Ethanol modulates apolipoprotein B mRNA editing in the rat

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Abstract We have studied the rat ethanol-liquid diet model for chronic ethanol modulation of lipid homeostasis, apolipoprotein (apo) B production and apoB mRNA editing. Male Wistar rats were fed one of three diets: *i)* regular chow, *ii)* an isocaloric liquid diet, or *iii)* isocaloric ethanol-liquid diet where ethanol accounts for 35.5% of the total calories, for up to 40 days. There was no difference in body weight or liver/body weight ratio among the three groups of animals at the end of the feeding period. Hepatic and plasma triglycerides were elevated in the ethanol-treated animals only, correlated with an accumulation of lipid particles in the liver of these animals. By DNA excess hybridization, the steady state mRNA levels of apoB and apoB mRNA-editing protein relative to actin were not significantly altered. The proportion of edited apoB mRNA; i.e., apoB-48 mRNA/(apoB-48 + B-100) mRNA, increased in a time-dependent manner from ~50% to 100% in the ethanol-treated group. It remained unchanged in the chow- and liquid diet-fed animals. The proportion of apoB-48/apoB-100 protein synthesis was determined by [^{35}S]methionine labeling followed by specific immunoprecipitation and SDS-polyacrylamide gel electrophoresis. The amount of newly synthesized apoB-48 increased from 30–50% to > 99% of the total apoB (apoB-48 + apoB-100). This increase in apoB-48 biosynthesis is reflected by an increase in circulating plasma apoB-48 from barely detectable to ~50% of total plasma apoB. Fractionation of plasma lipoproteins by fast protein liquid chromatography (FPLC) indicates that the ethanol-induced hypertriglyceridemia is completely accounted for by an increase in plasma very low density lipoprotein (VLDL). The proportion of apoB-48 as a percent of total apoB in the VLDL fraction increased from ~50% in controls to > 90% in ethanol-treated animals. Furthermore, there is a strong correlation between plasma triglyceride concentration and proportion of edited apoB-mRNA in the liver of ethanol-treated rats, but no direct correlation of the latter with intrahepatic triglyceride content. ■ Ethanol-treated rats represent a new model for studying the regulation of apoB mRNA editing by dietary factors in vivo.—**Lau, P. P., D. J. Cahill, H-J. Zhu, and L. Chan.** Ethanol modulates apolipoprotein B mRNA editing in the rat. *J. Lipid Res.* 1995. **36:** 2069–2078.

Supplementary key words apoB-100 • apoB-48 • plasma triglycerides • triglyceride metabolism

ApoB mRNA editing is a posttranscriptionally regulated process that is coincident with splicing and polyadenylation (1, 2). By this process, a single ribonucleotide (C to U) change in apoB-100 mRNA modifies codon (CAA) for glutamine-2153 to a translational stop codon (UAA), producing apoB-48 mRNA (reviewed in refs. 1 and 3). As a result of this tissue-specific process, translation of the edited apoB mRNA terminates prematurely; apoB-48, the translation product, contains 2152 amino acid residues instead of the 4536 residues in apoB-100. The major functions of apoB-100 include cholesterol uptake into hepatic and extrahepatic tissues by serving as a ligand for the low density lipoprotein (LDL) apoB/E receptor, as well as transporting triglycerides out of the liver. ApoB-48, which lacks the putative receptor binding domain, functions mainly in transporting triglycerides out of the small intestine in humans and from both small intestine and liver in rodents. It is an obligatory component of chylomicrons, and also has sufficient structural competence to direct the effective intracellular assembly and secretion of hepatic very low density lipoprotein (VLDL) (4). In a study by Baum, Teng, and Davidson (5), hepatic lipogenesis was modulated in the rat in vivo by a 48-h fast followed by high carbohydrate diet feeding for 24–48 h, a maneuver that produced a 30-fold increase in hepatic triglyceride content. Under these circumstances, hepatic apoB-100 synthesis became undetectable, being replaced by the almost exclusive production of apoB-48. This shift from the synthesis of apoB-100 to apoB-48 as the predominant apoB species was accounted for by an increase in the proportion of edited (apoB-48) mRNA from 37% in the fasting state to 79% and 91% after at 24 and 48 h of high carbohydrate refeeding, respectively (5).

Abbreviations: apo, apolipoprotein; FPLC, fast protein liquid chromatography; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; RT-PCR, reverse transcriptase- polymerase chain reaction; TCA, trichloroacetic acid; REPR, mRNA-editing protein.

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It was shown previously that isocaloric replacement of some of the carbohydrate of a liquid diet by ethanol produced a 5- to 10-fold increase in hepatic triglyceride concentrations in rats (6). We reasoned that such a shift in lipid homeostasis may modulate apoB mRNA editing. Therefore, we have investigated the effects of chronic alcohol ingestion on the extent of apoB mRNA editing and the biogenesis of apoB-100 and apoB-48 and their correlation with plasma and intrahepatic lipid values.

The mechanism of the ethanol-induced hyperlipemia has been attributed to an accumulation of lipids in blood that occurs when their rate of entry into the blood exceeds their rate of removal (for a review see ref. 7). In this report we show that chronic ethanol feeding in rats also increases the production of hepatic apoB-48 by increasing the proportion of edited apoB mRNA resulting in an increase in the proportion of apoB-48 in total plasma and VLDL. The ethanol-fed rat provides a novel animal model for dietary regulation of apoB mRNA editing and triglyceride metabolism.

