

Short- and long-term effects of ethanol administration in vivo on rat liver HMG-CoA reductase and cholesterol 7 α -hydroxylase activities

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Abstract Short- and long-term effects of ethanol on HMG-CoA reductase (EC 1.1.1.34.) and cholesterol 7 α -hydroxylase activities in rat liver have been investigated. Neither the reductase nor the hydroxylase activity as measured in vitro was significantly affected within 2 hr after a single intraperitoneal injection of ethanol (7 mmol per 100g body weight), whether tested at the diurnal low or the diurnal high point of activity. Although chronic ethanol feeding for 21 days did not affect the diurnal rhythm of either of these enzyme activities, it caused a 29% decrease in HMG-CoA reductase activity and a 56% decrease in cholesterol 7 α -hydroxylase activity at the diurnal high point. The same chronic ethanol feeding caused a moderate increase in serum cholesterol and a significant increase in hepatic cholesterol concentration. On the basis of these findings, it is suggested that the decreased rate of cholesterol degradation to bile acids may play a significant role in the accumulation of cholesterol in the liver after chronic ethanol feeding.

Supplementary key words cholesterol biosynthesis · degradation · rate limiting enzymes · diurnal variation · radiochemical assay

Chronic treatment with ethanol has been shown to increase esterified cholesterol concentration in the liver as well as blood cholesterol (1–3). The possible reasons for this effect of ethanol are thought to be either an increased rate of hepatic cholesterol synthesis, a decreased rate of its degradation to bile acids, or both. In an earlier study (3) it was claimed that hepatic cholesterol synthesis is increased and bile acid excretion is decreased in rats chronically treated with ethanol.

Liver is the major organ responsible for cholesterol biosynthesis and its degradation to bile acids except when cholesterol synthesis is repressed by the feeding of a diet high in cholesterol. It has been clearly demonstrated with the use of labeled cholesterol that at least 90% of cholesterol is excreted in the feces as a saponifiable component of the bile (4). It is obvious from this that the conversion

of cholesterol to bile acids is the major pathway for the degradation of cholesterol; excretion of cholesterol, per se or as coprosterol, constitutes only a minor pathway. Furthermore, since bile acids are not oxidized to CO₂ by animal tissues, they represent the true end products of cholesterol metabolism.

HMG-CoA reductase and cholesterol 7 α -hydroxylase are thought to be the rate-limiting enzymes of cholesterol biosynthesis and its degradation to bile acids, respectively (5–10). This conclusion is derived from the fact that the rate of cholesterol synthesis is known to vary in the same direction as the activity of hepatic HMG-CoA reductase under a variety of nutritional states (11). Similarly, a positive correlation exists between the activity of hepatic cholesterol 7 α -hydroxylase and the rate of bile acid production (8–10). Thus it is logical to expect that any effect of ethanol administration on cholesterol synthesis and degradation to bile acids might be reflected in the activities of HMG-CoA reductase and cholesterol 7 α -hydroxylase. Therefore, we have investigated the effects of both short- and long-term ethanol administration on these two enzymes in rat liver.

MATERIALS AND METHODS

[3-¹⁴C]HMG-CoA (18.5 mCi/mmol), [4-¹⁴C]cholesterol (56 mCi/mmol) and Triton X-100 (trade mark of Rohm & Haas) were from New England Nuclear, Boston, MA. [2-¹⁴C]Mevalonic acid lactone (18 mCi/mmol) was from Amersham, Arlington Heights, IL. Nonradioactive HMG-CoA was from P.L. Biochemicals, Milwaukee, WI. [7 β -³H]-5-cholestene-3 β ,7 α -

Abbreviations: HMG-CoA, β -hydroxy- β -methylglutaryl coenzyme A; Cutscum, isooctyl phenoxy polyoxyethylene ethanol; Triton X-100, alkyl phenoxy polyethoxy ethanol.

diol (7 α -hydroxycholesterol) was a gift from Dr. H. Danielsson, Karolinska Institutet, Stockholm. NADP, glucose-6-phosphate and glucose-6-phosphate-dehydrogenase were from Boehringer Co., New York; 2-mercaptoethanol was from Calbiochem, La Jolla, CA. Silica gel-G plates (500 μ m thickness) were from Analtech Inc., Newark, DE. Cutscum was from Fisher Scientific Co., Pittsburg, PA, and cholesterol (>99% pure) was from Sigma, St. Louis, MO.

