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NAFENOPIN-INDUCED PEROXISOME PROLIFERATION IN VITAMIN A DEFICIENT RATS

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Abstract—Induction of peroxisome proliferator responsive genes is thought to be mediated through binding of a peroxisome proliferator-activated receptor (PPAR) to specific peroxisome proliferator response elements in the upstream region of these genes. Binding of PPAR to the acyl-CoA oxidase promoter requires heterodimerization with the retinoid X receptor (RXR), and subsequent transactivation is strongest when ligands for both PPAR and RXR are present. Therefore, we hypothesized that depletion of ligand for the retinoid receptor would limit the induction of peroxisome proliferation in rats. Hepatic retinol content was reduced by more than 90% by feeding weanling rats a vitamin A deficient (VAD) diet for approximately 3 months. Nafenopin treatment for 7 days induced peroxisomal β-oxidation 18-fold in VAD rats compared with 16-fold in rats fed a vitamin A sufficient (VAS) diet. Nafenopin induced microsomal laurate hydroxylase and mitochondrial β-oxidation to comparable rates of specific activity in both VAD and VAS rats. However, the activities in VAD controls were significantly lower than in VAS controls, so the magnitude of the nafenopin-induced increases was greater in the VAD rats. Relative liver weights were increased nearly 2-fold in both VAS and VAD rats treated with nafenopin. Ultrastructural examination of the livers demonstrated that nafenopin increased the number and size of peroxisomes in both VAD and VAS rats. These data demonstrate that rats with severely depleted vitamin A stores remained responsive to the peroxisome proliferator nafenopin. Whether critical retinoid pools that supply RXR ligand (9-*cis*-retinoic acid) are spared in the vitamin A deficient rats remains to be determined.

Key words: vitamin A deficiency; peroxisome proliferation; nafenopin; rat

Peroxisome proliferation is a phenomenon that is produced by a wide variety of chemicals including hypolipidemic drugs, fatty acid analogs, pesticides, and other agents [1–3]. The phenomenon is characterized by an increase in size and number of liver peroxisomes, hepatomegaly, and the induction of numerous hepatic enzymes in rodents. The induction of peroxisome proliferation is now thought to be mediated through the activation of the PPAR†, a member of the steroid receptor superfamily [4]. Recently, this receptor was found to heterodimerize with the retinoid receptor, RXR [5, 6]. Heterodimerization was found to be required for binding to the peroxisome proliferator response element [5–7]. Furthermore, stimulation of the acyl-CoA oxidase promoter in transactivation assays was strongest in the presence of ligands for both PPAR and RXR, suggesting a role for retinoids in the induction of peroxisome proliferation [5, 6].

Vitamin A is an essential nutrient and must be supplied in the diet [8]. The liver serves as a storage depot for retinol and is involved in the maintenance of a constant retinol concentration in the circulation. At weaning, vitamin A reserves increase rapidly upon consumption of the diet [9]. Therefore, a vitamin A deficient state can be induced in weanling rats by feeding a diet lacking vitamin A. The liver stores become expended to maintain constant plasma levels and only when liver stores are severely depleted do circulating retinol levels decline [10].

Because of the reported role of retinoids in the induction of PPAR responsive genes [5–7], we hypothesized that vitamin A deficiency would attenuate the peroxisome proliferation response in rats. To test this hypothesis, weanling rats were made vitamin A deficient by feeding a VAD diet for approximately 3 months. The rats were given nafenopin for the final week of the experiment. We report here that the peroxisome proliferation response in rats was not attenuated by vitamin A deficiency.

MATERIALS AND METHODS

Materials. L-[1-^{14}C]Palmitoyl carnitine was obtained from New England Nuclear (Boston, MA). Sodium 1-[^{14}C]laurate was obtained from ICN (Irvine, CA). Nafenopin was a gift from Ciba-Geigy (Summit, NJ). All other chemicals were of the highest quality commercially available.

Animals. Male weanling Fisher 344 rats were

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† Abbreviations: PPAR, peroxisome proliferator-activated receptor: RXR, retinoid X receptor; VAD, vitamin A deficient; VAS, vitamin A sufficient, HPTLC, high-performance thin-layer chromatography; and DAB, 3,3′-diaminobenzidine tetrachloride.