MATERIALS AND METHODS

Animal treatment

We used the chronic ethanol rat model as described (7). Male Wistar rats initially weighing about 200–250 g were housed, three per cage, in temperature- and light-controlled rooms and fed Purina rat chow for at least 10 days prior to entry into the study protocol. Before establishing treatment groups, they were accustomed to drinking a liquid diet as their only source of nourishment. Baseline lipoproteins (see below) and body weights were measured. To exclude that malnutrition is a primary factor for development of chronic alcoholic fatty liver, a liquid diet pair-fed control group was included to monitor for the nutritional adequacy of the diets. Rats were randomly assigned to three dietary groups: *i)* untreated controls that were fed regular chows and drinking water, *ii)* isocaloric controls that were fed a control liquid diet, and *iii)* ethanol-treated that were fed an ethanol liquid diet (Bio-Serv Inc., Frenchtown, NJ), with ethanol making up 35.5% of the total calories. The control liquid diet in a liter contained protein 180 kcal, fat 350 kcal, carbohydrate 470 kcal; the ethanol diet contained protein 180 kcal, fat 350 kcal, carbohydrate 115 kcal, and ethanol 355 kcal which corresponds to 6.7% ethanol (v/v). Ethanol was incorporated in the liquid diet containing all required nutrients and offered as the only source of fluid and food as recommended by the manufacturer. Animals were gradually introduced to the ethanol diet as follows: the first 3 days, 1/3 ethanol and 2/3 control diet; 4th–7th day, 2/3 ethanol and 1/3 control diet, and from the 8th day on, pure ethanol diet. Ethanol consumption was finally increased to account for 35.5% of the total caloric requirement, a proportion comparable to moderate alcohol consumption in humans (7). The animals were treated for 15, 20, 30, and up to 40-day periods. At the end of the treatment period, they were weighed and killed and blood was collected by cardiac puncture. Tissues were collected and treated differently according to the subsequent manipulations. The total amount of liquid diet consumed was recorded.

Serum and hepatic triglyceride and cholesterol levels and tissue morphology

Serum cholesterol and triglyceride concentrations were determined enzymatically by using kits from Sigma Diagnostics. Hepatic lipid was extracted from liver homogenates as described by Folch, Lees, and Sloane Stanley (8) and total cholesterol and triglyceride were assayed enzymatically by using kits from Sigma, St. Louis, MO. Lipid particles in the fixed tissue (3% glutaraldehyde in PBS) of the control and ethanol-treated rats were also examined and counted under light microscopy and electron microscopy.

Steady-state levels of apoB and editing protein mRNA

Total RNAs were extracted from fresh or pulverized frozen tissue of large sections of liver (1 g) to minimize sampling variability by a guanidinium isothiocyanate/CsCl method (9). RNA concentration was determined by the orcinol colorimetric method (10). The integrity of the purified total RNA was assayed by formaldehyde–agarose (1.5%) gel electrophoresis, and 28S and 18S ribosomal RNAs were found to be intact under these conditions. Quantitation of steady-state mRNA level was determined by DNA-excess hybridization as described (11). Probes used were: oligonucleotide for rat apoB (including both apoB-100 and B-48) mRNA, TATCGCGTATGTCTCAAGTTGAGAGAGTTTTTCATTCAAGTT; for actin mRNA, GCCACACGCAGCTCATTGTAGAAGGTG; and for the rat apoB mRNA-editing protein mRNA (12), CAACGTGTTTGTTGGTGTTTTGGCTCGTGTGTCGCCAGAT. The oligonucleotides were end-labeled with γ[^{32}P]ATP (3000 Ci/mmol), and purified from 12% acrylamide/7 M urea sequencing gels. Total RNAs (50 µg) were incubated with the gel-purified, end-labeled oligonucleotides in 3 × aqueous buffer (3 M NaCl, 0.5 M HEPES, pH 7.5, and 1 mM EDTA) at 75°C for 10 min and reannealed at 55°C for 18 h. The tubes were topped off with mineral oil during the extended incubation. The hybridization reaction mixtures were digested with 100–300 U of S1 nuclease. Reactions were stopped and run on 12% acrylamide/7 M urea sequencing gels. The autoradiograms

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were scanned with a BioImage scanner to quantify the mRNA signals. The hybridization signals were linear with respect to increasing RNA concentrations. Furthermore, for individual reactions, the RNA samples were incubated with the specific oligonucleotide and the actin oligonucleotide in the same tubes so that the ratio of the specific message versus that of actin could be calculated.

