamide gel. The electrophoretic pattern obtained showed that a protein with a molecular weight of approximately 70 kDa (presumably the multifunctional protein) was dramatically increased in both vitamin A-adequate and -deficient mice after treatment with PFOA (Fig. 1).

Immunoblot analysis with antibodies against the multifunctional enzyme demonstrated that the maximal effect caused by PFOA on this protein was approximately the same in vitamin A-adequate and vitamin A-deficient mice, although vitamin A deficiency itself induced the control level of this enzyme somewhat (Fig. 2). The multifunctional enzyme levels, as quantified by light scanning (expressed in arbitrary units), were 0.9 ± 0.3 for vitamin A-adequate mice (lanes 1 and 2); 8.2 ± 0.1 for vitamin A-adequate mice + PFOA (lanes 3 and 4); 3.6 ± 0.1 for vitamin A-deficient mice (lanes 5 and 6); and 8.0 ± 0.7 for vitamin A-deficient mice + PFOA (lanes 7 and 8).

Effect of PFOA on Hepatic Microsomal Cytochrome P-450iva Activity (Lauric Acid ω- and ω-1 Hydroxylase) and on Cytosolic Palmitoyl-CoA Hydrolase Activity in Vitamin A-Adequate and -Deficient Mice

Vitamin A deficiency itself caused an increase in cytochrome P-450 IVA activity (lauric acid ω- and ω-1 hydroxylation) (Table 2). The induction of this enzyme by PFOA was less prominent in vitamin A-deficient than in vitamin A-adequate mice, resulting in approximately the same maximal values in both groups.

Cytosolic palmitoyl-CoA hydrolase was increased approximately 10-fold in both vitamin A-adequate and -deficient mice after treatment with PFOA (Table 2).

Effects of PFOA on the Number and Size of Hepatic Peroxisomes in Vitamin A-Adequate and -Deficient Mice

A morphometric study revealed that PFOA causes the same extent of hepatic peroxisome proliferation in vitamin A-adequate as in vitamin A-deficient mice, as determined by the increases in the number and size of hepatic peroxisomes (Table 3). Figure 3A–D shows four electron micrographs from this experiment. As illustrated, treatment with PFOA caused marked increases in the number and size of peroxisomes in both vitamin A-adequate and -deficient mice.

DISCUSSION

Three aspects of the findings here are of major importance:

1. Up-regulation of hepatic peroxisomal acyl-CoA oxidase in mice by the potent peroxisome proliferator perfluorooctanoic acid is highly dependent on the presence of an adequate level of vitamin A in the liver. This observation corroborates earlier *in vitro* studies—employing both transfected cell lines [6, 7] and another cell line that is itself responsive to peroxisome proliferators [23]—indicating that a heterodimer involving PPAR (presumably with its bound ligand) and RXR with