Table 1. Plasma and hepatic retinol concentrations in vitamin A sufficient (VAS) and deficient (VAD) rats

Feed	(n)	Treatment	Plasma retinol (μg/dL)	Hepatic retinol (μg/g tissue)
VAS	(8)	Vehicle	82.5 ± 12.5	410.3 ± 3.9
VAS	(8)	Nafenopin, 80 mg/kg	55.0 ± 6.6*	417.1 ± 26.3
VAD	(4)	Vehicle	12.2 ± 1.3*	27.1 ± 4.4*
VAD	(7)	Nafenopin, 80 mg/kg	17.6 ± 6.7*	46.8 ± 21.9*

Rats were fed a vitamin A sufficient or vitamin A deficient diet for approximately 3 months. The rats were then given acacia or 80 mg/kg nafenopin by gavage for the last 7 days. Values are the means ± SEM of 4–8 rats.

* Significantly different from VAS controls, $P < 0.05$.

obtained from Harlan–Sprague Dawley (Indianapolis, IN) at the age of 20 days. All animals were housed individually in stainless steel cages with a 12-hr light/dark cycle and allowed free access to water. The weanling rats were fed a vitamin A deficient diet (Purina diet No. 5822M) immediately upon arrival to prevent any further accumulation of retinol in the liver. Body weights and food consumption were monitored to aid in determining the onset of vitamin A deficiency [10]. When body weight gain ceased (approximately 60 days), the rats were anesthetized and plasma samples were taken from the orbital sinus to confirm the retinol-deficient status. VAS control rats were pair-fed a complementary diet containing vitamin A (Purina No. 5755M). After 14 weeks, VAD and VAS rats were given nafenopin by gavage in acacia at a dose of 80 mg/kg for 7 days. At necropsy, animals were anesthetized with isoflurane, and blood samples were taken from the vena cava. The livers were then excised and frozen in liquid nitrogen.

Analytical. Total retinol concentrations in saponified plasma and liver homogenates were measured by HPLC analysis as described by Ross [11] using retinol acetate as an internal standard. Plasma cholesterol and triglycerides were assessed on a Monarch automated system (Instrumentation Laboratory Co., Lexington, MA). Hepatic neutral lipids were extracted by the method of Folch *et al.* [12] and separated by HPTLC as described by Schmitz *et al.* [13]. Lipids were quantified by fluorescence densitometry [14]. Hepatic laurate hydroxylase activity was determined by the method of Giera and van Lier [15], using sodium 1-[^{14}C]-laurate as substrate. Mitochondrial β-oxidation was determined under conditions described by Turnbull *et al.* [16], using L-[^{14}C]palmitoyl carnitine as substrate and measuring acid-soluble metabolites [17]. Peroxisomal β-oxidation was determined as described by Lazarow [18], using 50 μM palmitoyl CoA as substrate.

Electron microscopy. Liver samples were fixed in aldehyde, rinsed in cacodylate (pH 7.4), and stained for peroxisomes with DAB solution, pH 9.7, in the dark. Samples were rinsed in cacodylate, fixed in potassium ferricyanide/osmium tetroxide, and dehydrated in ethanol. Sections were embedded in epoxy resin, cut, and mounted on copper grids and examined by transmission electron microscopy.

Statistical analysis. Statistical analysis was performed with a two-way analysis of variance method (diet and drug). P values less than 0.05 were considered significant.

RESULTS

Induction of vitamin A deficiency. Fisher 344 weanling rats were made vitamin A deficient by feeding a diet lacking vitamin A. After 60 days, the rats displayed the severely reduced weight gain that is characteristic of vitamin A depleted rats [10]. After 70 days on the VAD diet, body weight declined. Body weight gain in the pair-fed VAS rats ceased with no significant loss of body weight. Thus, despite pair-feeding, the body weights of VAD rats were less than those of VAS rats (65% of VAS control). The vitamin A status was confirmed by measuring retinol in plasma and liver. The plasma retinol concentration in rats fed the VAD diet was reduced to 15% of the level observed in control rats pair-fed the equivalent diet fortified with vitamin A (Table 1). Similarly, hepatic retinol stores were severely depleted after 3 months on the VAD diet (7% of pair-fed VAS controls).

Nafenopin-induced peroxisome proliferation. To determine if vitamin A depletion would attenuate the induction of peroxisome proliferation, VAD and VAS rats were given 80 mg/kg/day of nafenopin by gavage for 7 days. VAS rats had a nearly 2-fold increase in relative liver weight after 7 days of drug treatment compared with VAS control rats (Fig. 1A). VAD rats responded to nafenopin with an equivalent increase in relative liver weight. Nafenopin induced peroxisomal β-oxidation 16-fold in rats fed the VAS diet and 18-fold in rats fed the VAD diet (Fig. 1B). These findings were reproduced in a separate experiment with ciprofibrate (data not shown). Liver weight was induced approximately 2-fold after treatment with ciprofibrate (25 mg/kg) for 7 days in both VAS and VAD rats. Similar to the nafenopin experiment, the induction of peroxisomal β-oxidation by ciprofibrate tended to be greater in VAD rats compared with VAS rats.