Hepatic apoB (B-100 and B-48) synthesis

Tissue samples from all liver lobes were pooled and sliced to 0.1-mm-thick pieces by a tissue slicer. Tissue slices (400 mg/dish) were incubated for 2 h in 2 mL Eagle's medium supplemented by 1 unit/mL penicillin and 1 mg/mL streptomycin in the presence of 100 μCi [^{35}S]methionine. Tissue slices were homogenized in phosphate-buffered saline, 1% Triton, 2 mM methionine containing the following protease inhibitors added at the indicated final concentrations: PMSF (1 mM), benzamidine (1 mM), N-*p*-tosyl-L-lysine chloromethyl ketone (25 μM), leupeptin (100 μM), EDTA (5 mM), papain (2 μM). A 225,000 *g* supernatant fraction was prepared and stored at -80°C prior to immunoprecipitation. Aliquots of homogenate were saved for measurement of total protein concentration, trichloroacetic acid-insoluble radioactivity and for lipid extraction. Under these conditions, incorporation of [^{35}S]methionine into TCA-precipitable protein was linear (data not shown). Quantitative immunoprecipitation of apoB (B-100 and B-48) was performed using monospecific polyclonal sheep antisera directed against rat apoB in the following buffer (final composition): 100 mM NaCl, 50 mM LiCl, 5 mM EDTA, 50 mM Tris, 0.02% sodium azide, 0.5% Triton, 0.5% sodium deoxycholate, and 0.05% SDS, at pH 7.40. This buffer had been optimized for apoB immunoprecipitation, particularly in the presence of high lipid concentrations (5). ApoB-immune complexes precipitated by Gamma-Bind G (Pharmacia, LKB) were washed extensively and analyzed on denaturing SDS-PAGE (4–15% gradient gels, Bio-Rad).

Determination of the proportion of edited apoB mRNA in the liver

The extent of endogenous apoB mRNA editing was determined by using the complementary techniques of *i)* PCR-cloning-colony hybridization using allele-specific oligonucleotides (13, 14) and *ii)* RNA primer extension analysis using a modification of the method described by Wu et al. (14). Quantitation was found to be linear using either method within the range of detection as required in this study (2–100% UAA/(UAA + CAA)).

Briefly, RQ1 DNase (Promega) pretreated total RNA was used for RT-PCR with the oligonucleotide primers; GGAATTCTGAAAAACTCTCTCAAC and CGGATCCATAATTATCTCTAATTAT, to amplify the 65-mer spanning the CAA site in apoB mRNA. The single-band PCR cDNA product (65-mer) was excised from the low-melting 3% agarose gel and purified with the Magic PCR Preps kit (Promega). After removal of the residual dNTPs, whose presence might interfere with T-4 ligation or give false UAA stops in the primer-extension assay, the gel-purified products were used either for subcloning into pGEM3Z (Promega) or directly for the primer-extension assay. The B-Gln and B-Stop allele-specific oligonucleotides and the primers used for primer extension assay were those described by Wu et al. (14). In the PCR-cloning-colony hybridization assays, a minimum of 2,000 colonies was plated for sequence-specific oligonucleotide hybridization in each assay. The ability of this technique to correctly discriminate between apoB-100 and apoB-48-specific colonies was verified by double-stranded sequencing of randomly picked clones as described previously (14). The proportion of edited apoB mRNA of the control rat liver consistently fell between 51 and 56% (2, 14, and this study).

FPLC fractionation of plasma samples and assessment of apoB-100/apoB-48 in VLDL

The method of Jiao et al. (15) was used for FPLC fractionation of rat plasma. We used a system with two Superose-6-columns (Pharmacia LKB) connected in series, run isocratically in 1 mM EDTA, 154 mM NaCl, and 0.02% NaN_3 at a flow rate of 0.5 ml/min at room temperature. Fractions of 0.5 mL were collected and total cholesterol and triglyceride in each fraction were assayed enzymatically. VLDL, LDL, and HDL fractions were well separated under these conditions.

The proportions of plasma apoB (apoB-100 plus apoB-48) were determined by Western blotting of the SDS-PAGE (4–15% gradient) gels (Bio-Rad). Samples of 1 μl of the plasma of the ethanol-treated and control rats were treated with 2% SDS, 0.5%-Nonidet P-40 at room temperature without reduction or heating before electrophoresis at 15 mA and at room temperature. The gels were then transferred electrophoretically on a transblot apparatus (Bio-Rad) to nitrocellulose membranes. The membranes were immunostained with sheep anti-rat apoB antiserum and subsequently with ^{125}I-labeled protein G (Amersham). Membranes were washed extensively, dried, and exposed to Kodak XAR-5 X-ray films.

To analyze VLDL apoB, pooled FPLC fractions under the VLDL peak were precipitated with TCA before electrophoresis. The pellets were resuspended with 2% SDS, 10 mM EDTA, 5 mM DTT, 2% glycerol, and 0.01% pyronin Y, all dissolved in the running buffer (pH 8.3) (16). For analysis of plasma VLDL, rats were fasted overnight with free access to drinking water for 14–16 h before they were killed and blood was drawn around 9 AM. The hepatic apoB mRNA editing in the control

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TABLE 1. Effects of an ethanol liquid diet in rats

	Group (n)	Days of Treatment	Body Wt.	Liver/Body	Hepatic Lipid Total TG	Hepatic Lipid Total CH	Serum Lipid Total TG	Serum Lipid Total CH
		g	*g*	*%*	*μg/mg protein*		*mg/dL*	
Untreated control (6)	0	371 ± 9.0	14.00 ± 0.8	3.7 ± 0.08	7.0 ± 0.12	1.70 ± 0.48	45.3 ± 4.3	52 ± 9
Ethanol diet (3)	15	311 ± 9.6	9.27 ± 0.3	2.98 ± 0.03	23.7 ± 0.07[a]	2.20 ± 0.55	72.5 ± 2.8	118 ± 3[a]
Ethanol diet (3)	25	283 ± 1.49	9.53 ± 0.27	3.36 ± 0.10	13.2 ± 0.32[b]	1.65 ± 0.33	156.0 ± 4.0[a]	89 ± 3.5
Ethanol diet (6)	35	365 ± 29.6	13.25 ± 0.62	3.63 ± 0.20	15.3 ± 0.09[a]	2.50 ± 0.58	126.8 ± 3.0[a]	60 ± 5
Control diet (6)	35	368 ± 6.3	11.25 ± 0.57	3.05 ± 0.09	8.9 ± 0.07	0.95 ± 0.15	71.0 ± 4.25	75 ± 9.5

