Retinol and retinyl esters in parenchymal and nonparenchymal rat liver cell fractions after long-term administration of ethanol

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Abstract Chronic ethanol consumption reduces the liver retinoid store in man and rat. We have studied the effect of ethanol on some aspects of retinoid metabolism in parenchymal and nonparenchymal liver cells. Rats fed 36% of total energy intake as ethanol for 4-6 weeks had the liver retinoid concentration reduced to about one-third, as compared to pair-fed controls. The reduction in liver retinoid affected both the parenchymal and the nonparenchymal cell fractions. Plasma retinol level was normal. Liver uptake of injected chylomicron [3H]retinyl ester was similar in the experimental and control group. The transport of retinoid from the parenchymal to the nonparenchymal cells was not found to be significantly retarded in the ethanol-fed rats. Despite the reduction in total retinoid level in liver, the concentrations of unesterified retinol and retinyl oleate were increased in the ethanol-fed rats. Hepatic retinol esterification was not significantly affected in the ethanol-fed rats. Since our study has demonstrated that liver uptake of chylomicron retinyl ester is not impaired in the ethanol-fed rat, we suggest that liver retinoid metabolism may be increased.

Supplementary key words retinol • retinyl esters • vitamin A • liver stellate cell • acyl CoA:retinol acyltransferase (ARAT) • fatty liver

Chronic ethanol consumption reduces the liver retinoid level in rat, baboon, and man (1–3). Plasma retinol concentrations are, however, usually not decreased. Symptoms of vitamin A deficiency such as night blindness and hypogonadism may be seen in alcoholics when liver disease is present (4, 5). The reason why chronic ethanol consumption decreases the vitamin A concentration of the liver is unknown. It is not explained by malnutrition or inhibited absorption of retinol or β-carotene (1, 6), and fatty liver or hepatitis and cirrhosis is not necessarily present (3, 6).

Retinyl ester, reaching the liver on chylomicron remnants, is initially taken up by the parenchymal cells, and in the normal rat the vitamin is then to a large extent transferred to the perisinusoidal, stellate (fat-storing) cells of the liver (7, 8). These cells store a large fraction of the liver vitamin A (9–11). In vitamin A-deficient rats, the absorbed vitamin recently taken up by the parenchymal cells is, to a large extent, secreted into blood again (7). Leo and Lieber (12) reported that, in rats given moderately high supplements of vitamin A, a positive correlation was found between the number of stellate cells of the liver and the hepatic vitamin A, both in normal rats and after long-term administration of ethanol.

Since vitamin A absorption is normal in rats fed ethanol, the reduced level of vitamin A in liver may be due to a) reduced liver uptake of vitamin A via chylomicron remnants; b) reduced storage capacity of the parenchymal liver cells; c) reduced transfer of the vitamin from parenchymal to stellate cells; d) reduced storage capacity of the stellate cells; e) increased mobilization of retinol from liver; or f) increased retinol metabolism/increased secretion of retinol metabolites. The aim of the current study was to examine some of these possibilities by studying the fate of retinol newly taken up by the liver and the storage of retinol in rats with a chronic consumption of ethanol.

Abbreviations: HPLC, high-performance liquid chromatography; ARAT, acyl CoA:retinol acyltransferase.

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MATERIALS AND METHODS

Chemicals

[15-3H(n)] all-trans retinol with specific activity 13.6 Ci/mm mol was from New England Nuclear, Boston, MA. [11,12-(n)-3H] all-trans retinol with a specific activity 60 Ci/mm mol was from Amersham International, Buckinghamshire, England. Retinol (all-trans), retinyl acetate, retinyl palmitate, palmitoyl-CoA, oleoylchloride, bovine serum albumin (fatty acid-free), dithiothreitol, dimethylsulfoxide, butylated hydroxytoluene, and pyrogallol were from Sigma Chemical Co., St. Louis, MO. N,N'-Di-phenyl-p-phenylenediamine was from Eastman Kodak Co., Rochester, NY. All other chemicals were of standard commercial high purity. Retinyl oleate was prepared according to Huang and Goodman (13). Retinyl stearate was a gift from Hoffmann-La Roche, Inc., Basle, Switzerland.