Mitochondrial β-oxidation was increased by nafenopin in both VAS and VAD rats (Fig. 1C). The absolute rates of mitochondrial β-oxidation after nafenopin treatment were comparable in VAS and VAD rats. However, the activity in VAD controls

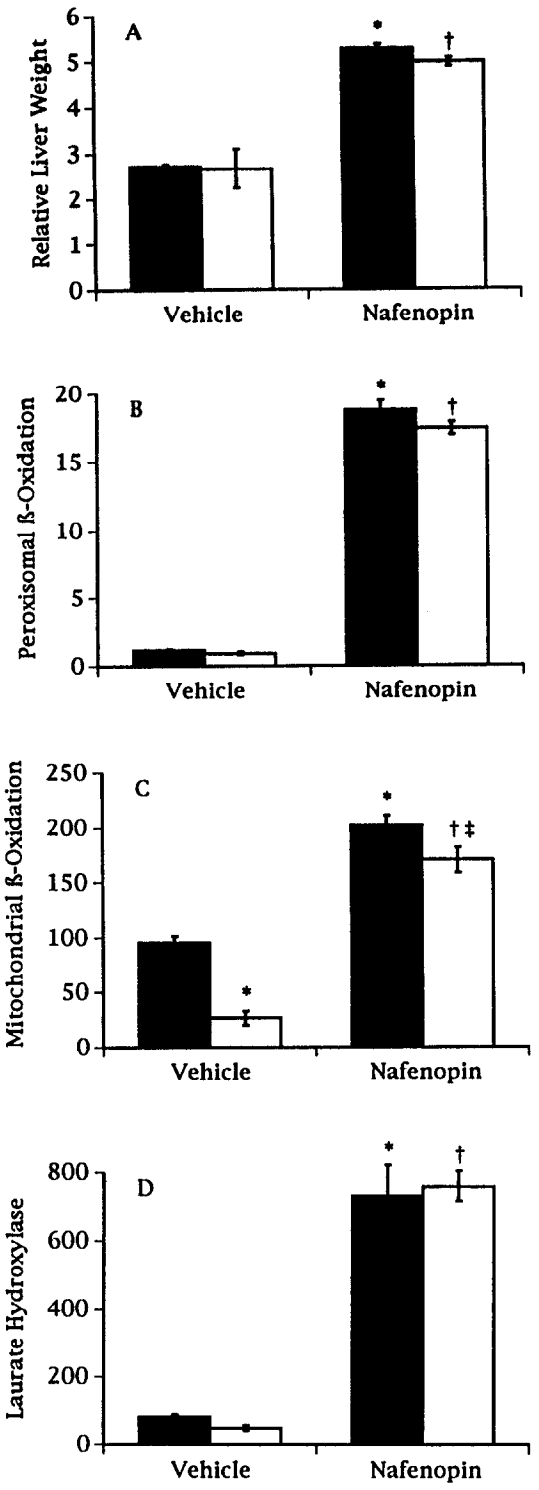

Fig. 1. Effect of nafenopin on liver weight and enzyme activity in vitamin A sufficient and vitamin A deficient rats. Vitamin A sufficient (■) or vitamin A deficient (□) rats were given nafenopin (80 mg/kg) by gavage for 7 days. Liver weight is expressed as g liver/100 g body weight (A), peroxisomal β-oxidation as μmol/g/min (B), mitochondrial β-oxidation as nmol/g/min (C), and laurate hydroxylase activity as nmol/mg/min (D). The bars represent the means ± SEM of 4–8 rats. Key: significantly different ($P < 0.05$) vs (*) VAS control, (†) VAD controls, or (‡) nafenopin-treated VAS rats.

was considerably less than in VAS controls. Thus, the magnitude of the nafenopin-induced increase was 6-fold in VAD rats and 2-fold in VAS rats. Similarly, laurate hydroxylase activity was lower in VAD controls compared with VAS controls. Nafenopin treatment induced laurate hydroxylase activity 9-fold in VAS rats and 16-fold in VAD rats, but absolute activities were essentially the same in the two groups (Fig. 1D).