Ethanol made up 35.5% of the total calories in the experimental diet. Animals were pair-fed the experimental (ethanol) or isocaloric control (ethanol-free) liquid diet. Abbreviations used are TG, triglyceride; CH, cholesterol.
[a]$P < 0.05$ vs. control diet.

rats was unchanged under these conditions (data not shown) and this manipulation did not interfere with our alcohol experiments, which is consistent with data reported by Baum et al. (5).

RESULTS

Effects of ethanol diet on body weight and liver weight, lipid content, and serum lipid concentrations

The effects of chronic ethanol administration on hepatic weight, lipid contents, and serum lipid compositions were analyzed prior to the molecular study. The two most conspicuous features of alcoholic fatty liver are the fat deposition and the organ enlargement. The hepatomegaly was attributed to an increase in lipid, protein and water retention (7). Our data showed that chronic ethanol administration did not significantly change the final body weight or liver/body weight ratio (**Table 1**), although the liver of ethanol-treated animals was enlarged and fat infiltration was apparent in the form of a whitish sheen morphologically. There was also an increase in hepatic triglyceride content and serum triglyceride level (Table 1), whereas hepatic cholesterol content and serum cholesterol level did not show any significant change. It had previously been demonstrated that the increase in hepatic cholesterol occurs mainly in the esterified fraction with little increase in free cholesterol in the rat (7). Under light microscopy, the number of lipid particles inside the liver cells increased 45- to 70-fold in ethanol-treated rats compared to the untreated controls. None of these changes were observed in controls pair-fed the isocaloric liquid diet.

Serum total triglyceride also showed a 280% increase from 45.3 ± 4.3 mg/dL (in untreated controls) to 126 ± 3 mg/dL (in ethanol diet-treated for 35 days) (Table 1). Plasma lipoproteins were fractionated on FPLC. Compared to controls, VLDL triglyceride showed a time-dependent rise after ethanol treatment (**Fig. 1**), whereas VLDL cholesterol was not significantly different between ethanol-treated rats and control rats (data not

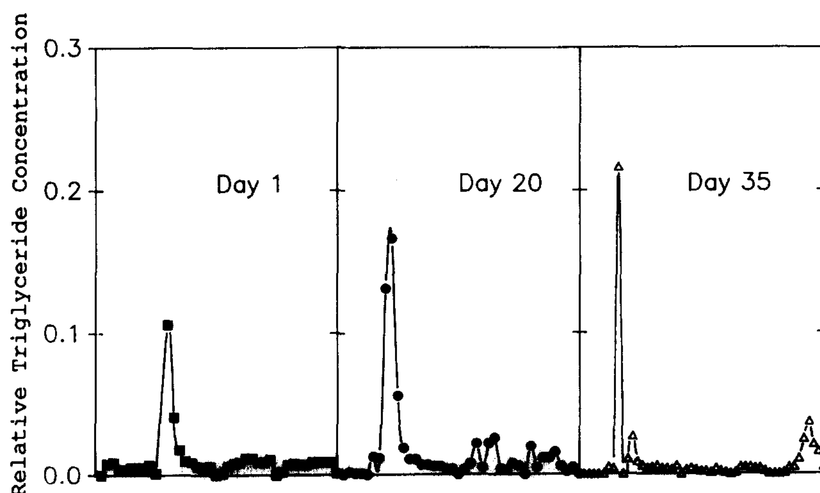

Fig. 1. FPLC fractionation of rat plasma. The FPLC separation system has two Superose-6 columns serially connected and run isocratically in 1 mM EDTA, 154 mM NaCl and 0.02% NaN_3 at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected and analyzed for triglyceride concentration. Representative profiles of three treatment periods (day 1, day 20, day 35) are shown with relative absorbance unit $A_{500\ nm}$.

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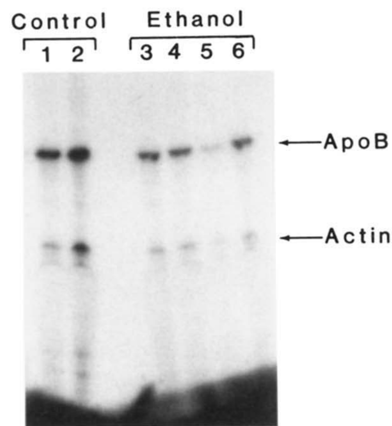

Fig. 2. Steady-state levels of apoB mRNA versus Actin mRNA. Lanes 1, 2: chow and isocaloric diet controls, respectively; lanes 3–6: four different ethanol-treated rats on day 35.