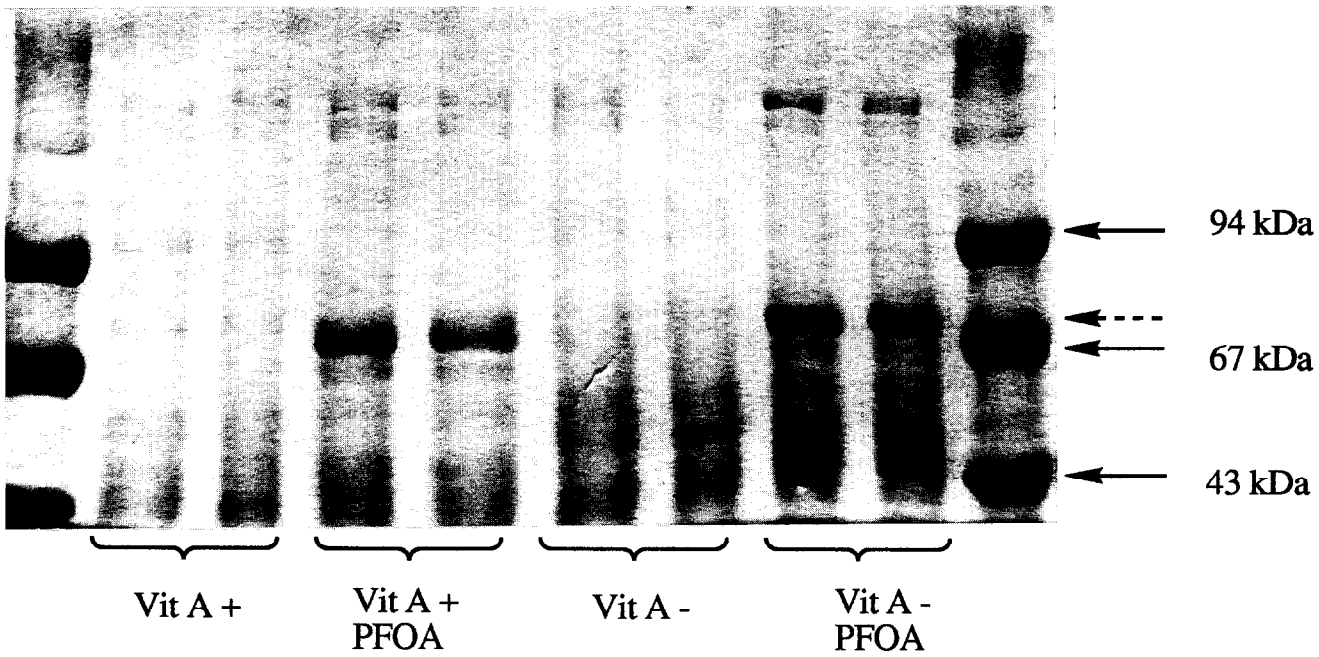

Fig. 1. SDS/polyacrylamide gel showing the effects of PFOA on a protein with a molecular weight of approximately 70 kDa (— — — →) in the 'mitochondrial' fraction from livers of vitamin A-adequate and -deficient mice. The acrylamide concentration in the gel was 7.5% and each well was loaded with 10 μg protein. The protein bands were detected by staining with Coomassie brilliant blue.

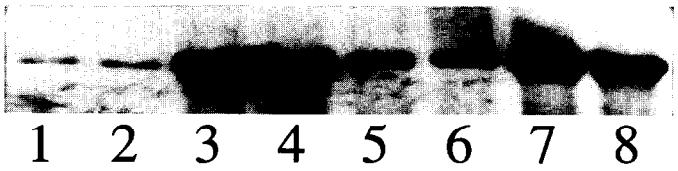

Fig. 2. Effect of PFOA (0.02%, 10 days) on the multifunctional protein in vitamin A-adequate and -deficient mice. Autoradiogram of immunoblot with antibodies against the multifunctional protein. Each well was loaded with 10 μg protein from the hepatic 'mitochondrial' fraction. Lanes 1 and 2 = vitamin A-adequate mice. Lanes 3 and 4 = vitamin A-adequate mice + PFOA. Lanes 5 and 6 = vitamin A-deficient mice. Lanes 7 and 8 = vitamin A-deficient mice + PFOA.

bound 9-*cis*-retinoic acid participates in this up-reguation, probably as a transcription factor.

A recent study on rats by Lawrence and coworkers [24] led them to a conclusion in disagreement with our own, i.e. that up-regulation of peroxisomal fatty acid β-oxidation (measured as the oxidation of palmitoyl-CoA) by the powerful peroxisome proliferator nafenopin occurred to an equal extent in vitamin A-adequate and -deficient animals. In presenting this conclusion, Lawrence and coworkers were careful to point out that their vitamin A-deficient animals still had approximately 6–11% of the control level of this vitamin in their livers, and that more pronounced vitamin A deficiency might have affected the up-regulation of peroxisomal fatty acid β-oxidation in response to treatment with nafenopin. In our opinion, this note of caution explains the apparent discrepancy between their findings and ours: Up-regulation of lauroyl-CoA oxidase (considered the rate-limiting enzyme in peroxisomal fatty acid β-oxidation) was eliminated in our investigation because the hepatic levels of vitamin A in our vitamin A-deficient mice were much lower.

Of course, other differences between our studies (e.g. the species investigated—we used mice and they rats, the peroxisome proliferator employed—perfluorooctanoic acid versus nafenopin, and the parameters investigated—peroxisomal lauroyl-CoA oxidase versus the total peroxisomal β-oxidation of palmitoyl-CoA) may also have contributed to the differences in our respective findings. However, it seems highly likely that the different degree of vitamin A-deficiency obtained is the major relevant factor.