Animals

Male rats of the Wistar strain were used. They were fed ordinary standard rat diet from Møelessentralen, Oslo, Norway, until their weight was 140-180 g (experimental rats 165 ± 10 g (mean ± SD, n = 19), controls 166 ± 12 g (mean ± SD, n = 16)). The rats were then given Bio-Mix 711 Iso-Cal liquid rat diets 0684 (Lieber/DeCarli, old formula) from Bio-Serv, Inc., Frenchtown, NJ. The experimental rats had 36% of the energy from fat, while the control diet dextrin-maltose replaced ethanol. Both diets had 35% of the energy from fat (olive oil 28.4 g/l, corn oil 8.5 g/l, safflower oil 2.7 g/l). The vitamin A intake, in the range 100-200 nmol/day, is considered adequate for rats (14). In the control diet dextrin-maltose replaced ethanol. Both diets had 35% of the energy from fat (olive oil 28.4 g/l, corn oil 8.5 g/l, safflower oil 2.7 g/l). The vitamin A intake, in the range 100-200 nmol/day, is considered adequate for rats (14). The rats were housed in separate cages (temperature 21-26°C, humidity 50-65%). They had light 12 hr during the day. The experimental rats had free access to their ethanol diet, to which they were gradually adapted over a period of 5 days. The controls were pair-fed. The average daily intake of ethanol by the test animals was 2.8-3.3 g. After a weight plateau or a small decrease in weight during the adaptation period, the rats generally increased steadily in weight. Despite pair-feeding — and in accordance with earlier observations (14) — the average weight gain in the control rats was somewhat higher than in the rats given ethanol. When they were killed after about 5 weeks on the diet, the weight was 235 ± 24 g (mean ± SD, n = 19) for the test animals and 264 ± 29 g (mean ± SD, n = 16) for the controls. The test animals were not obviously clinically affected by the constant ethanol intake, but after they had fasted the last night, many of them were aggressive, possibly because of alcohol abstinence. Under ether anesthesia the livers of the rats were perfused just before they were killed, as described below. Blood was collected for analysis of plasma retinol, and a lobe of the liver was saved before the collagenase treatment. One part of this liver sample was used for morphological studies, and one was homogenized in 0.25 M sucrose. An aliquot of the homogenate was centrifuged to isolate liver microsomes (15).

Collection of intestinal lymph. In vivo administration of [3H]retinyl ester

Collection of intestinal lymph was as described (16). The donor rat was given 630 nmol of [11,12-(n)-3H] all-trans retinol (100-200 μCi). About 99% of the radioactive retinol in the lymph was esterified, and about 99% of the radioactivity was in the d < 1.006 g/ml fraction (7). The lymph was injected intravenously to the control and ethanol-fed rats at intervals up to 4 hr before they were killed. The total radioactivity in plasma, whole liver, and parenchymal and nonparenchymal liver cells was determined by radioassay.

Preparation of liver cells

Total liver cell suspensions were prepared by a modified collagenase perfusion technique (17). The parenchymal cells were isolated by differential centrifugation (17). About 98% of the isolated parenchymal cells were viable as determined by the trypan blue exclusion test. The parenchymal cell suspension was contaminated with 1.1 ± 1.9% (mean ± SD, n = 7) endothelial cells and 4.6 ± 3.1% (mean ± SD, n = 7) Kupffer cells. Endothelial cells were identified by selective uptake of fluorescein-labeled ovalbumin and Kupffer cells by positive peroxidase staining (8). The parenchymal cell fractions from control rats and alcoholic rats were contaminated with 4.8 ± 1.1% (mean ± SD, n = 9) and 2.1 ± 1.2% (mean ± SD, n = 13) stellate cells, respectively. These data were based on quantitation of stellate cells from their vitamin A autofluorescence (8). Nonparenchymal liver cells were prepared from the total liver cell suspension by incubation with pronase, a treatment that destroys the parenchymal cells (18). The fraction of nonparenchymal cells from control rats and alcoholic rats contained 57.1 ± 2.0% (mean ± SD, n = 5) endothelial cells and 28.9 ± 1.5% (mean ± SD, n = 5) Kupffer cells. The fractions of nonparenchymal cells from control rats and alcoholic rats contained 12.2 ± 1.6% (mean ± SD, n = 9) and 8.2 ± 4.2% (mean ± SD, n = 8) autofluorescent stellate cells, respectively. Furthermore, it was assumed for both groups of rats that the total weight of liver was 4.1% of total body weight (7) and that the liver contained 125 × 10^6 parenchymal cells/g and 65 × 10^6 nonparenchymal cells/g (19).