Ultrastructural examination of DAB-stained livers showed that both VAD and VAS rats given nafenopin had increases in the number and size of peroxisomes as compared with the respective controls (Fig. 2). There were no other morphological abnormalities associated with administration of nafenopin. As compared with VAS rats, hepatocytes of VAD rats often had enlarged mitochondria that were round in shape and had normal cristae and matrix.

Nafenopin effects on plasma and hepatic lipids. Nafenopin decreased plasma triglycerides by 20% and plasma cholesterol by 30% in VAS rats (Table 2). The reduction in plasma triglycerides was less than expected, which may have been due to the food deprivation caused by pair-feeding. VAD controls had significantly lower plasma triglycerides and cholesterol than VAS controls. Nafenopin did not affect plasma triglyceride concentrations in VAD rats but increased plasma cholesterol.

The concentration of hepatic triglycerides was less in VAD controls than in VAS controls (Table 2). Nafenopin tended to decrease hepatic triglycerides regardless of vitamin A status, although the decrease was statistically significant only for the VAS rats. Nafenopin tended to decrease hepatic cholesterol slightly in VAS and VAD rats but the change was significant only in the VAD rats.

DISCUSSION

We have tested the hypothesis that vitamin A deficiency would limit the peroxisome proliferation response to nafenopin in rats. The basis for this hypothesis is the recently reported role of retinoids in the activation of PPAR responsive genes [5–7]. The markers of peroxisome proliferation that were examined, including peroxisomal β-oxidation, mitochrondrial β-oxidation, laurate hydroxylase and liver enlargement, were increased to at least an equivalent level by nafenopin in vitamin A deficient rats and vitamin A sufficient rats. Likewise, the morphological evaluation demonstrated increases in the size and number of peroxisomes in VAS and VAD rats.

The vitamin A deficiency affected some of the parameters independent of nafenopin treatment. Plasma triglycerides and cholesterol were decreased markedly in VAD controls versus VAS controls. Nafenopin treatment produced divergent effects on plasma lipids. Plasma triglyceride levels remained unchanged in VAD rats after nafenopin treatment, possibly because the levels were already maximally depressed. Plasma cholesterol was decreased by nafenopin in VAS rats. In contrast, nafenopin treatment increased the plasma cholesterol in VAD