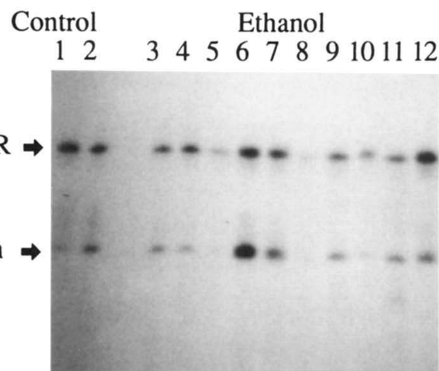

Fig. 3. REPR/Actin mRNA ratio determined by DNA-excess hybridization. Lanes 1–2, chow and isocaloric controls; lanes 3–5, 15 days; lanes 6–7, 20 days; lanes 9–12, 35 days.

shown). The level of VLDL triglyceride increased more than 2-fold from day 1 to day 35. Essentially all the serum triglyceride was contained in the VLDL fractions, and LDL and HDL did not have any detectable triglyceride (Fig. 1). Control animals that were fed the isocaloric liquid diet for 35 days had plasma lipid levels that were not significantly different from controls fed regular chow (Table 1). Therefore, ethanol ingestion causes an increase in serum triglyceride which is almost entirely accounted for by the increase in the VLDL triglyceride.

Effects of ethanol on the steady-state levels of the mRNAs for apoB and apoB mRNA-editing protein

The steady-state levels of apoB mRNA in the total rat liver RNA were quantified by quantitative DNA-excess oligonucleotide hybridization (11). By this technique, we found that total apoB mRNA content in ethanol-treated rats was unchanged compared to the regular chow- and isocaloric liquid diet-fed controls (**Fig. 2**). The relative mRNA concentration of apoB mRNA-editing protein (REPR) was determined by a similar technique. Because we found that the proportion of edited apoB mRNA was changed with the ethanol diet (see below), we measured the relative concentration of the mRNA for the editing protein by DNA-excess hybridization in the same RNA samples. We found that the level of apoB mRNA-editing protein (REPR) mRNA relative to actin mRNA remained unchanged (**Fig. 3**). Therefore, ethanol treatment apparently had no detectable effect on the relative amount of mRNAs for apoB or its editing protein compared to actin.

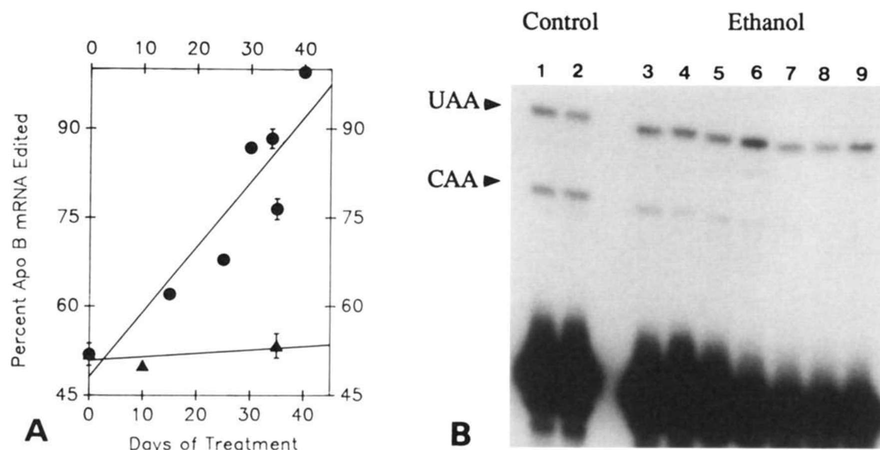

Fig. 4. Ethanol effects on endogenous apoB mRNA editing in rat liver. A: Data were obtained by two complementary methods (see Materials and Methods); they represent means ± SE for 3–6 animals. For some data points, the standard error bars fall within the symbol and are not evident. The ethanol-treated animals (closed circles) were compared with isocaloric liquid diet-fed controls (closed triangles). The day zero indicates untreated controls. B: Endogenous apoB mRNA editing assayed by the method of primer extension. Representations of ethanol-treated versus control animals: control (1) is untreated; (2) is isocaloric control for 35 days; (3)(4) are ethanol treated for 15 days; (5)(6) are those of day 25; (7) is day 35; and (8)(9) are day 40.

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Effects of ethanol on hepatic apoB mRNA editing

The proportions of edited apoB mRNA [i.e., apoB-48 mRNA/(apoB-48 + apoB-100)mRNA] were assayed by both PCR-cloning and primer-extension methods; their accuracy as well as reproducibility were verified (see Materials and Methods) as previously reported (14). For each data point, there were 3–6 animals in each treatment group and the standard errors are as indicated (**Fig. 4A**). After ethanol feeding, the proportions of hepatic edited apoB mRNA increased toward 100% (Figs. 4A, 4B). The pretreatment (day 1) proportion of edited apoB mRNA was 51.9 ± 1.9%; the proportions increased with the time of ethanol treatment; they were, on day 15, 62 ± 1.0%; day 25, 67.85 ± 1.2%; day 30, 86.8 ± 0.9%; day 32, 88.3 ± 1.66%; day 35, 76.4 ± 1.77%; and day 40, 99.5 ± 0.5%. The increase in the proportion of apoB-48 mRNA occurred in a roughly linear fashion over the course of 40 days of ethanol intake (Fig. 4). In the pair-fed controls where ethanol was replaced isocalorically by carbohydrate, no change in the proportion of edited (apoB-48) mRNA was evident (Fig. 4).