High performance liquid chromatography (HPLC)

The system consisted of a Constametric pump, Model III from Laboratory Data Control, Riviera Beach, FL or a Model 45 pump from Waters Associates, Milford, MA, and a detector, Model 440 from Waters Associates, monitoring at 313 nm. The columns used were a 5-μm Supelcosil LC-8 column, 25 cm × 4.6 mm i.d. from Supelco,
Quantitation of retinol

The procedures were carried out under dim light. Whole liver homogenate and separated parenchymal and nonparenchymal liver cells were treated for 30 min at 57°C with 2 ml of 10% KOH in 90% ethanol in the presence of 1% pyrogallol, and the retinol was extracted into 5 ml of hexane, containing butylated hydroxytoluene, 5 µg/ml. Two ml of water was then added. Four ml of the upper hexane phase was transferred to new glass vials, and the internal standard retinyl acetate was added in known amount. The hexane was then evaporated under a stream of N₂ at about 30°C in a water bath. The residue was dissolved in 100-200 µl of methanol, and an aliquot of 10-45 µl was injected onto the HPLC column. Plasma (100 µl) was thoroughly mixed (20 sec) with 0.4 ml of ethanol containing a known amount of retinyl acetate. One ml of hexane with butylated hydroxytoluene as described above and then 0.2 ml of water were added, the vials being vortexed after each addition. Methanol was added to the plasma samples to match the amount of methanol in the hexane extracts. Four ml of the upper hexane phase was transferred to new glass vials, and the internal standard retinyl acetate was added in known amount. The hexane was then evaporated under a stream of N₂ at about 30°C in a water bath. The residue was dissolved in 100-200 µl of methanol, and an aliquot of 10-45 µl was injected onto the HPLC column. Plasma (100 µl) was thoroughly mixed (20 sec) with 0.4 ml of ethanol containing a known amount of retinyl acetate.

Activity was measured as described (15) in parenchymal and nonparenchymal liver cell homogenates and in whole liver microsomes. The activities were expressed as nmol of ester formed per 10⁶ cells per min for the cell homogenates and per mg protein per min of the liver microsomes. When testing ARAT [15-³H(n)] all-trans retinol was used as the radioactive label.

Morphological studies

Five ethanol-fed animals and five control animals were examined. Parts of the livers were fixed in formaldehyde. Frozen sections (5-7 µm) were made and stained for fat with oil red O. Tissue was also dehydrated and blocked in paraffin, and sections (3-5 µm) were stained with hematoxylin-eosin.

Analysis of distribution between retinol and different retinyl esters

Whole liver and cell homogenates were extracted with ethanol and hexane. The homogenates (0.1-0.5 ml) were deproteinized with 96% ethanol, mixed thoroughly, and after a period of 30 min, 5 ml of hexane was added for extraction. The procedure was thereafter essentially as described above for “Quantitation of retinol.” The standard HPLC conditions for these analyses were elution of the LC-8 column (24) with water-methanol 5.95:10.90 (vol/vol) at a flow rate of 1.5 ml/min. Retinyl ester standards had been injected separately onto the same column to determine the positions of the three main ester peaks, retinyl palmitate, retinyl oleate, and retinyl stearate. In addition, small peaks of absorbing substances were found; most probably other retinyl esters were present (24-26). In the isolated cell fractions were 106% and 65% for the control rats, respectively. Assuming that the livers contained 125 x 10⁶ parenchymal and 65 x 10⁶ nonparenchymal cells per g wet weight, the recoveries of retinoids in the isolated cell fractions were 106% and 95% for the control and the ethanol-fed rats, respectively. The retinol level in plasma was not affected in the ethanol-fed rats.
and we could not detect any retinyl ester (when we examined for it) in blood from the animals.

**Uptake and transfer of vitamin A in liver**

Fig. 1 shows the fate of chylomicron [3H]retinyl ester injected intravenously. The disappearance of radioactivity from plasma was almost identical in the two groups of rats, revealing that the removal of chylomicron remnants was not altered significantly in the chronically ethanol-fed rats in the fasting state. Most of the radioactive retinoids were initially taken up by the liver which, after 50 min, contained about 90% of the injected radioactivity. Hepatic radioactivity then declined and, after 3-4 hr, about 50% of the injected dose was found in the liver. The rise and fall of radioactivity in livers were almost identical in the two groups of animals (Fig. 1).