Treatment of animals

Male rats (140–180 g) of the Sprague-Dawley strain were purchased from Carworth and were housed in wire cages in a room that was artificially illuminated from 0600 to 1800 hours during each 24-hr period.

Behavior of rat liver HMG-CoA reductase and cholesterol 7 α -hydroxylase

It is known that both of these enzymes exhibit diurnal variation in their activities (12–15). When lights are on from 0600 to 1800 hours, the low point in both activities occurs between 1200 and 1400 hours when the food intake is low; the high point occurs between 2200 and 2400 hours or about 4–6 hr after the onset of the major feeding period. The time of the day during which these enzyme activities reach their diurnal high is set by the time of feeding (16, 17) and, by reversing the feeding schedule, the activities of both enzymes were shown to reach diurnal highs within 4–6 hr after the start of the meal-feeding period (16, 17). The effects of ethanol on both of these enzymes at the diurnal low and high points of activity were studied by manipulating the feeding schedules of the animals as described below.

Short term effects of ethanol

Rats (160–180g) were divided into two groups. One group was fed a Wayne lab blox diet (composition: crude protein, 23.5%; crude fat, 5.0%; and carbohydrate 54.3%) on an ad libitum feeding schedule; the second group was meal-fed the same diet between 0900 and 1200 hours every day. All rats had free access to fresh tap water. At the end of 7 days the rats in each of the above groups were further subdivided into two subgroups, an ethanol group and a control group. Rats in the ethanol group received a single intraperitoneal injection of ethanol (7 mmol per 100 g body weight in 0.9% NaCl) at 1300 hours while the control rats received an equivalent volume of 0.9% NaCl. The rats were killed by decapitation at indicated time intervals. Hepatic HMG-CoA reductase and chole-

sterol 7 α -hydroxylase activities were determined as described below.

Long-term effects of ethanol

Rats (140–160g) were divided into three groups: control, ethanol-fed, and glucose-fed. Rats in the control and glucose-fed groups were pair-fed with those from the ethanol-fed group throughout the 21-day experimental period. The ethanol-fed rats received a 20% (w/v) mixture of ethanol in drinking water whereas the glucose-fed rats received an isocaloric mixture of 37.5% (w/v) glucose in water. In addition, each rat in the ethanol-fed group was fed by stomach tube a solution of 25% (w/v) ethanol in 0.9% NaCl (1ml per 100 g body weight) daily at 1700 hours. The control rats received an equivalent volume of 0.9% saline while the glucose-fed rats received an equicaloric amount of 47% (w/v) glucose in 0.9% NaCl. In order to adapt the rats in the ethanol-fed group to drink water containing ethanol, the ethanol concentration in the drinking water was kept at 10% (w/v) during the initial three days. The glucose-fed rats received an equicaloric amount of 18.7% (w/v) glucose in water during this period.

During the last 7 days of the experiment, pair-feeding was continued but all the rats were meal-fed between 0900 and 1200 hours daily in order that the two enzymes could be assayed near their diurnal peak of activity (16, 17). On the last meal-feeding day at least three rats from each group were killed by decapitation at 1100, 1400, and 1700 hours and the activities of hepatic HMG-CoA reductase and cholesterol 7 α -hydroxylase were determined as described below. Blood serum and a small piece of liver were saved for cholesterol determination.