Of interest in this connection is our observation that up-regulation of peroxisomal lauroyl-CoA oxidase in vitamin A-deficient mice fed perfluorooctanoic acid varied between 0 and 30% of the corresponding change in vitamin A-adequate animals (see Results). All of these deficient animals had a very low hepatic level of vitamin A, but this parameter may have varied between 0–2% of the control value in the different experiments performed here. Thus, 1% or so of the normal hepatic level of vitamin A may be sufficient to allow effective formation of the PPAR-(RXR-9-*cis*-retinoic acid) dimer.

2. Not all of the hepatic responses to perfluorooctanoic acid in mice are affected by vitamin A deficiency. The normal increases in the size and number of hepatic peroxisomes in mice fed perfluorooctanoic acid is in striking contrast to the lack (or decreased extent) of up-regulation of peroxisomal lauroyl-CoA oxidase in these same animals. Furthermore, the response of vitamin A-deficient mice to dietary perfluorooctanoic acid in terms of increase in liver-somatic index (g liver/g body weight), the decrease in mitochondrial size (as reflected in an increase in the amount of protein in the mitochondrial subfraction [25]), the increase in a 70 kDa protein and the multifunctional protein involved in peroxisomal fatty acid β-oxidation (which are presumably identical [26]), and the increase in cytosolic palmitoyl-CoA hydrolase activity are all normal. In the case of cytosolic and peroxisomal catalase and of cytochrome P-450 IVA, changes caused by vitamin A deficiency itself make it impossible to draw any firm conclusions concerning the response of these parameters to perfluorooctanoic acid treatment of vitamin A-deficient mice. However, the fact that the values obtained in treated vitamin A-adequate and -deficient mice are the same in all three of these cases might suggest that the responses of these parameters to perfluorooctanoic acid are not affected by vitamin A deficiency.

Lawrence and coworkers [24] also reported that in their vitamin A-deficient rats, the increase in relative liver weight, mitochondrial fatty acid β-oxidation, and lauric acid ω- and ω-1 hydroxylase activity, peroxisome proliferation (as judged by qualitative examination of electron micrographs), and decreases in plasma and hepatic lipid levels in response to nafenopin treatment were all the same as in control animals.

The fact that up-regulation of peroxisomal acyl-CoA oxidase in mouse liver in response to dietary perfluorooctanoic

TABLE 3. Changes in the number and size of hepatic peroxisomes in vitamin A-adequate and -deficient mice exposed to PFOA

	Vitamin A-adequate		Vitamin A-deficient	
	Control	PFOA	Control	PFOA
V	2.3 ± 0.5	21.3 ± 2.3	3.2 ± 0.3	28.0 ± 4.7
N	8.6 ± 3.6	33.2 ± 16	12.4 ± 5.1	36.4 ± 13.8
V/N	0.269 ± 0.05	0.680 ± 0.02	0.269 ± 0.04	0.771 ± 0.06

V = total volume density ("area") per unit area
N = number per unit area
V/N = mean volume density ("size") per peroxisome.
Each experimental group contained 5 animals, except for the vitamin A-deficient group treated with PFOA, which contained 4 animals. 45 micrographs were examined from each animal.