When liver parenchymal and nonparenchymal cells were separated (Fig. 2), we found that most of the radioactive retinoids were initially in the parenchymal cells in rats from both groups. During the next few hours the radioactive retinoids were gradually transferred from the parenchymal cells to the nonparenchymal cells. Although it may appear from Fig. 2 that the transfer to the nonparenchymal cells was somewhat delayed in the ethanol-fed rats, there was no statistically significant difference in the transfer regarding the two groups of animals.

**Retinol and retinyl esters in rat liver**

Fig. 3 shows typical HPLC recordings of the hepatic retinyl esters from one control rat and one animal given ethanol for 5 weeks. There was in the actual HPLC system an adequate separation of retinyl palmitate and oleate. Note that the relative amounts of these two esters were different in the two animals. Table 2 gives the mean and SD for the retinoid distribution between retinol and different retinyl esters in livers from ethanol-fed rats and control animals. In both groups of animals most of the retinoid was esterified. However, the relative distribution between free retinol and the main retinyl esters was different in the two groups of rats. In the normal rats, about 1% of the total retinol was unesterified, and the predominant ester, retinyl palmitate, constituted more than 80% of the total retinoids. In the ethanol-fed rats, the relative amounts of unesterified retinol were significantly increased, suggesting a higher rate of hydrolysis or a lower re-esterification activity than normal. Furthermore, retinyl oleate was considerably increased, with a corresponding decrease in retinyl palmitate, but not in retinyl stearate.

**Activity of acyl-CoA:retinol acyltransferase (ARAT)**

The relative high amounts of unesterified retinol in the livers from the ethanol-fed rats might be explained by a decreased esterification of retinol. The ARAT activity in the livers of the animals was therefore of interest. Table 3 shows that the ARAT activities in liver microsomes for

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### TABLE 1. Retinoids (retinol + retinyl ester) in whole liver, liver cells, and in plasma from normal rats and from rats given large doses of ethanol for 5-6 weeks

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*P < 0.0002, ethanol-fed rats versus controls.
both groups of rats were in the range we usually find (15), and we found no difference in ARAT activity in the microsomes from total liver or in the isolated cell fractions from the two groups of rats.

Morphological studies

The ethanol-fed animals had large amounts of lipids in the cytoplasm of the parenchymal cells. The lipids were located in small vacuoles in the cytoplasm. The lipids were evenly distributed in the lobules of the liver. Lipids were also seen in the cytoplasm of the nonparenchymal cells. The amount of lipids varied among the animals. No necrosis, fibrosis or inflammatory cells were seen in the liver tissue. The amount of hepatic lipids in the control animals was minimal compared to that of the ethanol-fed animals. No necrosis, fibrosis, or inflammatory cells were seen.

DISCUSSION

Chronic ethanol consumption markedly reduces the level of retinoids in the liver, both in man, baboon and rat (1-3, 6). The current study confirms this observation in the rat (Table 1). The reduction is probably not due to a decreased absorption of retinol, inasmuch as Grummer and Erdman (6) have found that retinyl acetate and β-carotene are absorbed in a normal way in ethanol-fed rats. Furthermore, the hepatic uptake of retinyl esters carried to the liver parenchymal cells by chylomicron remnants is not affected in the ethanol-fed rats (Fig. 1).

We found no signs of an increased mobilization of retinol from the liver. The plasma retinol concentration was normal, in accordance with earlier observations (1, 6, 12), and we did not detect any retinyl esters in blood plasma, which would suggest a leakage from the hepatic stores.

A reduced liver content of retinoids could be due to a reduced storage capacity in the parenchymal or in the nonparenchymal cells or in both. In a parallel study (30) we have shown that most of the retinoid content in the parenchymal cell fraction is due to contaminating stellate cells. Furthermore, in the nonparenchymal cell fraction, almost all the retinoid is found in stellate cells. These results suggest that 80% or more of the total retinoids in liver from normal rats is located in stellate cells (30). Hence, the present results most probably reflect a reduced storage of retinoids in stellate cells in ethanol-treated rats, in agreement with what Leo and Lieber demonstrated histologically (12).

The accumulation of lipids in the livers from the ethanol-fed rats was to a large extent found in the parenchymal cells. Since decreased liver retinoid store has been found in ethanol-treated rats with only slight hepatic lipoidosis (6), the increase in hepatic lipids in the current study can probably not explain the reduced retinoid storage.