Preparation of microsomes

Rat liver microsomes were prepared essentially as described previously (18), except the composition of the homogenizing solution was as follows: potassium phosphate buffer, pH 7.0, 100 mM; nicotinamide, 30 mM; EDTA, 1 mM; and 2-mercaptoethanol, 20 mM.

HMG-CoA reductase assay

The HMG-CoA reductase assay was based on the method of Shapiro et al. (19). A typical assay mixture consisted of the following in a volume of 150 μ l: [3-¹⁴C]HMG-CoA, 0.15 μ mol of the racemate (sp act 800 dpm/nmol); NADP, 1 μ mol; glucose-6-phosphate, 5 μ mol; potassium phosphate buffer, pH 7.0, 100 μ mol; dithiothreitol, 4 μ mol; glucose-6-phosphate-dehydrogenase, 1.0 unit. After

preincubation for 10 min at 37°C, a microsomal homogenate (50 μ l containing 0.2–1.0 mg of protein) was added and the incubation was continued for a further 30-min period at 37°C. The final volume of the incubation mixture was always 200 μ l. The reaction was terminated by adding 20 μ l of 12 N HCl. The reaction mixture was incubated for an additional 30 min at 37°C, in order to lactonize the radioactive mevalonate formed, and then it was centrifuged at 2000 *g* at 5°C for 10 min to pellet the denatured protein. A 150 μ l aliquot of the supernatant was carefully withdrawn and applied directly to a 2-cm track of a silica gel G thin-layer plate. Radioactive mevalonic acid lactone (R_f 0.60) was separated from HMG-CoA and free HMG acid (R_f 0.0–0.3) by developing the chromatogram to 15 cm in benzene–acetone 1:1 (19). Scraped sections of the gel (2 \times 2 cm) were assayed for radioactivity in a dioxane-based scintillation fluid in a Beckman LS-350 liquid scintillation spectrometer. Correction for counting efficiency was applied by the automatic external standard channel ratio method.

Cholesterol 7 α -hydroxylase assay

Cholesterol 7 α -hydroxylase activity was measured by a modification of a procedure described previously (20). Care was taken throughout the assay procedure to avoid light (21). The substrate, [4-¹⁴C]-cholesterol (sp act 3.0×10^3 dpm per nmol) was purified by thin-layer chromatography (22) just before use. The modification was as follows: the assay mixture (800 μ l) contained potassium phosphate buffer, pH 7.4, 100 μ mol; MgCl₂, 6 μ mol; NADP, 1 μ mol; glucose-6-phosphate, 5 μ mol; glucose-6-phosphate-dehydrogenase, 2 units; 2-mercaptoethanol, 20 μ mol; Cutscum, 0.75 mg; and [4-¹⁴C]cholesterol, 200 nmol and 6.0×10^5 dpm. After preincubation for 10 min at 37°C, 200 μ l of microsomal homogenate (2–3 mg of protein) was added and the incubation was continued for a further 30-min period with automatic shaking (100 oscillations per min). The reaction was terminated by adding 10 ml of chloroform–methanol–ethyl acetate 4:2:1. Two ml of water was added and the mixture was shaken vigorously for a minute and then centrifuged. The upper aqueous layer was carefully removed by aspiration and the lower chloroform layer was evaporated at 50°C under a stream of nitrogen. Under these conditions 90–95% of the steroids were recovered from the incubation mixture.

The dried steroid fraction was dissolved in 500 μ l of acetone and extracted again with 200 μ l of acetone. The acetone extract was chromatographed on a silica gel G thin-layer plate in a track of 0.05

$\times 2.0 \times 15$ cm; benzene–ethyl acetate 7:13 was the developing solvent (22).