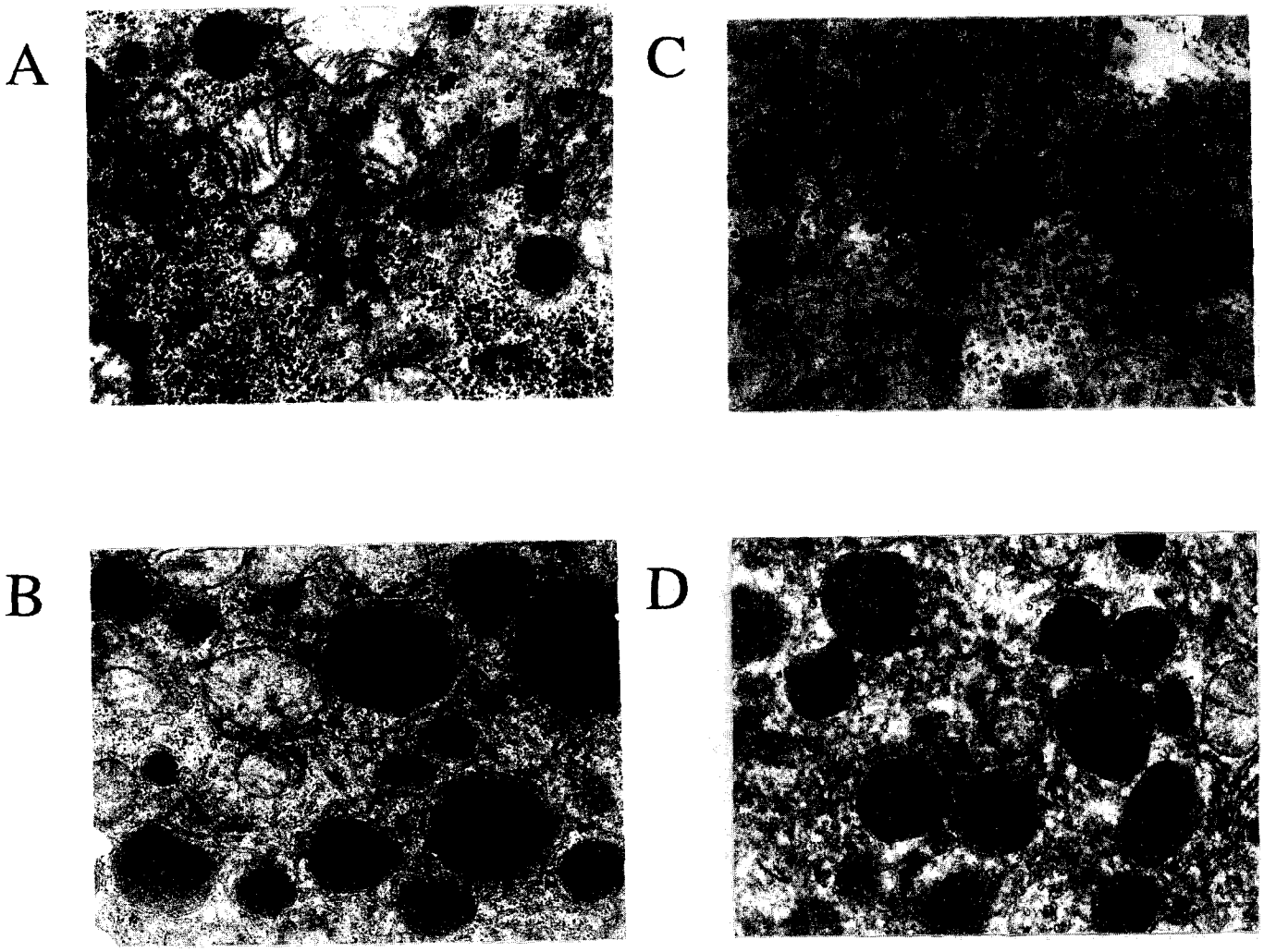

FIG. 3. Electron micrographs of livers from (A) a vitamin A-adequate mouse, (B) a vitamin A-adequate mouse treated with PFOA, (C) a vitamin A-deficient mouse and (D) a vitamin A-deficient mouse treated with PFOA.

acid is dependent on the hepatic vitamin A status, whereas several other responses are not, suggests that the different responses to this potent peroxisome proliferator are mediated by at least two different mechanisms. These mechanisms may be small variations on a central theme, e.g. transfection experiments have indicated that whereas enhanced transcription of the acyl-CoA oxidase gene mediated by the PPAR-RXR heterodimer requires the presence of the ligand for RXR [6, 7], this same heterodimer can function as a transcription factor for the gene for the multifunctional protein in the absence of the ligand for RXR [27]. Similarly, different forms of PPAR and RXR may form a series of heterodimers with differing functional requirements and/or affinities for 9-*cis*-retinoic acid and/or other metabolites of vitamin A. On the other hand, some of the mechanisms involved in many responses of rodent liver to peroxisome proliferators may be quite different from those commonly postulated at present. It is tempting to speculate that this may well be the case for the more complex responses (e.g. increased hepatocyte proliferation, decreased mitochondrial size, and increased biogenesis of peroxisomal membranes).

3. Vitamin A deficiency itself significantly increases hepatic levels of peroxisomal and cytosolic catalase and of cytochrome P-450 IVA in mice. The significance of this observation is presently unclear, but it may be speculated that vitamin A and/or its metabolites are somehow involved in the down-regulation of these proteins.

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