Fig. 2. Distribution of radioactivity between parenchymal (●-●-) and nonparenchymal liver cells (○-○-) after uptake of [3H]retinyl ester in liver. The percentage total liver radioactivity at the different times after injection (zero time) is shown. The points on the curve represent from one to six animals (one animal where no error bars are given).

Fig. 3. Typical HPLC profiles showing the retinyl esters in liver total homogenate from a control rat (upper panel) and an ethanol-fed rat (lower panel). A 5-μm Supelcosil LC-8 column was eluted with water-methanol 7.5:92.5 (vol/vol) at a flow rate of 1.5 ml/min, see Materials and Methods. The arrows indicate retinol (ROH), probable retinyl linoleate (RL?), retinyl palmitate (RP), retinyl oleate (RO), and retinyl stearate (RS), respectively. Retention time from injection of the sample is indicated.
The reduction of retinoids in the stellate cells of the ethanol-fed rats does not seem to be due to a failure in the transfer of recently incoming retinoids from the parenchymal cells to the nonparenchymal cells (Fig. 2). However, both the ethanol-fed animals and their controls in the present study showed a somewhat delayed transfer, as compared to our earlier studies (7). We do not know the reason for this, but the diet we used this time (as advocated by Lieber and collaborators) contained considerably more fat than our usual pelleted rat diet, and this may be of some importance.

An enhanced metabolism of retinol has been suggested as an explanation for the reduced liver retinoid store in ethanol-fed rats (31, 32). The livers of the alcoholic rats contained relatively more unesterified retinol than did the livers of control rats (Table 2). The higher level of retinol was not explained by different ARAT activity in the two groups. We have not tested whether the ethanol-treated rats had a higher activity of retinyl ester hydrolase.

An unexpected finding was a change in the acyl pattern of hepatic retinyl esters in the rats given large amounts of ethanol in their diet (31, 32). The decrease in retinyl palmitate and the increase in retinyl oleate may reflect a relative increase in retinyl palmitate hydrolase activity, or a relative increase in oleoyl CoA over palmitoyl CoA as substrate for ARAT in the alcoholic liver. Both groups of animals received considerable amounts of oleic acyl groups, as olive oil constituted about 70% of the dietary fat. Lieber, Spritz, and DeCarli (33) suggest increased retention of dietary fatty acids in liver in chronic ethanol consumption. Our laboratory is currently investigating the possible explanation for the retinyl ester acyl shift, and preliminary results seem to rule out dietary fat as a main reason (A. Nilsson and K. R. Norum, unpublished experiments).

The liver retinoid level was markedly reduced in rats given ethanol in their diet for several weeks. The reduction was probably not due to a reduced liver uptake of recently absorbed vitamin A, nor to altered transport of the vitamin from the parenchymal cells to the stellate cells. The high level of unesterified retinol in liver from ethanol-fed rats may suggest an increased hydrolytic activity. Based on the findings in the current study we think that the reduced retinoid level in the liver of ethanol-fed rats must be related in some way to an increased retinol metabolism. To further elucidate this, we plan to perform retinol turnover studies in rats given ethanol.

It is of great importance to obtain more knowledge about the intriguing interplay between the two alcohols, retinol and ethanol, in the liver, as such knowledge could help our understanding of several diseases in man. A role for retinoids in the development of ethanolic liver cirrhosis has been suggested (12). Furthermore, the probable increased risk of liver cancer (34) in alcoholics might be related to low retinoid stores in these patients (3), as adequate amounts of vitamin A are needed for normal cellular differentiation (35, 36). A further insight into the metabolic pathways of retinol and ethanol in liver may have a bearing on our knowledge of retinol metabolism.
per se, and on the knowledge of retinol's role in cellular differentiation in alcoholism and neoplastic diseases.

The authors thank Lizette B. Petersen, Kari Holte, and Grete M. Kindberg for skilled technical assistance. The study was supported by grants from the Norwegian Cancer Society, the Anders Jahre Foundation, The Norwegian Council on Cardiovascular Disease, the Norwegian Research Council for Science and the Humanities, and Nordisk Insulinfond. Magnhild Rasmussen is a Fellow of the Norwegian Council on Cardiovascular Disease. Rune Blomhoff is a Fellow of the Norwegian Cancer Society. Rune Blomhoff is a Fellow of the Norwegian Council on Cardiovascular Disease.

Manuscript received 19 October 1984.

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