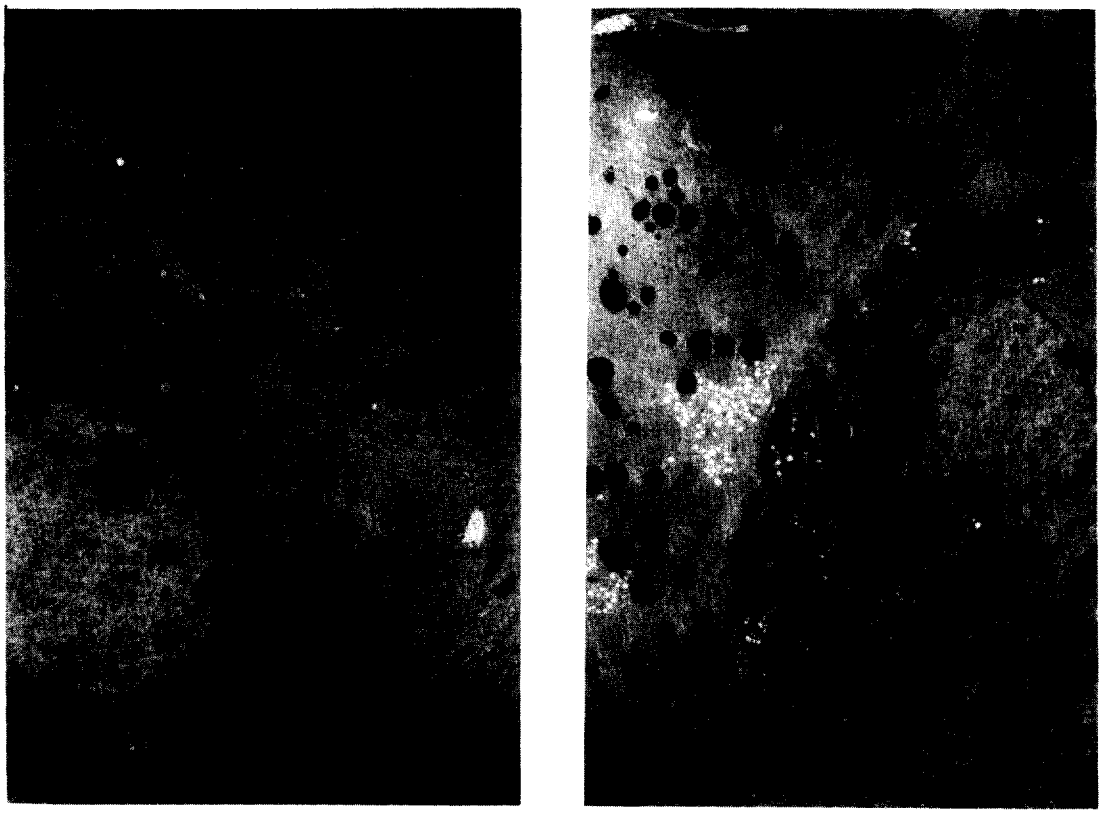

Fig. 2. Electron micrographs of livers from vitamin A deficient rats treated with nafenopin. Diaminobenzidine-stained peroxisomes from (A) a VAD control rat or (B) a VAD rat given nafenopin. Magnification: 3130.

Table 2. Effects of nafenopin on plasma and hepatic lipids in vitamin A sufficient (VAS) and deficient (VAD) rats

		Plasma lipids		Hepatic lipids	
Feed	Treatment	Cholesterol (mg/dL)	Triglycerides (mg/dL)	Cholesterol (mg/g)	Triglycerides (mg/g)
VAS	Vehicle	117 ± 3	131 ± 10	2.01 ± 0.06	15.33 ± 3.43
VAS	Nafenopin, 80 mg/kg	82 ± 3*	104 ± 8*	1.81 ± 0.07	10.64 ± 0.72*
VAD	Vehicle	43 ± 12*	49 ± 12*	2.45 ± 0.15*	8.74 ± 4.91*
VAD	Nafenopin, 80 mg/kg	81 ± 4†	45 ± 5‡	1.86 ± 0.04†	4.59 ± 1.76

Vitamin A sufficient and vitamin A deficient rats were given acacia or 80 mg/kg nafenopin by gavage for 7 days. Values are the means ± SEM of 4–8 rats.

Significantly different ($P < 0.05$) from: *VAS controls, †VAD controls, or ‡nafenopin-treated VAS rats.

rats. The underlying mechanisms for the divergent effects on plasma cholesterol are unclear.

The vitamin A deficiency also affected hepatic enzyme activities. Mitochondrial β-oxidation was decreased by 70% compared with VAS controls. Likewise, microsomal laurate hydroxylase activity was 35% lower in VAD control rats than in VAS controls. It is possible that the transcriptional control of genes involved in these processes is partially regulated by retinoid response elements. However, the changes in these parameters in VAD controls may also have been related to the debilitated state of the animals. The VAD animals stopped growing after approximately 60 days and their body weights were 35% less than VAS controls, despite the pair-feeding regimen. Even though the rats were in poor physical condition, mitochondrial β-oxidation as well as the other enzymatic activities were induced

markedly by nafenopin in VAD rats. The findings demonstrate the enormous expansive capacity of the liver in response to peroxisome proliferators even in health-compromised rats. This finding is consistent with the report that the livers of VAD rats retained their regenerative capacity after partial hepatectomy [19].

The observation that the response to nafenopin was independent of the retinol status in this study suggests that a retinoid ligand may not be required for the maximum induction of peroxisome proliferation *in vivo*. This is not consistent with *in vitro* data from co-transfection assays, which indicate that maximal induction of acyl-CoA oxidase promotor activity requires both the peroxisome proliferator and retinoid ligands [5, 6]. In the retinol-depleted state, it is possible that induction of peroxisome proliferator responsive genes could be mediated through other heterodimer partners for PPAR [20]. It is also possible that the critical pool of retinol used for synthesis of the RXR ligand, 9-*cis*-retinoic acid, may be resistant to depletion. This does not seem to be the case for the retinol pools required for weight gain, because this index of vitamin A status clearly indicated a vitamin A deficiency. It is noteworthy that even though the livers in the nafenopin-treated VAD rats grew to twice the size of the VAD controls, the hepatic retinol stores remained about the same on a per gram tissue basis. We would have expected the retinol concentration in the enlarged livers to be half of that found in the control livers since the diet was deficient of retinol. The source of the retinol that filled these stores is unknown.

These data demonstrated that rats with severely depleted vitamin A stores remained responsive to the hepatic effects of the peroxisome proliferator nafenopin. Further studies will be required to determine whether there is depletion of the pools of vitamin A used for 9-*cis*-retinoic acid synthesis and subsequently for RXR/PPAR-mediated activation of gene transcription.

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