Authentic 7 α -hydroxycholesterol ([7 β -³H]-5-cholestene-3 β ,7 α -diol) had an R_f value of 0.3 whereas cholesterol ([4-¹⁴C]-5-cholestene-3 β -ol) had an R_f value of 0.78, thus giving the two compounds a distinct separation in the solvent system used. The gel fraction corresponding to authentic 7 α -hydroxycholesterol was scraped off into counting vials and analyzed for radioactivity in a toluene-based scintillation fluid (23) with a Beckman LS-350 liquid scintillation spectrometer. Quenching due to the presence of the gel in the scintillator was negligible. Correction for counting efficiency was applied by the automatic external standard channel ratio method. Correction for any nonenzymatic formation of 7 α -hydroxycholesterol was accounted for by subtracting the counts (~ 150 dpm) from a blank tube incubated under identical conditions with an equivalent volume of boiled microsomal homogenate. Protein was determined by the modified biuret method (24) using 5% Triton X-100 to eliminate turbidity due to lipid. The enzyme activities are expressed as nmol of mevalonate or 7 α -hydroxycholesterol formed per mg of microsomal protein per hr.

Cholesterol determination

Total cholesterol in serum and liver was extracted as described previously (25) and determined by the *o*-phthalaldehyde procedure described by Rudel and Morris (26).

RESULTS AND DISCUSSION

In preliminary experiments the cholesterol 7 α -hydroxylase assay was checked for proportionality and reproducibility with respect to microsomal protein concentration, time of incubation, and substrate concentration. It was found that the rate of 7 α -hydroxycholesterol formation was linear up to a protein concentration of 5 mg/ml and up to 40 min of incubation. Addition of 200 nmol of cholesterol per ml of incubation mixture gave maximal reaction rates.

The results in **Table 1** clearly show that when rats were meal-fed from 0900 to 1200 hours, the activities of both HMG-CoA reductase and cholesterol 7 α -hydroxylase reached their diurnal highs within 4–6 hr after the start of the meal-feeding period, thus confirming the earlier observations (16, 17). Significantly, chronic ethanol treatment did not disturb the circadian rhythm of these two enzymes (**Table 1**). These results clearly show that the diurnal

TABLE 1. Effect of chronic ethanol feeding (21 days) on circadian rhythm of hepatic HMG-CoA reductase and cholesterol 7 α -hydroxylase activities in meal-fed rats

Time of Killing	Reductase Activity			Hydroxylase Activity		
	Control	Ethanol	Glucose-fed	Control	Ethanol	Glucose-fed
	<i>nmol mg⁻¹ hr⁻¹</i>			<i>nmol mg⁻¹ hr⁻¹</i>		
9 AM	2.40 \pm 0.56 (3) ^a	5.33 \pm 1.68 (3) ^b	6.25 \pm 0.83 (3) ^b	0.25 \pm 0.04 (3)	0.26 \pm 0.10 (3)	0.46 \pm 0.08 (3) ^b
11 AM	5.62 \pm 0.56 (3)	8.55 \pm 1.70 (5) ^b	10.37 \pm 1.91 (5) ^b	0.64 \pm 0.09 (3)	0.40 \pm 0.05 (6) ^d	1.10 \pm 0.31 (6) ^b
2 PM	9.62 \pm 0.69 (5)	8.55 \pm 1.70 (5) ^b	10.37 \pm 1.91 (5) ^b	1.05 \pm 0.27 (5)	0.40 \pm 0.05 (6) ^d	1.10 \pm 0.31 (6) ^b
5 PM	5.63 \pm 0.22 (3)	3.02 \pm 0.66 (3) ^c	8.39 \pm 2.56 (3) ^d	0.54 \pm 0.04 (3)	0.36 \pm 0.03 (3) ^d	0.59 \pm 0.19 (3) ^b

^a Each value is the mean \pm SEM. The number of animals used for each time period is indicated in parentheses.

^b Not significant as compared to the control group.

^c Significantly different from the control group $P < 0.02$.

^d Significantly different from the control group $P < 0.05$.

rise in the activity of both the reductase and the hydroxylase is controlled by the time of feeding.