The results of primer-extension assays on representative RNA samples from selected time points are displayed in Fig. 4B. A ^{32}P end-labeled rat 23-nucleotide oligonucleotide was used as the primer in the primer-extension assays. UAA corresponds to the B-48 mRNA extension product and CAA corresponds to the B-100 mRNA extension product. By this assay, control liver samples from rats that were receiving the regular chow (lane 1) or isocaloric liquid diet (lane 2) have approximately half of their apoB mRNA in the edited form. In the samples from ethanol-treated animals, the percent edited apoB mRNA/total apoB mRNA displayed a progressive increase from ~70% (day 25, lanes 3–5) to ~100% (day 40, lanes 6–9). The data presented in Figs. 4A and 4B indicate that ethanol treatment in rats in vivo up-regulates the proportion of edited apoB-48 mRNA relative to total apoB mRNA in the liver.

TABLE 2. Ethanol effects on apoB synthesis in liver slices

Diet	Immunoprecipitable ApoB cpm / TCA-Precipitable cpm × 100: ApoB-100	ApoB-48
Control	0.23 ± 0.057	0.346 ± 0.06
Ethanol		
Day 10	0.18 ± 0.06[a]	0.430 ± 0.058[a]
Day 27	ND	0.558 ± 0.215[a]
Day 35	ND	0.643 ± 0.117[a]

Values given as means ± SD; n = 3 animals per group, three determinations per animal; ND, not detectable.

[a]Student's *t*-tests show no significant differences of total apoB as compared to controls

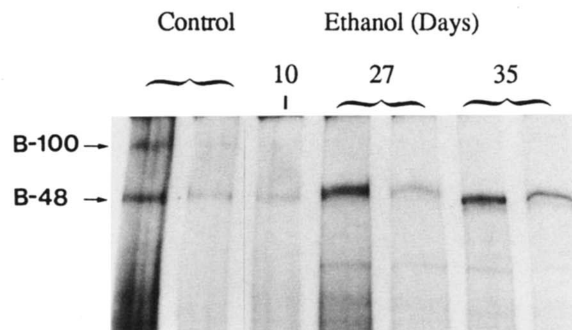

Fig. 5. Ethanol effects on rate of synthesis of apoB-48 versus apoB-100 in rat liver. The controls are isocaloric controls and those of the untreated; and the ethanol-treated ones are those up to day 35. Rat liver slices were labeled with [^{35}S]methionine for 2 h and radioactivity incorporated into newly synthesized apoB was immunoprecipitated by anti-rat apoB antiserum. The precipitated samples were fractionated on a 4–15% SDS-PAGE gel and analyzed by fluorography.

Effects of ethanol on hepatic synthesis of apoB (B-100 and B-48)

The increase in apoB-48 in total plasma and in plasma VLDL fractions can be attributed either to up-regulation of apoB-48 biogenesis or preferential secretion of the small molecular form (B-48) over the large molecular form (apoB-100) of apoB from hepatocytes in ethanol-treated rats, as ethanol is known to affect protein secretion (17). We found that total immunoprecipitable apoB as a percent of total (TCA) precipitable protein did not change significantly on days 10, 27, and 35 of ethanol treatment as compared to controls (**Table 2**). We also determined the relative synthetic rates of the two molecular weight forms of apoB in the rat liver under conditions where the incorporation of radiolabeled methionine into apoB was linear. When total liver slices were labeled with [^{35}S]methionine for 2 h and the radioactivity incorporated into newly synthesized apoB in the liver homogenates was immunoprecipitated by an anti-rat apoB antiserum and analyzed by SDS polyacrylamide gel electrophoresis, apoB-48 increased progressively to ~100% of total apoB synthesized by liver slices of ethanol-fed rats (**Fig. 5**); little if any apoB-100 was synthesized on day 27 and day 35. This is in contrast to control rat liver slices which synthesized substantial amounts of the total ^{35}S-labeled apoB as apoB-100 (Fig. 5). Quantitation of the radiolabeled bands indicated that over 99.5% of the apoB species synthesized by the ethanol-treated rats consisted of apoB-48 compared to approximately 30–50% in the untreated controls. We conclude that one major effect of ethanol treatment in rats is the greatly enhanced synthesis of apoB-48 relative to apoB-100 in the liver, which is consistent with the greatly increased apoB-48/apoB-100 mRNA ratio in the presence of a constant level of total apoB mRNA. Additionally, the

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total apoB synthesized as percent of total TCA precipitable proteins did not change significantly (Table 2).

Effects of ethanol on the relative concentrations of apoB-100 and apoB-48 in total plasma and VLDL

The relative concentrations of circulating plasma apoB-100 and apoB-48 were assessed by Western blot analysis. Total plasma from three control animals and two animals treated with the ethanol diet for 35 days were analyzed on 4–15% gradient polyacrylamide gels in SDS (Bio-Rad), transferred to nitrocellulose membrane electrophoretically, immunostained with monospecific sheep anti-rat apoB antibody, and analyzed by ^{125}I-labeled Protein G and autoradiography. In control animals, total plasma apoB consists almost exclusively of apoB-100 (**Fig. 6A**). In ethanol-treated animals, the relative amount of apoB-48 increased markedly and its molar concentration made up about 50% of the total plasma apoB. Therefore, ethanol treatment in vivo markedly increases the amount of circulating apoB-48 relative to apoB-100 in rat plasma.

Plasma VLDL in ethanol-treated rats are elevated (Fig. 1). ApoB-100-containing VLDL are metabolized to LDL, but VLDL containing apoB-48 cannot be converted to LDL (18, 19). We examined the ratio of apoB-48/apoB-100 in the VLDL fractions of the ethanol-fed rats versus the untreated controls. The pooled FPLC fractions containing the VLDL from the fasted rats were analyzed by 4–15% gradient SDS-denaturing gels. As shown in Fig. 6B, VLDL from two ethanol-treated rats had almost exclusively apoB-48, whereas VLDL from three control animals contained approximately equal amounts of apoB-100 and apoB-48. Therefore, the observed increase in the proportion of apoB-48 mRNA after ethanol treatment was reflected by an increase in the relative concentration of apoB-48 in total plasma as well as in VLDL.

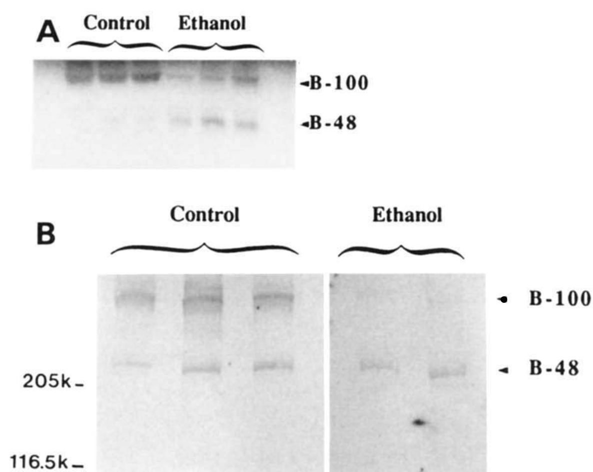

Fig. 6. A: Total plasma apoB in control and ethanol-treated rats studied by Western blot analysis. Control lanes are those of untreated and isocaloric controls; ethanol lanes are from animals treated with ethanol diet for 35 days. The gel was electro-transferred onto a nitrocellulose membrane. ^{125}I-labeled anti-rat apoB antiserum was used for immuno-detection. B: VLDL apoB in control and ethanol-treated rats studied by Western blot analyses. Control lanes are those of untreated and isocaloric controls and ethanol-treated are from animals treated with the experimental diet for 35 days. The VLDL fractions were separated by FPLC and TCA precipitated before electrophoresis was performed on a 4–15% SDS-polyacrylamide gel.

DISCUSSION

In this study, we showed that in the rat the hyperlipidemia and fatty infiltration in the liver resulting from chronic ethanol intake are associated with an increase in the proportion of edited apoB mRNA in the liver (Figs. 4A, 4B). This increase in apoB-48 mRNA with a reciprocal decrease in apoB-100 mRNA is reflected by changes in the biosynthetic rates of the two apoB species in vivo (Fig. 5), leading to a marked increase in circulating apoB-48/apoB-100 ratio in total plasma (Fig. 6A) as well as in the plasma VLDL fraction (Fig. 6B). The amounts of circulating apoB-100 and apoB-48 can be modulated intracellularly at a posttranslational level (reviewed in ref. 20) or extracellularly in the intravascular and interstitial compartments via receptor- and non-receptor-mediated pathways (21). The correlation of apoB mRNA editing with apoB-48 protein production is not absolute. For example, Teng et al. (22) found that in some tissues containing both edited and unedited apoB mRNA, only apoB-100 synthesis was detected. We have therefore examined both the amount of apoB mRNA editing and proportion of apoB-48 synthesis in the rat liver after ethanol ingestion. The fact that the higher relative amount of apoB-48 mRNA during chronic ethanol ingestion in the rat was associated with an increase in circulating apoB-48 relative to apoB-100 indicates that the up-regulation in the extent of apoB mRNA editing contributes significantly to the altered pathophysiological state induced by ethanol in this animal.

The hyperlipidemia associated with chronic ethanol administration in the rat involves mainly VLDL. Previous studies suggest that the clearance of plasma lipoproteins is unchanged after acute or chronic ethanol administration in the rat (for a review, see ref. 7). Therefore, the hyperlipidemia in the experimental animals is apparently not caused by an impaired LDL (apoB/E) receptor-mediated uptake of the apoB-48-rich VLDL particles. The two major sources of VLDL in the rat are the liver and the small intestine. The small intestine puts out increased amounts of VLDL in the lymph after ethanol infusion (23). However, the overall contribution of the small intestine to the hyperlipidemia

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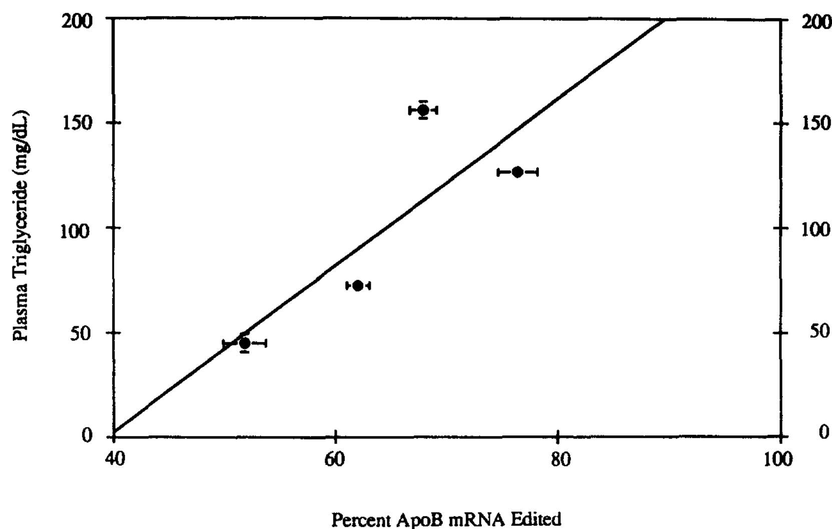