It is also obvious from Table 1 that both the reductase and the hydroxylase exhibited the same circadian rhythms in rats fed isocaloric amounts of glucose as in control or ethanol-fed animals. Several facts should be pointed out. A portal vein infusion of glucose was shown to stimulate cholesterol synthesis (27). Dietary carbohydrate is known to stimulate insulin secretion and we have previously shown that insulin causes a rapid stimulation of HMG-CoA reductase activity in rat liver (28). Also, it has recently been shown that glucose administration causes the stimulation of rat liver cholesterol 7 α -hydroxylase activity (29). In view of all these facts, we have deliberately stressed the comparison of reductase and hydroxylase activities from the ethanol-fed group only with those from the control group and not with those from the glucose-fed group, so that any stimulatory effects of glucose on these two enzyme activities would not obscure the interpretation of the results.

The data given in Table 2 show that both HMG-CoA

reductase and cholesterol 7 α -hydroxylase exhibited their typical diurnal low and high points of activity in rats fed ad libitum and meal-fed rats, respectively, under the experimental conditions employed. Furthermore, the activity of neither enzyme was significantly affected 30–120 min after an acute dose of ethanol, whether tested at the diurnal low point or at the diurnal high point of activity. These results imply that neither the rate of cholesterol synthesis nor its degradation is affected by an acute dose of ethanol within 2 hr.

Effects of chronic ethanol feeding for 21 days on body weights, liver weights, and cholesterol contents of liver and serum are shown in Table 3. During the initial 3–4 days of adaptation to drinking water containing ethanol, the rats in the ethanol group lost weight but, after this adaptation period, they gained an average 3.2 g per day as did the rats in the control group. This clearly indicates that the rats in the ethanol group had the same nutritional status as the control animals and did not suffer from any nutritional deficiency. Furthermore, the stomachs of all the rats in both the ethanol group

TABLE 2. Effects of an acute dose of ethanol administration on rat liver HMG-CoA reductase and cholesterol 7 α -hydroxylase activities

Time after Ethanol Administration	Reductase Activity		Hydroxylase Activity	
	Control	Ethanol	Control	Ethanol
<i>Minutes</i>	<i>nmol mg⁻¹ hr⁻¹</i>		<i>nmol mg⁻¹ hr⁻¹</i>	
Diurnal low ^a				
30	2.3 \pm 0.15 ^b	2.1 \pm 0.12	0.23 \pm 0.01	0.21 \pm 0.01
60	2.1 \pm 0.12	2.0 \pm 0.12	0.24 \pm 0.01	0.23 \pm 0.02
120	2.2 \pm 0.12	2.0 \pm 0.10	0.23 \pm 0.02	0.24 \pm 0.02
Diurnal high ^a				
30	15.2 \pm 0.6	16.1 \pm 1.6	1.17 \pm 0.15	1.24 \pm 0.16
60	13.9 \pm 0.9	14.5 \pm 1.3	1.18 \pm 0.13	1.32 \pm 0.16
120	14.0 \pm 0.9	13.6 \pm 0.8	1.22 \pm 0.12	1.18 \pm 0.11

^a Three animals per group for each time period.

^b Each value is the mean \pm SEM. None of the values in the ethanol-injected group was significantly different from the corresponding value in the control group.

TABLE 3. Effects of chronic ethanol feeding (21 days) on body weights, liver weights, and cholesterol concentrations in liver and serum

Treatment	Number of Animals	Body Weight		Liver Weight		Cholesterol	
		Initial	Final	g	g/100 g body weight	Liver mg/100 g	Serum mg/dl
Control	19	148 ± 2.7 ^a	210 ± 4.1	6.84 ± 0.27	3.23 ± 0.11	285 ± 6.0	76 ± 3.1
Ethanol	18	149 ± 2.5 ^b	193 ± 3.8 ^c	6.58 ± 0.27 ^b	3.43 ± 0.08 ^b	406 ± 21 ^d	90 ± 2.9 ^c

^a Each value is the mean ± SEM.

^b Not significant as compared to the control group.

^c Significantly different from the control group $P < 0.01$.

^d Significantly different from the control group $P < 0.001$.

and the control group were full at the time of kill, showing that none of the rats had suffered a starvation period. However, because of the initial loss in weight, the average final body weight of the rats in the ethanol group was significantly lower than that in the control group ($P < 0.01$).

Table 3 also shows that chronic ethanol treatment did not significantly alter the average liver weight from that of the control group whether expressed as total liver weight or as liver weight per 100 g body weight. Furthermore, there was no evidence of fatty infiltration by ethanol treatment. Similar results have been reported by Maddrey and Boyer (30) previously.

Hepatic cholesterol concentration was markedly increased (43%) in ethanol-fed rats as compared to the controls ($P < 0.001$). On the other hand, feeding of ethanol caused only a moderate increase (18%) in serum cholesterol concentration ($P < 0.01$). These results are in good agreement with those of Lefevre, DeCarli, and Lieber (3).

The effects of the chronic feeding of ethanol for 21 days on hepatic HMG-CoA reductase and cholesterol 7α -hydroxylase activities at the diurnal high point are presented in Table 4. HMG-CoA reductase activity was decreased by 29% in ethanol-treated rats as compared to the control rats ($P < 0.001$), thereby suggesting that chronic ethanol treatment inhibited hepatic cholesterol synthesis slightly. This conclusion is contrary to an earlier claim (3) based on [¹⁴C]acetate incorporation into cholesterol. It is possible that the apparent stimulation of cholesterol synthesis by ethanol observed by these earlier workers could have resulted from the two groups of rats being killed at different points in the diurnal cycle with respect to HMG-CoA reductase activity and cholesterol synthesis. In any event, the present study failed to find any stimulation of hepatic HMG-CoA reductase activity by chronic ethanol treatment under carefully controlled feeding conditions. We have already demonstrated (Table 1) that both the reductase and the hydroxylase reached

their diurnal high points of activity around 1400 hours in meal-fed rats regardless of whether or not they were chronically treated with ethanol.

Table 4 also shows that chronic ethanol treatment caused a 56% decrease in hepatic cholesterol 7α -hydroxylase activity as compared to the controls ($P < 0.001$). This implies that the rate of degradation of cholesterol to bile acids is markedly inhibited by chronic ethanol treatment. The conversion of cholesterol to bile acids and their subsequent excretion accounts for 90% of the degradation of cholesterol (4). Lefevre et al. (3) found that chronic feeding of ethanol to rats prolonged the half excretion time of bile acids and indirectly concluded that ethanol may impair the degradation of cholesterol to bile acids.

Assuming that cholesterol 7α -hydroxylase is the rate-limiting enzyme for cholesterol degradation to bile acids in chronic ethanol-treated rats as it appears to be in the normal rat (8–10), then the impaired activity of this key enzyme, as demonstrated in the present study, may play a major role in ethanol-induced accumulation of cholesterol. A block in cholesterol degradation, rather than increased synthesis, would be the major cause for cholesterol accumulation because it would affect not only the disposal of cholesterol synthesized de novo, but also the exogenous cholesterol derived from the diet. Interestingly, the primary defect in many of the lipid storage diseases is due to a block in degradation rather than to enhanced synthesis (31).

TABLE 4. Effect of chronic ethanol feeding (21 days) on rat liver HMG-CoA reductase and cholesterol 7α -hydroxylase activities at the diurnal high point

Treatment	Number of Animals	Reductase Activity	Hydroxylase Activity
		<i>nmol mg⁻¹ hr⁻¹</i>	
Control	19	9.87 ± 0.37 ^a	0.91 ± 0.07
Ethanol	18	7.03 ± 0.57 ^b	0.4 ± 0.03 ^b

^a Each value is the mean ± SEM.

^b Significantly different from the control group $P < 0.001$.

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