Fig. 7. Correlation of plasma triglycerides with percent apoB mRNA edited. Data are derived from experimental animals presented in Table 1 and Fig. 4. The line drawn was first-ordered, least-squared regression by Sigma-Plot, $r = 0.813$.

of chronic ethanol intake is relatively small compared to that from the liver. Hepatic VLDL production in the rat is stimulated by chronic ethanol administration, a process associated with an increased synthesis of the protein components in VLDL (7, 24). In this study, we showed that apoB mRNA (i.e., apoB-100 plus apoB-48 mRNA) level is not selectively stimulated by this dietary manipulation; this is in keeping with the observation that apoB mRNA transcription rates are generally not selectively changed by various dietary manipulations. Furthermore, the hyperlipidemia, mainly in the form of elevated VLDL, is associated with increased production of apoB-48 rather than apoB-100. Therefore, the ethanol-induced VLDL would likely be metabolized via an apoE-mediated pathway and would not result in any enhancement of the plasma LDL, which contains exclusively apoB-100 (19, 25).

In the adult rat liver and small intestine, apoB mRNA editing can be modulated by dietary and hormonal factors. When an animal is fasted and subsequently refed a high carbohydrate diet, the proportion of hepatic apoB-48 mRNA decreases from about 60–70% to about 30–40% during fasting, increasing to 80–90% in 2–3 days of refeeding (5, 26). Similarly, thyroid hormone treatment in hypothyroid rats also increases the relative amount of apoB-48 mRNA in the rat, concomitant with increased hepatic triglyceride synthesis and secretion (27, 28). A relationship between triglyceride metabolism and apoB mRNA editing in the rat liver is also suggested by the very strong correlation between the proportion of edited apoB mRNA and plasma triglyceride levels (**Fig. 7**, $r = 0.813$) observed in this study. However, there is no direct linear correlation between hepatic triglyceride content and the extent of apoB mRNA editing during the time course of chronic ethanol treatment ($r = 0.36$) although both are increased by ethanol treatment (Table 1). Whether hepatic triglyceride accumulation plays a direct role in modulating apoB mRNA editing in the rat remains unclear. The acute effects of dexamethasone-induced increase in hepatic triglyceride content is not accompanied by any change in the extent of apoB mRNA editing (29).

ApoB mRNA editing is a complex process. In the rat liver, it is an intranuclear process that occurs posttranscriptionally, coincident with RNA splicing and polyadenylation and is essentially complete before the mature apoB mRNA is exported into the cytoplasm (2). There is considerable evidence that multiple protein components are involved in the process (30–32). Recently, the cDNA for a 27-kDa apoB mRNA-editing protein was cloned from rat small intestine (12). This protein appeared to be a cytidine deaminase requiring zinc for activity (33). It is active against synthetic apoB mRNA substrates only in the presence of chicken intestinal extracts or S100 fractions from other mammalian tissues. Inui et al. (34) found that in the rat liver in vivo, alterations in cholesterol flux might modulate the complex interplay of the apoB mRNA-editing protein and complementation factor(s). The editing protein may not be necessarily rate-limiting because Funahashi et al. (35) demonstrated that in the rat, hepatic and kidney apoB mRNA editing both demonstrated a temporal dissociation from alterations in the abundance of editing protein mRNA. In this study, we found that the increased proportion of edited transcripts in the total hepatic apoB mRNA after ethanol feeding in the rat was not

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accompanied by any significant change in the level of the steady state mRNA level for the apoB mRNA-editing protein. A number of RNA-binding proteins have been described that bind to apoB mRNA in the vicinity of the editing site, including a 40 kDa nuclear protein described by Lau et al. (36) and a 60 kDa and a 43 kDa protein in the S100 extracts described by Harris et al. (32) and Navaratnam et al. (31). It is not clear whether these proteins are actually involved in the editing process or whether they are affected by ethanol treatment in the rat. Alternatively, if edited apoB mRNA is selectively stabilized and/or unedited mRNA selectively destabilized by ethanol feeding, the end result would be that edited mRNA would predominate in the ethanol-fed rat. Finally, we found that direct exposure of McAR7777 cells to ethanol did not affect the ratio of apoB-48/B-100 synthesized in these cells (data not shown). This is in contrast to the stimulation of apoB mRNA editing in primary rat hepatocytes by chronic insulin treatment (37). To date, only a handful of manipulations have been found to significantly affect hepatic apoB mRNA editing in the rat; these include thyroid hormone treatment (27), fasting and carbohydrate refeeding (5), and high-dose estrogen treatment (38). Here we document that chronic ethanol treatment in the rat is another useful model for studying the regulation of apoB mRNA editing in vivo.

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